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The C-terminus of rat L-histidine decarboxylase specifically inhibits enzymic activity and disrupts pyridoxal phosphate-dependent interactions with L-histidine substrate analogues

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INTRODUCTION

In mammals, the biogenic amine histamine plays an important role in a number of physiological processes, and while it is best known for its function in immune and inflammation responses [1], it has also been described as a regulator of gastric acid secretion in the stomach [2], and of circadian rhythms in the brain [3]. Histamine-mediated signalling is not restricted to these well described models, however, and recent studies with an HDC (L-histidine decarboxylase) knockout mouse model [4] have provided new insights into additional roles in angiogenesis, cellular differentiation of mast cells, and bone loss in osteoporosis [5–8].

While much is known about histamine in mammals, the dimeric PLP (pyridoxal phosphate)-dependent enzyme that produces it, HDC (EC 4.1.1.22) [9–11], has not been so well described. Nevertheless, recent studies have provided new insights into the process of histamine biosynthesis, and have identified a number of catalytic features that are shared with other PLP-dependent enzymes [12–15]. In the case of HDC, therefore, it is proposed that the dimeric form of the enzyme establishes an internal aldime whereby the PLP cofactor is bound to the enzyme backbone. During catalysis this bond is broken as PLP forms an external aldime bond with the substrate within the active site. This allows decarboxylation of the substrate to occur. The final step in the catalytic mechanism is breaking of the PLP–histamine bond, leading to the release of histamine and CO₂ product from the active site, and concomitant re-establishment of the internal aldime bond between PLP and the enzyme backbone.

Studies that have demonstrated the dimerization and PLP-dependence of native HDC have, for the most part, been performed on a C-terminally truncated ~54–55 kDa isoform [9–11,16]. To our knowledge, no such studies have ever been attempted with the full-length enzyme. Indeed, it was only after mammalian cDNAs had been sequenced that it became apparent that the ~54–55 kDa isoform is only a processed form of the enzyme. The primary translation product, on the other hand, has a molecular mass of ~74 kDa [16–18]. This full-length isoform becomes processed post-translationally to generate multiple C-terminally truncated isoforms, including major ~63, ~58, ~55 and ~36 kDa isoforms, as well as a number of other isoforms that are less well represented [19–22].

There are two contributing factors that help to explain why the ~74 kDa form of HDC was initially missed. First, the full-length enzyme contains degradation-promoting elements in the C-terminus and is extremely unstable [21–25]. Post-translational processing removes these regions, and in over 20 years of analysis only the processed forms of the enzyme, such as the stable ~54–55 kDa isoform described above, were purified reproducibly [9–11]. The second reason why the full-length isoform may initially have been overlooked relates to its apparent inactivity. There are now a number of studies proposing that C-terminal truncation is required for activity of HDC, and when the ~74 kDa isoform is expressed in vitro in the absence of significant processing, it is indeed inactive [18–22]. It is understandable, therefore, that early studies to isolate the enzyme failed to detect the full-length isoform, given both its instability and its apparent inactivity.

Abbreviations used: FL, FLAG; α-FMH, α-fluoromethylhistidine; GFP, green fluorescent protein; HA, haemagglutinin; HDC, histidine decarboxylase; HME, histidine methyl ester; Ni-NTA, Ni²⁺-nitritriacetate; PLP, pyridoxal phosphate.

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The ratio of processed to unprocessed isoforms differs between HDC-expressing tissues [19,21,22,26,27]. With such a multitude of differentially regulated isoforms, it has proven impossible to identify the specific isoforms that are contributing to catalysis at any given time. Certainly we are now in a position to say that catalysis is not dependent solely on the ∼54–55 kDa isoform, and that isoforms smaller than ∼53 kDa in size are catalytically inactive [22]; however, a number of processed isoforms between ∼53 and ∼74 kDa in size can be detected in the cell. Indeed, these are the isoforms that show the greatest change in expression during physiological regulation, and are potentially of greatest interest [22]. The relative activities of these isoforms in the absence of processing have not previously been established, and the point of C-terminal truncation at which the enzyme gains catalytic competence has yet to be defined. Considerable benefit could be gained from identifying active and inactive isoforms in the ∼53 to ∼74 kDa size range and, for the inactive ones, from establishing the parameter of catalysis in which they are deficient. This would assist in the identification of isoforms that are theoretically capable of contributing to catalysis in vivo, and provide important functional information regarding expression of the enzyme.

