Studies of *Leishmania major* Pteridine Reductase 1, a Novel Short Chain Dehydrogenase

James Luba

*University of Massachusetts Medical School*

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Studies of *Leishmania major* Pteridine Reductase 1, a Novel Short Chain Dehydrogenase

A Dissertation Presented

by

James Luba

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences,

Worcester, in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES

SEPTEMBER 1997

PHARMACOLOGY AND MOLECULAR TOXICOLOGY
Studies of *Leishmania major* Pteridine Reductase 1, a Novel Short Chain Dehydrogenase

A Dissertation Presented

by

James Luba

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I would like to thank my thesis advisor Dr. Larry Hardy. His guidance and support were invaluable to me during my time here. Dr. Hardy offered advice when necessary and gave me the room to make mistakes when appropriate. Thanks Larry.

Thanks also go to Dr. Neal Brown and Dr. George Wright who often took the time to provide advice and valuable criticism, although I was not a member of either of their labs.

And to my family, I could never have done this without your love and support. Thanks for being there in all those times of crisis and happiness.

Dedication

A La Dr. Alba E. Morales

"En mi cielo al crepúsculo cres como una nube

y tu color y forma son como yo los quiero.

Eras mía, cres mía, mujer de labios dulces

y viven en tu vida mis infinitos sueños."

Pablo Neruda
Abstract

Pteridine reductase 1 (PTR1) is an NADPH dependent reductase that catalyzes the reduction of several pterins and folates. The gene encoding this enzyme was originally identified in *Leishmania* based on its ability to provide resistance to the drug methotrexate (MTX). The DNA and amino acid sequences are known, and overproducing strains of *Escherichia coli* are available. PTR1 has been previously shown to be required for the salvage of oxidized pteridines (folate, biopterin, and others). Since *Leishmania* are folate and pterin auxotrophes, PTR1 is a possible target for novel anti-folate drugs for the treatment of leishmaniasis.

PTR1 catalyzes the transfer of hydride from NADPH to the 2-amino-4-oxo-pteridine ring system yielding 7, 8-dihydropteridines, and to the pteridine ring system of 7, 8-dihydropteridines yielding 5, 6, 7, 8-tetrahydropteridines. PTR1 shows a pH dependent substrate specificity. At pH 4.6 the specific activity of PTR1 is highest with pterins, while at pH 6.0 the specific activity of PTR1 was highest with folates.

The sequence of PTR1 is only 20-30% homologous to the sequences of members of the short chain dehydrogenase/reductase enzyme family. Although this is typical for members of this enzyme family, it does not allow for unambiguous classification in this family. In fact, when the DNA sequence of *PTR1* was first determined, PTR1 was classified as an aldoketo reductase. To classify PTR1 definitively, further biochemical characterization was required. To provide this information, the work described here was undertaken: (i) the stereochemical and kinetic course of PTR1 was determined; (ii)
residues important in catalysis and ligand binding were identified; and (iii) conditions for
the crystallization of PTR1 were developed.

**The stereochemistry of hydride transfer**

The use of [³H]-folate, showed that the ultimate product of PTR1 was 5, 6, 7, 8-
tetrahydrofolate. 4R-[³H]-NADPH and 4S-[³H]-NADPH were synthesized enzymatically
and used as the cofactor for the reduction of folate. PTR1 was coupled to thymidylate
synthase (TS), and tritium from 4S-[³H]-NADPH was transferred to thymidylate.
Therefore, the pro-S hydride of NADPH was transferred to the *s*<sup>i</sup>* face of dihydrofolate
(DHF; see figure 1-1). The transfer of the pro-S hydride indicates that PTR1 is a B-side
dehydrogenase which is consistent with its membership in the short chain dehydrogenase
(SDR) family.

**The kinetic mechanism of PTR1**

When NADPH was varied at several fixed concentrations of folate (and *vice-versa*) V/K (V<sub>max</sub>/K<sub>M</sub>) showed a dependence upon concentration of the fixed substrate.
This is consistent with a ternary complex mechanism, in contrast to a substituted enzyme
mechanism that exhibits no dependence of V/K on fixed substrate. Product inhibition
patterns using NADP<sup>+</sup> and 5-deazatetrahydrofolate (5dTHF, a stable product analog)
were consistent with an ordered ternary complex mechanism in which NADPH binds first
and NADP<sup>+</sup> dissociates last. However, an enzyme-DHF binary complex was detected by
fluorescence. Isotope partitioning experiments showed that the enzyme-DHF binary
complex was not catalytically competent whereas the enzyme-NADPH complex was.
Measurement of the tritium isotope effect on V/K (¹(T/V/K)) at high and low dihydrofolate
confirmed that PTR1 proceeds via a steady state ordered mechanism. Rapid quench analysis showed that dihydrofolate was a transient intermediate during the reduction of folate to tetrahydrofolate and that folate reduction is biphasic.

**Catalytic Residues of PTR1**

The amino acid sequences of dihydropteridine reductase and 3-α, 20-β, hydroxysteroid dehydrogenase were aligned to that of PTR1. Based on the results of the alignment, site directed mutagenesis was used to investigate the role of specific residues in the catalytic cycle of PTR1. Variant enzymes were screened based on their ability to rescue a dihydrofolate reductase (DHFR) deficient strain of *E. coli*. Selected PTR1 variants (some complementing and some non-complementing) were purified and further characterized. Tyrosine 193 of the wild type enzyme was found to be involved in the reduction of pteridines, but not in the reduction of 7, 8-dihydropteridines, and eliminated the substrate inhibition of 7, 8-dihydropteridines observed with the wild type enzyme. Both PTR1(K197Q) and PTR1(Y193F/K197Q) had decreased activity for all substrates and low affinity for NADPH. In contrast to the wild type enzyme, NADPH displayed substrate inhibition towards PTR1(K197Q). All PTR1(D180) variants that were purified were inactive except for PTR1(D180C), which showed 2.5% of wild type activity with DHF. The binary complexes of PTR1(D180A) and PTR1(D180S) with NADPH showed a decrease in affinity for folate. Based on the kinetic properties of the PTR1 variants, roles for Y193, K197, and D180 are proposed. In conjunction with D180, Y193 acts as a proton donor to N8 of folate. K197 forms hydrogen bonds with NADPH in the active
site and lowers the pKₐ of Y193. D180 participates in the protonation of N8 of folate and N5 of DHF.

**Crystallization of PTR1 and PTR1-ligand complexes**

The crystallization of PTR1 from *L. major* and *L. tarentolea* as unliganded and as binary and ternary complexes was attempted. Several crystal forms were obtained including *L. major* PTR1-NADPH-MTX crystals that diffracted to ~3.2 Å resolution. It was not possible to collect a full data set of any of the crystals. At their current stage, none of the crystal forms is suitable for structural work.
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<td>Aldoketo reductases</td>
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<td>ΔA₃₄₀</td>
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<td>DEAE</td>
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<td>Dihydropteridine reductase</td>
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<td>dTMP</td>
<td>Deoxythymidine 5'-monophosphate</td>
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<td>dUMP</td>
<td>Deoxyuridine 5'-monophosphate</td>
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<td>HEPES</td>
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<td>HSD</td>
<td>3α, 20β-Hydroxysteroid dehydrogenase</td>
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<td>7α-HSD</td>
<td>7α-Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>Mes</td>
<td>2-[N-Morpholino]ethanesulfonic acid</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>β-Nicotinamide adenine dinucleotide phosphate</td>
</tr>
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<td>NADPH</td>
<td>Reduced β-nicotinamide adenine dinucleotide phosphate</td>
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<td>O₁₂₉</td>
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<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>Abbreviation</td>
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<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
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<td>SDR</td>
<td>Short chain dehydrogenase</td>
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<td>5dTHF</td>
<td>5-Deazatetrahydrofolate</td>
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<td>TS</td>
<td>Thymidylate synthase</td>
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<td>T(V/K)</td>
<td>Tritium isotope effect on V/K</td>
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Chapter I

General Introduction

PTR1 is an NADPH dependent reductase, from Leishmania, that reduces pterins to 7, 8-dihydropterins and 7, 8-dihydropterins to 5, 6, 7, 8-tetrahydropterins (Figure I-1). These activities allow for the salvage of oxidized pterins and folates in Leishmania (Nare et al., 1997a). Overproduction of the enzyme leads to decreased susceptibility to several folate antagonists (Nare et al., 1997b). Because of this role, PTR1 is a possible drug target for the treatment of leishmaniasis. This chapter provides a brief discussion of the biology of Leishmania and the infectious disease leishmaniasis, followed by some background on PTR1 and a statement of those problems addressed by the experimental studies presented in this thesis.

Leishmania

The genus Leishmania, first described by Dr. William Leishman in 1900, are parasitic Protozoa of the Order Kinetoplastida, Family Trypanosomatidae (Peters et al., 1987). Leishmania exhibit a complex life cycle that is divided between phlebotomine sandflies (Diptera: Psychodidae: Phlebotominae) and mammalian hosts. Species in this genus are classified based on region, sandfly host, morphology, and more recently immunological reactivity, enzyme isoforms (Peters et al., 1987) and nuclear ribosomal RNAs (Fernandes et al., 1993). Several classification schemes have been suggested, but the evolutionary relationship between individual species of Leishmania is in flux at present. Currently, there are three clusters of subspecies in Leishmania. These clusters are the L. donovani, L. mexicana, and L. braziliensis clusters.
Figure I-1. The Pterin Ring System and the PTR1 Catalyzed Reaction. The numbering system of the pterin ring is shown in the small numbers adjacent to each atom. Shown in bold numbers is the Cahn-Ingold-Prelog system priority number of the atoms bound to C6 of the pterin ring. According to this system, the observer is looking down on the \textit{si} face of the sp\textsuperscript{2} carbon center C6. Shown below is the PTR1 catalyzed reduction of folate and biopterin to their tetrahydro forms.
**Life Cycle**

The two main stages in the life cycle of *Leishmania* are divided between phlebotomine sandflies and mammalian host, and promastigote and amastigote morphologies (Walters, 1993). Promastigotes are elongated motile cells with a single flagellum that reproduce by binary fission. This form is found in the digestive tract of sandflies. Amastigotes are smaller, rounded forms that lack flagella, and live inside of the macrophages of mammalian hosts. Once inside a human host, the mobile promastigotes attach to receptors on the surface of macrophages and are engulfed. Inside lysosomes, the promastigotes differentiate to amastigotes. These amastigotes reproduce by binary fission inside the macrophage. There is significant divergence in morphology and life cycle between different species of *Leishmania*, and within species based on the sandfly host (Walters et al., 1993).

**Leishmaniasis**

Leishmaniasis is the disease caused by *Leishmania*. It is estimated that > 100 million people suffer from some form of this disease, and the disease has an annual incidence of 12 million (Goodman and Gilman, 1990). Leishmaniasis is prevalent in South and Central America, the Mediterranean from Portugal to Turkey, the Middle East, Sub-Saharan Africa, China, and India. Children are the most susceptible population to leishmaniasis (WHO, 1984).

There are three main forms of leishmaniasis, cutaneous, mucosal, and visceral, that depend on the species of *Leishmania* causing the infection. Old World cutaneous leishmaniasis is caused by *L. major*, *L. tropica*, and *L. aethiopica*, and is characterized
by dry ulcers on the skin that may or may not respond to treatment or disappear on their own. Secondary infections of the lesions can occur. New World cutaneous Leishmaniasis is similar to the Old World disease but lesions are generally more severe. Species that cause New World leishmaniasis are *L. braziliensis*, *L. mexicana*, and *L. peruviana*. In India and East Africa patients cured of kala azar (visceral leishmaniasis) can manifest multiple nodular infiltrations of the skin, usually without ulceration, several years after their “cure” (Peters et al., 1987). Common names for different forms of cutaneous leishmaniasis are oriental sore (Old World), pian bois, chiclero’s ulcer, bay sore, and uta (WHO, 1984).

Mucocutaneous leishmaniasis, or espunida, is caused by *L. braziliensis* and is characterized by ulcerations similar to cutaneous leishmaniasis. Metastatic spread to the oronasal/ pharyngeal mucosa can occur during the presence of the primary lesion or up to 30 years later. The soft tissue of the nasopharyngeal cavity is progressively destroyed by ulceration. Pain may or may not be associated with the disease and secondary infections are common. Lesions do not heal spontaneously. The mutilation caused by mucocutaneous leishmaniasis can be severe both physically and psychologically. Death can occur as a result of bronchopneumonia or malnutrition (WHO, 1984).

Visceral leishmaniasis, or kala azar, is caused by *L. donovani*. Visceral leishmaniasis is characterized by fever, malaise, weight loss, and anorexia. This disease has a 90% mortality rate if left untreated (Peters et al., 1987).

By far the most common and effective treatment of the leishmaniasis is pentavalent antimony (Katzung, 1992). The two most commonly used forms are sodium
stibogluconate and meglumine antimoniate (Peters et al., 1987). Antimony, in either form, is given intravenously or intramuscularly for 20 days at a dose of 20 mg/kg/day (Katzung, 1992). This dosing regime can present logistical difficulties in rural settings, and these drugs can be expensive for the patients receiving treatment. Common side effects for these drugs are anorexia, vomiting, nausea, malaise, myalgia, headache, and lethargy. These side effects may prevent patients from completing a full course of therapy (Saenz, et al., 1991). The mechanism of action of Sb$^{5+}$ is unknown, but effects on overall energy production and cellular physiology have been observed. Second line drugs for use against recurrent or unresponsive infections are pentamidine, amphotericin B, and allopurinol (Peters, 1987). Because of the toxicity and limited efficacy of the few drugs now used, new therapies are urgently needed (WHO, 1984).

**Biochemistry of Leishmania**

*Leishmania* can take up glycerol, glucose, and a number of monosaccharides from the environment (von Brand et al., 1967). Enzymes from glycolosis (Poorman and Janovy, 1969; Janovy, 1972; Berens and Marr, 1977a, b; Berens et al., 1980), the pentose phosphate shunt (Berens et al., 1980; Ghosh et al., 1971) and the Krebs' cycle (Martin et al., 1976) have been identified in *Leishmania*. The significance of these pathways on energy production depends on the strain and the nutrition available in the surroundings (Peters, 1987). Amino acids also have an important role as energy and carbon sources in *Leishmania* (Zeledon, 1960a, b, c, d; Zeledon, 1967). Levels of polyamines fluctuate during the life cycle and have functions unique to trypanosomatids (Bacchi, 1981).
These organisms also contain many unique enzymes that recycle, salvage, or interconvert the precursors of DNA metabolism, and a dependence on pre-formed purines has been demonstrated (Nelson et al., 1979a, b). Enzymes in this category include phosphoribose transferases and purine and pyrimidine nucleosidases.

Pterin metabolism in *Leishmania* shows several differences from pterin metabolism in either mammals or bacteria. *Leishmania* are incapable of de novo synthesis of either folate or pterins (Kidder et al., 1958; Hunter et al., 1979) and must obtain both from their environment (Huennekens et al., 1978). At least two folate and pterin transport systems are known in *Leishmania* (Ellenberger, et al., 1987; Borst et al., 1995). Pterins can be converted into DHF via dihydrobiopterin (DHB, Borst et al., 1995). Furthermore, *Leishmania* can incorporate [³H]folate, but cannot incorporate [³H]PABA, into reduced cellular folate pools (Kovacs et al., 1989). *Leishmania* cannot synthesize purines and the only well established role for folate is in thymidylate synthesis. Since the nutritional requirement for methionine in the related trypanosomatid *Crithidia* depends upon low levels of dietary folate (Kidder, 1958) and *Leishmania* are known to contain high levels of intracellular 5-methyltetrahydrofolate (Scott et al., 1987), it seems likely that *Leishmania* can biosynthesize methionine by a folate dependent pathway, as occurs in other organisms. As in all other protists and most plants, dihydrofolate reductase (DHFR) and thymidylate synthase (TS) are fused into one enzyme (Coderre et al., 1983; Beverley, et al., 1986; Grumont, et al., 1986).

*Leishmania* cannot synthesize the pterin moiety from GTP and are dependent on the salvage of pterins from the host organism. The enzymatic cofactor biopterin, a pterin
that has been shown to be important for the growth of \textit{Leishmania}, has an essential but uncharacterized metabolic role in \textit{Leishmania} (Nare et al., 1997). In mammals biopterin is used in the hydroxylation of aromatic amino acids, cleavage of ether-linked lipids, and the synthesis of nitric oxide (Tietz et al., 1964; Blakley, et al., 1985; Kwon et al., 1989; Tayeh, \textit{et al.}, 1989). Trypanosomatids lack phenylalanine hydroxylase activity (Milstein \textit{et al.}, 1986) and use NADPH instead of tetrahydrobiopterin (THB) in the cleavage of ether-linked lipids (Ma \textit{et al.}, 1996). Dihydropteridine reductase (DHPR) activity has been isolated from \textit{Leishmania} (Hirayama \textit{et al.}, 1980), and a relationship between levels of reduced cellular biopterin and the sensitivity of \textit{Leishmania} to oxidants exists (S. Beverley, personal communication). This suggests some role(s) for biopterin that is independent of folate synthesis.

\textbf{Pteridine Reductase 1 (PTR1)}

Because of the problems associated with Sb\textsuperscript{5+} (toxicity, expense, inconvenience), the search for new treatments of leishmaniasis is ongoing. Anti-folates have been useful in the treatment of cancer (MTX), bacterial infections (trimethoprim and the sulfa-drugs), and malaria (trimethoprim, chloroguanine, and pyrimethamine), but no anti-folate has proven to be clinically effective against leishmaniasis. This inability to target folate metabolism could be the result of differences in folate metabolism in \textit{Leishmania} that confound conventional approaches. In order to model anti-folate resistance in trypanosomatids and gain possible insight into folate metabolism in \textit{Leishmania}, the mechanisms of resistance to the drug methotrexate (MTX) were examined in several laboratories (Kaur \textit{et al.}, 1988; Coderre, \textit{et al.}, 1983; Callahan \textit{et al.}, 1992; Borst \textit{et al.},
Three modes of resistance have been described. One mechanism is decreased uptake of MTX by the MTX/folate transporter (Kaur et al., 1988). A second mechanism involved amplification of the R region of chromosomal DNA. This region contains the *dhfr-ts* gene. Thus, amplification of the target of MTX overcomes the inhibitory effect of the drug (Coderre et al., 1983). A third mechanism for MTX resistance involved amplification of a different region of chromosomal DNA, the H region (Callahan et al., 1992; Papadopoulou et al., 1992). Previous work showed that amplification of H region DNA (which contains several open reading frames) conveyed heavy metal resistance, but the gene responsible for this resistance did not also convey MTX resistance (Callahan et al., 1991). Thus, a novel H region gene was responsible for MTX resistance. The gene (hmtx) responsible for resistance was identified through a transfection approach (Callahan et al., 1992). The mechanism of this resistance was not clear, however, as the gene product (HMTX) could act by chemically modifying, and thus deactivating, MTX, or could represent a new target for MTX.

No chemical modifications of MTX could be identified in the resistant cells. Furthermore, the predicted amino acid sequence of HMTX showed homology to numerous members of the polyol dehydrogenase/carbonyl reductase family, a family that contains aldo/keto reductases and short chain dehydrogenases. Based on these homologies HMTX was classified as an Aldoketo Reductase (AKR, Callahan et al., 1992) and also as a short chain dehydrogenase (SDR, Papadopoulou et al., 1992). These protein families have different folds and distinct sequences (Wilson et al., 1992; Bruce et al., 1994; Jornvall et al., 1995). Table I-1 compares features of both of these protein
families. The overall sequence homology in both protein families was only 20-30%, and no activity of HMTX had been identified.

