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# The Hydrogen Ion Equilibria of Chicken Heart Lactic Dehydrogenase*

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ABSTRACT: The electrometric titration of chicken heart lactic dehydrogenase (CHLDH) alone between pH 4.30 and 11.00 is compatible with the titration of practically all the carboxyl and lysyl groups of the enzyme, while only about one-third of the histidyl and tyrosyl groups are titrated. However, all the ionizable groups seem to titrate with plausible p$K$ values in the acid-denatured enzyme. The pH dependence of the reaction between CHLDH and *p*-nitrophenyl acetate is consistent with the ionization of about ten histidyl residues having a p$K$ of 7.00. In the presence of reduced diphosphopyridine nucleotide (DPNH), $DPN^+$, or 3-acetylpyridine analog of $DPN^+$ there is a shift of the electrometric titration curve toward the alkaline side. This finding confirms the results of the spectrophotometric titration of CHLDH in the presence of coenzymes. The reaction of chicken heart lactic dehydrogenase with *p*-nitrophenyl acetate in the presence of DPNH is consistent with the involvement of two classes of histidyl residues, one having a $k_2$ of 5.2 l. mole$^{-1}$ min$^{-1}$ and the other having a $k_2$ of 21.0 l. mole$^{-1}$ min$^{-1}$. It is postulated that steric phenomena are responsible for this change in the $k_2$ of the histidyl groups.

The study of the hydrogen ion equilibria of chicken heart lactic dehydrogenase (CHLDH)[1] alone and in the presence of coenzymes or coenzyme analogs should help to elucidate the nature of the binding site(s) of the coenzymes to lactic dehydrogenases. With this in mind, a titration curve between pH 4.30 and 11.00 was obtained for native CHLDH, alone and in the presence of $DNP^+$, DPNH, or $APDPN^+$, and between pH 2.00 and 12.00 for acid-denatured CHLDH. Specific methods were applied to determine the amide, histidine, and tyrosine content of the enzyme.

## Materials and Methods

CHLDH was dialyzed at 4° for about 48 hr against several changes of a suitable solvent. Enzyme concentrations were measured from the optical density at 280 m$\mu$ applying a molar extinction coefficient of $1.8 \times 10^5$; the adopted molecular weight was 140,000. Ovalbumin was prepared according to Sorensen (1917). PNPA was prepared according to the method of Chattaway (1931). Nessler reagent was purchased from Fisher Scientific Co. $DPN^+$ and DPNH were purchased from C. F. Boeringer and Sons, Mannheim, Germany.

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[1] The following abbreviations were used: CHLDH, chicken heart lactic dehydrogenase; $DPN^+$ and DPNH, oxidized and reduced diphosphopyridine nucleotide; $APDPN^+$, 3-acetylpyridine analog of $DPN^+$; PNPA, *p*-nitrophenyl acetate; PMB: *p*-mercuribenzoate.