In the present study we employed an experimental approach that allows for the substrate-binding properties of the different HDC isoforms to be studied. Our approach, which uses the substrate analogues α-FMH (α-fluoromethylhistidine) and HME (histidine methyl ester), suggests that the full-length isoform is deficient in interacting with L-histidine substrate. We mapped the point of C-terminal truncation at which the enzyme gains catalytic activity between amino acid residues 633 and 617.

**MATERIALS AND METHODS**

**Plasmid DNA constructs**

The pEP7-FL, pEP7-HA and pEP7-His constructs used in this study were generated by cloning double-stranded oligonucleotides, for which the sense strands are shown in Table 1, into the NheI and NotI sites of the pEP-empty vector, which has already been described [22]. For pEP7-GFP, the Sall–NotI fragments of pEGFP-N1 was cloned into the pEP7-FL empty vector, thus replacing the HA (haemagglutinin) tag with GFP (green fluorescent protein). These vectors contain CMV (cytomegalovirus) and T7 promoter sequences upstream, and respectively FL (FLAG), HA, 6×His and GFP sequences downstream, from the multiple cloning site. The HDC1/339 (i.e. HDC isoform comprising amino acids 1–339), HDC1/516, HDC1/557, HDC1/575, HDC1/600, HDC1/617, HDC1/633 and HDC1/656 (full-length HDC) vector inserts were generated by PCR amplification using the common sense primer -gggaacctgtcaccatagtagcgaccggccggggagtgaataccgtg- and antisense primers shown in Table 1, and the pCMV-HDC18 vector as template.

The pNET7-empty vector was generated by cloning a double-stranded oligonucleotide, for which the sense strand is shown in Table 1, into the NheI–BamHI sites of the pET11a vector. pNET7-HDC1/516His was generated by cloning a PCR product amplified from within the linear range of the activity curve using the common sense primer -gggaacctgtcaccatagtagcgaccggccggggagtgaataccgtg- and antisense primers shown in Table 1, and the pEP7-HDC1/516HA insert as a template, into the BamHI–NotI sites of the pNET7-empty vector.

**Cell culture and transfections**

For transient transfection experiments, Cos-7 cells were seeded at a density of 1.5 × 10⁶ cells per 100 mm dish. After 24 h the cells were transfected with 15 μg of test or empty vector (negative) plasmid DNA and 30 μl of Superfect reagent (Qiagen) overnight. Lysates to be analysed for enzyme activity or for standard immunoblotting of whole-cell lysates were harvested 24 h later by sonication in 200 μl of 0.1 M sodium phosphate buffer (pH 7.4). Protein concentrations were determined by the method of Bradford [27a].

**Assay of HDC activity**

Activity values (measuring radiolabelled CO₂ product released) were determined from within the linear range of the activity curve as described previously [21] using whole-cell lysates or coupled transcription/translation reticulocyte reactions (40 μl volumes). For transfected Cos-7 cell experiments, enzyme activities are shown above fractionated protein samples from the same experiment (means ± S.D.), and are representative of three independent experiments.