Expression of HMTX in *E. coli*, and subsequent purification of the enzyme, have allowed for some preliminary characterization (Bello et al., 1994). It was shown that HMTX (now Pteridine reductase 1 (PTR1)), was an NADPH dependent reductase that reduced biopterin and 7, 8-dihydrobiopterin at pH 4.6, and folate and 7, 8-dihydrofolate at pH 6.0. *Leishmania* lines with gene knockouts in either PTR1 or DHFR-TS showed that the ability to grow in diverse pterins correlated with the activity of PTR1, not DHFR (Nare, et al., 1997). Thus, PTR1 is the sole mediator of oxidized pterin salvage in *Leishmania*. PTR1 was 2000 times less sensitive to MTX than DHFR-TS, but its relative inactivity towards dihydrofolate (relative to DHFR) require overexpression of PTR1 to overcome MTX inhibition. Thus, PTR1 can compromise the inhibition of DHFR by MTX. Therefore, if an effective anti-folate is to be used in the treatment of leishmaniasis, it must inhibit both DHFR and PTR1, or be used in conjunction with a potent PTR1 inhibitor. A greater understanding of the mechanism of PTR1 would aid in the design of anti-folates that would be useful in the treatment of leishmaniasis.
Statement of Thesis Problem

This thesis research project is aimed at understanding the kinetic and chemical mechanism of PTR1. Towards this end:

1) the kinetic and stereochemical course of catalysis were determined (Chapter II);

2) the involvement in catalysis and ligand binding of several possible active site residues was evaluated (Chapter III); and

3) conditions for the crystallization of PTR1 and PTR1 binary and ternary complexes were developed, and preliminary characterization of crystals was carried out (Chapter IV).

As stated above, PTR1 has been suggested to be either an AKR or a SDR (Callahan et al., 1992; Papadopoulou et al., 1992). Results from Chapter II allowed the classification of PTR1 as a short chain dehydrogenase and provided the equations and assays necessary for subsequent studies of PTR1 variants (described in Chapter III) and the evaluation of PTR1 inhibitors (carried out by other members of the laboratory).

There are several conserved residues among members of the SDR family (Jornvall et al., 1995). Of these, a Y-K-S catalytic triad has been suggested. The tyrosine and lysine residues are part of a YXXXXK pentapeptide, while the S is from a different secondary structural element. PTR1 contains two YXXXXK pentapeptide motifs. Sequence alignments of PTR1 with DHPR and 3-α, 20-β hydroxysteroid dehydrogenase (HSD), two SDRs with known crystal structures, indicated that either YXXXXK motif from PTR1 might be homologous to those of DHPR and HSD. Furthermore, an aspartic
acid aligned to the conserved serine residue of DHPR and HSD. Results from Chapter III confirmed that only the more C terminal YXXXK pentapeptide and the aspartic acid residue were involved in catalysis and ligand binding. Based on the results presented in Chapter III, the crystal structures of the DHPR, HSD, and 7-α hydroxysteroid dehydrogenase (another SDR), and the electronic structure of pterins, it was possible to suggest a chemical mechanism for catalysis.

The crystal structures of several SDRs have been identified, but only one structure of a ternary complex is available. Thus, the structure of PTR1 in several liganded states would provide insight into the mechanism of SDRs. Furthermore, crystal structures of PTR1 would aid in the development of novel compounds for the treatment of Leishmaniasis. The results obtained from Chapter IV were a step towards obtaining the crystal structures of apo-PTR1 and PTR1 in several liganded states.
Table I-1. A Comparison of Members of the Aldoketo Reductase (AKR) Family with Members of the Short Chain Dehydrogenase (SDR) Family.

<table>
<thead>
<tr>
<th></th>
<th>AKR</th>
<th>SDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold</td>
<td>$(\alpha$-$\beta)_8$ barrel</td>
<td>$\alpha/\beta$ doubly wound with N-terminal Rossman fold</td>
</tr>
<tr>
<td>Stereochemistry</td>
<td>pro-$R^a$</td>
<td>pro-$S^a$</td>
</tr>
<tr>
<td>Catalytic Residues</td>
<td>Y/T or H/Q-K-D</td>
<td>Y-K-S catalytic triad</td>
</tr>
</tbody>
</table>

$^a$ Pro-$R$ and pro-$S$ refer to the pro-chiral hydride of C4 of the nicotinamide ring of NAD(P)H that is transferred by the enzyme.
Chapter II

Stereochemistry and Kinetic Mechanism

Introduction

Pteridine reductase 1 (PTR1) is a novel enzyme of pterin metabolism in the trypanosomatid parasite *Leishmania*. Isolation of the gene for PTR1 was aided by its amplification in some methotrexate-resistant strains of the protozoan (Beverley et al., 1984; Callahan & Beverley, 1992; Papadopoulou et al., 1992). Recent studies indicate that a major physiological role for PTR1 in *Leishmania* is the salvage of oxidized pterins and folates, for which this organism is auxotrophic (Bello et al., 1994; Nare et al., 1997a). PTR1 is likely responsible for the failure of antifolate therapeutic strategies targeted against dihydrofolate reductase (DHFR), since it catalyzes the same reaction as DHFR and is much less susceptible to inhibition by several clinically useful DHFR inhibitors (Bello et al., 1994; Hardy, et al., 1997). A detailed understanding of the mechanism of catalysis by PTR1 is therefore a desirable goal. This understanding will assist in the development of effective inhibitors of this enzyme, which may render *Leishmania* DHFR vulnerable as a drug target in this human parasite.

Analysis of the amino acid sequence of PTR1 led to suggestions (Callahan and Beverley, 1992; Papadopoulou et al., 1992) that the protein belongs to a large group of oxidoreductases, which includes the aldo-keto reductases (AKRs - Bruce et al., 1994) and the short chain dehydrogenases / reductases (SDRs - Jornvall et al., 1995). Because of the large evolutionary distance encompassed by both the AKR and SDR families, unambiguous assignment to one family or the other was not possible based upon amino
acid sequence similarities alone. For this assignment, stereochemical and kinetic information about the reaction catalyzed by PTRI was also required. Although both families of oxidoreductases utilize nicotinamide nucleotides as substrates and generally require no metal cofactors, AKRs and SDRs have distinct protein folds, differing stereochemical courses, and probably different catalytic mechanisms. The SDRs are B-side dehydrogenases and the AKRs are A-side dehydrogenases. The data presented here demonstrate that PTRI has the stereochemical course and kinetic characteristics typical of members of the SDR family. More than 50 members of the SDR family have been identified to date (reviewed by Jornvall et al., 1995). The proteins are frequently homodimers or homotetramers, possess a unique N-terminal \( \beta-\alpha-\beta \) unit which is involved in the binding of NADH or NADPH, and typically have a YXXXXK motif which has been implicated in catalysis. PTRI is a homotetramer (Nare et al., 1997), with two YXXK sequences and an N-terminal sequence which is highly homologous to SDR sequences corresponding to the \( \beta-\alpha-\beta \) unit.

One of the most well studied members of the SDR family is the mammalian enzyme, dihydropteridine reductase (DHPR). The three dimensional structures of DHPR (Su et al., 1993) and several other SDRs (3\( \alpha \), 20\( \beta \)-hydroxysteroid dehydrogenase - Ghosh et al., 1994; carbonyl reductase-Tanaka et al., 1996a; 7\( \alpha \)-hydroxysteroid dehydrogenase - Tanaka et al., 1996b) have been determined by X-ray crystallographic methods. The structures reveal a conserved core, with significant structural diversity arising from the highly variant C-terminal sequences of these proteins. A key feature, based upon both
kinetic and crystallographic evidence, is that the binding of the nicotinamide cofactor must occur in order to create a competent enzyme (Kiefer et al., 1996).

A strain of *E. coli* engineered to overproduce recombinant *Leishmania* major PTR1 has provided an abundant supply of the enzyme (Bello et al., 1994). Homogeneous PTR1 purified from this bacterial strain behaves identically with the enzyme isolated from the native protozoan (Nare et al., 1997a), and was employed in the studies described here.

**Materials and Methods**

**Materials**

$4S[^3]H\text{-NADPH}$ and $4R[^3]H\text{-NADPH}$ were generated by published methods using, respectively, glucose-6-phosphate dehydrogenase (Little, 1972) and malic enzyme (Reddy et al., 1995). $1[^3]H\text{-glucose-6-phosphate}$ was generated *in situ* from $1[^3]H\text{-glucose}$ (from Moravek Biochemicals) using hexokinase. $4S[^2]H\text{-NADPH}$ was generated by the same methods as $4S[^3]H\text{-NADPH}$, except that $1[^2]H\text{-glucose-6-phosphate}$ was generated *in situ* from $1[^2]H\text{-glucose}$. $2[^3]H\text{Malic acid}$ was generated by reduction of oxaloacetate with $[^3]H\text{NaBH}_4$ according to a published procedure (Lowenstein, 1961).

$4[^1]H_2\text{-[adenosine-}^{14}\text{C]}\text{-NADPH}$ was generated as follows. NAD$^+$ kinase (Sigma) was used to catalyze the phosphorylation of $[^{14}\text{C}]\text{NAD}^+$ (DuPont NEN) by ATP, according to a published procedure (Wang *et al.*, 1954). The $[^{14}\text{C}]\text{NADP}^+$ was reduced using glucose-6-phosphate and glucose-6-phosphate dehydrogenase, and purified by HPLC (Jeong & Gready, 1994). The purity of NADP$^+$, NADPH, folate, dihydrofolate (DHF), and methotrexate were routinely confirmed to be
>95% by HPLC. Folate was recrystalized from ethanol-water prior to use. DHF was either synthesized from folate by dithionite reduction (Futterman, 1957) or purchased from Schircks Laboratories (Jona, Switzerland). 5-Deaza-5,6,7,8-tetrahydrofolate (5dTHF) was a gift from Dr. C. J. Shih of Eli Lilly Research Laboratories. Methotrexate was purchased from Schircks or Sigma. [3',5',7,9-³H]Folic acid was from Moravek. The isolation of homogeneous recombinant Leishmania major PTR1 (Nare et al., 1997), E. coli thymidylate synthase (Hardy and Nalivaika, 1992) and E. coli DHFR (Baccanari et al., 1975) were done using methods previously described.

**HPLC methods**

Large scale separation of NADP⁺ and NADPH was conducted on a semi-preparative Exasil C18 column (250 x 10mm) essentially as described by Gready and coworkers (Jeong & Gready, 1994). For smaller quantities or for analytical purposes, NADP⁺ and NADPH were isolated by reverse phase HPLC on a 250 x 4.6mm C18 column eluted at one mL min⁻¹ with a mobile phase consisting of 5 mM TBAS, 5 mM potassium phosphate, pH 7.5, 28.5% methanol (HPLC buffer A). This HPLC system was also used for analysis of the folates following isotope trapping experiments. Non-radioactive NADP⁺/NADPH and folates were detected by monitoring UV absorbance at 260 nm and 295 nm, respectively, using a Waters model 481 LC spectrophotometer linked to an autointegrator (Waters 740 data module). The retention times during analytical runs were 12 and 60 min for NADP⁺ and NADPH, respectively. The radiolabelled nucleotides produced by the coupled action of PTR1 and thymidylate synthase were separated by reverse phase C18 HPLC using a mobile phase of 5 mM
tetrabutyl ammonium sulfate (TBAS), 5 mM potassium phosphate, pH 7.5, 8% methanol (HPLC buffer B). Proteins were removed from most samples prior to HPLC by passage through a Centricon-10™ unit.

The substrates and products from the rapid chemical quench experiments were quantified by HPLC using a radioactivity flow detector at Yale University with the assistance of Dr. Po Liang and Dr. Karen Anderson. The HPLC separation was performed using a BDS-Hypersil-C18 reverse phase column (250 x 4.6 mm, Keystone Scientific, Bellefonte, PA) with a flow rate of 1 mL/min. The HPLC effluent from the column was mixed with liquid scintillation cocktail (Mono-flow V, National Diagnostics) at flow rate of 4 mL/min. Radioactivity was monitored continuously using a Flo-One radioactivity flow detector (Packard Instruments, Downers Grove, IL). The analysis system was automated by the use of a Waters 712B WISP (Milford, MA) autosampler.

Stereochemical course

The stereochemistry at carbon 6 of the THF produced by PTR1-catalyzed reduction of 7, 8-DHF was determined by coupling the reaction to thymidylate synthase, an enzyme known to require the 6R isomer of 5,10-methylene-THF (Blakey, 1969; Fontecilla-Camps et al., 1979; Kaufman et al., 1963). The reaction was conducted under argon in a solution containing: 20 mM 4-(hydroxyethyl)piperazineethanesulfonic acid (HEPES), pH 7, 0.4 mM 2-[14C]dUMP (45 Ci/mol), 0.5 mM 4S-[3H]NADPH (680 Ci/mol), 0.2 mM folate, and 1 mM formaldehyde. (This concentration of formaldehyde was shown in a separate experiment to have no effect on the activity of either PTR1 or thymidylate synthase.) The reaction was initiated by addition of 2.8 μM PTR1 and 3.3
μM thymidylate synthase. Samples of 0.1 mL were removed from the reaction at 15 min intervals and frozen on dry ice. After samples were thawed and treated to remove proteins, the radiolabelled dUMP and dTMP were separated by HPLC and quantitated by liquid scintillation counting.

**Spectrophotometric methods and rate assays**

The concentrations of PTR1 determined using the molar absorbance at 280 nm calculated from Tyr+Trp content (26.8 mM⁻¹cm⁻¹) were identical to those measured by the Coommassie dye-binding method (Bradford, 1976). The spectrophotometric rate assay for NADPH-dependent reduction of pteridines catalyzed by PTR1 was performed at pH 7.0 and 30 °C. Although the pH optimum for the reduction of folate by PTR1 is 6.0, measurements were made at pH 7.0 to decrease the rate of non-enzymatic oxidation of NADPH. The assays were initiated by the addition of enzyme, and the rate was measured for 15 seconds, during which time less than 10% of substrates were consumed. Data was fit to equations 1-7 using the program Kaleidagraph™.

Initial velocities (v_i) were measured at several fixed concentrations of folate while NADPH was varied. Because the order of substrate binding was not known, initial velocities were also measured at several fixed concentrations of NADPH while folate was varied. 0.33 or 0.66 μM PTR1 was used in these measurements. Folate was varied from 0.5 to 16 μM and NADPH was fixed at 5, 10, 20, or 40 μM. NADPH was varied from 0.5 to 80 μM and folate was fixed at 0.75, 1, 1.5, 3, or 6 μM. Apparent values of the kinetic parameters (V_{app} and K_{M_{app}} for each varied substrate, S) were calculated by non-
linear least squares regression of the data (at the fixed concentrations of the second substrate) onto equation 1.

\[ V_i = \frac{V^{\text{app}}[S]}{K_M^{\text{app}} + [S]} \]  
\[ \text{Eq. 1} \]

In order to determine if the PTR1 catalyzed reaction proceeds through a substituted or ternary complex mechanism, the appropriate double reciprocal plots were examined (Cleland, 1963). All the initial velocity measurements were pooled and \( V_{\text{max}} \), the \( K_M \) for NADPH (\( K_{\text{MN}} \)), the \( K_M \) for folate (\( K_{\text{MF}} \)), and the \( K_d \) for NADPH (\( K_{\text{IN}} \)) were determined from plots of [folate] \( V^{\text{app}} \) and [folate] \( K_M^{\text{app}/V^{\text{app}}} \) fit to equations 2 and 3 (Cornish-Bowden, 1995).

\[ \frac{[\text{folate}]}{V^{\text{app}}} = \frac{K_{\text{MF}}}{V_{\text{max}}} + \frac{[\text{folate}]}{V_{\text{max}}} \]  
\[ \text{Eq. 2} \]

\[ \frac{[\text{folate}]K^{\text{app}}}{V^{\text{app}}} = \frac{K_{\text{IN}}K_{\text{MF}}}{V_{\text{max}}} + \frac{K_{\text{MN}}[\text{folate}]}{V_{\text{max}}} \]  
\[ \text{Eq. 3} \]

To determine the order of substrate binding the inhibition patterns of NADP\(^+\) and the stable product analogue 5dTHF were determined with respect to folate and NADPH.

For measurements with varied NADPH, folate was fixed at 30 \( \mu \text{M} \), NADP\(^+\) was fixed at 2.5, 5, or 10 \( \mu \text{M} \), and 5dTHF was fixed at 20, 40, or 80 \( \mu \text{M} \). For measurements with varied folate, NADPH was fixed at 90 \( \mu \text{M} \), NADP\(^+\) was fixed at 1, 2.5, or 5 \( \mu \text{M} \), and 5dTHF was fixed at 10, 20, or 40 \( \mu \text{M} \). \( V^{\text{app}} \) and \( K_M^{\text{app}} \) for folate and NADPH were
measured at several fixed concentrations of NADP$^+$ and 5dTHF. Data that showed competitive inhibition in double reciprocal plots were fit to equation 4.

$$v_i = \frac{V_0^{\text{app}} [S]}{K_0^{\text{app}}(1+[I]/K_i) + [S]}$$  \hspace{1cm} \text{Eq. 4}

Data showing noncompetitive inhibition in double reciprocal plots were fit to equation 5.

$$v_i = \frac{V_0^{\text{app}} [S]}{1 + [I]/K_i}$$  \hspace{1cm} \text{Eq. 5}

Data that showed uncompetitive inhibition were fit to equation 6.

$$v_i = \frac{V_0^{\text{app}} [S]}{1 + [I]/K_i}$$  \hspace{1cm} \text{Eq. 6}

$$\frac{K_0^{\text{app}}}{1 + [I]/K_i} + [S]$$

[S] was the concentration of the varied substrate, [I] was the concentration of inhibitor, $V_0^{\text{app}}$ was the apparent $V_{\text{max}}$ in the absence of inhibitor, $K_0^{\text{app}}$ was the apparent $K_M$ in the absence of inhibitor, and $K_i$ was the inhibition constant.

**Fluorescence measurements of ligand binding**

The binding of NADPH to PTR1 was monitored by fluorescence energy transfer from the protein to NADPH, using excitation at $\lambda_{\text{ex}}$ 295 nm and emission at $\lambda_{\text{em}}$ 450 nm. The binding of NADP$^+$ or DHF to PTR1 was measured by monitoring the quenching of the fluorescence emission of the protein, using $\lambda_{\text{ex}}$ 290 nm and $\lambda_{\text{em}}$ 340 nm. All fluorescence measurements were done at 30 °C, at pH 6, using a Farrand MK2
fluorescence spectrometer, with 5 μM PTR1 unless otherwise noted. The decreased intensity of PTR1's fluorescence emission at 340 nm which occurs upon addition of DHF was corrected for a small amount of absorbance at this wavelength by DHF. This correction was done by measuring the decreased intensity of fluorescence emission of a 7.5 μM solution of tryptophan in the same buffer. In the concentration range of DHF used (0 to 48 μM) the decrease due to absorbance was linear, with 0.18% of the light emitted being absorbed per μM DHF.

The quantitative analysis of the NADPH fluorescence by 0.5 μM PTR1 was similar to that described by Janin et al. (1969) for binding of NADPH to homoserine dehydrogenase. Briefly, the data above 5 μM NADPH increased linearly with NADPH concentration, and the values below this approached this line asymptotically. Differences between the extended line and the observed fluorescence arising from the NADPH-PTR1 complex from 0.05 μM to 0.95 μM NADPH were used to calculate the fraction of PTR1 bound to NADPH (Y). A plot of (1-Y) versus ([NADPH]_{total}/Y) yielded a line of slope K_d⁻¹.

The data from the quenching of PTR1 fluorescence by DHF were fit to equation 7:

\[ \Delta F = F_0 - F = F_0 \left( \frac{[\text{DHF}]}{K_d + [\text{DHF}]} \right) \]  

Eq. 7
where \( F \) and \( F_0 \) are the observed fluorescence intensity at all ligand concentrations and the observed intensity in the absence of ligand, respectively, \( K_d \) is the apparent dissociation constant, and \([\text{DHF}]\) is the concentration of dihydrofolate.

**Isotope Partitioning**

In order to determine if the binary PTR1-NADPH complex was productive, the complex was pre-formed by preparing a solution containing 20 or 40 \( \mu \text{M} \) PTR1 and 20 \( \mu \text{M} \) \(^{14}\text{C-adenosine}\)-NADPH (1.5 Ci/mol). This solution was then rapidly mixed with an equal volume of 80 \( \mu \text{M} \) DHF and 2 mM NADPH (the chase). The mixture was incubated at ambient temperature (ca. 22 °C) for 12 sec (sufficient for a single turnover), and quenched with 40 \( \mu \text{M} \) methotrexate. NADP\(^+\) was separated from NADPH by HPLC, and the HPLC fractions were analyzed by liquid scintillation counting. Controls for these experiments included reactions in the absence of PTR1, in the absence of the non-radiolabelled NADPH chase, and with the \(^{14}\text{C}\)-NADPH added to the chase instead of being pre-incubated with enzyme.

