thioester and an associate oxidation–reduction cofactor (*e.g.*, NAD), neither of which is dissociable from the enzyme surface. This proposal would require either two different reversible, direct, stereospecific hydride transfers or one reversible, direct, nonspecific hydride transfer. The direct internal hydride shift proposal is considered most likely at this time since it is analogous with the glyoxalase model system.

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The Role of the Tyrosyl Groups on the Mechanism of Action of Chicken Heart Lactic Dehydrogenase*

Giovanni Di Sabato†

ABSTRACT: About 10 of the 30 tyrosyl residues of chicken heart lactic dehydrogenase have been found free to titrat between pH 8 and pH about 11, with an average pK_{app} of 10.25. Addition of reduced diphosphopyridine nucleotide or its acetylpyridine analog shifts the pK_{app} of the free tyrosines to 10.70 and 10.90, respectively. Iodination of chicken heart lactic dehydrogenase causes a loss of enzymatic activity which is practically complete when about 4 moles of diiodotyrosine and 1–2 moles of monoiodotyrosine have been formed per mole of chicken heart lactic dehydrogenase. Reduced diphosphopyridine nucleotide seems to protect chicken heart lactic dehydrogenase from loss of activity due to the addition of iodine. Reduced diphosphopyridine nucleotide does not protect the iodinated enzyme from dissociation into subunits by sodium dodecyl sulfate. The iodinated enzyme is not denatured, as shown by its normal sedimentation coefficient, specific optical rotation, and rate of binding of *p*-mercuribenzoate. Moreover tyrosine is the only amino acid residue of chicken heart lactic dehydrogenase which reacts in substantial amounts with iodine. The evidence points to the tyrosyl residues of the enzyme as forming a part of the site of binding of the coenzymes.

Evidence has been provided (Di Sabato and Kaplan, 1963) for the involvement of four sulfhydryl residues in the binding of pyridine nucleotide coenzymes to lactic dehydrogenases. In an attempt to investigate if other amino acid residues participate in this binding, a study of the free and "buried" groups of chicken heart lactic dehydrogenase (CHLDH)[1] and their relationships with the pyridine nucleotide coenzymes has been undertaken. The present paper deals with the role played by the tyrosyl residues in the mechanism of action of CHLDH.

Iodination of lactic dehydrogenase from beef heart has been attempted by Neilands (1954) and by Nygaard (1956). These authors obtained some evidence for the binding of iodine to the sulfhydryl groups of the enzyme. More recently, Caplow (1961) studied the effect of iodination of beef heart lactic dehydrogenase on the fluorescence of the enzyme and found a decreased interaction between the iodinated enzyme and acetylpyridine analog of reduced diphosphopyridine nucleotide (AcPyDPNH). Unlike the above mentioned authors, who carried out the iodination in phosphate buffer near neutrality, we carried out the iodination of CHLDH at pH 9.5 according to the general procedure outlined by Hughes and Straessle (1950) by which maximal specificity in the iodination of the tyrosyl residues should be obtained.

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[1] The following abbreviations have been used: CHLDH; chicken heart lactic dehydrogenase; DPN^+, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; AcPyDPNH, 3-acetylpyridine analog of DPNH; SDS; sodium dodecyl sulfate; PCMB, *p*-mercuribenzoate.

