Histidine Decarboxylase of *Lactobacillus* 30a: Function and Reactivity of Sulfhydryl Groups†

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ABSTRACT: Two classes of sulfhydryl groups in histidine decarboxylase from *Lactobacillus* 30a can be differentiated by their reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Five cysteinyl residues (class I) of the native enzyme are titrated by DTNB as the pH of the reaction medium is increased from 6.5 to 7.5; the pH–rate profile for their reaction is described by a pK_a of 9.2. An additional five thiol groups (class II) are titrated only when denaturing agents are added above neutral pH. Histidine decarboxylase is completely inactivated by DTNB in a kinetically second-order process (K_{app} = 660 ± 20 M^{-1} min^{-1} at pH 7.6 and 25 °C) which occurs coincident with and at the same rate as modification of the five class-I SH groups of the enzyme, i.e., one thiol group per pyruvoyl prosthetic group. The competitive inhibitors, histamine and imidazole, markedly enhanced the reactivity of these cysteinyl residues toward DTNB; this enhancement is accompanied by a concomitant increase in the rate of inactivation. A single SH group in each of the five catalytic units of histidine decarboxylase is thus implicated as being critical for the expression of enzymatic activity.

Histidine decarboxylase from *Lactobacillus* 30a, in contrast to most other amino acid decarboxylases studied thus far, does not require pyridoxal 5′-phosphate for enzymatic activity (Rosenthaler et al., 1965). The native enzyme is a decamer composed of five pairs of nonidentical subunits; five of the ten polypeptide chains contain a pyruvoyl residue in amide linkage to a phenylalanine residue at the amino-terminal end (Riley and Snell, 1968, 1970). Results of borohydride trapping experiments (Recsei and Snell, 1970) show that the pyruvoyl residue participates in the catalytic mechanism of this enzyme by forming a Schiff-base intermediate with the α-amino group of the substrate histidine, thereby facilitating the decarboxylation reaction.

Histidine decarboxylase is devoid of disulfide bonds, but contains ten half-cystine residues (Chang and Snell, 1968a), two in each of the five peptide chains terminating in pyruvoyl residues (Riley and Snell, 1970). Earlier work (Chang and Snell, 1968a) showed that reaction of five to seven SH groups of histidine decarboxylase with iodoacetamide or *p*-chloromercuribenzoate inactivated the enzyme. The present paper describes the reactivity of the SH groups of histidine decarboxylase with DTNB[1] and provides evidence that a single thiol residue per active site is essential for the catalytic activity of the enzyme.

Materials and Methods

Crystalline histidine decarboxylase was isolated from acetone powders of *Lactobacillus* 30a cells by the procedure of Chang and Snell (1968b) as modified by Riley and Snell (1968). The enzyme gave a single band on electrophoresis on polyacrylamide gels (5%) in 0.075 M sodium phosphate (pH 7.2) and had a specific activity of 70–80 μmol of CO_2 evolved min^{-1} (mg of protein)$^{-1}$ at 37 °C. The concentration of purified enzyme was determined from its absorbance at 280 nm ($E_{1cm}^{1\%}$ 16.2; Riley and Snell, 1968). Molar concentrations were calculated based on a molecular weight of 190 000 (Chang and Snell, 1968a).

5,5′-Dithiobis(2-nitrobenzoic acid) from Aldrich Chemical Co. was twice recrystallized from glacial acetic acid before use. Histamine dihydrochloride and imidazole were products of Calbiochem; guanidine hydrochloride ("Ultra Pure") was from Mann Research Laboratories.

Histidine decarboxylase was assayed manometrically at 37 °C in 0.2 M ammonium acetate (pH 4.8) containing 8 mM L-histidine hydrochloride monohydrate (Nutritional Biochemical Co.) in a final reaction volume of 3 ml. Initial velocities were determined from measurements of CO_2 evolution at 1-min intervals over a 10-min period.

Titration of histidine decarboxylase with DTNB was carried out at 25 °C in 0.1 M potassium phosphate–1 mM EDTA in a total volume of 1.0 ml in 1-cm cuvettes; pH and reactant concentrations are specified for each experiment. Sodium pyrophosphate (0.1 M) was substituted for potassium phosphate when titrations were performed at high pH. Reactions were initiated by the addition of DTNB and monitored by following the change in absorbance at 412 nm as a function of time with a Gilford Model 2000 recording spectrophotometer equipped with a thermostated cell compartment. The number of SH groups titrated was calculated by use of a molar extinction coefficient of 13 600 M^{-1} cm^{-1} (Ellman, 1959). Titration of L-cysteine hydrochloride with DTNB showed that this value varied by only about ±3% in the pH range of 6 to 10. Pseudo-first-order rate constants (k_{SH}) were calculated by least-squares analysis from the slopes of plots of log ($SH_f - SH_t$) vs. time where SH_f is the sulfhydryl titer after completion of reaction and SH_t is the number of SH groups titrated at time t. Apparent second-order rate constants (k_2) were calculated from the relationship, $k_2 = k_{SH}/[DTNB]$.

