A Unique Catalytic and Inhibitor-Binding Role for Lys93 of Yeast Orotidylate Decarboxylase†

Jeffrey A. Smiley‡ and Mary Ellen Jones*

Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599-7260

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ABSTRACT: The presence of a proton-donating catalytic amino acid side chain in orotidylate decarboxylase (ODCase) was sought by site-directed mutagenesis. Replacement of yeast ODCase Lys93 with a cysteine resulted in a mutant protein (K93C) with no measurable activity, representing a decrease in activity by a factor of, at most, 2×10^{-8} times the activity of the wild-type enzyme. Treatment of this mutant protein with 2-bromoethylamine, designed to append Cys93 to yield *S*-(2-aminoethyl)cysteine, restored activity by a factor of at least 5×10^5 over the untreated mutant protein. Activity could not be restored by treatment with other brominated reagents designed to replace the ϵ-amino of *S*-(2-aminoethyl)Cys93 with a different functional group. The overall architecture of the K93C protein was not significantly changed, as judged by the similar dimerization properties (in the absence of ligands) of the mutant enzyme compared to the wild-type enzyme. The binding affinity of the substrate orotidylate was not measurably changed by the mutation, indicating that Lys93 has an essential role in catalysis which is mechanistically distinguishable from substrate binding. Apparently the mutation removes an integral portion of the active site and does not drastically affect the structural or substrate binding properties. However, the affinities of the mutant protein for the competitive inhibitors 6-azauridylate (6-azaUMP) and UMP are significantly altered from the pattern seen with the wild-type enzyme. The K93C protein has an affinity for the neutral ligand UMP which is greater than that for the anionic 6-azaUMP, in clear contrast to the preference for 6-azaUMP displayed by the wild-type enzyme. Lys93 is apparently critical for catalysis of the substrate to product and for the binding of anionic inhibitors; the data are discussed in terms of previously existing models for transition-state analogue inhibitor binding and catalysis.

The chemical mechanism by which orotidylate decarboxylase (ODCase,[1] EC 4.1.1.23) catalyzes the formation of uridylate (UMP) from orotidylate (OMP) is apparently different from any of the collection of common routes to decarboxylation seen with other enzymes [review, O'Leary (1992)]. The absence of a detectable cofactor and the observation of nonenzymatic rates of decarboxylation of various heterocyclic carboxylic acids resembling OMP led Beak and Siegel (1976) to propose a noncovalent mechanism, in which the enzyme protonates the substrate, stabilizes a zwitterionic intermediate, and catalyzes formation of a nitrogen ylide which results upon decarboxylation (Figure 1). The observation by Levine et al. (1980) that barbituric acid ribonucleotide (BMP) is a prodigious inhibitor of yeast ODCase ($K_i = 9 \times 10^{-12}$ M) and comparison of the structure of BMP to the proposed transition state for the reaction lent support to this mechanism. Levine et al. also showed quantitatively that the difference in inhibition constants of 6-azauridylate (6-azaUMP) versus the isosteric UMP was predominantly due to the lower pK_a of 6-azaUMP and the enzyme's preference for binding the anionic forms of these ligands, as first proposed by Handschumacher (1960).

Recent kinetic isotope effect studies with yeast ODCase have provided further support for this catalytic mechanism. The pH dependence of carbon isotope effects (Smiley et al., 1991) indicated a noncovalent reaction involving a proton-sensitive catalytic step, which became partially rate-determining at high pH, in accord with the Beak and Siegel mechanism. Acheson et al. (1990) provided evidence against an alternative mechanism involving covalent attachment at C-5 (Silverman & Groziak, 1982) with the observation of no significant secondary hydrogen isotope effect with [5-^{2}H]-BMP. A kinetic equation for the C-5 covalent mechanism, which included both measured isotope effects, was not solvable (Smiley et al., 1991), and this proposed covalent mechanism apparently does not function for yeast ODCase.

The key catalytic residue indicated by the pH-dependent carbon isotope effects appears to have a pK_a of $\sim$7 and must be protonated for activity. Increased affinity of the enzyme for anionic pyrimidine inhibitors, as mentioned above, suggested the presence of a positively charged enzyme functional group at the active site, responsible for attracting the anionic inhibitors. We suspected that this cationic amino acid side chain may be the same one implicated as the proton donor in the zwitterion mechanism. Removal of such a functional group by site-directed mutagenesis might produce a mutant enzyme with both reduced catalytic activity and altered relative affinities for 6-azaUMP versus UMP.

A survey of ODCase amino acid sequences[2] reveals significant similarity among sequences from even the most

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‡ Work done in partial fulfillment of the Ph.D. degree. Present address: Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

[1] Abbreviations: ODCase, OMP decarboxylase; OMP, orotidylate (orotidine 5′-monophosphate); UMP, uridylate (uridine 5′-monophosphate); BMP, barbituric acid ribonucleotide; 6-azaUMP, 6-azauridine 5′-monophosphate; K93C ODCase, yeast ODCase with the mutation Lys93 → Cys93; WT, wild-type ODCase; IPTG, isopropyl β-thiogalactoside; MOPS, 3-(*N*-morpholino)propanesulfonate.

[2] A comparison of ODCase sequences from 20 organisms has appeared since this work has been in progress (Kimsey & Kaiser, 1992); an earlier sequence comparison (Radford & Nix, 1987) and unpublished sequence comparisons in this laboratory were originally used for selecting amino acids for mutagenesis.

