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General Base Catalysis in a Glutamine for Histidine Mutant at Position 51 of Human Liver Alcohol Dehydrogenase†

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ABSTRACT: On the basis of the three-dimensional structure of horse liver alcohol dehydrogenase determined by X-ray crystallography, His 51 has been proposed to act as a general base during catalysis by abstracting a proton from the alcohol substrate. A hydrogen-bonding system (proton relay system) connecting the alcohol substrate and His 51 has been proposed to mediate proton transfer. We have mutated His 51 to Gln in the homologous human liver $\beta_1\beta_1$ alcohol dehydrogenase isoenzyme which is expected to have a similar proton relay system. The mutation resulted in an about 6-fold drop in V/K_b (V_{max} for ethanol oxidation divided by K_m for ethanol) at pH 7.0 and a 12-fold drop at pH 6.5. V/K_b could be restored completely or partially by the presence of high concentrations of glycylglycine, glycine, and phosphate buffers. A Brønsted plot of the effect on V/K_b versus the pK_a of these bases plus H_2O and OH^- was linear. Only secondary or tertiary amine buffers differed from linearity, presumably due to steric hindrance. These results suggest that His 51 acts as a general base catalyst during alcohol oxidation in the wild-type enzyme and can be functionally replaced in the mutant enzyme by general base catalysts present in the solvent. Steady-state kinetic constants for NAD^+ and the trifluoroethanol inhibition patterns were similar between the wild-type and the mutant enzyme. Differences in the inhibition constants (K_i) of caprate and trifluoroethanol below pH 7.8 and in the pH dependence of K_i can be explained by the substitution of neutral Gln for positively charged His.

Enhancement of chemical reactions by enzymes in many cases involves acid/base catalysis by functional groups in the enzyme. The imidazole side chain of histidine with a pK_a value around 7 is a particularly suitable acid/base catalyst (proton donor/acceptor) at pH 7; e.g., a histidine located in the active center of papain, chymotrypsin, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase has been suggested to act as a proton donor/acceptor (Fersht, 1984). In the horse liver alcohol dehydrogenase EE isoenzyme, the imidazole ring of His 51 is about 6 Å from the enzyme-bound alcohol substrate hydroxyl group. This distance precludes direct proton transfer, but the three-dimensional structure suggests that alcohol and His 51 are connected by a hydrogen-bonding system involving hydroxyl groups of Ser 48 and of the coenzyme nicotinamide ribose (Eklund et al., 1982). This hydrogen-bonding system has been proposed to mediate proton transfer and has been named the "proton relay system" (Eklund et al., 1982).

The EE isoenzyme of horse liver ADH[1] is a member of a family of mammalian isoenzymes termed class I ADH (Vallee & Bazzone, 1983). It is a dimer and contains two atoms of Zn^{2+} per subunit (Eklund et al., 1976). The horse liver EE isoenzyme shares 88% amino acid sequence identity with the human liver $\beta_1\beta_1$ isoenzyme (Hempel et al., 1984). Importantly, $\beta_1\beta_1$ contains the same active site residues as EE except for Ser 48 and Ile 318, which are conservatively replaced by Thr and Val, respectively, in $\beta_1\beta_1$ (Eklund et al., 1987). Computer modeling studies suggest that the hydroxyl of Thr 48 in $\beta_1\beta_1$ can form the same hydrogen bonds as Ser in the EE isoenzyme (Eklund et al., 1987). It is therefore probable that the proton relay system exists in $\beta_1\beta_1$.

We have mutated His 51 in the $\beta_1\beta_1$ isoenzyme to Gln and examined the steady-state kinetics with ethanol and NAD^+,

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[1] Abbreviations: ADH, alcohol dehydrogenase; Cap-Gapp, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]pyrazole; ACES, 2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid; TAPSO, 3-[*N*-[tris(hydroxymethyl)methyl]amino]-2-hydroxypropanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate.

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the inhibition constants of trifluoroethanol and caprate, and the effects of buffers on alcohol oxidation. These studies were designed to test the role of His 51 in alcohol dehydrogenase.