The rate of inactivation of histidine decarboxylase by DTNB was determined by periodic assay of quenched aliquots of the reaction mixture as outlined in the text.

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[1] The abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, the thionitrobenzoate anion of DTNB; PMB, *p*-chloromercuribenzoic acid; EDTA, ethylenediaminetetraacetic acid.

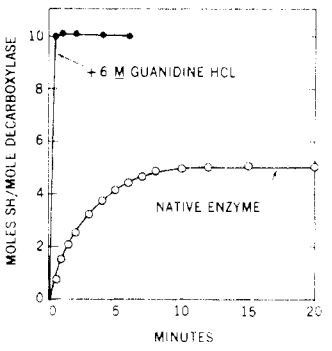

FIGURE 1: DTNB titration of histidine decarboxylase in the presence (●) and absence (○) of 6 M guanidine hydrochloride. Histidine decarboxylase (0.96 mg/ml, 5.07×10^{-6} M) was incubated at 25 °C in 0.1 M potassium phosphate-1 mM EDTA (pH 7.6) containing 0.5 mM DTNB.

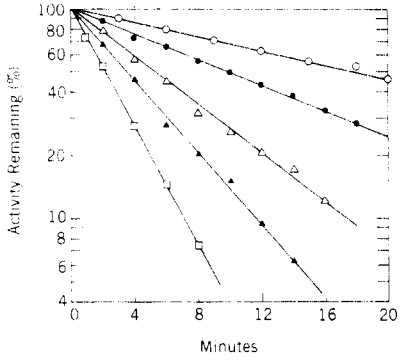

FIGURE 2: Pseudo-first-order plots for the inactivation of histidine decarboxylase (0.61 mg/ml, 3.21×10^{-6} M) by DTNB in 0.1 M potassium phosphate-1 mM EDTA (pH 7.6) at 25 °C. Aliquots (50 μl) were withdrawn at the times indicated, diluted 20-fold at 0 °C with 0.2 M ammonium acetate-0.1% bovine serum albumin (pH 4.8), and assayed spectrophotometrically: (○) 0.05 mM, (●) 0.1 mM, (△) 0.2 mM, (▲) 0.3 mM, (□) 0.5 mM DTNB.

Results

Titration of Native and Denatured Histidine Decarboxylase with DTNB. The time course for titration of histidine decarboxylase with DTNB at pH 7.6 and 25 °C in the presence and absence of guanidine hydrochloride is shown in Figure 1. With denatured enzyme, ten SH groups react instantaneously, in accord with the half-cystine content of the enzyme reported previously (Chang and Snell, 1968a). In the absence of denaturant, however, only five thiol residues are titrated; no further reaction occurs during prolonged incubation of the decarboxylase with DTNB under the conditions specified. Kinetic analysis of the titration of these five reactive SH groups (class I) in the presence of a 20-fold molar excess of DTNB indicates that the reaction can be defined by a single rate process ($k_{obsd} = 0.32$ min^{-1} at pH 7.6 and 25 °C). There appears, therefore, to be little distinction in reactivity toward DTNB among these five cysteinyl residues (one per protomer).

Inactivation of Histidine Decarboxylase by DTNB. Histidine decarboxylase can be completely inactivated by incubation with DTNB at pH 7.6 and 25 °C. As shown in Figure 2, loss of decarboxylase activity is first-order in active enzyme to at least 95% inactivation when DTNB is in excess. Moreover, the apparent first-order rate constant for inactivation (k_{inact}) calculated from data of Figure 2 is directly proportional to the concentration of DTNB up to 0.5 mM (Table I, columns 1 and 2), indicating that inactivation is also first-order with respect to DTNB and second-order overall ($k_2 = 660 \pm 20$ M^{-1} min^{-1} at pH 7.6 and 25 °C). Thus, there is no apparent saturation effect over the concentration range studied and no evidence for noncovalent complex formation between histidine decarboxylase and DTNB prior to inactivation. The rate of inactivation parallels the rate of liberation of the thionitrobenzoate anion during sulfhydryl titration of the enzyme, as shown by the identity in rate constants for the two processes (Table I). Thus, inactivation is directly proportional to the extent of reaction of the five class I SH groups with DTNB, and complete inactivation of the enzyme is associated with the loss of five thiol residues, i.e., of one catalytically critical SH group per pyruvoyl prosthetic group. The derivatized enzyme containing five DTNB-modified SH groups/190 000 daltons was found to be homogeneous by the criterion of polyacrylamide gel electrophoresis and also cross-reacted with rabbit antibodies to native enzyme.