Materials and Methods

CHLDH (L-lactate:diphosphopyridine nucleotide (DPN^+) oxidoreductase, EC 1.1.1.27), as used in previous studies (Di Sabato and Kaplan, 1963; Di Sabato and Kaplan, 1964; Pesce *et al.*, 1964; Di Sabato and Ottesen, 1965), was obtained as an ammonium sulfate paste as a gift from Dr. N. O. Kaplan, Brandeis University. The paste was dissolved in 0.1 M KCl or 0.4 M glycine–NaOH buffer, pH 9.45, and dialyzed for about 48 hr at 4° against several changes of the same solvent. Enzyme concentrations were measured from the optical densities at 280 mμ applying a molar extinction coefficient of 1.8×10^5. However, the protein concentration was measured at the isosbestic point for tyrosine ionization [278 mμ] (ϵ_M 17,600) in the experiments on tyrosine ionization and at 269 mμ (ϵ_M 14,600) in the experiments with iodinated enzyme. Although at 269 mμ there is some optical density change in the conversion of tyrosine to monoiodotyrosine and of tyrosine to diiodotyrosine, the assumption of 269 mμ as isosbestic point in the iodination of tyrosine does not introduce more than 2% error in the determination of protein concentration. The adopted molecular weight of the enzyme was 140,000. Reduced diphosphopyridine nucleotide (DPNH) was a commercial preparation obtained from C. F. Boeringer and Sons, Mannheim, Germany. AcPyDPNH was prepared according to Kaplan and Stolzenbach (1957) and was a kind gift from Dr. N. O. Kaplan, Brandeis University. Diethylamine was redistilled before use. Sodium dodecyl sulfate (SDS) was obtained from E. I. du Pont de Nemours and Co. and was recrystallized twice from ethanol. *p*-Mercuribenzoate (PCMB) and 3-monoiodotyrosine were purchased from Sigma Chemical Co. 3,5-Diiodotyrosine was a Hoffman-La Roche product obtained through the courtesy of Dr. L. Korsgaard Christensen, Gentofte Hospital, Copenhagen, Denmark. All other reagents were analytical grade.

Spectra were taken in a Beckman spectrophotometer Model DU equipped with thermospacers in order to maintain the temperature at the required value. Cuvets (1 ml) (10-mm light path) were used. pH values were measured by means of a Radiometer pH meter type PHM 22 and/or type 25 equipped with a scale expander; the pH standardization was made with 0.1 M sodium citrate and 0.05 M sodium borate after Sørensen at the temperature of the experiment (Clark, 1926).

Ultracentrifugal experiments were run at 20–21° for 90–120 min at 59,780 rpm in a Spinco Model E ultracentrifuge with a schlieren optical system; 12-mm K-F cells were used. Sedimentation coefficients were corrected to water at 20°. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter using 1-dm cells thermoregulated at 21°.

Spectral readings were taken every 2–3 mμ between 260 and 370 mμ. The ionization of the tyrosyl residues of CHLDH was measured from the increase in optical density at 295 mμ *vs.* a blank cell containing no enzyme; an extinction coefficient of 2300/mole of ionized tyrosine was applied (Crammer and Neuberger, 1943). The buffers employed in these experiments were the following: 0.01 M Tris–HCl between pH 7.4 and 9.0, 0.01 M sodium phosphate between pH 6.7 and 7.0, 0.01 M glycine–NaOH between pH 8.2 and 10.0, 0.01 M diethylamine–HCl between pH 9.8 and 11.5, and 0.03 M diethylamine–HCl between pH 10.9 and 11.6. Differences in ionic strength due to different buffer composition were "swamped out" with 0.1 M KCl, which was present in every sample. Spectra of CHLDH in the presence of coenzyme were taken on the same samples used for the spectrum of CHLDH alone; the coenzyme was added to both the experimental and the blank cell. In calculating the extinction coefficients of these samples, a small correction (usually no more than 2%) for dilution due to the added coenzyme was applied.

The iodination of CHLDH was carried out at about 4° with continuous magnetic stirring by addition of small amounts (usually 0.05 ml every 10 min) of iodinating solution to $6–10 \times 10^{-5}$ M CHLDH in 0.4 M glycine–NaOH buffer, pH 9.45. The iodinating solution was prepared just before use by dissolving 12.6 mg of I_2 and 36 mg of KI in 100 ml of water. The iodinated enzyme was then dialyzed at about 4° for 3–4 days *vs.* 2–3 daily changes of 0.05 M sodium phosphate buffer, pH 8.22. Occasionally a faint turbidity appeared which was easily spun down. Small amounts of 0.2 M sodium dihydrogen phosphate or 0.1 M glycine–NaOH buffer were added in order to adjust the pH to the desired values. Spectra were usually taken between 260 and 330 mμ with readings every 2–3 mμ. The iodination of CHLDH in the presence of DPNH was carried out at about 4° with continuous magnetic stirring by 4 or 5 additions of 0.01 ml of iodinating solution (prepared as described above) every 5 min to 0.5 ml of about 3×10^{-6} M enzyme. Activities were measured after 35 min since the first addition of iodinating solution.

