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THE PURIFICATION AND PROPERTIES OF OXIDIZED DERIVATIVES OF L-HISTIDINE AMMONIA-LYASE*

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SUMMARY

1. L-Histidine ammonia-lyase (L-histidine ammonia-lyase, EC 4.3.1.3) purified from *Pseudomonas fluorescens* is unstable in air. Treatment of the enzyme with 5,5'-dithio-bis-(2-nitrobenzoic acid) produces a stable derivative which retains a fraction of the original activity and which may be restored to full activity by treatment with dithiothreitol. The oxidation of histidine ammonia-lyase by O_2 and by H_2O_2 has also been investigated.

2. The kinetic behavior of 5,5'-dithio-bis-(2-nitrobenzoic acid)-oxidized histidine ammonia-lyase (TNB-histidine ammonia lyase) and its response to photosensitized oxidation and to thermal inactivation were all consistent with the existence of two classes of active sites.

3. EDTA was a competitive inhibitor of reduced histidine ammonia-lyase but had no effect on TNB-histidine ammonia-lyase. The maximum inhibition which could be achieved with EDTA was close to 90%. The interaction of EDTA and the reduced enzyme was rapid in the absence of L-histidine but was perceptably slow in its presence.

4. The time-course of the inhibition of histidine ammonia-lyase by borohydride was investigated under conditions which achieved a steady-state level of borohydride in the reaction mixture. The effects of pH and of a variety of small molecules on the borohydride-induced inactivation of the enzyme were explored. These data appear to be consistent with the proposal of an azomethine function at the active site.

INTRODUCTION

L-Histidine ammonia-lyase (L-histidine ammonia-lyase, EC 4.3.1.3), catalyzes the elimination of ammonia from L-histidine yielding the *trans* isomer of urocanate[1].

Abbreviations: DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); TNB-histidine ammonia-lyase, L-histidine ammonia-lyase oxidized by treatment with 5,5'-dithio-bis-(2-nitrobenzoic acid).

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Evidence has been obtained which suggests that an enzyme–ammonia intermediate is involved in the catalytic process and that its decomposition is the rate-limiting step[2]. The inhibition of histidine ammonia-lyase by a variety of carbonyl reagents and by treatment with borohydride has led to the proposal of the involvement of a carbonyl group at the active site[3]. Tritium-labelled borohydride introduced tritium onto the enzyme[3–5]. Tritiated alanine has been isolated from acid digests of enzyme so labelled[6]. This result is in accord with the proposal that the active site contains a residue of dehydroalanine[5,6]. Similar data have been reported which support the existence of dehydroalanine at the active site of phenylalanine deaminase[7]. The inhibition of histidine ammonia-lyase by sulfhydryl compounds[4,8–10], mercurials[4] and by EDTA (refs. 4, 8–10) has been reported. The amino acid composition, the physical parameters and some of the kinetic properties of the enzyme derived from a Pseudomonas species have recently been defined[4]. Crude extracts of this enzyme have repeatedly been observed to lose activity upon aging or purification and these losses have been found to be variably reversible by treatment with mercaptans[2–4,9,11].

We have attempted to avoid problems associated with the instability of histidine ammonia-lyase by converting it to a stable oxidized product which could then be purified, stored and converted back into a fully active form, at will, by subsequent reduction. In the course of these studies several interesting properties of the oxidized derivatives and of the reduced enzyme have come to light. It is the purpose of this report to describe and to document these aspects of the behavioor of histidine ammonia-lyase.

MATERIALS AND METHODS

All reagents were obtained from commercial sources, were the highest purity available and were used without further purification. Conductivity was measured with a radiometer CDM-2d. Measurements of absorbancy were made with either a Gilford Model 2000 or a Cary Model 15 spectrophotometer. Temperatures were controlled by the use of thermostated cell blocks. *Pseudomonas fluorescens* ATCC 11250 was obtained from the American Type Culture Collection. We were unable to obtain stable pure cultures directly from the lyophilized material supplied. This problem was circumvented by inoculating minimal media agar plates with cell suspensions cultured from the lyophilized material and subsequently isolating single clones which fluoresced pale green under ultraviolet light. *P. fluorescens* isolated in this way, was maintained in pure culture for several years without difficulty. The purity of the cultures was judged by examination of the cells under a phase contrast microscope and by the consistent production of a pale green fluorescent material by these cells. Several modifications of the culture conditions described by TABOR AND MEHLER[11] permitted a 3–5-fold increase in the yield of cells without significant sacrifice of the histidine ammonia-lyase activity of cell free extracts. Thus, L-histidine was replaced by a doubled quantity of DL-histidine, the concentration of bacto-yeast extract was doubled, and 13 ml of glycerol were added per l of medium, to serve as a carbon source.

Histidine ammonia-lyase activity was measured in terms of the rate of formation of urocanate as indicated by increased absorbancy at 277 mμ (ref. 9). Oxidized histidine ammonia-lyase was routinely assayed at pH 9.2 and at 25° in solutions con-

taining 0.10 M sodium pyrophosphate and 0.10 M L-histidine. Reduced histidine ammonia-lyase was prepared from oxidized histidine ammonia-lyase by incubating it with 0.010–0.025 M dithiothreitol at 25° for 50 min in a solution buffered at pH 7.8 by 0.10 M Tris–HCl. The reduced enzyme, thus generated, was stored in an ice bath where it was stable for several hours. Aliquots of this reduced enzyme were then added to the assay cuvettes such that the final concentration of dithiothreitol never exceeded 0.1 mM. Under these conditions the reduced enzyme exhibited a brief lag followed by a linear rate. This lag phase was lengthened when the concentration of dithiothreitol in the reduction mixture was raised and was diminished by increasing the concentration of L-histidine in the reaction cuvette. This lag was probably related to the inhibitory effect of high concentrations of dithiothreitol and to the measurably slow reversal of this inhibition upon dilution into the reaction cuvette. In any case, the linear phase was of long duration and was easily measured. One unit of histidine ammonia-lyase has been defined[11] as that amount causing an absorbance change of 0.001 per min under specific conditions of assay and is so defined in this report.