**Gel electrophoresis and immunoblot analysis**

Whole-cell lysates were fractionated on denaturing (samples containing 5% β-mercaptoethanol were boiled for 5 min; gels and buffers contained 0.1% SDS) or semi-denaturing (gels and buffers contained 0.1% SDS; samples were not boiled or reduced before electrophoresis) SDS/8% (w/v)-polyacrylamide gels at 4°C. Lysates to be fractionated on semi-denaturing gels were not frozen before electrophoresis. Fractionated gels were transferred to a PVDF membrane and exposed to autoradiographic film (for radiolabelled protein samples) or immunoblotted by standard

<table>
<thead>
<tr>
<th>Name of construct</th>
<th>Sense/antisense</th>
<th>Oligonucleotide sequence</th>
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</thead>
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</tr>
<tr>
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<tr>
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Co-immunoprecipitation of HDC monomer generated in reticulocyte cell lysates

pEP7-HDC1/656HA was used as template in a reticulocyte cell lysate reaction in the presence of non-radiolabelled methionine as described above. Expressed protein was immunoprecipitated using an anti-HA antibody and Protein A–Sepharose beads using previously described methods [22], except that buffer C (0.1 M sodium phosphate, pH 7.4, 1.0 mM PLP, 150 mM NaCl, 0.1% Triton X-100) was used for antibody incubation and washing steps. Immunoprecipitated, HA-tagged protein was resuspended in 30 µl of buffer C, and subsequently divided into two fractions and incubated for 2 h with either radiolabelled GFP or radiolabelled HDC1/656–FL, which had been expressed in reticulocyte cell lysate reactions in the presence of 35S-labelled methionine using the pEP7-GFP and pEP7-HDC1/656FL vectors as templates. The HA-tagged protein HDC1/656–HA was re-precipitated and washed multiple times in buffer A. Samples were fractionated on denaturing SDS/polyacrylamide gels and transferred to PVDF membranes. Membranes were dried and exposed to autoradiographic film to test for the co-immunoprecipitation of radiolabelled proteins, and subsequently used for immunoblotting with an anti-HA antibody to confirm equal immunoprecipitation of bait HDC1/656–HA protein.

RESULTS

The full-length HDC1/656 isoform is processed into multiple isoforms in vivo and is inactive when expressed in vitro

Post-translational processing of full-length rat HDC results in the production of a number of different sized isoforms. This is shown in Figure 1(A), where transient transfection of Cos-7 cells with pEP7-HDC1/656HA resulted in enzymic activity (Figure 1A, upper panel). However, due to the fact that post-translational processing of the primary translation product resulted in the production of numerous isoforms, it was not immediately apparent which isoforms were responsible for activity (Figure 1A, lower panel).

In reticulocyte cell lysate reactions, and in the absence of visible processing, the full-length isoform was inactive, and C-terminal truncation was required for activity (Figure 1B). Excessive truncation also inactivated the enzyme, however, and a ~36 kDa isoform (HDC1/339–HA), similar to that produced by in vivo processing in Cos-7 cells (Figure 1A), similarly lacked activity (Figure 1B).

The substrate analogues HME and α-FMH could theoretically be used to determine the catalytic stage in which the full-length HDC isoform is deficient

We wanted to understand better why the full-length HDC isoform is inactive and in what parameter of catalysis it is deficient. Three hypotheses were initially considered: first, that the C-terminus somehow acts to block interactions with the substrate and formation of the external aldimine with PLP; secondly, that substrate binding occurs, but there is an inability to decarboxylate the external aldimine within the active site; or thirdly, that substrate binding and decarboxylation occur, but the histamine and CO2 products fail to be released from the active site. Deficiencies in any of these steps could explain the apparent necessity for truncation.

Substrate analogues of L-histidine have been described that block the reaction at different catalytic stages [14,26–35]. It was proposed that exploiting the differential inhibition properties of these analogues might make it possible to determine the catalytic step at which the full-length HDC isoform is deficient. Scheme 1 highlights the basic features of this approach, with Scheme 1(A) showing the normal decarboxylation reaction when the substrate L-histidine is used, with the release of CO2 and histamine.
products. When the enzyme-activated inhibitor $\alpha$-FMH is used, decarboxylation of the analogue also occurs (Scheme 1B). In this case, however, the decarboxylated intermediates that are produced bind covalently to the enzyme backbone to inhibit the enzyme irreversibly [28,29,36–38]. Our enzyme assay, which measures the release of radiolabelled CO$_2$ product, shows that product is not being released from the active site of the full-length enzyme. If we could demonstrate that catalytic intermediates of $\alpha$-FMH are nevertheless accumulating in the active site, this might indicate that the full-length isosform is capable of decarboxylating the substrate, but is specifically deficient in product release.

