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A Cysteine Residue (Cysteine-116) in the Histidinol Binding Site of Histidinol Dehydrogenase†

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ABSTRACT: *Salmonella typhimurium* L-histidinol dehydrogenase (EC 1.1.1.23), a four-electron dehydrogenase, was inactivated by an active-site-directed modification reagent, 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl). The inactivation followed pseudo-first-order kinetics and was prevented by low concentrations of the substrate L-histidinol or by the competitive inhibitors histamine and imidazole. The observed rate saturation kinetics for inactivation suggest that NBD-Cl binds to the enzyme noncovalently before covalent inactivation occurs. The UV spectrum of the inactivated enzyme showed a peak at 420 nm, indicative of sulfhydryl modification. Stoichiometry experiments indicated that full inactivation was correlated with modification of 1.5 sulfhydryl groups per subunit of enzyme. By use of a substrate protection scheme, it was shown that 0.5 sulfhydryl per enzyme subunit was neither protected against NBD-Cl modification by L-histidinol nor essential for activity. Modification of the additional 1.0 sulfhydryl caused complete loss of enzyme activity and was prevented by L-histidinol. Pepsin digestion of NBD-modified enzyme was used to prepare labeled peptides under conditions that prevented migration of the NBD group. HPLC purification of the peptides was monitored at 420 nm, which is highly selective for NBD-labeled cysteine residues. By amino acid sequencing of the major peptides, it was shown that the reagent modified primarily Cys-116 and Cys-377 and that the presence of L-histidinol gave significant protection of Cys-116. The presence of a cysteine residue in the histidinol binding site is consistent with models in which formation and subsequent oxidation of a thiohemiacetal occurs as an intermediate step in the overall reaction.

L-Histidinol dehydrogenase (EC 1.1.1.23) catalyzes the terminal step in the microbial biosynthesis of histidine, the four-electron oxidation of the α-amino alcohol L-histidinol to histidine. Such four-electron dehydrogenases (three are known) are extremely interesting mechanistically because a single active site catalyzes two distinct types of oxidation, from the substrate alcohol to an intermediate aldehyde, and then to the product acid. *Salmonella* L-histidinol dehydrogenase is of additional interest because it is the product of the *hisD* gene, mutations in which are used in the Ames mutagenicity assay (McCann et al., 1975). Although L-histidinol dehydrogenase has been known for 30 years (Adams, 1955), and the homogeneous enzyme is available in large quantities (Yourno & Ino, 1968), little is known about the enzyme mechanism. Most recent speculation has focused on a proposed mechanistic similarity between L-histidinol dehydrogenase and UDP-glucose dehydrogenase, which utilizes a lysine-derived imine and a cysteine thiohemiacetal–thiol ester in its mechanism (Ordman & Kirkwood, 1977a). Like UDP-glucose dehydrogenase, L-histidinol dehydrogenase uses 2 mol of NAD^+ to oxidize its substrate and also proceeds via a tightly or covalently bound form of the intermediate aldehyde (Eccleston et al., 1979). In addition, both enzymes follow ordered mechanisms in which substrate binds before coenzyme, in contrast to nearly all other known dehydrogenases (Ordman & Kirkwood, 1977b; Gorisch, 1979; Burger & Gorisch, 1981a). However, the published stereochemistry of NAD^+ reduction is S(B) for both steps in the mammalian liver UDP-glucose dehydrogenase reaction (Krakow et al., 1963) but R(A) for both steps in the L-histidinol dehydrogenase reaction catalyzed by extracts of *Neurospora* (Davies et al., 1972). Since the fungal L-histidinol dehydrogenase is known to be homologous with that of *Salmonella* (Donahue et al., 1982), it is most likely that the latter enzyme also shows R(A) specificity.

Since thiohemiacetal intermediates are employed in the oxidation of aldehydes by liver aldehyde dehydrogenases (Feldman & Weiner, 1972) and glyceraldehyde-3-phosphate dehydrogenase (Harris & Waters, 1976), cysteine is a likely catalytic site residue for the second oxidation step of the L-histidinol dehydrogenase reaction. Earlier work on L-histidinol dehydrogenase identified a single cysteine sulfhydryl on the enzyme whose complete modification by *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide resulted in partial

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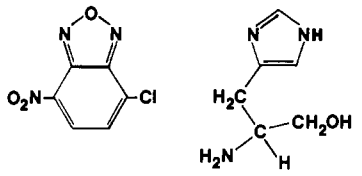
(60–80%) loss of activity (Wolf & Loper, 1969). Substrate protection could not be observed, however, suggesting that the modified cysteine was not located at the catalytic site. The peptide containing the labeled cysteine was isolated and sequenced (Bitar et al., 1977). The complete amino acid sequence of the enzyme (Kohno & Gray, 1981) revealed that the labeled residue was Cys-159 and revised the sequence of the peptide slightly.

The present work makes use of the resemblance between the substrate L-histidinol and 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl)[1] [nbf-Cl (Boulton et al., 1966)], a well



NBD-Cl **Histidinol**

characterized protein modification reagent. NBD-Cl is shown to be an active-site-directed modifier of a single cysteine group on L-histidinol dehydrogenase. Using pepsin degradation in 5% acetic acid to eliminate base-catalyzed NBD migration, we were able to identify the catalytic site sulfhydryl as Cys-116 in the primary structure of the protein.

