Wasserman, S. A., Daub, E., Grisafi, P., Botstein, D., & Walsh, C. T. (1984) *Biochemistry 23*, 5182.
Wells, J. A., Vasser, M., & Powers, D. B. (1985) *Gene 34*, 315.
Wiegand, G., & Remington, S. J. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 97.
Wild, J., Henning, J., Lobocka, M., Walczak, W., & Klopotowski, T. (1985) *MGG, Mol. Gen. Genet. 198*, 315.

Salmonella typhimurium Histidinol Dehydrogenase: Complete Reaction Stereochemistry and Active Site Mapping†

Charles Timmis Grubmeyer,* Salvatore Insinga, Mohit Bhatia, and Nader Moazami
Department of Biology, New York University, 100 Washington Square, New York, New York 10003
Received March 24, 1989; Revised Manuscript Received June 6, 1989

ABSTRACT: The stereochemistry of the L-histidinol dehydrogenase reaction was determined to be *R* at NAD for both steps, confirming previous results with a fungal extract [Davies, D., Teixeira, A., & Kenworthy, P. (1972) *Biochem. J. 127*, 335–343]. NMR analysis of monodeuteriohistidinols produced by histidinol/NADH exchange reactions arising via reversal of the alcohol oxidation reaction indicated a single stereochemistry at histidinol for that step. Comparison of vicinal coupling values of the exchange products with those of L-alaninol and a series of (*S*)-2-amino-1-alcohols allowed identification of the absolute stereochemistry of monodeuteriohistidinols and showed that histidinol dehydrogenase removes first the *pro-S* then the *pro-R* hydrogens of substrate histidinol. The enzyme stereochemistry was confirmed by isotope effects for monodeuteriohistidinols as substrates for the *pro-R*-specific dehydrogenation catalyzed by liver alcohol dehydrogenase. Active site mapping was undertaken to investigate substrate–protein interactions elsewhere in the histidinol binding site. Critical binding regions are the side-chain amino group and the imidazole ring, whose methylation at the 1- or 2-position caused severe decreases in binding affinity. Use of alternative substrates further clarified active site interactions with the substrate. Compounds in which the α-amino group was replaced by chloro, bromo, or hydrogen substituents were not substrates of the overall reaction at 1/10 000 the normal rate. The α-hydroxy analogue of histidinol was a substrate, with $k_{cat}/K_m = 0.1\%$ that of histidinol. Removal or alteration of the imidazole ring also prevented catalysis, resulting in complete inactivity with alaninol or thienylalaninol.

Salmonella typhimurium histidinol dehydrogenase (EC 1.1.1.23) catalyzes the four-electron dehydrogenation of L-histidinol to L-histidine using 2 mol of NAD, probably at a single active site (Adams, 1955). The enzyme is one of three known NAD-linked four-electron dehydrogenases [the two others are UDP-glucose dehydrogenase (EC 1.1.1.22) and 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34)]. Such enzymes are interesting mechanistically because they oxidize both a substrate alcohol and an intermediate aldehyde, reactions with quite different redox potentials. In the case of HMGR[1] the substrate is a thiol ester, and a cysteine–UDP glucuronic thiol ester is believed to be an intermediate for UDPGDH (Ridley et al., 1975). Such a thiohemiacetal–thiol ester oxidation is much closer in redox potential to the alcohol–aldehyde reaction. An active site cysteine has recently been identified in HDH (Grubmeyer & Gray, 1986), suggesting that a thiol ester may be involved in this enzyme as well.

To learn how HDH catalyzes two sequential oxidations, we have sought to understand the structure of the active site. Stereochemical studies on NADH produced by the HDH activity of a crude extract of *Neurospora* have been performed previously (Davies et al., 1972) and showed that the reaction was *R*(A) for both steps [UDPGDH is *S*(B) for both steps, and HMGR is *R*(A) for both steps; You, 1982]. However, HDH from yeast is known to be a multifunctional enzyme (Donahue et al., 1982), and the recent finding of opposite stereochemistry at NADH for liver and *Drosophila* ADH (Benner et al., 1985) suggested that additional stereochemical studies on HDH from *Salmonella* were important. We have also been able to use exchange reactions, NMR analysis, and isotope effects to determine the stereochemistry of the sequential hydrogen removals from histidinol, and thus to orient the coenzyme with respect to the substrate molecule in the first transition state, and with the hypothesized thiohemiacetal in the second transition state. Finally, we have also investigated the origins of the high substrate specificity of the histidinol dehydrogenase reaction by using histidinol analogues as competitive inhibitors and alternative substrates to map the functionally important binding interactions between substrate and enzyme.

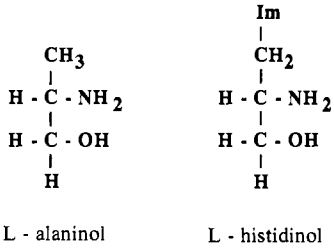
Materials and Methods

Materials. L-Histidinol, L-histidine, D-histidine, L-alaninol, histidine methyl ester, L-histidine hydroxamate, histamine, 2-methylimidazole, L-β-imidazol-4-yllactic acid, DL-triazol-

† This research was supported by grants from the NSF (DMB87-05583). The NMR Facility at New York University is supported by grants from the NIH and the NYU Cost-Sharing Instrumentation Fund.

[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDH, histidinol dehydrogenase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LADH, liver alcohol dehydrogenase; SMP, submitochondrial particles; UDPGDH, uridine diphosphoglucose dehydrogenase; YADH, yeast alcohol dehydrogenase. For terminology regarding labeling of histidinol, see Materials and Methods.

