Stereospecific Irreversible Inhibition of Histidine Ammonia-Lyase by L-Cysteine†

Claude B. Klee

ABSTRACT: Treatment of histidine ammonia-lyase at high pH and in the presence of O_2 with L-cysteine or L-homocysteine but not with the corresponding D enantiomers results in an irreversible inhibition of the enzyme. Other thiols are unable to substitute for these inhibitors. The inactivation is accompanied by the binding of 3.2–3.5 mol of cysteine/mol of enzyme tetramer and by subsequent spectral changes characterized by a new absorption peak at 340 nm. The inactivation is considered to be active site specific since L-histidine, but not D-histidine, and urocanic acid, the product of the enzyme reaction, have a protective effect.

The effects of sulfhydryl compounds on the enzymatic activity of histidine ammonia-lyase have been partially elucidated (Tabor and Mehler, 1955; Peterkofsky and Mehler, 1963; Rechler, 1969; Givot *et al.*, 1969; Soutar and Hassall, 1969; Hanson and Havir, 1972) and can be divided into four distinct classes. First, cysteine, as well as other thiols, reduces the four reactive -SH groups of the oxidized enzyme, which have been shown to be required for optimal activity (Givot *et al.*, 1969; Soutar and Hassall, 1969; Frankfater and Fridovich, 1970; Klee, 1970), and thereby activates the enzyme. Second, excess thiols in the assay mixture, particularly cysteine, have an inhibitory effect on the enzymatic activity (Peterkofsky and Mehler, 1963; Rechler, 1969) which has been attributed to the ability of thiols to bind metal ions (Cornell and Lien, 1970) which are also required for optimal activity (Mehler and Tabor, 1953; Peterkofsky and Mehler, 1963; Rechler, 1969; Givot *et al.*, 1969; Mildvan, 1970; Klee, 1972). Third, a specific, competitive, and reversible inhibition by L-cysteine has been observed (Givot *et al.*, 1969) and seems to indicate that this amino acid can bind at the active site of the enzyme. This competitive behavior led us to postulate that the fourth effect of L-cysteine, an irreversible inactivation of the enzyme (Klee, 1970), could be the result of an active site modification. The nature of the last two reactions of L-cysteine with histidine ammonia-lyase is the subject of this communication.

Experimental Section

Materials

Histidine ammonia-lyase used in these studies was prepared from *Pseudomonas* (ATCC 11,299b) by the published procedure[1] (Klee, 1970) and had a specific activity of 12–14 units/mg. The enzyme was assayed spectrophotometrically (Tabor and Mehler, 1955; Rechler, 1969) in 0.1 M Tris-HCl buffer (pH 9.0). When the enzyme was not reduced prior to assays, 1 mM mercaptoethylamine was added to the assay mixture (this concentration of thiol was shown not to be inhibitory), and the reaction rate was measured after 10 min to allow full reduction of the enzyme. The unit of enzyme is as defined previously (Klee, 1970). When [^{35}S]- and [U-^{14}C]-L-cystine with specific activities of 90–250 Ci/mol (Amersham/Searle Corporation) were used, they were diluted to a final specific activity of 0.3–1 Ci/mol with nonlabeled L-cysteine at pH 8.0 and incubated at 25° for 30 min under N_2 prior to the experiment.

Superoxide dismutase was prepared from human blood using the method of McCord and Fridovich (1969).

Methods

Preparation of L-Cysteine-Inactivated Enzyme. The enzyme (3 mg) in 2 ml of 0.05 M sodium carbonate–bicarbonate buffer (pH 10.5) (Gomori, 1955) containing 0.004 M L-cysteine was incubated at 25° in an open test tube with slow stirring. The reaction was monitored by successive assays of 1-μl aliquots for enzymatic activity and by following the appearance of the 340-nm absorption peak, a characteristic of the L-cysteine inactivated enzyme (Klee, 1970). The reaction was considered complete when the increase in absorption at 340 nm ceased, usually after 100–120 min. To remove the excess reagent, as well as any cysteine bound through disulfide bridges with the four reactive -SH groups of the enzyme, the reaction mixture was made 0.01 M in dithiothreitol and immediately passed through a 0.9 cm × 25 cm Sephadex G-25 column equilibrated and eluted with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl, 0.001 M EDTA, and 0.001 M dithiothreitol. The material excluded from the column can be freed of thiols by passage through the same G-25 Sephadex column equilibrated with 0.05 M potassium phosphate buffer (pH 7.5) or by overnight dialysis against 100 volumes of this buffer, and can be stored frozen at −20°. The modified enzyme was shown to contain 3.6–4.0 free -SH groups by titration with Ellman's reagent (Ellman, 1970), as is the case for the enzyme activated by reduction with other thiols (Klee, 1970).

The rate of release of radioactive label from the modified enzyme was measured by an assay based on the ability of Millipore filters to bind proteins. Histidine ammonia-lyase or its labeled derivative was retained (90–100%) on 0.22-μ Millipore filters (25 mm diameter), when applied in a 4-ml sample containing 40–70 μg of protein in 0.05 M potassium phosphate (pH 7.5) at 25°. To measure the effect of various pH levels or 6 M guanidine hydrochloride, the enzyme

† From the Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received June 21, 1974.*

[1] I thank Mr. David L. Rogerson for growing large batches of cells and carrying out the first steps of the large scale purification.