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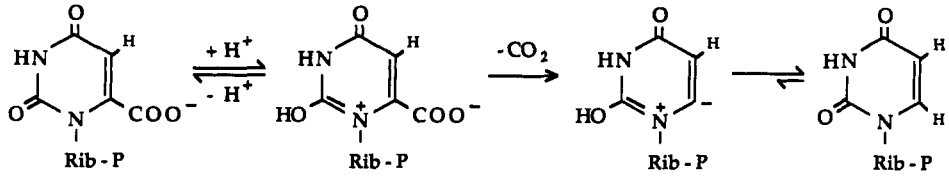

FIGURE 1: Zwitterionic mechanism for OMP decarboxylation by ODCase.

disparate organisms, but only a few amino acids which are invariant in all enzymes, which we considered would be the case for a catalytic proton-donating residue. Many of these invariant amino acids are found in a region of the protein with very high similarity among all sequences; one of these, Lys97 in the ODCase domain from bifunctional mouse UMP synthase (Ohmstede et al., 1986), was selected as a target for mutagenesis, as was the corresponding lysine of the yeast enzyme (Rose et al., 1983), Lys93.

The repertoire of amino acids naturally occurring in proteins includes no close analogues to lysine; arginine has a much higher basicity and is branched at the guanidine terminus, and no other polar amino acid extends to the length of the lysine side chain. Thus the changes one can effect upon a lysine residue by site-directed mutagenesis alone are not subtle changes. To overcome this limitation of nature, some investigators (Smith & Hartman, 1988; Planas & Kirsch, 1991) have utilized mutagenesis of critical lysine residues to cysteines, followed by chemical modification of the nonfunctional cysteine with 2-bromoethylamine to yield a partially active protein containing *S*-(2-aminoethyl)cysteine, an isostere of lysine. Our characterization of Lys93 → Cys93 (K93C) yeast ODCase includes the attempted restoration of catalytic activity by modification with 2-bromoethylamine, as well as similar reagents designed to append Cys93 with a number of various chemical moieties, in order to assess the function of this invariant amino acid.

MATERIALS AND METHODS

Site-Directed Mutagenesis of Mouse ODCase Domain. A vector for the expression of the functional mouse ODCase domain in *Escherichia coli* was constructed in this laboratory by Dr. Carol-Ann Ohmstede. An *Eco*RI–*Hind*III fragment containing the cDNA encoding the ODCase domain from the plasmid pODC (Ohmstede et al., 1986) was inserted into the parent vector pKK223-3 (Pharmacia), yielding the expression plasmid pODCtac. A portable translation initiation sequence (Pharmacia) was inserted just upstream from the start codon. This plasmid allows increased expression of cDNA's upon induction of log-phase cultures with IPTG. The plasmid can complement the auxotrophic strain JFS116 when grown in the absence of a pyrimidine source.

For mutagenesis of this cDNA, the *Eco*RI–*Hind*III fragment was excised from pODCtac and inserted into M13mp19 (Bethesda Research Laboratories). DNA fragments in these cloning procedures were isolated by agarose gel electrophoresis and purified by a glass powder adsorption method, using the GeneClean kit (Bio101). A construct carrying this fragment was introduced into strain CJ236 (*dut⁻ ung⁻*) for the production of uracil-containing phage DNA for mutagenesis (Kunkel et al., 1987). For mutagenesis of Lys97, a mixed oligonucleotide was designed to allow replacement of the Lys97 codon with a codon for any of the other 19 amino acids, plus a stop codon. The first two nucleotides in codon 97 of the mutagenic oligonucleotide were equimolar mixtures of all four bases; a mixture of G and C in the third position allows all amino acids to be represented while minimizing the degeneracy of the genetic code. This oligonucleotide was designed as follows (mismatched bases in bold type): K97X, 5′-AGACAGGNN-(G/C)TTCGCTG-3′.

Mutations were identified by dideoxy sequencing of the single-stranded phage DNA from plaques arising from transformation of *E. coli* strain DH5αF′*lac*I^Q (Bethesda Research Laboratories) with the mutagenesis reaction mixture. DNA fragments from RF DNA bearing mutations were reinserted into the pODCtac vector from which they were originally excised.

Mutated plasmids were introduced into competent JFS116 cells to assay the ability of these mutated ODCase cDNA's to complement this auxotrophic strain. This complementation assay gives a semiquantitative measure of the activity of mutated ODCase. Aliquots of saturated cultures of JFS116 carrying the wild-type and mutant plasmids, grown in LB medium (Difco), were used to inoculate cultures of the following sterile medium: 1× M9 salts (Maniatis et al., 1982), 0.2% casamino acids (Difco), 2% glucose, 25 μg/mL tryptophan, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 2 μg/mL thiamin hydrochloride, 50 μg/mL ampicillin, 50 μg/mL kanamycin, 12.5 μg/mL tetracycline, and 0.5 mM IPTG. Bacterial growth was monitored by a Klett Summerson photoelectric colorimeter.

Site-Directed Mutagenesis of Yeast ODCase. Lys93 of ODCase, corresponding to Lys97 of the mouse domain and encoded by the yeast plasmid pGU2 (Lue et al., 1987; Bell & Jones, 1991), was targeted for site-directed mutagenesis. The method of Kunkel et al. (1987) was again used: a 0.6-kilo-base-pair *Bam*HI–*Csp*45I fragment was ligated into a derivative of M13mp19 in which a *Csp*45I restriction site was created by a separate mutagenesis procedure. This construct was introduced into competent CJ236 cells, and uracil-containing, single-stranded phage DNA was harvested from a culture of phage-containing cells. The oligonucleotide used for mutagenesis was designed to change Lys97 to a cysteine, and so the sequence was as follows (mismatched bases indicated as above): K93C, 5′GAAGACAGATGTTTTGCTGAC-3′.