APDPN+ was obtained from P. L. Biochemicals, Inc. All other compounds were reagent grade. The solutions used in the electrometric titrations were made up with water which was freed from $CO_2$ by boiling it for 15–20 min. The normality of the KOH solutions used in the titration experiments was determined by titration to neutrality with a standard 0.1 N solution of HCl. This solution of KOH was used to determine the normality of the HCl solutions also used in these experiments. Constriction pipets (H. E. Petersen, Copenhagen, Denmark) were used throughout this work. The pH meters used for the titrations were of the type TTTla and type 25 (both equipped with scale expander) produced by Radiometer, Copenhagen, Denmark. The instruments were calibrated at 21° with 0.1 M sodium citrate at pH 4.96, with 0.05 M sodium borate at pH 9.20, and with the Radiometer precision buffer at pH 7.38 and 38°. The titration assembly consisted of a temperature-controlled, closed glass vessel having four holes in its lid for the glass electrode, the calomel electrode, the nitrogen inlet, and a thin polyethylene tubing from which the titrating solution was delivered manually by means of an "Agla" micrometer syringe. In a typical experiment of electrometric titration, a sample of CHLDH (about 15–20 mg of protein in 1.80 ml of 0.2 M KCl) was placed in the reaction vessel and flushed with nitrogen for 15–20 min before starting the titration. The titration was carried out at 21 ± 0.1° under nitrogen atmosphere by adding small amounts of about 0.2 N KOH or HCl (both in 0.2 M KCl) with magnetic stirring. Readings were taken usually every 0.2 pH unit. Suitable blanks were titrated before and after each set of measurements. Spectrophotometric titration of the tyrosyl groups was carried out as previously described (Di Sabato, 1965). Counting of groups was carried out as suggested by Tanford (1962). The empirical value of the electrostatic interaction factor ($w$) and the intrinsic p$K$ (p$K_{int}$) of any one type of titratable groups were calculated from the slopes and the intercepts at zero net charge, respectively, of plots of the left-hand side of the equation $\text{pH} - \log [x_i/(1 - x_i)] = \text{p}K_{int} - 0.868w\bar{Z}$ *vs.* $\bar{Z}$, where $x_i$ is the average degree of dissociation of any one titratable group of type i and $\bar{Z}$ is the average net charge per protein molecule. The theoretical value of $w$ was calculated from the equation $w = (\epsilon^2/DRkT)[1 - (KR/(1 + Ka))]$, where $\epsilon$ is the unit of electron charge, $D$ is the dielectric constant, $R$ is the radius of the sphere which represents the protein molecule, $a$ is the radial distance of closest approach to the center of this sphere of the center of the average ion of the salt which is being used to create the ionic strength and has been taken equal to $R + 2.5$ A, $k$ is the Boltzmann constant, $T$ is the temperature, and $K$ is the Debye–Hückel parameter (*cf.* Tanford, 1962).

Amide analysis was performed by first digesting CHLDH with 3 N $H_2SO_4$ at 110° for 4, 9, and 22 hr. Microdiffusion of the amide ammonia was carried out in plastic Conway dishes. The inner chamber contained 1.0 ml of 0.02 N HCl and the outer chamber contained 0.5–0.8 ml (1.5–2.0 mg) of protein digest,

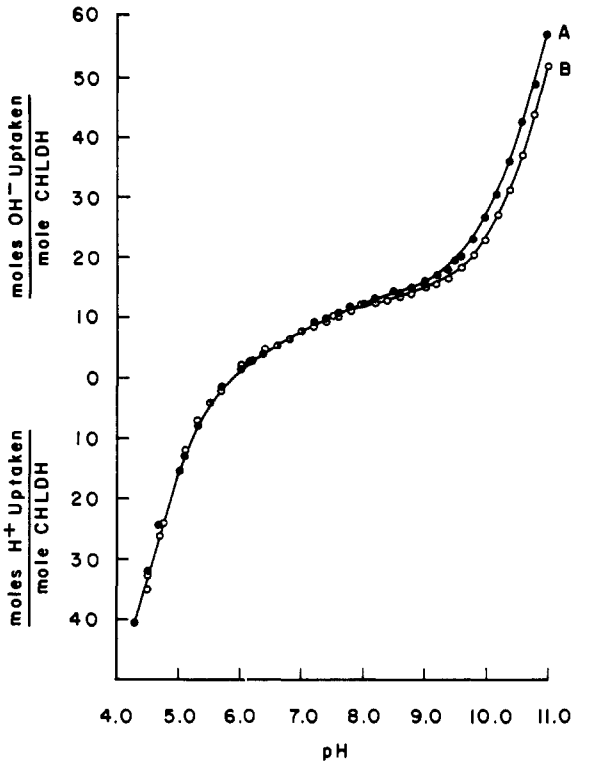


FIGURE 1: Titration curve of CHLDH alone (curve A) and CHLDH and DPNH (curve B) in 0.2 M KCl. CHLDH, 7–8 × $10^{-5}$ M; DPNH, 1.3 × $10^{-3}$ M; temperature, 21.0°.

to which enough KOH to neutralize $H_2SO_4$ and 1.0 ml of a saturated solution of potassium metaborate containing 10% KOH were added (Conway, 1962). Diffusion of the ammonia into HCl was usually allowed to proceed for 5 hr. Then 1.80 ml of water and 0.30 ml of Nessler reagent were added to the content of the inner chamber. The color intensity was read at 520 mμ in a Zeiss spectrophotometer *vs.* a blank containing all the ingredients except the enzyme digest. No difference was detected by varying the diffusion time from 5 to 10 hr. Ammonia analyses of standard solutions of $NH_4Cl$ were run in parallel with the actual amide determinations.