TABLE I: Correlation of Inactivation of Histidine Decarboxylase with SH Group Titration as a Function of DTNB Concentration.[a]

DTNB (mM)	Inact. Rate Constant k_{inact} (min^{-1})	SH Titration Rate Constant k_{SH} (min^{-1})	k_{SH}/k_{inact}
0.05	0.038		
0.1	0.065	0.071	1.09
0.2	0.128	0.133	1.04
0.3	0.193	0.192	1.00
0.5	0.322	0.327	1.02

[a] Experimental conditions and methods for determining the rate of inactivation are described in the legend of Figure 2. The rate of SH titration was determined from the increase in absorbance at 412 nm as a function of time (see Materials and Methods).

Sulfhydryl Group Reactivity in the Presence of Competitive Inhibitors. Histamine, a product of the decarboxylation reaction and a competitive inhibitor of histidine decarboxylase (Recsei and Snell, 1970), markedly enhances the rate of titration of enzyme SH groups by DTNB. The reaction is first-order in remaining thiol groups over an eightfold increase in histamine concentration. Moreover, the plots (not shown) extrapolate to a common intercept at the ordinate indicating that 4.7 SH groups are influenced in their reactivity toward DTNB at each concentration of histamine employed. Thus, histamine elicits no change in the number of cysteinyl residues titratable in the native enzyme but promotes a significant and equivalent enhancement in their reactivity toward DTNB. Kinetic experiments conducted as a function of DTNB concentration at constant levels of histamine showed that reaction of histidine decarboxylase with DTNB was a second-order process overall in the presence of the inhibitor.

The apparent second-order rate constant for DTNB-titration of class I SH groups is a linear function of histamine concentration over the range studied (Figure 3); the rate enhancement observed is approximately sixfold at the highest concentration of histamine (0.2 M) tested. Imidazole, another competitive inhibitor of histidine decarboxylase (Rosenthaler et al., 1965), exerts a similar but less potent effect, a threefold activation of SH-group reactivity recurring in the presence of 0.2 M imidazole (Figure 3). Here, too, no alteration in the SH titer was observed.

Effect of Histamine and Imidazole on Rate of Enzyme Inactivation. Experiments similar to that of Figure 2 but conducted in the presence of histamine or imidazole showed

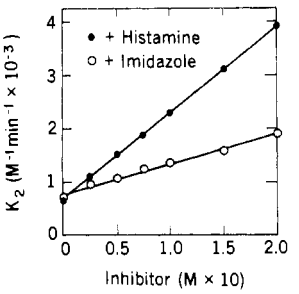

FIGURE 3: Variation of the apparent second-order rate constants (k_2) for titration of histidine decarboxylase with DTNB with increasing concentrations of histamine (●) or imidazole (○) at pH 7.6 and 25 °C. Histidine decarboxylase (0.66 mg/ml, 3.48 μM) was incubated at 25 °C in 0.1 M potassium phosphate–1 mM EDTA (pH 7.6) containing 0.3 mM DTNB and imidazole or histamine as indicated.

TABLE II: Correlation of DTNB Inactivation of Histidine Decarboxylase with SH Group Titration as a Function of Histamine and Imidazole Concentration.[a]

Competitive Inhibitor	Concn (mM)	SH Titration Rate Constant k_{SH} (min^{-1})	Inact. Rate Constant k_{inact} (min^{-1})
Histamine	0	0.065	0.070
	25	0.110	0.104
	50	0.150	0.131
	100	0.230	0.217
	200	0.388	0.367
Imidazole	0	0.069	0.065
	25	0.096	0.088
	50	0.107	0.103
	100	0.135	0.140
	200	0.191	0.181

[a] Histidine decarboxylase (0.66 mg/ml) was incubated with 0.1 mM DTNB in 0.1 M potassium phosphate–1 mM EDTA (pH 7.6) at 25 °C. Histamine or imidazole was included in the reaction mixture as indicated. The rate of SH titration was determined from the increase in absorbance at 412 nm as a function of time. Aliquots (50 μl) of the reaction mixture were removed periodically, diluted 20-fold with 0.2 M ammonium acetate–0.1% bovine serum albumin (pH 4.8) at 0 °C, and assayed for decarboxylase activity.