In order to determine if the binary complex between PTR1 and DHF was productive, 20 \( \mu \text{M} \) PTR1 was incubated with 20 \( \mu \text{M} \) \(^{3}\text{H}\)DHF (3 Ci/mol) to form the complex. After this solution was mixed with equal volume of 100 \( \mu \text{M} \) NADPH and 2 mM DHF and incubated for 12 sec, an analysis similar to that described above was done, with the appropriate HPLC system to separate DHF and THF. In order to determine whether the binary complex between PTR1 and folate was productive, a solution containing 20 \( \mu \text{M} \) PTR1 and 20 \( \mu \text{M} \) \(^{3}\text{H}\)folate (500 Ci/mol) was rapidly mixed with an
equal volume of 100 μM NADPH and 2 mM folate, and analyzed as described above. Controls were included to demonstrate that radiolabelled substrates were capable of conversion to products in the absence of a chase by unlabelled substrates, and to verify the stability of the radiolabelled products during the analysis.

**Kinetic Isotope Effects**

The deuterium isotope effect on $V_{\text{max}}$, $D_V$, and on $V_{\text{max}}/K_M$, $D(V/K)$ were measured using the spectrophotometric assay. $D_V$ and $D(V/K)$ are defined in the nomenclature of Northrop (1977). To minimize errors arising from slight variations in enzyme activity or assay conditions, data were collected at varying 4-[$^1$H]-NADPH and 4S-[$^2$H]-NADPH concentrations on the same day, using 30 μM folate. The data from the 4-deuterated cofactor was fit to equation 8:

$$v_i = \frac{[\text{NADPH}]}{V_{\text{max,D}}} + \frac{K_{\text{MN,D}}}{V_{\text{max,D}}}$$

Eq. 8

where $V_{\text{max,D}}$ and $K_{\text{MN,D}}$ were the values for $V_{\text{max}}$ and $K_M$ measured with 4S-[$^2$H]-NADPH. The kinetic parameters for 4-[$^1$H]-NADPH were obtained similarly.

The instability of both NADPH and THF resulted in difficulties during the measurement of $T(V/K)$. At pH 6.0, where PTR1 has the highest specific activity for DHF, NADPH and THF are readily oxidized. THF can be stabilized by 12% ascorbate, pH 10, but PTR1 is not active at this pH and is inhibited by ascorbate (data not shown). Nonenzymatic oxidation of NADPH was controlled by adjusting the pH of the reaction to 7.5. These initial attempts at measuring $T(V/K)$ were unsuccessful because of the instability of THF. Measurements of the fraction of NADPH consumed (by $A_{340}$, an
HPLC standard curve, and alcohol dehydrogenase assay) indicated that more NADPH was consumed than could be accounted for by the amount of DHF or folate added. This was likely the result of the non-enzymatic oxidation of THF to DHF, resulting in additional rounds of PTR1 catalyzed NADPH oxidation and loss of stoichiometry. Thus, a major problem was encountered in obtaining accurate, reproducible and valid estimates of f (fractional conversion of substrates to products). Ultimately, the problems arising from the instability of THF were circumvented by coupling the reduction of DHF by PTR1 with the methylation of dUMP by thymidylate synthase (TS). This method ensured that the concentration of DHF remained constant because any THF formed was immediately oxidized by TS to DHF. Measurement of the production of the stable product thymidylate (dTMP) allowed valid estimates of f to be made accurately and reproducibly. Changes were measured in the specific activity of residual 4S[^3]H]-NADPH and in the ratio of 2[^14]C-dTMP to the sum of 2[^14]C-dTMP and 2[^14]C-dUMP, as a way to monitor the consumption of NADPH. Measurements were made at 0.5 µM and 60 µM DHF. All reactions were done at 30 °C in 25 mM Tris pH 7.5, under N₂ and contained 300 µM 4S[^3]H]-NADPH (ca 15 µCi/µmol), 300 µM dUMP (3.3 µCi/µmol), 2 mM formaldehyde, 3 nM PTR1, and 3 nM TS(C50S) in a volume of 2 mL. Under these conditions, the rate of TS(C50S) catalysis was 10,000 times faster than PTR1 catalysis. The reaction was initiated by the addition of 0.5 µM folate or 60 µM DHF. The reaction was incubated for 1.5 hours for the measurements at 0.5 µM DHF, or 10-15 minutes for the measurements at 60 µM DHF. In order to quench the reaction and remove the enzymes, 0.66 mL of CCl₄ was added to the 2 mL reaction and the solution was mixed
rapidly. Proteins were denatured and partitioned into the CCl₄ phase. Phases were separated in a clinical centrifuge and the aqueous layer was removed with a Pasteur pipette.

In order to measure the initial specific activity of 4S[³H]-NADPH 100 µL of reaction solution was removed and diluted in 1 mL of HPLC buffer A with 30% MeOH. The absorbance at 340 nm of this dilution was measured seven times, and seven 100 µL aliquots were added to 5 mL of Ready Safe™ scintillation fluid. Samples were counted to 2σ values < 0.2%.

The final specific activity of 4S[³H]-NADPH was measured by purifying the remaining NADPH from the reaction mixture by HPLC, using the semi-preparative column described above with a mobile phase of HPLC buffer A with 30% MeOH. Fractions containing an A₂₆₀/A₃₄₀ ratio < 2.4 were pooled. The absorbance at 340 nm of the pool was measured seven times, and seven 100 µL aliquots were counted for ³H as described above.

The fraction of NADPH consumed was measured by quantitating the conversion of 2-[¹⁴C]-dUMP to 2-[¹⁴C]-dTMP. dUMP and dTMP were purified from the quenched reaction mixture by HPLC as described above. Fractions containing either dUMP or dTMP were mixed with 5 mL of Ready Safe™ scintillation fluid and counted for ¹⁴C to 2σ values < 0.2%. The fraction of conversion was calculated using the formula,

\[ f = \frac{\text{dpm dTMP}}{\text{dpm dUMP} + \text{dpm dTMP}} \times \frac{[\text{NADPH}]_0}{[\text{dUMP}]_0} \]  

Eq. 9
where dpmtMP represents the total $^{14}$C dpm in dTMP, dpmUMP represents the total $^{14}$C dpm in dUMP, $[\text{NADPH}]_0$ represents the initial concentration of NADPH and $[\text{dUMP}]_0$ represents the initial concentration of dUMP. The reactions at 60 μM DHF were allowed to proceed to 0.6-0.8 fractional conversions to allow for larger values of $R_s/R_o$. This, in turn, allowed for more precise estimates of the near unity $^T(V/K)$ values.

The tritium effect on $V/K$ was calculated using equation 10 (Cook & Cleland, 1981),

$$^T(V/K) = \frac{\log(1-f)}{\log[(1-f)(R_s/R_o)]}$$

Eq. 10

where $f$ is the the fraction of NADPH converted to NADP⁺ (as defined by equation 9), $R_o$ is the initial specific activity of NADPH and $R_s$ is the specific activity of NADPH at fractional conversion $f$.

**Rapid Chemical Quench Experiments**

All measurements were made using a Kintek RFQ-3 rapid chemical quench apparatus (Kintek Instruments, State College, PA). Each reaction was initiated by mixing 15 μL PTR1 (73 μM or 160 μM) and 1 μM NADPH with an equal volume of radiolabeled substrate (either 20 μM $[^3]$Hfolate or $[^3]$HDHF). The solutions were rapidly mixed and then aged (0.2 - 40 seconds for folate, or 0.005 - 60 seconds for DHF). The concentrations of enzyme and substrates cited below in the discussion of the presteady state kinetic results and in legend of figure II-10 are those after mixing and during the enzymatic reaction. The enzyme reactions were terminated by quenching with 67 μL of 0.78 N KOH to give a final concentration of 0.54 N KOH. The basic quench solution
contained 10% sodium ascorbate, pH 12.7, to prevent THF degradation after reactions were terminated. The quenched reaction solution was directly collected into an argon-purged vial for a Waters WISP autosampler, vortexed, and analyzed by HPLC in combination with radioactivity flow detection. The substrates and products were quantified by HPLC as described above. In order to ensure that the base was quenching the enzymatic reaction, a control was included with each experiment to insure that catalysis was being terminated. This involved adding the substrate to a premixed solution of base and enzyme. Samples were collected in vials containing an argon atmosphere to protect against oxidation during subsequent analysis. Control experiments were also done to establish the stability of the radiolabeled substrates and products under the quench conditions employed.

Results & Discussion

Stereochemical Course

The conversion of DHF and of folate to THF by PTR1 were accompanied, respectively, by the stoichiometric oxidation of one and two equivalents of NADPH to NADP⁺. The reduction of folate or DHF by 4S[^3]H]-NADPH catalyzed by PTR1 yielded non-radioactive NADP⁺. In contrast, tritium was retained by NADP⁺ when 4R-[^3]H]NADPH was used. The opposite results were observed when 4R and 4S[^3]H]-NADPH were used for the reduction of DHF catalyzed by *E. coli* DHFR. The latter result was consistent with the previously described chirality preference for DHFR (Blakley *et al.*, 1963; Pastore & Friedkin, 1962). Thus, PTR1 transfers the 4S prochiral hydrogen from NADPH.
It was important to determine whether carbon or nitrogen is the hydrogen acceptor during PTR1-catalyzed transfer of a hydride equivalent from NADPH to pteridine substrates. Early experiments using HPLC analyses to monitor the fate of tritium from 4S[^3]H-NADPH during PTR1-catalyzed reduction of folate showed that most of the tritium appeared in THF, but small variable amounts also appeared in solvent water (data not shown). Control experiments indicated that this was likely due to non-enzymatic oxidation of[^3]H-NADPH,[^3]H-THF, or both, during the reaction and/or HPLC analysis, but two precedents from related systems indicated a need for a more conclusive determination of the fate of the tritium. The NADH-dependent reduction of quinoid 7,8-dihydrobiopterin catalyzed by the mammalian enzyme DHPR (Kaufman, 1963) has been suggested to resemble the reduction of a flavin (Whitley et al., 1993), in that the hydrogen transferred from NADH is freely exchangeable with solvent protons (Ararego et al., 1979). Electrolytic or zinc metal reduction of fully oxidized pterins proceeds via a 2 electron, 2 proton process (Kwee & Lund, 1973). The electrolytic process yields directly a 5,8-dihydropterin intermediate, which generally rearranges rapidly to a more stable 7,8-dihydropterin. It was also important to determine the stereochemistry at carbon 6 of the THF produced by PTR1-catalyzed reduction. A coupled enzyme system was devised to settle both the site and stereochemistry of the PTR1-catalyzed hydrogen transfer to the pteridine.

Both the transfer of hydrogen from NADPH to carbon and the resulting stereochemistry were demonstrated by coupling PTR1 to thymidylate synthase, an enzyme known to utilize exclusively the 6R isomer of 5,10-methylene-5,6,7,8-THF
(Blakley, 1969; Fontecilla-Camps et al., 1979; Kaufman et al., 1963). Thymidylate synthase catalyzes the transfer of tritium from C6 of 6[^3]H-methylene-THF to form the stable methyl group of thymidylate. The stereoisomer of THF which corresponds to the
Figure II-1. The Stereochemistry of PTR1 Catalyzed Hydride Transfer to DHF.
6R isomer of methylene-THF has S stereochemistry at carbon 6. Together, PTR1 and thymidylate synthase effectively catalyzed the time-dependent transfer of tritium from 4S-[3H]NADPH into thymidylate. The conversion of 2-[14C]-2'-deoxyuridylate to [3H, 14C]-thymidylate was 42%, 61%, and 100% complete at 30, 60, and 240 min under the conditions used; no thymidylate was produced when either PTR1 or thymidylate synthase was omitted. Thus, PTR1-catalyzed reduction of the 5,6 double bond of DHF produces 6S-THF, the isomer produced by DHFR (Fontecilla-Camps et al., 1979). This result is consistent with the observation that expression of PTR1 will permit the growth of an E. coli strain which is deficient in both DHFR and thymidylate synthase (thyA Δfol) on a medium lacking purines and methionine (Chapter III). The 6S diastereoisomer of methylene-THF (formed from the 6R isomer of THF) is microbiologically inactive, and incompetent as a substrate for several methylene-THF-dependent enzymes (Kaufman et al., 1963). Thus, the absolute stereochemistry of the hydride transfer to DHF catalyzed by PTR1 must be as shown in figure II-1. A similar result, indicating "natural" stereochemistry at C6 of 5,6,7,8-tetrahydrobiopterin produced by PTR1-catalyzed reduction of biopterin, was previously demonstrated by the efficient coupling of the reactions catalyzed by PTR1 and phenylalanine hydroxylase (Nare et al., 1997).

Initial Velocity and Product Inhibition Patterns

The steady state kinetic analysis employed folate as the pteridine substrate. The rapid quench studies described below showed that DHF is an intermediate in the reduction of folate to THF. It would have been preferable to employ DHF, which only undergoes one reductive reaction, rather than folate which undergoes two. However, the
steady state kinetic analysis with DHF is complicated by the significant substrate inhibition seen with this pteridine (Nare et al., 1997). Folate was chosen instead of biopterin because the specific activity of PTR1 is higher for folate at a pH where there is negligible non-enzymatic oxidation of NADPH (pH 7.0).

When the initial velocities from varied NADPH at several fixed concentrations of folate (and vice versa) were analyzed by a Hanes-Woolf plot (Cornish-Bowden, 1995) the lines intersected in quadrant II or III (Fig. II-2). In this plot, the Y-intercept of the line is the apparent $K/V (1/V/K)$ of the enzyme. For a substituted enzyme mechanism, the apparent $V/K$ will be independent of the concentration of the fixed substrate and the data will intersect on the Y axis. Whereas, for a ternary complex mechanism the apparent $V/K$ will be dependent on the concentration of the fixed substrate and the data will intersect to the left of the Y intercept at $-K_d^{NADPH}$ (Cornish-Bowden, 1995). This indicated that PTR1 catalysis proceeds through a ternary complex mechanism. Although the intersection is not well determined, it is clear that these lines did not intersect at the origin. Therefore, the ternary complex mechanism is likely to be neither an equilibrium ordered nor random equilibrium mechanism. Values for $V_{max}$, the $K_M$ for folate, the $K_M$ for NADPH, and the $K_d$ for NADPH were derived from Fig. II-3 A and II-3 B and shown in Table II-1. The value of $K_M$ for NADPH obtained in the absence of inhibitors ($1.4 \pm 0.2 \, \mu M$) is about ten fold less than the value reported earlier (Nare et al., 1997). The higher values reported earlier may have been due to inhibition by a low level NADP$^+$ contaminant ($K_i = 1 \pm 0.2 \, \mu M$) of the NADPH used previously. It must also be noted that the previous experiments were done at a lower pH and different concentration of folate.
Figure II-2. Initial Velocities of the PTR1 Catalyzed Reduction of Folate. When NADPH was varied at several fixed concentrations of folate, the lines intersected in quadrant three. Data is presented as a Hanes-Woolf plot. Data was generated as described in Materials and Methods. The concentrations of folate were: closed circles 8 \mu M; open triangles 6 \mu M; closed squares 4 \mu M; open diamonds 3 \mu M; open squares 1.5 \mu M; closed diamonds 2 \mu M; closed triangles 1 \mu M; open circles 0.75 \mu M.
Figure II-3A. Secondary Plots of Steady State Parameters Generated From Figure II-2.

A. A graph of [folate]/$V_{\text{max}}$ as a function of [folate]. The data was fit to equation 2 to generate the line shown in the figure.
Figure II-3B. A Graph of $[\text{folate}]K/V$ as a Function of $[\text{folate}]$. The data was fit to equation 3 to generate the line shown in the figure. The fits were weighted by the standard deviation of each point shown as error bars.
Table II-1. Steady State Parameters of PTR1<sup>a</sup>.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( V_{\text{max}} ) (nmoles/mg s)</th>
<th>( V/K ) (( \mu \text{M}^{-1} \text{s}^{-1} ))</th>
<th>( K_M ) (( \mu \text{M} ))</th>
<th>( K_d ) (( \mu \text{M} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>7.5 (± 0.6)</td>
<td>0.16 (± 0.04)</td>
<td>1.4 (± 0.3)</td>
<td>3 (± 1)</td>
</tr>
<tr>
<td>Folate</td>
<td>7.5 (± 0.6)</td>
<td>0.26 (±0.06)</td>
<td>1.0 (±0.2)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Assays were done at pH 7.0, 30 °C, as described in Materials and Methods.
The order of substrate binding was examined through product inhibition studies. The inhibition of PTR1 by product NADP⁺ is strictly competitive with NADPH (Fig. II-4 A, Table II-1), indicating that NADPH and NADP⁺ compete for the same form of the enzyme. The inhibition of PTR1 by product NADP⁺ is non-competitive vs. folate (Fig. II-4 B, Table II-1). The inhibition by the product analogue 5dTHF was uncompetitive vs. NADPH and noncompetitive vs. folate (Figs. II-4 C and D, Table II-1). These results are consistent with catalysis proceeding mainly via the ordered ternary complex pathway shown in figure II-5, with binding of NADPH preceding that of the oxidized pteridine substrate, and dissociation of the reduced pteridine product preceding that of NADP⁺.

**Ligand Binding**

The enhanced fluorescence of NADPH at 450 nm upon excitation at 295 nm in the presence of PTR1 presumably arises from energy transfer between the enzyme and bound NADPH (Fig. II-6A). The fluorescence of the bound NADPH is quenched upon addition of methotrexate. Addition of DHF (Fig. II-7) quenched the intrinsic tryptophan fluorescence of the free enzyme. Although the enzyme's fluorescence also was decreased upon addition of folate or methotrexate to solutions containing PTR1 alone, most or all of these decreases were due to absorbance. Folate and methotrexate each absorb more strongly at 340 nm than does DHF. The effects of folate or methotrexate on the intensity of PTR1's fluorescence intensity was nearly identical to their effects on the fluorescence of pure tryptophan. These results suggest either that the affinities of folate and methotrexate for PTR1 alone are much lower than that of DHF, or that formation of
**Figure II-4.** Effects of the Product NADP⁺ and the Product Analog 5dTHF Upon the Initial Velocities \(v_i\) of the PTR1-Catalyzed Reduction of Folate (30 μM) by NADPH. Measurements were made using the spectrophotometric assay described in Materials and Methods. Double reciprocal plots are given at several fixed concentrations of product or product analogue. When folate was varied NADPH was held at 90 μM. When NADPH was varied folate was held at 16 μM. **A.** Initial velocities of the PTR1 catalyzed reduction of folate at fixed folate, varied NADPH, and several fixed concentrations of NADP⁺. The [NADP⁺] concentrations were: open triangles, 0 μM; open diamonds, 2.5 μM; open squares, 5 μM, open circles, 10 μM.
Figure II-4 B. Initial Velocities of the PTR1 Catalyzed Reduction of Folate at Fixed Folate, Varied NADPH, and Several Fixed Concentrations of 5dTHF. The [5dTHF] concentrations were: open triangles, 0 µM; open diamonds, 20 µM; open squares, 40 µM; open circles, 80 µM.
Figure II-4 C. Initial Velocities of the PTR1 Catalyzed Reduction of Folate at Fixed NADPH, varied Folate, and Several Fixed Concentrations of NADP+. The [NADP+] were: open triangles, 0 µM; open circles, 1 µM; open squares, 2.5 µM.
Figure II-4 D. Initial Velocities of the PTR1 Catalyzed Reduction of Folate at Fixed NADPH, Varied Folate, and Several Fixed Concentrations of [5dTHF]. The [5dTHF] concentrations were: open triangles, 0 µM; open diamonds, 10 µM; open squares, 20 µM; open circles, 40 µM.
Table II-2. Steady State Parameters of PTR1 in the Presence of Products.

<table>
<thead>
<tr>
<th>Varied Substrate</th>
<th>Inhibitor(^a)</th>
<th>Mode of Inhibition(^b)</th>
<th>(K_i) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>NADP(^+)</td>
<td>C</td>
<td>1 (± 0.2)</td>
</tr>
<tr>
<td>NADPH</td>
<td>5dTHF</td>
<td>U</td>
<td>39 (± 6)</td>
</tr>
<tr>
<td>Folate</td>
<td>NADP(^+)</td>
<td>N</td>
<td>1.06 (± 0.08)</td>
</tr>
<tr>
<td>Folate</td>
<td>5dTHF</td>
<td>N</td>
<td>41 (± 12)</td>
</tr>
</tbody>
</table>

\(^a\) Because of the instability of THF, the product analogue 5dTHF was used.