Materials and Methods

β_1 cDNA Mutagenesis. The isolation of the human ADH cDNA coding for the β_1 subunit has been reported by Edenberg et al. (1989). The cDNA was cloned into the *Eco*RI site of M13HinEco1 (Edenberg et al., 1987), and the noncoding 5'-end was deleted to obtain a 13-base-pair distance between Shine–Dalgarno sequence and start codon in the expression vector (Hurley et al., 1990). Mutagenesis was performed according to the method of Taylor et al. (1985), using the Amersham mutagenesis kit. The codon for amino acid 51 was changed from CAC (His) to CAA (Gln) by using a 28-mer oligonucleotide which was synthesized on an Applied Biosystems 380B DNA synthesizer. Mutants were identified by sequencing of single-stranded M13 DNA (Tabor & Richardson, 1987). The mutated cDNA insert was excised with *Eco*RI, isolated from vector DNA by electrophoresis on low-melting agarose (International Biotechnologies, New Haven, CT), and ligated into the *Eco*RI-cut and dephosphorylated expression plasmid vector pKK223-3 (Pharmacia, Uppsala, Sweden). The recombinant expression vector DNA was transformed into *Escherichia coli* JM105 cells (Yanisch-Perron et al., 1985). Recombinant vector DNA for double-stranded sequencing (Chen & Seeburg, 1985) of the cDNA insert was obtained from a large-scale plasmid DNA preparation by cesium density gradient centrifugation (Maniatis et al., 1982). Sequencing at this stage ensured that no other mutations had occurred and was performed with synthetic primer oligonucleotides. *E. coli* JM105 cells containing the expression vector were frozen in liquid nitrogen (Maniatis et al., 1982) and stored at −80 °C.

Expression and Isolation of ADH. A starter culture of 25 mL of LB media (Maniatis et al., 1982) containing 50 μg/mL ampicillin was inoculated with 1 mL of frozen *E. coli* JM105 cell stocks. After approximately 6–10 h of cell growth, this culture was used to inoculate a 4.8- or 9.6-L culture of modified TB media (Tartof & Hobbs, 1987) containing 12 g of peptone, 12 g of yeast extract, and 4 mL of glycerol per liter, plus 17 mM KH_2PO_4, 72 mM K_2HPO_4, and 50 μg/mL ampicillin. At an A_{600} of 0.4–0.7, 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to induce expression. The cells were harvested after approximately 10 h of growth by using the Millipore Pellicon cassette concentrator equipped with a 0.45-μm membrane. The concentrated cells were centrifuged at 5000*g* for 10 min, and the cell pellets were lysed in a bead beater (Bio-Spec, Bartlesville, OK) in 10 mM Tris buffer, pH 8.0, containing 0.1 mM phenylmethanesulfonyl fluoride. The lysis buffer as well as all buffers used during enzyme isolation were degassed by purging for 10 min with He and contained 3 mM DTT, 1 mM EDTA, and 1 mM benzamidine. The purification of ADH enzyme from the cell lysates was performed essentially as described for the isolation of ADH from human livers (Lange et al., 1976; Burnell et al., 1989; Hurley et al., 1990). Briefly, the lysed cells were centrifuged for 35 min at 100000*g*. The supernatant was filtered through a DEAE-cellulose batch (DE 52, Whatman, Hillsboro, OR), made 100 mM in Tris buffer, pH 8.0, and applied to an Agarose-AMP affinity column (Sigma) in the same buffer. Enzyme was eluted with 100 mM Tris, pH 8.0, containing 0.5 M NaCl and 0.5 mM NADH. After dialysis against one of the following column buffers, enzyme was purified to homogeneity either by chromatography over Cap-Gapp–Sepharose (Lange et al., 1976) in 100 mM Tris buffer, pH 8.0, or over CM-cellulose (Whatman) in 10 mM Tris buffer, pH 8.6 (Burnell et al., 1989). The pure enzyme was dialyzed against 10 mM sodium phosphate buffer, pH 7.2, and, if necessary, concentrated with an Amicon stirred cell concentrator.

Enzyme activity was followed spectrophotometrically in 100 mM glycine buffer, pH 10.0, containing 33 mM ethanol and 2.5 mM NAD^+ by using a Gilford Response or a Cary 219 spectrophotometer. Enzyme activity units are expressed as micromoles of NADH produced per minute on the basis of an absorbance of NADH of 6.22 mM^{-1} cm^{-1} at 340 nm. SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) was performed by using a Hoefer Mighty Small apparatus. Protein concentration was determined by the Bio-Rad protein assay with bovine serum albumin as standard (Bradford, 1976). Isoelectric focusing was performed according to the conditions described by Burnell et al. (1989).

Steady-State Kinetics. All enzyme assays were performed at 25 °C in a volume of 1 or 3 mL. Production of NADH was monitored spectrophotometrically or fluorometrically by using a Gilford Response or Cary 210 spectrophotometer or an Aminco filter fluorometer. All buffer compounds were purified by crystallization from water, and buffers were titrated to pH with NaOH. The activities of the conjugate base in a buffer at a given buffer concentration and pH were calculated according to Ellis and Morrison (1982). All assays were done in duplicate or triplicate with different enzyme amounts. Kinetic constants were calculated from initial rates by using the computer programs HYPER, SEQUEN, COMP, and UNCOMP, and pK_a values were calulated from pH-dependent data with the program HABELL (Cleland, 1979). If not stated otherwise, the standard errors for kinetic parameters were 20% or less. The significance of differences between kinetic constants at the 95% level was tested by using Student's *t* test. Maximal activity (V) is expressed as turnover number (s^{-1}) using a molecular weight of 40000 per subunit, each having one active site. pK_a values of buffers at 25 °C were taken from Good et al. (1966), Ferguson et al. (1980), and Segel (1968).