RESULTS

Purification of the enzyme

40 g of a frozen paste of *P. fluorescens* was suspended in 160 ml of 1 mM Tris–HCl at pH 7.8 and was treated for 15 min at 5° with a 20-kcycle Branson Sonic Oscillator at a power setting of 8. The sonicate was clarified by centrifugation at 40 000 × *g* for 30 min at 3°. To the supernatant solution 50 ml of 10% streptomycin sulfate was added. The precipitated nucleic acids were removed by centrifugation and the supernate brought to 80° and held at this temperature for 15 min, following which, it was cooled rapidly and was clarified by centrifugation. The pH was adjusted to 8.8 and 50 ml of 0.01 M 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) at pH 7.5 were added rapidly under continuous stirring. The pH was then readjusted to 8.8 and the mixture incubated at 25° for 30 min, after which the pH was lowered to 6.0. Ethanol fractionation was then performed at 20° and with the pH maintained at 6.0. The precipitate which formed between 24 and 44% (v/v) ethanol was collected by centrifugation and suspended in a minimal volume of 0.10 M Tris–HCl at pH 7.8. This suspension was clarified by centrifugation and the slightly turbid supernate applied directly to a 4.2 cm × 34 cm column of Bio-Gel agarose 0.5 M which had been equilibrated at 4° with 0.10 M Tris–HCl buffer which had a pH of 7.5 at 25°. A flow rate of 10.0 ml/cm^2 per h was applied and those fractions (5.0 ml each) which had specific activities exceeding 2000 were pooled and placed directly on a 2.2 cm × 20 cm column of DEAE-Sephadex A-25 which had been equilibrated at 4° with 0.10 M Tris–HCl buffer which had a pH of 7.8 at 25°. Several column volumes of this buffer were passed through the column, followed by successive elutions with 2.5 column volumes of this buffer containing first 0.135 M KCl, then 0.185 M KCl and finally 0.25 M KCl. The results of these chromatographic procedures are illustrated in Fig. 1. As is evident in Fig. 1B, this procedure yielded three peaks of histidine ammonia-lyase activity. These are referred to as TNB-histidine ammonia-lyase I, II and III and their ratios of reduced to oxidized activities were 8.0, 15.5 and 18.0, respectively. TNB-histidine ammonia-lyase II represented 65% of the total activity and its specific activity ranged from 35 000 to 38 800. Peak tubes containing TNB-histidine ammonia-lyase II

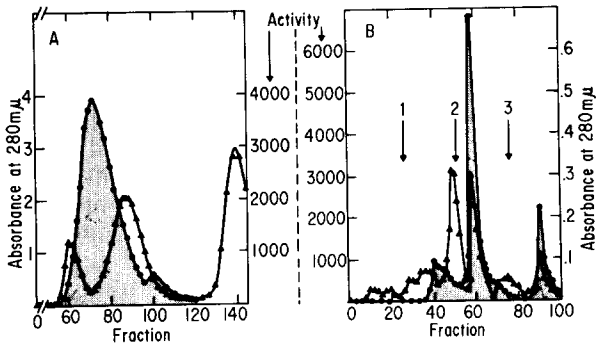


Fig. 1. A. Gel-exclusion chromatography of DTNB-treated histidine ammonia-lyase. 25 ml of TNB-histidine ammonia-lyase, processed through the ethanol fractionation step, was passed through a 4.2 cm × 34 cm column of Agarose 0.5 M in 0.10 M Tris–HCl at 4° and at pH 7.8. Fractions (5.0 ml) were assayed for histidine ammonia-lyase activity (●—●) and for absorbance at 280 mμ (▲—▲). The stippled area represents histidine ammonia-lyase activity. B. Ion-exchange chromatography of TNB-histidine ammonia-lyase. Fractions off of the agarose column whose specific activities exceeded 2000 were pooled and were absorbed onto a 2.2 cm × 20 cm column of Sephadex A-25 equilibrated at 4° with 0.10 M Tris–HCl at pH 7.8. This column was then eluted with this buffer to which the following was added: 0.135 M NaCl at Arrow 1, 0.185 M NaCl at Arrow 2 and 0.250 M NaCl at Arrow 3. Fractions (5.0 ml) were assayed for histidine ammonia-lyase activity and for absorbance at 280 mμ. The stippled area represents histidine ammonia-lyase activity.

had specific activities of 42 000. It must be noted that reduction raised these specific activities by a factor of 15.5. The specific activity of TNB-histidine ammonia-lyase II after reduction was therefore similar to the specific activity achieved by WILLIAMS AND HIROMS[12] and GIVOT *et al.*[5] and was greater, by a factor of 10, than the specific activity reported by RECHLER[4]. The results of this purification procedure are summarized in Table I.

TABLE I

RESULTS OF THE PURIFICATION OF HISTIDINE AMMONIA-LYASE

The results of the procedure described in the text are here tabulated. Specific activities are computed in all cases as oxidized activities, that is as activities after DTNB treatment.

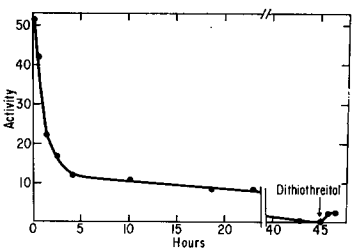
Step	*Total activity* ($A_{277\ m\mu} \times 10^3$/*min*)	*Total protein* (*mg*)	*Specific* activity* ($A_{277\ m\mu} \times 10^3$/*min per mg*)	*Purification** (*-fold*)	*Recovery* (*%*)
Crude sonicate	$8.2 \cdot 10^6$	3800	140	1.0	—
10% streptomycin sulfate	$8.2 \cdot 10^6$	3300	160	1.1	100
Heat at 80° for 15 min	$7.2 \cdot 10^6$	800	720	5	88
DTNB oxidation	$5.8 \cdot 10^5$	800	720	5	88
24 and 44% ethanol fractionation	$2.8 \cdot 10^5$	164	1 000	14	51
Bio-Gel A 0.5 M agarose column	$1.53 \cdot 10^5$	15.7	10 000	70	28
DEAE-Sephadex column	$1.1 \cdot 10^5$	3.1	35 500	250	20

* Computed as oxidized activity.

