An Investigation of the Contribution Made by the Carboxylate Group of an Active Site Histidine–Aspartate Couple to Binding and Catalysis in Lactate Dehydrogenase†

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ABSTRACT: The influence of aspartate-168 on the proton-donating and -accepting properties of histidine-195 (the active site acid/base catalyst in lactate dehydrogenase) was evaluated by use of site-directed mutagenesis to change the residue to asparagine and to alanine. Despite the fact that asparagine could form a hydrogen bond to histidine while alanine could not, the two mutant enzymes have closely similar catalytic and ligand-binding properties. Both bind pyruvate and its analogue (oxamate) 200 times more weakly than the wild-type enzyme but show little disruption in their binding of lactate and its unreactive analogue, trifluorolactate. Neither mutation alters the binding of coenzymes (NADH and NAD^+) or the pK of the histidine-195 residue in the enzyme–coenzyme complex. We conclude that a strong histidine–aspartate interaction is only formed when both coenzyme and substrate are bound. Deletion of the negative charge of aspartate shifts the equilibrium between enzyme–NADH–pyruvate (protonated histidine) and enzyme–NAD^+–lactate (unprotonated histidine) toward the latter. In contrast to the wild-type enzyme, the rate of catalysis in both directions in the mutants is limited by a slow hydride ion transfer step.

The coupling of an aspartate with an active site histidine residue is a feature of several enzymes, among them chymotrypsin (Blow et al., 1969), phospholipase A_2 (Dijkstra et al., 1981), and malate dehydrogenase and lactate dehydrogenase (Birktoft & Banaszak, 1983). In the widely studied serine proteases, the histidine of the pair is coupled to the reactive serine-195 residue and stabilizes the oxy anionic form of the serine by acting as a base. This promotes the serine attack on the amide bond to form a transient tetrahedral intermediate. In the subsequent rearrangement to generate the acylated serine, the histidine acts as an acid which transfers a proton to the leaving amino group. It is suggested that the aspartate contributes to catalytic efficiency by raising the pK of the histidine to neutrality (Fersht & Sperling, 1973) and by anchoring it in the most favorable orientation for acid/base catalysis [for description of mechanism, see Stamato et al. (1986)].

In lactate and malate dehydrogenase the active site histidine also acts as an acid/base catalyst, accepting a proton from the substrate hydroxyl group in the conversion of hydroxy acid to keto acid and donating one to the carbonyl oxygen in the reverse direction (see Figure 1). Its accompanying aspartate residue has been identified in the crystal structures of these dehydrogenases (Birktoft & Banaszak, 1983; Grau et al., 1981) and is present in the sequence of all 16 known LDHs[1] and all 4 known MDHs (J. J. Holbrook and J. Birktoft, unpublished alignments). To provide an empirical test of the contribution of the aspartate-168 residue to LDH catalysis, we have mutated the residue to asparagine and to alanine. In the former case the asparagine residue could still form a hydrogen bond which will orient histidine-195, but the formal negative charge has been removed. In the case of alanine there will be neither the possibility of a hydrogen bond nor a negative charge effect.

Materials and Methods

Mutagenesis. Mutants of the lactate dehydrogenase gene from *Bacillus stearothermophilus* (Barstow et al., 1986) were generated by the oligonucleotide mismatch procedure (Winter et al., 1982) in M13 with the mutagenic oligonucleotide as the primer for in vitro chain extensions. Wild-type and mutant LDHs (Asp-168 → Asn and Asp-168 → Ala) were expressed in the pKK223-3 plasmid in *Escherichia coli* as previously described (Barstow et al., 1986).

Enzyme Purification. The wild-type enzyme was purified on oxamate–Sepharose (Clarke et al. 1985c), and the mutants were purified on Blue Sepharose-F3GA and DEAE-Sepharose (Hart et al., 1987) owing to their poor binding of oxamate and, therefore, lack of adhesion to oxamate–Sepharose.

Steady-State Kinetics. Steady-state measurements were made by following the change in absorbance at 340 nm in the NADH/NAD^+ conversion. All assays were at 25 °C and in 100 mM triethanolamine hydrochloride buffer. Determinations of k_{cat} and K_m for pyruvate were made at pH 6 at saturating NADH concentrations (0.2 mM) and in the presence of 5 mM FBP. The same parameters for lactate were measured at pH 8 at saturating NAD^+ concentrations (10 mM) and in 20 mM FBP. We define k_{cat} as the rate of turnover of the enzyme at saturating coenzyme and substrate concentrations. Enzyme concentrations used were 25 nM (sites) for pyruvate as substrate and 100 nM for lactate.