Reagents. Reagents were from the following sources: NaOH, Mallinckrodt, St. Louis, MO; ethanol, Midwest Grain, Pekin, IL.; NAD^+, grade I, Boehringer, Mannheim, FRG. All other chemicals were from Sigma, St. Louis, MO.

Results

Expression and Purification of Enzymes. Between 1.5 and 3.5 units (μmol/min) of ethanol-oxidizing activity per liter of bacterial culture of the mutant (β51Gln) and 1.2–2.2 units of the wild-type (β51His) enzyme were obtained in the cell homogenates. Chromatographic purification with either Cap-Gapp–Sepharose or CM–cellulose as the final step yielded enzymes which were homogeneous on SDS–polyacrylamide gels with a subunit molecular weight of 40000 (not shown). Overall yields of pure enzyme were 5–10% for the wild type and 17–40% for the mutant enzyme. Once purified to homogeneity, both enzymes were stable at 4 °C for at least 2 weeks. On isoelectric focusing gels there was a different pattern depending on the final step in the purification. For both the wild-type and the mutant enzyme, a single band was seen after Cap-Gapp chromatography, and two closely spaced bands after CM chromatography. There was no apparent difference in the p*I* between the wild-type and the mutant forms. The K_m values for NAD^+ and ethanol, the inhibition constant of NAD^+, the maximal velocity, tested at pH 7 and 10, and the effect of buffer bases on V/K_b showed no significant difference between single- and double-banded enzyme preparations. A similar heterogeneity in isoelectric focusing had been described for the $\beta_3\beta_3$ ADH isoenzyme purified from

Table I: Steady-State Kinetic Constants for Reduction of NAD^+ with Ethanol[a]

constant	enzyme	pH 7	pH 8	pH 9	pH 10
V (s^{-1})	β51His	0.066	0.066	0.085	0.15
	β51Gln	0.13[b]	0.12[b]	0.19[b]	0.43[b]
V/K_a (s^{-1} mM^{-1})	β51His	11	19	32	13
	β51Gln	5.7	13	26	33[b]
K_{ia} (μM)	β51His	53	19	7.5	10
	β51Gln	33	36	25[b]	30[b]

[a] Maximal standard errors of single experiments were 8% (V), 40% (V/K_a), and 35% (K_{ia}). Buffers used are ACES (pH 7), glycylglycine (pH 8), and glycine (pH 9 and 10), all at 50 mM. [b] Significantly higher ($p < 0.05$) in β51Gln compared to β51His. All other values are not significantly different.

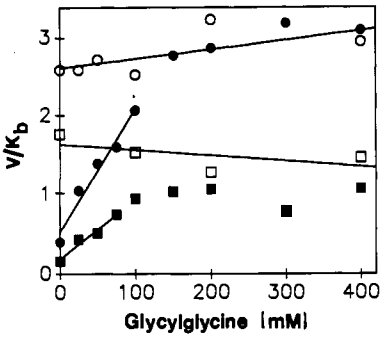

FIGURE 1: Effect of glycylglycine on ethanol oxidation. Assay buffer contains 10 mM ACES and varying concentrations of glycylglycine. NAD^+ is 2.5 mM. V/K_b is given in s^{-1} mM^{-1}. Open circles represent wild-type (β51His) values at pH 7.0; filled circles, mutant (β51Gln) at pH 7.0; open squares, β51His at pH 6.5; filled squares, β51Gln at pH 6.5.

human livers (Burnell et al., 1989).

Steady-State Kinetic Characterization. The kinetic parameters for the reduction of NAD^+ (substrate A) with ethanol (substrate B) were determined by varying both the ethanol and NAD^+ concentration. Kinetic constants[2] were obtained by fitting initial rates to the equation for the sequential mechanism:

$$v = \frac{V[A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]} \quad (1)$$

Table I lists V/K_a, K_{ia}, and V at different pH values for the wild type (β51His) and the mutant (β51Gln). The values of either enzyme vary less than 6-fold over a range of 4 pH units; thus none of these parameters exhibits a marked pH dependency. The differences in the parameters between wild type and mutant are less than 4-fold. Nonetheless, V is significantly higher in the mutant than in the wild type at all pH values.

Effect of Bases on Ethanol Oxidation. Steady-state kinetic parameters for the oxidation of ethanol were determined by varying ethanol at 2.5 mM NAD^+. Lineweaver–Burk plots were linear in all cases, and initial rates fit a simple Michaelis–Menten equation (Cleland, 1979). To confirm that 2.5 mM NAD^+ was actually saturating at different buffer concentrations, the apparent K_m of NAD^+ at low ethanol concentration ($0.5K_b$) was determined for the highest concentration of each buffer or of Na_2SO_4. The apparent K_m was found to be at least 10-fold below the NAD^+ concentration.