0006-2960/89/0428-8174$01.50/0



L - alaninol L - histidinol

ylalanine, YADH, LADH, $NaBH_4$, and $NaBD_4$ were purchased from Sigma. Pyrazole, imidazole, 1,2,4-triazole, tetrazole, ethanolamine, and 4-(hydroxymethyl)imidazole were from Aldrich. Histidine amide was from Bachem, Torrance, CA. GAPDH and NAD were from Boehringer. (4-^{3}H)NAD was from Amersham. All inorganic chemicals used were of reagent grade. Histidinol dehydrogenase was prepared from cells of *S. typhimurium his01242* as described previously (Yourno & Ino, 1968; Grubmeyer & Gray, 1986). The protein appeared homogeneous on SDS–PAGE and showed a specific activity of 12–15 units/mg, in agreement with observations made by others (Burger & Gorisch, 1981).

Synthetic Methods. Methyl esters of organic acids were made by dissolving the compound in methanol and adding dry HCl gas for 2–10 h. Methanol and HCl were then removed by evaporation under reduced pressure. Dideuteriohistidinol was synthesized by $NaBD_4$ reduction of histidine methyl ester as outlined by Andersson and Wolfenden (1982). Such reductions of amino acid esters are known to proceed with retention of stereochemistry at the amino carbon (Seki et al., 1965). D-Histidinol, (*S*)-3-(4-imidazolyl)-1,2-propanediol, and 3-(2-thienyl)alaninol were synthesized by the same procedure using the appropriate methyl esters and $NaBH_4$. Amino alcohol products were purified initially following the procedure of Andersson and Wolfenden (1982) and then by a cation-exchange column (Dowex 50) eluted with a gradient of 0–5 M HCl, and after evaporation of solvent under reduced pressure, by anion-exchange chromatography on Dowex AG-1-X8 ("hydroxide" form) to remove contaminating acid.

The preparation of (H,D)histidinol and (D,H)histidinol (the order of letters refers to the order in which the hydroxymethyl hydrogen atoms are removed in the overall HDH reaction) was carried out with the use of histidinol dehydrogenase exchange reactions (Grubmeyer et al., 1987). For (H,D)-histidinol the reaction mixture (100-mL final volume) contained 1 mM (D_2)histidinol, 1 mM NAD, 10 mM NADH in 50 mM sodium glycine, and 0.1 mM $MnCl_2$, pH 9.0. Histidinol dehydrogenase (3 mg) was added to start the reaction, which was allowed to proceed 1–2 h at 30 °C. The product was purified initially on Dowex 50, using elution by a gradient of 0–5 M HCl, and then by anion exchange as described for (D_2)histidinol. For (D,H)histidinol, the reaction medium was identical except that (D_2)histidinol was replaced by (H_2)-histidinol, and (4(*R*)-^{2}H)NADD (Viola et al., 1979) at 6 mM was added to replace NADH. The NADD was purified before use on a 2.5 × 30 cm column of TSK-DEAE, eluted with a 0–0.2 M NaCl gradient in 50 mM Tris-HCl, pH 8.0.

4-(2-Chloroethyl)imidazole (as for its bromo analogue; Jolley and Yankeelov, 1972) and 3-(4-imidazolyl)-2-chloro-1-propanol (Beyerman et al., 1977) were synthesized and purified as described. *O*-Acetyl-4-(hydroxymethyl)imidazole was synthesized by the reaction of acetyl chloride with (hydroxymethyl)imidazole in chloroform plus pyridine for 2 h at 50 °C. 3-(4-Imidazolyl)-1-propanol was synthesized and purified as described by Kivits and Hora (1975) from 2-methoxy-3-bromotetrahydropyran (Gaydou, 1972) that had been purified by fractional distillation. DL-4-(4-Imidazolyl)-3-amino-2-butanone was synthesized from L-histidine by the Dakin–West reaction as described (Smissman & Weis, 1971). The compound was purified on a column of cellulose eluted with propanol/1 N acetic acid, 3:1, concentrated under reduced pressure, and further purified by paper chromatography using the same solvent.

The purity of all imidazole compounds was assayed by employing the chromatography and detection systems described by Ames and Mitchell (1952), with the exception that thin-layer chromatography plates of cellulose (Machery-Nagel, 0.1 mm) were used in place of paper. Ninhydrin reagent was employed for detection of other compounds. Proton NMR was used to verify the identity of all synthetic products. Spectra were taken in D_2O on a Nicolet/GE QE-300 FT NMR operating at 300 MHz.

Methods. Chiral (4-^{3}H)NADH for stereochemical analysis was prepared from (4-^{3}H)NAD and purified as described in Grubmeyer et al. (1987) using HDH or GAPDH to produce (4(*S*)-^{3}H)NADH and (4(*R*)-^{3}H)NADH, respectively. NADH degradation reactions were carried out in 1-mL reaction mixtures containing 50 μmol of Tris-HCl, 0.1 μmol of (^{3}H)NADH, and either 50 μg of YADH with 37 μmol of acetaldehyde or 0.6 mg of beef heart SMP. Reactions were allowed to proceed at 30 °C in quartz cuvettes until A_{340nm} had gone to zero, with appropriate correction for light scattering. DEAE chromatography for separating pyridine nucleotides (Figure 1) was performed as described previously (Grubmeyer et al., 1987).

Oxidation of labeled histidinols by LADH was followed spectrophotometrically in 1-mL reaction mixtures containing 50 μmol of sodium glycine, pH 9.0, 3 μmol of NAD, and 2.5 mg of LADH (Sigma A6128). After 5 min at 30 °C in a quartz cuvette, 1 μmol of histidinol was added and A_{340} was monitored for 5–10 min. To eliminate residual ethanol, each component of the reaction mixture was twice lyophilized in a Savant Speed-Vac prior to assay. In the case of LADH solutions, lyophilization was from solutions in 50 mM sodium phosphate buffer, pH 7.5.