Oxidation and reduction of histidine ammonia-lyase

Histidine ammonia-lyase which had been fully activated by reduction with thiol compounds was found to be markedly unstable when exposed to air in the absence of the thiol reductant. Thus, TNB-histidine ammonia-lyase II was incubated with 0.025 M dithiothreitol in 0.10 M phosphate buffer at pH 6.8 and at 25° for 40 min. It was then separated from the thiol compound by passage through a 1 cm × 12 cm column of Sephadex G-50. This was done in the absence of O_2 by equilibrating the column with 0.10 M phosphate buffer at pH 6.8 which had been swept free of O_2 by a stream of N_2. The enzyme sample, its density increased by admixture of 10% glycerol, was placed on the column through an overlying layer of anaerobic buffer. Any O_2 which might have diffused into the overlying buffer during this operation would have been kept from contact with the reduced enzyme because the enzyme would progress down the column more rapidly than the O_2. This reduced enzyme gave rates of urocanate production which were linear within a few seconds of mixing of enzyme and L-histidine. Exposure of this reduced enzyme to air caused a loss of activity which, as is illustrated in Fig. 2, was biphasic, ultimately total and not significantly reversed by treatment with dithiothreitol. In contrast, oxidation of the histidine ammonia-lyase in crude extracts, by exposure to air, caused a loss of activity which was reversed by subsequent reduction. The difference in these two situations probably relates to the presence of sulfhydryl compounds in the crude extracts which could, during the course of oxidation, form mixed disulfides with groups on the enzyme.

When relatively crude preparations of histidine ammonia-lyase, which had been subjected to the streptomycin and heat treatment steps of the purification procedure, were treated with H_2O_2 a rapid decrease of activity ensued. The histidine ammonia-lyase derivative thus generated retained 8% of the initial activity, was relatively stable in air and could be restored to the initial activity, by reduction with H_2S. Fig. 3 illustrates these results. Whereas DTNB-oxidized derivatives of histidine ammonia-lyase retained the ability of being restored to full activity upon subsequent



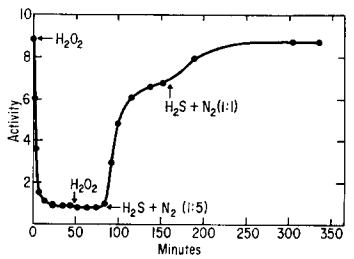


Fig. 2. Aerobic inactivation of reduced histidine ammonia-lyase. Reduced histidine ammonia-lyase was separated from supporting mercaptans by gel-exclusion chromatography, as described in the text. An aliquot (1.0 ml) containing 24 700 units of activity and 48 μg of protein was exposed to air at 25° and was assayed at intervals. A parafilm seal was used to minimize evaporation. At the arrow dithiothreitol was added to a final concentration of 0.025 M.

Fig. 3. Oxidation and reduction of histidine ammonia-lyase. 1.0 ml of an extract of *P. fluorescens* which had been processed through the streptomycin and heat treatment steps of the purification procedure was treated with 1.0 μmole of H_2O_2 at "zero time". At 48 min an additional μmole of H_2O_2 was added. At 80 min an atmosphere of 20% H_2S, 80% N_2 was applied. At 160 min the composition of the atmosphere was changed to 50% H_2S, 50% N_2. Aliquots (10 μl) were removed at intervals for histidine ammonia-lyase assay.

reduction with dithiothreitol, the H_2O_2-oxidized enzyme gradually lost this capacity over a period of several weeks at 4°.

Kinetic behavior

TNB-histidine ammonia-lyase II did not follow the expectations of classical Michaelis–Menten kinetics. Reciprocal plots of initial velocities as a function of substrate concentration were not linear, and guanidine, which is known to inhibit histidine ammonia-lyase[13], had the effect of increasing the nonlinearities observed. These effects are illustrated in Fig. 4. Similar nonlinear reciprocal plots were seen with TNB-histidine ammonia-lyases I and III and with H_2O_2-oxidized histidine ammonia-lyase. In contrast, and in agreement with RECHLER[4], histidine ammonia-lyase which

$$\frac{1}{v} = \frac{\frac{K_1K_2}{[S]^2} + \frac{K_1 + K_2}{[S]} + 1}{V_1\left(\frac{K_2}{[S]} + 1\right) + V_2\left(\frac{K_1}{[S]} + 1\right)} \tag{1}$$

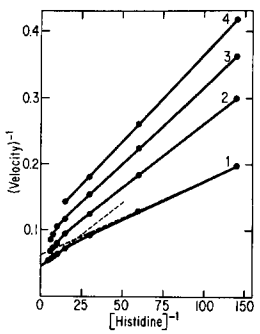
$$\frac{1}{v} = \frac{\frac{K_1K_2}{[S]^2} + \frac{2K_2}{[S]} + 1}{V_1\left(\frac{2K_2}{[S]}\right) + 2V_2} \tag{2}$$

had been reduced by incubation with dithiothreitol, exhibited the classical linear behavior over a wide range of substrate concentrations. The points shown in Fig. 4 are empirical, whereas Line 1 is a theoretical line calculated on the basis of Eqns. 1 and 2 with values for K_1 and K_2 of 15 and 70 mM in the case of Eqn. 1 and of 24.8 and 42.7 mM for the case of Eqn. 2. Eqns. 1 and 2 are similar to those derived by KISTIAKOWSKY AND ROSENBERG[14] to describe the kinetic behavior of urease. Eqn. 1 is based on a model which assumes two types of independent active sites with differing affinities for substrate and a state of quasi-equilibrium. K_1 and K_2 may be Michaelis constants. Eqn. 2 is based on the assumption of pairs of identical but interacting sites where the substrate affinity of one member of each pair is influenced by the state of occupancy of the second member of the pair.

