Expression, purification and kinetic characterization of wild-type human ornithine transcarbamylase and a recurrent mutant that produces 'late onset' hyperammonaemia

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Ornithine Transcarbamylase Deficiency, an X-linked disorder, is the most common cause of inherited urea cycle disorders. Approx. 90 mutations that produce reduced levels of ornithine transcarbamylase (OTCase) activity have been identified in patients [Tuchman (1993) *Hum. Mutat.* **2**, 174–178; Tuchman and Plante (1995) *Hum. Mutat.* **5**, 293–295]. A model of the three-dimensional structure of OTCase, developed on the basis of its homology to the catalytic subunit of *Escherichia coli* aspartate transcarbamylase (ATCase) [Tuchman, Morizono, Reish, Yuan and Allewell (1995) *J. Med. Genet.* **32**, 680–688], and in good agreement with the crystal structure of *Pseudomonas aeruginosa* OTCase [Villeret, Tricot, Stalon and Dideberg (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10762–10766], indicates that many mutations that produce severe clinical symptoms are at the active site or buried in the interior of the protein. However, one of the few recurrent mutations, R277W, an alteration that produces a milder phenotype of ornithine transcarbamylase deficiency, is located in the model in a loop remote from the active site that is

analogous to a similar loop (the 240's loop, a flexible loop of the catalytic chain of *Escherichia coli* aspartate transcarbamylase, comprised of residues 230–250) of ATCase. Human wild-type OTCase and the R277W mutant have been cloned and over-expressed in *E. coli* and a rapid and efficient purification method utilizing the bisubstrate analogue, *N*³-(phosphonacetyl)-L-ornithine, has been developed and used to purify both proteins. Gel chromatography indicates both are trimeric. The pH dependence of the kinetic parameters of the wild-type enzyme is similar to that of *E. coli* OTCase [Kuo, Herzberg and Lipscomb (1985) *Biochemistry* **24**, 4754–4761], suggesting that its catalytic mechanism is similar, although its maximal activity is approx. 10-fold less. Compared with the wild-type, the R277W mutant has nearly 70-fold lower affinity for L-ornithine, shows no substrate inhibition, and its thermal stability is reduced by 5 °C. Its reduced affinity for L-ornithine, which in turn results in lower activity at physiological concentrations of ornithine, as well as its reduced stability, may contribute to the clinical effects that it produces.

INTRODUCTION

The most common disorder of inherited hyperammonaemia is X-linked Ornithine Transcarbamylase Deficiency resulting from defects in ornithine transcarbamylase (ornithine transcarbamylase, OTCase, EC 2.1.3.3), an enzyme of the urea cycle that catalyses the formation of citrulline and inorganic phosphate from carbamoyl phosphate and L-ornithine [1]. Many different mutations in the human ornithine transcarbamylase gene result in dysfunctional enzymes that give rise to hyperammonaemia and subsequent brain damage. Severe mutations in males may cause death in the newborn period while the symptoms of males with milder mutations may not become apparent until later in life. Approx. 90 mutations in the ornithine transcarbamylase gene that produce reduced OTCase activity have been identified [2,3].

Aspartate transcarbamylases (ATCase, EC 2.1.3.2) and OTCases from various species appear to form a family of proteins in which the three-dimensional structure is conserved despite variability in sequence and function [4–7]. Human OTCase shares 27% amino acid sequence identity and 46% similarity with the catalytic subunit of *Escherichia coli* ATCase, for which the three-dimensional structure in the holoenzyme has

been determined by X-ray crystallography [PDB:8AT1] [8]. We previously used the ATCase structure to develop a homology-based model of human OTCase [9] that is in good agreement with the recently reported crystal structure of *Pseudomonas aeruginosa* OTCase (Figure 1) [5]. In addition, we mapped the mis-sense mutations that result in clinical symptoms on the model (Figure 2). Many of these alterations are located at the active site or in the interior of the protein, where they would be expected to interfere with catalysis, folding or stability. Some, however, are located in regions of the molecule that do not have an obvious functional role. Hence, further structural and biochemical studies are required to understand how these mutations give rise to dysfunctional OTCase enzymes and Ornithine Transcarbamylase Deficiency.