Enzymatic activities were measured from the reduction of pyruvate by the standard method already described (Di Sabato and Kaplan, 1963). PCMB titration was usually carried out at 25° according to Boyer (1954) and applying a molar extinction coefficient of 7.6×10^3 at 250 mμ for the mercaptide formation. The molar ratio PCMB/enzyme was 10. CHLDH was treated with SDS according to the procedure already described (Di Sabato and Kaplan, 1964). The molar ratio SDS/CHLDH was about 350. Amino acid analyses were carried out in a Beckman-Spinco Model 120 automatic amino acid analyzer. Acid hydrolysis was carried out in sealed evacuated tubes for 24 or 48 hr at 110° in 6 N HCl. Alkaline hydrolysis was performed according to Neumann *et al.* (1962) in 15% KOH for 16–24 hr at 110°.

Results

Figure 1 shows the increase in molar extinction coefficient at 295 mμ as a function of pH for CHLDH alone (curve A), CHLDH in the presence of DPNH (curve B) and in the presence of AcPyDPNH (curve C). The left ordinate represents the increase in molar extinction coefficient of the enzyme at 295 mμ over the extinction coefficient at pH 8; the right ordinate represents the

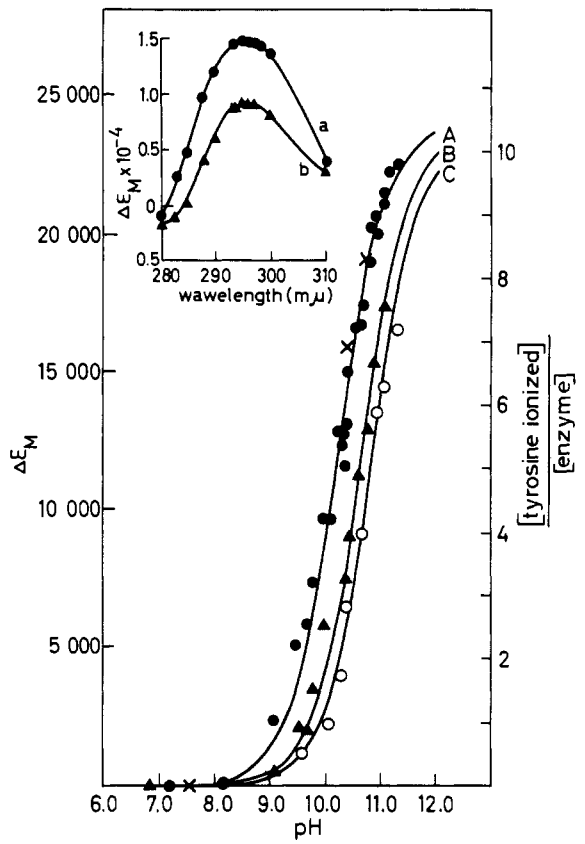

FIGURE 1: pH dependence of the ionization of the tyrosyl residues of CHLDH alone and in the presence of coenzymes. Left ordinate: increase in ϵ_M at 295 mμ over the value at neutral pH; right ordinate: number of tyrosyl residues ionized per mole of enzyme on the basis of ϵ_M 2300/mole of tyrosine. The enzyme ($5–8 \times 10^{-6}$ M) was originally in 0.1 M KCl. The buffers used (all 0.01 M, unless otherwise indicated) were: Tris–HCl, pH 7.4–9.0; sodium phosphate, pH 6.7–7.0; glycine–NaOH, pH 8.2–10.0; diethylamine–HCl, pH 9.8–11.5; 0.03 M diethylamine–HCl, pH 10.9–11.6. Curve A: CHLDH alone; curve B: CHLDH + 1.1–3.3×10^{-4} M DPNH; curve C: CHLDH + 0.6–1.5×10^{-4} M AcpyDPNH. Insert: difference spectrum between neutral pH and pH 10.50 for (a) CHLDH alone and (b) CHLDH + DPNH. Same experimental conditions as above.