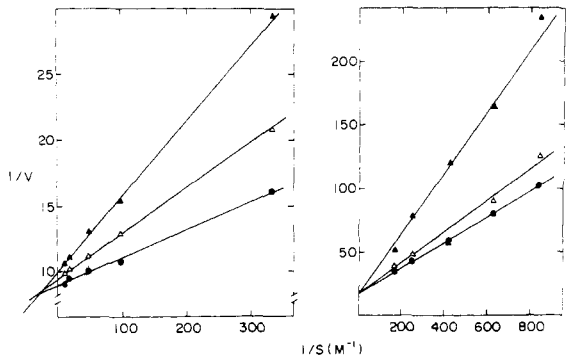

FIGURE 1: Effect of D- and L-cysteine on histidine ammonia-lyase. Left: mixed inhibition by D- and L-cysteine in the presence of added cadmium. The assays were carried out in the absence of mercaptoethylamine with reduced enzyme (0.3 μg/ml) as described in Materials and Methods in the presence of 0.1 mM Cd^{2+}. The reaction was started by the addition of substrate. V is expressed as the increase in the number of absorbancy units at 277 nm/min. Right: competitive inhibition by L-cysteine in the absence of added metal. The assays were as described above but Cd^{2+} was replaced by 1 mM EDTA and the protein concentration was 1.7 μg/ml. (●) No addition; (▲) 0.4 mM L-cysteine; (△) 0.4 mM D-cysteine.

(0.4–0.7 mg/ml) was first incubated at the indicated pH or in the presence of the reagents. The reaction was stopped by a tenfold dilution with the above buffer. The diluted protein solution was immediately passed through the Millipore filter, and washed three times with the same buffer. The filter or the filtrates were counted in a Triton toluene-containing solvent (Patterson and Greene, 1965). This technique is particularly suitable for the multiple samples necessary for rate measurements and requires very little enzyme.

Performic acid oxidation was as described by Hirs (1967). Reduction and carboxymethylation were carried out according to Sela *et al.* (1959), using 0.002 M dithiothreitol instead of mercaptoethanol.

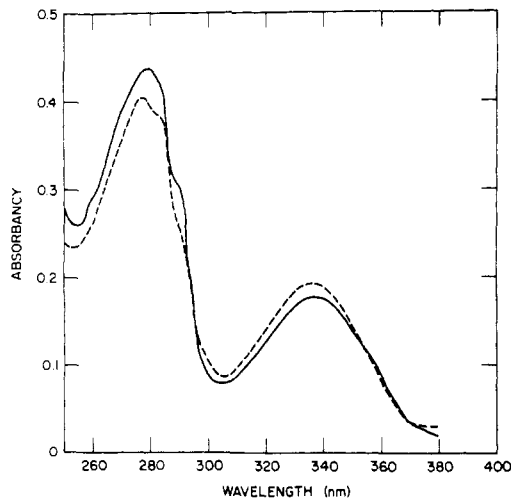

FIGURE 2: Uv absorption spectra of L-cysteine-inactivated enzyme in the presence and absence of guanidine hydrochloride. The spectra were measured in a Cary Model 11 spectrophotometer using a 1-cm pathlength quartz cuvet. The protein was measured by the method of Lowry *et al.* (1951) using the correction factor previously published (Klee and Gladner, 1972). [^{14}C]-L-Cysteine-inactivated enzyme prepared as described in Materials and Methods contained 3.2 mol of [^{14}C]-L-cysteine/mol of enzyme tetramer. The spectrum was measured in 0.05 M potassium phosphate buffer (pH 7.5) at a protein concentration of 2.6 mg/ml and is corrected to a solution of 1 mg/ml (—). The same solution of enzyme was diluted fourfold with a solution of guanidine hydrochloride to a final concentration of 6 M and the spectrum measured under these conditions was corrected to 1 mg of protein/ml (- - - - -). (Addition of 1 mM EDTA and 1 mM dithiothreitol to the latter did not result in any significant changes.)

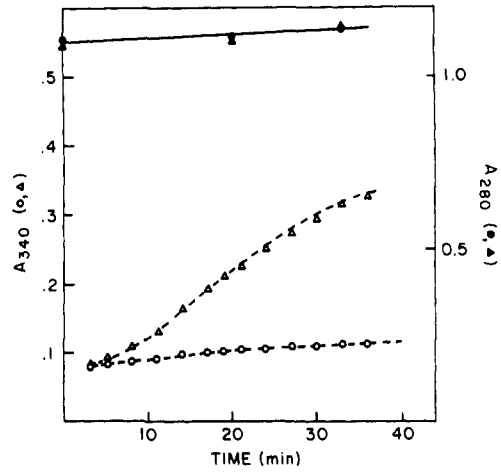

FIGURE 3: Time course of spectral changes of histidine ammonia-lyase during treatment with L- and D-cysteine. The enzyme (1.15 mg/ml) in 0.05 M sodium carbonate-bicarbonate buffer (pH 10.7) was incubated at 25° in the presence of 10 mM L-cysteine (△, ▲) or 10 mM D-cysteine (○, ●). Absorption at 280 nm (▲, ●) and 340 nm (△, ○) was recorded as a function of time. The specific activity of the starting material was 13.5 units/mg. After dialysis to remove excess reagent, the samples treated with L-cysteine and D-cysteine had specific activities measured in the presence of 1 mM mercaptoethylamine of 0.8 and 11.6 units/mg, respectively.