One of the few recurrent mutations occurs in exon 8 of the OTC gene, where the mutation of CGG to TGG produces the R277W mutant. This mutation generally does not produce any symptoms until adulthood, when a severe clinical episode may unexpectedly occur. Although Arg-277 is well conserved across species, it is located in the homology model in a loop remote from the active site (Figures 1 and 2). This loop is homologous to a functionally important region of *E. coli* ATCase, the 240's loop (a flexible loop of the catalytic chain of *E. coli* ATCase,

Abbreviations used: ATCase, aspartate transcarbamylase; OTCase, ornithine transcarbamylase (enzyme); OTC, ornithine transcarbamylase (gene); PALO, *N*³-(phosphonacetyl)-L-ornithine; IPTG, isopropyl- β -D-thiogalactoside.

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Figure 1 Superposition of the homology model of human OTCase (light grey trace) and the crystal structure of *P. aeruginosa* OTCase (thin dark-grey trace)

The loop inserted in bacterial OTCases is shown as a thick dark-grey trace; the corresponding sequence in human OTCase is shown as a thick light-grey trace. The r.m.s. difference between backbone atoms is 2.9 Å. The Figure was generated with the program RasMol [37].

Figure 2 Homology-based model of the three-dimensional structure of human OTCase based upon the crystal structure of the catalytic subunit of *E. coli* ATCase in the holoenzyme [PDB:8AT1] [8]

The three-fold axis lies on the left side of the molecule, approx. vertical in the plane of the page. Interchain interfaces are formed on the left, and in front of the subunit displayed. The locations of mutations that give rise to neonatal (●) or late onset (○) OTC deficiency are shown. Arg-277 is indicated by the arrow.

comprised of residues 230–250), which forms an intersubunit contact in the T state and swings in towards the active site in the R state to bring the two domains of the catalytic chain closer together [10]. It is also adjacent to an extended loop found in *E. coli* OTCases and all sequenced catabolic *Pseudomonas* and *Neisseria* OTCases (Figure 1).

We have cloned, overexpressed, purified to homogeneity and studied the kinetic parameters of mature human OTCase over a range of pH and compared them with those of *E. coli* OTCase, with which human OTCase shares 59% sequence identity. We

have also cloned and purified to homogeneity the recombinant R277W mutant and compared its kinetic parameters and thermal stability with those of the wild-type enzyme. The results indicate that the clinical effects of R277W may depend upon a combination of reduced affinity for L-ornithine, which in turn results in reduced catalytic activity at physiological concentrations of ornithine, as well as reduced stability.

EXPERIMENTAL

Construction of OTCase expression vector (pETOTCII)

Plasmid pSPROTC4, with the mature human OTC cDNA minus the first four codons beyond the leader sequence (codon 37–355) inserted in pSP64, was obtained from Dr. Arthur Horwich at Yale University. An *EcoRI*–*SacI* fragment from pSPROTC4 containing the cDNA was cloned into a T7-*lac* expression vector (pET21a+, Novagen) forming the pETOTCI plasmid. In addition to lacking the codons for the first four amino acids of mature OTCase, the new construct also contains an additional T7-*Tag* sequence downstream to the promoter. To remove the T7-*Tag* sequence and incorporate the missing codons, a short fragment overlapping the promoter and the cDNA (150 bp) was amplified by PCR. The forward primer contained the first four codons and the cDNA sequence with an *NdeI* site at its 5' end while the reverse primer contained an *SauI* site. The expression vector and PCR fragment were digested with *NdeI* and *SauI* and the digested fragment was ligated into the vector creating a new plasmid (pETOTCII) containing the entire sequence of the mature OTCase with a methionine codon (ATG) at its 5'-end downstream to a T7 promoter and a *lac* operator. The incorporated fragment was sequenced to exclude PCR errors.

