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Residue N84 of Yeast Cystathionine β-Synthase is a Determinant of Reaction Specificity ☆

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ABSTRACT

Cystathionine β-synthase (CBS) catalyzes the pyridoxal 5'-phosphate (PLP)-dependent condensation of L-serine and L-homocysteine to form L-cystathionine in the first step of the reverse transsulfuration pathway. Residue N84 of yeast CBS (yCBS), predicted to form a hydrogen bond with the hydroxyl moiety of the PLP cofactor, was mutated to alanine, aspartate and histidine. The truncated form of yCBS (ytCBS, residues 1-353) was employed in this study to eliminate any effects of the C-terminal, regulatory domain. The $k_{cat}/K_m^{L\text{-}Ser}$ of the N84A, N84D and N84H mutants for the β-replacement reaction is reduced by a factor of 230, 11000 and 640, respectively. Fluorescence resonance energy transfer between tryptophan residue(s) of the enzyme and the PLP cofactor, observed in the wild-type enzyme and N84A mutant, is altered in N84H and absent in N84D. PLP saturation values of 73%, 30% and 67% were observed for the alanine, aspartate and histidine mutants, respectively, compared to 98% for the wild-type enzyme. A marginal β-elimination activity was detected for N84D ($k_{cat}/K_m^{L\text{-}Ser} = 0.23 \pm 0.02\ M^{-1}\ s^{-1}$) and N84H ($k_{cat}/K_m^{L\text{-}Ser} = 0.34 \pm 0.06\ M^{-1}\ s^{-1}$), in contrast with wild-type ytCBS and the N84A mutant, which do not catalyze this reaction. The ytCBS-N84D enzyme is also inactivated upon incubation with L-serine, via an aminoacrylate-mediated mechanism. These results demonstrate that residue N84 is essential in maintaining the orientation of the pyridine ring of the PLP cofactor and the equilibrium between the open and closed conformations of the active site.

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Cystathionine β-synthase (CBS; E.C. 4.2.1.22.) catalyzes the pyridoxal 5'-phosphate-(PLP) dependent condensation of L-serine (L-Ser) and L-homocysteine (L-Hcys) to form L-cystathionine (L-Cth) [1]. The first half reaction of the CBS-catalyzed β-replacement comprises abstraction of the C_α proton of L-Ser, followed by β-elimination of the hydroxyl group. Reaction of the resulting aminoacrylate intermediate with L-Hcys and release of the L-Cth product regenerates the internal aldimine form of the enzyme [2]. Mechanistic studies relying on the spectral properties of catalytic intermediates are precluded in human CBS (hCBS) because of the overlapping absorbance spectra of the PLP and heme cofactors. Therefore, as yeast CBS (yCBS; CYS4/Ygr155w) catalyzes the same reaction as the human enzyme and shares 47% amino acid sequence identity in the catalytic domain, but does not contain heme, it provides a useful proxy for the human enzyme [2,3]. The structure of the truncated form of hCBS, lacking the C-terminal, regulatory domain, provides a model for studies on yCBS, for which no structure is available [4,5].

The interactions between PLP and active-site residues, which maintain a catalytically-productive orientation of the cofactor, include hydrogen bonds to N1 (the pyridinium nitrogen), O3' (the hydroxyl moiety) and the phosphate group [6]. The residue that interacts with the N1 position of the cofactor plays a central role in modulating the electronic properties of the pyridine ring [6]. In PLP-dependent enzymes of the β-family this residue is a serine, as exemplified by hCBS and *Salmonella typhimurium* *O*-acetylserine sulfhydrylase (stOASS) and tryptophan synthase (stTrpS) [4,5,7,8]. While the residue that interacts with N1 is strongly conserved within each of the fold types of PLP enzymes, greater variability is found at the position that interacts with O3'. For example, crystal structures show that the phenolic group of the PLP cofactor is within hydrogen-bonding distance of a histidine (H217) in *Rhodobacter capsulatus* 5-aminolevulinate synthase (rcALAS) and a tyrosine (Y225) in *Escherichia coli* aspartate aminotransferase (ecAATase), which both belong to the large, α-family of PLP-dependent enzymes, an asparagine in hCBS (N149) and stOASS (N71), both members of the β-family, and an arginine (R136) in *Bacillus stearothermophilus* alanine racemase, of the small alanine racemase family [4,7,9–12]. The specific role of the residue interacting with PLP-O3' depends on the nature of the reaction catalyzed. For example, while Y225 of ecAATase lowers the pK_a of the internal aldimine, β-subunit

Abbreviations: ALAS, 5-aminolevulinate synthase; AATase, aspartate aminotransferase; L-Cth, L-cystathionine; CBL, cystathionine β-lyase; CBS, cystathionine β-synthase; hCBS, human CBS; yCBS, yeast CBS; ytCBS, truncated yCBS (residues 1 - 353); DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); L-Hcys, L-homocysteine; LDH, L-lactate dehydrogenase; OASS, *O*-acetylserine sulfhydrylase; PLP, pyridoxal 5′-phosphate; TrpS, tryptophan synthase

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residue Q114 (βQ114) is involved in the partitioning of the reactive aminoacrylate intermediate and allosteric communication in stTrpS [13,14]. The N84A,D,H site-directed mutants were constructed to probe the role of this residue in yCBS. The truncated form of yCBS (ytCBS, residues 1-353) was employed to avoid sample heterogeneity caused by cleavage of the labile regulatory domain and to facilitate interpretation of the data in the context of other studies that have investigated the roles of specific active-site residues of ytCBS [15,16]. The histidine and aspartate substitutions of ytCBS-N84 were selected to maintain the potential for polar contacts within the active-site, in contrast with N84A, while modifying the capacity of this residue to form hydrogen bonds. Characterization of these mutants demonstrates that residue N84 is involved in binding of the L-Ser substrate and plays an important role in maintaining a catalytically-productive orientation of the PLP cofactor and partitioning of the reactive aminoacrylate intermediate.