When the data shown in Fig. 4 were plotted as a function of guanidinium concentration on logarithmic coordinates[15,16] a family of gently curved lines were obtained which exhibited slopes of less than 1.0 over the range of guanidinium investigated and which approached slopes of 1.0 only at very low concentrations of this inhibitor. This could be explained by models which propose two populations of sites which differ only with respect to affinity for the inhibitor or pairs of interacting sites in which case occupancy of one site diminishes affinity at the other.

Effects of pH

It is clear from the data on Line 1 of Fig. 4 that kinetic data taken at substrate concentrations below 0.05 M would fit a straight line and that apparent values of K_m and V could then be obtained from lines representing such a restricted range of substrate concentration. The effects of pH on these kinetic constants could then be studied. This was done and the results so obtained are illustrated in Figs. 5A and 5B.



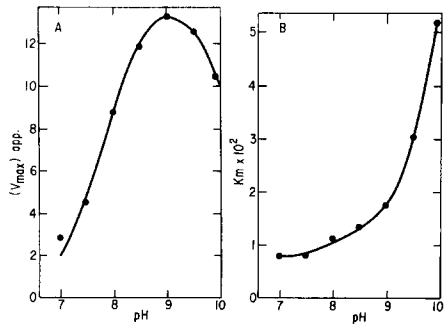


Fig. 4. Kinetic behavior of TNB-histidine ammonia-lyase II. The activity of TNB-histidine ammonia-lyase II is here expressed as a function of the concentration of L-histidine. Reaction mixtures contained 0.36 μg/ml of enzyme and the indicated concentrations of L-histidine in 0.10 M sodium pyrophosphate buffer at 25° and pH 9.2. The points on Line 1 were obtained in the absence of guanidinium whereas the points on Lines 2, 3 and 4 were obtained in the presence of 0.008, 0.020 and 0.040 M guanidinium, respectively. Velocity is given in units of absorbance increase at 277 mμ per min times 1000. Line 1 is a theoretical line drawn with the aid of Eqns. 1 and 2 in the text. The dashed lines represent limiting slopes.

Fig. 5. The effect of pH on apparent V and on K_m. Kinetic studies were performed at 25° in 0.10 M sodium pyrophosphate buffer at the indicated pH. Reaction mixtures contained 0.36 μg/ml of TNB-histidine ammonia-lyase II. Velocity is expressed in units of absorbance increase at 277 mμ per min times 1000. In A the points represent extrapolated maximum velocities whereas the line was drawn from the second Michaelis function using $pK_1 = 7.9$ and $pK_2 = 10.2$.

Fig. 5A presents apparent V as a function of pH. The points are empirical whereas the line was calculated from the expression:

$$V_{app} = \frac{V}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}}$$

V is the maximum velocity attainable were all of the enzyme in the active ionized form, and the denominator term is the second Michaelis function[17] for a substance which can undergo two ionizations. V was assumed to be 15.5 and pK_1 and pK_2 were assumed to be 7.9 and 10.2, respectively. The effect of pH on K_m is shown in Fig. 5B. In view of the possible effects of neighboring charged groups on the dissociation constants of ionizable groups on a protein and in the absence of additional pertinent information it would be impossible to reliably deduce the chemical nature of these activity-limiting ionizable groups from their effective pK_a's.

Inactivation of histidine ammonia-lyase by borohydride

It appeared useful to study the effects of a variety of agents on the sensitivity of histidine ammonia-lyase towards borohydride. A steady-state concentration of borohydride would facilitate comparisons and this was achieved by a continuous infusion of borohydride as already described[18]. Reaction mixtures contained 20 μg of TNB-histidine ammonia-lyase II in 0.5 ml of 0.10 M sodium pyrophosphate buffer at the specified pH and temperature. The rate of infusion of borohydride was 2.56 μl/min. For the experiments at pH 8.2, the infusate was 0.05 M borohydride in 0.08 M NaOH; whereas, for experiments at pH 6.5, it was 0.005 M borohydride in 0.10 M

NaOH. In the latter situation 5-μl aliquots of 0.05 M H_2SO_4 were added at 2-min intervals to counter the pH change otherwise caused by the alkaline infusate. The reaction mixture was stirred continuously and 25-μl aliquots were removed at intervals for assay. Under these conditions a balance is quickly arrived at between the rate of introduction of borohydride by infusion and those reactions, such as hydrolysis, which eliminate it from the reaction mixtures[18,19]. This results in a steady-state level of borohydride in the reaction mixtures. It was observed that this procedure caused a transient activation of the TNB-histidine ammonia-lyase II followed by a rapid inactivation. The activation phase was associated with the reductive activation of the TNB–enzyme analogous to the process which occurred when TNB-histidine ammonia-lyase was activated by reduction with dithiothreitol. Since it was desired to study the reductive inactivation caused by borohydride, unobscured by the reductive activation, the 25-μl aliquots removed from these reaction mixtures were routinely diluted into 2.5 ml of 0.833 mM H_2O_2 in 0.10 M sodium pyrophosphate buffer at pH 9.2 and incubated at 25° for 30 min before assaying for residual histidine ammonia-lyase activity. This treatment with H_2O_2 had no effect upon the residual activity of TNB-histidine ammonia-lyase but did serve to reverse any reductive activation which might have occurred. Since the rate of infusion of borohydride was known and since the rate constants for the acid-catalyzed hydrolysis of borohydride have been published[19] it was possible to calculate that the steady-state level of borohydride achieved was 0.85 mM at pH 8.2 and was 1.67 μM at pH 6.5.