Experimental Procedures

Materials

NBD-Cl was obtained from Sigma, St. Louis, MO (C5261), and was used without further purification. Stock solutions of 10 and 100 mM were made in ethanol. NAD (free acid) was obtained from Boehringer or Sigma. Pepsin, L-histidinol, and all other biochemicals were from Sigma. Inorganic compounds were reagent grade, obtained through Fisher. Bacterial growth media were from Difco.

Methods

Salmonella typhimurium hisO1242, a mutant strain which overproduces enzymes of the histidine operon, was obtained from Dr. John Roth, University of Utah. Cells were grown in a Lab-Line high-density fermenter (Model 29500) using a medium containing (per liter) 20 g of Bacto-tryptone, 10 g of yeast extract, 5 g of NaCl, 5 mL of 0.1 M $CaCl_2$, 5 mL of 1 M $MgSO_4$, and 1 mL of 10 mM $Fe(NH_4)(SO_4)_2$. Three liters of this medium was inoculated with 1 L of a culture of bacteria grown overnight with shaking in a water bath. The high-density cultures were aerated at a flow rate of 20 L of air/min, with the culture temperature maintained at 35 °C. Glycerol additions (100 mL, 50% solution) were made each 0.5 h, and the pH was maintained at approximately 7–7.5 with the addition of 1 M K_2HPO_4 and, when necessary, 1 M KOH. After growth (monitored by light scattering) had slowed, cells were harvested by centrifugation at 10000*g* for 15 min, using a Sorvall GSA rotor. This procedure yielded approximately 200 g of cells/4 L of culture.

L-Histidinol dehydrogenase was purified and crystallized from cells stored at −20 °C following the method of Yourno and Ino (1968), with the sole exception that buffer E (20 mM Tris, 20 mM succinate, 0.1 mM $MnCl_2$, and 0.75% NaCl, pH 6.2) was used with no added NaCl. The method, which involves $(NH_4)_2SO_4$ precipitation, a heat step, and chromatography on DEAE-Sephadex, yielded enzyme that crystallized readily and appeared homogeneous when electrophoresed on acrylamide gels run in the presence or absence of SDS.

Enzyme was prepared for assay by centrifugation of a small portion of the suspended crystals at 14000*g* for 1.5 min in an Eppendorf microfuge. The supernatant was drawn off by using a pipet, and the crystals were dissolved in "modification buffer", consisting of 0.5 mM $MnCl_2$ and 0.1 M $K_2B_4O_7$, adjusted to pH 9.0 with HCl.

The protein content of solutions of L-histidinol dehydrogenase was determined spectrophotometrically by using a value of 0.478 for the extinction coefficient of a 1 mg/mL solution at 280 nm (Yourno, 1968). Enzyme concentration is expressed as a molarity using the known molecular weight of the enzyme subunit, 45 823 (Kohno & Gray, 1981; Burger et al., 1979). Where noted, concentration is also expressed as molarity of the dimer.

Enzyme activity was assayed by continuously monitoring the absorbance at 340 nm of product NADH in the assay system described by Loper (1968), consisting of 1 mL of 50 mM glycine–NaOH, 10 mM NAD^+, 2 mM L-histidinol, and 0.5 mM $MnCl_2$, pH 9.2. This pH value provided optimal activity. Enzyme (1–3 μg) was added to start the reaction, which was carried out at 30 °C in the thermostated cuvette holder of a Perkin-Elmer 552A spectrophotometer.

One unit of enzyme activity is defined as that amount of enzyme which catalyzes the oxidation of 1 μmol of L-histidinol/min under the defined conditions. Homogeneous enzyme used in these studies showed activity of 10–12.5 units/mg. This specific activity compares well with published values [see Burger & Gorisch (1981b)].

Modification studies were carried out at 30 °C, either in test tubes in a Thermolyne block heater or in quartz cuvettes in the spectrophotometer. The buffers and concentrations of enzyme and modifiers are described in the footnotes to the tables and the legends to the figures.

In the experiments of Figure 3, enzyme was freed of substrate L-histidinol and excess modifier by use of the centrifuge column method described by Penefsky (1977), except that Bio-Gel P-10 was used in place of Sephadex G-50, in order to avoid formation of borate–polyol complexes.

Preparation of NBD-Labeled Enzyme for Peptide Analysis. For a typical experiment, L-histidinol dehydrogenase (4.1 mg, 90 nmol of subunit) was dissolved in 1.0 mL of modification buffer at 30 °C, and a sample was assayed for activity. NBD-Cl (50 nmol) was added and allowed to react completely (approximately 40 min). The extent of modification was monitored at 420 nm by using a millimolar extinction coefficient for the cysteine–NBD adduct of 13.6, and enzyme activity was redetermined. An identical sample was modified in the presence of 2 mM L-histidinol. Each sample was applied to a column (1.5 × 25 cm) of Sephadex G-50 equilibrated with 5% acetic acid and eluted with 5% acetic acid. Fractions containing protein were pooled and stored on dry ice while awaiting analysis.