The reaction between CHLDH and PNPA was carried out at 21° in a Zeiss spectrophotometer with 2–6 × $10^{-6}$ M enzyme and 1 × $10^{-3}$ or 1 × $10^{-4}$ M PNPA in 0.14 M sodium phosphate buffer. The splitting of PNPA was measured from the increase in optical density at 400 mμ; the reaction was followed for 30–300 min depending on its velocity. First-order rate constants were calculated from the slopes of linear plots of $\log (a/(a - x))$ *vs.* time, where $a$ is the optical density obtained when PNPA is completely split into *p*-nitrophenolate ion (end point) and $x$ is the optical density given at 400 mμ by *p*-nitrophenolate ion at each time of the reaction after correction for spontaneous hydrolysis of PNPA in the same experimental

TABLE I: Parameters Used to Compute the Theoretical Curves.

| | Carboxyl | | Histidine | | $NH_2$ Terminal | | Tyrosine | | Lysine | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $n_i$[a] | $pK_{int}$ | $n_i$[a] | $pK_{int}$ | $n_i$[a] | $pK_{int}$ | $n_i$[a] | $pK_{int}$ | $n_i$[a] | $pK_{int}$ |
| CHLDH alone[b] | 120 | 4.26 | 10 | 7.00 | | | 11 | 9.94 | 90 | 10.60 |
| CHLDH + DPNH[b] | 120 | 4.30 | 10 | 7.00 | | | 11 | 10.38 | 90 | 10.73 |
| CHLDH + $DPN^+$[b] | 120 | 4.23 | 10 | 7.00 | | | 11 | 10.03 | 90 | 10.75 |
| CHLDH + $APDPN^+$[b] | 120 | 4.32 | 10 | 7.00 | | | 11 | 10.14 | 90 | 10.71 |
| Denatured CHLDH | 120 | 4.10 | 30 | 6.00 | 4 | 7.80 | 30 | 9.80 | 90 | 10.60 |
| CHLDH[c] | 125[d] | | 30 | | | | 30 | | 95 | |

[a] $n_i$ is expressed in moles of amino acid residue per mole of CHLDH. [b] $w = 0.007$ for the acid branch; $w = 0.011$ for the alkaline branch. For further details see text. [c] Values obtained from amino acid analysis. [d] Values corrected for amide (asparagine + glutamine) content (118 residues/molecule of CHLDH). For details see text.

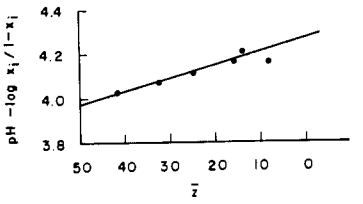


FIGURE 2: Plot of $pH - \log[x/(1 - x_i)]$ *vs.* $\bar{Z}$ for carboxyl groups of CHLDH titrated in 0.2 M KCl. Same experimental conditions as reported in Figure 1.

conditions, in the absence of enzyme. The optical density at the end point of the reaction was determined in separate experiments by completely splitting PNPA in 1 N KOH and by adding a small amount of this solution to a cuvet containing the buffer at the pH at which the reaction between CHLDH and PNPA was carried out. Second-order rate constants were calculated by dividing the first-order rate constants by the molar concentration of the enzyme.

PMB was treated with CHLDH in the same experimental conditions as already described (Di Sabato and Kaplan, 1963). The enzyme was $8 \times 10^{-6}$ M; PMB was $1.8 \times 10^{-4}$ M.

## Results

To explore the accuracy of the electrometric titration, preliminary experiments were carried out by titrating ovalbumin (about 20 mg) in 0.1 M KCl. Good agreement was found between our results and those published by Cannan *et al.* (1941).

A solution of CHLDH containing 15–20 mg of enzyme in 1.80 ml of 0.2 M KCl has a pH of 5.80. No attempt was made to determine the "true" isoionic point of the protein because of its insolubility in distilled water.