The kinetics experiments were performed in 1-mL assay mixtures containing 50 μmol of glycine–NaOH, 0.5 μmol of $MnCl_2$, 10 μmol of NAD, and L-histidinol at 10-, 12.5-, 16.6-, 30-, and 50-nmol levels. For K_i calculations, assays were performed at four levels of inhibitor. The assay medium was adjusted to pH 9.2 shortly before use and allowed to equilibrate for 5 min at 30 °C in the thermostated cuvette holder of a Perkin-Elmer 552 spectrophotometer. Enzyme (1 μg) was added to start the assay, which was monitored for 2–3 min at 340 nm. Rates were calculated from chart recordings and used to construct Lineweaver–Burk plots, from which kinetic data were obtained. Computer programs (Cleland, 1979) were used to calculate K_i values. A greater than (>) sign in Table III indicates that only a single concentration of inhibitor was used (1/5 the K_i value reported) against five levels of histidinol and showed no detectable inhibition. The concentration of L-histidinol solutions used for kinetics experiments was determined enzymatically as described previously (Grubmeyer et al., 1987).

The reaction buffer used to investigate potential alternative substrates was identical with that used for kinetic experiments with the substrate present at levels ranging from 100 μM to 100 mM. HDH was added at 1 mg/mL and absorbance monitored at 340 nm for 1 h. This technique can readily detect 0.1 turnover of the enzyme (A = 0.01). To measure reduction of DL-4-(4-imidazolyl)-3-amino-2-butanone, the assay mixture contained 0.1 mM NADH, 0.5 mM $MnCl_2$, 50 mM tri-

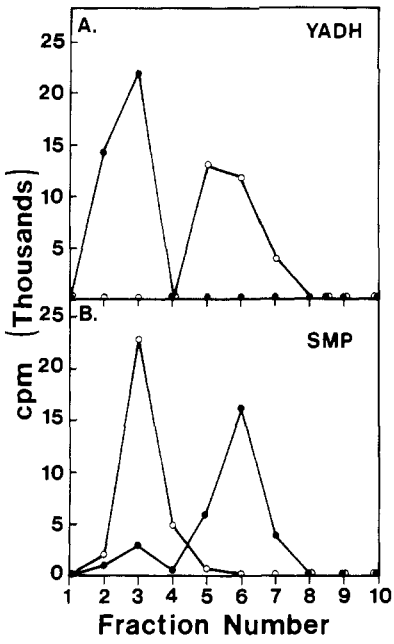


FIGURE 1: Chromatography of reaction products for (^{3}H)NADH degradation. (^{3}H)NADH produced by the action of HDH (solid circles) or GAPDH (open circles) on (4-^{3}H)NAD and nonradioactive substrate was degraded by the indicated enzyme, and the products were applied to DEAE chromatography columns as described under Materials and Methods. The columns were eluted with 50 mM Tris-HCl, and fractions were assayed by liquid scintillation counting. (A) Degradation by YADH. (B) Degradation by NADH oxidase (SMP).

ethanolamine hydrochloride, pH 8.0, and substrate at 10–250 μM.

Results

Stereochemistry of Reduction at NAD. The stereochemistry of the reduction at C4 of NAD was determined by reducing (4-^{3}H)NAD with unlabeled histidinol in the histidinol dehydrogenase reaction and enzymatically analyzing the (^{3}H)-NADH produced by using classical procedures. Degradation reactions were chosen that produced NAD and uncharged products (ethanol or H_2O) and the mixtures separated by using anion-exchange chromatography (Grubmeyer et al., 1987). When (^{3}H)NADH produced by HDH was used with acetaldehyde as substrate for the *R*-specific enzyme YADH (You, 1982), ^{3}H-labeled ethanol was produced (Figure 1). In contrast, when the (^{3}H)NADH was used as a substrate for the *S*-specific NADH dehydrogenase of the mitochondrial electron transport chain (You, 1982), ^{3}H-labeled NAD was the product. Exactly opposite results were obtained with both degradations when (4(*R*)-^{3}H)NADH produced by the GAPDH reaction replaced the (^{3}H)NADH produced by the action of HDH. The results clearly showed that only (4-(*S*)-^{3}H)NADH had been produced by histidinol dehydrogenase, demonstrating that both of the NAD reductions catalyzed by histidinol dehydrogenase are *R*(A), as previously observed in a crude extract of *Neurospora* (Davies et al., 1972).

Determination of Stereochemistry of Dehydrogenation at Histidinol. HDH catalyzes hydrogen exchange reactions between histidinol and NADH that appear to arise solely by reversal of the first oxidation step (Grubmeyer et al., 1987). Figure 2 shows the upfield region of NMR spectra of histidinol (Figure 2A), dideuterated histidinol (Figure 2D), and the two monodeuteriohistidinols purified from the exchange reactions between (D_2)histidinol and NADH (Figure 2B), and between (H_2)histidinol and (4(*R*)-^{2}H)NADD (Figure 2C). It is clear