Results

Effect of D- *and* L-*Cysteine on Histidine Ammonia-Lyase Activity.* Thiols are known to have an inhibitory effect on the enzymatic activity of histidine ammonia-lyase (Peterkofsky and Mehler, 1963; Rechler, 1969; Givot *et al.*, 1969; Cornell and Lien, 1970). The effect of D- and of L-cysteine on the activity of the enzyme is shown in Figure 1. To eliminate stimulation by cysteine due to the reduction of the enzyme sulfhydryl groups, the enzyme was reduced with dithiothreitol and freed of excess thiols prior to assays. When the activity was measured in the presence of Cd^{2+} (left), both thiols were inhibitory, although D-cysteine was a less potent inhibitor than L-cysteine. The mixed type of inhibition observed in this case can be attributed to the metal chelating properties of cysteine (Cornell and Lien, 1970). When the assays were done in the presence of EDTA to eliminate cysteine inhibition of the stimulatory effect of metal ions (right), inhibition by D-cysteine was almost undetectable and only the competitive component of the inhibition by L-cysteine was observed.

Irreversible Inactivation of Histidine Ammonia-Lyase by L-*Cysteine.* The inhibition described above is reversed by dialysis and observed at low concentrations of cysteine and pH below 9.0. Prolonged incubation with higher concentrations of L-cysteine at pH above 9.0, followed by removal of the cysteine by dialysis prior to assay, results in an irreversible inactivation of the enzyme, as well as in spectral changes characterized by the appearance of a new peak at 340 nm (Klee, 1970). These changes in absorption are stable in 6 M guanidine hydrochloride (Figure 2) and are specific for L-cysteine (Figure 3). Incubation with the L enantiomer resulted in the characteristic increase in absorption at 340 nm, whereas no more than 10% of this increase was observed with the D isomer. After dialysis to remove excess reagent, the L-cysteine-treated enzyme had lost 94% of its enzymatic activity and its D-cysteine counterpart, only 14%

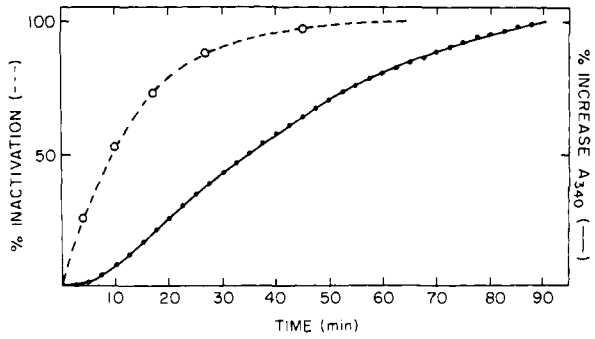

FIGURE 4: Time course of increase at 340 nm and of inactivation of histidine ammonia-lyase in the presence of L-cysteine. The incubation mixture was 0.05 M sodium carbonate-bicarbonate buffer (pH 10.5) containing 0.004 M L-cysteine and 2 mg of protein/ml. The absorption at 340 nm was followed in the Cary Model 11 spectrophotometer at 25°. At various times 2-μl aliquots were assayed for enzymatic activity in the presence of thiol, as described in Materials and Methods.

TABLE I: Oxygen Requirement for Inactivation of Histidine Ammonia-Lyase by L-Cysteine.

Treatment[a]	Activity	
	Units/ml	%
No addition (O_2)	20.2	100
No addition (N_2)	20.3	100
0.05 M L-cysteine (O_2)	3.7	18
0.05 M L-cysteine (N_2)	20.6	102

[a] The enzyme (1.6 mg/ml) was incubated at 25° in 0.1 M Tris-HCl buffer (pH 9.0)–0.1 M NaCl in a final volume of 50 μl with the additions shown in the table. Anaerobic incubations were done in narrow (0.4 cm diameter) short test tubes; the samples were flushed with N_2 and kept closed. Aerobic incubations were in wider test tubes (0.8 cm diameter) and kept open. After 2 hr activity was measured after appropriate dilution in the presence of 1 mM mercaptoethylamine. [The latter was included in order to reduce any formed mixed disulfides which cause a decreased activity (Klee, 1970).]

(see legend to Figure 3). It is unlikely that the small reactivity of the D stereoisomer is due to contamination by L-cysteine.[2]

In contrast to the lag period observed in the development of the increased absorption at 340 nm (Figures 3 and 4), the inactivation of the enzyme was immediate and linear (Figure 4). Thus, the change in absorption is a secondary reaction. Since the native enzyme contains a chromophoric group which absorbs near 315 nm (Klee, 1970), it is possible that the new chromophore at 340 nm is formed as a result of a modification of this group. The 315-nm chromophore appears to be unrelated to the tyrosine or tryptophan residues since the pH-dependent changes of this chromophore do not affect the absorption at 280 or 290 nm. The tryptophan and tyrosine contents, measured spectrophotometrically (Edelhoch, 1967), were identical for both forms

[2] The solutions of D- and L-cysteine (products of Calbiochem) were tested for SH content with 5,5′-dithiobis(2-nitrobenzoic acid), and the amino acid concentration was determined by amino acid analysis after performic acid treatment. The content of L-cysteine in the D-cysteine solutions was measured with L-amino acid oxidase and was less than 0.5%. I thank Dr. Edmund W. Hafner for performing the last measurement.