Digestion and Separation of Peptides. Samples of labeled protein, as desalted on Sephadex G-10, were digested with pepsin. To the protein solution (1–1.5 mg/mL in 5% acetic acid, v/v) was added 5% by volume of a solution of pepsin (2 mg/mL in 10 mM HCl to which a small crystal of NaCl had been added). The mixture was incubated at 37 °C, and samples of 200 μL were removed at intervals for analysis.

Peptide fractionation was carried out by using a Hewlett-Packard Model 1084B chromatograph, with variable-wave-

[1] Abbreviations: NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; hol, L-histidinol; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

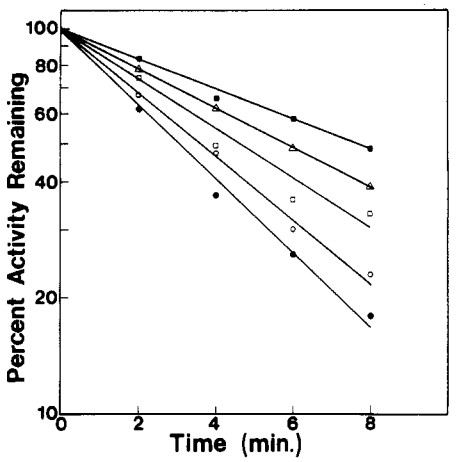


FIGURE 1: Inactivation of L-histidinol dehydrogenase by NBD-Cl and protection by L-histidinol. Enzyme (0.1 mg) was dissolved in 0.5 mL of modification buffer containing the indicated concentration of L-histidinol. NBD-Cl was added to a concentration of 0.1 mM. L-Histidinol concentrations were (●) none, (○) 20, (□) 60, (△) 100, and (■) 160 μM.

length detector monitoring the column effluent at 420 nm. The column used was a large pore VYDAC C18 (25 cm × 0.46 cm, 5-μm particle size, end-capped). Peptides were eluted with a linear gradient of from 10% B to 100% B in 40 min at a flow rate of 1 mL/min. Buffer A was 0.1% trifluoroacetic acid in water; buffer B was 0.1% trifluoroacetic acid in 60% acetonitrile (v/v). Major absorbance peaks were collected manually into polypropylene tubes (Falcon), and the solutions were dried in a Speed-Vac (Savant).

Sequence analysis was carried out on major peaks without attempting further purification. Samples were applied to the cup of a Beckman Model 890D sequencer, with 2 mg of polybrene (Tarr et al., 1978) which had been previously subjected to several rounds of degradation to remove impurities. A 0.2 M Quadrol program was used. Conversion of the extracted anilinothiazolinone amino acids was effected by incubating at 80 °C for 10 min in 200 mL of 1 M HCl containing 0.3 mg/mL dithiothreitol. The resulting PTH-amino acids were dissolved in 50 μL of 0.1% trifluoracetic acid in 20% acetonitrile (v/v) and analyzed by HPLC as described elsewhere (Winge et al., 1984).

Results

The similarity of structure between NBD-Cl and L-histidinol suggested that the former might bind specifically to the active site of L-histidinol dehydrogenase and modify the cysteine group proposed to take part in the catalytic mechanism (Ordman & Kirkwood, 1977a).

In pH 9.0 borate buffer containing 0.5 mM $MnCl_2$, 0.1 mM NBD-Cl caused the rapid pseudo-first-order inactivation of L-histidinol dehydrogenase (Figure 1). In the absence of NBD-Cl the enzyme activity was stable in this buffer for at least several days (not shown). The presence of low levels of L-histidinol during NBD-Cl treatment resulted in a reduction of the rate of inactivation (Figure 1); 10 mM NAD^+ had no effect on the rate of inactivation either in modification buffer or in 50 mM glycine–NaOH, pH 9.2 (not shown).

The concentration of substrate L-histidinol required to protect the enzyme from NBD-Cl inactivation was determined from a replot of the data of Figure 1, in which the half-time for inactivation was plotted as a function of L-histidinol concentration (not shown). The intercept gave a $K_{protect}$ of 110 μM. This may be compared with the K_m for L-histidinol of 15 μM at pH 9.2 in the presence of 10 mM NAD^+ (Loper & Adams, 1965; C. T. Grubmeyer, unpublished results).