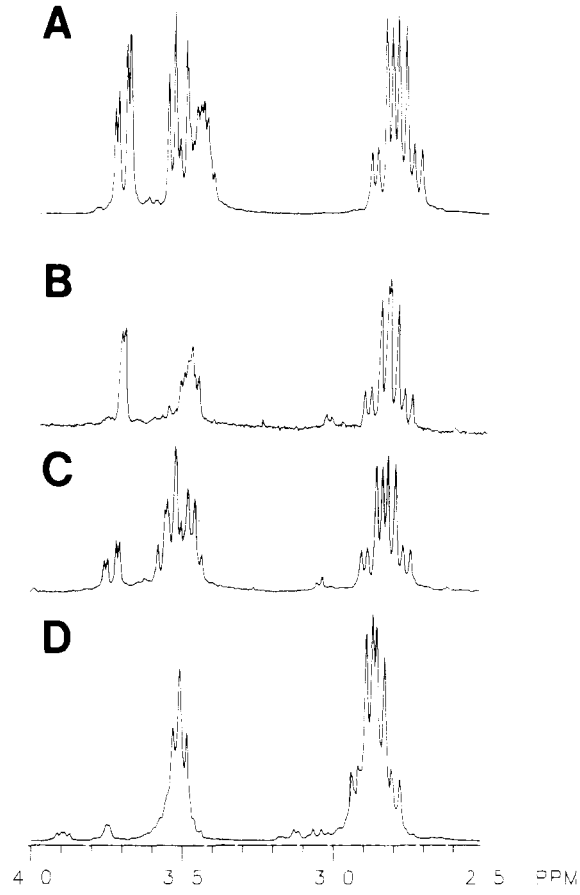


FIGURE 2: ^{1}H NMR of labeled histidinols. Spectra taken in D_2O at a pD of 8.0. Total of 512 acquisitions, 300 MHz. (A) (H_2)-Histidinol; (B) (H,D)histidinol; (C) (D,H)histidinol; (D) (D_2)histidinol.

from a comparison of Panels A and D of Figure 2 that two complex peaks are observed for the hydroxymethyl hydrogens, one fused with the α-H, and the other about 0.2 ppm downfield (3.55 and 3.75 ppm). The two peaks represent chemical shift differences for the two hydrogens that result from their position α to a chiral center. (H,D)Histidinol produced via the (D_2)histidinol/NADH exchange reaction is specifically enriched by hydrogen in the downfield position, indicating a single stereochemistry in the exchange product, confirming previous specific activity results with ^{3}H exchanges (Grubmeyer et al., 1987). Integration of the spectrum of this compound indicated a 80% substitution by hydrogen. Similarly, integration of the spectrum of the (D,H)histidinol (Figure 2C) produced by (H_2) histidinol/NADD exchange showed a 60% exchange-out of hydrogen from the downfield position, with 40% remaining (H_2)histidinol (the geminal splitting of the downfield peak indicates that it arises from remaining (H_2)-histidinol and not a monodeuteriohistidinol; the complexity of the upfield signal results from the presence of (H_2)histidinol and (D,H)histidinol).

Table I summarizes the coupling values for the upfield and downfield hydrogens of histidinol. When an 11–12-Hz geminal coupling is taken into account, the couplings between the α-H and the upfield and downfield hydroxymethyl H are respectively 7 and 3 Hz. Although these coupling values doubtless arise from the solution conformation of histidinol, it is not a trivial matter to deduce or predict the conformation of such nonrigid compounds. However, for L-alaninol, the exact homologue of the three-carbon histidinol side chain, the stereochemical assignments from the NMR have been made by using LADH-catalyzed exchange reactions (Diziol et al., 1980) which proceed via known stereochemistry. In this case, the

Table I: NMR of Amino Alcohols[a]

compound	pH	ppm A	ppm B	couplings (Hz) $J_{A(gem)}$	couplings (Hz) $J_{B(gem)}$	couplings (Hz) J_{HCND-A}	couplings (Hz) J_{HCND-B}
histidinol	8.0	3.77	3.61	11.66	11.58	3.00 ± 0.04	6.89 ± 0.21
	2.0	3.80	3.65				
(H,D)histidinol	8.0	4.11					
	2.0	3.81				3.43	
(D,H)histidinol	8.0						
	2.0		3.81				6.69
leucinol	8.0	3.76	3.54	12.37	12.34	3.56 ± 0.01	6.94 ± 0.11
methioninol	8.0	3.87	3.69	12.19	11.98	3.39 ± 0.01	6.80 ± 0.23
valinol	8.0	3.81	3.61	12.38	12.35	3.57 ± 0.00	7.82 ± 0.14
tyrosinol	8.0	3.71	3.53	12.10	11.93	3.67 ± 0.01	6.68 ± 0.04
phenylalaninol	8.0	3.72	3.54	11.64	11.78	3.13 ± 0.04	6.51 ± 0.18
prolinol	8.0	3.85	3.74	11.64		3.26 ± 0.30	
2-amino-1-butanol	8.0	3.78	3.59	12.19	12.12	2.72 ± 0.09	6.77 ± 0.14
alaninol	8.0	3.73	3.53	12.11	12.21	3.81 ± 0.11	7.15 ± 0.11
	6.0	3.75	3.51	12.26	12.27	3.75 ± 0.07	7.20 ± 0.06
	4.0	3.81	3.60	11.97	11.99	3.72 ± 0.14	7.13 ± 0.28
	2.0	3.87	3.66	11.89	11.91	3.70 ± 0.00	7.26 ± 0.01

[a] All compounds have *S* stereochemistry at the amino carbon.

Table II: Oxidation of Deuterated Histidinols in the LADH Reaction[a]

compound	rate (nmol/min)	compound	rate (nmol/min)
(H,H)histidinol	10	(H,D)histidinol	3.4
(D,D)histidinol	3.3	(D,H)histidinol	9

[a] Methods given in text.

downfield proton is equivalent to *pro-S* and upfield to *pro-R*. L-Alaninol shows the same splitting of upfield and downfield hydroxymethyl hydrogens as L-histidinol (Table I), strongly suggesting that in the two compounds the coupling values have the same physical origin. To test this hypothesis further, we examined a variety of accessible L-amino alcohols by NMR. As shown in Table I, all evidence the same pattern of splitting of the hydroxymethyl hydrogens. We conclude that in this series of 2-amino-1-alcohols conformational constraints discussed later dictate the NMR spectrum and that the chirality of monodeuteriohistidinol can be reliably ascertained by analogy with alaninol. Thus, the downfield hydrogen is *pro-S*, and the upfield is *pro-R*. Further, the (H,D)histidinol of Figure 2B is *R*, that of (D,H)histidinol in Figure 2C is *S*, and the first dehydrogenation step in the HDH reaction goes by *S* stereochemistry at histidinol.