Fig. 6 illustrates some of the results of this technique applied at pH 8.2. Line 1 was a control for possible effects of the hydrolysis product of borohydride and was obtained by infusing sodium borate in place of sodium borohydride, all other conditions being identical. Curve 2 illustrates the results of infusing borohydride, while Curve 3 demonstrates the facilitation of the inactivation by borohydride which was caused by the presence of 0.05 M imidazole in the reaction mixture. Control experiments demonstrated that imidazole, *per se*, or imidazole *plus* borate did not cause inactivation of the enzyme. Under these conditions the rate of inactivation by borohydride was a first order dependent of the amount of activity remaining. The rate of borohydride inactivation could therefore conveniently be expressed in terms of a pseudo first order rate constant. The effects of a variety of compounds on the rate of inactivation of TNB-histidine ammonia-lyase II under various conditions are summarized in Table II. One must consider the possibility that imidazole enhanced the effectiveness of borohydride by decreasing the acid-catalyzed hydrolysis of this compound, rather than by any effect on the enzyme itself. This was checked by investigating the effect of imidazole on the inactivation of acetoacetic decarboxylase in the presence of acetoacetate[18]. Since, in this case, the rate of inactivation is known to be a function of the concentration of borohydride and since imidazole had no effect, we may conclude that imidazole does not affect the rate constants for the hydrolysis of borohydride and must therefore exert its influence directly on the enzyme. The same is presumed to have been true for the other compounds used.

Photosensitized inactivation

TNB-histidine ammonia-lyase was rapidly inactivated when exposed to light in the presence of rose bengal[20]. Reaction mixtures contained 40 μg of TNB-histidine ammonia-lyase and variable amounts of rose bengal in 1.1 ml of 0.10 M phosphate

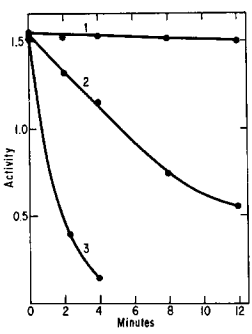
TABLE II

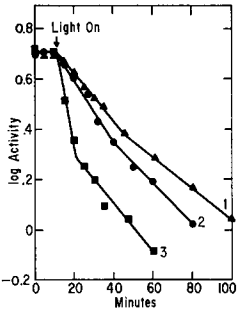
INACTIVATION OF TNB-HISTIDINE AMMONIA-LYASE II BY BOROHYDRIDE

Pseudo first order rate constants for the inactivation of TNB-histidine ammonia-lyase II under the general conditions described in Fig. 6 but with a variety of other agents present, are here tabulated. The effects of pH were also investigated.

*Conditions**	*Pseudo first order rate constant* $\times 10^{-3}$ *(min*$^{-1}$*)*	
	pH 8.2	*pH 6.5*
1. TNB-histidine ammonia-lyase II + 0.05 M hydroxylamine	0.034	3.0
2. TNB-histidine ammonia-lyase II + 0.05 M histidine	0.064	4.4
3. TNB-histidine ammonia-lyase II + 0.0125 M urocanate	0.079	20.0
4. TNB-histidine ammonia-lyase II + 0.025 M EDTA	0.088	5.1
5. TNB-histidine ammonia-lyase II + 0.05 M KNO_3	0.097	9.9
6. TNB-histidine ammonia-lyase II	0.104	13
7. TNB-histidine ammonia-lyase II, 0°	0.104	37
8. TNB-histidine ammonia-lyase II + 3.0 M urea	0.170	—
9. TNB-histidine ammonia-lyase II + 0.05 M histidinol	0.210	41
10. TNB-histidine ammonia-lyase II + 0.05 M guanidine	0.391	32
11. TNB-histidine ammonia-lyase II + 0.025 M imidazole	0.670	—
12. TNB-histidine ammonia-lyase II + 0.025 M imidazole + 0.05 M KNO_3	0.670	—
13. TNB-histidine ammonia-lyase II + 0.05 M imidazole	0.850	65

* All reactions were performed at 25° unless otherwise specified.





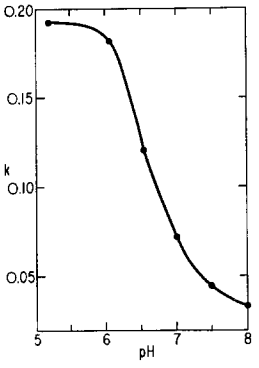


Fig. 6. Inactivation of TNB-histidine ammonia-lyase II by infusion of borohydride. Reaction mixtures contained 0.01 mg of enzyme in 0.5 ml of 0.10 M sodium pyrophosphate at pH 8.2 and 25°. Small aliquots were removed at intervals for assay. Curve 1 was obtained when the infusate was 0.05 M sodium borate in 0.08 M NaOH. Curve 2 was obtained when the infusate was 0.05 M sodium borohydride in 0.08 M NaOH. Curve 3 was obtained with the same infusion as in curve 2 but in this case the reaction mixture contained 0.05 M imidazole.

Fig. 7. Photo-inactivation of TNB-histidine ammonia-lyases I, II and III. Reaction mixtures contained 40 μg of enzyme and 0.5 μg of rose bengal in 1.1 ml of 0.10 M potassium phosphate buffer at pH 8.8 and 0°. Illumination was performed as described in the text and 10-μl aliquots were removed at intervals for assay in terms of urocanate production followed at 277 mμ as described in RESULTS.