Curve A of Figure 1 shows a titration of CHLDH in 0.2 M KCl. The titration was reversible between pH 11.00 and 5.80 and between pH 4. 0 and 5.80. The alkali uptake became time dependent and the titration was irreversible at about pH 11.3–11.4, possibly because of denaturation of the protein. Similarly, time-dependent uptake of acid was noticed at pH 4.3–4.0. Also in this case the titration became irreversible and turbidity appeared.

Curve B of Figure 1 shows the titration of CHLDH in the presence of DPNH. Similar titrations (not shown) of CHLDH in the presence of $DPN^+$ or $APDPN^+$ were also carried out. Also in the presence of coenzymes the titrations were started from pH 5.80. The pH of the solutions was adjusted to this value whenever necessary. In the presence of coenzymes, the pH at which the acid uptake began to be time dependent was 0.4–0.6 unit lower than in the titration alone.[2]

The curves shown in Figure 1 are theoretical. They were computed to fit the experimental points as described under Materials and Methods, using the parameters reported in Table I and assuming that no ions other than $H^+$ are bound to the protein. The experiments in the presence of $DPN^+$ or $APDPN^+$ showed an agreement between theoretical curves and experimental points as good as or better than the experiments reported in Figure 1. However, it should be pointed out that the parameters reported in Table I do not provide a unique solution of fitting the experimental data of Figure 1. Empirical electrostatic factors ($w$) of 0.007 and 0.011 were used to compute the acid and alkaline branch, respectively, of the titration curves. Figure 2 shows the plot from which $w$ factor and $pK_{int}$ were calculated for the computation of the theoretical line corresponding to the titration of the carboxyl groups of CHLDH alone. Figure 3 shows

[2] The enzyme in the presence of DPNH was titrated between pH 5.80 and 4.30 in less than 5 min. On the other hand, it was shown in separate experiments that the half-time for the destruction of DPNH at pH 4.0 is about 11 min. Therefore, we feel that most of the DPNH is not destroyed over the pH range of the acid titration of the enzyme.

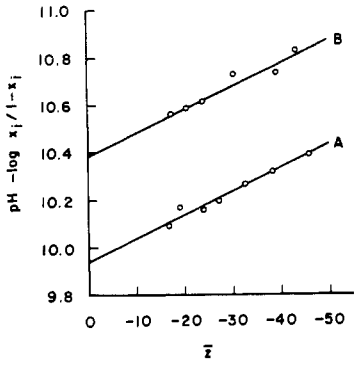


FIGURE 3: Plot of pH − log $[x_i/(1 - x_i)]$ *vs.* $\bar{Z}$ for tyrosyl groups of CHLDH alone (curve A) and CHLDH plus DPNH (curve B) titrated in 0.2 M KCl. Same experimental conditions as reported in Figure 1.

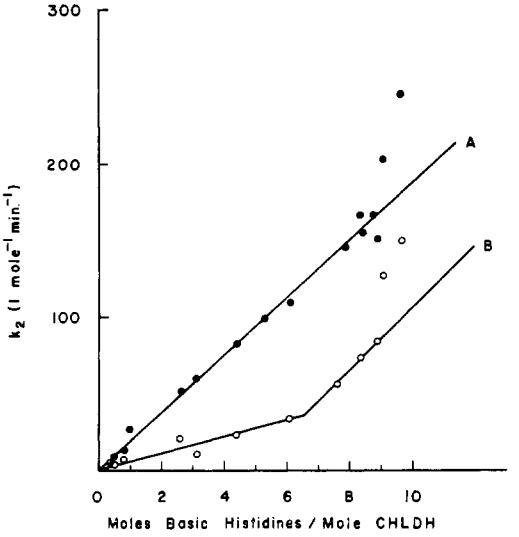


FIGURE 4: Second-order rate constant for the reaction of CHLDH alone (curve A) and CHLDH plus DPNH (curve B) with PNPA as a function of the molar content of basic histidyl residues per mole of CHLDH. For further details see text. CHLDH, 2-6 × $10^{-6}$ M, PNPA, 1 × $10^{-4}$ and 1 × $10^{-3}$ M, 0.14 M sodium phosphate buffer; temperature, 21.0°.

similar plots for the spectrophotometric titration of CHLDH alone (curve A) and CHLDH in the presence of DPNH (curve B). The theoretical *w* factor for the molecule of CHLDH was calculated to be 0.014 on the basis of a molecular radius of 37.9 A. A few titrations of CHLDH alone were carried out in 1.0 M KCl. The empirical *w* factor for the alkaline branch was 0.010; no value could be obtained for the acid branch of the titration curve owing to instability of the enzyme at pH values below about 5.0. The theoretical *w* factor for the enzyme in 1.0 M KCl was calculated to be 0.012.