1. Methods

1.1. Reagents

L-Cth [*S*-(2-amino-2-carboxyethyl)-L-homocysteine], β-nicotinamide adenine dinucleotide (NADH, reduced form), N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid (AMPSO), N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), N-Tris (hydroxymethyl)methyl-3-aminopropanesulfonic Acid (TAPS), L-Ser and L-Hcys thiolactone were purchased from Sigma. Dithiothreitol (DTT), ampicillin, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and imidazole were obtained from Fisher Scientific and L-lactate dehydrogenase (LDH) and protease inhibitor (Complete EDTA-free) tablets were from Roche Diagnostics. Restriction endonucleases and T4 DNA ligase were from New England Biolabs. Nickel-nitrilotriacetic acid (Ni-NTA) resin was from Qiagen. L-Hcys was prepared from the thiolactone according to the method of Kashiwamata and Greenberg [17]. Cystathionine β-lyase (CBL) was expressed and purified as described previously [18].

1.2. Construction, Expression and Purification of ytCBS mutants

Overlap-extension polymerase chain reaction (PCR), with the pSECseq1 (CGG TTC TGG CAA ATA TTC TGA AAT GAG CTG) and pSECseq7r (GCC CGC CAC CCT CCG GGC CGT TGC TTC GC) flanking primers and either the N84Af (CCT ACT TCT GGT GCG ACC GGT ATC GGT CTA GC), N84Df (CCT ACT TCT GGT GAT ACC GGT ATC GGT CTA GC) or N84Hf (CCT ACT TCT GGT CAT ACC GGT ATC GGT CTA GC) mutagenic primers and their reverse complements, was employed to introduce the N84A, N84D and N84H site-directed mutations in the pTSECb-His plasmid. This plasmid contains the gene encoding the truncated form of yCBS (ytCBS; residues 1-353), lacking the regulatory domain, and with a C-terminal, 6-His affinity tag [15]. The amplification product was digested with *Bam*HI and *Pst*I, inserted at the corresponding sites of the pT-SECb-His vector and transformed into the *Escherichia coli* strain DH10B (Gibco BRL) *via* electroporation (Gene Pulser, BioRad). The N84A, N84D and N84H site-directed mutants were expressed and purified via Ni-NTA affinity chromatography (Qiagen), as previously described for the heterologous expression of 6-His tagged ytCBS [15].

1.3. Steady-State Kinetics

Enzyme activity was measured in a total volume of 1 mL with a model HP8453 spectrophotometer (Agilent) or in 0.1 mL with a Spectramax model 340 microtiter plate spectrophotometer (Molecular Devices) at 37 °C. The assay buffer was comprised of 50 mM Tris, pH 8.6, and 20 μM PLP. A background rate, for all components except the ytCBS enzyme, was recorded for each sample before initiating the reaction with the addition of ytCBS. Data was fit by nonlinear regression with the program SAS (SAS Institute, Cary, NC). The assay employing cystathionine β-lyase and lactate dehydrogenase (CBL-LDH) as coupling enzymes, in which formation of L-Cth is detected continuously, was employed to monitor the ytCBS β-replacement activity. The LDH and 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) assays were employed to monitor the β-elimination of L-Ser, producing pyruvate and NH_3, and the hydrolysis of L-Cth, to L-Ser and L-Hcys, respectively [15,18]. The k_{catE} and $K_{mE}^{L\text{-}Ser}$ and the k_{catR} and $K_{mR}^{L\text{-}Cth}$ for the β-elimination of L-Ser and the L-Cth hydrolysis reactions, respectively, were determined from the fit of the data to the Michaelis-Menten equation. The R and E subscripts denote the reverse-physiological L-Cth-hydrolysis and the β-elimination of L-Ser, respectively. The k_{cat}/K_m for each reaction was obtained independently from Eq. (1).

$$\frac{v}{[E]} = \frac{k_{cat}/K_m \times [L\text{-}Cth]}{1 + [L\text{-}Cth]/K_m} \tag{1}$$

The kinetic parameters of the β-replacement reaction of ytCBS-N84A were determined from the fit of the data to Eq. (2), where the F subscript denotes the physiological, β-replacement activity and $F_1^{L\text{-}Hcys} = 1 + [\text{L-Hcys}]/K_{tF1}^{L\text{-}Hcys}$ and $F_2^{L\text{-}Hcys} = 1 + [\text{L-Hcys}]/K_{tF2}^{L\text{-}Hcys}$ [19]. Eq. (2) was rearranged by dividing all terms by $K_{mF}^{L\text{-}Ser}$ or $K_{mF}^{L\text{-}Hcys}$ to obtain independent values for $k_{catF}/K_{mF}^{L\text{-}Ser}$ and $k_{catF}/K_{mF}^{L\text{-}Hcys}$, respectively.

$$\frac{v}{[E]} = \frac{k_{catF}[L\text{-Ser}][L\text{-Hcys}]}{K_{mF}^{L\text{-Hcys}}[L\text{-Ser}] + K_{mF}^{L\text{-Ser}}F_1^{L\text{-}Hcys}[L\text{-Hcys}] + F_2^{L\text{-}Hcys}[L\text{-Ser}][L\text{-Hcys}]} \tag{2}$$

The kinetic parameters for the β-replacement reaction of the N84D and N84H mutants was determined from the fit of the data to Eq. (3), which incorporates terms (k_{catE} and $K_{mE}^{L\text{-}Ser}$) for the β-elimination activity observed for these enzymes [15]. The E subscript denotes the non-physiological, β-elimination of L-Ser, to produce pyruvate and ammonia. The values of k_{catE} and $K_{mE}^{L\text{-}Ser}$, determined via the β-elimination assay, were substituted into Eq. (3), thereby reducing the number of kinetic parameters to be determined. Eq. (3) was rearranged by dividing all terms by $K_{mF}^{L\text{-}Ser}$ or $K_{mF}^{L\text{-}Hcys}$ to obtain independent values for $k_{catF}/K_{mF}^{L\text{-}Ser}$ and $k_{catF}/K_{mF}^{L\text{-}Hcys}$, respectively.