\(^b\) The modes of inhibition were: C, competitive; U, uncompetitive; N, noncompetitive.

The assays were done at pH 7.0, 30 °C as described in Materials and Methods.
Figure II-5. The Ordered Ternary Complex Mechanism of PTR1.

\[ \text{NH} \xrightarrow{F} \text{E-NH} \xrightarrow{\text{E-NH-F}} \text{E-N}^+\text{-DHF} \xrightarrow{\text{DHF}} \text{E-N}^+ \xrightarrow{\text{N}^+} \text{E} \]
either a PTR1-folate complex or a PTR1-methotrexate complex does not much alter the enzyme's fluorescence.

The linear dependence of the NADPH-dependent fluorescence intensity observed at subsaturating NADPH concentrations in the presence of 5 μM PTR1 (Fig. II-6B) indicated that NADPH must bind with a dissociation constant \( K_d \ll 5 \text{ μM} \). The value of \( K_d \) was determined to be 0.46 (±0.02) μM from a quantitative analysis of the fluorescence enhancement observed using 0.5 μM PTR1 (data not shown). The \( K_d \) estimated in the fluorescence titration is six fold lower than the rather poorly defined value (3 ± 1) estimated from the steady state kinetic analysis. Recently, a study of PTR1 from \textit{L. tarentolae} (Wang \textit{et al.}, 1997) was published. This report did not address the kinetic mechanism of PTR1 and reported a \( K_d \) for NADPH of 130 μM. The source of the discrepancy between the value reported here and the value reported by Wang (1997) is unclear, but may be related to the fact that \textit{L. tarentolae} PTR1 was conjugated to MalE for purification. A \( K_d \) of 130 μM for NADPH is inconsistent with previously reported values for SDRs and the reported value for the \( K_M \) of NADPH (17 μM) in Wang (1997).

The two intersecting lines shown in figure II-6B describe the fluorescence of NADPH upon binding to PTR1 and of excess NADPH. The intersection of the two lines (at 3.2 μM) provides an estimate of the PTR1 active sites available for NADPH binding. Values of the ratio between the concentration of binding sites and the protein concentration, from this and other titrations, were consistently between 0.4 and 0.6.

The apparent \( K_d \) for DHF binding to the free enzyme, derived by fitting the fluorescence quenching data shown in Fig II-7 to eq. 7, is 10 (±2) μM. Since the steady
Figure II-6A. Fluorescence Emission Spectra of Solutions Containing *L. major* PTR1 (5 μM, dashed line), NADPH (5 μM, dotted line), or Both (solid line). Excitations were done with a 5 nm band of radiation centered at 295nm; the emission slit was also 5nm.
**Figure II-6B.** The Increases in Fluorescence Intensity (F) at 450nm Were Used to Analyze NADPH binding to PTR1. The open triangles show the data for NADPH alone. These measurements were made at a higher detector sensitivity than the spectra shown in (A). The data measured in the presence of 5 µM PTR1 (solid circles) above and below 4 µM NADPH were fit to separate straight lines, intersecting at 3.2 µM.
Figure II-7. Quenching of PTR1 Fluorescence by DHF. Excitation and emission wavelengths were 290nm and 340nm, respectively. The reduction of emission intensity due to absorbance by DHF at 340nm (broken line), quantitated using a solution of 7.5 μM tryptophan, was linear over this concentration range. Values for the observed fluorescence (F) of a 5 μM solution of PTR1 (squares) were subtracted from this line. These differences (ΔF, circles) were fit by nonlinear regression to eq 7, yielding K_d = 10 (±1.6) μM. The solid line shows the theoretical curve for this fit.
state kinetics presented above and the isotope partitioning results discussed below indicate that the enzyme-NADPH complex is the only productive binary complex, the PTR1-DHF complex lies off the reaction pathway. The \( K_d \) for DHF binding to the free enzyme is very close to the \( K_i \) for substrate inhibition by DHF (Nare et al., 1997a). At least part of that inhibition may be due to the formation of a non-productive PTR1-DHF complex.

**Isotope Partitioning**

The steady-state kinetic results are consistent with an ordered ternary complex mechanism. In this model, NADPH must bind to PTR1 first, followed by binding of the pteridine substrate. After both substrates are bound, the redox chemistry ensues, the reduced pteridine dissociates from the ternary complex of enzyme and products, and NADP\(^+\) dissociates last (Fig. II-5). However, with DHF at least, the data from fluorescence quenching indicates that pteridine binding to PTR1 may occur without NADPH binding. It was therefore important to test whether such a binary PTR1-pteridine complex could be catalytically competent. The partitioning approach developed by Rose and associates ("isotope trapping", Rose, 1980, Rose et al., 1974) was employed to make this determination.

Treatment of a mixture of \([^{14}C]-\text{NADPH}\) and PTR1 (5 \( \mu \text{M} \) each) with 40 \( \mu \text{M} \) DHF and a 200-fold excess of non-radioactive NADPH resulted in the conversion of about half (mean of four independent experiments 46\%, standard deviation 13\%) of the \([^{14}C]-\text{NADPH}\) to NADP\(^+\) (Fig. II-8). An insignificant amount of radioactive NADP\(^+\) was formed when the \([^{14}C]-\text{NADPH}\) was added to PTR1 at the same time as the DHF and
Figure II-8. Analysis of the Partitioning of $[^{14}\text{C}]$NADPH-PTR1 Complex. A sample containing $[^{14}\text{C}]$-NADPH and PTR1 (5 μM each) was mixed manually with an equal volume of 80 μM DHF and 2 mM non-radioactive NADPH. After twelve seconds, the reaction was quenched by addition of methotrexate. Protein was removed from the reaction mixture, and a sample was analyzed by HPLC and liquid scintillation counting (solid line) as described in Materials and Methods. The dashed line shows a similar analysis with a sample in which the $[^{14}\text{C}]$NADPH was mixed with the enzyme simultaneously with the DHF and non-radioactive NADPH.
non-radioactive NADPH. Similar results were obtained when 100 μM DHF was used as the trap, or when DHF was replaced by folate (100 μM). In contrast with these results, [³H]-folate or [³H]-DHF pre-mixed with PTR1 was not trapped upon the addition of the mixture to NADPH and excess non-labeled pteridine substrate. On the single turnover time scale, significant conversion of [³H]-folate or [³H]-DHF to reduced radioactive products only occurred when the enzyme-pteridine mixture was added to NADPH in the absence of non-radioactive folate or DHF.

The results of the partitioning experiments show that a significant fraction of the enzyme-NADPH complex is catalytically competent, but enzyme-pteridine binary complexes probably are not. These results are consistent with obligatory binding of NADPH to PTR1 prior to pteridine binding for a catalytically productive ternary complex. They are inconsistent with a random mechanism in which either NADPH or pteridine can bind first. Saturating concentrations of folate or DHF trapped only half of the PTR1-NADPH complex, rather than all of it. In control experiments with the [¹⁴C]-NADPH, all of this substrate was capable of being converted to product. The fraction of [¹⁴C]-NADPH trapped was essentially identical at 40 μM or 100 μM DHF, or at 100 μM folate, concentrations which are ca. twenty times the $K_M$ values for these substrates. Moreover, doubling the concentration of PTR1 did not increase fraction of [¹⁴C]-NADPH trapped. This may indicate that only ca. half of the enzyme-NADPH binary complex is catalytically competent. It is noteworthy that the fraction of active sites seen by fluorescence titration of PTR1 with NADPH (0.6) is similar to the fraction of PTR1-[¹⁴C]NADPH complex which could be trapped.
**Kinetic Isotope Effects**

At subsaturating DHF, a significant tritium effect (1.8(±0.4)) on V/K for the PTR1-catalyzed reduction of DHF was observed. At saturating DHF, the value of this isotope effect, \( T(V/K) \), was near unity (Table III-3). It is important to note that under the conditions outlined in Materials and Methods all 5, 10-methylene-THF produced was rapidly and quantitatively converted back to DHF, based on the relative rates of PTR1 and TS catalysis. Therefore, any isotope effect on the reaction catalyzed by TS could not have interfered with the measurement of \( T(V/K) \) on PTR1. The fact that high concentrations of the second substrate reduce the observed value of \( T(V/K) \) to unity requires that PTR1 is governed by a steady state ordered ternary complex mechanism (Cook and Cleland, 1981).

A value of 1.6 (± 0.2) was measured for \( D(V) \) for folate (Fig. II-9). This indicates that the rate of conversion of the ternary enzyme-NADPH-pteridine complex to product is only partially limited by the chemistry of hydride transfer. The turnover rate must also be partially determined by other catalytic steps.

The value of \( D(V/K) \) is indistinguishable from unity (0.9 ± 2), as expected, since the parameter was measured at 30 μM folate, which would saturate the enzyme with the second substrate.

**Presteady State Kinetics**

The reduction of 10 μM folate by an excess of the PTR1-NADPH complex (80 μM) was accompanied by the transient appearance of DHF (Fig. II-10A). This is a direct demonstration that DHF is an intermediate in the conversion of folate to THF.
### Table II-3. Measurement of $T(V/K)$ of the PTR1 Catalyzed Reduction of DHF$^a$.

<table>
<thead>
<tr>
<th>DHF (µM)</th>
<th>$R_0$ (µCi/µmole)$^b$</th>
<th>$R_s$ (µCi/µmole)$^b$</th>
<th>$f^c$</th>
<th>$T(V/K)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>11.9 (± 0.5)</td>
<td>13.5 (± 0.4)</td>
<td>0.24 (± 0.03)</td>
<td>1.8</td>
</tr>
<tr>
<td>0.5</td>
<td>13.95 (± 0.15)</td>
<td>15.0 (± 0.3)</td>
<td>0.23 (± 0.009)</td>
<td>1.3</td>
</tr>
<tr>
<td>0.5</td>
<td>12.75 (± 0.15)</td>
<td>15.1 (± 0.2)</td>
<td>0.27 (± 0.005)</td>
<td>2.1</td>
</tr>
<tr>
<td>60</td>
<td>18.75 (± 0.35)</td>
<td>19.35 (± 0.35)</td>
<td>0.62 (± 0.01)</td>
<td>1.04</td>
</tr>
<tr>
<td>60</td>
<td>9.0 (± 0.2)</td>
<td>9.7 (± 0.2)</td>
<td>0.841 (± 0.007)</td>
<td>1.04</td>
</tr>
</tbody>
</table>

The definitions of $R_0$, $R_s$, and $f$ are given in Materials and Methods. Measurements were made at pH 7.5, 30 °C, as described in Methods and Materials.

b Represents the mean of seven determinations.

c Represents the mean of three to five determinations.
Figure II-9. Deuterium Isotope Effects on the PTR1 Catalyzed Reduction of Folate.

The data was collected at pH 6.0, 30 °C using the spectrophotometric assay described in Materials and Methods. The data is presented as a Hanes-Woolf plot and fit to eq. 2 to generate the lines. The open circles represent initial velocities when NADPH was varied at 30 mM folate. The open circles represent initial velocities when 4S-[3H]NADPH was varied at 30 mM folate.
Surprisingly, the time course for the conversion of folate to THF was not well described by monophasic but rather biphasic kinetics. The theoretical curve shown in Fig. II-10A for disappearance of folate is the sum of two exponentials. The two processes have apparent rate constants of 3 sec$^{-1}$ and 0.15 sec$^{-1}$ and approximately equal amplitudes (59% and 41% for the rapid and slow phases respectively). Note that these pseudo-first order rate constants are not comparable to turnover numbers, since these studies were done under conditions of excess enzyme, but they can be compared to the encounter constants (see below). The time course of THF appearance was also poorly described by a single exponential (Fig. II-10A). Similar results were obtained using 10 μM folate and 37 μM PTR1-NADPH complex (data not shown), with rate constants ca. half the values obtained using 80 μM enzyme. At both enzyme concentrations, DHF was formed from folate and disappeared on a time scale consistent with its competence as a kinetic intermediate. The reduction of 10 μM DHF by an excess of the PTR1-NADPH complex also occurred with biphasic kinetics (Fig. II-10B), with rapid and slow phases (rate constants 1.5 sec$^{-1}$ and 0.03 sec$^{-1}$) of similar amplitudes, 38% and 62% respectively.

We interpret the biphasic kinetics for reduction of folate or DHF by excess PTR1 as consistent with the existence of two forms of the enzyme-NADPH complex. Since all of the pteridine substrate was sequestered, with approximately half reduced slowly despite the presence of a large excess of enzyme, both forms of the enzyme-NADPH complex must bind to folate (or DHF) with nearly equal avidity. This idea is consistent with the fact that only ca. half of the PTR1-NADPH complex could be trapped in the isotope partitioning experiments. The steady state kinetics are dominated by the rapidly
Figure II-10A. Rapid Quench Kinetic Analysis of PTR1-Catalyzed Reduction of (A) Folate. The concentrations of PTR1 and NADPH were 80 μM and 0.5 μM, respectively. The theoretical curve for the disappearance of folate is a sum of two exponentials with time constants of 3 sec\(^{-1}\) and 0.15 sec\(^{-1}\), and approximately equal amplitudes (59% and 41% for the rapid and slow phases respectively). The symbols are: open squares, [folate]; closed triangles, [DHF]; open circles [THF] at a given time point. The theoretical curve for the appearance of THF is a sum of two exponentials with time constants of 0.8 sec\(^{-1}\) and 0.07 sec\(^{-1}\), and amplitudes of 39% and 61% for the rapid and slow phases, respectively.
Figure II-10B. Rapid Quench Kinetic Analysis of PTR1 Catalyzed Reduction of Dihydrofolate. The theoretical curves for the disappearance of DHF (and appearance of THF, not shown) are each a sum of two exponentials with time constants of 1.5 sec\(^{-1}\) and 0.03 sec\(^{-1}\), and amplitudes of 38% and 62% for the rapid and slow phases, respectively. The closed circles represent [DHF] at a given time point.
reacting forms of the enzyme-NADPH complex. The apparent second order rate constants for the faster phases of folate and DHF reduction (assuming that only half of the enzyme present participates in this phase) are 0.1 (±0.02) μM⁻¹ sec⁻¹ and 0.04 (±0.02) μM⁻¹ sec⁻¹ respectively. These values are comparable to the values of V/K obtained from the steady state kinetics (0.26 and 0.06 μM⁻¹ sec⁻¹ respectively).

Comparison of PTR1, SDRs, and DHFR

The stereochemical course and kinetic mechanism of PTR1 are very similar to those which have been described for many members of the SDR family. The preference of PTR1 for transfer of the pro-5 hydrogen from NADPH is observed for all other members of the SDR family which have been studied (Alizade et al., 1976; Armarego, 1979; Arnold et al., 1976; Benner et al., 1985; Betz & Warren, 1968; Do Nascimento & Davies, 1975; Jarabak & Talalay, 1960; Nakayama & Sawada, 1986). Members of the AKR family (Bruce et al., 1994) have been observed, like DHFR, to transfer the A-side hydrogen of NADH or NADPH (Deyashiki et al., 1995; Feldman et al., 1977; Flynn et al., 1975; Pineda et al., 1989). This stereochemical result, therefore, resolves the ambiguity regarding whether PTR1 is a member of the SDR family or the AKR family. Amino acid sequence comparisons (Callahan & Beverley, 1992; Papadopoulou et al., 1992) indicated that PTR1 belongs to a superfamily of oxidoreductases, but relying solely on sequence comparisons is risky. A comparison of the sequences and atomic structures of several AKRs and SDRs reveals that several proteins with the SDR fold lack the YXXXXK motif in their primary sequences (Dessen et al., 1995; Rafferty et al., 1995), and two AKRs each have a YXXXXK sequence in a surface site distant from the catalytic
cleft (Borhani et al., 1992; Wilson et al., 1992; Hoog et al., 1994). The steady state ordered ternary complex kinetic mechanism for PTR1 (Fig. II-5) is analogous to mechanisms reported for both SDRs (such as Drosophila alcohol dehydrogenase (Winberg & McKinley-McKee, 1994) and DHPR (Poddar and Henkin, 1984)) and AKRs (such as aldose reductase (Grimshaw et al., 1995 and references cited therein)). The results of several site-directed mutant forms of PTR1 (Chapter III) further supports structural homology between PTR1 and other SDRs.

DHPR, a mammalian member of the SDR family, is unusual in that it requires a non-ground state substrate, quinoid dihydrobiopterin. Since the π electron structure of quinoid dihydrobiopterin resembles the structure found in oxidized flavins, the mechanism of DHPR catalysis has been suggested to resemble the reduction of flavin observed in flavoproteins (Whitley et al, 1993) such as glutathione reductase (Pai et al, 1982). This is supported by the release of $^3$H into solution when 4S-$[^3]$H-NADH is oxidized by DHPR (Armarego, 1979). In contrast to DHPR, PTR1 does not utilize quinoid dihydrobiopterin as a substrate (Bello et al., 1994). The hydride transfer mechanism used by PTR1 and the reduction of ground state substrates resembles catalysis by DHFR, despite the fact that PTR1 is likely to be structurally homologous to DHPR and not to DHFR. (DHFR has no apparent structural similarity to either the AKRs or the SDRs). Most SDRs transfer hydride directly to (from) the carbon of a carbonyl (alcohol) moiety.

A complex kinetic mechanism has been observed for DHFR (Fierke et al., 1987). Either NADPH or DHF may bind to DHFR first in the progression to a catalytically
competent ternary complex, and the products may dissociate in either order. However, the most rapid pathway during steady state turnover (and therefore the catalytic circuit of highest conductance) is one in which NADPH binds not only prior to DHF, but before dissociation of the THF produced from the previous turnover. Rapid dissociation of the THF product requires dissociation of NADP+ and binding of a fresh molecule of NADPH. In contrast to DHFR, PTR1 must bind to NADPH first, and likely releases NADP+ after the release of the reduced pteridine. As in the case of DHPR (Kiefer et al., 1996), the creation of functional enzyme requires that PTR1 bind to NADPH, but unlike DHPR, a binding site on PTR1 for at least one pteridine substrate (DHF) exists in the absence of NADPH.

Several pieces of evidence seem to indicate that two forms of PTR1-NADPH binary complex exist. Trapping of the PTR1-[14C]NADPH complex by folate or DHF results in the oxidation of only ca. one-half of the radiolabelled NADPH to product. The stoichiometry of NADPH binding sites evidenced by fluorescence enhancement with PTR1 was 0.5 (±0.1); another population of sites could be present which was not made evident by this method. Finally, the biphasic kinetics of folate or DHF reduction by excess PTR1-NADPH complex is also consistent with the existence of at least two forms of this binary complex. The physical meaning and physiological relevance of this is not clear. PTR1 has been shown to exist as a tetramer in solution (Bello et al 1994, Le Blanc et al., 1997). Although no evidence for cooperativity was observed, the tetrameric state of PTR1 may be involved in the formation of multiple PTR1-NADPH complexes with different characteristics. It is possible that a slow structural re-arrangement of the form
of the PTR1-NADPH complex which does not appear to turn over rapidly is required to allow catalysis, or that this form is a slowly dissociating dead-end complex. The available data do not distinguish between these possibilities. We are not aware of any reports of similar behavior by other SDRs. In particular, isotope trapping experiments with the DHPR-NADH binary complex demonstrated stoichiometric conversion of that complex to \( \text{NAD}^+ \) by saturating quinoid dihydrobiopterin (Poddar and Henkin, 1984).

A conformational change has been postulated to limit at least partially the rate of catalysis by DHPR (Poddar and Henkin, 1984). The crystal structures of the binary and ternary complexes of 7α-hydroxysteroid dehydrogenase show that large conformational changes occur upon substrate binding to the E-NADPH complex (Tanaka et al., 1996b). Given the low magnitude of \( D^V \) for PTR1 catalyzed reduction of folate, a conformational change and/or product release step may be largely rate determining for this enzyme.