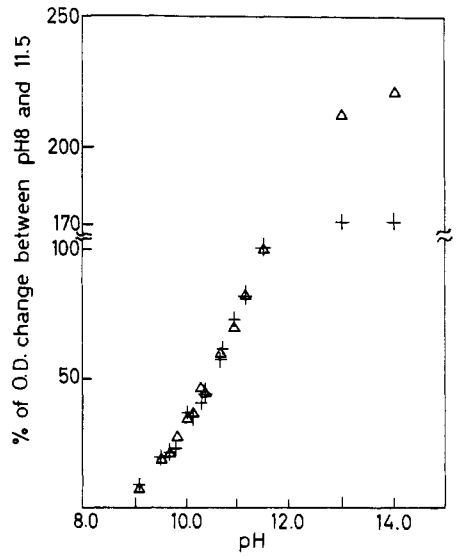

FIGURE 2: Relative increase of the optical density of CHLDH at 288 (+) and 295 mμ (△) in function of pH, expressed as a percentage of the change in optical density observed between neutral pH and pH 11.5. For further details see text. Same experimental conditions as in Figure 1.

moles of tyrosine ionized per mole of enzyme, assuming an extinction coefficient of 2300/mole of ionized tyrosine. The curves in Figure 1 are theoretical; in fitting a theoretical curve to the experimental data several combinations of the two parameters, number of tyrosines ionizing and pK_{app}, were tried. Best agreement was obtained with the set, maximum ϵ_M 24,000 (10.4 tyrosines ionizing) and pK_{app} = 10.25. The maximum ϵ_M of 24,000 has also been used for calculating curves B and C with pK_{app} of 10.70 and 10.90, respectively. In obtaining the results shown in Figure 1, no differences were detected in the pH regions where two different buffers overlapped, or by increasing the concentration of the diethylamine–HCl buffer from 0.01 to 0.03 M. The points of curves B and C were obtained with CHLDH saturated with DPNH and AcPyDPNH, respectively. The saturation point was found by successive additions of coenzyme until no further spectral modifications at 295 mμ were detected. However, more than one addition of coenzyme was necessary only at the highest pH values.[2] The insert in Figure 1 shows that the maximum increase in optical density in the spectrophotometric titration of CHLDH both in the absence and in the presence of DPNH takes place at 294–296 mμ; moreover the maximum quenching in the presence of DPNH also takes place at around 295 mμ. Similar results have been obtained with AcPyDPNH. A few points of the reverse titration curve have also been determined, starting from pH 11.0 to 11.1, and they fall nicely on the forward titration curve (crosses in curve A of Figure 1). However, at pH values higher than about 11.3, the titration curve is not reversible. In a few experiments, CHLDH was back-titrated with glycine from pH 11.8 to 9.2. Twenty-nine tyrosyl residues were titrated in this pH range, of which 21 were between pH 11.3 and pH 9.2. In order to gain further evidence for the absence of denaturation of the enzyme at pH values lower than 11.3, the molar extinction coefficients of CHLDH at 288 and 295 mμ, expressed as per cent of the increase in

[2] In interpreting the curves in the presence of coenzymes, it should be pointed out that the decrease in optical density at 295 mμ cannot be due to an aspecific oxidation of the coenzymes by CHLDH, because the enzyme was shown in separate experiments to be completely deprived of this activity.

absorbancy occurring between neutral pH and pH 11.5, have been plotted *vs.* pH (Figure 2). It is evident that the increase in optical density as a function of pH is proportionally the same at the two wavelengths under consideration. Had the enzyme undergone denaturation, a perturbation of the spectrum of the tryptophan residues (288 mμ) would very probably have occurred with consequent spectral modifications unrelated to the tyrosine ionization (295 mμ). On the other hand, it is evident (Figure 2) that upon alkali denaturation of the enzyme a "dissociation" between the optical density at 288 and 295 mμ takes place.