$$\frac{v}{[E]} = \frac{k_{catE}K_{mF}^{L\text{-Hcys}}[L\text{-Ser}] + k_{catF}[L\text{-Ser}][L\text{-Hcys}]}{K_{mE}^{L\text{-Ser}}K_{mF}^{L\text{-Hcys}} + K_{mF}^{L\text{-Hcys}}[L\text{-Ser}] + K_{mF}^{L\text{-Ser}}F_1^{L\text{-}Hcys}[L\text{-Hcys}] + F_2^{L\text{-}Hcys}[L\text{-Ser}][L\text{-Hcys}]} \tag{3}$$

1.4. Fluorescence Spectroscopy

Fluorescence spectra were acquired with a Cary Eclipse spectrofluorimeter (Varian) at 37 °C in 50 mM Tris, pH 8.6. To investigate the possibility of energy transfer from one or more of the four tryptophan residues of ytCBS to the PLP, a probe of cofactor orientation, the fluorescence spectrum ($\lambda_{ex} = 298$ nm) of 20 μM enzyme was recorded from 480-560 nm, with excitation and emission slit widths of 5 nm, in the absence and presence of 50 mM L-Ser [20]. Differences in the efficiency of energy transfer from tryptophan residues to the PLP cofactor have been observed in several PLP-dependent enzymes. For example, Frederiuk and Shafer [21] and McClure *et al.* [20], working on D-serine dehydratase and stOASS, respectively, have both suggested that formation of the external aldimine results in a change in the conformation of the cofactor within the active site which increases energy transfer from tryptophan residues of the protein to the PLP cofactor. Therefore, energy transfer from tryptophan residues to the PLP cofactor provides a probe of changes to cofactor orientation for investigations of the role of active-site residues. The apparent dissociation constant for the enzyme-aminoacrylate (E-AA) complex due to L-Ser association with free enzyme ($K_{d(app)}^{L\text{-}Ser}$) was determined by the protocol described by Jhee et al. [19], where a 1.0 μM solution of

ytCBS enzyme was titrated with aliquots of L-Ser, and the increase in fluorescence at 540 nm ($\lambda_{ex} = 460$ nm), due to formation of the aminoacrylate (AA) intermediate, was monitored. The change in fluorescence at 540 nm (ΔF_{max}) was plotted versus [L-Ser] and fit to Eq. (4) [19].

$$\Delta F = \frac{\Delta F_{\max}[L-Ser]}{K^{L-Ser}_{d(app)} + [L-Ser]} \quad (4)$$

The method of Adams [22] was adapted for determination of the PLP saturation of wild-type and mutant enzymes. The enzymes were diluted (to 10 μM) in 200 μL of 5 mM phosphate buffer, pH 7.4. Following the addition of an equal volume of 11 % trichloroaceteic acid and incubation for 15 min at 50 °C, 140 μL of 3.3 M K_2HPO_4 and 50 μL of 0.02 M KCN were added and samples incubated at 50 °C for a further 25 min prior to the addition of 70 μL of 28% H_3PO_4 and 640 μL of 2 M potassium acetate, pH 3.8. The PLP concentration of the samples was determined from comparison of the fluorescence intensity at 425 nm ($\lambda_{ex} = 325$ nm) to a standard curve of 0-20 μM PLP. The PLP saturation of each enzyme was determined from the ratio of the PLP concentration to the concentration of enzyme monomer.

1.5. Absorbance Spectroscopy

Spectra were recorded from 250-1100 nm with a model HP8453 spectrophotometer (Agilent) at 37 °C. The internal aldimine of wild-type ytCBS and the N84A, N84D and N84H site-directed mutants were titrated *versus* pH. The pH of a solution of enzyme in 5 mM TAPS (p*K*a 8.4, pH 7.2), containing 0.5 M KCl, was varied by successive additions of 0.5 M AMPSO (p*K*a 9.0, pH 10.6) below pH 9.0, 0.5 M CAPS (p*K*a 10.4, pH 11.5) between pH 9.0- 10.5 and 1.0 M NaOH above pH 10.5. The enzyme solution was drawn through a 0.2-μm filter, to reduce light scattering from precipitate, and the pH of the solution was determined prior to each absorbance measurement. The reaction of a 14 μM solution of each of the N84 mutants with 0.1-100 mM L-Ser in 50 mM Tris, pH 8.6, was monitored for 120 min to observe the formation of the reaction intermediates.

2. Results

2.1. Steady state kinetic parameters

The protein expression levels of the N84A (84 mg/L), N84D (162 mg/L) and N84H (83 mg/L) site-directed mutants were comparable to wild-type ytCBS (36 mg/L), demonstrating that the mutants adopt a stable, folded conformation. The k_{catF}/K^{L-Ser}_{mF} of the β-replacement activity is reduced by 230, 11000 and 640-fold, respectively, for ytCBS-N84A, N84D and N84H, compared to the wild type enzyme (Table 1). The subscripts F, E and R in the kinetic parameters listed in Table 1 denote the physiological β-replacement reaction, the β-elimination of L-Ser, not catalyzed by the wild-type enzyme, and the reverse-physiological hydrolysis of L-Cth, respectively. The ~2 order-of-magnitude decrease in the k_{catF}/K^{L-Ser}_{mF} of N84A is the result of an 11-fold increase in K^{L-Ser}_{mF} and 20-fold decrease in k_{catF}. In contrast, the K^{L-Hcys}_{mF} value of N84A is only 2-fold greater than the wild-type enzyme (Table 1), suggesting that while residue N84 interacts directly or indirectly with L-Ser, the L-Hcys binding site of this mutant is unperturbed. The $K^{L-Ser}_{d(app)}$ of the N84A mutant is increased 2-fold, compared to the wild-type enzyme, suggesting an indirect role for N84 in substrate binding (Table 1). The k_{catR} of the reverse-physiological L-Cth hydrolysis reaction is reduced 145-fold for both the N84A and N84D mutants and 100-fold for N84H. Although the N84D ($k_{catE}/K^{L-Ser}_{mE} = 0.23 \pm 0.02$ M^{-1} s^{-1}) and N84H ($k_{catE}/K^{L-Ser}_{mE} = 0.34 \pm 0.06$ M^{-1} s^{-1}) mutants possess a marginal β-elimination activity, the k_{catE} of this reaction is 25 and 1300-fold, respectively, lower than the k_{catF} of the β-replacement reaction. The N84A mutant, like the wild-type enzyme, does not catalyze the conversion of L-Ser to pyruvate and ammonia (Table 1).