In contrast, the substrate analogue HME has been well described to enter the active site and form an external aldimine with PLP, but, unlike $\alpha$-FMH, it is not decarboxylated (Scheme 1C). Catalytic intermediates produced from this analogue act to inhibit the enzyme reversibly, and their accumulation within the active site can be used as a measure of substrate binding and an ability to form the external aldimine, but does not reflect an ability to decarboxylate [15,33,39]. If we could demonstrate that catalytic intermediates of HME accumulate in the active site of the full-length enzyme, this might indicate that the primary translation product is deficient specifically in decarboxylation.

Substrate analogues alter the electrophoretic mobility of recombinant HDC1/516, indicative of the accumulation of catalytic intermediates

The binding of substrate analogues within the active site alters the absorption spectrum properties of the PLP cofactor. Ideally, therefore, we would use highly purified extracts of recombinant full-length enzyme to study spectrophotometric properties in the presence and absence of the two substrate analogues $\alpha$-FMH and HME. However, in accordance with previous studies, we experienced difficulties in isolating full-length rat HDC, with insufficient material recovered to allow for classical biochemical or biophysical analysis [15,24,39]. Nevertheless, it has recently been demonstrated [40] that the accumulation of HME and $\alpha$-FMH intermediates within the active site alters the fractionation properties of an HDC isoform on semi-denaturing polyacrylamide gels. This is shown in Figure 2A, where there is a clear change in fractionation properties after incubation of partially purified recombinant HDC1/516–His with $\alpha$-FMH and HME. No change was observed when the native L-histidine substrate was used (lane labelled ‘His’ in Figure 2A), indicating that rapid decarboxylation was occurring without the accumulation of intermediates. While the exact molecular basis for each of the bands observed has yet to be characterized fully, the changes in electrophoretic mobility observed can nevertheless be used as an indicator of successful interactions with the respective substrate analogues. This approach has only ever been shown for purified fractions of recombinant enzyme, however, and as already mentioned, we were unable to purify sufficient quantities of the full-length enzyme to perform such an analysis. Nevertheless, we wondered whether it would be possible to detect changes in the electrophoretic mobility of proteins in a heterogenous mixture, such as the reticulocyte cell lysate reactions used in Figure 1(B).

To test this, we expressed the active HDC1/516–FL protein in reticulocyte cell lysate reactions in the presence of $\alpha$-FMH. This treatment inhibited the enzyme (Figure 2B, top panel). When lysates were electrophoresed on semi-denaturing gels, the pattern of fractionation was slightly different from that observed for the purified fractions in Figure 2A, in as much as higher-molecular-mass bands were also observed in the absence of $\alpha$-FMH (see Figure 2B, middle panel, Control lane). Nevertheless, the incubation of expressed protein with $\alpha$-FMH still resulted in a change in the pattern of electrophoretic mobility (Figure 2B, middle panel, compare Control and $\alpha$-FMH lanes). Denaturing

Figure 1 In the absence of post-translational processing, the ~74 kDa HDC primary translation product is inactive

(A) Cos-7 cells were transfected with the pEP7-HA empty (−ve) or pEP7-HDC1/656HA vector. Lysates were analysed for HDC activity (upper panel; means ± S.D.) or fractionated for immunoblotting with an anti-HDC antibody (lower panel). Molecular-mass markers are shown on the left. Major and minor arrows on the right correspond to HDC immunoreactive bands. (B) pEP7-HA empty (−ve), pEP7-HDC1/339HA, pEP7-HDC1/516HA and pEP7-HDC1/656HA vectors were used as templates in coupled transcription/translation reactions. Unlabelled expression reaction products were analysed for enzyme activity (upper panel; means ± S.D.). $^{35}$S-radiolabelled reaction products were fractionated on denaturing SDS/polyacrylamide gels (lower panel).
L-Histidine decarboxylase interactions with L-histidine substrate analogues