Binding of Substrate Analogues. The K_d for oxamate was determined in an equilibrium experiment by measuring the quench in the fluorescence of bound NADH as the binary

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[1] Abbreviations: FBP, fructose 1,6-bisphosphate; LDH, L-lactate dehydrogenase; MDH, L-malate dehydrogenase.

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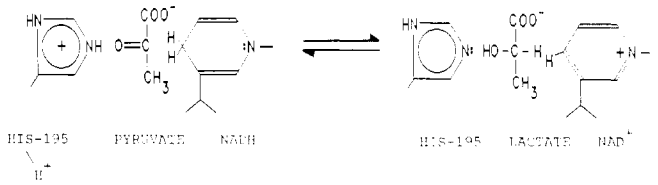

FIGURE 1: Acid/base catalysis of lactate dehydrogenase reaction.

complex was titrated with oxamate [see Clarke et al. (1985a)]. Conditions were pH 6, 1 mM FBP, 12 μM enzyme sites, and 10 μM NADH, oxamate was added in small aliquots, and the fluorescence at 450 nm was monitored continuously (excitation 340 nm).

Primary Isotope Effects. Deuterium isotope effects on k_{cat} in the pyruvate-to-lactate direction were determined in rapid-mixing, single-turnover experiments (Clarke et al., 1985b, 1986) using a Hi-Tech stopped-flow spectrometer (Hi-Tech, Salisbury, Wilts, U.K.) and measuring the decrease in A_{340} as NADH is converted to NAD^+. The rate constants were compared for reactions in which NADH and specifically deuteriated NADH (NADD, [*nicotinamide*-4-^{2}H]NADH) were used as coenzyme. The conditions were pH 6, 35 μM enzyme sites, 30 μM NADH, 1 mM FBP, and varying pyruvate concentrations from 0.5K_m to 2.5 K_m. The measurements were made in transient rather than steady-state experiments owing to the insensitivity of the former to small amounts of tight-binding NADH breakdown products generated in the synthesis of the deuteriated coenzyme. Deuterium isotope effects on the maximal reaction velocity in the lactate-to-pyruvate direction were determined in the steady-state conditions described above, with isotopically labeled lactate [$CH_3CD(OH)COO^-$] as substrate.

Coenzyme Binding. NADH binding was followed by measuring the increase in the static polarization of the dihydronicotinamide ring fluorescence as enzyme was added to free NADH [H/V ratio of free NADH is 0.78, and that of enzyme-bound NADH is 0.58 (Clarke et al., 1985a)]. The K_d for NAD^+ was determined at pH 6 and at pH 8 by measuring its competition with NADH binding in the wild-type enzyme and with the asparagine-168 mutant. The apparent K_d for NADH was determined in the presence of 40, 80, 120, and 200 μM NAD^+. At pH 6 and pH 8 a plot of apparent K_d versus [NAD^+] gave a dissociation constant for NAD^+ of 70 μM (±20 μM) for both the wild-type and the mutant enzyme.

RESULTS

Effects of Mutations on Steady-State Kinetic Constants. Table I shows the influence of the amino acid substitutions on the steady-state reaction velocity and on substrate binding at saturating concentrations of coenzyme. Both mutations cause a drop in k_{cat} for both pyruvate reduction by NADH (10–40-fold) and lactate oxidation by NAD^+ (around 10-fold). The mutations also produce a substantial increase in the K_m for pyruvate (around 100-fold). In both mutant enzymes the lactate K_m is almost unchanged, and surprisingly, in all the above properties it appears to make little difference whether aspartate-168 is substituted by asparagine or by alanine.

Measurements of the pH Dependence of Pyruvate Binding. The binding of pyruvate in LDHs to form a stable ternary complex (E–NADH–pyruvate) demands that the histidine-195 residue is protonated. This is the only amino acid pK that is reflected in the pH dependence of the K_m for pyruvate and for lactate; in the latter case, the group must be unprotonated (Holbrook & Ingram, 1973; Holbrook & Stinson, 1973). Given this, the simplest explanation of the weaker binding of pyruvate to the mutant enzymes would be that the pK of histidine-195 had dropped, so that at pH 6 (the condition used for measurement of pyruvate K_m above) the group is largely unprotonated in the binary E–NADH complex and the proportion of enzyme that can receive pyruvate is small.