2.2. Formation of the aminoacrylate intermediate

The degree of PLP saturation of the wild-type, N84A, N84D and N84H enzymes is 98%, 73%, 30% and 67%, respectively, demonstrating that binding of the cofactor to the mutants is impaired, but not precluded. Comparison of the spectra of the wild-type and N84A mutant (Fig. 1) demonstrates that while the 412-nm peak, corresponding to the ketoenamine tautomer of the PLP cofactor, of N84A is only 53% the intensity of the same peak in the wild-type enzyme, the absorbance of N84A at 330 nm, corresponding to the enolamine tautomer, is 62% greater than the wild-type enzyme. A role for the corresponding N149 of hCBS in maintaining the balance between the ketoenamine and enolamine tautomers has also recently been suggested based on investigation of the Fe^{II}-CO complex of human CBS [23]. Weeks et al. [23] proposed that the tautomeric shift observed in hCBS is caused by loss of the hydrogen bond between O3' of the PLP cofactor and the side chain residue N149 due to disruption of a series of interactions resulting from reduction of the heme iron and switching of the C52 axial ligand for CO.

In contrast with the wild-type enzyme and the N84A and N84H mutants, formation of the aminoacrylate intermediate, with characteristic peaks at 320 and 460 nm, is not observed for the N84D mutant at L-Ser concentrations up to 100 mM and over a period of 2 h (Fig. 1). The broad, unresolved PLP spectrum of ytCBS-N84D decreases in intensity at ~412 nm, corresponding to the internal aldimine, and increases at ~320 nm upon reaction L-Ser (Fig. 2). The possibility that the ~320-nm species is indicative of substrate-induced inactivation, previously reported for the Q157H and Y158F mutants of ytCBS, was investigated [15]. Following a 3-h incubation of 20 μM N84D with 50 mM L-Ser, the remaining L-Ser was removed by dialysis in 5 mM Tris, pH 8.6. The spectrum of the enzyme was unchanged by the dialysis step. The pH was subsequently adjusted to 11.5 and an increase in absorbance at 424 nm, resulting from a vinylglyoxylic acid moiety linked to the C4' atom of the cofactor, was recorded [24–26]. The presence of the diagnostic 424-nm band following alkaline treatment, observed for ytCBS-N84D, but not the wild-type, N84A or N84H enzymes, demonstrates that N84D alone undergoes substrate-mediated inactivation. Addition of 0.1-100 mM L-Ser to the N84A and N84H mutants results in a decrease in absorbance at 412 nm and a concomitant increase in intensity at 460 and 320 nm, consistent with formation of the external aldimine of aminoacrylate (Fig. 1), as observed for the wild-type enzyme [27]. The linear increase in intensity at 320 nm, upon reaction of ytCBS-N84H with 50 mM L-Ser (Fig. 3), not observed for the wild-type and ytCBS-N84A enzymes, is due to the accumulation of pyruvate resulting from the β-elimination activity of this enzyme (Table 1). The N84D mutant possesses a similar level of β-elimination activity as N84H, but the increase in intensity at 320 nm (Fig. 2) observed for N84D is also due to the mechanism-based inactivation of this mutant. The 460-nm peak observed upon incubation of ytCBS-N84H with 50 mM L-Ser (Fig. 3) decreased over the course of the 2-h incubation with a concomitant increase in intensity in the 410-420-nm region.

2.3. Equilibrium spectroscopic studies

Absorbance spectra of the N84A, N84D and N84H site-directed mutants were recorded over the pH range of 7.1-12.5. The pK_a of the Schiff base nitrogen of the internal aldimine of ytCBS, which is >11 for the wild-type enzyme, is unchanged in the N84 mutants [18]. Similar results were observed for the alanine and aspartate mutants of residue S289, which interacts with N1 of the cofactor, while the corresponding S377D mutation in stTrpS results in a reduction of the pK_a of the internal aldimine to 7.63 ± 0.06 [16,28]. The decrease in

Table 1
Steady-State kinetic parameters for ytCBS and the N84A, N84D and N84H mutants.[a]

	ytCBS[b]	N84A[c]	N84D[c]	N84H[c]
L-serine + L-homocysteine → L-cystathionine				
k_{catF} (s^{-1})	17 ± 1	0.87 ± 0.09	0.0205 ± 0.0006	1.2 ± 0.1
$K_{mF}^{L\text{-}Ser}$ (mM)	0.7 ± 0.2	8 ± 2	9 ± 1	30 ± 7
$K_{mF}^{L\text{-}Hcys}$ (mM)	0.21 ± 0.04	0.46 ± 0.09	0.08 ± 0.01	0.7 ± 0.1
$k_{catF}/K_{mF}^{L\text{-}Ser}$ (M^{-1} s^{-1})	(2.5 ± 0.6) x 10^4	110 ± 20	2.3 ± 0.3	39 ± 7
$k_{catF}/K_{mF}^{L\text{-}Hcys}$ (M^{-1} s^{-1})	(8 ± 1) × 10^4	1900 ± 200	250 ± 30	1700 ± 100
$K_{iF1}^{L\text{-}Hcys}$ (mM)	1.0 ± 0.4	10 ±	8 ± 5	5 ± 2
$K_{iF2}^{L\text{-}Hcys}$ (mM)	15 ± 7	5 ± 1	n.d.	11 ± 4
$K_{d(app)}^{L\text{-}Ser}$ (mM)[d]	15.1 ± 0.6	34 ± 4	n.d.	n.d.
L-serine → pyruvate + NH_3				
$k_{catE}^{L\text{-}Ser}$ (s^{-1})	n.d.	n.d.	0.00082 ± 0.00002	0.00094 ± 0.00004
$K_{mE}^{L\text{-}Ser}$ (mM)	n.d.	n.d.	3.5 ± 0.4	2.7 ± 0.5
$k_{catE}/K_{mE}^{L\text{-}Ser}$ (M^{-1} s^{-1})	n.d.	n.d.	0.23 ± 0.02	0.34 ± 0.06
L-cystathionine → L-homocysteine + L-serine				
k_{catR} (s^{-1})	1.03 ± 0.02	0.0071 ± 0.0002	0.0072 ± 0.0009	0.0104 ± 0.0007
$K_{mR}^{L\text{-}Cth}$ (mM)	0.14 ± 0.01	0.022 ± 0.004	5 ± 1	2.1 ± 0.3
$k_{catR}/K_{mR}^{L\text{-}Cth}$ (M^{-1} s^{-1})	(7.5 ± 0.4) x 10^3	330 ± 50	2.4 ± 0.1	5.0 ± 0.5