Scheme 1 L-Histidine substrate analogues inhibit HDC enzyme activity at different catalytic stages

SDS/PAGE (Figure 2B, lower panel) confirmed that only a single radiolabelled protein was being expressed in the reticulocyte reactions, so it is quite possible that some of the extra bands observed on the semi-denaturing gels were representative of interactions with other reticulocyte cell proteins. Nevertheless, they did not prevent the detection of interactions between the substrate analogue and the enzyme.

Although the mechanism of action of α-FMH has been well described [13,15,28,29,39], we needed to be sure that the changes observed for the enzyme in reticulocyte cell lysates related specifically to the active site. Given that HDC-mediated catalysis depends on PLP, it made sense also to determine whether changes in electrophoretic mobility were influenced by concentrations of the specific active-site cofactor, and reactions were supplemented with 1.0 mM PLP (see Figure 2B, middle panel, right-hand lane). This treatment strongly promoted the observed changes in electrophoretic mobility associated with α-FMH, and involved the clear recruitment of protein from the lower-molecular-mass band.

Together, these studies indicated a specific relationship between HDC expressed in reticulocyte cell lysates, the substrate analogue α-FMH, the active-site cofactor PLP, and a change in electrophoretic mobility detectable on semi-denaturing gels.

α-FMH does not alter the electrophoretic mobility of the full-length HDC1/656 isoform

In the previous sections, we detected interactions between the HDC enzyme and a substrate analogue in a heterogeneous experimental model. We wished to use this approach to test the ability of full-length HDC to interact with the α-FMH substrate analogue. Truncated HDC1/516–HA and full-length HDC1/656–HA were expressed in reticulocyte cell lysate reactions in both the presence and the absence of α-FMH. Radiolabelled reaction products were fractionated on semi-denaturing and denaturing SDS/polyacrylamide gels (Figure 2C, upper and lower panels respectively). In contrast with the HDC1/516–HA isoform, incubation of HDC1/656–HA with α-FMH had no effect on electrophoretic properties under semi-denaturing conditions (Figure 2C, upper panel, compare two right-hand lanes).

Specific C-terminal sequences block interactions with the substrate analogue

We wondered how specific this effect is. For example, was the effect associated with particular amino acid sequences encoded by the C-terminal tail of HDC, or would the presence of non-HDC protein sequences placed at this location in the enzyme also inhibit...
Figure 2 Semi-denaturing gels can be used to test interactions between HDC isoforms and L-histidine substrate analogues

(A) Samples of 200 µg of recombinant HDC1/516–His were incubated in the absence (Control) or presence of 1 mM α-FMH, 20 mM L-histidine (His) or 20 mM HME for 1 h. Lysates were fractionated on semi-denaturing (upper panel) or denaturing (lower panel) SDS/polyacrylamide gels for immunoblotting with an anti-HDC antibody. Arrow B highlights changes in electrophoretic mobility in the presence of substrate analogues. (B) pEP7-HDC1/516L was used as template in coupled transcription/translation reactions in the presence or absence of 1.0 mM α-FMH and 1.0 mM PLP as indicated. Reactions were not supplemented with PLP except where indicated. Unlabelled reaction products were used to determine enzyme activity. Products from radiolabelled reactions performed in parallel were fractionated on semi-denaturing (middle panel) or denaturing (lower panel) SDS/polyacrylamide gels. ‘−ve’ denotes reactions where the pEP7-FL empty vector was used as template. (C) pEP7-HDC1/516HA and pEP7-HDC1/656HA vectors were used as templates in coupled transcription/translation reactions in the presence or absence of 1.0 mM α-FMH as indicated. All reactions were supplemented with 1.0 mM PLP. Radiolabelled reaction products were fractionated on semi-denaturing (upper panel) or denaturing (lower panel) SDS/polyacrylamide gels.