Construction of R277W plasmid (pETOTCIV)

An expression vector for the R277W (CGG to TGG) human OTCase mutant was constructed by PCR site-directed mutagenesis as described below. Sense and anti-sense overlapping 19-mer oligonucleotides containing the mutation at the 10th position were synthesized. These primers and two additional flanking

primers were used to amplify two fragments of the ornithine transcarbamylase gene (OTC) by PCR containing *KpnI* and *SpeI* restriction sites respectively. The two fragments were combined in one PCR reaction to amplify a common DNA fragment containing the R277W mutation and the *KpnI* and *SpeI* sites at the 5' and 3' ends respectively. This fragment 'cassette' was cloned into pETOTCII. The cloned fragment was sequenced to verify the mutation and to exclude PCR errors.

GroES and GroEL plasmid (pGroESL)

Plasmid pGroESL, derived from pACYC184 expressing *E. coli* chaperonin genes GroES and GroEL, was obtained from Dr. Ian Krause, University of Colorado.

Synthesis of *N*^δ-(phosphonacetyl)-L-ornithine (PALO)

PALO, a compound first described by Mori et al. [11], was synthesized in 23 % overall yield in four steps starting from *N*^α-(benzyloxycarbonyl)-*N*^δ-(*t*-butoxycarbonyl)-L-ornithine, essentially as described by Alewood et al. [12]. Catalytic hydrogenation for final deprotection required analytical-grade glacial acetic acid and fresh 10 % palladium on charcoal catalyst; yields were best (nearly quantitative) on a scale of 1 g or less. A key co-intermediate, dibenzylphosphonoacetic acid, was prepared as outlined by Koppel and Kinnick [13]. Dibenzyl phosphite was deprotonated with *n*-butyl-lithium in dry tetrahydrofuran (69 % yield), then methylated at phosphorus, again treated with *n*-butyl-lithium in dry tetrahydrofuran and the resultant lithium salt was quenched by adding the cold (< -50 °C) solution under vigorous stirring to finely powdered dry ice (51 % yield after extractive workup). NMR supported the structures of all intermediates as well as the final product, melting points agreed with the literature, and C₁₈ reversed-phase HPLC eluted with 0.1 % aqueous trifluoroacetic acid/acetonitrile gradients was used to verify purities which were >98 %.

Preparation of PALO affinity column

Dry epoxy-activated Sepharose 6B (2.5 g; Pharmacia), washed with 1 litre of distilled water on a sintered glass filter, was reacted with 80 μmol of PALO in 25 ml of 0.1 M NaOH at 37 °C overnight in a shaker/incubator. Unbound PALO was removed by washing with 1 litre of 0.1 M NaOH, and the remaining reactive sites on the resin were blocked by incubating overnight in 1 M ethanolamine, pH 8.0, at 37 °C. The matrix was poured into a 1.5 cm diameter column, thoroughly washed with a buffer containing 20 mM Tris acetate/0.5 M KCl/2 mM EDTA, pH 7.4, and equilibrated with 20 mM Tris acetate/0.25 M KCl/2 mM EDTA, pH 7.4. The capacity of a 20 ml column exceeds 17 mg.

Protein purification

Log phase cultures of *E. coli* HMS174 in LB broth were transformed with pETOTCII or pETOTCII + pGroESL, in the presence of 100 μg/ml ampicillin or 100 μg/ml ampicillin + 35 μg/ml chloramphenicol respectively and were incubated overnight at 37 °C. A 10 ml aliquot was used to inoculate 1 litre of culture which was grown at 25 °C until an *A*₆₀₀ of 0.4–0.8 was reached. After induction overnight with 0.01 mM isopropyl-β-D-thiogalactoside (IPTG), the cells were harvested by centrifugation, resuspended in 10 ml of 20 mM Tris acetate/100 mM NaCl/2 mM EDTA/100 μM PMSF, pH 7.4 per gram of cells at 4 °C, and disrupted by sonication on ice for 15 min. The lysate was centrifuged for 10 min at 4 °C and

18000 g (*r*_{av}, 8.26 cm) and the supernatant was incubated with 2 mg of DNaseI, 2 mg of RNaseA and 5 mM MgCl₂ for 30 min at room temperature. The protein that precipitated between 1.6 and 3.6 M (NH₄)₂SO₄ was collected by centrifugation, dissolved in a column buffer consisting of 20 mM Tris acetate/250 mM KCl/2 mM EDTA, pH 7.4 (3 ml/g of precipitate), and dialysed overnight against the same buffer. It was then loaded onto the PALO column, pre-equilibrated with the buffer, and the column was continuously washed until all unbound protein was eluted. OTCase was eluted with 10 mM carbamoyl phosphate in column buffer. The column was reconditioned with 3 vol. of column buffer containing 2 M KCl and re-equilibrated with column buffer. Fractions containing OTCase activity were pooled, and stored in 50 % v/v glycerol at -20 °C. Protein concentrations were determined with a dye-binding protein assay (Bio-Rad) using bovine serum albumin as a standard.