[a] Kinetic parameters for the β-replacement, β-elimination and L-Cth hydrolysis activities are denoted by the subscripts F, E and R, respectively. Kinetic measurements were carried out in 50 mM Tris, pH 8.6, containing 20 μM PLP at 37 °C. n.d. denotes that activity was not detectable.

[b] From reference [15].

[c] β-replacement conditions: 0.4 μM CBL, 1.4 μM LDH, 300 μM NADH, 0.041-11.6 mM L-Hcys, 0.25-180 mM L-Ser, and 7.7 μM N84A, 51-103 μM N84D or 5.1-10.3 μM N84H. Data were fit to Eq. (2) (N84A) and Eq. (3) (N84D and N84H). β-elimination conditions: 1.25 mM NADH, 1.3 μM LDH, 0.1-120 mM L-Ser and 33 μM ytCBS, 15 μM N84A, 46 μM N84D or 23 μM N84H. Hydrolysis of L-Cth to L-Ser and L-Hcys conditions: 2 mM DTNB, 0.01-6.4 mM L-Cth and 15 μM N84A, 46 μM N84D or 23 μM N84H. β-elimination and L-Cth hydrolysis data were fit to the Michaelis-Menten equation and Eq. (1).

[d] $K_{d(app)}^{L\text{-}Ser}$ values were determined by the increase in fluorescence at 540 nm (λex = 460 nm), due to the formation of AA, resulting from a titration of 1.0 μM ytCBS enzyme with aliquots of L-Ser [19]. Data were fit to Eq. (4).

absorbance at 412 nm, corresponding to the ketoenamine form of the internal aldimine, at pH >11 is concomitant with the appearance of the ~330-nm absorbance of the enolamine form, for the wild-type, N84A (Fig. 4) and N84H enzymes. In contrast, at elevated pH an increase in absorbance at ~412 nm is observed for ytCBS-N84D (Fig. 4). The appearance of a similar, ~406-nm absorbance observed at pH ~13 for the Q114N mutant of the β-subunit of stTrpS (stTrpS-βQ114N) was shown to be comprised of 390 and 424-nm peaks, corresponding to the enolamine form of the internal aldimine and aminoacrylate-inactivated cofactor, respectively [14].

Fluorescence spectra (λ_{ex} = 298 nm and λ_{em} = 460-560 nm) of the wild-type and mutant enzymes were recorded in the absence and presence of 50 mM L-Ser (Fig. 5). Upon excitation at 298 nm, a peak is observed at ~500 nm in the emission spectrum of the wild type enzyme that is likely the result of Förster energy transfer from tryptophan residue(s) of the enzyme to the PLP cofactor. A similar effect has been observed for stOASS [20]. The ~500-nm peak of the N84A mutant is identical to the wild-type enzyme, while that of N84H shows a 67% increase in intensity (Fig. 5), suggesting that the orientation of the cofactor is altered by replacement of N84 with histidine. This corresponds to the observed β-elimination activity of ytCBS-N84H, but not N84A. The ~500-nm fluorescence intensity of ytCBS-N84D is 43% of the wild-type enzyme, in agreement with the 30% PLP saturation of this mutant. Addition of 50 mM L-Ser to the wild type and N84A and N84H enzymes results in a shift in the fluorescence emission peak from 500 to 540 nm (Fig. 5). The ratio of the intensity of the 500 and 540-nm peaks, corresponding to the internal aldimine and the external aldimine of aminoacrylate, respectively, of the wild type, N84A and N84H enzymes is 0.95, 1.1 and 3.5, respectively, providing further evidence of differences in the orientation of the PLP cofactor within the ytCBS-N84H active site. The shift in fluorescence emission to 540 nm is not observed for the N84D mutant, which does not form an observable aminoacrylate intermediate upon reaction with L-Ser (Figs. 1 and 2).

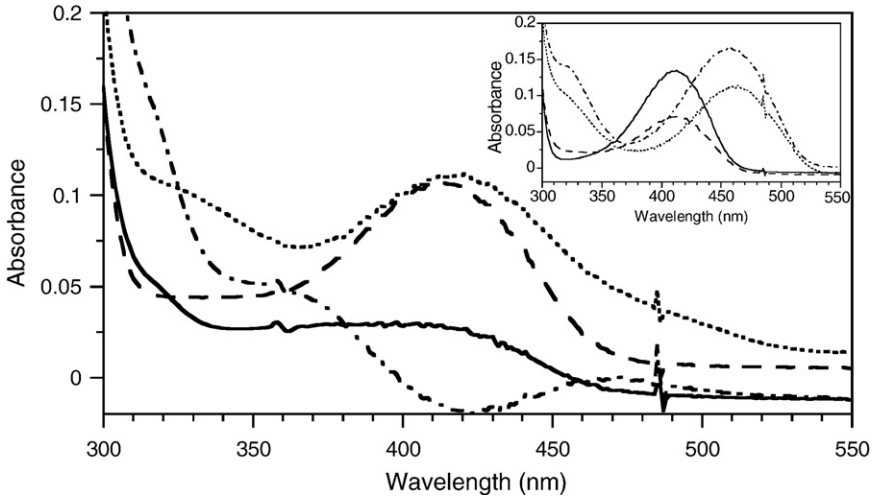

Fig. 1. Spectra of 14 μM ytCBS-N84D (–) and N84H (- - -) alone and after 2 h incubation with 50 mM L-Ser (-·-·-· N84D; ··· N84H). Inset: wild-type ytCBS (–) and N84A (- - -) alone and after 2 h incubation with 50 mM L-Ser (-·-·-· ytCBS; ··· N84A). Conditions: ytCBS or N84A,D,H mutants (14 μM) in 50 mM Tris, pH 8.6 at 37 °C.