TABLE II: Effect of Sulfhydryl Compounds on the Activity of Histidine Ammonia-Lyase in the Presence and Absence of Substrate.[a]

Expt	Additions	Enzyme Activity[b] (Units/ml) 0 Time	120 Min	% Loss
1	None	4.9	5.4	0
2	L-Cysteine, 5 mM	4.7	<0.05	100
3	L-Cysteine, 5 mM, EDTA, 1 mM	4.5	<0.05	100
4	L-Cystine, 5 mM	5.4	5.3	2
5	β-Mercaptoethanol, 5 mM	5.0	4.7	6
6	Thioglycolate, 5 mM	4.7	5.7	0
7	Dithiothreitol, 5 mM	3.9	5.4	0
8	Mercaptoethylamine, 5 mM	3.8	3.3	13
		0 Time	10 Min	% Loss
9	L-Homocysteine, 9 mM[c]	16.8	4.9	70
10	L-Cysteine, 9 mM[c]	15.4	2.1	86
		0 Time	60 Min	% Loss
11	L-Cysteine, 1 mM[d]	1.7	0.4	76
12	L-Cysteine, 1 mM, L-histidine, 150 mM	1.7	1.6	5
13	L-Cysteine, 1 mM, D-histidine, 150 mM	1.9	0.5	74
14	L-Cysteine, 1 mM, urocanate, 150 mM	1.9	1.2	37

[a] The enzyme (0.45 mg/ml, experiments 1–8; 1.5 mg/ml, experiments 9 and 10; 0.15 mg/ml, experiments 11–14) was incubated at 25°, in the presence of air, in 0.05 M sodium carbonate–bicarbonate buffer, pH 10.2, in a final volume of 20 μl. Additions were as indicated in the table. The pH of all of the solutions was adjusted prior to addition to the incubation mixtures. [b] Enzyme activity was measured after the incubation times shown in the table with 2–3-μl aliquots in a standard assay mixture containing 1 mM mercaptoethylamine and is expressed as units of enzyme per ml of incubation mixture. [c] This experiment was carried out for only 10 min in view of the ease of oxidation of L-homocysteine. After this time the SH content of L-cysteine was 8 mM, that of homocysteine was 3.6 mM. Homocysteine thiolactone was used for this study and was converted to homocysteine by treatment with 1 M NaOH for 1 min prior to starting the experiment. Cysteine was treated in a similar fashion. [d] The inactivation in the presence of substrate or product was carried out at pH 9.8 instead of pH 10.2 in 0.05 M sodium carbonate–bicarbonate buffer.

of the enzyme and the spectra were not significantly different from that of an appropriate mixture of *N*-acetyltryptophanamide and *N*-acetyltyrosinamide in 80% methanol except for a peak at 310 nm for the native enzyme and 340 nm for the cysteine-treated enzyme. Chemical determinations after hydrolysis in 6 N HCl, 3.5% thioglycolate (Matsubara and Sasaki, 1969), or 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Liu, 1973) were also identical for both forms of the enzyme, but the tryptophan recoveries were too low (80%) to rule out a difference of less than 2–3 mol of tryptophan/mol of enzyme tetramer.

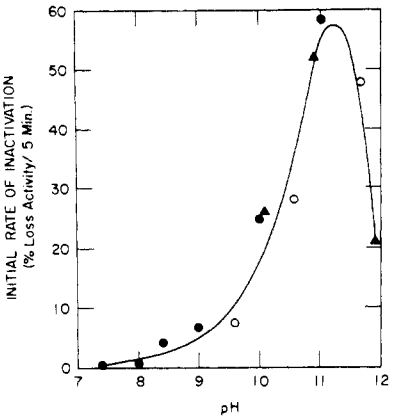

FIGURE 5: pH dependence of the rate of inactivation of histidine ammonia-lyase by L-cysteine. The enzyme (0.3 mg/ml) was incubated in 0.05 M Tris-HCl buffer (above pH 10.7, the pH was adjusted with NaOH), containing 0.02 M L-cysteine in a final volume of 50 μl in open test tubes. The different symbols represent different experiments. Activity of control samples incubated without cysteine was unchanged throughout the entire pH range. At various times 2-5-μl aliquots were assayed for activity in the presence of thiol as described in Materials and Methods. The initial rate of the loss of activity plotted on the ordinate was determined from these time courses and is expressed as the per cent loss of activity in 5 min of incubation with L-cysteine at the pH indicated in the abscissa.

Irreversible inactivation of the enzyme by L-cysteine requires the presence of O_2 (Klee, 1970). When the incubation with thiol was carried out in an atmosphere of N_2, no inactivation was observed (Table I). In the absence of L-cysteine, the enzyme was completely stable under both conditions. In large scale experiments, in which the exposed liquid surface is relatively small, the incubation mixture was continuously shaken to ensure adequate oxygenation. Incubation of the enzyme or cysteine separately with O_2 followed by mixing and further incubation of the mixture under anaerobic conditions did not result in any significant inactivation. Varying the enzyme concentration, incubation in the dark, addition of superoxide dismutase, or of other free radical scavengers (data not shown), or EDTA (Table II) had little, if any, effect on the inactivation. Catalase had a small but irreproducible protective effect.