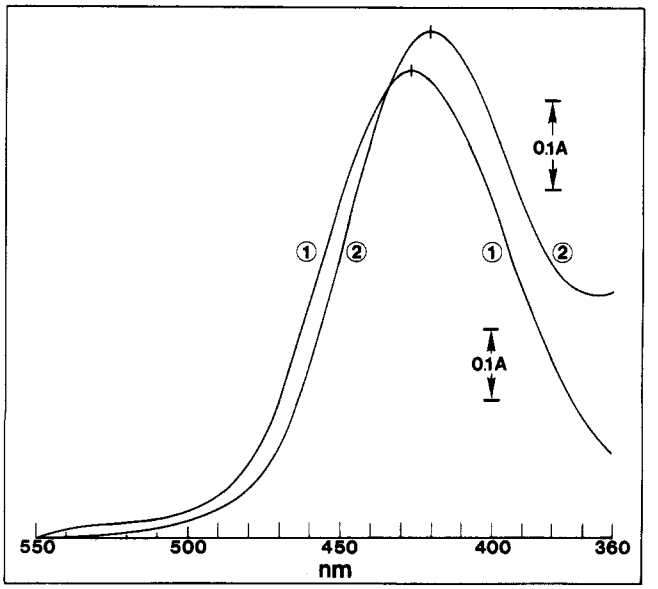


FIGURE 2: Spectrum of NBD-Cl-modified L-histidinol dehydrogenase. Line 1: L-histidinol dehydrogenase (18 nmol) was dissolved in 1.0 mL of modification buffer, and a base-line spectrum was taken against 1.0 mL of the same buffer. NBD-Cl (50 nmol) was added to both solutions, and the enzyme was allowed to react at 30 °C until activity was less than 10% of the starting value (about 30 min), and then the solution was scanned again. Line 2: 1 μmol of *N*-acetylcysteine in the same buffer was allowed to react for 5 h with 50 nmol of NBD-Cl at 30 °C and then scanned.

If NBD-Cl acts as a substrate analogue to interact with the L-histidinol binding site before causing inactivation, then substrate analogues should also decrease the rate of inactivation. Histamine, an excellent competitive inhibitor of L-histidinol dehydrogenase, K_i = 40 μM, also protected against NBD-Cl inactivation, $K_{protect}$ = 200 μM (not shown). Imidazole, a weaker competitive inhibitor, K_i = 1.5 mM, protected against NBD-Cl with a $K_{protect}$ of 4.4 mM (not shown).

Since NBD-Cl is capable of reacting with amino groups (Ferguson et al., 1975), it was important to show that the apparent protection by L-histidinol was not a result of its competing reaction with NBD-Cl. In control experiments 0.1 mM NBD-Cl was allowed to react with 0.2 mM L-histidinol in modification buffer. The reaction, which required 18 h to go to completion, resulted in a spectral peak at 475 nm, as expected for modification of an amino group, as well as a peak at 390 nm. During the time scale of the experiments of this paper, this free-solution reaction is insignificant.

The rate of NBD-Cl inactivation of L-histidinol dehydrogenase followed hyperbolic saturation kinetics with respect to NBD-Cl concentration (not shown), indicating that a step involving noncovalent binding of NBD-Cl to a saturable site preceded covalent modification. The K_{app} for the noncovalent binding was 0.25 mM. At saturating NBD-Cl the half-time for inactivation was 1.2 min, k_{inact} = 0.6 min^{-1}.

The residue modified by NBD-Cl was initially determined by spectral observations on the modified enzyme. Figure 2 shows a spectral scan of a partially modified enzyme. A clear peak at 420 nm was observed, indicative of sulfhydryl modification (Birkett et al., 1970). The spectrum of NBD-Cl-modified *N*-acetylcysteine, also shown in Figure 2, shows an absorbance peak centered on 425 nm. Peak absorbance at 420 nm was also observed by Hartl and Roskoski (1982) for NBD-Cl modification of sulfhydryl groups on the catalytic subunit of cAMP-dependent protein kinase. Modification by NBD-Cl of amino or tyrosyl groups provides spectral features

Table I: Reactivation of NBD-Cl-Inactivated L-Histidinol Dehydrogenase[a]

preparation	activity (%)
untreated	100
NBD-Cl modified	39
NBD-Cl, 0.1 mM mercaptoethanol	113
NBD-Cl, 0.1 mM dithioerythritol	70

[a] L-Histidinol dehydrogenase (18.4 nmol) in 1 mL of 0.5 mM $MnCl_2$ and 0.1 M $K_2B_4O_7$, pH 9.0 (unreacted enzyme), was reacted with 30 nmol of NBD-Cl at 30 °C for 1 h (NBD-treated enzyme). To 100-μL portions of this inactivated enzyme were added β-mercaptoethanol or solid dithioerythritol to 0.1 M, and incubation was continued for 10 min. Portions of 2 μL were assayed for enzymatic activity.

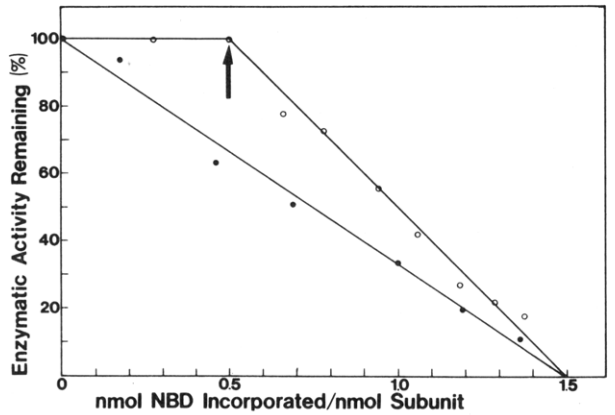


FIGURE 3: Stoichiometry of NBD-Cl modification. L-Histidinol dehydrogenase was dissolved in 1.0 mL of modification buffer, a 2-μL sample was assayed for enzymatic activity, and NBD-Cl was added as noted below. The extent of modification was followed spectrally at 420 nm, and samples were taken for assay of enzymatic activity. Closed circles: 100 nmol of NBD-Cl was added to 4.2 nmol of L-histidinol dehydrogenase. Open circles: the experimental procedure was identical except that 2 mM L-histidinol was present. After modification of 0.5 mol of sulfhydryl/mol of enzyme subunit (arrow), the solution was divided into 150-μL portions and passed through centrifuge columns (Methods) of Bio-Gel P-10. The pooled column effluents were then brought to 1.0 mL and again assayed for enzymatic activity and absorbance at 420 nm to correct for recovery of protein. A separate enzyme assay established that no L-histidinol had passed through the columns. The resulting solution was then treated with 20-nmol portions of NBD-Cl. The extent of modification was followed at 420 nm, and 2-μL samples were taken for assay of enzymatic activity.

easily distinguished from those observed here [see Ferguson et al. (1975)].