the interaction with α-FMH? We have recently demonstrated that HDC1/477 is the minimal unit of C-terminally truncated HDC that retains catalytic activity, and that residues 478–656 of the protein sequence of HDC are not required for catalysis [22]. We removed this non-essential domain and generated a chimaeric protein in which the specific HDC residues 478–656 (∼20 kDa) were replaced by GFP (∼27 kDa). The chimaeric HDC1/477–GFP protein was expressed in coupled transcription/translation reactions in the presence and absence of α-FMH, and reaction products were fractionated on semi-denaturing gels. These studies showed that replacing the C-terminal sequence of HDC with a non-specific GFP sequence restored the ability of HDC to interact with α-FMH, and reaction products were fractionated on semi-denaturing gels. These studies demonstrated for the first time that the C-terminus contains specific sequences that disrupt HDC-mediated catalysis, and localized the inhibitory domain in a very general manner to the region of the protein comprising residues 478–656. We wanted to determine more specifically what sequences are involved, and to identify the minimal C-terminal truncation that is required to remove the block. We also wanted to study the manner (gradual or immediate) in which decarboxylation competence is acquired.

A series of C-terminally truncated isoforms was generated and expressed in reticulocyte cell lysate reactions in the presence and absence of α-FMH. Radiolabelled reaction products were fractionated on semi-denaturing gels. The electrophoretic mobility of the HDC1/656–HA and HDC1/633–HA isoforms was unaltered by the substrate analogue (Figure 3B). The HDC1/617–HA isoform, on the other hand, and all subsequent truncated isoforms, exhibited a change in electrophoretic mobility in response to interactions with the analogue. For the HDC1/617–HA isoform this change was quite weak and indicated a gradual transition to being able to interact with the analogue. Gradually acquiring the ability to interact with α-FMH coincided exactly

C-terminal truncation between amino acid residues 633 and 617 allows interactions with the substrate analogue to be established

These data demonstrated for the first time that the C-terminus contains specific sequences that disrupt HDC-mediated catalysis, and localized the inhibitory domain in a very general manner to the region of the protein comprising residues 478–656. We wanted to determine more specifically what sequences are involved, and to identify the minimal C-terminal truncation that is required to remove the block. We also wanted to study the manner (gradual or immediate) in which decarboxylation competence is acquired.
Figure 3  Specific sequences in the C-terminus of HDC inhibit interactions with the substrate analogue α-FMH

(A) pEP7-HDC1/477GFP and pEP7-HDC1/656HA vectors were used as templates in coupled transcription/translation reactions in the presence or absence of 1.0 mM α-FMH as indicated. All reactions were supplemented with 1.0 mM PLP. Radiolabelled reaction products were fractionated on semi-denaturing (upper panel) or denaturing (lower panel) SDS/polyacrylamide gels. ‘−ve’ denotes reactions where the pEP7-HA empty vector was used as template. (B) pEP7-HDC1/516HA, pEP7-HDC1/557HA, pEP7-HDC1/575HA, pEP7-HDC1/617HA, pEP7-HDC1/633HA and pEP7-HDC1/656HA vectors were used as templates in parallel transcription/translation reactions in the presence or absence of 1.0 mM α-FMH as indicated. All reactions were supplemented with 1.0 mM PLP. Radiolabelled reaction products were fractionated as described for (A). (C) pEP7-HDC1/516HA, pEP7-HDC1/575HA, pEP7-HDC1/617HA, pEP7-HDC1/633HA and pEP7-HDC1/656HA vectors were used as templates in parallel transcription/translation reactions as indicated. All reactions were supplemented with 1.0 mM PLP. Unlabelled expression reactions were analysed for enzyme activity (upper panel; mean ± S.D.). Radiolabelled reaction products expressed in parallel were fractionated on denaturing SDS/polyacrylamide gels to ensure equal expression of samples.

with progressive acquisition of catalytic activity in enzyme assays (Figure 3C).