While the experiments reported above were carried out at 21°, the tyrosine ionization was also investigated at 10.0 and 31.0°. The temperature dependence of the pK of the tyrosyl residues seems to be about 0.2 pH unit/10°; from this value the ΔH of ionization was calculated to be about 8 kcal and the ΔS of ionization about −18 eu. Similar experiments were carried out with CHLDH in the presence of DPNH; in this case the differences in pK seem to be smaller than in the absence of coenzyme.

In order to obtain more information about the role of the tyrosyl residues in the mechanism of action of CHLDH, the enzyme was treated with iodine which, under suitable experimental conditions, should specifically react with the tyrosyl residues (Hughes and Straessle, 1950). A change in pK of the phenolic groups of tyrosine is obtained upon iodination, monoiodotyrosine having a pK of 8.2 and diiodotyrosine a pK of 6.4 (Herriot, 1947). The observed loss in enzymatic activity was correlated to the spectral changes occurring at different pH values at 311 and 325 mμ. These two wavelengths were chosen for the following reasons: (a) at both of these wavelengths the spectral contribution from both ionized and un-ionized tyrosine is very small and therefore the optical density is due almost exclusively to mono- and diiodotyrosine; (b) the absorbtion maximum of diiodotyrosine is at 311 mμ; however, at this wavelength there is a strong spectral contribution from monoiodotyrosine; (c) the optical density at 325 mμ is contributed mainly by diiodotyrosine, and the monoiodotyrosine contribution does not amount to more than 15%.[3] Table I shows the differences in molar extinction coefficient at 311 and 325 mμ between iodinated and noniodinated CHLDH at various pH values for different samples at different degrees of inactivation. It is evident that the differences in extinction coefficients tend to level off at high pH (10.0–11.0). Unfortunately spectra at pH values lower than about 6.5 could not be taken because of turbidity developing in the samples.

Figure 3A shows the differences in molar extinction coefficients at 311 and 325 mμ between iodinated and noniodinated CHLDH at high pH (10.5–10.9) plotted *vs.* loss in enzymatic activity; it is evident that a linear relationship exists between the two quantities.

TABLE I: Difference Molar Extinction Coefficients at 311 and 325 mμ between Iodinated and Noniodinated CHLDH at Different pH Values.[a]

Activity Lost (%)	pH	$\Delta\epsilon_M \times 10^{-4}$ At 311 mμ	$\Delta\epsilon_M \times 10^{-4}$ At 325 mμ
10	9.45	0.30	0.16
	10.20	0.22	0.18
	10.58	0.39	0.26
27	6.70	0.25	0.15
	10.00	0.81	0.37
	10.66	0.75	0.35
36	9.45	1.08	..
	9.92	1.01	..
	10.58	1.11	0.80
59	6.50	0.38	0.20
	6.90	0.62	0.30
	7.40	0.63	0.42
	8.20	0.76	0.54
	9.50	1.49	0.82
	10.60	1.62	0.91
	10.92	1.62	0.95
64	6.90	0.71	0.94
	9.52	1.95	1.03
	10.40	1.96	1.02
	10.90	1.95	1.06
76	9.45	2.41	1.68
	10.45	2.43	1.70
87	6.90	1.58	1.31
	7.40	1.82	1.39
	8.29	2.08	1.56
	9.27	2.43	1.69
	10.46	2.58	1.81
	10.95	2.60	1.82
89	7.40	2.03	1.58
	8.20	2.25	1.66
	9.42	2.64	1.80
	10.59	2.68	1.85
95	6.90	1.27	0.86
	9.52	2.68	1.59
	10.40	3.15	2.09
	10.90	3.15	2.11

[a] The enzyme ($5–8 \times 10^{-6}$ M) was originally in 0.05 M sodium phosphate buffer, pH 8.22; lower pH values were obtained by addition of 0.2 M sodium dihydrogen phosphate, higher pH values by addition of 0.1 M glycine–NaOH buffer; temperature, 21°.