Further evidence for the identity of the modified group was provided by the finding that the inactivation caused by NBD modification of L-histidinol dehydrogenase was reversed by the addition of reagents containing sulfhydryl groups. Table I shows that L-histidinol dehydrogenase that had been 60% inactivated by NBD-Cl was completely reactivated by the addition of 0.1 M mercaptoethanol. Dithioerythritol at 0.1 M produced a 50% reactivation of the inhibited enzyme.

The stoichiometry of sulfhydryl group modification was determined by using the known extinction coefficient of its reaction product with *N*-acetylcysteine at 420 nm (Birkett et al., 1970). Complete loss of activity was associated with the modification of 1.5 of the 7 total (Kohno & Gray, 1981) cysteine residues per monomer (Figure 3, closed circles).

In order to determine if modification of a single essential sulfhydryl per monomer was responsible for inactivation, we performed modification in the presence of L-histidinol and then removed the protecting agent and treated the enzyme again with NBD-Cl (Figure 3, open circles). With substrate protection, modification was limited to 0.5 bound NBD per monomer with no loss of activity. Additional modification occurred extremely slowly. After removal of L-histidinol and unreacted NBD-Cl by the centrifuge column method (Penefsky, 1977; see Methods), titration of the eluted enzyme with NBD-Cl demonstrated that rapid inactivation of the enzyme was associated with modification of an additional 1 mol of sulfhydryl/mol of subunit (2 mol of sulfhydryl/mol of dimer).

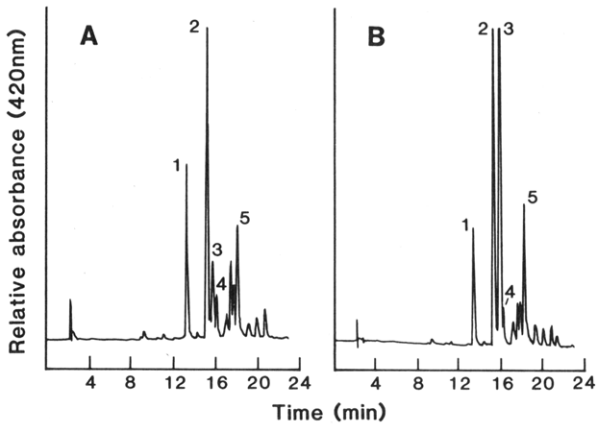


FIGURE 4: HPLC elution profile of pepsin digests of NBD-labeled L-histidinol dehydrogenase. Conditions: VYDAC C18 column, 5-μm particle size, end-capped, 300-Å pores, 4.6 mm × 25 cm; flow 1.0 mL/min, ambient temperature; gradient from 10% B to 100% B in 40 min; buffer A, 0.1% trifluoroacetic acid in water; buffer B, 0.1% trifluoroacetic acid in 60% acetonitrile (v/v); monitored at 420 nm. (A) Enzyme labeled with NBD-Cl in the presence of 2 mM L-histidinol (0.48 mol of NBD/mol of subunit); (B) enzyme labeled in the absence of L-histidinol (0.41 mol of NBD/subunit).

Under our conditions of storage and pepsin digestion, the NBD label appeared to be stably bound to cysteine residues. The absorbance profiles of HPLC runs monitored at 420 nm, which is highly selective for the cysteine derivative, contained a series of sharply defined peaks (Figure 4). Monitoring at 214 nm (general for peptide bonds) showed an expectedly very complex pattern of peaks (data not shown). In contrast, monitoring at 475 nm (selective for N-modified derivatives) revealed only small peaks corresponding to those seen at 420 nm and no new ones (data not shown). The same pattern at 420 nm was obtained with enzyme that had been digested within 24 h of labeling or after a further 2 weeks of storage at −20 °C. Yields of the various peaks changed very little after 1 h of digestion. This stability demonstrates the usefulness of pepsin digestion for analysis of NBD-labeled proteins.

Panels A and B of Figure 4 illustrate typical profiles of peptic digests of enzyme labeled in the presence and absence of L-histidinol, respectively. Substitution levels were 0.48 and 0.41 mol of NBD/mol of subunit in the two cases, but total protein loading was higher in panel B (1.67×). It is apparent that the dominant peak 3 in the digest of unprotected enzyme is greatly diminished in the digest of enzyme protected by L-histidinol.

In earlier experiments digestions were carried out with a different batch of pepsin. The position and yield of peak 3 was essentially the same, but the relative intensities of peaks 1 and 2 were greatly altered, as were those of peaks 4 and 5. We were able to identify the Cys residues associated with each of peaks 1–5 but did not attempt to characterize any others (Table II). In all experiments peaks 1–5 accounted for more than 75% of the total integrated absorbance at 420 nm.