Fig. 8. The effect of pH on the photo-inactivation of TNB-histidine ammonia-lyase III. Reaction mixtures contained 40 μg of TNB-histidine ammonia-lyase III and 0.50 μg of rose bengal in 1.1 ml of 0.10 M potassium phosphate buffer at the indicated pH and at 0°. Pseudo first order rate constants of the rapid phase of photo-inactivation are here plotted as a function of pH.

buffer in a 1 cm × 7.5 cm tube immersed in a 100-ml beaker of cracked ice. The photoreactor was a pipetting lamp (Arthur H. Thomas, Co.) whose light source was a 15-W fluorescent tube. The sample in the beaker of ice was placed inside the pipetting lamp, which rested on its back, and the open face of the lamp was covered with aluminum foil. Relative light intensities inside the lamp were estimated with a G.E. Exposure Meter Model PR-2. Light intensities were varied by partially masking the fluorescent tube with black tape. Samples were removed at intervals, diluted with buffer and kept in the dark until assayed for residual histidine ammonia-lyase activity. Sampling during a dark period which preceeded the onset of illumination, demonstrated that there was no loss of activity in the dark and allowed each reaction mixture to serve as its own control. The rates of photoinactivation were directly proportional to light intensity and to the concentration of rose bengal in the ranges studied.

When TNB-histidine ammonia-lyases I, II and III were illuminated at pH 8.8 in the presence of 0.46 μg/ml of rose bengal, rapid and biphasic loss of activity resulted. TNB-histidine ammonia-lyase III was more labile under these conditions, than was TNB-histidine ammonia-lyase II which in turn was more labile than TNB-histidine ammonia-lyase I. These results, which are illustrated in Fig. 7 suggested that each of these enzyme fractions contained two populations of active sites whose labilities to photo-inactivation were distinctly different. If these duplicities of photosensitive sites corresponded to the active sites which generated the deviations from classical Michaelis–Menten behavior illustrated in Fig. 4; then we might expect to largely eliminate the kinetic peculiarities by partial photo-inactivation of these enzymes. Thus, destruction of more photo-labile sites should leave only the resistant sites which, if then catalytically homogeneous, would exhibit linear reciprocal plots of kinetic data. This was tested. Aliquots of TNB-histidine ammonia-lyases I, II and III were photo-inactivated to the point of 60% loss of activity. The dye was then removed by dialysis in the dark and the kinetic behavior of these samples then investigated. In each case partial photo-inactivation eliminated the nonlinearities previously seen in the Lineweaver–Burk plots.

The effects of pH on the rapid phase of the photo-inactivation of TNB-histidine ammonia-lyase III in the presence of 0.46 μg/ml of rose bengal are illustrated in Fig. 8. Similar curves were generated when the slow phase of photo-inactivation was plotted as a function of pH. TNB-histidine ammonia-lyases I and II responded to variation of pH in a similar way. Changes of dye concentration or of light intensity did not appreciably alter the response to pH.

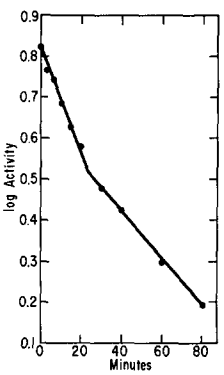
Thermal inactivation of TNB-histidine ammonia-lyase II

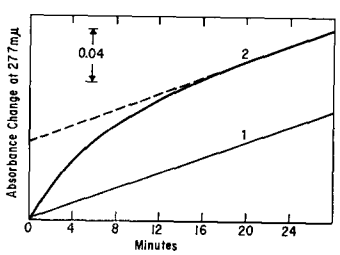
If TNB-histidine ammonia-lyase contained two distinct populations of catalytic sites which differed in lability towards photo-inactivation and which exhibited different catalytic constants, then it appeared possible that they might also differ in thermal stability. TNB-histidine ammonia-lyase II at a concentration of 0.05 mg/ml in 0.08 M sodium pyrophosphate at pH 8.2 was heated to 80°. At intervals, 50-μl aliquots were removed for assay. Evaporation was minimized by performing the heating in 0.6 cm × 5.0 cm tubes which were closed with a rubber septum. Samples were removed with a microsyringe whose needle was inserted through the septum. That thermal inactivation was a biphasic process is clearly illustrated by the results in Fig. 9.

Inhibition by EDTA

It has been reported[4] that aged histidine ammonia-lyase is insensitive to EDTA but that histidine ammonia-lyase which has been activated by treatment with mercaptans is inhibited by EDTA to a maximum degree of approx. 90% inhibition. We have similarly observed that TNB-histidine ammonia-lyase is unaffected by the presence of EDTA but becomes susceptible to EDTA inhibition after incubation with dithiothreitol and that the maximum inhibition which can be achieved, in the latter case, is 90%. It appears very unlikely that the inhibition of reduced histidine ammonia-lyase by EDTA is related to a functional metal prosthetic group, since dialysis against 1 mM EDTA at pH 6.8 followed by dialysis against buffer was without effect on the activity of TNB-histidine ammonia-lyase or of reduced histidine ammonia-lyase. Furthermore 10 mM, Mn^{2+}, Cu^{2+}, Mg^{2+} or Co^{2+} had no effect on the activity of histidine ammonia-lyase and, a variety of chelating agents, other than EDTA, were found to be without effect on the activity of reduced histidine ammonia-lyase. Thus 1 mM citrate, or 0.1 mM *o*-phenanthroline, 2,9-dimethyl-*o*-phenanthroline, 8-hydroxyquinoline and dithizone were without effect on the activity of reduced histidine ammonia-lyase. Pyrophosphate, which is an effective chelating agent, was used in routine assays of histidine ammonia-lyase and it did not inhibit.

The order of mixing of EDTA, substrate, and enzyme had a profound effect on the subsequent behavior of the system. Thus, if EDTA was added to the enzyme before the substrate, the inhibition was observed immediately upon initiation of the reaction with substrate. If, on the other hand, EDTA was added to the enzyme after the substrate, the inhibition developed slowly. These results, which are illustrated in





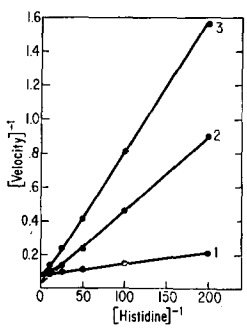


Fig. 9. Thermal inactivation of TNB-histidine ammonia-lyase II. TNB-histidine ammonia-lyase II at a concentration of 50 μg/ml in 0.08 M sodium pyrophosphate buffer at pH 8.2 was incubated at 80° and aliquots were removed for assay at intervals. The logarithm of the residual activity is here presented as a function of time.