The mutated DNA fragment was excised from RF DNA and ligated into a 10.3-kilo-base-pair fragment of pGU2, which was produced from a partial *Csp*45I digest of *Bam*HI-linearized plasmid and isolated by agarose gel electrophoresis. The presence of the mutation in the reconstructed plasmid was verified by reinsertion of the 0.6-kilo-base-pair fragment into the M13 vector and dideoxy sequencing. Mutated pGU2 was introduced into competent 15C yeast (Lue et al., 1987) by a modified spheroplast protocol, with selection for the *leu2-d* gene of pGU2.

Partial Purification of K93C ODCase. Mutant ODCase was produced by galactose induction of log-phase yeast 15C cells (Lue et al., 1987). Cell growth and protein purification steps up to and including ammonium sulfate fractionation and dialysis were the same as described previously for wild-type ODCase (Bell & Jones, 1991). Further purification of the mutant enzyme by column chromatography with AffiGel Blue (Amicon) and CM52 cellulose (Whatman) was also attempted by methods previously described (Bell & Jones, 1991). Purity of the mutant protein was judged by silver stain (Livingstone & Jones, 1987) of electrophoresed SDS–

polyacrylamide gels (Laemmli, 1970). Protein concentrations were determined by the dye binding method of Bradford (1976), using bovine serum albumin (Sigma) as a standard.

Chemical Modification of K93C. Wild-type and K93C ODCases were diluted to 0.2 mg/mL in 50 mM sodium phosphate, pH 7.0, containing 10% glycerol and 2 mM EDTA, and dialyzed (1:300) against this buffer for 1 h to remove dithiothreitol from the purification steps. Urea (ultrapure, ICN Biomedicals, Inc.) was added in small amounts over 10 min with gentle mixing to a final concentration of 7.5 M. Control experiments involved addition of buffer to give the same final volume. Aliquots of 710 μL were separated, and 50-μL aliquots of 0.76 M solutions of the following sulfhydryl modification reagents, prepared in the above buffer, were added: 2-bromoethylamine hydrobromide, 2-bromoethanol, (2-bromoethyl)trimethylammonium bromide, 2-bromoacetamide (Aldrich). Addition of the reagent lowered the urea concentration to a final value of 7.0 M. Following incubation of 1 h at 25 °C, dithiothreitol was added to the reaction mixtures to a final concentration of 50 mM, and the mixtures were dialyzed (1:300) separately against three changes of the above buffer plus 50 mM dithiothreitol. Following dialysis, the enzyme solutions were chilled on ice briefly before assay for decarboxylase activity.

Sucrose Density Gradient Centrifugation. Experiments were performed essentially as described previously (Bell & Jones, 1991), with the exception that fewer molecular weight markers were used. Each protein sample (10 μL) contained only carbonic anhydrase (4 μg; MW = 31 000) and bovine serum albumin (0.5 μg; MW = 66 000) as molecular weight standards. K93C ODCase (MW = 29 000) was diluted to a final concentration of 0.15 mg/mL with 50 mM Tris–HCl, pH 7.5, 10% glycerol, 2 mM EDTA, and 5 mM dithiothreitol. Gradients (5 mL of an 8–25% sucrose solution plus 10% glycerol) were centrifuged at 4 °C for 40 h at 250000*g* in a Beckman SW65 rotor or 42 H at 240000*g* in a Beckman SW 50.1 rotor. Fifty 100-μL fractions were collected. The positions of K93C ODCase and the marker proteins were determined by qualitative analysis of electrophoresed SDS–polyacrylamide gels (Laemmli, 1970) containing gradient fractions and standards (see Figure 2). The monomer–dimer form of ODCase (wild type and K93C) was determined by its apparent molecular weight, roughly equal to that of either carbonic anhydrase or bovine serum albumin.

Assays of ODCase activity were performed using [*carboxy*-^{14}C]OMP (New England Nuclear-DuPont) and the $^{14}CO_2$ collection method of Prabhakararao and Jones (1975). Assays were performed at room temperature (25 ± 1 °C) using 25 mM MOPS, pH 7.0. Reaction mixtures contained 1.0×10^4 cpm of [*carboxyl*-^{14}C]OMP and a total OMP concentration of 50 μM.

RESULTS

Activity of Lys93 Mutants of Mouse ODCase Domain. Sequencing of phage DNA from plaques arising from the random mutagenesis of Lys97 identified 15 of the possible 19 amino acid substitutions at this position of the mouse ODCase domain, including the positively charged amino acids arginine and histidine, which could be considered conservative changes. Additionally, a silent mutation of the Lys97 codon and a stop codon were identified. These mutations are listed in Table I. Of these 17 mutations, only the silent mutation resulted in an altered pODCtac which complemented JFS116 pyrimidine auxotrophy. Thus, no amino acid substitution resulted in a protein with appreciable activity, since less than 1% of wild-type ODCase activity is sufficient to allow growth in these conditions, judging from mutations of two cysteines which were found not to be obligatory for partial activity (Smiley, 1991).

Table I: Amino Acid Substitutions at Mouse ODCase Lys97 and Attempted Growth of JFS116 Transformed with Corresponding pODCtac Mutants

amino acid at position 97	incub (h)	ending Klett reading[a]	amino acid at position 97	incub (h)	ending Klett reading[a]
WT	17	165	His	31.5	3
Lys[b]	17	130	Gly	37	2
Ile	39	0	Asn	37	2
Met	39	6	Thr	36	5
Leu	39	0	Ala	36	0
Val	39	4	Asp	36	0
Arg	31.5	8	Gln	36	0
Tyr	31.5	7	Ser	36	0
Cys	31.5	3			

[a] Ending Klett reading is the value taken at the indicated time minus the reading at the beginning of the incubation. [b] AAA codon in wild-type plasmid is changed to AAG (Lys).