To determine the pK of His-195 in the binary active site of all three enzymes, the apparent K_m for pyruvate was measured at pHs between 5.5 and 9 (see Figure 2). In all three cases the relationship of pH to K_m shows that the pK of a single group controls pyruvate binding; the pK of this group is 7.1 (±0.2). Thus the pK of histidine-195 and its state

Table I: Effects of Mutations on Catalytic Constants and Ligand Binding[a]

		enzyme		
part	constant	Asp-168 (wild type)	Asn-168 (mutant)	Ala-168 (mutant)
A	K_m(pyruvate) (mM)	0.06	10	3.3
	k_{cat}(pyruvate) (s^{-1})	250	20	5.5
B	K_m(lactate) (mM)	40	120	80
	k_{cat}(lactate) (s^{-1})	9	0.12	0.09
	K_d(oxamate) (mM)	0.06	11	9
	K_i(trifluorolactate)	30	60	50
C	k_H/k_D(pyr → lac)	1.0	2.4	2.5
	k_H/k_D(lac → pyr)	1.8	3.7	3.6

[a] (Part A) K_m and k_{cat} values for pyruvate and lactate were measured at pH 6 and pH 8, respectively, in steady-state conditions at saturating concentrations of NADH and NAD^+ (see Materials and Methods). (Part B) The K_d for oxamate was determined by fluorometric titration of the enzyme–NADH complex as described under Materials and Methods. The K_i for trifluorolactate was measured by steady-state analysis of its competitive inhibition with lactate as substrate. The apparent K_m for lactate was measured at 0, 30, 60, and 120 mM trifluorolactate in the assay conditions described above. A plot of K_m (apparent) versus trifluorolactate concentration was constructed, and the K_i was given by the intercept on the horizontal axis. (Part C) Primary kinetic isotope effects using deuterium-substituted NADH and pyruvate as substrate were determined in single-turnover experiments as described under Materials and Methods. Here the effect is given as the ratio of the catalytic rate constants (extrapolated to infinite pyruvate concentrations) in experiments where NADH and NADD ([*nicotinamide*-4-^{2}H]NADH) were used as coenzyme. The K_m values for pyruvate measured from these transient kinetic results were the same as those derived from steady-state experiments, and deuterium substitution had no measurable effect on these constants. The effect of deuterium substitution of the lactate substrate on V_m in the direction of lactate oxidation was determined from steady-state measurements extrapolated to infinite lactate concentrations. In no case did isotopic substitution have any clear effect on the K_m value for lactate.

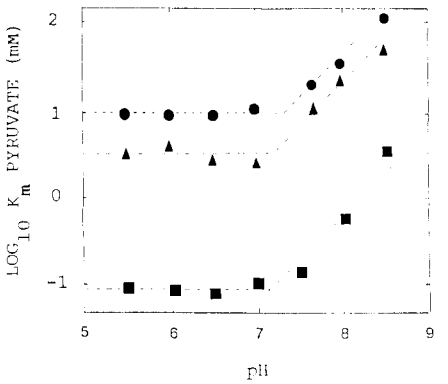

FIGURE 2: Determination of the pK of histidine-195 by the pH dependence of the pyruvate K_m. The K_m for pyruvate was determined in steady-state assays performed over a range of pH in conditions otherwise as described under Materials and Methods. At higher pH values the concentration of FBP was increased to 20 mM to ensure maximal activation of enzyme activity (Clarke et al., 1986b). Over the pH range shown there was no change in the maximal reaction velocity. Symbols: wild type (■), Asn-168 (●), and Ala-168 (▲).

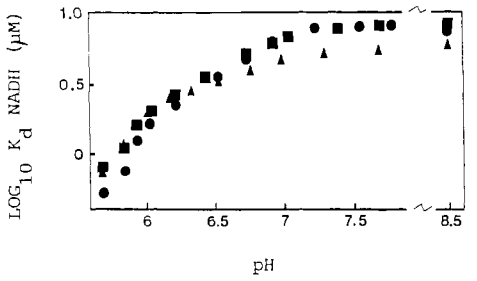

FIGURE 3: Influence of pH on binding constant for NADH. The K_d for NADH was determined by measuring the polarization of fluorescence of an NADH solution which was titrated with enzyme from a concentrated stock as described previously (Clarke et al., 1985a). The experiment was performed in 100 mM triethanolamine hydrochloride buffer (adjusted to the given pH) and with 4 μM NADH in the cuvette. Symbols: wild type (■), Asn-168 (●), and Ala-168 (▲). The binding constant of NAD^+ was determined at pH 6 and pH 8 by the competition experiments described under Materials and Methods. At both these pH values, all three enzymes showed a K_d of 70 (±20) μM.

of protonation in the binary complex are undisturbed by the replacement of aspartate-168 by either asparagine or alanine.