To verify our NMR experiments, labeled histidinols were also used as substrates for LADH which goes via *R* stereochemistry at the hydroxymethyl carbon. The oxidation of histidinol by LADH shows a K_m for histidinol of 10 mM and is completely inhibited by the LADH inhibitor iodopyrazole (unpublished observations). In keeping with previous observations by Andersson and Wolfenden (1982), Table II shows that LADH demonstrates a 3-fold isotope effect with (D_2)-histidinol under our reaction conditions. A similar reduction in velocity is caused by (H,D)histidinol, but not by (D,H)-histidinol, which behaves like (H_2)histidinol. Results are in agreement with the conclusions from the NMR and show that (H,D)histidinol is *R*, that (D,H)histidinol is *S*, and that histidinol dehydrogenase removes first the *pro-S* hydrogen of histidinol substrate.

Active Site Mapping. We have previously found that histidinol can bind to two sites on the HDH dimer with $K_D = K_m = 10\ \mu M$ (Grubmeyer et al., 1987). Imidazole was found to be a competitive inhibitor of HDH versus histidinol, K_i = 1.5 mM (Table III). Methylation at the 1- (equivalent to 3-) or 2-position resulted in a loss of binding strength. Methylation at the 4- (equivalent to 5-) position was without effect on the binding interaction of imidazole. 3-Methylhistidine did not appear to be an inhibitor at 10 mM concentrations. The alternative five-membered rings pyrrole, 1,2,4-triazole, thiazole, and tetrazole were all poor inhibitors at pH 9.2. DL-3-(2-Thienyl)alaninol was a poor inhibitor, K_i = 70 mM, and DL-(1,2,4-triazolyl)alanine did not detectably inhibit at 5 mM levels, suggesting K_i values above 25 mM.

L-Alaninol, a side-chain analogue, was a poor inhibitor, K_i = 200 mM. Ethanolamine was at least as poorly bound as

Table III: K_i Values for Competitive Inhibitors[a]

compound	K_i (mM)
Ring Analogues	
imidazole	1.5
1-methylimidazole	20
2-methylimidazole	14
4-methylimidazole	1.3
4-(hydroxymethyl)imidazole	3.3
O-acetyl-4-(hydroxymethyl)imidazole	0.63
pyrrole	>200
1*H*-tetrazole	120
thiazole	63
1,2,4-triazole	19
DL-3-(1,2,4-triazolyl)alanine	>25
DL-3-thienylalaninol	70[b]
DL-3-methylhistidine	>50
Side-Chain Analogues	
L-alaninol	200
ethanolamine	>200
Histidine Analogues	
L-histidine	1.0
D-histidine	22
N-acetyl-L-histidine	5.0
urocanic acid	28
L-histidine hydroxamate	0.20
L-histidinamide	0.80
L-histidine methyl ester	1.3
DL-4-(4-imidazolyl)-3-amino-2-butanone	0.005
2-(4-imidazolyl)acetate	14
D-histidinol	0.2
Amino-Modified Analogues	
histamine	0.035
N-formylhistamine	0.19
L-4-(2-chloroethyl)imidazole	12
imidazol-4-yl-2-chloropropanol	0.45
L-3-(4-imidazolyl)-1,2-propanediol	0.42
3-(4-imidazolyl)-1-propanol	14

[a] Assays were performed as described under Methods. [b] Kinetics indicate mixed-type inhibition.

alaninol, and neither compound enhanced the inhibition caused by imidazole (not shown).

L-Histidine was also a competitive inhibitor versus L-histidinol, K_i = 1 mM, about 100-fold less potent than histidinol. L-Histidine hydroxamate and the uncharged analogue L-histidine methyl ester had K_i values of 0.2 mM and 1.3 mM, respectively. L-Histidine amide had a K_i of 0.8 mM. The compound DL-4-(4-imidazolyl)-3-amino-2-butanone was a potent inhibitor, K_i = 5 μM.

In order to determine the relative roles of the amino and alcohol group in the binding of histidinol, compounds lacking the terminal carbon were also tested. Histamine was a potent inhibitor, K_i = 0.04 mM, while (chloroethyl)imidazole had a K_i of 1.2 mM. (*S*)-3-(4-Imidazolyl)-2-chloro-1-propanol, in which the α-amino group has been replaced by the chloro group, showed a K_i of 0.45 mM. (*S*)-4-(4-Imidazolyl)-1,2-propanediol gave a K_i of 0.42 mM.

Alternative Substrates. The substrate requirements for catalysis by HDH were explored with the use of alternative substrates. The side-chain analogues ethanolamine and alaninol were not substrates at 1/100 000 the normal rate with histidinol. These compounds also did not behave as substrates in the presence of 10 mM imidazole. The compound DL-3-(2-thienyl)alaninol, in which the five-membered ring lacks hydrogen bond capacity, was also not a substrate.

Replacing the α-amino group also dramatically affected catalysis. The chloro compound (*S*)-3-(4-imidazolyl)-2-chloro-1-propanol was not a substrate, nor was its racemic bromo analogue. D-Histidinol was also not a substrate, as observed earlier (Loper & Adams, 1965). Most revealingly, 3-(4-imidazolyl)-1-propanol was also not a substrate. The compound (*S*)-3-(4-imidazolyl)-1,2-propanediol was a substrate with a rate of 0.08 s^{-1}, K_m = 0.16 mM, k_{cat}/K_m = 0.5 $\times$ 10^3 M^{-1} s^{-1}. The corresponding parameters for histidinol are 11.5 s^{-1}, 0.01 mM, and 1.2 $\times$ 10^6 M^{-1} s^{-1}.