C-terminal sequences also disrupt interactions with the substrate analogue HME

These data suggested that the full-length isofrom of HDC is unable to accumulate decarboxylated intermediates of α-FMH, and identified the specific sequences that appear to be responsible for this. Next, we wanted to determine whether this reflected an inability to bind the substrate and form an external aldimine within the active site, as might be indicated by whether or not the protein is capable of interacting with the substrate analogue HME (Scheme 1C). Thus far, our experiments with reticulocyte cell lysates were performed by adding α-FMH directly. When HME was added to active HDC1/516–HA under similar experimental conditions, however, there was no effect on electrophoretic mobility. Our studies with the recombinant protein HDC1/516–His (Figure 2A) indicated that this active form of the enzyme should indeed interact with HME. We wondered, therefore, whether this apparent inconsistency with the heterogeneous lysates was a reflection of the irreversible versus reversible inhibition properties of α-FMH and HME respectively.

Reticulocyte cell lysate reactions contain free L-histidine for protein synthesis, and thus would be expected to reversibly displace any intermediates of the HME analogue that accumulated in the active site. Decarboxylated intermediates of α-FMH, on the other hand, would not be affected in this way. To test for this, we expressed the His-tagged HDC1/516–His and HDC1/656–His isoforms in the reticulocyte cell lysates. The His-tagged proteins were pulled down with Ni-NTA–agarose beads and washed once with a low-stringency sodium phosphate buffer to remove excess histidine. After elution from the beads with EDTA (as opposed to imidazole), HDC isoforms were incubated with HME for 1 h and fractionated on semi-denaturing gels. It was immediately apparent that the mobility of the active HDC1/516–His isoform was changed by this treatment (Figure 4A, upper panel, HME lane). In contrast, that of the full-length isoform was not (Figure 4B, upper panel, HME lane). The full-length primary translation product thus appears to be deficient in binding of substrate and formation of the external aldimine.

The full-length HDC isoform is capable of homodimerization

Thus far, we had worked to identify steps in the catalytic reaction that the full-length HDC isoform was unable to perform.
Having demonstrated that it was deficient in the most basic step of substrate binding to PLP in the active site, we wondered whether this in fact reflected an inability of the full-length isoform to dimerize. The dimerization properties of HDC isoforms have not been well studied, and the possibility that C-terminal sequences might disrupt monomer interactions has not adequately been addressed. To test this hypothesis, unlabelled HDC1/656–HA was expressed in reticulocyte cell lysates in the presence of non-radiolabelled methionine. HA-tagged protein was immunoprecipitated with an anti-HA antibody and Protein A–Sepharose, and incubated subsequently with radiolabelled HDC1/656–FL or radiolabelled GFP. HA-tagged protein was re-precipitated and fractionated to look for co-immunoprecipitation. A specific radioactive HDC1/656–FL band was detected, and demonstrated that the full-length isoform is indeed capable of forming specific homodimers (Figure 5, upper panel, right lane). Thus the C-terminus of HDC does not disrupt dimerization. The observed deficiencies in catalysis therefore do not appear to be associated with the tertiary arrangement of monomers, but with the catalytic and substrate-binding mechanism of the enzyme.

**DISCUSSION**

Our primary objective at the outset of this study was to understand why C-terminal truncation appears to be required for the enzymic activity of HDC, and to identify the parameter of catalysis in which the full-length isoform is deficient. Hypotheses relating to the dimerization, substrate binding, substrate decarboxylation and product release properties of the enzyme were proposed and tested. The results from these studies lead us to believe that the full-length isoform is dimerization-competent, but that specific sequences located primarily between residues 617 and 633 (but extending through to residue 660) act to disrupt interactions with substrate and substrate analogues.