Purification of the R277W mutant was performed as outlined above, except that the concentration of KCl in the column buffer was decreased to 200 mM.

Analytical gel chromatography

The hydrodynamic radius of wild-type and R277W OTCase was determined with a calibrated 10 mm × 150 mm Sephacryl S-300 (Pharmacia) column in 20 mM Tris acetate/200 mM KCl/2 mM EDTA, pH 7.4. Partition coefficients (*σ*) for a set of proteins with known Stokes' radii, wild-type OTCase and the R277W mutant were determined using Blue Dextran and glycine for the void and included markers [14]. Stokes' radii for wild-type and R277W OTCase were determined from a plot of *σ* versus log(Stokes' radius). Protein concentration ranged between 0.1 and 0.6 mg/ml.

Enzyme assays

OTCase activity was measured with the colorimetric assay of Pastra-Landis et al. [15], which detects the formation of L-citrulline. Unless otherwise indicated, the assay buffer was 50 mM Tris acetate/2 mM EDTA, pH 8.3. Standard curves for L-citrulline were generated in each experiment as this assay is sensitive to ambient conditions [15,16]. One unit of enzyme catalyses the formation of 1 μmol/min of L-citrulline at 25 °C.

RESULTS

Expression and purification

Although approx. one-quarter of the cellular protein synthesized following induction in cells transformed with pETOTCII was OTCase, approx. one-half of it was sequestered in inclusion bodies. Soluble OTCase was maximized by optimizing IPTG concentrations and the duration and temperature of incubation. Growth in the presence of betaine or sucrose, which has increased yields in other expression systems, was not effective. Attempts to recover active OTCase from the inclusion bodies through the use of detergents and/or guanidine hydrochloride or urea, followed by overnight dialysis during which the denaturant was incrementally diluted, were unsuccessful. Although the OTCase in inclusion bodies could be solubilized by hexadecyltrimethyl ammonium bromide, the protein was inactive. Greatly increasing the renaturation time (> 45 h), as described by Marshall and Cohen [17] and Murakami et al. [18] for bovine and rat OTCase respectively, resulted in enzyme with approx. 20 % of the specific activity of the soluble protein purified by affinity chromatography. By comparison, chaperonin-mediated renaturation results in an 80 % recovery of activity after a 10 min incubation [19].

Figure 3

(A) SDS/PAGE gel of samples from various stages of the purification of human OTCase stained with Coomassie Blue. Lane 1, cell lysate; lane 2, cell supernatant; lane 3, supernatant after precipitation in 1.6 M $(\text{NH}_4)_2\text{SO}_4$; lane 4, sample applied to PALO column; lane 5, fractions eluted in wash from PALO column; lane 6, pooled fractions eluted with carbamoyl phosphate; lane 7, purified R277W. Lanes 1–3 were diluted 10-fold to minimize overloading. (B) Elution profile from PALO column in wild-type OTCase purification: (i) application of sample to the column and wash, (ii) application of 10 mM carbamoyl phosphate, and (iii) application of 2 M KCl. The small peak appearing after (iii) has an $A_{260/280}$ of 2 and probably represents nucleic acids.

Attempts to purify OTCase by ion exchange chromatography on DEAE-Sephadex resulted in large losses in activity, as reported by Kalousek et al. [20], although losses were reduced by using a DEAE fast-flow column, and decreasing the pH to 7 to prevent adsorption of OTCase to the matrix. $(\text{NH}_4)_2\text{SO}_4$ precipitation and affinity chromatography on a PALO affinity column proved more efficient and resulted in high yields. SDS protein electrophoretic gels of samples from various stages of the purification are shown in Figure 3. Co-expression of OTCase with GroES and GroEL allowed up to 90 % of the active protein to be recovered. The efficiency of the PALO affinity column was increased approx. 2.4-fold by incorporating a step in which nucleic acids were digested and removed by dialysis before application of the sample to the column. Dithiothreitol and β -mercaptoethanol were not used in the purification, since they inactivate the enzyme.