Peak 3, selectively suppressed by L-histidinol protection, was clearly identified as having at least residues Val-108–Cys-116. Three independently obtained samples were analyzed in the sequencer. From an early digest of unprotected enzyme labeled to 1.50 mol/mol of subunit, approximately 500 pmol of peptide was sequenced for 12 steps. A single sequence Val-

Table II: Sequences of Labeled Peptides 1–5[a]

peak	digest	sequence (position)	Cys
1	A	complex mixture, see text; main components—Tyr (373)-Thr-Ala-Thr-Cys-Ser; Val (316)-Ala-Ile-Ser-Asn-Glu; Ile (94)-Glu-Thr-Phe	377
1	B	complex; Tyr at first step in 15–20% yield of major peaks	(377)
2	B	Tyr (373)-Thr-Ala-Thr-Xxx-	377
3	A	Val (108)-Glu-Thr-Gln-Pro-Gly-Val-Arg-Cys-	116
3	C	Val (108)-Glu-Thr-Gln-	116
3	B	Val (108)-Glu-Thr-Gln-Pro-Gly	116
4	A	Ile (6)-Asp-Trp-Asn-Ser-Cys-Ser-Pro-	11
5	B	two peptides in approximately 3:1 ratio—Leu (192)-Ala-Phe-Gly-Ser; Ile (6)-Asp-Trp-Asn-(Ser)-	11

[a] Digests: A, unprotected enzyme labeled to 1.50 mol/mol of subunit; B, unprotected enzyme labeled to 0.58 mol/mol of subunit; C, protected enzyme labeled to 0.50 mol/mol of subunit. Assignments in parentheses indicate that results were compatible, but not definitive.

Glu-Thr-Gln-Pro-Gly-Val-Arg-Cys- was obtained, though the yield of Cys was low; no identifiable PTH amino acid was obtained beyond this point. In L-histidinol-protected sample containing 0.50 mol of NBD/mol of subunit, the corresponding peak was greatly reduced. Only four cycles were run, and the terminal sequence Val-Glu-Thr-Gln- was obtained at a level of approximately 100 pmol. The great reduction in yield is fully consistent with this peptide being the actual labeled peptide and not merely a fortuitous coeluting unlabeled one. A third sample from a late digest gave the same terminal sequence, establishing correspondence between the old and new digests.

Peaks 1 and 2 appear to be different digestion products arising from labeled Cys-377. In an early digest peak 1 was dominant. Sequence analysis for six steps showed that this peak was a mixture of three major components, plus minor ones. One of the three major amino acids at the first step was Tyr. The major amino acids released at subsequent steps were compatible only with its assignment as Tyr-373, corresponding to a peptide containing Cys-377. Cys was obtained at the expected position. The other likely major components were peptides beginning with Val-316 and Ile-94. Peak 1 was greatly reduced in later digests, and we did not obtain enough to attempt repurification of the Cys-containing peptide. However, the presence of the Cys-377 peptide in this peak was suggested by the observation of Tyr in approximately 15–20% yield compared to those of the major components. Peak 2, which was dominant in later digests, was essentially clean and gave the sequence Tyr-Thr-Ala-Thr-X. In a like manner peaks 4 and 5 were both found to correspond to Cys-11, the former being obtained in significant amounts only in the early digest.

Relative yields of the identified peptides 1 and 2 (Cys-377), 3 (Cys-116), and 4 and 5 (Cys-11) varied according to both the absolute level of substitution and to whether L-histidinol was present during labeling (Table III). In three samples of labeled enzyme that had been protected by L-histidinol (NBD = 0.41–0.50 mol/mol of subunit) approximately 60–65% of the total label was found in Cys-377, with another 20–25% present in Cys-11. Only 5–10% was in Cys-116. In unprotected enzyme it appeared that Cys-377 labeled more readily at low loadings but that it saturated more rapidly than the others. Cys-116 was specifically labeled in unprotected enzyme and was the major labeled Cys at high NBD loading levels. Only at the highest loading was Cys-11 significantly labeled; at lower levels inclusion of L-histidinol clearly had no protective effect.

From yields of PTH-amino acids in the sequence analysis we estimate that total recovery of NBD label in peptides was greater than 50% (from intact protein to peptides recovered from HPLC). Although it is possible that all of the missing label represents a unique peptide that was not recovered from HPLC, we regard it as unlikely because of the very reproducible patterns obtained after 1–72 h of digestion, and the likelihood of pepsin having degraded the protein into relatively small fragments.

Table III: Labeling of Cysteines by NBD-Cl[a]

	total NBD	(1 + 2) Cys-377	(3) Cys-116	(4 + 5) Cys-11	total % (1–5)
−hol	0.10	0.044	0.024	0.007	75
	0.40	0.173	0.109	0.038	80
	0.54	0.158	0.219	0.065	82
	0.58	0.219	0.215	0.057	85
	1.50	0.429	0.575	0.364	83
+hol	0.41	0.201	0.044	0.067	76
	0.48	0.241	0.043	0.076	75
	0.50	0.300	0.029	0.070	80

[a] Figures in columns 2–5 represent loading of NBD in mol/mol of subunit, in total, and on individual Cys residues as indicated by HPLC peaks 1–5. Column 6 indicates the total of recovered absorbance that was present in peaks 1–5. The first five samples (−hol) were labeled in the absence of L-histidinol; the remainder (+hol) were labeled in the presence L-histidinol. Values for individual Cys residues are based on the assumption that recovered label is distributed in the same manner as total label.