The Dakin–West reaction between L-histidine and acetic anhydride gives the ketone DL-4-(4-imidazolyl)-3-amino-2-butanone. This compound is reduced by HDH in the presence of NADH. Under the conditions employed, with subsaturating NADH at pH 8.0, the K_m for reduction was 45 μM, with a V of 0.015 s^{-1} and k_{cat}/K_m = 0.3 $\times$ 10^3 M^{-1} s^{-1}.

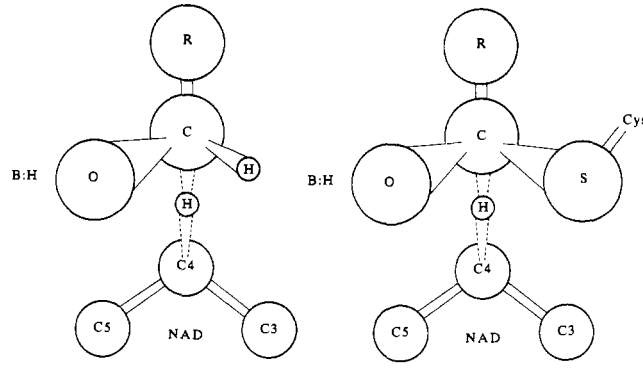
Discussion

The major result of this study is a complete stereochemical description of the histidinol dehydrogenase reaction, consisting of two hydride transfers to the *pro-R* position of NADH, sequentially removing the *pro-S* and then the *pro-R* hydrogen of the histidinol substrate. We have also been able to use inhibitors and alternative substrates to map some of the enzyme–ligand interactions at the catalytic site.

The NADH stereochemistry was readily approached by using classical methods. The result indicated *R* stereochemistry for both half-reactions, confirming previous results on a fungal extract (Davies et al., 1972). All three known NAD-linked four-electron dehydrogenases use a single stereochemistry at NAD for both steps.

The determination of the stereochemistry of dehydrogenation at histidinol rests partially on our analysis of NMR results to assign the prochiral histidinol hydroxymethyl hydrogens. Although stereochemistry determinations based on rationalization of NMR results have occasionally proven problematic in the past (Anet, 1960), we believe a firm ground exists for analogy between alaninol and histidinol. The primary evidence for analogy lies in the fact that the compounds are exact homologues over all the relevant atoms and in the identity of coupling values for upfield and downfield hydroxymethyl

Scheme I: Proposed Transition States for First and Second Hydride Transfers[a]



[a] For further details, see text.

hydrogens. Microwave spectroscopy (Ellingsen et al., 1978) and dipole moment measurements (Millefiori et al., 1980) have established that the conformation of alaninol is determined by an intramolecular amino–hydroxyl hydrogen bond, a common occurrence in small molecules (Wilson & Smith, 1987). It is thus logical that, other factors being equal, such conformations would hold for higher 2-amino-1-alcohols. That the pattern of NMR splittings we observe is similar for alaninol and histidinol might still be a fortuitous result of alaninol's conformation being governed by other forces. Our work shows, however, that very similar observed values hold for a wide series of 3-substituted 2-amino-1-alcohols whose side chains differ greatly in bulk and hydrogen bond capability. We thus conclude that a feature common to all these compounds governs their solution conformation and that the stereochemical assignments for histidinol can be made by analogy to alaninol.

We confirmed our NMR work by examination of isotope effects. LADH is known to react via *R* stereochemistry at the hydroxymethyl carbon, although a minor proportion of *S* stereochemistry has been observed after prolonged LADH-catalyzed equilibration of octanol with NADH (Shapiro et al., 1983). Similar prolonged equilibration of L-alaninol goes via a single stereochemistry (Diziol et al., 1980). Although the LADH intrinsic deuterium kinetic isotope effect of about 5 is masked with most good substrates (Cook & Bertagnolli, 1986), Andersson and Wolfenden (1982) have shown that a substantial kinetic isotope effect is expressed during the oxidation of 2-amino-1-alcohols. We observed a 3-fold effect of deuteration on the reaction rate at 1 or 10 mM histidinol (the K_m for histidinol in the LADH reaction is 10 mM), and results showed that the (D,H)histidinol thought to be *R* showed an isotope effect similar to (D_2)histidinol while that thought to be *S* behaved like (H_2)histidinol. It has been shown (Dutler et al., 1986) that LADH can, under some conditions, catalyze the complete HDH reaction to yield product histidine. Under the conditions of our experiment formation of histidine was not observed (by TLC or quantitation of NADH production) and appears not to be a factor in interpretation.

The stereochemistry results permit us to diagram some features of the two transition states in the HDH reaction (Scheme I), although much remains to be learned. The key features of Scheme I are that NAD is positioned in the same geometrical relation to substrate in the two transition states and that an active site base occupies the same location in both cases. We have accomplished this by proposing that an intermediate thiohemiacetal, formed between histidinaldehyde and an enzyme cysteine residue, is the substrate for the second reaction. Although a lysine carbinolamine, serine or threonine

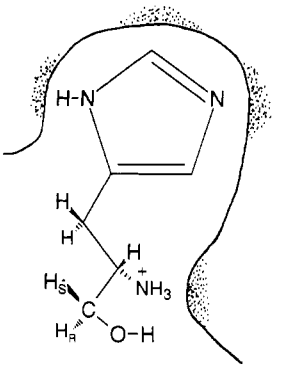
hemiacetals, or an aldehyde hydrate could each accomplish the same geometry for the second oxidation, we have shown that an active site cysteine (Cys-116) occurs in *Salmonella* HDH and is conserved in the yeast enzyme (Grubmeyer & Gray, 1986). The similarity in structure, and the similar redox potentials for oxidation of alcohols and thiohemiacetals, suggests that histidinol dehydrogenase may have solved the problem of catalyzing two different oxidation reactions by employing a thiohemiacetal intermediate to make them structurally and chemically similar. It should be added that we do not know the identity of the active site base used in the HDH reaction, nor do we know if proton and hydride transfer are concerted or stepwise as shown. Although a Zn^{2+} is bound to HDH and is essential for activity (this laboratory, unpublished results), the nature of its role is unknown, and it has been omitted from our picture.