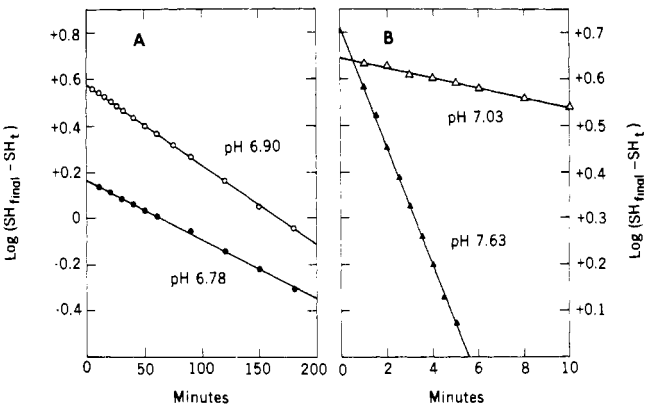

FIGURE 4: Pseudo-first-order plots for the titration of histidine decarboxylase (0.39 mg/ml, 2.05×10^{-6} M) by DTNB (0.5 mM) in 0.1 M potassium phosphate–1 mM EDTA buffers at the pH values indicated and 25 °C. Note the change in scales in parts A and B of the figure.

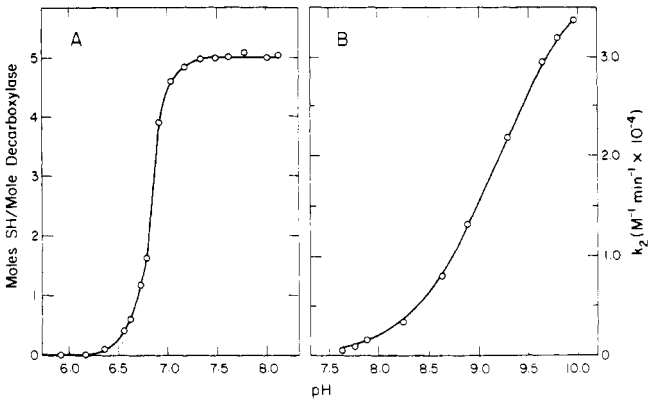

FIGURE 5: (A) Effect of pH on the total number of sulfhydryl groups titratable with DTNB in histidine decarboxylase. The number of thiol groups titrated was calculated from the ordinate intercepts of plots shown in Figure 4 or from the ΔA_{412} after completion of the reaction. Values determined by these two methods agreed within ±2%. (B) The effect of pH on the rate of titration of class I thiol groups of histidine decarboxylase (0.34 mg/ml, 1.79×10^{-6} M) by DTNB (0.1 mM). Reactions were carried out at 25 °C in 0.1 M sodium pyrophosphate–1 mM EDTA buffers at the pH values indicated. Experimental points (○) represent the observed second-order rate constant (k_2). The solid line represents a theoretical curve calculated from eq 2 (see text).

the expected acceleration in the pseudo-first-order loss of decarboxylase activity. In addition, the enhancement in inactivation rate exactly paralleled the increase in reactivity of class I SH groups when the histamine or imidazole concentration was varied at a fixed level of DTNB (Table II). These results clearly demonstrate a direct relationship between the inhibitor-induced state of activation of these five thiol residues and their essentiality for enzymatic activity.