PTR1 shows significant differences in mechanism and primary structure from DHPR and DHFR. These unique features of PTR1 may make this enzyme vulnerable to selective inhibition, a desirable goal for the development of antifolate therapeutics for treatment of leishmaniasis.
Chapter III
Residues Important in PTR1 Catalysis

Introduction

Recently, a greater understanding of the structure and mechanism of SDRs has been obtained through X-ray crystallographic studies (Ghosh et al., 1994; Kiefer et al., 1996; Tanaka et al., 1996a; Tanaka et al., 1996b; Varughese et al., 1994; Varughese et al., 1992), site directed mutagenesis (Agarwal et al., 1995; Ensor et al., 1991; White et al., 1995), and kinetic studies (Luba et al., 1997; McKinley-McKee et al., 1991). This family consists of more than 50 different enzymes from both prokaryotes and eukaryotes (reviewed by Jornvall et al., 1995). All SDRs, in which this has been studied, are B side dehydrogenases/reductases and have an ordered ternary complex kinetic mechanism in which nucleotide cofactor binds first and dissociates last. Despite a wide range of substrates and low sequence homology within the SDR family, a common fold has been observed in the crystal structures of SDRs (Jornvall et al., 1995). A notable feature of this fold is a β-α-β Rossman Fold domain that has been shown to be the nucleotide cofactor binding site. A second common feature of the SDR family is a conserved YXXXK motif. Studies using site directed mutagenesis have shown that both these residues are required for catalysis. Crystal structures of 7α-hydroxysteroid dehydrogenase (Tanaka et al., 1996b), DHPR (Varughese et al., 1992), 3α, 20β-hydroxysteroid dehydrogenase (HSD) (Ghosh et al., 1994), and mouse lung carbonyl reductase (MLCR) (Tanaka et al., 1996a) showed that the active site tyrosine residue is positioned near both the site of reduction/oxidation on the substrate and C4 of the
nicotinamide ring of the cofactor, while the active site lysine residue forms hydrogen bonds with O2' and O3' of the nicotinamide ribose. A less strictly conserved serine residue (S139 of 20βDH, S136 of MLCR, and S146 of 7α-SDH) was also shown to interact with substrate in the case of the steroid dehydrogenases and MLCR (Tanaka et al., 1996a). These data led to the proposal of a general mechanism for the SDR family consisting of a YKS catalytic triad. In this mechanism the triad acts in the following manner: the active site tyrosine acts as a proton donor/acceptor; the active site lysine serves to hold the nicotinamide cofactor in the correct orientation, and to lower the pKₐ of the active site tyrosine residue; the serine acts to stabilize the reaction intermediate through hydrogen bonding (Jornvall et al., 1995). Interestingly, the DHPR variant DHPR(Y146F,K150Q) retained 50% of the specific activity of the wild type enzyme. The crystal structure of this DHPR variant revealed that an ordered water molecule occupied the position of the hydroxyl group of Y146 in the wild type enzyme (Kiefer et al., 1996).

As with all other members of the SDR family, PTR1 transfers the pro-S hydrogen of C4 of the nicotinamide ring of NADPH to substrate, and exhibits an ordered ternary complex kinetic mechanism in which NADPH binds first and NADP⁺ dissociates last (Chapter II). PTR1 from L. major and L. donovonae contain two characteristic YXXXX "catalytic pentapeptides" consisting of Y152 and K156 and Y193 and K197 (Callahan et al., 1992; Papadopoulou et al., 1992). One of these motifs may be artifactual, or PTR1 may contain two catalytically active pentapeptide motifs. The gene for PTR1 from L. mexicana and L. tarentolae were sequenced (by Mr. Mark Landis) in order to determine
if both pentapeptides were conserved. Subsequent alignment of these sequences indicated that both YXXXK pentapeptides were conserved in PTR1 sequences.

PTR1 displays some significant differences from other SDRs. The serine of the catalytic triad has been replaced by an aspartic acid. Furthermore, unlike DHPR reductase, PTR1 can bind at least one substrate without NADPH bound (although this complex is not productive) (Chapter II). Thus, PTR1 has diverged from other members of the SDR family and may provide insight into the general mechanism of this family.

In the work described here, mutations of both YXXK motifs of PTR1 and of D180 (the residue corresponding to the conserved serine of other SDRs) were constructed and the variant proteins were characterized for their ability to complement a DHFR deficient E. coli strain. Several variant enzymes were purified and characterized for their catalytic and ligand binding properties. Of the two YXXXK motifs, only the Y193-K197 pentapeptide was found to be catalytically important. PTR1(Y193F) showed little effect on the enzyme's ability to bind ligands, significantly reduced ability to reduce fully oxidized folate and biopterin, and, surprisingly, increased ability to reduce 7,8-dihydrofolate. PTR1(K197Q) and the doubly mutated PTR1(Y193F/K197Q) showed very little catalytic activity and large increases in $K_d$ values for NADPH. Neither PTR1(D180A) nor PTR1(D180S) were able to reduce any substrate tested and the binary complex with NADPH showed decreased affinity towards folate. PTR1(D180C) retained a fraction of wild type ability to reduce dihydrofolate. Based on these results a mechanism for PTR1 catalyzed reduction of pterins and 7, 8-dihydropterins is presented.
Materials & Methods

Materials

PTR1 and PTR1 variants were purified as described previously (Bello et al., 1994). NADPH, folate, and biopterin were available from Sigma/Aldrich. Dihydrofolate and dihydrobiopterin was purchased from Schircks Laboratories (Jona, Switzerland). NADPH, folate, dihydrofolate, biopterin, and dihydrofolate were routinely confirmed to be >95% pure by HPLC. Oligonucleotides were purchased from Genesys.

Mutagenesis

Mutants were generated by Ellen Nalivaika using the Sculptor™ kit from Amersham. Mutagenesis was carried out using a single stranded copy of the PTR1 gene in a Bluescript™ backbone. The variant genes for PTR1 were then transferred to pET-3a using Bam-H1 and Nde-I. The following oligonucleotides were used to promote mutant formation:

Y152-F, 5' - TAGCGCCCTTCTTCTTGTAGAAGG-3';
K156-R or Q, 5' - TTCTTGATTC(A/G)GGCGTTCGCGCAT-3';
Y193-F, 5' - CACCATATTACCATGGCCAAGG-3';
K196-Q, 5' - ATATACCCATGCCCA(G/A)GGGGCGTT-3';
D180-A, 5' - ACATGGTCCGCTGCCATGAC-3';
D180-E, 5' - ACATGGTCCAGCCATGAC-3';
D180-N, 5' - ACATGGTCAACGCCATGAC-3';
D180-S, 5' - AACATGGTCCACGCCATGAC-3';
D180-H, 5' - ACATGGTCCACGCCATGAC-3';
D180-C, 5'-AACATGGTCTGCGCCATGAC-3'.

Letters in bold face indicate the mutation. Final verification of constructs was achieved by dideoxy DNA sequencing using the Sequenase™ kit from U. S. Biochemical, or by using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit from Perkin Elmer.

**Complementation of DHFR-deficient *Escherichia coli***

*E. coli* strain NM522 thyA Δfol::kan (Δlac Δpro supE, hsd R5 F'(lac(φ-Z, ΔM15)+ pro+ thyA Δfol::kan)) (Howell et al., 1988), or LH18Δfol, was transformed with the plasmids described above using standard methods. The viability of the Δfol strain requires that thyA also be present. Strains containing a plasmid bearing a PTR1 variant were patched onto solid minimal media (M9 salts, 4% glucose (w/v), 1mM MgSO4, 5 μg/ml thiamin, 0.1 μg/ml thymidine, 100 μg/mL ampicillin, 25 μg/ml kanomycin, 1.5% (w/v) Noble Agar) and plates of supplemented media (minimal media with 50 μg/ml methionine, 50 μg/ml glycine, 20 μg/ml adenosine, and 1 μg/ml pantothenic acid). The plates were incubated at 37 °C overnight and inspected for the appearance of colonies.

The ability of LH18Δfol, alone and with wild type PTR1, to grow on minimal and supplemental media were used as negative and positive controls. This experiment was done with the aid of Ms. Ellen Nalivaika.

**Purification and properties of PTR1 variants**

The expression level of all PTR1 variants was checked using SDS-PAGE. In all cases, the level of expression was equivalent to the expression seen with the wild type enzyme. PTR1 was purified as described in Bello et al., 1994.
The molecular weight of the PTR1 variants was determined through molecular exclusion FPLC. Five to ten milligrams of a PTR1 variant was loaded onto a Pharmacia Superose™ 12 HR 10/30 column equilibrated in 20 mM acetate, pH 5.3, 20 mM β-mercaptoethanol (β-ME), and 0.2 M NaCl. The column was run at 2.5 mL/min, and the retention volume of each of several PTR1 variants was determined. The retention volumes of the variants were compared to the retention volume of wild type PTR1. Molecular weights were calculated from a laboratory standard curve. Catalase, Mut H, T4 DNA Polymerase, thymidylate synthase, β-lactoglobulin, carbonic anhydrase, dihydrofolate reductase, blue dextran, and bovine serum albumin were used as molecular weight standards.

Sequence Alignment

The amino acid sequences of PTR1 from all sources was deduced from DNA sequences using the program MacVector™. Sequence alignments to PTR1 from L. major based only on sequence and the alignment of the four PTR1 sequences were aligned using the multi sequence alignment algorithm Clustal W (Thompson et al., 1994) at http://biology.ncsa.uiuc.edu. Manual alignments based on secondary structural elements were made using an alignment of DHPR and HSD generated from the published structures (Varughese et al., 1992; Ghosh et al., 1994). PTR1 sequences were aligned to DHPR and HSD such that insertions were placed between secondary structural elements.

Measurement of Kinetic Parameters

Initial velocity measurements were done using a spectrophotometric assay described previously (Chapter II). Initial velocities were the rates of the reaction during
the first 10% of conversion of substrate to product. In order to determine $K_M$ and $V_{max}$ values for DHF, 0.08-0.16 μM PTR1(Y193F) was used and DHF was varied between 2 and 100 μM in the presence of 100 μM NADPH. In order to determine $K_M$ and $V_{max}$ values for NADPH, 0.08-0.16 μM PTR1(Y193F) was used and NADPH was varied between 1 and 60 μM in the presence of 60 μM DHF. For PTR1(Y152F) and PTR1(K156Q), biopterin was varied between 3 and 60 μM at 90 μM NADPH. Assay times for PTR1(K197Q) were extended from 15 seconds to five minutes.

In cases where substrate saturation was seen the initial velocity data were fit to equation 1.

\[
v_i = \frac{V_{max} [S]}{K_M + [S]} \tag{equation 1}
\]

In cases where substrate saturation was not achieved, the initial velocity data was fit to equation 2.

\[
\frac{1}{v_i} = \frac{K_M [S]}{V_{max}} + \frac{1}{V_{max}} \tag{equation 2}
\]

Steady state data for PTR1(K197Q) that displayed substrate inhibition was fit to equation 3.

\[
v_i = \frac{V_{max} [S]}{K_M + [S] + \frac{[S]^2}{K_I}} \tag{equation 3}
\]

For enzyme variants with very low activity upper limits of enzyme activity were established by incubating 33 μM variant PTR1 with 90 μM NADPH and 30 μM folate or
biopterin, or 5 μM dihydrofolate or dihydrobiopterin. The ΔA₃₄₀ of the reaction was measured for one hour. Because of the amount of enzyme used in each measurement and variability in the rates of non-enzymatic oxidation of NADPH, the lowest specific activity that can be measured under these conditions is 0.007 nmoles/mg s for folate, 0.003 nmoles/mg s for dihydrofolate, and 0.03 nmoles/mg s for biopterin and dihydrobiopterin.

The pH dependence of PTR1(Y193F) was examined, by Ms. Wendy Matthews, in a pH range from 4-8. Initial velocities were measured using the spectrophotometric assay described above. Dihydrofolate was varied from 0.4-20 μM in the presence of 100 μM NADPH. At each pH, 0.64 μM enzyme was used. The lack of any dependence of initial velocities upon initial NADPH concentration demonstrated that 100 μM NADPH was saturating over this pH range.

Data that showed one apparent pKa were fit to equation 4

\[ k_{\text{obs}} = \frac{k_{\text{lim}}}{1 + 10^{pK_a - \text{pH}}} \]

where \( k_{\text{lim}} \) is the rate constant that was examined (\( k_{\text{cat}} \) or \( k_{\text{cat}}/K_M \)).

**Fluorescence Assays of Ligand Binding**

Measurements of \( K_d \) values were done as described previously (Chapter II). In the cases of PTR1(K197Q) and PTR1(Y193F, K197Q) there was no evidence from the fluorescence signal that the enzyme binding was saturated at NADPH concentrations up to 64 μM. At concentrations higher than 64 μM, the fluorescence of NADPH was no longer linear. To set a lower limit on the \( K_d \) for NADPH, a line, whose slope was equal
to the slope of the line for fluorescence of NADPH in the absence of protein, was modeled into the data such that it passed through the data point at 64 μM. Values from the modeled line were used to calculate values of fraction NADPH bound and the data was handled as described previously (Chapter II).

**Results**

**Sequence Alignment of PTR1**

The amino acid sequences of PTR1 from four different species of *Leishmania* aligned to dihydropterdine reductase and 3α, 20β-hydroxysteroid dehydrogenase are shown in Figure III-1. The sequence of PTR1 from *L. major* differs from that previously reported in the region between amino acid 162 and 170. Three nucleotides were inserted into the DNA sequence of this region resulting in a local frame shift. The new amino acid sequence is reported here. The sequences of PTR1 from *L. tarentolea*, *L. mexicana*, and *L. donovana* were 83%, 96%, and 91% identical to the *L. major* sequence.

There are two conserved YXXXX pentapeptides in all known PTR1 sequences. The first YXXXX pentapeptide consists of Y152 and K156 and the second consists of Y193 and K197. When the alignment was done strictly based on amino acid sequence using Clustal W (Thompson *et al.*, 1994), the first YXXXX motif aligned to the corresponding YXXXX motif of DHPR while the second aligned to the YXXXX motif of HSD (data not shown). However, when the amino acid sequence of *L. major* PTR1 was manually aligned to the previously reported alignment of DHPR and HSD generated from a superposition of crystal structures (Jornvall *et al.*, 1995 and Tanaka *et al.*, 1996b), only the second pentapeptide aligned to the corresponding conserved motif in DHPR or
HSD. Because of this ambiguity, it was important to examine the effects of amino acid substitutions in both of these motifs.

Aspartic acid 180 of *L. major* PTR1 aligns to serine 139 of HSD (Fig. III-1). This serine residue of HSD corresponds to a conserved serine in most other short chain dehydrogenases (Jornvall *et al.*, 1995). PTR1, DHPR, and a naturally occurring variant form of ribitol dehydrogenase from *Klebsiella* are the only known members of the SDR protein super family without a serine at this position. DHPR has an alanine at this position while the ribitol dehydrogenase has a valine (Lin *et al.*, 1994).

The amino acid sequence of DHPR was 10% identical to *L. major* PTR1 in the structure based alignment. The DHPR Y146 XXX K150 motif differs from the corresponding motif in PTR1 only by glycine 147. This region contains the most identities between the two sequences. The amino acid sequence of HSD was 20% identical to *L. major* PTR1 in the structure based alignment. Members of the SDR family are typically 15 – 30% identical (Jornvall *et al.*, 1995). Clusters of identical amino acids were located in the N terminal region of the polypeptide (13% identity between *L. major* PTR1 and DHPR and 33% identity between *L. major* PTR1 and HSD), corresponding to the Rossman Fold of DHPR and HSD (Varughese *et al.*, 1992, Ghosh *et al.*, 1994) and in α helix F to β sheet F, the region containing the YXXXXK catalytic pentapeptide (29% identity between *L. major* PTR1 and DHPR and 40% identity between *L. major* PTR1 and HSD). The latter region ends with a highly conserved proline residue (P233 of PTR1). The region between β sheet F and α helix G contains very little homology between the PTR1 sequences and DHPR and HSD. This
Figure III-1. The Amino Acid Sequences of PTR1 from *L. major* (*L. maj.*), *L. donovanae* (*L. don.*), *L. mexicana* (*L. mex.*), and *L. tarentolae* (*L. tar.*) with Dihydropteropteridine Reductase and 3-α, 20-β Hydroxysteroid Dehydrogenase. Residues in red are conserved in all amino acid sequences, residues in green are conserved in four out of six of the amino acid sequences, residues in blue are conserved in a minority of amino acid sequences, and black residues are not conserved. A – indicates a deletion or insertion. The secondary structural elements of DHPR and HSD are shown above and below the alignment.
region corresponds to the substrate-binding loop in the SDR family (Tanaka et al., 1996b).

**Complementation of DHFR-deficiency in *Escherichia coli***

The ability of a PTR1 variant to complement the DHFR-deficiency of an *E. coli* Δfol strain was used as an initial screen of activity. Complementation indicates the variant enzyme retains the ability to reduce DHF to THF. As shown in Table III-1, six PTR1 variants could rescue the Δfol strain and therefore retained their ability to reduce DHF. PTR1 (K197Q) did not rescue the Δfol *E. coli* strain, while both Y152F and K157 rescued the Δfol strain. The variant enzyme PTR1 (Y193F) can complement a DHFR− strain, even though this tyrosine residue corresponds to the active site tyrosines of dihydropteridine reductase and 3α, 20β-hydroxysteroid dehydrogenase.

**Purification and physical properties of PTR1 variants**

Because of the limited information available from complementation studies, several PTR1 variants were purified for further analysis. All purified PTR1 variants (Y152F, K156Q, Y193F, K197Q, Y193F/K197Q, D180A, D180S, and D180C) behaved in the same manner as wild type enzyme during anion exchange chromatography (data not shown). Molecular exclusion chromatography showed that the Y193F, K197Q, Y193F/K197Q, and all D180 variants had approximately the same elution volume as wild type enzyme (159, 161, 159, and 159 mL, respectively) under conditions where a 30 kD protein eluted at 215 mL. These elution volumes indicate that these variants are, like wild type PTR1, homotetramers (Nare et al., 1997a).
Table III-1. Complementation of an *E. coli Δfol* Strain by PTR1 Variants.

<table>
<thead>
<tr>
<th>PTR1 Variant</th>
<th>Growth on Selection Media&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
</tr>
<tr>
<td>Y152F</td>
<td>+</td>
</tr>
<tr>
<td>K156Q</td>
<td>+</td>
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<td>K156R</td>
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<td>D180A</td>
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<td>D180S</td>
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<tr>
<td>Y193F</td>
<td>+</td>
</tr>
<tr>
<td>K197Q</td>
<td>-</td>
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</table>

<sup>a</sup> A + indicates the appearance of > 10 colonies on the plate of selection media. A – indicates that no colonies appeared on the plate of selection media.
Effects of the Mutations on Kinetic Parameters

Y152F

Replacement of tyrosine 152 with a phenylalanine resulted in little change in the specific activity towards any substrate as measured by the spectrophotometric assay described in Materials and Methods (Table III-2). A slight (1.7 fold) increase in the value of $V_{\text{max}}$ for biopterin and no significant change in the value of $V_{\text{max}}$ for folate was observed. Likewise, the $K_M$ for biopterin showed a 2.4 fold increase while the $K_M$ values for folate and NADPH remained unchanged (data not shown).

K156Q

Replacement of lysine 156 with glutamine also resulted in little change in the specific activity towards any substrate as measured by the spectrophotometric assay (Table III-2). A 1.9 fold decrease in the value of $V_{\text{max}}$ for biopterin was observed. There was no significant change in the value of $V_{\text{max}}$ for folate. The $K_M$ value for biopterin showed a 3.8 fold increase, while the $K_M$ values for folate and NADPH remained unchanged (data not shown).

Y193F

Decreases of 1,000 fold and at least 700 fold in the specific activity of PTR1(Y193F) with folate and biopterin, as measured by the spectrophotometric assay, were observed. The specific activities with dihydrofolate and dihydrobiopterin of PTR1(Y193F) were 10 times higher and 10 times lower than the specific activities of the wild type enzyme (Table III-1).
Table III-2. Specific Activity of PTR1 (nmol/mg s) with Folates and Biopterins. a.