[2] V denotes the maximal activity (V_{max}) at saturating alcohol and NAD^+ concentrations; K_a and K_b are the Michaelis constants for NAD^+ and alcohol, respectively; and K_{ia} is the inhibition constant for NAD^+ (eq 1). K_i (eq 2) is the slope inhibition constant for caprate or trifluoroethanol with ethanol as the varied substrate.

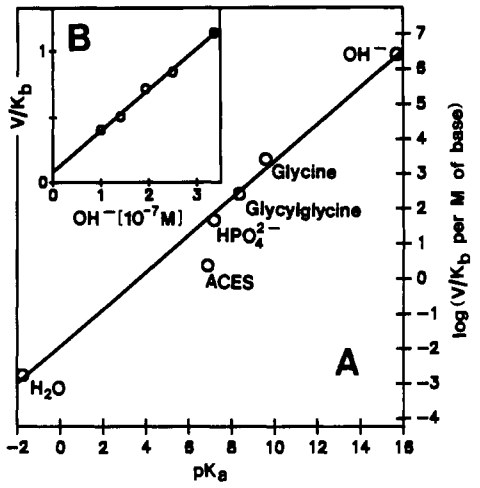

FIGURE 2: (A) Brønsted plot for the increase in V/K_b (s^{-1} mM^{-1}) per M of buffer base or OH^-. The y axis value for H_2O is taken from the y-axis intercept in panel B, divided by 55 M. The line represents a fit to data points except for ACES. (B) Effect of OH^- on ethanol oxidation by the mutant (β51Gln). The assay buffer was 50 mM TAPSO, pH 7.0–7.5. Assay conditions and unit of V/K_b are described in Figure 1.

In experiments examining the effects of buffer bases, 10 mM ACES was included to extend buffering capacity beyond the pK_a of the buffer. The influence of varying concentrations of glycylglycine on V/K_b is shown in Figure 1. In the absence of glycylglycine (i.e., in 10 mM ACES only), V/K_b was about 6-fold higher in the wild type (β51His) than in the mutant (β51Gln) at pH 7.0. With increasing concentration of glycylglycine, V/K_b was approximately constant in the wild type but increased in the mutant enzyme up to the wild-type value, with no further increase at glycylglycine concentrations above about 200 mM. Similarly, at pH 6.5 the value of V/K_b was about 12-fold lower in the mutant than in the wild type and approximated the wild-type value with increasing concentrations of glycylglycine up to about 200 mM. Below 100 mM, the slope of the increase in V/K_b per M glycylglycine at pH 6.5 (7.3 ± 1.0) was about 2-fold less than the slope at pH 7.0 (15.1 ± 1.6) in the mutant enzyme (Figure 1). An effect on V/K_b was seen for several other buffers (phosphate, glycine, and ACES) tested at pH 7.0 in the mutant enzyme. In the wild-type enzyme, the effect of all buffers was at least 8-fold less than that in the mutant as shown for glycylglycine in Figure 1. No effect on V/K_b was found with TAPSO and MOPS tested up to a concentration of 200 mM. The effect of the buffers was not a consequence of changes in ionic strength, since Na_2SO_4 varied between 16 and 100 mM did not affect V/K_b.

Since the mutant enzyme appears to be susceptible to general base catalysis, OH^- and H_2O, which are the conjugate bases of H_2O and H_3O^+, respectively, are expected to affect V/K_b. Since TAPSO had no influence on V/K_b for the mutant enzyme, the effect of pH between 7.0 and 7.5 on V/K_b was examined in 50 mM TAPSO buffer. The effect of OH^- on V/K_b was found to be linear (Figure 2B), allowing extrapolation to an OH^- concentration of zero, where 55 M H_2O is the only proton acceptor. V/K_b of the wild-type enzyme was independent of pH in this range. Figure 2A shows a Brønsted plot, where the increase in V/K_b per M activity of base (conjugate base of buffers, and OH^-) is plotted against the pK_a values. The y axis value of H_2O in Figure 2A is taken from the y-axis intercept in Figure 2B, expressed per M H_2O. Figure 2A shows that the bases H_2O, HPO_4^{2-}, glycylglycine, glycine, and OH^- exhibit a linear Brønsted relationship with