Coenzyme Binding. It has been shown that the protonation state of the active site histidine is mildly sensitive to the binding of NADH in the 2-hydroxyacid dehydrogenases (Stinson & Holbrook, 1973; Lodola et al., 1978). A proposed explanation (Birktoft & Banaszak, 1983) was that, on binding the hydrophobic dihydronicotinamide ring, the reduction of the local "apparent" dielectric will favor the formation of the overall electroneutral His^+/Asp^- couple and that this explains the relatively stronger binding of NADH in comparison with NAD^+ in the binary complexes of these enzymes at pHs where the histidine is protonated.

To look at the effect of the histidine–aspartate pair on the binding of NAD^+ and NADH, we have determined their binding constants over a broad range of pH in both the wild-type and mutant enzymes (see Figure 3). We find that neither the affinity of binding nor its pH dependence is influenced significantly by the amino acid replacements. The results are consistent with NADH binding being tightened 10-fold by the protonation of a group with a pK of about 6 (measurements of NADH binding below pH 5.7 are unreliable owing to coenzyme instability). By analogy with pig MDH and pig LDH (Stinson & Holbrook, 1973; Lodola et al., 1978), we suggest that this reflects the pK of histidine-195 in the apoenzyme. Like the other 2-hydroxyacid dehydrogenases, the binding of NAD^+ to this bacterial enzyme (K_d = 70 μM) is independent of pH over the 6–8 range (see legend to Figure 3). These observations do not support the view that electroneutrality in the His–Asp couple favors NADH binding; in the mutants NADH binding appears to be enhanced when the histidine is protonated, despite the absence of the neutralizing aspartate residue.

Stability of Ternary Complexes Containing Substrate Analogues. Both lactate and pyruvate have unreactive, isosteric analogues (trifluorolactate and oxamate) which have been used extensively for evaluation of the stability of the respective ternary complexes and for crystallization (Holbrook et al., 1975; Pogolotti & Rupley, 1973; White et al., 1976). The binding constants of these analogues are extremely close to the K_m values for the respective substrates, and the difference in energy between the LDH–NADH–oxamate complex and the LDH–NAD^+–trifluorolactate complex is the same as that across the hydride transfer step in catalysis (Whitaker et al., 1974; C. C. O'Neal and J. A. Rupley, personal communication). To consolidate our findings on the effect of the

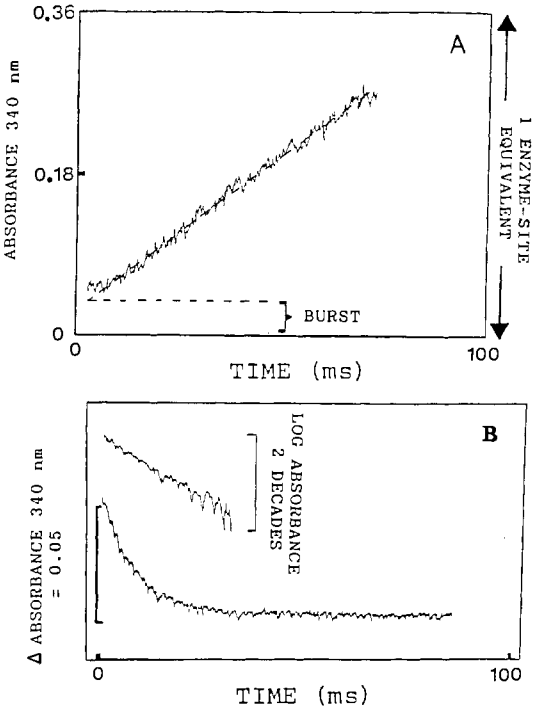

FIGURE 4: Rapid kinetics of lactate oxidation. (A) A solution containing 116 μM wild-type LDH (sites), 150 mM lactate, and 20 mM FBP was mixed rapidly in a stopped-flow spectrometer with one containing 1.5 mM NAD^+ and 20 mM FBP, and the absorbance at 340 nm was recorded. Both solutions contained 100 mM triethanolamine hydrochloride/NaOH, pH 9. The imposed time constant was 0.6 ms. (B) A solution of 20 mg/mL horse liver alcohol dehydrogenase containing 4 mM acetaldehyde and a solution of 40 μM LDH sites containing 40 μM NADH were rapidly mixed. The absorbance at 340 nm was recorded as the NADH dissociated from the LDH and was oxidized by the ADH/acetaldehyde mixture. The first-order rate constant for NADH turnover in an experiment in which LDH was omitted, but which was otherwise identical, was also 100 s^{-1}, showing that NADH must dissociate from the enzyme at a rate which is at least equal to this. The experiments were performed in 100 mM triethanolamine hydrochloride/NaOH, pH 8, at 25 °C.