Discussion

The major observation reported in this paper is the identification of a unique sulfhydryl at the active site of L-histidinol dehydrogenase. From the known amino acid sequence of the protein the position of the sulfhydryl can be assigned as Cys-116 [the order of peptides in the molecule is that assigned in numbering the tryptic peptides reported in Kohno and Gray (1981) (Barnes, Gray, Keller, and Kohno, unpublished observations)]. Several lines of evidence lead to the conclusion that the NBD-Cl-modified sulfhydryl is at the active site, most importantly the observation that L-histidinol, histamine, and imidazole at low levels provided excellent protection against modification. This protection strongly suggests competition between NBD-Cl and other ligands for a site on the enzyme.

The $K_{protect}$ for the protecting agents was in all cases higher than the K_m or K_i obtained in kinetic experiments. The difference was 7-fold in the case of L-histidinol, 5-fold for histamine, and 3-fold for imidazole. One reason for this discrepancy is the fact that NBD-Cl competes with ligand for the L-histidinol binding site. A correction for this competition can be made by using the formula:

$$K_{protect(true)} = K_{protect(obs)}/(1 + [modifier]/K_{app})$$

The equation yields corrected values of $K_{protect}$ of 80 μM for L-histidinol, 140 μM for histamine, and 3 mM for imidazole. This represents a discrepancy of 5-fold for L-histidinol, 3.5-fold for histamine, and 2-fold for imidazole. These differences appear to be minor and may arise from differences between the K_m and K_D for L-histidinol and differences between the assay and modification media.

It is of interest that the cysteine which is protected by L-histidinol is not that with highest intrinsic reactivity. At low

NBD ratios Cys-377 labels more rapidly than Cys-116 (Table III). However, labeling of this residue is not affected by L-histidinol, and it saturates below 0.5 mol/mol of subunit. One possible explanation is that Cys-377 may be located close to the twofold axis so that only one residue per dimer may be accessible. Cys-11 was strongly labeled only in one highly modified protein (1.50 mol of NBD/mol of subunit).

Two possible locations for Cys-116 could be considered: at the imidazole binding portion of the catalytic site or at that part which interacts with the α-aminopropanol side chain. The structural similarities between NBD-Cl and L-histidinol suggested that the furazan ring of NBD-Cl would bind to the imidazole site, placing C-4 of the benzene ring at a location which could be occupied by the oxidizable carbon of L-histidinol during catalysis. The expected binding of NBD-Cl was demonstrated by a rate saturation effect for inactivation, and the low K_{app}, 0.25 mM, indicated favorable interaction. Hartl and Roskoski (1982) observed a similar K_{app} for the binding of NBD-Cl that led to the inactivation of the catalytic subunit of cAMP-dependant protein kinase, where NBD-Cl may act as an adenine analogue. In the case of L-histidinol dehydrogenase, it is unlikely that the benzene-derived ring of NBD-Cl would bind to the imidazole site of the enzyme, since the site is quite specific. Thus, phenylalaninol is neither a substrate nor an effective inhibitor for the catalytic reaction of L-histidinol dehydrogenase (Loper & Adams, 1965), and work in this laboratory has determined that methylation of the imidazole ring at the 1-, 2-, or 3-positions leads to a dramatic decrease in the potency of competitive inhibitors (C. T. Grubmeyer, unpublished results).

The amino acid sequence of the complex L-histidinol dehydrogenase protein of the yeast *Saccharomyces* is known from DNA sequencing (Donahue et al., 1982). A comparison of the amino acid sequence of the two enzymes using the newly developed program FASTP (Lipman & Pearson, 1985) revealed 45% homology in a 419-residue region of the yeast protein. Only two of the seven Cys residues of the *Salmonella* enzyme are conserved in the yeast enzyme (Cys-116 and -153 are conserved as Cys-470 and -507, respectively). This is consistent with an active site location for Cys-116. Cys-377 and Cys-159, which from this and other work (Bitar et al., 1977) appear to be nonessential, are not conserved in the yeast sequence.

The existence of a catalytic site sulfhydryl for L-histidinol dehydrogenase is consistent with a proposed mechanism (Ordman & Kirkwood, 1977a) that involves formation and oxidation of a thiohemiacetal as the second dehydrogenation step in the overall reaction. This type of reaction is common among the aldehyde dehydrogenases and appears to occur in UDP-glucose dehydrogenase (Ridley et al., 1975). It is important to note that while the current work has demonstrated the existence of a catalytic site sulfhydryl group, the chemical involvement of the group in catalysis of the L-histidinol dehydrogenase reaction remains to be determined. In particular there is ambiguity concerning whether HDH might exhibit half-of-the-sites reactivity toward NBD-Cl. On the one hand, the substrate protection experiments (Figure 3, open circles) show that there is a 1:1 stoichiometry between enzyme inactivation and NBD uptake per subunit after removal of substrate. On the other hand is the finding that, at the highest loading, with complete inactivation, Cys-116 is only labeled to about 60%, with extensive labeling of Cys-11 occurring also (Table III). Although significant reaction at this residue was observed in only the one experiment, the results are consistent with a model in which there is half-of-the-sites reactivity at Cys-116 and a concomitant increase in susceptibility of Cys-11.