Production of Yeast K93C ODCase. Following mutagenesis, construction of the mutant yeast plasmid, and verification of the mutation, the mutant plasmid pGU2/K93C was introduced into yeast strain 15C. The abundance of K93C protein was evident to the same extent in the yeast lysate as the wild-type protein from cultures carrying pGU2. The mutant protein was precipitated by ammonium sulfate to the same degree as the wild-type protein in parallel experiments. When the next step in the published purification (Bell & Jones, 1991), Affi-Gel Blue column chromatography, was attempted, the mutant protein was found to emerge in the first wash fractions, and so the high degree of purification effected for the wild-type protein by specific elution of the bound enzyme with 6-azaUMP could not be used. Chromatography on carboxymethylcellulose also resulted in no apparent binding of the mutant protein to this resin. However, the pooled wash fractions from the Affi-Gel Blue chromatography containing K93C ODCase apparently represented a modest purification step, since other proteins in the lysate remained bound to the resin and were eluted only with high KCl, as in the published method (Bell & Jones, 1991). The fractions containing K93C ODCase were pooled, concentrated, and found to contain greater than 80% pure K93C ODCase.

Enzymatic Activity of K93C ODCase. Despite rigorous attempts, no ^{14}C above background could be detected in the $^{14}CO_2$ displacement assay for ODCase activity with this mutant protein. The minimum detectable level of 0.025 nmol of product (10 cpm in a reaction mix containing 10^4 cpm and 25 nmol of total OMP) was not observed in a 100-min assay containing 160 μg of K93C protein and 50 μM total OMP. The maximum activity for K93C ODCase is thus 1.6×10^{-6} nmol min^{-1} μg^{-1}; using a value of 75 nmol min^{-1} μg^{-1} for wild-type ODCase (Bell & Jones, 1991), the factor of this decrease in activity is 2×10^{-8} (or possibly a smaller factor, since only a upper limit of activity can be calculated).

Restoration of Activity with 2-Bromoethylamine. Since neither urea denaturation 2-bromoethylamine modification, nor the combination of these two treatments drastically reduced ODCase activity in the wild-type enzyme (Table II), this method was attempted for restoration of enzyme activity to the inactive K93C enzyme. With the mutant protein, 2-bromoethylamine treatment alone did not produce a modified protein capable of producing measurable cpm in the $^{14}CO_2$ displacement assay, but the combination of urea and 2-bromoethylamine (followed by renaturation of the protein by removal of urea) yielded an active modified protein. Following

Table II: Restoration of Activity to K93C ODCase by Chemical Modification with Bromoethylamine[a]

enzyme + condition	cpm[b]	protein (μg)	time (min)	activity[c] (nmol min^{-1} μg^{-1})
WT	380	0.05	1.0	19.0 (100)
WT + bromoethylamine	370	0.05	1.0	18.5 (97)
K93C + bromoethylamine	0	10	20	0 (0)
WT	3500	0.24	2.0	18.2 (100)
WT + urea	2900	0.24	2.0	15.1 (83)
K93C + urea	0	2.4	10	0 (0)
WT + urea + bromoethylamine	2240	0.24	2.0	11.7 (64)
K93C + urea + bromoethylamine	1570	2.4	10	0.16 (0.90)

[a] Conditions of modification reactions and assays are described in Materials and Methods. [b] Minus background counts. [c] Values in parentheses are percent of control.

modification for 1 h in the presence of urea as described above, the K93C protein regained 0.16 nmol min^{-1} μg^{-1} of ODCase activity (Table II). No detectable activity could be observed in separate reactions with 2-bromoethanol, 2-bromoacetamide, or trimethyl(2-bromoethyl)ammonium bromide.

Sedimentation of K93C ODCase in Sucrose Density Gradients. In the absence of ligands, K93C ODCase sedimented in sucrose gradients with properties very similar to the wild-type protein (Bell & Jones, 1991). At high protein concentrations, the mutant protein sedimented as a 60 000 molecular weight species; when the concentration was lowered, the protein traveled as a species with an apparent molecular weight only slightly greater than carbonic anhydrase. This low protein concentration (1.5 μg per 5-mL gradient) was used in the presence of ligands to observe the ability of each ligand to cause full or partial dimer formation. Various ligand concentrations caused varying degrees of shifts toward dimer formation of K93C ODCase. The results obtained here varied slightly from the results of Bell and Jones (1991); specifically, the wild-type enzyme was found to be dimerized at lower ligand concentrations. Bell and Jones did not search for the minimal ligand concentration required to generate dimer formation. In addition, another variable between the experiments reported here and the earlier studies was the amounts of protein molecular weight markers used, which were minimized here. Sample gels of gradient fractions electrophoresed with K93C ODCase and marker proteins are shown in Figure 2; the peak positions of the wild-type and mutant protein and the markers in the respective gradients with various ligand concentrations are compiled in Table III.