aspartate substitutions on K_m values, we have compared the dissociation constants of these analogues in all three enzymes. The results (Table I) show the great similarity between values for substrate K_m and analogue affinity (either K_d for oxamate or K_i for trifluorolactate). Furthermore, the deletion of aspartate-168 greatly destabilizes the oxamate ternary complex but has very little effect on that containing trifluorolactate. This confirms the selective quality of the mutations in preferentially reducing the stability of the complexes which contain a protonated histidine-195.

Transient Kinetics and Rate-Determining Steps. Wild-type *B. stearothermophilus* LDH, in common with those from mammalian sources, shows a rate-limiting step in the direction of pyruvate reduction which is a structural rearrangement of the enzyme–NADH–pyruvate complex. Consequently, the catalytic rate constant in this direction is insensitive to deuterium substitution of the transferred hydrogen on NADH (Clarke et al., 1986). In the direction of lactate oxidation, mechanistic interpretations of rapid kinetic results with the wild-type enzyme are less straightforward. When the apoenzyme is rapidly mixed with saturating concentrations of NAD^+ and lactate, there is an instant (<2 ms) formation of 0.1 (±0.05) equiv of an E–NADH–pyruvate complex followed by a steady-state linear phase with a rate of 8.5 s^{-1} (see Figure 4A). This rate-determining step is not NADH dissociation since this occurs at a rate greater than 100 s^{-1} (see Figure 4B). We infer that the predominant steady-state complex (i.e., 0.9

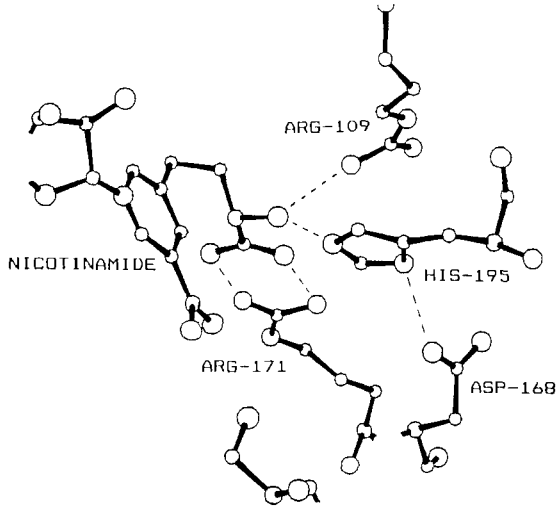

FIGURE 5: Active site of native LDH. The figure shows the structure of the active site in the LDH ternary complex. The view was generated on FRODO graphics by building (J.J.H., unpublished) the *B. stearothermophilus* amino acid sequence (Wirz et al., 1983) into the structural coordinates of the homologous pig H_4 S-lac-NAD^+ complex (Grau et al., 1981). The ligand used in this ternary complex has the lactate molecule covalently linked to NAD^+ by a methylene bridge connecting the substrate C3 atom with the coenzyme nicotinamide C3.

site equiv) is one containing NAD^+ and lactate and that the slowest step in lactate oxidation occurs after a rapid redox step (the equilibrium favoring E–NAD^+–lactate). The wild-type enzyme shows a small reduction (44%) in k_{cat}/K_m when the C2 hydrogen of lactate is replaced by deuterium (see Table I).

Unlike the wild-type enzyme, both of the mutant LDHs (Asn-168 and Ala-168) show a fairly large deuterium isotope effect on the value of k_{cat} in both reaction directions (see Table I). The magnitude of this effect shows that the carbon–hydrogen bond breaking step limits the maximal rate both of lactate oxidation and of pyruvate reduction in the mutant enzymes.