A titration curve of acid-denatured CHLDH between pH 2 and 12 was also obtained. The theoretical line was calculated on the basis of the number of titratable residues found by amino acid analysis and on the basis of plausible values of p*K*. These parameters are reported in Table I. The number of residues of each ionizable type, as determined by amino acid analysis, was also recorded for comparison. It should be pointed out that the $n_i$ value reported in Table I for the carboxyl groups of CHLDH, as found by amino acid analysis, has been obtained by subtracting the amide content of the enzyme from the combined aspartic and glutamic acid content. About 118 amide residues (glutamine + asparagine) were found to be present per molecule of CHLDH and this amount was the same, within experimental error, at 4, 9, and 22 hr of digestion of the enzyme with $H_2SO_4$.

The data obtained from the spectrophotometric titration of the tyrosyl residues were used to calculate the alkaline branch of the titration curves. Spectrophotometric titration curves of the tyrosyl residues of CHLDH alone and in the presence of DPNH or 3-acetylpyridine analog of DPNH have already been published (Di Sabato, 1965). It should be pointed out that in that paper the apparent p*K* (p$K_{app}$) of the tyrosyl groups was given.

The data in Table I show that only about one-third of the histidyl groups of CHLDH ionize with a normal p*K*. In order to have more information on this point, the reaction between PNPA and CHLDH was studied at different pH values. The advantage of this method lies in the fact that uncharged (basic) amino groups and charged thiol groups react with PNPA, while the carboxyl groups are relatively inactive (Koltun *et al.*, 1958, 1959; Jencks and Carriuolo, 1960). Thus, the method is a means of measuring the ionization of the histidyl groups at pH values at which the fraction of un-ionized primary amino groups and of ionized thiol groups is negligible (Breslow and Gurd, 1962). Curve A of Figure 4 is a plot of the second-order rate constant (per mole of enzyme) for the reaction between PNPA and CHLDH *vs.* the average number of uncharged histidine residues present per molecule of enzyme. This number was calculated on the basis of the parameters reported in Table I for the histidyl residues of CHLDH ($n_i$ = 10 and p$K_{int}$ = 7.00). It is evident that a linear relationship exists between the second-order rate constant for the reaction between PNPA and CHLDH and the amount of basic histidyl residues present in CHLDH up to about nine histidyl residues, corresponding to a pH of about 8.0. Above this pH, $k_2$ rises sharply, as is to be expected for the involvement of the primary amino groups and/or of the thiol groups of the enzyme in the reaction with PNPA. On the other hand, the fact that the line passes through the origin is consistent with the assumption that the carboxyl groups do not appreciably contribute to $k_2$. From the slope of the line, it may be calculated that the average $k_2$ per basic histidine group is 18.7 l. mole$^{-1}$ min$^{-1}$. This value may be compared with that of 28.3 l. mole$^{-1}$ min$^{-1}$ reported by Bender and