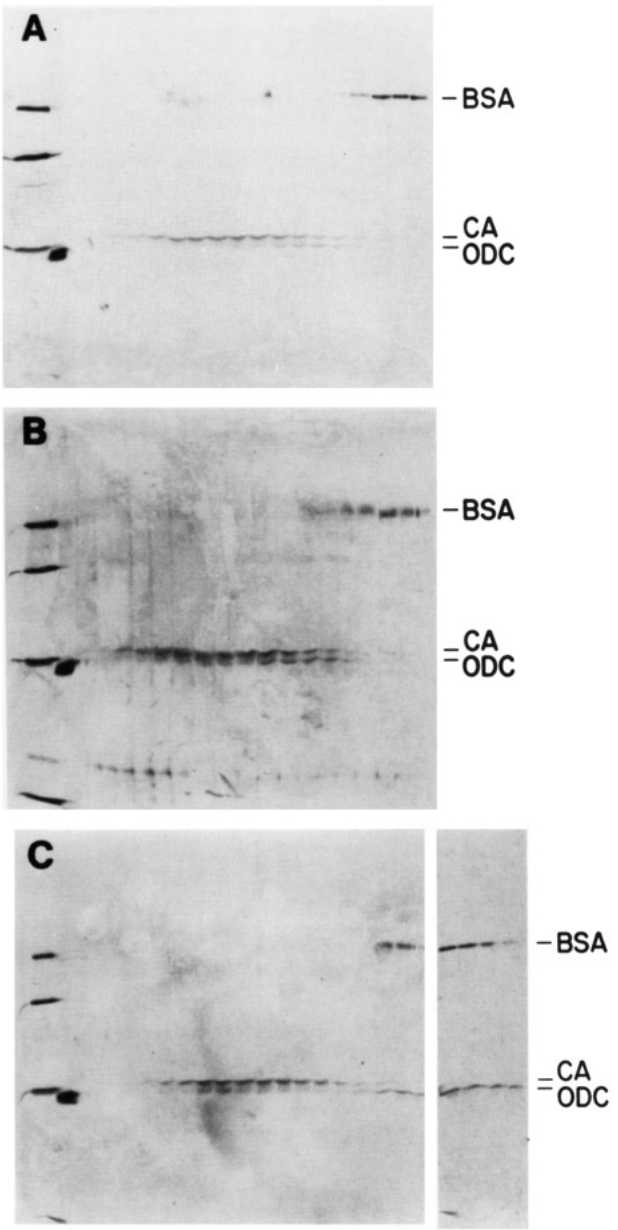

FIGURE 2: SDS–polyacrylamide gels of fractions from sucrose gradient centrifugation of K93C yeast ODCase in the presence and absence of mononucleotides. The first column on the left of each gel contains low molecular weight markers (Sigma): bovine serum albumin (66 000), porcine heart fumarase (48 500), bovine erythrocyte carbonic anhydrase (29 000), bovine milk β-lactoglobulin (18 400), and bovine milk α-lactalbumin (14 200). The second column has a sample of pure wild-type yeast ODCase. The remaining samples are fractions 15–32 (A and B) or 15–36 (C). Key: BSA, bovine serum albumin; CA, carbonic anhydrase, ODC, yeast K93C ODCase. Conditions are described in Materials and Methods. In (A), no nucleotide was present. In (B) and (C), the nucleotides present were 5 μM 6-azaUMP and 0.5 μM UMP, respectively. ODCase is predominantly a monomer in (A) and (B) and predominantly a dimer in (C).

DISCUSSION

Mutagenesis of Mouse ODCase Domain Lys97 and Yeast ODCase Lys93. The observation that any of the 15 amino acid replacements for lysine at position 97 led to an inability of mutated pODCtac to complement JFS116 in pyrimidine-free media suggests that Lys97 of the mouse ODCase domain plays a very specific role in enzyme function. Efforts to purify these mutant proteins from *E. coli* by column chromatography were unsuccessful, and so attention was shifted to mutagenesis and production of the more easily obtainable yeast ODCase, in which the invariant lysine is located at position 93. The high degree of similarity between the amino acid sequences for the mouse ODCase domain of UMP synthase and monofunctional yeast ODCase (Ohmstede et al., 1986; Kimsey & Kaiser, 1992) suggests that these two lysines carry out the same enzymatic function.

The relative purity of the K93C yeast enzyme afforded the opportunity to accurately gauge the decrease in catalytic activity resulting from replacement of Lys93 with cysteine. However, no activity could be detected, even in the most sensitive assays possible, and only an upper limit of 1.6×10^{-6} nmol min^{-1} μg^{-1} for the activity of the mutant protein could be calculated. The activity is thus decreased by a factor of 2×10^{-8} times that of the wild-type enzyme (or a reciprocal value of 5×10^{7}), a magnitude one would expect for removal of a side chain which plays an indispensible role in catalysis. This result substantiates the finding from the mutations of mouse ODCase, which suggested that lysine plays a role in ODCase catalysis that cannot be mimicked by any other naturally occurring amino acid.

Table III: Sucrose Density Gradient Sedimentation Data for Wild-Type (WT) and K93C ODCases with Various Ligand Concentrations

enzyme (μg)	ligand (μM)[a]	peak positn[b]	MW markers[c]	aggreg state[d]
K93C (100)	none	30	22–30	dimer
K93C (1.5)	none	24	21–30	monomer
K93C (1.5)	OMP (2)	30	24–32	dimer
K93C (1.5)	OMP (4)	31	23–32	dimer
WT (1.5)	6-azaUMP (0.5)	38	27–38	dimer
K93C (1.5)	6-azaUMP (0.5)	26	23–30	monomer
K93C (1.5)	6-azaUMP (120)	32	25–33	dimer
WT (1.5)	UMP (80)	31	24–32	dimer
WT (1.5)	UMP (60)	25	24–31	monomer
K93C (1.5)	UMP (60)	31	22–31	dimer
K93C (1.5)	UMP (0.5)	33	24–33	dimer