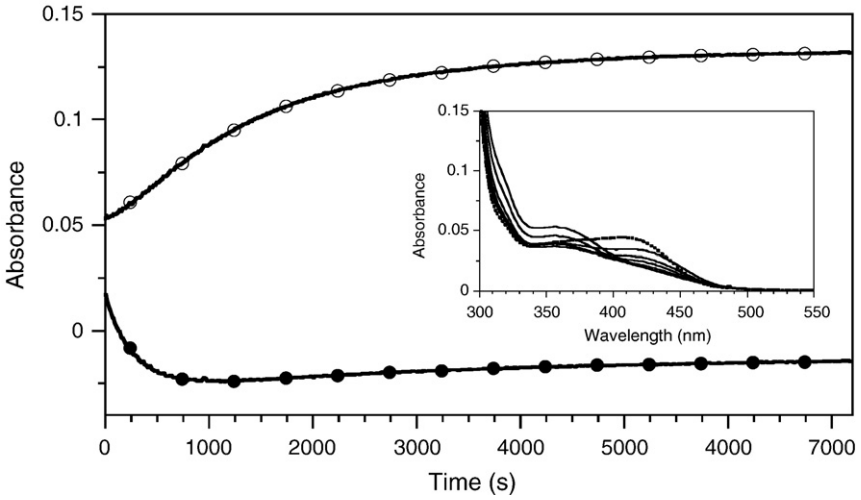

Fig. 2. The time course of the reaction of N84D with L-Ser. Absorbance was monitored at 320 nm (open circles) and 412 nm (closed circles) following the addition of 50 mM L-Ser to 14 μM N84D. Inset: time-resolved spectra immediately prior to the addition of L-Ser (dotted line) and 0.2, 1, 2, 3.5, 11, and 24 min (solid lines) after the addition of 50 mM L-Ser. Conditions as in Fig. 1.

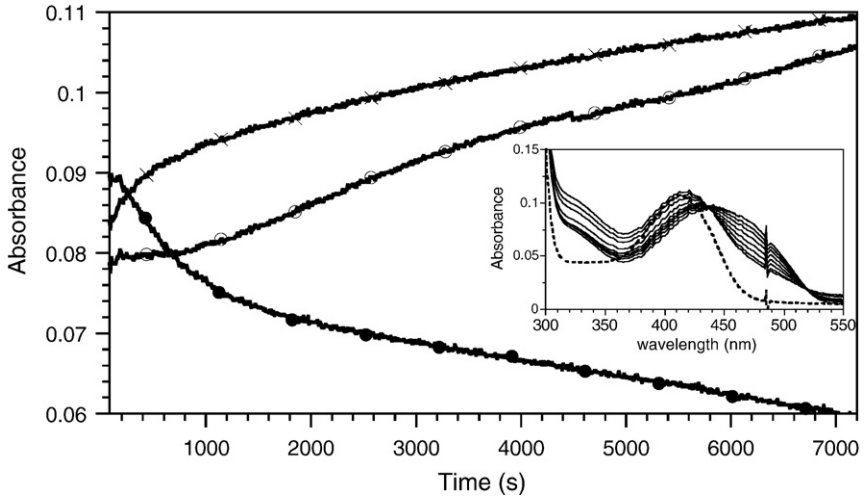

Fig. 3. The time course of the reaction of N84H with L-Ser. Absorbance was monitored at 320 nm (open circles), 412 nm (×) and 460 nm (closed circles) following the addition of 50 mM L-Ser to 14 μM N84H. Inset: time-resolved spectra immediately prior to the addition of L-Ser (dotted line) and 0.7, 2.3, 6, 10, 17, 33, 67, 100 and 120 min (solid lines) after the addition of 50 mM L-Ser. Conditions as in Fig. 1.

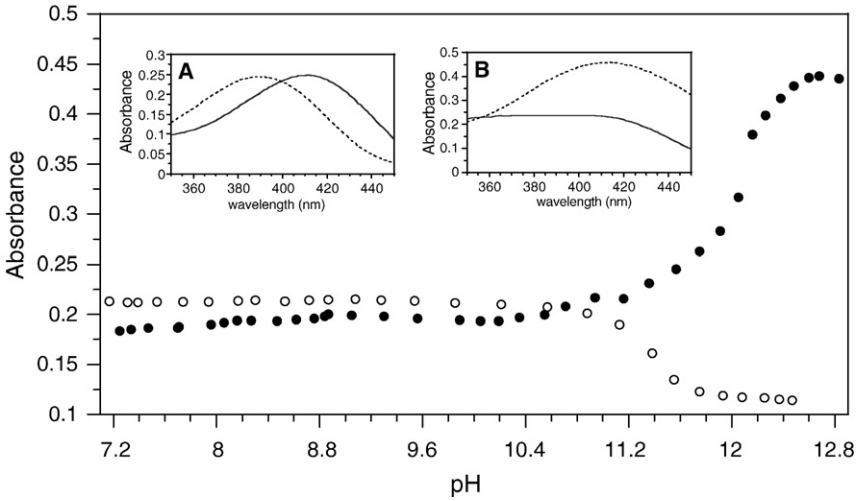

Fig. 4. Spectrophotometric titration of 35.5 μM ytCBS-N84A (open circles) and 48 N84D (closed circles) versus pH at 425 nm. (Inset A) UV-visible spectra of ytCBS-N84A at pH 7.5 (—) and 12.5 (---). (Inset B) UV-visible spectrum of ytCBS-N84D at pH 7.5 (—) and 12.5 (---).