Discussion

The environment of aspartate-168 and its relationship to the active site of lactate dehydrogenase are shown in Figure 5. The substrate binding site lies at one side of the imidazole ring of histidine-195, and the "paired" aspartate lies at the other. The most striking effect of replacing this acidic group by either asparagine or by alanine is to destabilize, selectively, the ternary complex containing pyruvate or its analogue oxamate. It has been shown that LDHs cannot form the E–NADH–pyruvate or E–NADH–oxamate complexes unless the imidazole group of histidine-195 is protonated. This is confirmed in this bacterial LDH by the K_m for pyruvate being controlled by a single group with a pK of about 7. The most direct explanation of this result is that the adjacent negative charge of aspartate-168 lowers the free energy of a ternary complex, in which the histidine is positively charged, by some 3 kcal/mol (the amount of energy required to account for the increase in the K_d of oxamate and in the K_m of pyruvate when the aspartate residue is substituted). In contrast to this, the formation of a stable E–NAD^+–lactate or trifluorolactate complex requires an unprotonated, neutral histidine, and thus in these ternary complexes the mutations have little influence on stability, as reflected in K_m and K_i, respectively. An inevitable consequence of this relative change in the stabilities of the ternary complexes is that the "on-enzyme" reaction equilibrium (E–NADH–pyruvate $\rightleftharpoons$ E–NAD^+–lactate) in the

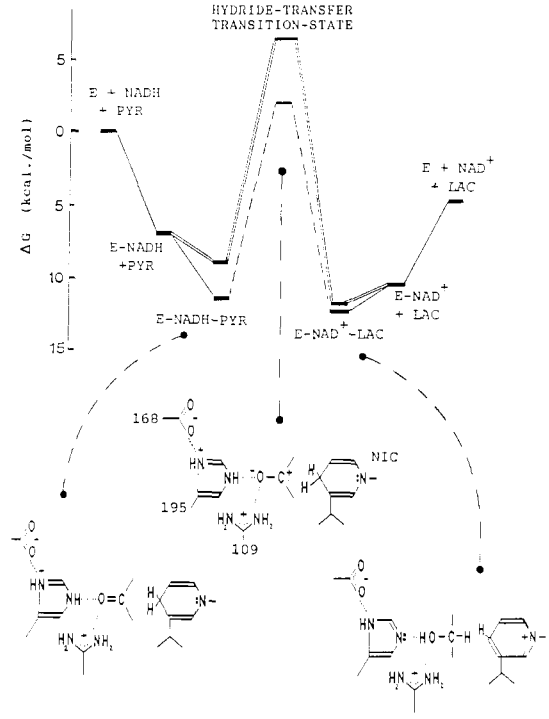

FIGURE 6: Energy profile for catalysis by wild-type and mutant LDH. The energy values are calculated from the kinetic data at pH 8 and represent the asparagine-168 mutant (=) and the wild-type enzyme (--). The energy of free enzyme, pyruvate, and NADH is arbitrarily set at zero. The energy of the hydride-transfer transition state in the wild-type enzyme is not known, but with a rate constant for the process of >620 s^{-1}, its energy must be less than that shown (dashed lines). Represented below the profile are proposed structures (not stereographic) for the indicated ternary states showing the aspartate residue in situ. Its deletion is shown to raise the energy of the E–NADH–pyruvate and transition-state complexes (Abbreviations: NIC, nicotinamide group; LAC, lactate; PYR, pyruvate).

absence of aspartate-168 is no longer balanced; it is shifted markedly toward NAD^+ and lactate (see Figure 6).

One possible advantage of coupling a catalytically essential histidine residue to an acid group is in fixing its orientation in the active site so that it is in the correct position for substrate binding. Were this the case, the effect of substituting an aspartate for an asparagine should be less severe than replacing it by alanine, since the former mutation will still allow the formation of a hydrogen bond with the imidazole ring. Our results do not support this suggestion as there is no significant difference between the catalytic behavior of the asparagine and alanine mutants.

Previous investigations of coenzyme binding in both LDH and MDH have shown that the enzyme–NADH interaction is pH sensitive, being favored by the protonation of histidine-195. The pK of this residue is around 6.4 in apo-MDH (cytoplasmic enzyme) and 6.5 in apo-LDH (pig heart enzyme); these pK's are shifted to 7.4 in the MDH–NADH complex and to 6.8 in the LDH–NADH complex. Consistent with this, NADH binding is tightened 10-fold in MDH and 2-fold in LDH when the active site histidine is protonated. This pattern is reflected in the *B. stearothermophilus* LDH; between pH 5.7 and pH 8 there is a 6-fold weakening of NADH binding. One explanation of this general effect has been that when the imidazole group is protonated, the histidine–aspartate couple has an overall charge of zero and this will promote binding of the hydrophobic dihydronicotinamide ring (Birktoft & Banaszak, 1983). The fact that replacement of aspartate by uncharged residues does not influence either the tightness or pH dependence of NADH binding argues against this

"electroneutrality" suggestion.