<table>
<thead>
<tr>
<th>PTR1</th>
<th>Folate</th>
<th>DHF</th>
<th>Biopterin</th>
<th>DHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>14.3 (± 0.3)</td>
<td>2.3 (± 0.02)</td>
<td>21 (± 5) b</td>
<td>16 (± 4) b</td>
</tr>
<tr>
<td>Y152F</td>
<td>12 (± 1)</td>
<td>3.0 (± 0.02)</td>
<td>12 (± 0.03)</td>
<td></td>
</tr>
<tr>
<td>K156Q</td>
<td>17 (± 1)</td>
<td>1.9 (± 0.02)</td>
<td>11 (± 1)</td>
<td></td>
</tr>
<tr>
<td>Y193F</td>
<td>0.017 (± 0.003)</td>
<td>23.3 (± 0.9)</td>
<td>&lt; 0.03</td>
<td>1.3 (± 0.3)</td>
</tr>
<tr>
<td>K197Q</td>
<td>0.01 (± 0.003)</td>
<td>&lt; 0.003</td>
<td>&lt; 0.03</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>Y193F/K197Q</td>
<td>&lt; 0.007</td>
<td>&lt; 0.003</td>
<td>&lt; 0.03</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>D180A</td>
<td>&lt; 0.007</td>
<td>&lt; 0.003</td>
<td>&lt; 0.03</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>D180C</td>
<td>0.1</td>
<td>0.1 c</td>
<td>ND d</td>
<td>ND d</td>
</tr>
<tr>
<td>D180S</td>
<td>&lt; 0.007</td>
<td>&lt; 0.003</td>
<td>&lt; 0.03</td>
<td>&lt; 0.03</td>
</tr>
</tbody>
</table>

a Measurements were made at 30 °C and pH 4.7 for biopterins and pH 6.0 for folates as described in Materials and Methods.

b Values were taken from Bello et al., 1994.

c Measurements were made at 40 μM DHF because of the relative inactivity of PTR1(D180C).

d ND indicates that activity measurements could not be made because of the low activity of PTR1(D180C) and the high background of NADPH oxidation under the conditions of the assay.
Table III-3. Values of $K_d$ for PTR1 and PTR1 Variant Enzymes and Enzyme NADPH Binary Complexes$^a$.

<table>
<thead>
<tr>
<th>PTR1 variant</th>
<th>NADPH + E (μM)</th>
<th>MTX + E-NADPH (μM)</th>
<th>Folate + E-NADPH (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>0.46 (± 0.02)</td>
<td>1.6 (± 0.1)</td>
<td>43 (± 2)</td>
</tr>
<tr>
<td>Y193F</td>
<td>8 (± 1)</td>
<td>4.8 (± 0.4)</td>
<td></td>
</tr>
<tr>
<td>K197Q</td>
<td>&gt; 24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y193F, K197Q</td>
<td>&gt; 24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D180A</td>
<td>0.46 (± 0.07)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D180C</td>
<td>1.48 (± 0.09)</td>
<td>62 (± 9)</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>D180S</td>
<td>0.34 (± 0.06)</td>
<td>&gt; 48</td>
<td>&gt; 64</td>
</tr>
</tbody>
</table>

$^a$ All data was collected at 30 °C in 10 mM MES, pH 6.0, 10 mM KCl using the fluorescent titration assay described in Chapter II.

$^b$ No interaction between ligand E-NADPH could be detected by fluorescence.

$^c$ Folate was a substrate, so the Kd for folate binding to the PTR1(D180C)-NADPH complex could not be measured.
In the case of wild type PTR1, both dihydrofolate and dihydrobiopterin are potent substrate inhibitors (Chapter II; Nare et al., 1997a). Typical Michaelis-Menten behavior of PTR1(Y193F) was observed for reduction of DHF (Fig. III-2) and for reduction of DHB (data not shown). This indicates that no substrate inhibition involving either substrate was observed for PTR1(Y193F). Values of $V_{max}$ for these substrates were 23 and 1.3 nmoles/mg s respectively.

The $K_M$ for dihydrofolate of PTR1(Y193F) was 3 fold higher than that determined for wild type PTR1 (Nare et al., 1997a). The $K_d$ for folate of the PTR1(Y193F):NADPH binary complex is shown in Table III-3. This is 17 fold greater than the $K_M$ for folate of the wild type enzyme. The $K_d$ for binding of methotrexate (MTX) to the E:NADPH binary complex was increased 3-fold (Table III-3).

The pH dependence of $k_{cat}$ and $k_{cat}/K_M$ of DHF were measured for PTR1(Y193F). The specific activity of wild type PTR1 was mediated by two apparent $pK_a$ values of 3.8 and 8.1 (Nare et al., 1997). Catalysis of PTR1(Y193F) is also mediated by two apparent $pK_a$ values, but only the more basic $pK_a$, a $pK_a$ value of 6, was determined (Fig. III-3).

**K197Q**

The specific activities for folate and biopterin were, respectively, 1,433 fold and at least 600 fold lower than the values for wild type PTR1 as measured by the spectrophotometric assay. The specific activities for dihydrofolate and dihydrobiopterin were reduced compared to wild type PTR1 at least 750 and 500 fold, respectively (Table III-2). DHF was the only substrate for which this PTR1 variant had significant activity.

An interesting feature of this variant enzyme is that NADPH shows substrate inhibition in
Figure III-2. Rates of Dihydrofolate Reduction by PTR1(Y193F). Data were collected at 30 °C in 20 mM 2-[N-morpholino] ethane-sulfonic acid (Mes) pH 6.0, 20 mM DTT, as described in Materials and Methods. Initial velocities were measured by following the decrease in A₄₀₀ over a 10 second period. Each data point represents the average of at least two determinations. The error bars refer to the standard deviation of the determinations. The solid line represents a fit of the data to equation 1.
**Figure III-3.** pH Profile of Dihydrofolate Reduction Catalyzed by PTR1(Y193F). All data were collected as described in Materials and Methods. An open triangle represents values of $k_{\text{cat}}$ for PTR1(Y193F). A closed circle represents $k_{\text{cat}}/K_M$ values for PTR1(Y193F). All data points represent the average of at least two determinations. For PTR1(Y193F), p$K_a$ values of 6 were obtained for both $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ by fits of the values for each kinetic parameter to equation 4. The error bars represent the error of each parameter determined at a given pH.
steady state assays with dihydrofolate (Fig. III-4). This is in contrast to the wild type enzyme where significant substrate inhibition was observed with dihydropteridine substrates, but not with cofactor (Chapter II). Although $K_{MN}$, $V_{\text{Max}}$, and $K_i$ could not be determined with any certainty, $V/K_{MN}$ ranged from 0.00014 to 0.0002 $\mu$M$^{-1}$s$^{-1}$ when $K_M$ and $K_i$ were varied from 21 to 5000 $\mu$M and 0.1 to 2 $\mu$M. This represents ca. 1000-fold decrease in $V/K_M$ compared to the wild type value (0.16 $\mu$M$^{-1}$ s$^{-1}$).

The $K_d$ for NADPH of PTR1(K197Q) increased by at least 50 times (> 24 $\mu$M) relative to wild type PTR1, based on fluorescence data (Table III-3).

**Y193F/K197Q**

Substitution of both phenylalanine for tyrosine 193 and glutamine for lysine 197 resulted in a variant with properties similar to those of PTR1(K197Q). The specific activities for folate and biopterin were reduced compared to wild type PTR1 at least 2000 and 700 times, respectively. The specific activities for dihydrofolate and dihydrobiopterin were reduced compared to wild type PTR1 at least 750 and 500 times, respectively (Table III-2).

The $K_d$ for NADPH of PTR1(Y193F, K197Q) showed at least a 52 fold increase (> 24 $\mu$M) relative to wild type PTR1 (Table III-2).

**D180A and D180S**

Replacement of aspartic acid 180 with either alanine or serine resulted in decreases in the specific activities for folate and biopterin of at least 2150 and 3250 times, respectively, compared to wild type PTR1. The specific activities for
**Figure III-4.** NADPH is a Substrate Inhibitor of PTR1(K197Q). PTR1(K197Q) shows substrate inhibition by NADPH when assayed with dihydrofolate. Data was collected at 30°C in 20 mM acetate pH 5.0, 20 mM DTT. This is the pH were the highest specific activity was seen. Initial velocities were measured by following the decrease in $A_{340}$ over a 5 minute period. Data were collected at 30°C in 20 mM acetate pH 5.0, 20 mM DTT. The specific activity of PTR1(K197Q) was highest at this pH. All data points represent the mean of at least two measurements. Values of $K_M$ and $K_i$ were estimated in order to model the data using equation 2.
dihydrofolate and dihydrobiopterin were reduced compared to wild type PTR1 at least 2100 and 4800 times, respectively (Table III-2).

The value of the $K_d$ for NADPH of PTR1(D180A) and PTR1(D180S) were both three fold higher than that seen with wild type PTR1. The $K_d$ for methotrexate of the PTR1(D180S)-NADPH binary complex was 39 fold higher than the value measured for the PTR1-NAPDH binary complex (Table III-3).

No interaction between methotrexate and the PTR1(D180A)-NADPH binary complex could be detected by fluorescence. It is unclear whether the PTR1(D180A)-NADPH binary complex lacks a binding site for methotrexate or if the fluorescent properties of PTR1(D180A) are different from those of wild type enzyme such that the binding of methotrexate to the PTR1(D180A)-NADPH binary complex cannot be detected by fluorescence quenching.

Plots of the fraction of PTR1(D180A or D180S)-NADPH bound versus concentration of folate showed that the E-NADPH binary complexes were not saturated up to 64 µM (Fig. III-5). Therefore, the $K_d$ for folate of the PTR1(D180A or D180S)-NADPH binary complexes must be greater than 64 µM (Table III-3). The $K_d$ values for folate of these D180 variants is at least 40 times greater than the value for wild type.

**D180C**

Replacement of aspartic acid 180 with cysteine resulted in a PTR1 variant that retained 2.5% of wild type enzyme specific activity towards folate (Table III-2).

A plot of initial velocity versus the concentration of dihydrofolate was linear up to 80 µM dihydrofolate, indicating that the $K_M$ for DHF was greater than 80 µM (Fig. III-6).
Figure III-5. Folate Does Not Saturate the PTR1(D180S)-NADPH Complex up to 64 μM. 5 μM PTR1 with 5 μM NADPH was titrated with folate. The subsequent quenching of emission at 450 nm (with excitation at 295 nm) was monitored. Data were collected at 30 °C in 20 mM Mes, 40 mM KCl as described in Materials and Methods.
Since saturation was not achieved, the data could not be fit to equation 1. A double reciprocal plot of the data (equation 2) yielded a value for V/K of 0.00025 μM⁻¹ s⁻¹. This V/K value is 640 fold lower than that of the wild type enzyme (Table II-1).

**Discussion**

**Sequence Alignment**

Although the substrate of PTR1 more closely resembles the substrate of DHPR than it does the substrate of HSD, the amino acid sequence of PTR1 is more similar to that of HSD than it is to the amino acid sequence of DHPR. This relationship is borne out in the mechanism of these enzymes as well. PTR1 and HSD transfer a hydride ion to a carbon atom whereas it is unclear whether DHPR transfers a hydride ion to a nitrogen or carbon atom. The most divergent PTR1 sequence was obtained from *L. tarentolae*. This species of *Leishmania* infects lizards while the other species infect human hosts. This divergence of hosts is consistent with the divergence in sequence.

Although both tyrosine 152 and lysine 156 are conserved in the four available PTR1 sequences, substitutions of either had only minor effects on the activity of PTR1. Therefore, Y152 and K156 are not critical residues for structure or catalysis. In contrast, substitutions of tyrosine 193 and lysine 197 had significant effects on the activity of PTR1. The pentapeptide containing Y193 and K197 aligned to the active site pentapeptide of DHPR and HSD. Thus, tyrosine 193 and lysine 197 form the catalytic pentapeptide of PTR1. However, unlike results observed for other SDRs (Ensor *et al.*, 1991; White *et al.*, 1995; Agarwal *et al.*, 1995; Kiefer *et al.*, 1996) replacement of tyrosine 193 with a phenylalanine did not result in a complete loss of enzymatic activity.
**Figure III-6.** PTR1(D180C) Retains Some Ability to Reduce Dihydrofolate. Data were collected as described in Materials and Methods at 30 °C in 20 mM Mes pH 6.0, 20 mM DTT. V/K was determined from the slope of the double reciprocal plot of the data. The line represents a fit of the data to equation 3. At least two measurements were made at each concentration of dihydrofolate.
The serine 139 of HSD is conserved in most SDRs. DHPR naturally contains an alanine residue (A131) at this position and can tolerate a serine substitution without loss of activity (Kiefer et al., 1996). Aspartic acid 180 of PTR1 aligns to these residues and the reductase activity of the enzyme did not tolerate the substitution of either serine or alanine at this position. Since all the PTR1(D180) variants examined had similar physical properties, were tetramers, and retained the ability to bind NADPH, it is likely that no major structural change occurred as a result of the D180 substitutions. Therefore, aspartic acid 180 seems likely to be important in catalysis and/or substrate binding.

Proline 233 of PTR1 (P178 of DHPR and P182 of HSD) is absolutely conserved in the SDRs shown here and is highly conserved within the SDR super family (Tanaka et al., 1996b, Jornvall et al., 1995). Structures of the binary and ternary complexes of 7α-HSD show that the corresponding proline, and another more C terminal proline (P203 of HSD), isolate the motion of the substrate binding loop from the rest of the polypeptide backbone during the binding of substrate (Tanaka et al., 1996b). The sequence of PTR1 does not contain a proline residue that corresponds to P203 of HSD. Proline 255 of PTR1 is conserved and aligns to proline 206 of HSD. Based on this it is reasonable to predict that the substrate-binding loop of PTR1 is contained between amino acids 233 and 255.

Role of Y193

Results from previous studies of SDRs employing X-ray crystallography and site-directed mutagenesis have provided evidence that the conserved tyrosine residue acts as a general acid/base during catalysis. This conclusion is consistent with the above data
except in the case of dihydropteridine reduction. PTR1(Y193F) displayed a 10 fold increase in dihydrofolate reductase activity and a small decrease in dihydrobiopterin reductase activity. Thus, the tyrosine hydroxyl group is not absolutely required for the reduction of dihydrofolate, but is required for the reduction of fully oxidized pteridines. Therefore, the most likely role for tyrosine 193 is the protonation of N8 during the reduction of the C7-N8 double bond (Fig. III-7A).

Besides its role in catalysis, tyrosine 193 appears to have a role in binding substrate to the enzyme-NADPH binary complex. The $K_d$ values for both MTX and folate of the PTR1(Y193F) binary complex were higher than the $K_d$ and $K_M$ values for the wild type enzyme (Table III-1). Tyrosine 193 may interact with a substrate molecule through hydrophobic stacking with the pteridine moiety of dihydrofolate, or form some hydrogen bond(s) with dihydrofolate. The fact that a phenylalanine residue prevents substrate inhibition by 7,8-dihydropteridines supports an interaction mediated by hydrogen bonding of the hydroxyl group of tyrosine 193 of the apoenzyme with the 7,8-dihydropteridine.

Replacement of tyrosine 193 with phenylalanine also resulted in a variant that was no longer inhibited by 7,8-dihydropteridines. It was shown previously that dihydrofolate can bind to wild type PTR1 in the absence of NADPH (Chapter II). The results with PTR1(Y193F) indicate that tyrosine 193 is involved in this interaction. As shown in Fig. III-7B, a possible hydrogen bond could occur between the hydrogen of N8 with a non-bonding $sp^3$ electron pair of the tyrosine hydroxyl. An interaction between the tyrosine of apoenzyme and substrate was observed in the crystal structure of apo-HSD with
Figure III-7A. A Proposed Mechanism of PTR1 Catalyzed Folate Reduction. Tyrosine 193 and aspartic acid 180 are in close enough proximity to hydrogen bond, although the bond is not shown. PTR1 catalyzed reduction of folate. Aspartic acid 180 donates a proton to O4 of folate. Tyrosine 193 donates a proton to N8 of folate, and subsequently accepts a proton from O4. Protonation at N8 results in carbonium ion character at C7 that facilitates hydride ion transfer.
carbenoxolone bound in the enzyme active site. In this structure the hemisuccinate terminal oxygen forms a hydrogen bond (2.8 Å) with tyrosine 152 of HSD, similar to the interaction proposed here for DHF and Y193 of PTR1.

Role of K197

Crystal structures of other SDRs support a role for the conserved lysine that involves correctly positioning the nucleotide cofactor and interacting with the conserved tyrosine residue such that its pKₐ is lowered. In holo-7α-HSD the distances from the amino group of lysine 163 to the hydroxyl group of tyrosine 152 and the O2' and O3' atoms of the nicotinamide ribose are respectively 2.8, 3.1, and 5.1 Å (Tanaka et al., 1996b). Similar distances between side chain functional groups are also seen in HSD and DHPR (Varughese et al., 1992, Ghosh et al., 1994). The results obtained for PTR1(K197Q) and PTR1(Y193F; K197Q) support both of these ideas. Large, but not precisely defined, increases of the Kₐ for NADPH of PTR1(K197Q) and PTR1(Y193F/K197Q) were observed. In contrast to results observed with DHPR, the combination of both the Y193F and K197Q substitutions did not restore any activity.

There is evidence for two kinetically different (fast and slow) PTR1-NADPH binary complexes (Chapter II). It is unclear whether the slow binary complex is incapable of catalysis and must dissociate, or is capable of catalysis but must undergo additional conformational changes that are not required for turnover of the fast binary complex. The substrate inhibition observed from the K197Q and the suggested role of this lysine residue variant is consistent with either hypothesis. The absence of the constraint on the nicotinamide ribose provided by the bifurcated hydrogen bond from
K197 may result in an increase of "slow" PTR1(K197Q)-NADPH binary complexes by causing delays in catalytically important conformational changes, or allow for the stabilization of more PTR1-NADPH dead end complexes through more non-productive binding modes for NADPH.

**Role of D180**

As stated previously most other SDRs contain a serine residue at this position. Crystal structures of several members of the SDR family show that the amino acid residue corresponding to aspartic acid 180 lies within 2.9-3.8 Å of the hydroxyl group of the catalytic tyrosine with and without co-factor bound (DHPR, HSD, 7α-HSD) (Varughese, et al., 1992; Ghosh et al., 1994; Tanaka et al., 1996b) and 2.6 Å away from the substrate in the ternary complex of 7α-HSD (Tanaka et al., 1996b). Thus, this residue could act to stabilize the transition state during catalysis. The results presented above show a large decrease in activity and an increased K_d for folate of PTR1(D180X)-NADPH complexes. This implies that D180 is important in catalysis and interacts with pteridines in the ternary complex. Possible roles for aspartic acid 180 include acting as a proton donor to N5, N8, or O4 of the pteridine ring system during catalysis, forcing the transition state through an energy minimum, or stabilizing the transition state (as is the putative role of the conserved serine residue). We suggest that D180 facilitates hydride transfer to folate and DHF through direct transfer of a proton to O4, or acts to raise the pK_a of O4 so that it could accept a proton. Protonation of O4 leads to protonation of N8 of folate and N5 of DHF which facilitates hydride transfer to C7 and C6 (Fig. III-7A and B).
**Figure III-7B.** A Proposed Mechanism of PTR1 Catalyzed DHF Reduction. Tyrosine 193 and aspartic acid 180 are in close enough proximity to hydrogen bond, although the bond is not shown. PTR1 catalyzed reduction of dihydrofolate. Aspartic acid 180 donates a proton to O4 of dihydrofolate which is transferred to N5. Protonation at N5 results in carbonium ion character at C6 that facilitates hydride ion transfer.
Possible Catalytic Mechanism of PTR1

Tyrosine 193 is only involved in the reduction of folate, thus it is shown as the acid which supplies a proton for N8. Aspartic acid 180 interacts with substrate and is likely to be in the proximity of tyrosine 193. Quantum mechanical calculations predict that protonation at either N8 or O4 of pteridines results in localized carbonium ion character at C7 (Gready, 1984). Protonation at either N5 or O4 of 7, 8-dihydropteridines results in localized carbonium ion character at C6 (Gready, 1984). Therefore protonation at any of these groups would facilitate hydride transfer. The solution pKₐ of N8 of the pterin ring is 1.69 (Rokos et al., 1973). This is comparable to a calculated value of 2.3 (Gready, 1984). A pKₐ value of > 0 was calculated for O4 (Gready, 1984). The solution pKₐ for N5 of the 7, 8-dihydropterin ring is 4.17 (Pfleiderer, 1966). Calculated solution pKₐₜ values for N5 and O4 of the 7,8-dihydropterin ring are 4.2 and 3.7, respectively (Gready, 1984). The pKₐ of N5 of dihydrofolate in solution is 2.6, but this pKₐ increases by four orders of magnitude to 6.5 in the ternary complex of DHFR (Chen et al., 1994). A significant increase in the pKₐ of N5 of DHF occurs on the active site of DHFR. Thus, although the pKₐ is very low there is precedent for the perturbation of a pKₐ on the active site of an enzyme.