Although the three known four-electron dehydrogenases are frequently considered to be closely related evolutionarily, the stereochemistry and protein sequence data suggest this is not the case. Although HDH is *R* at NADH and *S* at histidinol, UDPGDH is *S* at NADH and is *R* at substrate alcohol (Ridley & Kirkwood, 1973), while HMGR, like HDH, is *R* at NADPH and *S* at substrate for the alcohol dehydrogenase step (Rogers et al., 1983); in contrast to HDH, HMGR uses NADPH and the substrate is the CoA thiol ester, which is reduced. Stronger evolutionary evidence is provided by amino acid sequence comparisons. No similarity was observed between the *Salmonella* HDH (Kohno & Gray, 1981) and the 537 carboxyl-terminal residues of human HMGR (Luskey & Stevens, 1985) by using the homology program FASTP (Lipman & Pearson, 1985). Similarly, a cysteine-containing active site peptide sequenced from UDPGDH (Franzen et al., 1981) shows no detectable homology to any of the cysteine-containing sequences of HDH, although we note sequence similarity between the former and nopaline dehydrogenase (Bevan et al., 1983). It does not appear that the evolutionary relationship among the three known four-electron dehydrogenases is any closer than between them and other NAD-linked enzymes. Since their shared ability to perform four-electron oxidations can in theory result from the action of a single catalytic site, there is no compelling reason to group them as a "family" in any rational taxonomy scheme.

It has been clear for some time that histidinol dehydrogenase shows a high degree of substrate specificity that arises from specific binding interactions (Loper & Adams, 1965). Such specificity is essential if the enzyme is to avoid oxidizing sugars and other polar alcohols. An analysis of data obtained from specificity studies (summarized in Scheme II) allows important conclusions to be drawn regarding the nature of substrate binding to histidinol dehydrogenase.

Our data show that the imidazole portion of the binding site contributes considerably to the overall binding energy and is highly specific. The analysis assumes that imidazole's interaction with the active site is an appropriate model for binding of the imidazole ring of histidinol. Imidazole itself might be able to bind in orientations not available to histidinol due to the anchoring effect of the alaninol side chain. It is clear, for example, that in imidazole a 2-fold symmetry exists that makes the ring faces equivalent and causes a factor of 2 error in our use of imidazole's K_i as a model for histidinol binding. If the enzyme bound imidazole solely by van der Waals interactions, then an additional 5-fold symmetry would exist in imidazole that is not present in histidinol. However, our data indicate that methylation of the 4-position does not result in a loss of binding affinity while, in direct contrast, methylation at the

Scheme II: Binding Interactions in the HDH Active Site[a]



[a] Stippled areas indicate proposed regions of substrate contact, based on inhibition experiments.

1- (equivalent to 3-) or 2-position causes a dramatic loss of inhibitor potency, suggesting that imidazole does not rotate freely in the active site and implying close interactions between the protein and the 1-, 2-, and 3-positions of imidazole. Since five-membered rings that would appear to be good structural imidazole analogues were far weaker inhibitors, it is suggested that very specific electronic interactions occur in binding of imidazole.

It is apparent that most of the side-chain binding interaction occurs at the amino group. This becomes clear when the K_i of histamine (35 μM) is compared with that of (chloroethyl)imidazole (1.2 mM) which is itself identical with that of 4-methylimidazole. Similarly, imidazolylchloropropanol (K_i = 0.45 mM) is 45-fold more weakly bound than histidinol K_D = 10 μM (Grubmeyer et al., 1987). Although the result suggests that the amino group can contribute nearly 3 kcal of binding energy, we found that L-alaninol itself was a very weak inhibitor of histidinol dehydrogenase.

The low relative binding strength of product histidine as compared to histidinol and histamine might arise from negative charge or steric factors at the oxidized carbon. However, the nearly equal K_i values for histidine, its neutral amide, and the methyl ester suggest that the negative charge on histidine is not critical.

The high specificity for L-histidinol was reinforced by our work with synthetic substrates. As observed by others (Loper & Adams, 1965), D-histidinol was not a substrate. The amino group was critical for function and could not be successfully replaced by hydrogen [in 3-(4-imidazolyl)-1-propanol] or by chloro or bromo groups [as in 3-(4-imidazolyl)-2-chloro-1-propanol]. The latter compounds, which have been employed as active-site-directed modifiers of LADH (Dahl et al., 1979), were synthesized with the hope of generating suicide substrates for HDH; however none of the alcohols, the acids, or the methyl esters detectably inactivated the enzyme. When the amino group was replaced by a hydroxyl, poor substrate activity was observed, suggesting a key role for this amino group in catalysis. The ring-modified compounds tested were all inactive as substrates, in agreement with their very poor inhibitory activities.

Kirkwood's group has proposed (Eccleston et al., 1979) that histidinol dehydrogenase has separate subsites for the alcohol and aldehyde dehydrogenase reactions and that intermediate histidinaldehyde is passed between these two sites, perhaps as a lysine-derived imine. Lysine modification studies have been interpreted as supportive of this idea (Burger & Gorisch, 1981). Our studies to date have not detected evidence for two distinct types of sites. First, but perhaps fortuitously, both

steps in the histidinol dehydrogenase reaction employ the same stereochemistry at NAD. Second, all inhibitors we employed were competitive versus histidinol, implying that none was able to bind to a separate site occupied by intermediate histidinaldehyde. Finally, it has long been known that mutations in the *hisD* gene fall into two major groups that complement in vivo to produce active enzyme (Greeb et al., 1971), suggesting that the mutations might characterize regions involved in the two HDH half-reactions. However, we have recently used in vitro complementation of purified mutant HDH to show that neither mutant class catalyzed the NAD/NADH exchange reaction reflective of the first oxidation (Lee & Grubmeyer, 1987).