Perhaps more intriguing than the lack of effect of the mutations on coenzyme binding is that they do not cause a drop in the pK of histidine-195 in the binary (E–NADH) complex. It is difficult to suggest environments in which an imidazole group will not be made more basic by being bonded to a strong acid; yet the replacement of the acid by neutrals induces no measurable acid shift in the histidine pK. Given that the mutations have a considerable influence on the stability of the enzyme–NADH–pyruvate complex, the conclusion is unavoidable that a strong histidine–aspartate bond is formed only in the ternary complex. In the hydroxyacid dehydrogenases, binding of substrate to the active site causes removal of bulk solvent from the vicinity of the His–Asp system, thus effectively placing the general acid/base in an environment of decreased polarity (Birktoft & Banaszak, 1983). The evidence for desolvation and structural rearrangement on substrate binding comes from three sources. First, the crystallographic structures of apo-LDH and ternary LDH show that a loop of polypeptide (residues 98–110) packs down over the active site when substrates are bound (Grau et al., 1981); second, between the binary (E–NADH) and ternary (E–NADH–oxamate) forms of LDH there is a reduction in the volume of the protein measurable by ultracentrifugation (Criddle et al., 1968); third, on binding oxamate there is a large change in heat capacity, consistent with a tightening of the protein structure (Schmid et al., 1976). A marked desolvation is likely to increase the strength of an imidazole–carboxylate interaction and may account for its effect only being manifested in the ternary structure.

Finally, there is a change in the rate of catalysis and in the nature of the rate-limiting step when there is no aspartate to pair with histidine-195. In the wild-type enzyme the rate of pyruvate reduction is limited by a unimolecular rearrangement of the E–NADH–pyruvate complex, and lactate oxidation is limited by a step occurring after hydride transfer (the observed k_{cat} of 10 s^{-1} will be a product of the equilibrium constant of hydride transfer and the rate of this slow step). In the two mutant enzymes the reaction mechanism is much simpler, the rate-limiting catalytic step in both directions being the transfer of the hydride ion.

A representation of the energetics of the reaction is given in Figure 6. Note that in the case of the wild-type enzyme the rate of hydride transfer is not directly measurable, so the height of the energy barrier for this process is unknown. However, if hydride transfer to pyruvate is slowed 2.5-fold by use of NADD and the measured k_{cat} is not influenced by this, then the transfer must occur at a rate of, at least, $2.5k_{cat}$ (i.e., >620 s^{-1}). We can therefore put a lower limit on the rate. It is notable that the equilibrium position of hydride transfer in the mutant enzyme (Asn-168) calculated from the relative stability of the ternary complexes [K_{eq} = 150, a value derived from the energy difference of 3 kcal/mol (see Figure 6) between these two species] is reassuringly close to that calculated from the ratio of the rates of the slowest steps in each reaction direction (K_{eq} = 170).

The immediate conclusion from the energy profile is that the wild-type aspartate-168 residue stabilizes both the E–NADH–pyruvate complex and the hydride-transfer transition-state complex. We attribute the stabilization of the ground-state complex to the aspartate residue maintaining a protonated histidine-195, and by analogy, we suggest that the reduction in free energy of the transition state is due to the same effect (see Figure 6). This is consistent with the development of negative charge on the C2 oxygen of the substrate during the transition state, which is stabilized by the positive charge of histidine-195. The electron-deficient C2 carbon is then prone to nucleophilic attack by a hydride ion donated by the dihydronicotinamide ring. Such a mechanism is supported by our previous observation (Clarke et al., 1986) that the free energy of the transition state is reduced by the positive charge of arginine-109, a group that is also within hydrogen-bonding distance of the substrate C2 oxygen in the LDH ternary complex. Furthermore, arginine-109 (strong base) and histidine-195 (weak base) come very close together only in the catalytically active ternary complex (see Figure 5). In these circumstances the arginine residue will tend to force deprotonation of the acid/base histidine leading to the collapse of the enzyme–NADH–pyruvate complex as the proton is lost to the bulk solvent rather than to the carbonyl oxygen. The presence of the aspartate will counterbalance this effect.