[a] The ligand concentrations studied ranged between 0.5 and 200 μM for UMP, 0.5 and 500 μM for 6-azaUMP, and 2 and 100 μM for OMP. If all of the wild-type or K93C ODCase were in a single 100-μL fraction, the protein concentration would be 5×10^{-7} M. Since the ODCase was distributed over 7–10 fractions this value would be lower in each fraction. [b] Values indicate the position of maximum density of either wild-type or K93C ODCase in fractions of sucrose gradients containing the indicated ligand. [c] Values indicate the peak positions of maximum density of the molecular weight markers carbonic anhydrase and bovine serum albumin, respectively; i.e., 22–30 indicates that the carbonic anhydrase peak was at tube 22 and the bovine serum albumin peak was in tube 30. [d] Designation indicates the *predominant* form of the respective enzyme in the gradient, judging from its apparent molecular size.

Beak and Siegel (1976) estimated an enzymatic rate enhancement (k_{cat}/k_{uncat}) of 4×10^{10} for rat liver ODCase versus the uncatalyzed reaction and an enhancement of 4×10^8 for the catalytic step which displaces the substrate toward the zwitterionic intermediate of the enzyme-catalyzed reaction.[3] The K93C mutation may thus abolish the entire displacement of the substrate toward this intermediate (Figure 1).

To address the effect of the mutation on the overall architecture and function of the inactive protein, we examined the sedimentation properties of K93C ODCase in sucrose gradients at various protein concentrations [cf. Bell and Jones (1991)]. In the absence of ligands, the aggregation state of the mutant protein closely resembled that of the wild-type enzyme. At high enzyme concentrations (200 μg/gradient), both proteins sedimented with an apparent molecular weight of approximately 60 000, indicative of virtually complete dimer formation. When the enzyme concentration was lowered to 2 μg/gradient, the monomer–dimer equilibrium was shifted nearly completely to the monomer for both species. These data indicate that the K93C mutation has removed an essential feature of the enzyme's catalytic apparatus which does not result in a drastic change from the overall structure of the wild-type enzyme.

Restoration of Catalytic Activity to K93C ODCase by Chemical Modification. Initial efforts to restore activity to native K93C ODCase with 2-bromoethylamine were unsuccessful, but activity could be restored upon sequential denaturation of the protein with 7 M urea and chemical modification, followed by renaturation by dialysis. The enzymatic activity which resulted from modification of urea-denatured K93C ODCase with 2-bromoethylamine revealed that the decrease in activity probably resulted solely from removal of this critical lysine residue and not from an undetected mutation elsewhere in the sequence. Since the restored activity represents $\sim 1 \times 10^{-2}$ times that of the wild-type enzyme in a parallel experiment, treatment with 2-bromoethylamine causes an increase in the catalytic activity of K93C ODCase of at least 5×10^5. The magnitudes of the decrease in activity upon mutation and the degree of restored activity are quite similar to those seen for similar manipulation of restored activity are quite similar to those seen for similar manipulation of the catalytic Lys258 in aspartate aminotransferase (Planas & Kirsch, 1991).

The restoration of activity by 2-bromoethylamine also allowed the opportunity to append Cys93 with a variety of reagents and examine the catalytic potential of modified proteins with various chemical moieties replacing the ε-amino terminus of Lys93, thus further expanding the selection of chemical substitutions available through mutagenesis alone. However, no other brominated reagent was able to restore detectable activity to the K93C protein. Apparently none of the functional groups at the end of the appended Cys93 side chain in this experiment—hydroxyl, quaternary amine, or amide—is able to function in the same way as the primary amine, and so the presence of a titratable proton appears necessary at the end of a side chain the length of the lysine side chain in the wild-type enzyme. We cannot rule out the possibility that the ineffective sulfhydryl reagents have less reactivity at Cys93 or that the mutant protein modified with the ineffective reagents may not properly refold upon renaturation. However, we suspect that much of the (5×10^5)-fold activity increase seen with the bromoethylamine-modified K93C protein is due to the properly placed amino group.

If the presence of a dissociable proton is indeed the required function of this side chain, then the complete absence of activity in the 2-bromoethanol-modified mutant protein is somewhat surprising. The possibility that the proton dissociation of Lys93 is enhanced in such a way that cannot be mimicked by *S*-(hydroxyethyl)Cys93 is discussed at the end of this section.

Binding of Ligands by K93C ODCase As Determined by Sedimentation. Several lines of evidence lead to the conclusion that the binding of ligands which shifts the monomer–dimer equilibrium toward dimer formation at low ODCase concentrations occurs at the active site of yeast ODCase. The formation of a 5.1S dimer of bifunctional mouse UMP synthase was found to be directly related to the degree of saturation of ODCase competitive inhibitors (Traut et al., 1980); this result was interpreted as an indication of dimerization upon active site ligand binding. Data on the sedimentation of yeast ODCase in the presence of various ligand concentrations (Bell & Jones, 1991) indicate a similar relationship between active site binding and dimerization and suggest that saturation at the active site is necessary for full dimer formation.

In this study, UMP at 80 μM, ~5 times the K_i at the pH studied [calculated from Levine et al., (1980)], was the lowest concentration measured which caused a shift in the apparent molecular weight of wild-type ODCase to give virtually full dimer formation (Table III). The wild-type enzyme was found to dimerize in the presence of 0.5 μM 6-azaUMP; the equilibrium between monomer and dimer could not be clearly observed with 6-azaUMP since the K_i value for this ligand is lower than the concentration of protein needed for these sucrose gradient experiments.