In order to estimate the amounts of mono- and diiodotyrosine formed at different degrees of enzyme inactivation, a pair of simultaneous equations of the type

$$M_1 = \frac{(k_a\epsilon_{2b}) - (k_b\epsilon_{2a})}{(\epsilon_{1a}\epsilon_{2b}) - (\epsilon_{2a}\epsilon_{1b})} \text{ and } M_2 = \frac{(k_b\epsilon_{1a}) - (k_a\epsilon_{1b})}{(\epsilon_{1a}\epsilon_{2b}) - (\epsilon_{2a}\epsilon_{1b})}$$

[3] The spectra of free mono- and diiodotyrosine were determined in separate experiments and they were found in accord with the spectra previously published (Herriot, 1948).

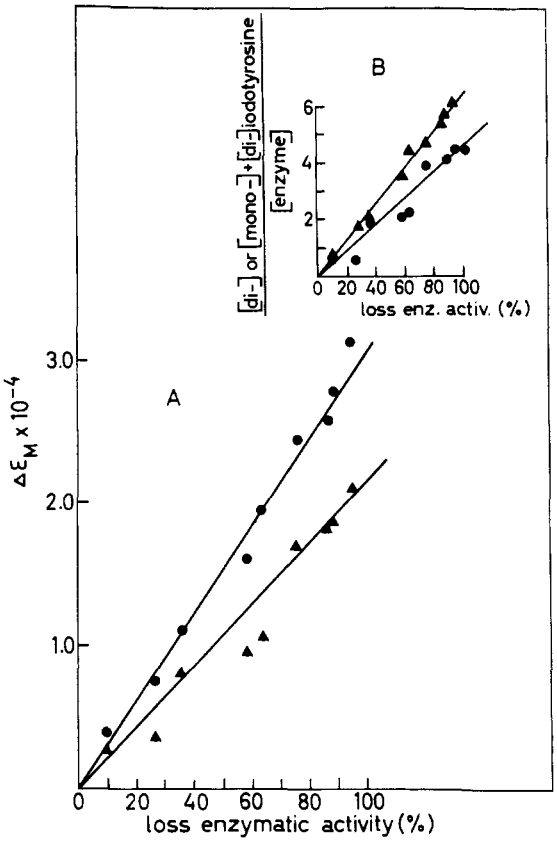

FIGURE 3: Dependence of the loss in enzymatic activity of CHLDH from the extent of iodination. A: loss in enzymatic activity of CHLDH as a function of the difference in molar extinction coefficient at high pH (10.5–10.9) between iodinated and noniodinated samples at 311 mμ (●) and 325 mμ (▲); B, loss in enzymatic activity of iodinated CHLDH as a function of the moles of diiodotyrosine (●) and mono- plus diiodotyrosine (▲) formed per mole of enzyme. For further details see text. Same experimental conditions as reported in Table I.

was solved (*cf.* Beaven and Holiday, 1952). In these equations, M_1 and M_2 are the gram moles of mono- and diiodotyrosine, respectively, per gram of enzyme; k_a and k_b are the difference in extinction coefficients (expressed in grams/liter) between iodinated and noniodinated CHLDH at high pH (10.5–10.9) at 311 and 325 mμ, respectively; ϵ_{1a} and ϵ_{2a} are the increases in molar extinction coefficients at 311 mμ for the ionization of mono- (3390) and diiodotyrosine[3] (5440); ϵ_{1b} and ϵ_{2b} are the increases in molar extinction coefficients at 325 mμ for the ionization of mono- (640) and diiodotyrosine[3] (4180). The results of these calculations are reported in Figure 3B, where the loss of enzymatic activity of iodinated CHLDH has been plotted *vs.* moles of diiodotyrosine and moles of mono- plus diiodotyrosine formed per mole of enzyme. The data clearly indicate the existence of a linear relationship between the amount of tyrosyl residues iodinated and loss in enzymatic activity of CHLDH.