Although the exact mechanism of DHFR remains unclear, there is evidence for keto-enol tautomerism of O4 during the DHFR catalyzed reduction of DHF (Bystroff et al., 1990; Basran et al., 1995; Lee et al., 1996). This tautomerism leads to protonation of N5 of DHF through a proton shuttle consisting of ordered water molecules (Bystroff et al.; 1990, Basran et al., 1995). Furthermore, a conserved aspartic or glutamic acid is
involved in protonation of O4 of DHF, but the exact role of this acidic residue is still debated. It is possible that PTR1 catalysis is similar to that of DHFR. Figures III-7A and III-7B show a possible mechanism for the PTR1 catalyzed reduction of folate and dihydrofolate.

Initial protonation at O4 during the reduction of folate and DHF requires only one binding orientation for both folate and DHF, while providing activation for hydride transfer to both C7 and C6 of the substrate pteridine. This mechanism does require some movement of the nicotinamide ring of NADPH to facilitate hydride transfer to C6 and C7. The activities of PTR1(D180E) and PTR1(D180C) are both consistent with the proposed role for aspartic acid 180 as both residues can donate a proton and stabilize an enol transition state.

Based on these results and the results presented above, it is likely that tyrosine 193 acts as a proton donor, that is activated by lysine 197, as is the case for other members of the SDR family. Aspartic acid 180 drives the reaction forward by directly or indirectly protonating O4 of the pteridine substrate.
Chapter IV
Attempts to Crystallize PTR1

Introduction

The crystal structures of several members of the SDR family have been solved. From these structures, a common fold for this family has emerged. This fold forms a one domain subunit consisting of seven (HSD, 7α-HSD, and MLCR) or eight (DHPR) α/β strands in a doubly wound arrangement (Orengo et al., 1994). In these structures, αE and αF are responsible for the interactions between subunits along one axis and αG and βG along a second axis. These enzymes also contain the β–α–β nucleotide binding domain typical of dehydrogenases in general (Rossman et al., 1975). The pyrophosphate moiety of co-factor is bound by a GxxxGxG sequence. Nucleotide specificity is determined by a lysine and an arginine (NADPH) or aspartic acid (NADH) (Tanaka et al., 1996a).

One of the most interesting structural features of the SDR family is its wide diversity of substrates. This family contains alcohol, ribitol, steroid, and pteridine reductases (Jornvall et al., 1995). The comparison of the binary (E-NADP+) and ternary (E-NADPH-reaction product) complexes of 7α-HSD revealed that a change in the Cα positions of the substrate binding loop (between βF and αG) occurred, and the largest position change was 8.5 Å. The remaining structural elements retained their position (Tanaka et al., 1996b). Sequence comparisons show that the substrate binding loop contains a high degree of diversity (Jornvall et al., 1995). Based on this observation, it is likely that the sequence of the loop between βF and αG determines substrate specificity.
This conclusion would be strengthened by corroborating structures of binary and ternary complexes from other members of the SDR family.

The results from kinetic and mutagenic studies described in the previous chapters permit several predictions about the interactions between PTR1 and its substrates. Tyrosine 193 is predicted to interact with aspartic acid 180 and N8 of the pteridine substrate through a hydrogen bond that results in protonation of N8. Lysine 197 forms a bifurcated hydrogen bond with the nicotinamide ribose of NADPH and lowers the pKₐ of tyrosine 193 through a charge-charge interaction. By analogy with 7α-HSD, aspartic acid 180 is predicted to form a hydrogen bond with tyrosine 193 in the absence of pteridine substrate. In the PTR1 ternary complex aspartic acid 180 is predicted to form a hydrogen bond with and protonate O4 of the pteridine substrate. Ultimately, direct evidence for or against these predictions would result from the three dimensional structure of PTR1 in a variety of liganded states. Towards this end, the crystallization of PTR1 from *L. major* and *L. tarentolae*, and several different PTR1 binary and ternary complexes was attempted.

In order to ensure that the best possible crystals were grown, the quality of the protein solution was maximized. An additional step consisting of molecular exclusion chromatography was added to the purification of PTR1 from *L. major* and *L. tarentolae*. The PTR1 purifications were done as rapidly as possible, and protein that was not immediately used was stored at −80 °C in the presence of 20% glycerol. Upon thawing and prior to setting up a crystal screen the solutions of PTR1 were spun in a microcentrifuge at the maximum setting to remove any precipitate.
The quality of the crystal screen solutions was also maximized. Solutions were made from a private set of stock solutions no older than two to three months. The stock solutions were passed through a 2 μm filter to sterilize them and remove any contaminating particles. All of the solutions in a crystal screen were made on the same day from the same stock solutions. When available to the lab, Crystal Screen I™ (CSI) (Jancarik et al., 1991) and Crystal Screen II™ (CSII) (Cudney et al., 1994) were purchased from Hampton Research. Prior to a screen the crystallization solutions were passed through a 2 μm filter. Where noted, after the addition of protein the crystallization solution was passed through 0.1 μm filters. Crystal trays were cleared of dust using compressed air prior to screening.

The sparse matrix screen, CSI, of salts, pH and precipitants was used to identify initial crystallization conditions. When suitable crystals were not attained from the initial screen, a screen consisting of 50% dilutions of those solutions which had caused protein precipitation was attempted. In most cases crystals were attained from at least one of these screens. The solutions that yielded crystals of PTR1 were optimized through subtle variations of salt and precipitant concentration and pH. It was observed that cacodylate buffer pH 6 – 6.5, with calcium acetate between 125 – 200 mM, and PEG 3350 or 8000 yielded the best crystals of *L. major* PTR1 when grown at room temperature.

**Materials and Methods**

*L. major* PTR1 was purified as described previously (Bello et al., 1994). *L. tarentolae* was purified as described below. Crystal Screen I, Crystal Screen II, and 24 well standing drop trays were purchased from Hampton Research. All salts, buffers and
precipitants, except polyethylene glycol (PEG) of various molecular weights, were purchased from Sigma/Aldrich. Dry PEG was purchased from Fluka. Microfiltration units were available from Millipore.

**Purification of *L. tarentolae* PTR1**

The plasmid for the production of *L. tarentolae* PTR1 was supplied by Dr. Stephen Beverley at Harvard University. Bacteria containing an over-expression plasmid for PTR1 were induced using the same method used for the induction of *L. major* PTR1. Inductions were done in 12 L batches.

Throughout the purification, the temperature of the protein solution was maintained at 4 °C. 20 g of frozen cell paste was resuspended in 30 mL (1.5 g/mL) of 50 mM Tris pH 7.0, 1mM phenylmethanesulfonyl fluoride (PMSF) (buffer A). The resuspended pellet was passed through a French Pressure cell, at 1,100 psi, twice in order to lyse the bacteria. The cell debris was removed by centrifugation at 35,000 g for 20 minutes. An equal volume of 3% streptomycin sulfate was added to the decanted supernatant. This mixture was stirred gently for 45 minutes at 4°C. The resulting precipitate was removed by centrifugation at 8,000 g for ten minutes. The supernatant was brought to 45% saturation (w/v, 326 g/L) of ammonium sulfate. The mixture was stirred gently for 45 minutes at 4 °C. The resulting precipitate was recovered by centrifugation at 8,000 g for ten minutes. The pellet was resuspended in 20 mL of buffer A. The concentration of ammonium sulfate was reduced by dialysis for 18 hours against a liter of 20 mM Tris, pH 7.0, 20 mM β-ME (buffer B). The dialyzed material was loaded onto a 200 mL diethylaminoethyl (DEAE) column (2.8 mm X 37 mm)
equilibrated in buffer B. The column was washed with buffer B, at -1mL/min, and the 
A$_{280}$ of the eluent was monitored. When the A$_{280}$ returned to baseline, bound proteins 
were eluted from the column with a liter gradient of 0 to 0.3 M NaCl in buffer B.

Fractions were assayed for PTR1 activity, using the spectrophotometric method described 
previously (Chapter II), and for purity using SDS-PAGE. Active fractions that were > 
75% pure were pooled and dialyzed against one liter of 20 mM Tris, pH 7.0, 1 mM 
PMSF (buffer C). The dialyzed pool was loaded onto a 90 mL Blue Sepharose CL-6B 
column (2.8 mm X 18 mm) equilibrated in buffer C. The column was washed with buffer 
C until the A$_{280}$ of the eluent was 0. Bound proteins were eluted with a 600 mL gradient 
of 0 to 0.3 M sodium potassium phosphate in buffer C. Fractions were assayed for 
activity, using the spectrophotometric assay described previously, and for purity using 
SDS-PAGE. Fractions > 90% pure were pooled and loaded onto a 2 L (6.5 X 90 cm) 
Sephadex G-100 column equilibrated in buffer C. Protein was eluted with buffer C at 1.4 
ml/min. Fractions were evaluated for A$_{280}$ and peak fractions were pooled. The protein 
solution was homogeneous by SDS-PAGE, even when the gel was overloaded. The pool 
was concentrated to 3 mg/mL, brought up to 20% glycerol, 20% β-ME, and stored at -80 
°C until needed. Typically, a 20 g cell pellet yielded 50 mg of purified PTR1 and 40% of 
the total activity of the crude extract.

Prior to use in crystallization trials, the buffer was changed to 20 mM Tris, pH 
7.5, 1 mM PMSF and the protein concentrated to 18.5 mg/mL.
Standing Drop Crystallization Screens

The wells of a standing drop tray were blown free of dust using compressed air.

0.8 mL of crystallization solution were placed into the moat of all the wells in the tray.

One row at a time, 5 μL of protein solution was placed into the tower of the wells. 5 μL of crystallization solution was added to the protein in the tower. Once all drops of protein were mixed with crystallization solution, the tray was sealed with a single piece of packing tape. The following protein concentrations were used for crystallization screens: *L. major* PTR1 (apoenzyme), 6.5 mg/mL; PTR1-NADPH, PTR1-NADPH-MTX, PTR1-NADP⁺, or PTR1-NADP⁺-MTX, 3.5 mg/mL; all complexes of *L. tarentolae* PTR1 and the apoenzyme, 18.5 mg/mL.

*L. major* PTR1-NADPH and PTR1-NADPH-MTX Complexes

0.5 mL of 0.3 mM PTR1, in 20 mM Na Acetate, pH 5.3, 20 mM β-ME, was added to 5 mL of 0.6 mM NADPH, or 0.6 mM NADPH and 0.6 mM MTX, in 20 mM Tris, pH 7.0, 20 mM β-ME. The volume of this solution was reduced to 0.5 mL using a Centricon 10™ unit. This solution was suitable for use in crystallization screens. Crystal trays were set-up and stored under N₂. Crystallization screens done under N₂ were conducted at ambient temperature (20-22 °C).

*L. major* PTR1-NADP⁺ and PTR1-NADP⁺-MTX Complexes

Sufficient amounts of a 10X stock of NADP⁺, and MTX when described, were added to a solution of 0.3 mM PTR1 to produce 1:1 ratios of PTR1, MTX, and NADP⁺. The solution was equilibrated on ice for 20 minutes and used in crystallization trials.
L. tarentolae PTR1-NADPH, PTR1-NADPH-MTX, PTR1-NADPH-O129, PTR1-NADP+ and, PTR1-O129 Complexes

All complexes of L. tarentolae PTR1 were made by mixing 0.6 mM PTR1 with 1.2 mM ligand in a 1:1 ratio. The solutions were allowed to equilibrate on ice for 20 minutes. The chemical structure of O129 is shown in figure IV-1.

Crystallization Strategies

The initial screen for crystallization conditions was the standard CSI sparse matrix screen of pH, salt, and precipitant shown in table IV-1 (Jancarik et al., 1991). Approaches towards optimizing conditions are shown in figure IV-1.

L. major PTR1

Based on conditions obtained from CSI, several more screens were designed as shown in Results.

L. major PTR1-NADP+-MTX

After the initial screen, 50% dilutions of those CSI solutions which had yielded a precipitate were screened.

L. major PTR1-NADP+

Conditions derived from screens of PTR1-NADP+-MTX were examined for their ability to crystallize the PTR1-NADP+ complex. The effect of filtration on crystallization was examined by passing the protein-crystallization solution through 0.1 μm filters. Optimizing the concentration of NADP+ and macro-seeding were also attempted in 0.125 M Mg Acetate, 0.05 M cacodylate, pH 6.6, 7.5% PEG 8K.
<table>
<thead>
<tr>
<th>CSI Solution No.</th>
<th>Salt</th>
<th>Buffer</th>
<th>Precipitant</th>
</tr>
</thead>
<tbody>
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<td>0.2 M CaCl₂</td>
<td>0.1 M Acetate</td>
<td>30% MPD</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>0.4 M NaK Tararate</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>0.4 M NH₄ Phosphate</td>
</tr>
<tr>
<td>4</td>
<td>0.2 M Na Citrate</td>
<td>0.1 M Tris</td>
<td>2.0 M NH₄SO₄</td>
</tr>
<tr>
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<td>0.2 M MgCl₂</td>
<td>0.1 M Tris</td>
<td>40% MPD</td>
</tr>
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<td>0.2 M Na Citrate</td>
<td>0.1 M Tris</td>
<td>20% PEG 3350</td>
</tr>
<tr>
<td>7</td>
<td>0.2 M Na Citrate</td>
<td>0.1 M Cacodylate</td>
<td>1.35 M Na Acetate</td>
</tr>
<tr>
<td>8</td>
<td>0.2 M NH₄ Acetate</td>
<td>0.1 M Cacodylate</td>
<td>30% Isopropanol</td>
</tr>
<tr>
<td>9</td>
<td>0.2 M NH₄ Acetate</td>
<td>0.1 M Citrate</td>
<td>20% PEG 3350</td>
</tr>
<tr>
<td>10</td>
<td>0.2 M NH₄ Acetate</td>
<td>0.1 M Acetate</td>
<td>20% PEG 3350</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>0.1 M Citrate</td>
<td>1.0 M NH₄ Phosphate</td>
</tr>
<tr>
<td>12</td>
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<td>0.1 M Heps</td>
<td>30% Isopropanol</td>
</tr>
<tr>
<td>13</td>
<td>0.2 M Na Citrate</td>
<td>0.1 M Tris</td>
<td>30% PEG 400</td>
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<tr>
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<td>1.35 M LiSO₄</td>
</tr>
<tr>
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<td>0.1 M Tris</td>
<td>20% PEG 3350</td>
</tr>
<tr>
<td>18</td>
<td>0.2 M Mg Acetate</td>
<td>0.1 M Cacodylate</td>
<td>20% PEG 8K</td>
</tr>
<tr>
<td>19</td>
<td>0.2 M NH₄ Acetate</td>
<td>0.1 M Tris</td>
<td>30% Isopropanol</td>
</tr>
<tr>
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<td>0.1 M Cacodylate</td>
<td>30% MPD</td>
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<td>22</td>
<td>0.2 M Na Acetate</td>
<td>0.1 M Tris</td>
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<td>23</td>
<td>0.2 M MgCl₂</td>
<td>0.1 M Heps</td>
<td>30% PEG 400</td>
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<td>0.1 M Acetate</td>
<td>20% Isopropanol</td>
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<tr>
<td>25</td>
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<td>0.1 M Imidazole</td>
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<td>30% MPD</td>
</tr>
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<td>27</td>
<td>0.2 M Na Citrate</td>
<td>0.1 M Heps</td>
<td>20% Isopropanol</td>
</tr>
<tr>
<td>28</td>
<td>0.2 M Na Acetate</td>
<td>0.1 M Cacodylate</td>
<td>20% PEG 8K</td>
</tr>
<tr>
<td>29</td>
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<td>0.1 M Heps</td>
<td>0.8 M NaK Tararate</td>
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<td></td>
<td>20% PEG 8K</td>
</tr>
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<td>20% PEG 3350</td>
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<td></td>
<td>2.0 M NH₄SO₄</td>
</tr>
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<td></td>
<td>4.0 M Na Formate</td>
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<td>2.0 M Na Formate</td>
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<td>35</td>
<td>0.1 M Heps</td>
<td></td>
<td>1.6 M NaK Phosphate</td>
</tr>
<tr>
<td>36</td>
<td>0.1 M Tris</td>
<td></td>
<td>6% PEG 8K</td>
</tr>
<tr>
<td>No.</td>
<td>Buffer Components</td>
<td>Concentrations</td>
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<tr>
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<td>---------------------------</td>
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<tr>
<td>37</td>
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<td>6% PEG 4K</td>
<td></td>
</tr>
<tr>
<td>38</td>
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<td>1.35 M NaKPhosphate</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>2 M NH₄SO₄</td>
<td>2% PEG 400</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.1 M Hepes</td>
<td>20% Isopropanol +</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>0.1 M Citrate</td>
<td>15% PEG 3350</td>
<td></td>
</tr>
<tr>
<td>42</td>
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<td>10% Isopropanol +</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td></td>
<td>15% PEG 3350</td>
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<tr>
<td>44</td>
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<td>45</td>
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<td>47</td>
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<td></td>
</tr>
<tr>
<td>48</td>
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<td>2.0 M NH₄SO₄</td>
<td></td>
</tr>
<tr>
<td>49</td>
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<td>2.0 M NH₄SO₄</td>
<td></td>
</tr>
<tr>
<td>50</td>
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<tr>
<td></td>
<td></td>
<td>12% PEG 8K</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations are: MPD, 2-methyl-2, 4-pentanediol; PEG, polyethylene glycol. The pH of the buffers used were: acetate, pH 4.6; citrate, pH 5.6; cacodylate, pH 6.6; HEPES, pH 7.5; imidizole, pH 7.5; Tris, pH 8.5.
Figure IV-1. The Crystallization Strategy Employed for PTR1 and PTR1-Ligand Complexes. In most cases crystals suitable for diffraction analysis were obtained from the sparse matrix screen or from 50% dilutions of the sparse matrix screen.
L. major PTR1-NADPH-MTX

Promising crystals were obtained from CSI 46 (0.2 M Zn Acetate, 0.1 M cacodylate, pH 6.6, 14% PEG 8K). This solution was optimized for pH and salt and precipitant. The diffraction quality of crystals grown from these screens was examined.

L. major PTR1-NADPH

Conditions that yielded crystals of the PTR1-NADPH-MTX complex were also screened for crystallization PTR1-NADPH binary complex.

L. tarentolae PTR1

Some promising results were obtained from the initial screen of conditions. The conditions of Crystal Screen 2 (CS2) were also examined at 4 and 20°C (Table IV-2). 50% dilutions of those CS2 solutions which had yielded a precipitate were also screened. These solutions were 1, 2, 5, 6, 8, 9, 10-15, 17-19, 22-32, 34, 35, 37-40, 42-45, and 48.

L. tarentolae PTR1-NADP+

CSII was used to screen this binary complex.

L. tarentolae PTR1-NADPH-MTX

Initial conditions for optimization attempts were produced from CSI.

L. tarentolae PTR1-O129, PTR1-NADPH-O129, PTR1-NADP⁺-O129

No conditions beyond CSI and CSII were examined.
Table IV-2. Solutions of the Sparse Matrix Screen CSII (Cudney et al., 1994)\textsuperscript{a}.