With the premise that dimerization is induced by ligand binding only at the active site, the dimerization of K93C ODCase can be used as a means to assess the affinity of this catalytically inactive protein for different ligands. The level of OMP necessary to induce dimer formation of K93C ODCase (Table III) is indicative of a binding constant which is not significantly changed from the K_m of the wild-type enzyme.

[3] The correct values for these estimated rate enhancements [compounds 3 and 7, Beak and Siegel (1976)] are 4×10^{10} and 4×10^8, not 4×10^{12} and 4×10^{10} (P. Beak, personal communication).

This is determined from the observation that dimer formation of the mutant protein is predominant in the presence of 2 μM OMP and essentially complete in the presence of 4 μM OMP (a further verification that the structure of K93C ODCase is relatively unperturbed). Assuming that the degree of saturation necessary for dimerization of the mutant is the same as for the wild-type enzyme, and using the data from the wild-type enzyme that dimerization is virtually complete in this assay at a ligand concentration 5-fold higher than the inhibition constant for UMP, the approximate dissociation constant calculated for OMP with the K93C enzyme would be 0.8 μM. The K_m for wild-type ODCase has been measured in the range of 0.5–1.0 μM, varying with the assay conditions. This observation indicates that the predominant effect of the K93C mutation on the catalytic ability of ODCase is not an effect on binding (K_m), but is instead an effect on k_{cat}, and that Lys93 participates in a catalytic step which follows substrate binding.

Although the mutant enzyme's binding affinity for OMP is not significantly changed from that of the wild-type enzyme, the affinity of K93C ODCase for the inhibitors UMP and 6-azaUMP have been altered considerably from those seen with the wild-type enzyme (Table III). The affinity of the mutant enzyme for UMP is higher than that for 6-azaUMP, in sharp contrast to the affinities of the wild-type enzyme. In fact, the minimum levels of 6-azaUMP and UMP necessary for dimerization of the wild-type enzyme are nearly reversed with the K93C mutant.

Proposed Role in Catalysis and Inhibitor Binding for Yeast ODCase Lys93. The following proposed catalytic role of Lys93 in yeast ODCase unifies the data on the altered affinities of K93C ODCase for ligands, as judged by sedimentation properties in the presence of these ligands, with the drastic loss of decarboxylase activity of mutant ODCase lacking this integral lysine residue. Our data suggests that Lys93—a residue which is invariant in all known ODCase sequences—is located at the active site and participates in catalysis in such a way that is not involved simply with substrate binding. Restoration of enzymatic activity to the inactive K93C mutant yeast ODCase with 2-bromoethylamine, but not with other similar sulfhydryl-modifying reagents, suggests that the titratability of a proton on Lys93, and not simply the positive charge or the polarity, is necessary for a functional catalytic site. In addition to its indispensible catalytic role, Lys93 is also involved in the wild-type enzyme's preference for anionic inhibitors and apparently contacts the differentially charged portions (C-2–C-4) of 6-azaUMP and UMP. Isotope effect studies (Smiley et al., 1991) evidenced a noncovalent, proton-dependent catalytic step kinetically distinguishable from substrate binding and preceding decarboxylation. We thus propose that the ϵ-amino group of Lys93 is the critical catalytic residue responsible for protonating the substrate OMP in a mechanism similar to that first proposed by Beak and Siegel (1976).

Our data with the K93C mutant enzyme lead to a model for ligand binding which involves contact of Lys93 with the C-2–C-4 side of OMP, UMP, and 6-azaUMP, including protonation of OMP which leads to catalysis. The loss of decarboxylase activity and the reversed affinities for 6-azaUMP and UMP of the K93C enzyme can be explained by this model, assuming that the active site is predominantly hydrophobic[4] with the significant exception of the amino group

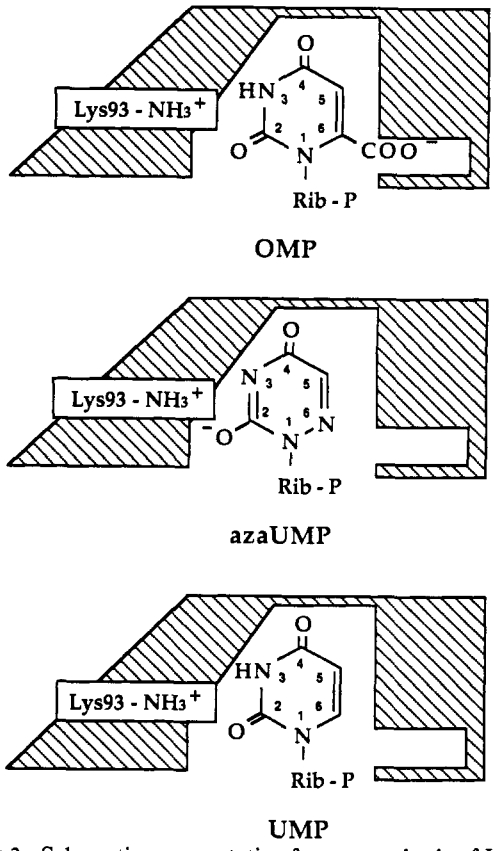

FIGURE 3: Schematic representation for proposed role of Lys93 in catalysis and inhibitor binding. Lys93 is postulated to donate the catalytic proton to O-2 of the substrate OMP. Lys93 is also proposed to be the key protein functional group which attracts the anionic forms of 6-azaUMP ($pK_a = 7.0$, one tautomeric form of anion shown) and UMP ($pK_a = 9.5$, neutral form shown).

of Lys93. This model is depicted in Figure 3. The positive charge of Lys93 of the wild-type enzyme is aligned with the negative charge of 6-azaUMP at the active site, and the favorable charge interaction allows the anionic ligand ($pK_a = 7.0$) to bind tightly. By contrast, UMP is predominantly neutral at the pH studied ($pK_a = 9.5$), has a much smaller degree of favorable charge interaction, and thus displays a more modest affinity.