As shown in Figure 5, the rate of inactivation is dependent on the pH of the reaction mixture. A maximum rate was observed at pH 11.0, which suggests that the reactive form of cysteine is the RS^- species. At this pH, complete inactivation of the enzyme could be achieved in less than 2–3 hr with concentrations of 1–5 mM L-cysteine. Above pH 11.0, a sharp decrease in rate of inactivation was observed, perhaps due to the instability of cysteine in alkali (Cecil and McPhee, 1959). In the absence of added thiol, the enzyme was completely stable for 1 hr at 25° at pH 11.8.

None of the other thiols tested (mercaptoethylamine, mercaptoethanol, dithiothreitol, or thioglycolate) or L-cystine (Table II) was able to replace L-cysteine, with the exception of L-homocysteine. The latter compound is difficult to study, however, because of its ease of oxidation at high pH or lactonization below pH 10. The strict specificity of the reaction for L-cysteine suggests that the reaction depends on the binding of the thiol to the active site of the enzyme and indeed urocanic acid or L-histidine, but not D-histidine, has a protective effect (Table II).

Binding of L-Cysteine to Histidine Ammonia-Lyase. To obtain direct evidence for the binding of L-cysteine to the enzyme, the inactivation was carried out with radioactive cysteine. [^{35}S]-L-Cysteine and [U-^{14}C]-L-cysteine gave essentially the same results. An experiment with [^{35}S]-L-cysteine is summarized in Table III. Inactivation was allowed to proceed for 15 min (sample II) (before any extensive spectral absorption change occurred) and to completion (sample III). The two samples were analyzed for ^{35}S content, enzymatic activity, and absorption at 340 nm after gel filtration in the presence of excess unlabeled thiol to remove excess reagents and any disulfide-linked cysteine. As described earlier, the 340-nm absorption change lags behind the loss of activity. Note that the latter loss closely parallels the incorporation of radioactive label. The reaction therefore occurs in at least two steps: the fast binding of cysteine with the resultant inactivation of the enzyme, followed by a slower secondary change resulting in the appearance of the new 340-nm peak. This secondary reaction does not require free cysteine; thus, when sample II (incubated for 15 min with cysteine and rapidly freed of excess cysteine by gel filtration) is incubated further at pH 10.5, its residual activity remains unchanged but its absorbance at 340 nm rises and

TABLE III: Correlation between Loss of Activity, Increased Absorption at 340 nm, and Incorporation of [^{35}S]Cysteine during Inactivation of Histidine Ammonia-Lyase.

	Incubation Time[a] (Min)	Activity Units/mg	$A_{340\ nm}$	Cysteine Incorporated nmole/nmole of Tetramer
I	0	15	0.042	
II	15	5 (67%)[b]	0.068 (14%)[c]	2.4 (69%)[a]
III	115	<0.2 (>98%)[b]	0.230 (100%)[c]	3.5 (100%)[d]

[a] The incubation of the enzyme with L-cysteine was as described in Materials and Methods. [^{35}S]-L-Cysteine had a specific activity of 760 cpm/nmol. Sample I was tested for activity and absorption at 340 nm prior to addition of L-cysteine. After 15 min of incubation, 1 ml of reaction mixture was made 0.01 M in dithiothreitol and freed of reagent by gel filtration as described in Materials and Methods (sample II). The remaining enzyme solution was incubated for an additional 100 min and treated in a similar fashion (sample III). Enzymatic activity, absorption at 340 nm, and incorporation of [^{35}S]cysteine were measured on the material eluted from the columns and dialyzed against 0.05 M potassium phosphate buffer (pH 7.5). [b] The numbers in parentheses represent the per cent loss of activity. [c] The numbers in parentheses represent the per cent of maximum increase at 340 nm. Since no further increase was observed after 115 min, this increase in absorption was taken as 100%. [d] The numbers in parentheses represent the per cent of maximum increase in incorporation.

levels off at 60% of the value observed with completely inactive enzyme. This 60% value correlates well with the 67% loss of activity and 69% of total cysteine incorporation.

Complete inactivation of the enzyme was accompanied by the binding of 3.5 mol of cysteine/mol of enzyme tetramer. This stoichiometry was obtained consistently with various preparations of [^{35}S]- and [^{14}C]-L-cysteine. Furthermore, when the modified enzyme was tested for its ability to incorporate ^{3}H upon treatment with Na[^{3}H]BH_4, a reagent believed to react with the active site of the enzyme (Givot *et al.*, 1969; Wickner, 1969), only 0.3 mol of ^{3}H was bound/mol of enzyme compared to 2.2 mol for the native enzyme. Conversely, enzyme previously inactivated by $NaBH_4$ did not undergo the spectral changes induced in the native enzyme by treatment with L-cysteine (data not shown).

As shown in Table IV, the bound cysteine was not released from the enzyme by treatment with thiols followed by gel filtration. Under these conditions the four reactive SH groups were reduced and titratable by Ellman's reagent; the modified enzyme was, however, completely inactive and maintained its absorption peak at 340 nm. After acid hydrolysis of the labeled enzyme, 82% of the radioactivity in the acid hydrolysate was recovered as cysteine under the proline peak in the amino acid analyzer [cysteine is known to elute with proline under these conditions (Moore and Stein, 1963)] and 18% with cystine (Table IV). No other radioactive peaks were detected, particularly at the known positions of the two lanthionine diastereoisomers which elute before and with glutamic acid under these conditions.