Finally, we note that identification of NBD-labeled peptides has suffered from the base-catalyzed migration of NBD. Recently, reduction of the nitro group to the amine was successfully employed to stabilize an NBD–tyrosine peptide (Andrews et al., 1984). The use of pepsin under acidic conditions represents another technique for stabilizing the adduct. While our work was in progress, another report appeared in which the same strategy was employed (Sutton & Ferguson, 1985).

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Registry No. NBD-Cl, 10199-89-0; EC 1.1.1.23, 9028-27-7; L-Cys, 52-90-4; L-histidinol, 4836-52-6; histamine, 51-45-6; imidazole, 288-32-4.

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Leaving Group Dependence in the Phosphorylation of *Escherichia coli* Alkaline Phosphatase by Monophosphate Esters†

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ABSTRACT: Values of k_{cat} and K_m have been measured for the *Escherichia coli* alkaline phosphatase catalyzed hydrolysis of 18 aryl and 12 alkyl monophosphate esters at pH 8.00 and 25 °C. A Brønsted plot of log (k_{cat}/K_m) (M^{-1} s^{-1}) vs. the pK of the leaving hydroxyl group exhibits two regression lines:

$$\log (k_{cat}/K_m) = -0.19\ (\pm 0.02)\ pK^{ArOH} + 8.14\ (\pm 0.15)$$

$$\log (k_{cat}/K_m) = -0.19\ (\pm 0.01)\ pK^{ROH} + 5.89\ (\pm 0.17)$$

Alkyl phosphates with aryl or large lipophilic side chains are not correlated by the above equations and occupy positions intermediate between the two lines. The observed change in effective charge on the leaving oxygen of the ester (−0.2) is very small, consistent with substantial electrophilic participation of the enzyme with this atom. Cyclohexylammonium ion is a noncompetitive inhibitor against 4-nitrophenyl phosphate substrate at pH 8.00, and neutral phenol is a competitive inhibitor (K_i = 82.6 mM); these data and the 100-fold larger reactivity of aryl over alkyl esters are consistent with the existence of a lipophilic binding site for the leaving group of the substrate. The absence of a major steric effect in k_{cat}/K_m for substituted aryl esters confirms that the leaving group in the enzyme–substrate complex points away from the surface of the enzyme. Arguments are advanced to exclude a dissociative mechanism (involving a metaphosphate ion) for the enzyme-catalyzed substitution at phosphorus.

The presently accepted kinetic scheme for the catalytic action of alkaline phosphatase from *Escherichia coli* possesses four sequential steps passing through a central, phosphoryl-enzyme (eq 1). The phosphoryl group ($-P_i = -PO_3^{2-}$) becomes co-

$$E + XOP_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}XOP_i \underset{k_{-2}[XOH]}{\overset{k_2}{\rightleftharpoons}} E{-}P_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E{\cdot}HOP_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + HOP_i \quad (1)$$

valently attached to serine-102 in the single peptide chain constituting a monomer of the dimeric enzyme (Coleman & Chlebowski, 1979; Bradshaw et al., 1981; Sowadski et al., 1985; Coleman & Gettins, 1983). There is now considerable information concerning the constituents of the active site from X-ray crystallographic, NMR spectroscopic, and chemical work providing ground-state spacial data.

Knowledge of the change in charge on the leaving atom in transfer of the phosphoryl group from the substrate to the enzyme would provide useful data to describe the molecular mechanism of the catalysis. Measurement of the change in charge requires knowledge of the polar effect on the transition state for the phosphorylation step as well as a calibrating polar effect on an equilibrium for phosphoryl group transfer where the charge change may be defined. The most appropriate calibrating equilibrium is the hydrolysis of the monophosphate dianion (eq 2) for which the polar effect has recently been

$$X{-}O{-}P_i \overset{K}{\rightleftharpoons} X{-}O{-}H + H{-}O{-}P_i \quad (2)$$

$$X{-}O{-}H \overset{K_a}{\rightleftharpoons} X{-}O^- + H^+ \quad (3)$$

determined (Bourne & Williams, 1984a). All measurements of charge are referred to the ionization of the hydroxyl species (eq 3) where the charge change is defined as unity (Williams, 1984a).

The rate-limiting step for the enzyme-catalyzed hydrolysis at low substrate concentrations is phosphorylation when the added leaving group concentration is negligible ($k_{-2}[XOH] \ll k_3$). The kinetic equation for k_{cat}/K_m simplifies to eq 4 (Ko & Kézdy, 1967). The free energy difference between ground

$$k_{cat}/K_m = k_2/K_s = k_1k_2/(k_{-1} + k_2) \quad (4)$$

state (E + XOP_i) and transition state of the phosphorylation step is essentially measured by k_{cat}/K_m; the effect of polar substituents on k_{cat}/K_m will therefore directly depend on the charge change (Williams, 1984; Hine, 1959; Jencks, 1971) by virtue of the change in energy of interaction between charge and the substituent. Previous work (Williams et al., 1973) has shown that there is little polar effect of substituents in the

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