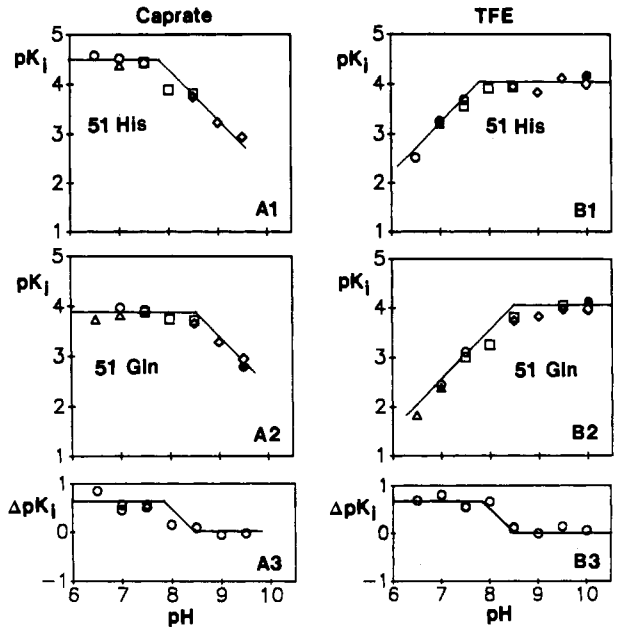

FIGURE 3: pH dependence of inhibition constants of substrate analogues. K_i was determined from the inhibition of ethanol oxidation by caprate (panels A1 and A2) or trifluoroethanol (panels B1 and B2). All buffers were 50 mM. NAD^+ was 2.5 mM. Lines are the asymptotes of fitted pH-dependent functions. Symbols for buffers are triangle for ACES, open circle for phosphate, square for glycylglycine, diamond for glycine, and filled circle for carbonate. The pH dependence of the difference in the inhibition constants between wild type and mutant is shown in panels A3 and B3. The pK_i values and asymptotes of fitted equations in panels A2 and B2 are subtracted from those in panels A1 and B1, respectively.

a slope of 0.53 ± 0.015. A deviation from the Brønsted relationship by about 1 log unit was seen for ACES.

pH Dependence of Inhibition of Activity by Trifluoroethanol and Caprate. The inhibition constants[2] of caprate and trifluoroethanol were determined in kinetic experiments by varying the ethanol and inhibitor concentration with saturating NAD^+. Initial rates were fit to the equation:

$$v = \frac{V[B]}{K_b(1 + [I]/K_i) + [B]} \quad (2)$$

A competitive inhibition pattern was obtained at all pH values. For both enzymes, the pH dependences of the inhibition constants fit single-pK_a protonation equations (eq 3 for trifluoroethanol, eq 4 for caprate). In the wild-type enzyme,

$$\log K_i = \log C + \log (1 + [H]/K_a) \quad (3)$$

$$\log K_i = \log C + \log (1 + K_a/[H]) \quad (4)$$

trifluoroethanol and caprate exhibited identical pK_a values of 7.8 ± 0.1 (Figure 3, panels A1 and B1). In the mutant enzyme, the pK_a values of both inhibitors were shifted to 8.5 ± 0.1 (Figure 3, panels A2 and B2). A shift was also seen in the pK_i of these inhibitors. This is evident from panels A3 (caprate) and B3 (trifluoroethanol) of Figure 3 where the fitted asymptotes describing the pK_i values of the mutant (panels A2 and B2) were subtracted from those of the wild type (panels A1 and B1, respectively). Above pH 8.5, the difference in pK_i (ΔpK_i) between wild type and mutant for either caprate or trifluoroethanol was virtually zero. Below pH 7.8, there was a shift by about 0.7 pK_i units.

Substrate Binding Order As Determined by Trifluoroethanol Inhibition. Earlier product inhibition studies conducted at pH 7.5 with the $\beta_1\beta_1$ wild-type enzyme purified from human livers were consistent with an ordered BiBi mechanism with coenzyme binding first (Bosron et al., 1983). As expected for this mechanism, an uncompetitive inhibition pattern was obtained for the wild-type enzyme at pH 7 when NAD^+ and the inhibitor trifluoroethanol were varied at a subsaturating ethanol concentration. This uncompetitive inhibition was obtained at both pH 7 and 10 with both the wild-type and the mutant enzyme.

Discussion

The primary sequence of the $\beta_1\beta_1$ isoenzyme of human liver ADH differs from that of the horse liver ADH EE isoenzyme at 48 positions. Computer modeling has shown that all side-chain differences between these enzymes can be accommodated in the three-dimensional structure without major rearrangements of the protein backbone (Eklund et al., 1987). Preliminary X-ray crystallographic studies of the $\beta_1\beta_1$ isoenzyme refined to 3.3-Å resolution indicate almost identical protein backbone folding (Hurley et al., unpublished results). In the three-dimensional structure of the horse EE enzyme–NAD^+–bromobenzyl alcohol complex, Ser 48 is positioned in the inner part of the active-site pocket in hydrogen-bonding distance to the alcohol oxygen. Distances suitable for hydrogen bonds are also found between the hydroxyl of Ser 48 and the 2′-hydroxyl of the coenzyme nicotinamide ribose, and between this and a nitrogen of the imidazole of His 51 (Eklund et al., 1982) as shown schematically in Figure 4. This hydrogen-bonding system has been proposed to transfer a proton from the alcohol substrate to His 51. In the ternary complex, the alcohol hydroxyl is completely shielded from solvent and probably cannot directly transfer its proton to solvent, whereas His 51 is solvent-exposed (Eklund et al., 1982). Computer modeling suggests that the hydroxyl of the substituted Thr 48 in $\beta_1\beta_1$ can form the same hydrogen-bonding system as Ser (Eklund et al., 1987).