<table>
<thead>
<tr>
<th>CSII Solution No.</th>
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<th>Buffer</th>
<th>Precipitant</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>2.0 M NaCl</td>
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<td>10% PEG 6K</td>
</tr>
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<td>0.5 M NaCl, 0.01 M</td>
<td>MCH</td>
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<td>25% Ethylene Glycol</td>
</tr>
<tr>
<td>4</td>
<td>2.0 M NH\textsubscript{4}SO\textsubscript{4}</td>
<td>35% Dioxane</td>
<td>5% Isopropanol</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.0 M Imidizole, pH 6.5</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>10% PEG 1K, 10% PEG 8K</td>
<td></td>
</tr>
<tr>
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<td>1.5 M NaCl</td>
<td>10% Ethanol</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.2 M NaCl</td>
<td>2.0 M NaCl</td>
<td></td>
</tr>
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<td>0.01 CCH</td>
<td>0.1 M Acetate</td>
<td>30% MPD</td>
</tr>
<tr>
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<td>0.1 M CdCl\textsubscript{2}</td>
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<tr>
<td>12</td>
<td>0.2 M NH\textsubscript{4}SO\textsubscript{4}</td>
<td>30% PEG 400</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>30% PEG MMEE 2K</td>
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</tr>
<tr>
<td>14</td>
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<td>0.1 M Citrate</td>
<td>2.0 M Ammonium Sulfate</td>
</tr>
<tr>
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</tr>
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<td>0.1 M Citrate</td>
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<td>21</td>
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<td>12% PEG 20K</td>
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</tr>
<tr>
<td>22</td>
<td>1.6 M NH\textsubscript{4}SO\textsubscript{4}</td>
<td>30% Jeffamine M-600</td>
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</tr>
<tr>
<td>23</td>
<td>0.05 M CsCl\textsubscript{2}</td>
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<td></td>
</tr>
<tr>
<td>24</td>
<td>0.01 CCH</td>
<td>0.1 M MES</td>
<td>30% PEG MMEE 5K</td>
</tr>
<tr>
<td>25</td>
<td>0.2 M NH\textsubscript{4}SO\textsubscript{4}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>0.1 M MES</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Solution 1</td>
<td>Solution 2</td>
<td>Buffer 1</td>
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<td>------------</td>
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</tr>
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<td>30% MPD</td>
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<td>0.1 M HEPES</td>
<td>20% Jeffamine M-600</td>
</tr>
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<td>0.1 M HEPES</td>
<td>2.0 M Ammonium formate</td>
</tr>
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<td>20% PEG 10K</td>
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<td>0.1 M Tris</td>
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</tr>
<tr>
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<td>0.1 M Tris</td>
<td>25% tert-butanol</td>
</tr>
<tr>
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<td>1.0 M Li₂SO₄</td>
</tr>
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<td>20% Ethanol</td>
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<td>0.1 M NaCl</td>
<td>0.1 M Bicine</td>
<td>20% PEG MMEE 2K</td>
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<td>2% Dioxane</td>
<td>0.1 M Bicine</td>
<td>2.0 M MCH</td>
</tr>
<tr>
<td>42</td>
<td>0.1 M NaCl</td>
<td>0.1 M Bicine</td>
<td>10% PEG 20K</td>
</tr>
</tbody>
</table>

Abbreviations are: CTMAB, cetyl trimethylammoniumbromide; MCH, magnesium chloride hexahydrate; MPD, 2-methyl-2, 4-pentanediol; CCH, cobalt chloride hexahydrate; PEG, polyethylene glycol; PEG MMEE, polyethylene glycol monomethylether; FCH ferric chloride hexahydrate, MSH, magnesium sulfate heptahydrate; ZSH, zinc sulfate heptahydrate; CSO, cadmium sulfate octahydrate. The pH of the buffers used were: acetate, 4.6; citrate, 5.6; MES, 6.5; HEPES, 7.5; Tris, 8.5; bicine, 9.0
Data Collection and Analysis

Data was collected using 1.54 Å Cu Kα radiation generated by a rotating anode (Rigaku RU200) and a Rigaku R-axis II™ area detector. Data was analyzed using the software package Denzo. Data were copied to 8 mm video tape for long term storage.

Results

L. major PTR1

Promising semi-crystalline precipitates were observed from CSI solutions:

17 (0.2 M Li₂SO₄, 0.1 M Tris pH 8.5, 20% PEG 8K);
35 (0.1 M HEPES, pH 7.5, 1.6 M Na, K Phosphate);
36 (0.1 M Tris, pH 8.5, 6% PEG 8K);
48 (0.1 M Tris, pH 8.5, 2.0 M Ammonium Sulfate).

To optimize CSI 17, pH was varied from 7.0 to 8.5, in 0.5 unit increments, PEG 3350 was varied from 5 to 30% (w/v) in 5% increments, at 0.2 M Li₂SO₄. This screen was done at both 4 and 20 °C. In order to optimize the salt of CSI 17, pH was held at 8.0, PEG 3350 was held at 15%, and Li₂SO₄, MgSO₄, LiCl, and MgCl₂ were varied from 0 to 100 mM in 20 mM increments. After one day, solutions of 2.0 Li₂SO₄, 0.1 M Tris, pH 8.0, and 15-30% PEG 3350 produced micro-crystals that grew into plates and starbursts (needles radiating from a central point) after a month.

To optimize CSI 35, pH was varied from 7.0 to 8.5 in 0.5 unit increments and NaKHPO₄ was varied from 1.1 to 1.6 M in 0.1 M increments. This screen was done at 4 and 20 °C. 0.1 M HEPES pH 7.5, 1.4 M NaK phosphate yielded small poorly formed crystals. No diffraction data were collected on these crystals.
To optimize CSI 36, pH was varied from 7.0 to 8.5 in 0.5 unit increments, PEG 8K was varied from 3 to 8% in 1% increments. 0.1 M Tris, pH 8.0, and 8% PEG 8K yielded small crystals. No diffraction data were collected on these crystals.

To optimize CSI 48, pH was varied from 7.0 to 8.5 in 0.5 unit increments and ammonium sulfate was varied from 0.5 to 3.0 M in 0.5 M increments. Solutions containing 2.5 M ammonium sulfate, at each pH examined, yielded very small crystals. No diffraction data were collected on these crystals.

Another set of screens consisted of 50% dilutions of CSI solutions that yielded precipitates. At 4 °C these solutions were 4, 5, 8, 9, 10, 14, 15, 18, 20, 22, 24, 27, 28, 30, 31, 32, 33, 40, 41, 45, 46, 47, 48, and 50. At 20 °C these solutions were 1, 4, 5, 6, 8, 9, 10, 11, 12, 14-22, 24, 26-34, 38-43, 45-50. A 50% dilution of CSI 31 yielded crystals. The diffraction pattern of a crystal grown under these conditions contained only faint widely spaced data, suggesting that this was a salt crystal.

*L. major* PTR1-NADP<sup>+</sup>-MTX

Since no CSI solutions yielded crystals, a screen of 50% dilutions of CSI solutions that yielded precipitate was done. At 4 °C these solutions were 1, 4, 6, 9-15, 18-20, 22, 28, 30-34, 38, 40, 45, 47, and 50. At 20 °C these solutions were 1, 4, 5, 6, 8, 9, 10, 12, 13, 14, 15, 17, 18-22, 24-28, 29-35, 38-43, and 46-50.

Small crystals were obtained from 50% CSI 6 (0.1 M Na Citrate, 0.05 M cacodylate, pH 6.6, 10% PEG 3350) after a week.

50% CSI 6 was optimized by varying pH from 7.0 to 8.5, in 0.5 unit increments, and MgCl<sub>2</sub> from 0 to 0.125 mM, in 0.25 mM increments at 10% PEG 3350. A protein
crystal that diffracted weakly to 4 Å resolution was grown from 0.05 M HEPES, pH 7.0, 0.1 M MgCl₂, 10% PEG 3350. These crystals were pyramidal in shape and were typically 0.25 mm wide and 0.125 mm long. Not enough data was obtained to estimate the space group, however. A screen of additives (K Acetate, NH₄ Acetate, Na Acetate, LiCl, CaCl₂, DTT, ethanol, methanol, isopropanol, glycerol, and spermidine) in CSI 6 was done in an effort to improve the resolution of the diffraction data, but the quality of these crystals was never improved.

Showers of micro-crystals were obtained from 50% CSI 18 (0.1 M Mg Acetate, 0.05 M cacodylate, pH 6.6, and 10% PEG 8K).

50% CSI 18 was optimized by fixing the pH at 4.6, 5.5, 6.6, and 7.0 and varying Mg Acetate from 25 to 125 mM in 25 mM increments at 10% PEG 8K. No crystals were grown from this screen.

A solution of 50% CSI 46, 0.1 M Ca Acetate, 0.5 M cacodylate pH 6.6, and 7% PEG 8K, yielded a shower of microcrystals.

50% CSI 46 was optimized by varying pH and Ca Acetate as in CSI 18. PEG 3350 was varied from 0 to 10% at pH 7.0 and 100 mM MgCl₂ in order to examine the effects of PEG 3350 on crystallization. No crystals were grown from this screen.

**L. major PTR1-NADP⁺**

For this binary complex, 50% CSI 6 was optimized by varying pH from 7.0 to 8.5, in 0.5 unit increments, and MgCl₂ from 0 to 0.125 mM, in 0.25 mM increments at 10% PEG 3350.
A screen based on 50% CSI 18 (at 7.5% PEG 8K, JL292) was also done. This screen varied PEG 8K from 2.5 to 8% in 0.5% increments at pH 6.6 and 125 mM Mg Acetate.

Conditions as described above for the PTR1-NADP⁺-MTX complex also yielded crystals of the PTR1-NADP⁺ binary complex. These crystals were not improvements on those obtained with the PTR1-NADP⁺-MTX complex. Further screening of precipitant and pH in smaller increments did not improve the quality of these crystals.

**L. major PTR1-NADPH-MTX**

Crystals were obtained from CSI 46 (0.2 M Ca acetate, 0.1 M cacodylate, pH 6.6, and 14% PEG 8K).

CSI 46 was optimized by varying pH from 6.2 to 6.6, in 0.2 unit increments, and PEG 8K from 10 to 20%, in 2% increments, at 0.2 M Ca Acetate. Salt concentration was also optimized by varying Ca Acetate from 125 to 250 mM, in 25 mM increments, and pH as described above at 10% PEG 8K. An additional screen consisting of 125-250 mM Ca Acetate and 8 to 9.5% PEG 8K at pH 6.2 was also done to optimize the salt component. The best crystals were grown from 0.2 M Ca Acetate, 0.1 M Cacodylate, pH 6.6, 10% PEG 8K. These crystals were 0.1 mm wide and 0.25 mm long. Two hour still X-ray photographs were taken at 30, 45, 75, 90, 110, and 135°. Four hour 1° oscillation photographs were taken, but the crystal decayed in the beam before much information could be gained. Based on analysis of the stills, the best estimation of the space group was orthorhombic. The estimated cell dimensions were a = 138 Å, b = 95 Å, c = 105 Å, and α = β = γ = 90°. Intensities were observed out to ~ 3.2 Å.
Crystals 1.2 mm long and 0.2 mm wide were grown from 0.1 M Tris, pH 8.6, 0.2 M Na Acetate, 18% PEG 3350. 1.5° oscillations showed that these crystals diffracted to ~3.2 Å. Analysis of these oscillations showed that the data were consistent with a unit cell of a = 80 Å, b = 103 Å, c = 246 Å, and α = β = γ = 90° in a primitive orthorhombic space group.

Crystals 0.25 mm long, 0.15 mm wide, and 0.1 mm thick were grown from 0.1 M cacodylate, pH 6.2, 0.2 M Ca Acetate, and 10% PEG 8K. Three 3 hour 1° oscillations were analyzed. The unit space cell parameters were a = 96 Å, b = 107 Å, c = 140 Å, and α = β = γ = 90°. The space group was estimated as primitive orthorhombic.

Macro-seeding was attempted in 0.1 mM cacodylate, pH 6.2, 0.2 M Ca Acetate, 10% PEG 8K. An identical screen was done with Mg Acetate in place of Ca Acetate. No crystals were obtained from this screen.

**L. major PTR1-NADPH**

Attempts to grow crystals of this complex from:
0.1 mM cacodylate pH 6.2, 0.2 M Ca Acetate, 10% PEG 8K;
0.1 mM cacodylate pH 6.6, 0.2 M Ca Acetate, 10% PEG 8K;
0.1 mM cacodylate pH 6.6, 0.2 M Mg Acetate, 16% PEG 8K;
0.1 mM cacodylate pH 6.2, 0.2 M Ca Acetate, 8.5% PEG were done. No crystals or precipitates were obtained.

**L. tarentolae PTR1**

Promising results were obtained from the following CS solutions:
2 (0.4 M Na K Tartrate);
4 (0.1 M, 2.0 M Ammonium Sulphate);
22 (0.2 M Na Acetate, 0.1 M Tris, pH 8.5, 20% PEG 3350); and
39 (2.0 M Ammonium Sulphate, 0.1 M HEPES, pH 7.5, 2% PEG 400).

Solutions 2 and 4 yielded needle like crystals.

CSI 22 was optimized for salt and precipitant by varying Na Acetate from 25 to
125 mM, in 25 mM increments, and PEG 3350 from 5 to 20% in 5% increments, at pH
8.6. Precipitant and pH were optimized by varying PEG 3350 from 2.5 to 15%, in 2.5% increments, and pH at 7.0, 7.4, 8.0, and 8.6 at 0.2M Na Acetate. Salt and pH were
optimized by varying Na Acetate from 0.05 to 0.2 M, in 0.025 M increments, and pH as
described above at 20% PEG 3350. The screen of Na Acetate and and PEG 3350 yielded
highly twinned crystals at 5 and 10% PEG 3350. The screen of pH and PEG 3350
yielded needle like crystals from solutions containing 12 and 15% PEG 3350. The screen
of salt and pH yielded semi-crystalline solids from solutions containing 0.05 and 0.075 M
Na Acetate.

CSI 39 was optimized for salt and precipitant by varying NH₄SO₄ from 0.25 to
1.5 M, in 0.25 M increments, and PEG 400 from 0.5 to 2.0%, in 0.5% increments at pH
7.5. Salt and pH were optimized by varying NH₄SO₄ from 0.25 to 1.5 M, in 0.25 M
increments, and pH from 6.5 to 8.0, in 0.5 M increments, at 2% PEG 400. Precipitant
and pH were optimized by varying PEG 400 from 0.25 to 1.5%, in 0.25% increments,
and pH as described above at 2 M NH₄SO₄. From the screen of PEG 400 and ammonium
sulfate, 0.1 M HEPES, pH 7.0, 1.25 M ammonium sulfate, and 1% PEG 400 yielded
clusters of needle like crystals. No crystals were obtained from the other screens based on CSI 39.

From a screen of 50% dilutions of CSI solutions which had yielded precipitate, 50% CSI 6 (0.1 M MgCl2, 0.05 M Tris, pH 8.5, 10 % PEG 3350), 50% CSI 18 (0.1 M Mg Acetate, 0.05 M cacodylate, pH 6.6, 10% PEG 8K), 50% CSI 28 (0.1 M Na Acetate, 0.05 M cacodylate, pH 6.6, 10% PEG 8K), 50% CSI 40 (0.05 M citrate, pH 5.6, 10% isopropanol, 7.5 % PEG 3350), and 50% CSI46 (0.1 M Ca Acetate, 0.05 M cacodylate, pH 6.6, 7% PEG 8K) yielded needles or promising semi-crystalline precipitates.

Passage of the 50% CSI 46 crystallization solution (50% CSI 46 and protein) through 0.1 µm filter membranes resulted in a single crystal that was 0.6 mm long and 0.6 mm wide. A crystal grown under these conditions was mounted and four still photographs of 30 minute exposure times at 0, 45, 90, and 135° were taken. Based on these data a primitive tetragonal space group with unit cell dimensions of a = b = 124 Å, c = 180 Å, and α = β = γ = 90° was predicted.

*L. tarentolae* PTR1-NADP⁺

Solutions from Crystal Screen 2 that yielded precipitate were diluted 50% and used in a new screen. These solutions were 1, 2, 5, 6,8-15, 17-19, 23-29, 32, 34, 35, 37, 40, and 42-45 from CS2.

Highly twinned crystals were obtained from:

50% CS2 26 (0.01 M Zn sulfate heptahydrate, 0.1 MES, pH 6.5, 30% PEG monomethylether 5000);
50% CS2 45 (0.01 M Ni chloride, 0.1 M Tris, pH 8.5, 20% PEG monomethylether 2000).

**L. tarentolae PTR1-NADPH-MTX**

After the initial screens, CSI solutions:

6 (0.2 M MgCl₂, 0.1 M Tris, pH 8.5, 20% PEG 8K);

17 (0.2 M Li₂SO₄, 0.1 M Tris, pH 8.5, 20% PEG 3350);

22 (0.2 M Na Acetate, 0.1 M Tris, pH 8.5, 20% PEG 3350)

were optimized for salt, pH and precipitant by varying pH from 7.0 to 8.5, in increments of 0.5 units, and PEG 3350 and PEG 8K from 10 to 20%, in 2% increments, in the presence of 0.2 M Na Acetate, CdCl₂, Ca Acetate, NH₄ Acetate, Mg Acetate, or Zn Acetate. Crystals suitable for diffraction analysis were grown from 0.2 M Ca Acetate, 0.1 M cacodylate, pH 6.6, 10% PEG 8K. These crystals grew to a maximum length of 1.2 mm and a maximum width of 0.2 mm. Ten one hour 0.5° oscillations starting at 0° were taken and the diffraction data out to 3.2 Å was collected. Based on this data, the space group was estimated as primitive orthorhombic with a unit cell of a = 87 Å, b = 107 Å, c = 256 Å, and α = β = γ = 90°. Further processing of this data indicated that the reflections were elongated, suggesting that these crystals were highly mosaic along one axis.

Further optimization of PEG 3350 was attempted. In the presence of 0.2 M Mg Acetate, PEG 3350 was varied from 14.2 to 15.6%, in 0.2% increments, at pH 7.0 and 16.2 to 17.6%, in 0.2% increments, at pH 7.5. In the presence of 0.2 M Na Acetate, PEG 3350 was varied from 16.2 to 17.6%, in 0.2% increments, at pH 8.6. In the presence of
0.2 M NH₄ Acetate, PEG 3350 was varied from 16.2 to 17.6%, in 0.2% increments, at pH 8.6. Na Acetate and PEG 3350 were optimized by varying Na Acetate from 0.1 M to 0.25 M, in 0.05 M increments, and PEG 3350 from 16.2 to 17.6%, in 0.2% increments, at pH 8.6. Only semi-crystalline precipitates were obtained from these screens.

*L. tarentolae* PTR1-O129, PTR1-NADPH-O129, PTR1-NADP⁺-O129

Only precipitation was obtained from screens with CSI and CSII.

**Discussion**

There are four distinct enzyme forms in the catalytic cycle of PTR1 (E, E-NADPH, the catalytic ternary complex, and E-NADP⁺). The crystallization of each of these complexes was attempted, although pteridine inhibitors were used in place of substrates. Structures of several different enzyme forms would be very useful as the only SDR for which the structures of the binary and ternary complexes are available 7α-HSD (Tanaka et. al., 1996b). These structures indicate that a conformational change occurs upon substrate binding. It would be interesting to compare the conformational change, if one occurs at all, of PTR1 with that of 7α-HSD.

The most promising crystal forms were *L. major* PTR1-NADP⁺-MTX grown from 0.2 M Ca Acetate, 0.1 M cacodylate, pH 6.6, 10% PEG 8K and *L. tarentolae* PTR1 grown from 50% CSI 46. Several strategies have not been fully exploited in the crystallization attempts of PTR1. Conditions for collecting diffraction data at −150 °C were not identified. The addition of 5-20% glycerol to the crystal mother liquor dissolved crystals of *L. major* PTR1-NADP⁺-MTX. These crystals were stable in 30% PEG 400, but vitreous ice did not form upon flash freezing. Micro-seeding may aid in
the growth of any of the liganded states of the \textit{L. major} enzyme. This is especially true for the \textit{L. major} PTR1-NADP$^+$-MTX crystals as these were abandoned because of their small size and weak diffraction. Another approach involves partial proteolysis of PTR1. This technique has proved useful for the crystallization of several proteins (Noel \textit{et al.}, 1993; Lambright \textit{et al.}, 1994). Proteolysis may have the most effect for the apo-enzyme as the co-factor and substrate binding domains may have more freedom of motion. This "looseness" may interfere with crystallization.

The variants of PTR1 examined in chapter III present a unique opportunity. Unlike results with other members of the SDR family, substitution of the catalytic tyrosine with phenylalanine resulted in an enzyme that can still reduce 7, 8-dihydropteridines. The structure of PTR1(Y193F)-NADPH-folate or biopterin would be the structure of an active variant enzyme with a substrate bound in the active site.
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