Fig. 10. Effect of L-histidine on the inhibition of EDTA. Reaction mixtures contained reduced histidine ammonia-lyase, 0.33 mM EDTA and 16.7 mM L-histidine in 3.0 ml of 0.10 M sodium pyrophosphate buffer at pH 9.2 and 25°. In the case of Line 1 substrate was the last component added to the reaction mixture whereas in the case of Line 2 EDTA was the last component added.

Fig. 11. Kinetic analysis of the inhibition of histidine ammonia-lyase by EDTA. Reaction mixtures contained reduced histidine ammonia-lyase and the indicated concentrations of L-histidine in 0.10 M sodium pyrophosphate buffer at pH 9.2. Line 1 was obtained in the absence of EDTA whereas Line 2 was obtained in the presence of 0.167 mM EDTA and Line 3 in the presence of 0.333 mM EDTA.

Fig. 10, indicate that the enzyme is modified as a consequence of its interaction with L-histidine and that this modified enzyme reacts with EDTA much more slowly than the resting enzyme. Fig. 11 demonstrates that the inhibition of reduced histidine ammonia-lyase by EDTA was competitive with respect to substrate and that EDTA imposed nonlinearities in the reciprocal plots of the kinetic data obtained at very high concentrations of L-histidine. Vaguely similar effects of EDTA have been observed by PETERKOFSKY AND MEHLER[8]. The K_i for EDTA computed from its effects on the slopes of Lines 2 and 3 in Fig. 11 are 25 and 28 μM, respectively.

DISCUSSION

Reduced histidine ammonia-lyase is prone to oxidation and inactivation when exposed to air in the absence of supporting mercaptans. This behavior has been seen with a variety of enzymes and has in general been associated with particularly labile sulfhydryl groups. Thus, the D-lactic dehydrogenase of *Escherichia coli*[21], a bacterial dioxygenase acting on 3-hydroxypyridines[22], ficin[23] and papain[24] are all in this category. Investigators have usually attempted to circumvent the air oxidation of such enzymes by performing all manipulations in the presence of high concentrations of mercaptans. An alternate approach would involve reversibly masking the labile groups by conversion to stable derivatives. Such a strategy can be successfully resorted to for purposes of purification only if there is some way to assay the modified enzyme. Thus the mercury salt of papain[25] is a convenient form for recrystallization and storage of this enzyme but is useless for purposes of purification, since it retains none of the activity of the native enzyme. In this respect the case of histidine ammonia-lyase is a happy one, since oxidized derivatives, whether prepared by treatment with H_2O_2 or with DTNB, retain a small but reproducible fraction of the activity of the native enzyme and may therefore be assayed and purified. Histidine ammonia-lyase which had been oxidized by treatment with DTNB could be fully reactivated, by treatment with dithiothreitol, even after prolonged storage. The H_2O_2-oxidized enzyme, in contrast, gradually lost the ability of being reactivated. This is similar to the response of glyceraldehyde-3-phosphate dehydrogenase to H_2O_2 (ref. 26), in which case loss of activity was proportional to loss of sulfhydryl-group titer and could be reversed by prompt treatment with excess thiol but not by delayed treatment with thiol. The air oxidation of purified, reduced histidine ammonia-lyase like that of reduced papain[27] was not reversible by thiols. It appears likely that DTNB reacts only with thiol groups of histidine ammonia-lyase and serves to protect them from oxidation by O_2 which may proceed beyond the disulfide stage. In a like manner DTNB has been used to protect the essential sulfhydryl group of lactic dehydrogenase[28]. The oxidation of histidine ammonia-lyase by H_2O_2 could well have involved oxidation of methionine residues. Thus the oxidation of a single methionine residue to the level of the sulfoxide has been shown to be the only effect of H_2O_2 on subtilisin and was associated with a decrease in enzymic activity[29]. In a similar fashion H_2O_2 oxidized a methionine residue of pituitary adrenocorticotrophin to the sulfoxide with a concomitant loss of biological activity. This effect was reversed by reduction with mercaptans[30].

The separation of three TNB-histidine ammonia-lyases shown in Fig. 1 could have been due to varying degrees of derivitization with DTNB. It should be noted

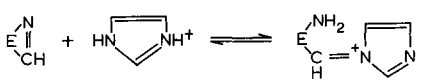
that an excess of DTNB was used in preparing these TNB derivatives, in order to minimize the possibility of polymerization[31] and that increasing the time of incubation of the enzyme with DTNB did not alter the ratios of derivatives obtained.

Whereas the kinetic behavior of reduced histidine ammonia-lyase did fit the Michaelis–Menten equation; the data obtained with TNB-histidine ammonia-lyase II was more complex and could be explained on the basis of multiple substrate binding sites.