The overall yield of active OTCase was 90 %, with a specific activity of $40.2 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$; 17 mg of pure protein was obtained from 1 litre of culture. While the host strain of *E. coli* has an intact copy of the *argI* gene, it is induced only when arginine is depleted, and control experiments indicated that

Figure 4 Determination of hydrodynamic radius

Calculated Stokes' radii for wild-type ($\square$) and R277W OTCase ($\circ$) and the catalytic trimer of *E. coli* ATCase ($\triangle$) are shown. Proteins used for calibration ($\bullet$) were cytochrome *c*, myoglobin, ovalbumin, bovine serum albumin, aldolase, catalase and ferritin.

< 0.8 % of the OTCase activity obtained was due to contamination by the bacterial enzyme (results not shown). Purified OTCase can be stored in 20 mM Tris acetate/2 mM EDTA, pH 7.4 with 50 % glycerol at -20°C without loss of activity for several months.

A similar procedure was used to purify the R277W mutant, except that the concentration of KCl in the buffer for the PALO column was reduced from 250 to 200 mM to increase specific binding while still minimizing binding of other molecules. Pure protein (10 mg) was obtained from 1 litre of culture. Yield of this mutant was significantly lower at every step in the purification, so that the final yield was only 25 %.

Analytical gel chromatography

The Stokes' radius of purified wild-type and R277W OTCase are $37.0 \pm 1.1 \text{ \AA}$ and $37.8 \pm 1.1 \text{ \AA}$ respectively. These values are in good agreement with the Stokes' radius of the catalytic trimer of *E. coli* ATCase (Figure 4). The Stokes' radius calculated by modelling the OTCase trimer as an oblate ellipsoid with principal axes of 81 $\text{\AA}$ and 49.5 $\text{\AA}$ (derived from the homology model) is 35.1 $\text{\AA}$. This value does not account for hydration of the protein. With a hydration of 0.3 cm^3 of water/ cm^3 of dry protein, the calculated Stokes' radius is 37.5 $\text{\AA}$.

Inhibitors and activators

The activity of OTCase is sensitive to a variety of agents. Like *E. coli* OTCase [21], human OTCase is inactivated by Zn(II), although at lower (micromolar) concentrations (results not shown). Micromolar dithiothreitol and millimolar β -mercaptoethanol also inhibit human OTCase (results not shown). Enzyme activity increases with ionic strength, up to $I = 0.1 \text{ M}$. This ionic strength effect accounts in part for the 4–6-fold increase in activity, relative to 50 mM Tris acetate, pH 8.3, observed in buffers that have high ionic strengths, such as the tripartite buffer used here in the pH studies or the borate buffer used in the thermal stability experiments.

Enzyme kinetics

Titration curves for L-ornithine and carbamoyl phosphate at pH 8.3 for both wild-type and R277W OTCase are shown in

Figure 5 Effect of substrate concentrations on the velocity of the reaction catalysed by wild-type (■) and R277W mutant (○) OTCase at 25 °C in 50 mM Tris acetate/2 mM EDTA, pH 8.3

When [carbamoyl phosphate] was varied (upper panel), [L-ornithine] was fixed at 4 mM and 50 mM for the wild-type and mutant enzymes respectively. When [L-ornithine] was varied (lower panel), [carbamoyl phosphate] was fixed at 4.8 mM. In the absence of substrate inhibition, the curves were fitted to the Hill equation:

$$V = \frac{V_{\max} [S]^{nH}}{K' + [S]^{nH}} \quad (1)$$

where $K' = (K_m^{app}) \approx [S]_{0.5}^{nH}$

Curves showing substrate inhibition were fit to an equation describing cooperative substrate binding and partial non-competitive substrate inhibition:

$$V = \frac{V_{\max} + \frac{V_i [S]^2}{K_i^2}}{1 + \frac{K'}{[S]^{nH}} + \frac{[S]^2}{K_i^2}} \quad (2)$$

where K_i is the dissociation constant for inhibitory substrate binding and V_i is the limiting velocity at maximal substrate inhibition [16]. The non-linear least-squares program NONLIN [38] was used throughout. K_m values for carbamoyl phosphate were 0.05 ± 0.01 and 0.15 ± 0.01 mM for wild-type OTCase and the R277W mutant respectively. $V_{\max}$ values were 41.3 ± 1.4 and 74.1 ± 1.4 $\mu\text{mol}/\text{mg} \cdot \text{min}$ respectively. The maximal conversion of carbamoyl phosphate to citrulline was 37% at the lowest concentrations used. Control experiments showed the time-dependent conversion of carbamoyl phosphate to citrulline to be linear up to 97% substrate consumption. K_m values for L-ornithine were 0.11 ± 0.02 and 6.85 ± 1.3 mM, while $V_{\max}$ values were 62.5 ± 3.4 and 84.3 ± 3.7 $\mu\text{mol}/\text{mg} \cdot \text{min}$ for wild-type and mutant respectively. The L-ornithine titration for wild-type shows pronounced substrate inhibition, with a V_i of 23.6 ± 1.5 $\mu\text{mol}/\text{mg} \cdot \text{min}$ and a K_i of 2.5 ± 0.4 mM. The inset replots the ornithine titration curves at physiological concentrations of ornithine.

Figure 6 pH dependence of the kinetic parameters of the wild-type enzyme

Each point is taken from a complete titration curve in which [L-ornithine] is varied, while [carbamoyl phosphate] was fixed at 4.8 mM. Panels **A** and **C**: K_m and k_{cat} values were calculated by using equations (1) or (2). Open squares in panels **A** and **B** show the pH dependence of *E. coli* OTCase from [28]. Concentrations of the zwitterionic forms of L-ornithine at each pH were obtained using the equation:

$$\text{Subs}_{\text{tot}} = \frac{10^{\text{pH} - \text{p}K_{a2}}}{1 + 10^{\text{p}K_{a1} - \text{pH}} + 10^{\text{pH} - \text{p}K_{a2}} + 10^{2\text{pH} - \text{p}K_{a2} - \text{p}K_{a3}}}$$

where $\text{p}K_{a1}$, $\text{p}K_{a2}$, and $\text{p}K_{a3}$ are 1.7, 8.7 and 10.8 [39] and are used to calculate K_m values for the zwitterionic forms of L-ornithine (panel **B**). Panel **D**: $k_{\text{cat}}^{\text{zwitterion}}/K_m$ calculated from panels **B** and **C**. The buffer was 51 mM 2-(*N*-morpholino)ethanesulphonic acid, 1 mM *N*-ethylmorpholine and 51 mM diethanolamine.

Figure 5. Wild-type OTCase shows pronounced substrate inhibition with L-ornithine, while R277W does not. There is also slight substrate inhibition of the wild-type enzyme, but not of the R277W mutant, by carbamoyl phosphate. Apparent K_m values for the wild-type enzyme (0.05 ± 0.01 mM for carbamoyl phosphate and 0.11 ± 0.02 mM for ornithine) were similar to those seen for other OTCases [20,22–26]. The R277W mutation reduces affinity for L-ornithine by nearly 70-fold while decreasing affinity for carbamoyl phosphate only three-fold. Although the fitted value of $V_{\max}$ for R277W is slightly larger than for the wild-type enzyme (84.3 ± 3.7 $\mu\text{mol}/\text{min}$ per mg versus 62.5 ± 3.4 $\mu\text{mol}/\text{min}$ per mg), the greatly reduced affinity of the mutant for ornithine results in the activity of the mutant being substantially lower than the wild-type enzyme at physiological concentrations (see inset to Figure 5).