On the basis of suggestions of Eklund et al. (1982) and Cook and Cleland (1981) concerning the horse EE enzyme, the role

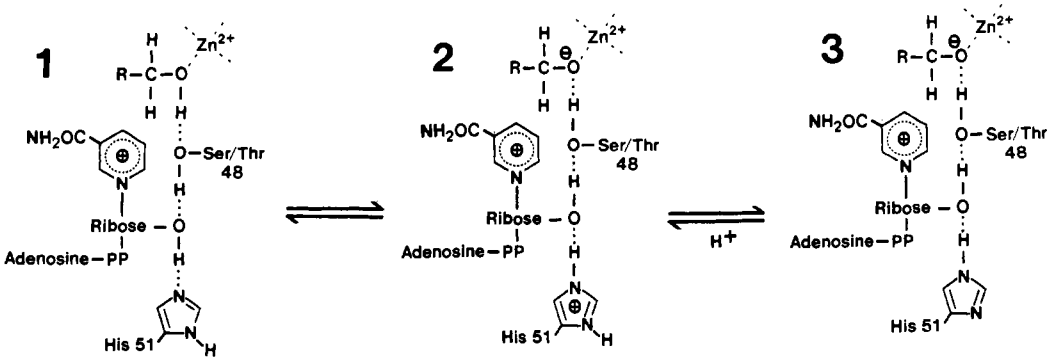

FIGURE 4: Scheme for hydrogen bonding and ionization of the proton-relay system.

of the proton relay system in the interconversion of the ternary complexes is summarized in Figure 4. Amino acid position 48 is given alternatively as Ser and Thr for the horse EE and the human $\beta_1\beta_1$ isoenzyme, respectively. During interconversion of structure 1 to the tautomeric structure 2, a rearrangement of hydrogen and covalent bonds results in abstraction of a proton from the alcohol substrate and addition of a proton to the imidazole of His 51. The proton can subsequently be released from His 51 to the solvent (structure 3). Deprotonation of zinc-bound alcohol is facilitated by the drop in pK_a of its hydroxyl, which is brought about by the positively charged active site zinc and, to a lesser extent, by the proximity of the positively charged NAD^+ nicotinamide ring. The pK_a values for different alcohols bound to zinc in ADH have been found to be between 4.3 and 6.4 and thus exhibit pK_a values depressed by about 9 units compared to the values in solution (Kvassman & Petterson, 1978, 1980; Kvassman et al., 1981).

We have replaced His 51 in the $\beta_1\beta_1$ enzyme (wild type) with glutamine (mutant enzyme) by site-directed mutagenesis. Gln was chosen since it is similar to histidine in hydrophilic character of the side chain (Wolfenden et al., 1981), in size, and in the ability to form hydrogen bonds. We therefore expected that functional differences between the wild type and mutant would be related to the ability of His 51 to act as a proton acceptor and/or to electrostatic effects of the imidazole cation.

His 51 in the horse EE isoenzyme contributes to coenzyme binding by forming a hydrogen bond to the coenzyme ribose hydroxyl as shown in Figure 4 (Eklund et al., 1982). V/K_a and K_{ia} of the wild-type β51His and mutant enzyme β51Gln (Table I) indicate essentially unchanged or only slightly decreased affinity for coenzyme. The 2- to 3-fold difference in V (Table I) suggests that coenzyme is somewhat less tightly bound in the mutant, since the NADH dissociation rate was reported to be limiting for V in $\beta_1\beta_1$ (Bosron et al., 1983; Hurley et al., 1990).

We also compared wild-type and mutant enzyme with respect to inhibition by trifluoroethanol and caprate. These inhibitors are competitive with ethanol in both the horse EE (Shore et al., 1974) and human $\beta_1\beta_1$ enzymes. Binding of trifluoroethanol to the substrate site of the horse EE enzyme has been confirmed by X-ray crystallography (Plapp et al., 1978). Binding of substrates and substrate analogues to the horse EE isoenzyme is generally characterized by two pK_a values. The pK_a for the dissociation rate constant is dependent on the alcohol structure (6.4, 6.4, 5.4, 4.5, and 4.3 for benzyl alcohol, ethanol, 2-chloroethanol, 2,2-dichloroethanol, and trifluoroethanol, respectively) and has been attributed to the ionization of the alcohol itself (Kvassman & Petterson, 1978, 1980; Kvassman et al., 1981). The pK_a of 7.6 for the association rate constant is independent of the alcohol structure and has been assigned to ionization of the zinc-bound water molecule which is displaced by alcohol (Kvassman et al., 1981). It was proposed that this pK_a also may reflect the concerted protonation/deprotonation of zinc-bound water and His 51 due to coupling through hydrogen bonds (Figure 4, structures 1 and 2) (Kvassman et al., 1981; Cook & Cleland, 1981). The pK_a of 7.6 is also seen for equilibrium binding of caprate and trifluoroethanol in the horse EE isoenzyme (Shore et al., 1974), and the corresponding value in the $\beta_1\beta_1$ isoenzyme is 7.8 (Figure 3, panels A1 and B1). In the mutant enzyme (β51Gln), this pK_a is shifted to 8.5 (Figure 3, panels A2 and B2). The small shift could be due to a perturbation of the pK_a of zinc-bound water by the side chain at position 51, the pK_a being lower in the wild-type enzyme due to the positive charge on His 51. Coincident with the shift in pK_a, the mutation resulted in a small shift in the inhibition constants of caprate and trifluoroethanol (K_i) below pH 7.5 (Figure 3, panels A3 and B3). We interpret this shift as a result of electrostatic interaction of His 51 with the anionic inhibitors. The magnitude of the shifts in pK_a and pK_i (0.7 pK unit) seems compatible with a perturbation by an electrostatic field effect over the distance of approximately 6 Å between His 51 and the zinc-bound alcohol or water molecule, since an empirical function for electrostatic effects in proteins derived by Mehler and Eichele (1984) suggests a shift by about 1 pK_a unit for this distance.