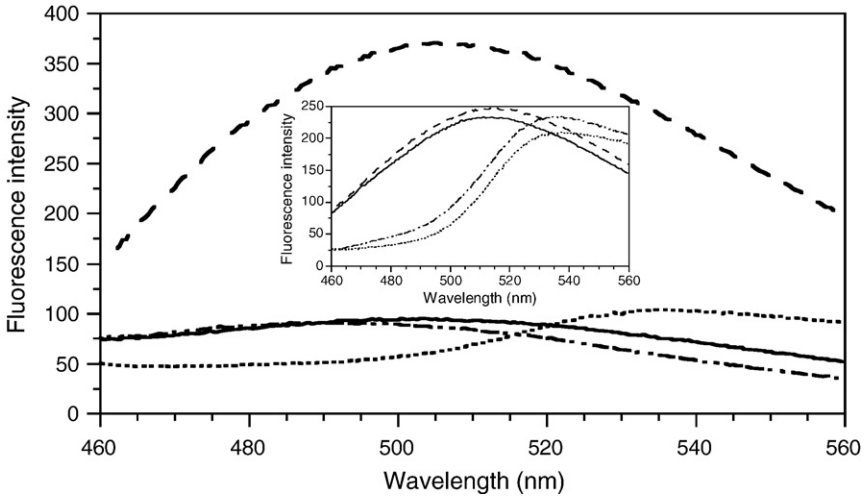

Fig. 5. Fluorescence spectra ($\lambda_{ex} = 298$ nm) of 20 μM ytCBS-N84D (—) and N84H (- - -) alone and in the presence of 50 mM L-Ser (-·-·-· N84D; ··· N84H). Inset: wild-type ytCBS (—) and N84A (- - -) alone and in the presence of 50 mM L-Ser (-·-·-· ytCBS; ··· N84A). Conditions as in Fig. 1.

3. Discussion

The amino acids that interact with the N1 and O3' positions of PLP regulate the distribution of electrons within the cofactor as well as its position within the active site [29]. An interaction between the pyridinium nitrogen of the cofactor and a conserved aspartate or serine residue is common to PLP enzymes of the α and β-families, respectively [4,7,8,30]. In contrast, there is variation in the identity of the amino acid which hydrogen bonds to the phenolic O3' atom. Characterization of the ecAATase-Y225F, ecAATase-N194A, meALAS-H282A and stTrpS-βQ114N site-directed mutants has demonstrated that the specific role of the residue interacting with PLP-O3' is variable and may include modulation of the pK_a of the Schiff base nitrogen, substrate and cofactor binding and maintenance of the equilibrium between the open and closed conformations of the active site [13,14,31,32]. Given the diversity of roles proposed and the differences in the interactions observed for hCBS-N149 and stTrpS-βQ114, the ytCBS-N84A,D,H mutants were constructed to probe the function of this residue in yCBS.

The catalytic efficiency of ytCBS-N84A is reduced, as demonstrated by the 230 and 145-fold decreases in the $k_{catF}/K_{mF}^{L\text{-}Ser}$ and $k_{catR}/K_{mR}^{L\text{-}Cth}$ of the β-replacement and L-Cth hydrolysis reactions, respectively (Table 1). A similar effect is observed for the corresponding meALAS-H282A and ecAATase-Y225F mutants demonstrating that removal of the hydrogen bond to O3' of the PLP cofactor reduces the catalytic capacity of these enzymes [13,31]. Turbeville et al. [31] suggest that the hydrogen bonding network of meALAS-H282, which interacts with PLP-O3', assists in maintaining a catalytically productive orientation of the cofactor within the active site. While a 20-fold increase in $K_m^{L\text{-}Ser}$ and an 11-fold decrease in k_{cat} are observed for

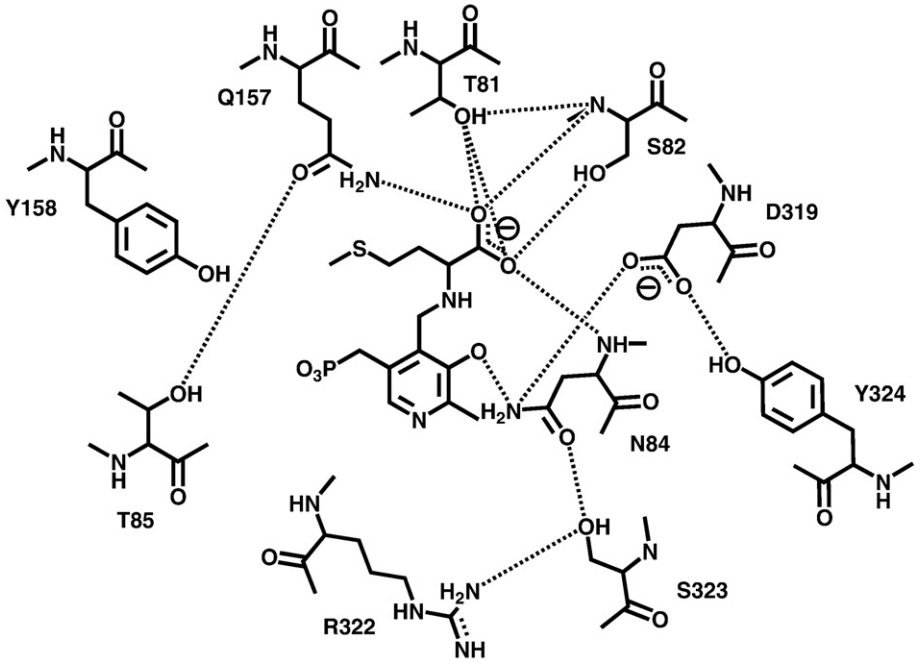

Fig. 6. Proposed active site contacts in the L-Met external aldimine yCBS complex. The interactions are based on the structures of hCBS and the stOASS-K41A mutant complex with L-Met [4,33]. The dotted lines represent putative hydrogen bond distances of ≤3.3 Å between heteroatoms. Residue labels are those of yCBS.