ACKNOWLEDGMENTS

Dr. Barbara Stitt performed homology searches on the Rockefeller University Computing Facility. Kim-Wai Chu synthesized several of the compounds and performed some of the inhibition assays. We thank Dr. Peter Coleman for the gift of beef heart SMP. Purchase of the FPLC apparatus was funded by the New York University Challenge Fund.

REFERENCES

Adams, E. (1955) *J. Biol. Chem. 217*, 325–344.
Ames, B. N., & Mitchell, H. K. (1952) *J. Am. Chem. Soc. 74*, 252–253.
Andersson, L., & Wolfenden, R. (1982) *Anal. Biochem. 124*, 150–157.
Anet, F. A. L. (1960) *J. Am. Chem. Soc. 82*, 994–995.
Benner, S. A., Nambiar, K. P., & Chambers, G. K. (1985) *J. Am. Chem. Soc. 107*, 5513–5517.
Bevan, M., Barnes, W. M., & Chilton, M.-D. (1983) *Nucleic Acids Res. 11*, 369–385.
Beyerman, H. C., Buijen van Weelderen, A. W., Maat, L., & Noordam, A. (1977) *Recl. Trav. Chim. Pays-Bas 96*, 191–193.
Burger, E., & Gorisch, H. (1981) *Eur. J. Biochem. 118*, 125–130.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.
Cook, P. F., & Bertagnolli, B. L. (1986) in *Pyridine Nucleotide Coenzymes* (Dolphin, D., Avramovic, O., & Poulsen, R., Eds.) Part A, pp 405–447, Wiley, New York.
Dahl, K. H., McKinley-McKee, J. S., Beyerman, H. C., & Noordam, A. (1979) *FEBS Lett. 99*, 308–312.
Davies, D. D., Teixeira, A., & Kenworthy, P. (1972) *Biochem. J. 127*, 335–343.
Diziol, P., Haas, H., Retey, J., Graves, S. W., & Babior, B. M. (1980) *Eur. J. Biochem. 106*, 211–224.
Donahue, T. F., Farabaugh, P. J., & Fink, G. R. (1982) *Gene 18*, 47–59.
Dutler, H., Ambar, A., & Donatsch, J. (1986) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., & Zeppezauer, M., Eds.) pp 471–483, Birkhäuser, Boston.
Eccleston, E. D., Thayer, M. L., & Kirkwood, S. (1979) *J. Biol. Chem. 254*, 11399–11404.
Ellingsen, B., Marstokk, K.-M., & Mollendal, H. (1978) *J. Mol. Struct. 48*, 9–23.
Franzen, B., Carrubba, C., Feingold, D. S., Ashcom, J., & Franzen, J. S. (1981) *Biochem. J. 199*, 599–602.
Gaydou, E. M. (1972) *Tetrahedron Lett.*, 4055–4058.
Greeb, J., Atkins, J. F., & Loper, J. C. (1971) *J. Bacteriol. 106*, 421–431.
Grubmeyer, C. T., & Gray, W. R. (1986) *Biochemistry 25*, 4778–4784.
Grubmeyer, C. T., Chu, K.-W., & Insinga, S. (1987) *Biochemistry 26*, 3369–3373.
Jolley, C. J., & Yankeelov, J. A., Jr. (1972) *Biochemistry 11*, 164–169.
Kivits, G. A. A., & Hora, J. (1975) *J. Heterocycl. Chem. 12*, 577.
Kohno, T., & Gray, W. R. (1981) *J. Mol. Biol. 147*, 451–464.
Lee, S. Y., & Grubmeyer, C. T. (1987) *J. Bacteriol. 169*, 3938–3944.
Lipman, D. J., & Pearson, W. R. (1985) *Science (Washington, D.C.) 227*, 1435–1441.
Loper, J. C., & Adams, E. (1965) *J. Biol. Chem. 240*, 788–795.
Luskey, K. L., & Stevens, B. (1985) *J. Biol. Chem. 260*, 10271–10277.
Millefiori, S., Raudino, A., & Zuccarello, F. (1980) *Z. Phys. Chem. 123*, 67–73.
Ridley, W. P., & Kirkwood, S. (1973) *Biochem. Biophys. Res. Commun. 54*, 955–960.
Ridley, W. P., Houchins, J. P., & Kirkwood, S. (1975) *J. Biol. Chem. 250*, 8761–8767.
Rogers, D. H., Panini, S. R., & Rudney, H. (1983) in *3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase* (Sabine, J. R., Ed.) pp 57–75, CRC Press, Cleveland.
Seki, H., Koga, K., Matsuo, H., Ohki, S., Matsuo, I., & Yamada, S. (1965) *Chem. Pharm. Bull. 13*, 995–1000.
Shapiro, S., Arunachalam, T., & Caspi, E. (1983) *J. Am. Chem. Soc. 105*, 1642–1646.
Smissman, E. E., & Weis, J. A. (1971) *J. Med. Chem. 14*, 945–947.
Viola, R. E., Cook, P. F., & Cleland, W. W. (1979) *Anal. Biochem. 96*, 334–340.
Wilson, E. B., & Smith, Z. (1987) *Acc. Chem. Res. 20*, 257–262.
You, K. S. (1982) *Methods Enzymol. 87*, 101–126.
Yourno, J., & Ino, I. (1968) *J. Biol. Chem. 242*, 3273–3276.