Turnquest (1957) for the reaction between imidazole (p$K$ = 7.04) and PNPA; Jencks (1958) reports a value of 29.4 l. mole$^{-1}$ min$^{-1}$ for the same reaction. When the reaction between PNPA and CHLDH was carried out in the presence of DPNH, about six histidine residues/mole of CHLDH seemed to react with PNPA with an average $k_2$ of 5.2 l. mole$^{-1}$ min$^{-1}$ and about three histidine residues with an average $k_2$ of 21.0 l. mole$^{-1}$ min$^{-1}$ (curve B of Figure 2). Also in the presence of DPNH there is a sharp rise in the second-order rate constant above pH about 8.0. About 10–15% loss in enzymatic activity was detected upon incubation of CHLDH with PNPA for 24 hr at pH 6.90. A two- to threefold increase in the enzyme concentration does not increase the second-order rate constant by more than 10%, at pH 6.90, 7.20, 7.49, and 7.75. This demonstrates that the rate of the reaction between CHLDH and PNPA does not depend strongly on enzyme concentration. Moreover, the rate constant varies within the limits of experimental error upon a tenfold variation of the PNPA concentration at pH 6.90, 7.20, and 7.87. In two experiments carried out at pH 6.90, for example, the rate of splitting of PNPA by CHLDH was followed for 120 min; the enzyme was $7.0 \times 10^{-6}$ M in both cases and PNPA was $1 \times 10^{-3}$ and $1 \times 10^{-4}$ M, respectively. The second-order rate constants were found to be 80 l. mole$^{-1}$ min$^{-1}$ at the higher PNPA concentration and 84 l. mole$^{-1}$ min$^{-1}$ at the lower PNPA concentration. In the first case one molecule of enzyme had split on the average about seven molecules of PNPA after 120 min, while in the second case about 0.7 molecule of PNPA was split by one molecule of CHLDH; in both cases, however, the rates were linear for the duration of the experiment. The relative independence of $k_2$ for the reaction between PNPA and CHLDH in spite of the wide variations of the PNPA/CHLDH ratio indicates the essentially catalytic nature of the process.

In some experiments, about 8 moles of PMB was allowed to bind/mole of CHLDH at pH 6.90; the PMB-bound enzyme was then treated with PNPA at pH 6.70; the $k_2$ for this reaction was found to be 90 l. mole$^{-1}$ min$^{-1}$, while the corresponding $k_2$ for the reaction between PMB-untreated CHLDH and PNPA was found to be 65 l. mole$^{-1}$ min$^{-1}$. A correction was applied in these experiments for the increased splitting of PNPA occurring in the presence of PMB. Essentially the same data have been obtained at pH 6.90 with enzyme in which about four sulfhydryl groups were blocked with PMB.

## Discussion

The titration curve of native CHLDH was limited to the range between pH 4.30 and 11.00 by the stability of the enzyme. Over this pH range only part of the carboxyl and lysyl groups were titrated. Therefore, the parameters we have used in the calculation of the ionization of these two types of groups must be taken with some reservation. However, the parameters reported in Table I probably describe the titration curve of the enzyme also below pH 4.30 and above pH 11.00. This is indicated by the good fitting of the theoretical curve to the experimental points and by the fact that the number of carboxyl and lysyl groups assumed to ionize in the native enzyme is the same as in the denatured enzyme.

About 20 out of 30 histidyl residues of CHLDH do not titrate with a normal p$K$ value. It is possible that these groups are situated in hydrophobic regions and are involved in hydrogen bonding, so that they titrate only upon denaturation of the molecule. An alternative explanation is that the p$K$ of these histidyl groups is only perturbed by some pH units. In this case the histidine titration will interfere with that of other groups on the enzyme. If the histidyl groups are not completely buried, it is conceivable that these groups may "trigger" the denaturation of CHLDH when they are titrated.

A linear relationship exists between the second-order rate constant for the reaction CHLDH–PNPA and the moles of un-ionized histidyl residues present per mole of enzyme (Figure 4). On the other hand, the same theoretical parameters ($n_i$ = 10 and p$K_{int}$ = 7.00) have been used to calculate the amounts of un-ionized histidyl residues reported in the abscissa of Figure 4 and to fit the theoretical line to the experimental points of the neutral portion of the electrometric titration. It seems, therefore, that those groups which react with PNPA between pH 6.0 and 8.0 are the same as those which are titrated in this pH range in the electrometric titration. However, as already mentioned, the second-order constant for the splitting of PNPA by CHLDH rises sharply above about pH 8.0. This is very probably due to the reaction of the un-ionized primary amino groups and/or of the ionized thiol groups of the enzyme with PNPA.