pH Dependence of SH-Group Reactivity. Two effects of pH on the reactivity of histidine decarboxylase sulfhydryl groups with DTNB are apparent (Figure 4). First, the rate of the disulfide exchange reaction with DTNB is markedly dependent on pH as evidenced by a progressive and dramatic increase in the rate (11-fold) between pH 6.8 and 7.6. Secondly, the total number of reactive thiols, as calculated from the ordinate intercept, increased from 1.6 at pH 6.8 to 5.3 equiv per mol of enzyme at pH 7.6. A plot of the number of DTNB-titratable SH groups as a function of pH (Figure 5A) shows that essentially no sulfhydryl groups titrate below pH 6.5. The SH titer then increases rapidly between pH 6.5 and 7.5 to reach a maximum of five cysteinyl residues per 190 000 g of protein; no additional thiols can be titrated when the pH of the reaction medium is increased in the range 7.5 to 10.0. These observations are indicative of a pH-dependent conformational change which occurs with a sharp transition at pH 6.9 and opens the protein structure sufficiently to permit reaction of the five class-I SH groups with DTNB. Class II thiols are not exposed to solvent as a consequence of this transition. The two presumed conformational states of the enzyme do not appear to be influenced by either histamine or imidazole. No SH groups can be titrated (during reaction times up to 12 h) at pH 6.0 in the presence of either inhibitor (0.2 M) nor can any variation from five reactive thiols be detected at pH 7.6 under the same conditions. Furthermore, the midpoint in the transition from one conformational state to the other is at 6.9 whether or not histamine or imidazole is included in the incubation mixtures. Addition of guanidine hydrochloride (6 M) to histidine decarboxylase at pH 6 also elicits no exposure of SH groups for reaction with DTNB; in contrast, both class-I and class-II thiols become susceptible to titration in the presence of 6 M denaturant at pH 7.6 (see Figure 1).

Although the number of SH groups titrated does not increase above pH 7.5 (Figure 5A), their rate of reaction with DTNB increases markedly (Figure 5B). The sigmoidal pH–rate profile may be interpreted as a dependence of the rate constant, k_2, on the mole fraction of the mercaptide ion, the reactive species required for nucleophilic attack at the disulfide bond of DTNB (cf. Fava et al., 1957). Thus, for reaction 1

$$\begin{array}{l} \text{E-S}^- + \text{DTNB} \xrightarrow{k_0} \text{E-S-TNB} + \text{TNB}^- \\ \quad \Big\Updownarrow K_a \\ \text{E-SH} \end{array} \qquad (1)$$

where E-SH and E-S$^-$ represent the protonated and unprotonated forms, respectively, of the class-I thiols of histidine decarboxylase, E-S-TNB, the catalytically inactive mixed-disulfide enzyme derivative, and TNB$^-$, the liberated thionitrobenzoate ion, the observed second-order rate constant (k_2) should vary according to the relationship shown in eq 2

$$k_2 = \frac{k_0}{1 + [\text{H}^+]/K_a} \qquad (2)$$

where K_a is the dissociation constant for the reactive SH groups and k_0 is the intrinsic second-order rate constant corresponding to complete ionization of the thiol groups. The solid curve shown in Figure 5B represents the theoretical variation of k_2 with pH, calculated from eq 2 by employing the values of $k_0 = 3.95 \times 10^4$ M^{-1} min^{-1} and pK_a = 9.20 (i.e., $K_a = 6.3 \times 10^{-10}$ M). The excellent fit of the experimental data (open circles) with the theoretical curve supports the proposal that the pK_a of 9.2 describes the ionization of the class-I SH groups of histidine decarboxylase and that the rate of the interchange reaction over this pH range depends primarily on the concentration of their ionized form.[2] This pK_a value compares favorably with those normally ascribed to cysteinyl residues in proteins (Edsall, 1943).

Discussion

Histidine decarboxylase (mol wt 190 000) contains five each of two dissimilar subunits. Each of the five larger subunits contains one pyruvoyl and two cysteinyl residues; the smaller subunit contains neither of these groups (Riley and Snell, 1970). Abundant evidence indicates a catalytic role for the pyruvoyl residues: (1) the enzyme is inactivated by carbonyl reagents and by borohydride reduction (Rosenthaler et al., 1965; Riley and Snell, 1968); (2) following borohydride reduction in the presence of [^{14}C]histamine, radioactivity equivalent to five histamine residues is fixed per mole of enzyme, and the secondary amine formed by reduction of the Schiff's base of histamine with pyruvate can be isolated from the acid hydrolysate of the reduced enzyme (Recsei and Snell, 1970); (3) similar treatment of the enzyme in the presence of histidine leads to isolation of two reduced Schiff's bases, one derived from histidine and one from histamine. Enzymatic action thus appears to involve formation and decarboxylation of a Schiff's base of histidine on the enzyme, leading directly to the Schiff's base of histamine (Recsei and Snell, 1970). No other catalytically essential residues have been identified in previous work.