Exposure of the labeled enzyme to denaturing conditions

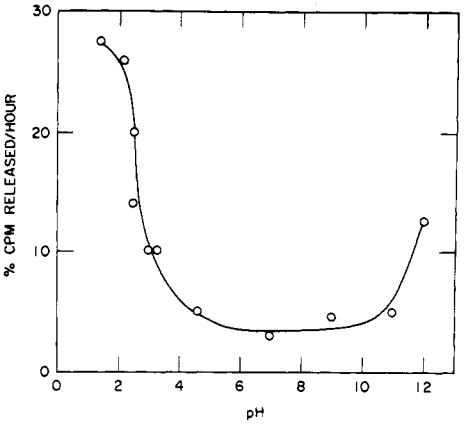

FIGURE 6: Effect of pH on the stability of L-cysteine adduct to histidine ammonia-lyase. ^{35}S-Labeled enzyme (0.4–0.7 mg/ml) was incubated for 4 hr at 25° in the following buffers: 0.13 M HCl, KCl buffer (pH 1.4); 0.05 M glycine HCl buffer (pH 2–3); 0.05 M sodium acetate buffer (pH 4.0); 0.05 M potassium phosphate (pH 7.0); 0.1 M Tris-HCl (pH 9.0); 0.05 M sodium carbonate–bicarbonate buffer (pH 10.5); and 0.1 N NaOH (pH 13). The release of radioactivity from 40- to 70-μg aliquots was measured at several time intervals for each sample as described in Materials and Methods. The release of counts exhibited pseudo-first-order kinetics over the time course measured. The percentage of total counts released after 1 hr is plotted against pH.

such as pH below 3 (Figure 6 and Table IV), pH above 12 (Figure 6), 6 M guanidine hydrochloride (Table IV), or 1% sodium dodecyl sulfate and mercaptoethanol (data not shown) was followed by a dissociation of the radioactive label (70–80%) without any change in the 340-nm absorption. However, after reduction in 8 M urea and carboxy-

TABLE IV: Identification of Labeled Material Released from Enzyme–L-Cysteine Adduct under Denaturing Conditions and Acid Hydrolysis.[a]

Expt. No.	Treatment	Labeled Derivatives Recovered after Acid Hydrolysis	Labeled Derivatives Released after Denaturing Conditions
1	None	Cysteine (82%) Cystine (18%)	
2	0.01 M Dithiothreitol[b]		Cysteine (5%)
3	6 M Guanidine·hydrochloride[b]		
	0.01 M Dithiothreitol		Cysteine (78%)
4	Acid treatment[c]		Cysteine (78%)
5	Carboxymethylation	Cysteine (63%) Cystine (8%) Carboxymethylcysteine (29%)	Carboxymethylcysteine (6–15%)
6	Performic acid oxidation	Cysteic acid (100%)	Cysteic acid (54%)

[a] The ^{35}S-labeled enzyme prepared as described in Materials and Methods was subjected to the treatments indicated in the table. An aliquot of the labeled enzyme was then acid hydrolyzed for 20–24 hr without prior removal of released radioactivity. The labeled derivatives in the hydrolysate were identified with an automatic amino acid analyzer. The radioactivity was measured in the fractions collected after ninhydrin reaction. The recovery of radioactivity was between 65 and 85%. The material eluting at the position of proline was taken as cysteine (Moore and Stein, 1963). Alternatively, the radioactive material released by the various treatments was separated from the enzyme by passage through a column of Sephadex G-25 (1 × 30 cm), equilibrated with 0.02 M Tris-HCl (pH 8) containing 1 mM EDTA and 0.1 M NaCl (experiments 2 and 3), 0.2 M acetic acid (experiment 4), 50 mM NH_4HCO_3 (experiment 5), or by precipitation with 6% trichloroacetic acid (experiment 6). The released radioactive material (in experiments 2 and 3 the salts were removed by chromatography on Bio-Rad AG 11A8 (50–100 mesh) was lyophilized and identified by thin-layer chromatography with three different solvents after mixing with [^{3}H]cystine and excess cysteine at pH 8 (experiments 2–4), with carboxymethylcysteine (experiment 5), and with [^{3}H]cysteic acid (experiment 6). [b] The ^{35}S-labeled enzyme (0.4 mg/ml) was incubated for 1 hr at 25° in 0.06 M Tris-HCl (pH 9.0) in the presence of dithiotheitol with and without guanidine hydrochloride as indicated. [c] The ^{35}S labeled enzyme (1.4 mg/ml) was brought to pH 1.9 with 5 N HCl and maintained at this pH for 4 hr at 25°.

methylation (Sela *et al.*, 1959) (this treatment did not yield a complete denaturation of the enzyme), no more than 15% of the labeled cysteine was dissociated from the enzyme as *S*-carboxymethylcysteine (Table IV), and when the carboxymethylated enzyme was subjected to acid hydrolysis under strict anaerobiosis, as shown in Table IV, 63% of the radioactivity was recovered as cysteine in the amino acid analyzer. Performic acid oxidation converted all of the radioactive material to cysteic acid, as determined by thin-layer chromatography in three different solvents and amino acid analyses after acid hydrolysis, but only 50% of it was found to be dissociated from the enzyme before acid hydrolysis.