Equations similar to those derived by KISTIAKOWSKY AND ROSENBERG[14] did fit the data satisfactorily. That TNB-histidine ammonia-lyase possessed two distinct populations of active sites was further supported by the biphasic responses exhibited during rose bengal-sensitized photo-inactivation and during thermal inactivation studies and by the observation that partial photo-inactivation eliminated the kinetic peculiarities. Whether these distinct populations of active sites coexist on a single species of protein molecules or on physically separable molecules is, as yet, unknown. It is, however, possible to put forth a hypothesis which is consistent with the available data and which could be tested. Let us tentatively suppose that there is a single catalytic species (histidine ammonia-lyase) which is capable of self-association. Further suppose that this association reaction reduces catalytic efficiency and imposes differences upon the units which they did not exhibit when monomeric. If oxidation favored the self-association reaction, then it would cause a partial loss of activity and would impose the kinetic peculiarities described above. Oxidation by H_2O_2, like that by DTNB, should lead to only a partial loss of activity; which it did. Furthermore the H_2O_2-oxidized histidine ammonia-lyase was observed to undergo a time- and temperature-dependent activation upon dilution[32] which is consistent with a dissociation reaction. As shown in Fig. 2, the oxidation of purified and fully reduced histidine ammonia-lyase in air was a biphasic process, the rapid phase of which could have been associated with a polymerization of oxidized subunits which yielded oligomers with a definite residual activity. If EDTA also favored the self-association of histidine ammonia-lyase then EDTA should inhibit reduced histidine ammonia-lyase but not oxidized histidine ammonia-lyase, because the former can associate whereas the latter already has done so. EDTA inhibition of the reduced histidine ammonia-lyase should leave a residual activity which would be resistant to further suppression by EDTA because the oligomers retain some fraction of the activity of the monomers. EDTA should also impose kinetic pecuilarities as does oxidation and, as shown in Fig. 11, this was the case. It is also conceivable that the association imposes upon the individual units the observed differences in susceptibility to photo-oxidation and to thermal denaturation. Finally, if EDTA favors the association while substrate opposes it, then one might have an explanation for the competition between EDTA and L-histidine and for the slowness with which EDTA inhibition manifests itself when substrate was allowed prior access to the enzyme.

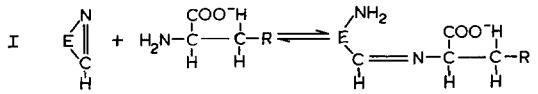
Raising the pH raised the rate of photosensitized inactivation of TNB-histidine ammonia-lyase (Fig. 8) in a manner reminiscent of a titration curve of a compound whose pK_a was 6.7. Although this pK_a might be suggestive of an imidazolium residue, the direction of change was the opposite to that which would be expected for an imidazolium. Thus, the free base form of the imidazole ring of histidine ammonia-lyase is sensitive for photosensitized oxidation whereas the protonated form is resistant[20]. We are thus unable to specify the chemical nature of the group whose ionization hinders the photo-oxidation of the enzyme.

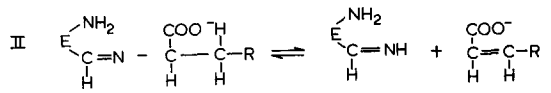
The borohydride-infusion studies are potentially very informative. The enzyme was very rapidly inactivated by the minuscule steady-state levels of borohydride maintained by the continuous infusions. The rate of borohydride inactivation was much more rapid at pH 6.5 than at pH 8.2. Nitrate protected against borohydride inactivation at pH 6.5 but had no effect at pH 8.2. Lowering the temperature from 25 to 0° had no effect on the borohydride inactivation at pH 8.2 but profoundly augmented the rate of borohydride inactivation at pH 6.5. Hydroxylamine slowed the rate of borohydride inactivation of the enzyme whereas imidazole accelerated it. These diverse effects could be explained were the reducible group on the enzyme an azomethine rather than a carbonyl. The existence of an azomethine involving the α-amino group of dehydroalanine and an unspecified carbonyl group at the active site of histidine ammonia-lyase has recently been proposed[5]. The reduction of carbonyl groups by borohydride is relatively slow and independent of pH, whereas the reduction of azomethines proceeds more rapidly and is facilitated by lowering the pH (ref. 33). Protonation of the proposed azomethine at pH 6.5 would facilitate the binding of the borohydride anion by an electrostatic interaction. In this case monovalent anions could compete with borohydride at this cationic site and thus protect the site against reduction by the borohydride. This sort of behavior was seen in the case of the reduction by borohydride of the Schiff's base intermediate involved in the catalytic action of acetoacetic decarboxylase[18]. Raising the pH to 8.2 should discharge the proton on the Schiff's base in which case the basis for the electrostatic binding of the borohydride anion would no longer exist and protection by monovalent anions would cease to occur. Hence the protection by nitrate against the inactivation of histidine ammonia-lyase by borohydride at pH 6.5 but not at 8.2. By analogy with the case of acetoacetic decarboxylase[18] where the binding of monovalent anions such as borohydride was found to be augmented by lowering the temperature; we might expect that the inactivation of TNB-histidine ammonia-lyase by borohydride should be accelerated by lowering the temperature at pH 6.5 but not at pH 8.2. This is the expectation because at pH 6.5 there is a positive charge on the azomethine and hence the basis for anion binding, whereas at pH 8.2 neither the charge nor the basis for anion binding would exist. Hydroxylamine hindered the inactivation of the enzyme by borohydride whereas imidazole facilitated this reaction. The controls which were performed indicated that these effects were due to interactions between these compounds and the enzyme; rather than being due to possible effects on the steady-state level of borohydride in the reaction mixtures. Facilitation of borohydride inactivation of the enzyme such as that caused by imidazole could be explained on the basis of a displacement of the endogenous amino group by the added nucleophile and the consequent generation of an azomethine more susceptible to reduction by borohydride. Thus:



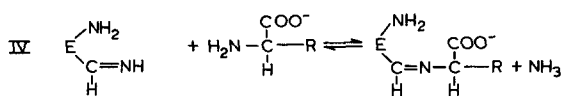
Protection against borohydride inactivation, such as that provided by hydroxylamine, would similarly be explained if the compound formed by the displacement of the endogenous amino group by hydroxylamine were less readily reduced by borohydride. If the active site of histidine ammonia-lyase is an azomethine rather than a

free carbonyl then the interaction with substrate would involve a transaldimination reaction in which the amino group of L-histidine displaced the endogenous amino group. There are precedents for such a proposed mechanism[34]. Elaboration of this proposal leads to the following reactions:





We may also consider the possibility that the enzyme–ammonia compound generated by Reaction II is itself capable of reaction with another molecule of substrate as follows:



If the rate of Reaction IV were similar to the rate of Reaction I, we would then have an explanation for the failure of ammonia to inhibit the histidine ammonia-lyase reaction.

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