These studies would ideally have been performed using homogeneous preparations of purified protein; however, purification of the full-length isoform from native sources is notoriously difficult. The low abundance of HDC-expressing cells, coupled with the instability of the full-length isoform, suggests that it will always be problematic to purify the ~74 kDa isoform in this way, not just on account of its instability, but also on account of its apparent lack of solubility [15,24,39]. Attempts to overexpress and isolate the full-length mammalian enzyme from bacteria as part of the present study also failed repeatedly (HDC1/656 isoforms with N- or C-terminal glutathione S-transferase or C-terminal 6 x His tags; results not shown). In each case the quantities of soluble protein recovered were insufficient, and while it was possible to increase this amount by employing chemical detergents, these conditions additionally inactivated the active C-terminally truncated HDC1/516–His isoform described in Figure 2(A), which was always expressed and purified in parallel.

It became increasingly apparent, therefore, that it would not be possible to study the dimerization, substrate binding and product release properties of the full-length isoform if we continued to depend solely on homogeneous preparations, and the traditional biochemical and biophysical approaches. We therefore chose to work with a reticulocyte cell lysate model, where solubility and stability were less of a problem, and in the absence of significant post-translational processing we would still be able to study the endogenous catalytic properties of expressed isoforms. It was relatively straightforward with this model, therefore, to demonstrate the dimerization properties of ~74 kDa monomers of HDC, but we also needed to employ methodologies that allowed
interactions with the substrate analogues HME and α-FMH to be detected in a heterogeneous protein environment. It was possible to detect these interactions by monitoring changes in electrophoretic mobility under semi-denaturing fractionation conditions. We specifically demonstrated that the observed changes, which were robust and highly reproducible, were indicative of interactions with the PLP cofactor within the active site. The exact molecular basis for all of the bands observed on the semi-denaturing gels remains unclear. Nevertheless, the molecular interactions of HME and α-FMH with active HDC isoforms have been well described, and the observed changes in electrophoretic mobility occurred only when these substrate analogues were used. Specific L-dopa analogues that inhibit the related dopa decarboxylase enzyme had no effect. Furthermore, not only were interactions not observed with the inactive full-length HDC isoform, but, in experiments to truncate or replace the C-terminus (Figure 3), restoring the ability to interact with a substrate analogue coincided exactly with the ability to bind and decarboxylate the native L-histidine substrate.

Employing this approach to study the catalytic properties of different HDC isoforms, and identifying the catalytic parameter in which the full-length isoform appears to be deficient, underlines the use and simple applicability of this novel approach. The relevance of this method for studying catalytically competent isoforms in heterogeneous tissue samples, and the potential to identify the isoforms that are binding substrate under different physiological conditions, is self-evident. In relation to the present study, however, a number of our observations are particularly noteworthy. For example, we demonstrated that specific HDC protein sequences in the C-terminus inhibit interactions with the substrate analogue. The manner in which interactions with substrate analogues are acquired slowly is, we believe, informative, and has important physiological implications. For example, the progressive acquisition of activity as the enzyme is C-terminally truncated is possibly suggestive of the unwinding of a relatively large inhibitory element, such as a helix or other secondary structure. This in contrast with truncation of the enzyme between residues 477 and 472, which results in the immediate and absolute loss of activity [22]. Therefore, while we are now in a position to state that HDC isoforms of between 70 kDa and 54 kDa contain endogenous activity, the specific activities of isoforms that retain some of the inhibitory structure between residues 633 and 575 are comparatively low. The ~63 kDa isoform, whose expression in rat stomach is regulated during feeding, is a case in point [22], and the changes in isoform expression that occur during physiological regulation need not necessarily be proportional to changes in enzymic activity.

T.C.W. is supported by NIH grant DK48077. J.V.F is supported by a Portuguese FCT grant (SRH/F/BPD/14639/2003). F.S.-J. is supported by grant SAF2002-2586 (Ministry of Science and Technology, Spain) and by REMA (Ministry of Health, Spain). I.F. was the recipient of a travelling scholarship from the Spanish Ministry of Education. We thank I.M. Rosenberg, R. Colucci and T.M. Wac for useful discussions.

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Received 9 October 2003/6 April 2004; accepted 19 April 2004
Published as BJ Immediate Publication 19 April 2004, DOI 10.1042/BJ20031553