We also compared the inhibition pattern obtained with trifluoroethanol when NAD^+ was varied at subsaturating ethanol concentrations both at pH 7 and 10. The uncompetitive inhibition pattern obtained is consistent with an ordered mechanism, whereas an equilibrium or steady-state random mechanism would have a noncompetitive pattern (Fromm, 1979). Thus, when compared to the wild type, the mutant exhibits similar values of NAD^+ kinetic constants and an identical kinetic mechanism.

The most interesting difference between wild type and mutant is seen for the steady-state kinetic parameter V/K_b which in the mutant was about 6-fold lower at pH 7.0 and about 12-fold lower at pH 6.5 compared to the wild-type value. This decrease could be restored by addition of a buffer such as glycylglycine in Figure 1. The increase in V/K_b per M glycylglycine was higher at pH 7.0 than at pH 6.5 (Figure 1), demonstrating that the effect is mediated by the conjugate base of the buffer. The saturation of the increase above about 200 mM glycylglycine may indicate that rate constants sensitive to proton transfer are no longer limiting or that glycylglycine forms a saturable complex with enzyme. Five of the bases tested (H_2O, HPO_4^{2-}, glycylglycine, glycine, and OH^-) formed a linear Brønsted relationship, whereas ACES deviated by about 1 log unit (Figure 2), and MOPS and TAPSO had no effect on V/K_b. Generally, the best linear relationships with Brønsted plots are obtained for homologous bases of similar chemical structure; however, in many cases satisfactorily linear Brønsted plots are obtained for hydrogen-bonding nitrogen or oxygen bases including H_2O and OH^-, even if the bases belong to different chemical classes (Jencks, 1969). The deviations from the Brønsted relationship in the case of ACES (Figure 2) and of TAPSO and MOPS most probably result from steric hindrance, because they are secondary (ACES, TAPSO) or tertiary (MOPS) amine buffers. That a buffer effect is not seen with the wild-type enzyme suggests that His 51 acts as the proton acceptor, whereas, in the mutant, a buffer or solvent base substitutes for this function.

The role of amino acid side chains as acid/base catalysts has been tested in proteins other than liver ADH. In the yeast ADH-I, which has 25% sequence identity to the mammalian ADH's (Jörnvall et al., 1978), Thr 45 and His 48, which have been aligned with Ser 48 and His 51 of the horse EE isoenzyme (Jörnvall et al., 1987b), may form a proton-relay system similar to the one in the mammalian enzyme. Mutation of His 48 to Gln has been reported to drastically change the pH dependence of alcohol oxidation. In the Gln mutant, log (V/K_b) increased linearly with pH over a pH range 5.5–10 with a slope of approximately unity, suggesting that alcohol oxidation was subject to hydroxyl ion catalysis in the mutant enzyme (Plapp et al., 1987). In aspartate aminotransferase, Lys 258 is situated in the active site and has been postulated to be responsible for proton transfer. When Ala was substituted for

Lys, the mutant enzyme had a decreased activity which could be restored by base catalysts added to the buffer, which is similar to our observation with the Gln 51 mutant of alcohol dehydrogenase. In the mutant aminotransferase, the effect of the bases conformed to the Brønsted relationship after correction for the molecular volume, indicating that a steric factor was limiting the accessibility of the bases to the active site (Toney & Kirsch, 1989). In carbonic anhydrase, mutation of His 64 to Ala resulted in a decreased catalytic activity which could be restored by high concentrations of buffers. Similar to the results reported here, not all buffers enhanced enzyme activity, possibly due to steric hindrance (Tu et al., 1989).