It seems reasonable to attribute the splitting of PNPA by CHLDH to the histidyl residues of the enzyme rather than to other kinds of residues for the following reasons. (a) The reaction is catalytic in nature over most of the pH range tested (see Results). (b) The carboxyl groups of CHLDH do not show appreciable reaction with PNPA (*cf.* Koltun *et al.*, 1958, 1959; Jencks and Carriuolo, 1960). (c) A p$K_{int}$ of 7.00 is plausible for the histidyl residues of a protein (*cf.* Tanford, 1962). (d) The second-order rate constant for the reaction between PNPA and CHLDH is similar to that reported by Bender and Turnquest (1957) and Jencks (1958) for the reaction between PNPA and imidazole. Moreover the $k_2$ value of 18.7 l. mole$^{-1}$ min$^{-1}$, as its log 1.27, corresponds to a p$K$ of 7.05 in the Brønsted plot reported by Koltun *et al.* (1963) for the reaction of a series of imidazoles with PNPA. (e) The thiol group of cysteine reacts with PNPA about ten times faster than histidine (Ogilvie *et al.*, 1964). (f) The rate of the reaction between PNPA and PMB-bound CHLDH at pH 6.70 is about 40% faster than for the enzyme not treated with PMB. Although the reason for this increase in reactivity is uncertain, these experiments suggest that cysteine residues are not involved in the splitting of PNPA by CHLDH between

pH 6.0 and 8.0. In this respect it should also be pointed out that the p$K$ of the cysteine residues in proteins is about 9.0 (Tanford, 1962).

About 11 out of 30 tyrosyl groups are titrated in the native enzyme. This is shown both by the electrometric and the spectrophotometric titration curves. As already reported (Di Sabato, 1965) titration of practically all the tyrosyl groups present in CHLDH takes place in the alkali-denatured enzyme. It should be pointed out that the spectrophotometric titration of the tyrosyl groups and the electrometric titration become time dependent at the same pH; namely, at about pH 11.4. The considerations we have made on the abnormal titration of the histidyl residues also apply to the tyrosyl groups. An approximately equal number of histidyl and tyrosyl residues do not titrate with a normal p$K$. It is conceivable that these groups may be involved in tyrosylhistidyl hydrogen bonds, possibly in hydrophobic regions of the molecule.

Failure of trinitrobenzensulfonic acid to react with the $\alpha$-amino groups of native CHLDH under the same conditions in which reaction was detected with denatured CHLDH (G. Di Sabato, unpublished experiments) was the main reason why no titration of amino-terminal groups has been taken into consideration in the computation of the titration curve of native CHLDH. The linear dependence of the rate of PNPA splitting by CHLDH alone on the average amount of basic histidine residues present per mole of enzyme up to pH 8.0 is consistent with this view. If the four amino-terminal groups (one per each subunit) of CHLDH had been exposed to the solvent, one would expect a substantial fraction of them to be in the basic form, and therefore react with PNPA, at pH values below 8.0. It could be, however, that the amino-terminal groups of CHLDH, although exposed to the solvent, have a relatively high p$K$ or that their rate of reaction with PNPA is small compared to the rate of reaction of the histidyl residues.

Masking of histidyl and/or tyrosyl groups in proteins is not unusual. Steinhardt and Beychock (1964), who recently have reviewed a number of proteins from this point of view, report masking of a substantial number of histidyl residues in carbonic anhydrase, hemoglobin, sperm whale metmyoglobin, and peroxidase. Carbonic anhydrase, chymotrypsin, chymotrypsinogen, conalbumin, myosin, meromyosin, ovalbumin, ribonuclease, taka-amylase A, and trypsinogen are reported to have different fractions of their tyrosine content "buried" inside the protein structure.

An empirical $w$ factor of 0.011 has been used to fit the alkaline branch of the titration curve of native CHLDH to the experimental points. The theoretical $w$ factor has been calculated to be 0.014; a difference of about 20% must be considered within the error of calculation. On the other hand, the theoretical $w$ factor used to fit the acid branch of the titration curve was 0.007; a 50% difference is certainly outside the limits of error of the calculation. Conformational changes of the protein at acid pH, ion binding to the protein, and anomalous values of $pK_{int}$ are all possible explanations of this discrepancy.

The shift toward the alkaline side of the $pK_{int}$ of the tyrosyl groups of CHLDH in the presence of coenzymes has already been discussed in detail in a previous paper (Di Sabato, 1965). It should be pointed out here that the binding of DPNH to the enzyme causes approximately the same shift in $pK_{app}$ (from 10.25 to 10.70) as in $pK_{int}$ (from 9.94 to 10.38).