The postulated lack of favorable charge interaction of the K93C mutant enzyme is in accord with the reversal of affinities for 6-azaUMP and UMP in this model. The anionic 6-azaUMP has no favorable charge interaction with the mutated active site, and so its affinity is weakened accordingly. UMP, by contrast, is neutral both in solution and at the active site and thus remains bound to be the less polar K93C active site with more affinity than with the wild-type enzyme.

The model in Figure 3 involves a modification of the Levine et al. (1980) model for substrate and inhibitor binding. In the proposed active site model presented in Figure 3, the ϵ-amino group would contact the substrate on the uncharged side of the OMP molecule, and interact with the charged side of 6-azaUMP and UMP, and so these inhibitors would be preferentially aligned at the active site in the same way as the substrate. Thus, the charged portions of OMP (the carboxylate) and the inhibitors 6-azaUMP and UMP (the C-2–C-4 side) would not be facing the same portion of the enzyme active site, as suggested in the model of Levine et al.

In the active site model presented here, BMP could interact with Lys93 in a forward or reverse orientation, either of which could lead to a highly stabilized enzyme–inhibitor complex (Figure 4). If the pyrimidine ring of BMP binds in a forward orientation (relative to OMP, panel A of Figure 4), then the

[4] Additional support for a hydrophobic active site comes from the observation of lowered ^{13}C isotope effects in ODCase catalysis upon addition of glycerol to the reaction medium (Smiley et al., 1991), an effect which is expected for enzymes with hydrophobic active sites.

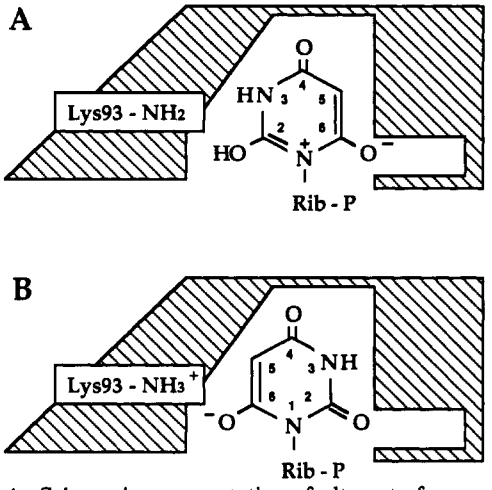

FIGURE 4: Schematic representation of alternate forms of BMP binding at ODCase active site. (A) BMP binds with uncharged C-2–C-4 side of pyrimidine facing Lys93 and becomes protonated, leading to zwitterion resembling catalytic intermediates. (B) Charged portion of BMP aligns with Lys93, yielding favorable charge interaction.

inhibitor could undergo protonation similar to the substrate and form a zwitterion which is very similar to the zwitterionic intermediates of the catalytic pathway (Figure 1). On the other hand, if a BMP molecule were to bind at the active site in a reverse conformation (panel B), then the C-4–C-6 side of the ring could form a charge interaction with Lys93, as postulated for 6-azaUMP and UMP. The differences in affinities of BMP, 6-azaUMP, and UMP would then be attributable to the abilities of the uncharged portions of the rings—amide, imine, and alkene, respectively—to contact other portions of the active site, as was proposed by Levine et al.

If Lys93 is indeed the titratable functional group with a pK_a of ~7, this dissociation constant would be quite low [cf. Fersht (1985)] even considering that a lysyl ϵ-amino group acting as a proton donor would be expected to have a lower dissociation constant. It is interesting to note that the preceding amino acid in the yeast protein sequence is an arginine and is conserved in all eucaryotic sequences but not in bacterial ODCases. Ample precedent exists in the literature for proximal cationic side chains which lower the pK_a of active site lysines; this effect has been seen in acetoacetate decarboxylase (Kokesh & Westheimer, 1971; Schmidt & Westheimer, 1971) and more recently in mandelate racemase (Neidhart et al., 1991). Possibly Arg92 of the yeast enzyme acts to enhance the acidity of the amino group of Lys93, thereby increasing the catalytic potential of the enzyme. This effect may explain the inability of 2-bromoethanol-modified K93C ODCase to regain catalytic activity; a charge repulsion exerted by Arg92 upon Lys93, leading to an efficient proton donor, may be recreated by 2-bromoethylamine modification of K93C ODCase but not by similar treatment with 2-bromoethanol. Such a mechanism is, of course, speculative at this point, and we anticipate that the acquisition of structural data from ODCase crystals (Bell et al., 1991) will reveal this enzyme mechanism in even more detail.

To summarize, evidence has been presented for a catalytic role of Lys93 of yeast ODCase as a proton donor in catalysis. Catalytic activity is reduced to an undetectable level by substitution of Lys93 with a cysteine, yet the mutant enzyme dimerizes, via ligand binding at the active site, in the presence of substrate at a concentration which reflects a binding constant which is not changed considerably from the K_m of the wild-type enzyme. Catalytic activity can be partially restored by chemical modification of the mutant enzyme with 2-bromoethylamine, but not with other analogous brominated reagents, indicating a specific role for the proton-donating ability of the Lys93 side chain. In addition to its catalytic role, Lys93 is also shown to be critical for the enzyme's enhanced binding of anionic inhibitors.

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