The results described here strongly indicate that the SH group of one of the two cysteine residues present per active center of the enzyme is directly or indirectly essential for catalysis. Thus, only five of the ten SH groups can be titrated by DTNB at pH values up to 10; these five SH groups (class I) are equivalent in reactivity toward DTNB and catalytic activity of the enzyme is lost in direct proportion to the number modified. In addition, their reactivity toward DTNB is greatly increased by competitive inhibitors of the decarboxylase, such as histamine and imidazole, and presumably, therefore, by histidine itself. The other five SH groups (class II) of the enzyme become accessible to DTNB only upon denaturation of the enzyme at alkaline pH.

The increased rate of modification of class-I SH groups in the presence of histamine or imidazole suggests that binding of the substrate or other imidazoles to the histidine-binding site of histidine decarboxylase enhances the reactivity of the DTNB-sensitive thiols. The failure to demonstrate a saturation effect for activation of class-I SH groups by histamine or imidazole at pH 7.6 probably reflects very weak binding of these inhibitors above neutral pH. Previous kinetic studies (Recsei and Snell, 1970) showed that the K_M value for L-histidine is 250 times greater at pH 7.6 than at the optimum pH of 4.8; moreover, the K_I value for histamine at pH 4.8 ($\simeq$11 mM) is 30 times greater than the K_M value for histidine. If the affinities for histidine and histamine are affected similarly by pH, the maximum concentration of histamine used here would be less than 0.1 its K_I value at pH 7.6 and thus far below the concentration where saturation could be observed. The activating effects observed here, although large, are therefore far less than those to be expected at saturation of the enzyme. One possible mechanism for this activating effect would be a substrate (or analogue)-induced conformational effect that increases reactivity of this SH group. Substrate-induced conformational changes have been invoked previously to explain the increase in SH group reactivity observed in the presence of added ligands with phosphoglucomutase (Yankeelov and Koshland, 1965), gluconate-6-phosphate dehydrogenase (Rippa et al., 1966), aspartate aminotransferase (Karni-Katsadimas et al., 1969), carbamoyl-phosphate synthetase (Anderson and Marvin, 1970), formyltetrahydrofolate synthetase (Nowak and Himes, 1971), and ATP:glutamine synthetase adenylyltransferase (Wolf and Ebner, 1972).

A second possible explanation for the increase in reaction rate elicited by histamine or imidazole is that the imidazole ring participates as a general-base catalyst assisting the disulfide interchange between DTNB and class-I thiols: abstraction of a proton from the SH group by imidazole would facilitate formation of the reactive thiolate anion for nucleophilic attack at the disulfide bond of DTNB. This interpretation requires a close proximity between the imidazole ring and these sulfhydryl groups and would place the SH group at the active site of the enzyme, i.e., in a position permitting a role in catalysis of decarboxylation. Such proximity is also indicated by the observation that histidine methyl ester, a noncompetitive active-site-directed inhibitor, prevents titration of some of these groups (Lane et al., 1971, 1976).

Although a definitive assignment of a role for these essential sulfhydryl groups would be premature on the basis of the evidence presently available, one possibility is that they provide an acid–base function which assists in the electron transfers that are postulated to occur during catalysis of decarboxylation by this enzyme (Riley and Snell, 1968; Recsei and Snell, 1970). This role is in accord with the observed pK_a of 9.2 describing their ionization and is consistent with the kinetic studies of Recsei and Snell (1970) which showed that ionizable groups with pK_a values between 2.9 and 7.6 cannot play a major cat-

[2] A similar interpretation has been invoked by Kemp and Forest (1968) to explain the high reactivity of a single SH group of phosphofructokinase toward DTNB on the basis of an abnormally low pK_a value (pK_a about 6).

alytic role in decarboxylation of histidine. A similar participation by other residues with functional groups of high pK_a (e.g., lysine or tyrosine), of course, cannot be ruled out.

A pH-dependent shift in the conformational state of histidine decarboxylase is indicated by the effect of pH on the number of DTNB-reactive SH groups of the enzyme. This transition occurs sharply at pH 6.9 and results in the exposure of the five class-I thiols to solvent (Figure 5A). This interpretation is consistent with previous observations. For example, an opening of the protein structure above neutral pH is indicated by a decrease in the sedimentation coefficient of the enzyme from 9.2 S at pH 4.8 to 7.6 S at pH 8.5 (Chang and Snell, 1968a). That this pH-dependent conformational change may be accompanied by the appearance of cooperative subunit interactions within the enzyme decamer is suggested by the shift from Michaelis–Menten to sigmoidal kinetics without appreciable change in maximal velocity that occurs at pH values above 7.0 (Recsei and Snell, 1970).

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