The gel chromatography results suggest that the reduced affinity for L-ornithine of R277W is not a result of a change in the state of association of the protein.

pH dependence

Figure 6 shows the pH dependence of the apparent kinetic parameters for L-ornithine, determined in a tripartite buffer designed to maintain constant ionic strength over the pH range

Figure 7 Thermal stability of wild-type (■) and R277W (○) OTCase

Enzyme diluted to 0.045 mg/ml in 50 mM borate/50 mM KCl/2 mM EDTA, pH 8.3 was incubated at the indicated temperatures for 10 min, then quickly chilled on ice and assayed within 15 min. The solid lines through the data were calculated assuming a two-state transition from a fully active to a fully inactive species of the form:

$$f_{\text{active}} = e^{k(T - T_m)}$$

where T_m is the temperature at the midpoint of the transition, and k is a fitting constant. The temperatures at which 50% of the activity of the wild-type and mutant protein is lost are 56 ± 1 °C and 51.5 ± 1 °C respectively.

5–11 [27]. The apparent dependence of k_{cat} on pH is bell-shaped with a maximum at around pH 8; however, OTCase gradually loses activity when incubated above pH 8.5 or below pH 6.5. The apparent K_m shows little variation over pH 7.5–10, but increases sharply with decreasing pH below pH 7.0.

This pH dependence is similar to that reported for crude homogenates of human liver and purified *E. coli* and bovine OTCase and has been interpreted as indicating that L-ornithine binds as a zwitterion, with its side chain uncharged [1,28,29]. The pH dependence of the calculated K_m values for the zwitterion is the opposite of that for the apparent K_m values, remaining constant over the pH range 6–8.5 and increasing at high pH (Figure 6b). These values agree closely with those reported for *E. coli* OTCase [28].

Thermal stability

Figure 7 compares the thermal stability of wild-type OTCase and R277W as monitored by enzymic activity. The mutation reduces the temperature at which 50% of the enzyme activity is lost under these conditions by approx. 5 °C, from 56 ± 1 °C to 51.5 ± 1 °C.

DISCUSSION

Ornithine Transcarbamylase Deficiency has been well studied in terms of its clinical severity, the range of enzyme deficiency and the location of mutations in the OTC gene of affected families. However, biochemical analyses of wild-type and mutant human enzymes are sparse by comparison with OTCases from *E. coli* and other organisms that can be obtained more readily. Extensive kinetic analysis has been performed on bovine OTCase [17,29,30]. Use of the efficient overexpression system and purification procedure for recombinant human OTCase described here will enable detailed investigations of the molecular basis of ornithine

transcarbamylase deficiency. Clinically important mutations can be created readily and expressed in *E. coli* for further characterization. The specificity of the PALO affinity column allows purification with yields of 90% for wild-type, and can be used to purify mutants such as R277W that are able to bind one or both substrates.

Although the crystal structure of human OTCase is not yet known, the crystal structures of *E. coli* ATCase and *P. aeruginosa* OTCase provide useful insights, since the OTCases and ATCases of various species form a family of proteins in which three-dimensional structure is highly conserved [4–7,9,31]. The crystal structure of the anabolic *P. aeruginosa* OTCase and *E. coli* ATCase have very similar folds, with an r.m.s. difference in common backbone atom positions in the range 0.3–5.1 Å [5]. Similarly, the overall r.m.s. difference in backbone atoms between our homology model of human OTCase and *P. aeruginosa* OTCase is 2.9 Å. The homology of the carbamoyl phosphate binding domains of OTCases and ATCases has also been demonstrated functionally; Houghton et al. have shown that a chimaeric enzyme comprising the carbamoyl phosphate binding domain of *E. coli* OTCase and the aspartate binding domain of *E. coli* ATCase has ATCase activity [4].

Eight of the 12 residues at the active site of *E. coli* ATCase are conserved in human OTCase. Although residues involved in binding ornithine have not been well characterized, Asp-263, Ser-267, Met-268, Gly-269, His-302, Cys-303, Leu-304, and Pro-305 are highly conserved in all OTCase sequences and align with residues involved in aspartate binding in ATCase and hence are likely to be implicated in binding ornithine. The aspartate in *P. aeruginosa* that is equivalent to Asp-263 in the human enzyme has been postulated to bind the amino group of ornithine [5]. S-Thioethylamine modification of OTCase has shown the existence of a methionine that affects binding of norvaline, but not of carbamoyl phosphate [30]. The invariance of Met-268 in all OTCases, and its location in the ornithine domain of the homology model and crystal structure make it a possible candidate.