Discussion

The observations presented in this paper indicate that L-cysteine has a double specificity for the active center of the enzyme. Its structural analogy with the substrate which results in stereospecific binding and its ability to interact with the active center residue [which is perhaps related to its reactivity toward carbonyl groups (Schubert, 1937; Rather and Clarke, 1937)] make it a very powerful and specific active site reagent for this enzyme. Such a double specificity of cysteine has been described previously for some pyridoxal phosphate enzymes (Jakoby and Bonner, 1953; Hoare and Snell, 1957; Shaltiel *et al.*, 1969).

The strict requirement for L-cysteine as opposed to D-cysteine shows that stereospecific binding is a prerequisite for inactivation. Complete inactivation is accomplished by the binding of 1 mol of cysteine/mol of enzyme subunit. The competitive inhibition observed with L-cysteine and the protection against inactivation by the substrate L-histidine and urocanic acid but not by D-histidine suggest that cysteine binds at the active site of the enzyme. The cysteine binding data, therefore, suggest the presence of four active sites/mol of enzyme tetramer. It has previously been shown that the four subunits are otherwise identical (Soutar and Hassall, 1969; Klee and Gladner, 1972). Irreversibility of the inactivation by cysteine may be associated with the reaction leading to the spectral changes observed following the binding of the reagent. However, since cysteine removal requires strong denaturing conditions, prior irreversible step cannot be ruled out as yet.

The bound cysteine can be removed only after disruption of the native structure of the enzyme by exposure to extremes of pH or to high concentrations of guanidinium chloride. One way in which this could happen is to have the cysteine buried in the interior of the protein, perhaps between subunits.

The roles of both oxygen and high pH in the inactivation process are unclear. The formation of disulfides or of sulfoxide products would be reversed by reducing agents. Furthermore, if the cysteine were oxidized it would not be recovered as such after acid hydrolysis. On the other hand, exposure of the enzyme to oxygen in the absence of cysteine does not eliminate the oxygen requirement for the subsequent inactivation. Apparently an oxidative step is an integral part of the mechanism of the cysteine–enzyme interaction. Perhaps cysteine inactivation requires the presence of an available carbonyl function so as to allow the formation of a thiazolidine-like derivative.[3]

Carbonyl groups have been postulated earlier on the basis of inactivation by carbonyl reagents (Smith *et al.*, 1967). However, in contrast to some other enzymes, where the presence of a keto acid prosthetic group has been clearly demonstrated (Riley and Snell, 1968; Hodgins and Abeles, 1967, 1969; George and Phillips, 1970; Wickner *et al.*, 1970; Lynch and Phillips, 1972), no carbonyl group has been identified in histidine ammonia-lyase, despite many studies showing inactivation with a variety of carbonyl reagents (Givot *et al.*, 1969; Wickner, 1969; Frankfater and Fridovich, 1970)[4]. Furthermore, the presence of dehydroalanine in the active center has been postulated since [^{3}H]alanine is recovered after reduction with Na^3HBH_4, but less than in the expected yield (Wickner, 1969).[4] Reaction of dehydroalanine with cysteine should lead to lanthionine (Gross *et al.*, 1969). Our experiments show that, under our conditions, no lanthionine is formed. The lability of the bound ^{3}H after borohydride reduction (Givot *et al.*, 1969) and the products obtained after inactivation by nitromethane led Givot *et al.* to postulate a more complex structure at the active site. Dehydroalanine, it was proposed, is attached through its amino group to a carbonyl in a Schiff base. Possibly at high pH and in the presence of O_2, this carbonyl is regenerated and thus made available for reaction with cysteine.[5] Since inactivation of the enzyme by cysteine prevents ^{3}H incorporation from Na^3HBH_4, it is likely that both reagents interact with similar or closely related residues. Since phenylalanine ammonia-lyase and threonine dehydratase have been shown to present very similar characteristics (Havir and Hanson, 1968,1973; Hanson and Havir, 1970; Hodgins, 1971; Parkhurst and Hodgins, 1972; Cohn and Phillips, 1974), it would be interesting to test cysteine as an inactivator of these enzymes.

Glycogen phosphorylase reacts with cysteine in a manner which is strikingly similar to that of histidine ammonia-lyase. In the case of phosphorylase, as with histidine ammonia-lyase, L- but not D-cysteine reacts to form a complex containing 1 molecule of bound cysteine/subunit (Shaltiel *et al.*, 1969). The bound cysteine is believed to form, in the case of phosphorylase, a thiazolidine with pyridoxal phosphate which is apparently buried within the protein at subunit interfaces (Hedrick *et al.*, 1969). Similarly also to histidine ammonia-lyase, the bound cysteine is removed from the native enzyme only with difficulty. Thus although the two kinds of reaction with cysteine are not identical, they show a number of common features. The major differences may be related to the need, in the case of histidine ammonia-lyase, to generate a free carbonyl function with which the cysteine can interact. Further work will be necessary to confirm this hypothesis and elucidate the nature of the postulated carbonyl function of histidine ammonia-lyase.

Acknowledgments

I thank Mr. Lawrence A. LaJohn for his skillful technical assistance and Mr. George Poy, who kindly performed the amino acid analyses.