ytCBS-N84A (Table 1), no β-elimination or substrate-induced inactivation are detected. Additionally, the fluorescence resonance energy transfer from tryptophan residue(s) to the internal aldimine and the external aldimine of aminoacrylate forms of the cofactor is similar to that of the wild-type enzyme (Fig. 5). This suggests that, despite the loss of the hydrogen bond to O3' of the cofactor, the orientation of PLP in the internal aldimine and the external aldimine of aminoacrylate, as well as the equilibrium between the open and closed conformations of the active site, of the N84A mutant is similar to that of the wild-type enzyme (Fig. 5). Therefore, while elimination of the hydrogen bond between PLP-O3' and N84 of ytCBS likely alters the movement and orientation of the cofactor during the catalytic cycle, the change is subtle. The observed change in the kinetic parameters of ytCBS-N84A may also be due to repositioning of the backbone of this residue as a result of elimination of the tethering hydrogen bonds of the N84 side chain. This could cause the weakening or loss of the proposed hydrogen bond between the α-carboxylate of the substrate and the backbone NH moiety of N84 [33].

The histidine and aspartate substitutions of ytCBS-N84 were selected to modify the capacity of this residue to form hydrogen bonds. The 640 and 100-fold reductions in the $k_{catF}/K_{mF}^{L\text{-}Ser}$ and $k_{catR}/K_{mR}^{L\text{-}Cth}$ of the β-replacement and reverse-physiological activities of N84H are similar to those observed for N84A (Table 1). However, a change in the orientation of the PLP cofactor within the active site, compared to the wild-type enzyme and ytCBS-N84A, are observed for N84H (Fig. 5). A marginal β-elimination activity ($k_{catE}/K_{mE}^{L\text{-}Ser} = 0.34 \pm 0.06\ M^{-1}\ s^{-1}$) is also detected for ytCBS-N84H, suggesting a corresponding change in active-site dynamics.

The $k_{catF}/K_{mF}^{L\text{-}Ser}$ and $k_{catR}/K_{mR}^{L\text{-}Cth}$ of N84D are reduced by 3 and 4 orders of magnitude, respectively. This drastic reduction in catalytic efficiency, compared to the wild-type enzyme and the N84A and N84H mutants, the 3-fold reduction in PLP-saturation, the lack of accumulation of an aminoacrylate intermediate (Figs. 1 and 2) and the observed inactivation of this mutant demonstrate that the cofactor is not bound in a productive orientation and that the equilibrium between the open and closed conformations of the active site is disrupted. The side chain $O_{\delta 1}$ and $N_{\delta 2}$ atoms of hCBS-N149, which corresponds to N84 of yCBS, are 3.24 and 3.28 Å from N380($N_{\delta 2}$) (yCBS-S323) and the carboxylate moiety of D376 (yCBS-D319), respectively (Fig. 6) [4]. The introduction of a carboxylate group in place of the amide moiety of N84 would disrupt the hydrogen bonding network, linking the mobile loop of residues (T81-S82-G83-N84-T85), which binds the α-carboxylate group of the substrate, with residues D319, R322, S323 and Y324 at the C-terminus of the catalytic domain (Fig. 6). It is interesting to note that although >100 distinct homocystinuria-associated mutations of hCBS have been reported, only one of these nine residues (G83) corresponds to the site of a disease-linked mutation (hCBS-G148R) [34]. A common feature of the ytCBS-N84D and stTrpS-βQ114N mutants is that they both undergo mechanism-based inactivation and are purified as a mixture of active and inactivated enzyme, due to reaction with serine during expression or purification (Fig. 4) [14]. The partitioning of aminoacrylate between nucleophilic attack on C4' of the PLP cofactor, resulting in inactivation, and the reaction with water to produce pyruvate and ammonia is dependent on active-site dynamics that enable the release of aminoacrylate [14]. Although an active-site residue could facilitate the attack of aminoacrylate on the PLP cofactor, this explanation was discounted as an inactivation mechanism for the βQ114N mutant of stTrpS because there are no carboxylate residues suitably placed to enable electrostatic catalysis in the active site of this enzyme [14]. Therefore, the observed inactivation of the stTrpS-βQ114N mutant by aminoacrylate is likely due to a change in the equilibrium between the open and closed conformations of the active site, as proposed for the Q157H and Y158F mutants of ytCBS [15]. Blumenstein et al. [14] suggest that, although wild-type stTrpS and the βQ114N mutant possess a similar level of β-elimination side reaction activity, the mutant is uniquely inactivated because it lacks the ability to efficiently release aminoacrylate from the active site [14]. In contrast with stTrpS-βQ114N, the carboxylate moiety of the aspartate side chain of the ytCBS-N84D mutant may stabilize the developing positive charge of the enamine nitrogen during the attack of aminoacrylate on C4' of the PLP cofactor, thereby facilitating the inactivation of the enzyme.

While a β-elimination activity has been observed for 10 (T81A, S82A, N84D, N84H, T85A, Q157A, Q157E, Q157H, Y158F and S289) of the 20 site-directed mutants, targeting 13 active-site residues (Fig. 6), reported for ytCBS, aminoacrylate-mediated inactivation has been reported for only three (N84D, Q157H and Y158F) [15,16,35]. PLP enzymes catalyzing β-replacement reactions have evolved to facilitate the transaldimination reaction with substrates and products, but not aminoacrylate, thereby minimizing the β-elimination side reaction, which produces pyruvate and ammonia, and inactivation by aminoacrylate. The ability to selectively facilitate the transaldimination of substrates and products and diminish the corresponding reaction with aminoacrylate is likely a function of conformational constraints imposed by the enzyme [14]. The data presented demonstrate that residue N84 of ytCBS is a determinant of cofactor positioning and of reaction specificity, via its role in the regulation of aminoacrylate partitioning to favor the β-replacement reaction over the release of aminoacrylate, via β-elimination.

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