The similarity of the kinetic parameters of *E. coli*, bovine and human OTCase, including their pH dependence, suggests that their active sites are structurally very similar and it is likely that human OTCase, like the *E. coli* and bovine enzymes, binds the ornithine zwitterion with its side chain uncharged [1,28,29].

Despite the similarities, there are functional and structural differences between the human and bacterial enzymes. Values of V_{max} for human OTCase are approx. 10-fold lower than those reported for *E. coli* OTCase and human OTCase appears to be inactivated by Zn(II) at concentrations approx. 100-fold lower than *E. coli* OTCase. In addition to many specific sequence differences, some bacterial enzymes, including the *E. coli* and the catabolic *P. aeruginosa* OTCases, also have an insertion of approx. 19 amino acids in the vicinity of residues 306–307 of the human enzyme, in a loop that extends away from the putative ornithine binding site formed by residues 301–305 (Figure 1). Given these differences, studies of the human enzyme are important to better understand the function of this region of the protein.

In the homology-based model for human OTCase, Arg-277 is positioned in a loop remote from the active site (Figure 2), raising the question of how a substitution of this residue gives rise to defective enzyme function, albeit generally not clinically apparent until after the newborn period and with effects less severe than those of many other mutations. The effect of the R277W mutation in human OTCase is similar to the W243I mutation in the same region of the molecule in *E. coli* OTCase [32]; both increase the apparent K_m for L-ornithine with little

change in the affinity for carbamoyl phosphate. Our value for the K_m of R277W for L-ornithine is similar to that obtained with a crude preparation of patient liver [33] under slightly different assay conditions [27]. The modest decreases in affinity and stability are consistent with the nature of the clinical effects of this mutation. Although the R277W mutant can catalyse the formation of citrulline at rates greater than that of the wild-type given sufficiently high concentrations of ornithine, estimates of the ornithine concentration in the mitochondrial matrix range from 0.3 mM to 1 mM [34,35]. At these concentrations, the velocity of R277W is 12 to 24% of wild-type (Figure 5, inset).

The location of the mutation in the homology model suggests a structural basis for its effects on the catalytic properties of the enzyme. The loop in which Arg-277 is located is analogous to the 240's loop of *E. coli* ATCase. This loop plays a critical role in the catalytic mechanism of ATCase: in the T state, it functions as the contact between the two catalytic subunits, while in the R state, it swings towards the active site to close the two domains of the catalytic chain around the substrates [36]. While the intersubunit contact is not present in the catalytic subunit of ATCase or in OTCase, domain closure would be expected still to be an essential part of the catalytic mechanism. Interestingly, the long insertion in bacterial OTCases forms a loop adjacent to the 240's loop analogue, and may also constrain motion of this loop. In *P. aeruginosa* OTCase, the Arg-277 homologue is fully visible on the surface of the molecule, but with only 30% of the solvent accessibility of a fully exposed arginine. Although the mutation to a more hydrophobic residue may also affect the conformation of this region, the Arg-277 homologue is in close contact with a conserved aspartate (Asp-196). This aspartate is approx. 98% buried, suggesting that the altered pH dependence reported by Hata et al. for R277W [33] may be due to greater difficulty in deprotonating this buried aspartate in the absence of Arg-277. The human R277W mutant has a similar pH dependence.

The sheer number of OTC mutations that have been identified clinically is not only of clinical relevance but also of biochemical importance since many of the changes are at sites with no apparent functional role. These mutations warrant further investigation. The complex linkages between folding, assembly and catalytic activity that result from the trimeric structure of OTCase, in which the active site is formed by both interdomain and interchain interactions, may be in part the reason why mutations of OTCase are the most commonly detected inheritable urea-cycle disorders. Fully understanding how these mutations give rise to clinical symptoms will require a combination of experiments *in vitro* of the type performed here, experiments with crude homogenates (such as have already been carried out with the R277W mutant to determine that purification has not altered the enzymological properties of the enzyme), as well as analyses *in vivo*. Careful studies of structure-function relationships in the purified enzyme are an essential part of this process.

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