This catalytic arrangement, whereby a stongly polarizing positive charge acts in concert with a proton-donating/accepting group, is seen in the active sites of ADH, MDH, and LDH, all of which achieve a carbonyl/alcohol interconversion. In all these enzymes it is histidine which serves as the donor/acceptor, the role of the polarizing group being variously taken by Zn^{2+} in ADH and arginine in the others.

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Registry No. LDH, 9001-60-9; NAD, 53-84-9; NADH, 58-68-4; pyr, 127-17-3; lac, 50-21-5; Asp, 56-84-8; His, 71-00-1; Asn, 70-47-3; Ala, 56-41-7; oxamate, 471-47-6; trifluorolactate, 684-07-1; deuterium, 7782-39-0.

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Purification and Characterization of Human Liver Sorbitol Dehydrogenase†

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ABSTRACT: Sorbitol dehydrogenase from human liver was purified to homogeneity by affinity chromatography on immobilized triazine dyes, conventional cation-exchange chromatography, and high-performance liquid chromatography. The major form is a tetrameric, NAD-specific enzyme containing one zinc atom per subunit. Human liver sorbitol dehydrogenase oxidizes neither ethanol nor other primary alcohols. It catalyzes the oxidation of a secondary alcohol group of polyol substrates such as sorbitol, xylitol, or L-threitol. However, the substrate specificity of human liver sorbitol dehydrogenase is broader than that of the liver enzymes of other sources. The present report describes the stereospecific oxidation of (2*R*,3*R*)-2,3-butanediol, indicating a more general function of sorbitol dehydrogenase in the metabolism of secondary alcohols. Thus, the enzyme complements the substrate specificities covered by the three classes of human liver alcohol dehydrogenase.

The most complex pattern of alcohol dehydrogenase isozymes has been established for human liver (Vallee & Bazzone, 1983). Multiple forms include three classes (about 60% residue identity), nonallelic (about 90% residue identity) and allelic (more than 95% residue identity) class I homodimers as well as hybridized heterodimers between the individual subunits of class I isozymes (Jörnvall et al., 1987a). Primary structures of all class I isozymes of alcohol dehydrogenase from human liver have been determined (Jörnvall et al., 1987b) as well as those of the other two classes (Höög et al., 1987; Kaiser et al., 1988). The alcohol dehydrogenase family includes yet another enzyme, sorbitol dehydrogenase (L-iditol:NAD^+ 5-oxidoreductase, EC 1.1.1.14), since the primary structure of the corresponding enzyme from sheep liver demonstrated its sequence homology with alcohol dehydrogenases (Jeffery et al., 1981; Jörnvall et al., 1981). Despite the structural and mechanistic similarities, sorbitol dehydrogenase differs distinctly from the mammalian alcohol dehydrogenase in regard to the following: (i) it is a tetramer rather than a dimer; (ii) it contains one instead of two zinc atoms per subunit (Jeffery et al., 1984a); (iii) on the basis of model studies (Eklund et al., 1985), a glutamic acid residue is thought to replace one of the typical cysteine ligands of the catalytic zinc atom in alcohol dehydrogenase. Though sorbitol dehydrogenase activity was detected in many other species and tissues (Gerlach & Hiby, 1974), including human brain, lens, erythrocytes, and liver (O'Brien et al., 1983; Jedziniak et al., 1981; Barretto et al., 1985; Nealon & Rej, 1983), to date, the enzyme from sheep liver remains the only one for which structural information exists. Therefore, the present study describes the isolation and characterization of sorbitol dehydrogenase from human liver tissue. This not only will allow for an extensive and rigorous structure/function comparison within this family of enzymes from one source but also will contribute to reveal the metabolic role of sorbitol dehydrogenase, which has remained largely enigmatic. Furthermore, sorbitol dehydrogenase has previously attracted considerable interest, since its enzymatic activity has been linked to formation of cataracts and neuro-, retino-, and nephropathies (Gabbay, 1973), circumstances which have been adduced to explain the pathogenesis of diabetic complications (Greene et al., 1987).

This report demonstrates that the enzymes and isozymes from the three classes of human liver alcohol dehydrogenase do not catalyze the oxidation of sorbitol and, further, that a unique sorbitol dehydrogenase is responsible for this reaction. The major form of this human liver sorbitol dehydrogenase has been purified to homogeneity and shown to have a substrate specificity extending to secondary alcohols other than polyhydric alcohols (sugar alcohols).

Experimental Procedures

Materials. NAD^+, NADH (both grade III), the dyes Reactive Green 19 (Green A), Reactive Green 5 (Procion H-4G, Green B), and Coomassie Brilliant Blue G, β-D-

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