[3] Treatment of the enzyme with [^{14}C]phenylhydrazine resulted in irreversible inactivation and incorporation of 2.3 mol of reagent/mol of enzyme tetramer. Inactivation of the reduced enzyme with [^{35}S]sulfite was accompanied by incorporation of 2.2–3.0 mol of sulfur/mol of enzyme (1.2–1.5 mol were recovered as cysteic acid after acid hydrolysis) (C. B. Klee, unpublished observations).

[4] The yield of [^{3}H]alanine never exceeded 1.5 mol/mol of enzyme tetramer under various pH conditions; 50% of the ^{3}H incorporated exchanged with H_2O during acid hydrolysis. Even greater exchange (75–80%) was observed after exposure to denaturants prior to acid hydrolysis (unpublished observations).

[5] The rate of inactivation with phenylhydrazine was also dependent on high pH and decreased under anaerobic conditions (unpublished observations).

References

Cecil, R., and McPhee, J. R. (1959), *Advan. Protein Chem. 14,* 256.

Cohn, M. S., and Phillips, A. T. (1974), *Biochemistry 13,* 1208.

Cornell, N. W., and Lien, L. L. (1970), *Physiol. Chem. Phys. 2,* 523.

Edelhoch, H. (1967), *Biochemistry 6,* 1948.

Ellman, G. L. (1970), *Arch. Biochem. Biophys. 82,* 70.

Frankfater, A., and Fridovich, I. (1970), *Biochim. Biophys. Acta 206,* 457.

George, D. J., and Phillips, A. T. (1970), *J. Biol. Chem. 245,* 528.

Givot, I. L., Smith, T. A., and Abeles, R. H. (1969), *J. Biol. Chem. 244,* 6341.

Gomori, G. (1955), *Methods Enzymol. 1,* 138.

Gross, E., Morell, J. L., and Craig, L. C. (1969), *Proc. Nat. Acad. U. S. 62,* 952.

Hanson, K. R., and Havir, E. A. (1970), *Arch. Biochem. Biophys. 141,* 1.

Hanson, K. R., and Havir, E. A. (1972), *Enzymes, 3rd Ed. 7,* 75.

Havir, E. A., and Hanson, K. R. (1968), *Biochemistry 7,* 1904.

Havir, E. A., and Hanson, K. R. (1973), *Biochemistry 12,* 1583.

Hedrick, J. L., Shaltiel, S., and Fisher, E. H. (1969), *Biochemistry 8,* 2422.

Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 197.

Hoare, D. S., and Snell, E. E. (1957), *in* International Symposium on Enzyme Chemistry, Tokyo, Mimeograph Report No. 110.

Hodgins, D. S. (1971), *J. Biol. Chem. 246,* 2977.

Hodgins, D. S., and Abeles, R. R. (1967), *J. Biol. Chem. 242,* 5158.

Hodgins, D. S., and Abeles, R. H. (1969), *Arch. Biochem. Biophys. 130,* 274.

Jakoby, W. B., and Bonner, D. M. (1953), *J. Biol. Chem. 205,* 709.

Klee, C. B. (1970), *J. Biol. Chem. 245,* 3143.

Klee, C. B. (1972), *J. Biol. Chem. 247,* 1398.

Klee, C. B., and Gladner, J. A. (1972), *J. Biol. Chem. 247,* 8051.

Liu, T. Y. (1973), 163rd National Meeting of the American Chemical Society Boston, Mass., Abstract AGFD-013.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Lynch, M. C., and Phillips, A. T. (1972), *J. Biol. Chem. 247,* 7799.

Matsubara, H., and Sasaki, R. M. (1969) *Biochem. Biophys. Res. Commun. 35,* 175.

McCord, Y. M., and Fridovich, I. (1969), *J. Biol. Chem. 244,* 6049.

Mehler, A. H., and Tabor, H. (1953), *J. Biol. Chem. 201,* 775.

Mildvan, A. S. (1970), *Enzymes, 3rd Ed. 2,* 445.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.

Parkhurst, J. R., and Hodgins, D. S. (1972), *Arch. Biochem. Biophys. 152,* 597.

Patterson, M. S., and Greene, R. C. (1965), *Anal. Chem. 37,* 854.

Peterkofsky, A., and Mehler, L. N. (1963), *Biochim. Biophys. Acta 73,* 159.

Rather, S., and Clarke, H. T. (1937), *J. Amer. Chem. Soc. 59,* 200.

Rechler, M. M. (1969), *J. Biol. Chem. 244,* 551.

Riley, W. D., and Snell, E. E. (1968), *Biochemistry 7,* 3520.

Sela, M., White, F. H., Jr., and Anfinsen, C. B. (1959), *Biochim. Biophys. Acta 31,* 417.

Shaltiel, S., Hedrick, J. L., and Fisher, E. H. (1969), *Biochemistry 8,* 2429.

Schubert, M. P. (1937), *J. Biol. Chem. 121,* 539.

Smith, T. A., Cordelle, F. H., and Abeles, R. H. (1967), *Arch. Biochem. Biophys. 120,* 724.

Soutar, A. K., and Hassall, H. (1969), *Biochem. J. 114,* 79P.

Tabor, H., and Mehler, A. H. (1955), *Methods Enzymol. 2,* 228.

Wickner, R. B. (1969), *J. Biol. Chem. 244,* 6550.

Wickner, R. B., Tabor, C. W., and Tabor, H. (1970), *J. Biol. Chem. 245,* 2132.