His 51 and Ser/Thr 48 are conserved in all known sequences of class I mammalian alcohol dehydrogenase isoenzymes, which share an overall sequence homology of 81% or more (Jörnvall et al., 1987a; Crabb & Edenberg, 1986; Edenberg et al., 1985; Trezise et al., 1989), and even in the distantly related yeast and plant ADH enzymes (Jörnvall et al., 1987b). It is interesting to note, however, that mammalian alcohol dehydrogenases of classes II and III, which share about 60–70% amino acid sequence homology with the class I isoenzymes, have conserved Ser/Thr 48, but not His 51. The human class II and III ADH isoenzymes have a Ser and a Tyr, respectively, at position 51 (Höög et al., 1987; Kaiser et al., 1988, 1989; Julià et al., 1988). Thus, there is no effective proton donor/acceptor at this position, which raises questions as to whether there is complete shielding of the reaction center in the ternary complex of the class II and III isoenzymes, and if so, how the proton in these enzymes is transferred to the solvent, and whether there is a general buffer base effect on V/K_b for these enzymes.

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Domain Structure of the Large Subunit of *Escherichia coli* Carbamoyl Phosphate Synthetase. Location of the Binding Site for the Allosteric Inhibitor UMP in the COOH-Terminal Domain†

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ABSTRACT: The large subunit of *Escherichia coli* carbamoyl phosphate synthetase (a polypeptide of 117.7 kDa that consists of two homologous halves) is responsible for carbamoyl phosphate synthesis from NH_3 and for the binding of the allosteric activators ornithine and IMP and of the inhibitor UMP. Elastase, trypsin, and chymotrypsin inactivate the enzyme and cleave the large subunit at a site approximately 15 kDa from the COOH terminus (demonstrated by NH_2-terminal sequencing). UMP, IMP, and ornithine prevent this cleavage and the inactivation. Upon irradiation with ultraviolet light in the presence of [^{14}C]UMP, the large subunit is labeled selectively and specifically. The labeling is inhibited by ornithine and IMP. Cleavage of the 15-kDa COOH-terminal region by prior treatment of the enzyme with trypsin prevents the labeling on subsequent irradiation with [^{14}C]UMP. The [^{14}C]UMP-labeled large subunit is resistant to proteolytic cleavage, but if it is treated with SDS the resistance is lost, indicating that UMP is cross-linked to its binding site and that the protection is due to conformational factors. In the presence of SDS, the labeled large subunit is cleaved by trypsin or by V8 staphylococcal protease at a site located 15 or 25 kDa, respectively, from the COOH terminus (shown by NH_2-terminal sequencing), and only the 15- or 25-kDa fragments are labeled. Similarly, upon cleavage of the aspartyl–prolyl bonds of the [^{14}C]UMP-labeled enzyme with 70% formic acid, labeling was found only in the 18.5-kDa fragment that contains the COOH terminus of the subunit. Thus, UMP binds to the COOH-terminal domain. Since the binding sites for IMP and UMP overlap, most probably IMP also binds in this domain. The protection from proteolysis by ornithine suggests that ornithine binds in the same domain. Acetylglutamate (the allosteric activator of the ureotelic enzyme) binds to the homologous COOH-terminal domain of the rat liver enzyme [Rodriguez-Aparicio, L., et al. (1989) *Biochemistry 28*, 3070–3074]. Thus, the COOH-terminal domain appears to be the regulatory domain of the carbamoyl phosphate synthetases. To account for the effects of the allosteric effectors on the binding of ATP, we propose a scheme where the two halves of the large subunit form a pseudohomodimer by complementary isologous association, thus placing the NH_2 half, which is involved in the binding of the molecule of ATP that yields P_i, close to the regulatory domain.

Carbamoyl phosphate synthetase catalyzes the first committed step in the urea, arginine, and pyrimidine biosynthesis pathways. Carbamoyl phosphate synthetase is therefore a good candidate for metabolic control, and indeed, the enzyme from a number of sources is subject to allosteric regulation [reviewed in Rubio et al. (1983b)]. The nature of the effectors depends on the physiological function of the synthetase. For example, the ureotelic enzyme is activated by *N*-acetyl-L-glutamate (acetylglutamate)[1] (Hall et al., 1958), whereas the *Escherichia coli* enzyme, which is involved in both pyrimidine and arginine synthesis, is activated by IMP and ornithine and inhibited by UMP (Meister & Powers, 1978).

The activation of the rat liver mitochondrial enzyme (carbamoyl phosphate synthetase I) has been investigated extensively (Alonso & Rubio, 1983; Rubio et al., 1983a; Guadalajara et al., 1987; Britton & Rubio, 1988; Rodriguez-Aparicio et al., 1989; Britton et al., 1990). We have shown that a single molecule of acetylglutamate is bound per enzyme molecule (M_r 160000). The affinity for acetylglutamate is greatest for

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[1] Abbreviations: acetylglutamate, *N*-acetyl-L-glutamate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; ATP_A, the ATP molecule that yields P_i in the enzyme reaction; ATP_B, the molecule that provides the phosphate group of carbamoyl phosphate.

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