In the presence of DPNH three out of nine histidyl groups of CHLDH react with PNPA with an average $k_2$ of 21.0 l. mole$^{-1}$ min$^{-1}$ which is similar to the average $k_2$ of 18.7 l. mole$^{-1}$ min$^{-1}$ for the reaction between CHLDH alone and PNPA. The other six histidyl groups of CHLDH react with PNPA with an average $k_2$ of 5.2 l. mole$^{-1}$ min$^{-1}$. On the other hand, no difference could be detected in the pH range 6–8 between the electrometric titration of CHLDH alone and that of CHLDH and DPNH. We are, therefore, inclined to believe that the six histidyl residues reacting slowly with PNPA become partially masked when DPNH binds to the enzyme. The steric factor deriving from this partial masking is not evident in the electrometric titration because of the very small steric requirement of the proton. However it becomes evident in the reaction with PNPA owing to the greater steric requirement of this compound. It is difficult to decide if this masking is due to hindrance of the histidyl groups of the enzyme by the bound coenzyme or to conformational changes taking place in the enzyme molecule upon binding of the coenzyme (Di Sabato and Kaplan, 1964, 1965; Di Sabato and Ottesen, 1965).

It is possible that shifts in p$K$ and masking of a limited number of carboxyl and/or lysyl groups also take place upon binding of the coenzymes to CHLDH. However, these phenomena would be difficult to detect owing to the large number of carboxyl and lysyl groups present in CHLDH. For this reason, the small differences in $n_i$ and $pK_{int}$ of the carboxyl and lysyl residues in the absence and in the presence of coenzymes (Table I) are difficult to interpret.

In conclusion, the most important findings reported in this paper are the following. The electrometric titration shows that about two-thirds of the tyrosyl and histidyl residues of CHLDH titrate with an abnormal p$K$. The spectrophotometric titration (Di Sabato, 1965) and the results of the reaction of PNPA with CHLDH are consistent with these findings. The shift in $pK_{app}$ of the tyrosyl groups upon binding of coenzymes shown by spectrophotometric titration (Di Sabato, 1965) has been confirmed by electrometric titration. The binding of DPNH to the enzyme modifies the reactivity of the histidyl groups of the enzyme toward PNPA, probably because of steric phenomena.

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# Formation of an Adduct with Tris(hydroxymethyl)aminomethane during the Photooxidation of Deoxyribonucleic Acid and Guanine Derivatives*

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ABSTRACT: Rabbits immunized with deoxyribonucleic acid (DNA) photooxidized in Tris buffer in the presence of methylene blue and complexed with methylated bovine serum albumin produced antibodies specific for a photoproduct. The lack of serologic activity of DNA photoxidized in bicarbonate buffer suggested that Tris was participating in the formation of the photoproduct. Using the specific antibody in conjunction with suitable radioisotopic and electrophoretic techniques, it was shown that (1) Tris is incorporated into DNA during photooxidation, and (2) guanosine 5′-phosphate photooxidized in Tris gives three electrophoretically separable products. Two of these photoproducts have incorporated Tris into their structures, but only one of these Tris adducts is capable of inhibiting the immune reaction. The ability of photooxidized guanine residues to condense with molecules such as Tris may permit the controlled modification of DNA *in vitro* but will make photodynamic studies carried out *in vivo* difficult to interpret without knowledge of the intracellular macromolecular and ionic environment surrounding the photooxidized residues.

The chemical structure of the photoproducts which are produced in nucleic acids irradiated with visible light in the presence of methylene blue and $O_2$ has yet to be described, although evidence implicating destruction of the guanine residue exists (Simon and Van Vunakis, 1962; Wacker *et al.*, 1963; Simon, 1963). When rabbits were immunized with complexes formed between methylated bovine serum albumin and deoxyribonucleic acid (DNA) photooxidized in the presence of methylene blue and 0.1 M Tris buffer, pH 8.5, antibodies specific for an altered guanine photoproduct were produced (Seaman *et al.*, 1965, 1966). At appropriate antiserum dilutions, there is no serologic reaction between anti-$DNA_{(PO\text{-}Tris)}$[1] and nonirradiated DNA, and the serologic activities of both native and denatured DNA increase with time of irradiation. During the course of these studies, it became apparent that

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