In order to obtain a better insight into the problem of the role played by the tyrosyl residues in the mechanism of action of CHLDH, the enzyme was iodinated in the presence of different amounts of DPNH and the enzymatic activity was measured. The results of these experiments, carried out at two different degrees of iodination, are reported in Table II. Clearly, DPNH protects CHLDH from inactivation by iodine. However, it has been demonstrated in separate experiments that the addition of iodine to DPNH in 0.4 M glycine–NaOH buffer, pH 9.45, causes modifications of the DPNH spectrum in the 260- and 340-mμ regions; these spectral modifications, which are similar to those described by Kosower and Klinedinst (1956) for the methylpyridinium iodide complexes, have been interpreted as due to a DPNH–iodine complex. It could therefore be that less iodination of the enzyme occurs in the presence of DPNH simply because the coenzyme complexes iodine and thus less of it is available to iodinate the enzyme. However, if we assume that each molecule of DPNH reacts with one molecule of iodine, it is clear that in the experiments reported in Table II the bulk of the iodine was free and that the decrease in the loss of enzymatic activity observed in the presence of DPNH is significant.

TABLE II: Effect of DPNH on the Loss in Enzymatic Activity of CHLDH upon Iodination.[a]

[Iodine]/[CHLDH] Added	[DPNH]/ [CHLDH]	Loss of Enzymatic Activity (%)
27	0	70
	2	57
	4	32
	6	28
	10	13
34	0	75
	2	65
	4	55
	6	43
	10	36

[a] Medium, 0.4 M glycine–NaOH buffer, pH 9.45; enzyme, ~3 × 10^{-6} M; temperature of iodination, ~4°. For further details see text.

Di Sabato and Kaplan (1964) demonstrated that PCMB-bound CHLDH (unlike untreated CHLDH) is not protected by DPNH from dissociation by SDS. This was interpreted as due to the fact that DPNH could not bind to PCMB–CHLDH because the sulfhydryl groups of the enzyme were blocked by the mercurial

reagent, and the conclusion was therefore drawn that the sulfhydryl groups are part of the site of binding of DPNH to the enzyme. The same experimental approach was applied in the present paper in order to demonstrate the involvement of the tyrosyl residues in the binding of DPNH to the enzyme. With this in mind, iodinated and noniodinated enzyme were treated with SDS in the presence and in the absence of DPNH. In Figure 4 are shown the pictures of the ultracentrifugal runs of CHLDH alone (A), iodinated CHLDH alone (B), CHLDH + SDS (C), iodinated CHLDH + SDS (D), CHLDH + SDS + NADH (E), and iodinated CHLDH + SDS + NADH (F). It is evident that: (a) SDS dissociates both CHLDH (*cf.* Di Sabato and Kaplan, 1964) and iodinated CHLDH into more slowly sedimenting subunits; (b) DPNH partially protects CHLDH from such dissociation, as shown by the appearance of two peaks, the faster having a normal sedimentation coefficient (*cf.* Di Sabato and Kaplan, 1964); (c) DPNH does not protect iodinated CHLDH from dissociation by SDS, and the enzyme in these conditions has the same sedimentation coefficient as the slower peak of the noniodinated enzyme. The same results have been obtained both in 0.05 M sodium phosphate buffer, pH 8.22, and 0.06 M sodium phosphate buffer, pH 7.20. The sedimentation coefficients of the noniodinated CHLDH in these experimental conditions have already been reported (Di Sabato and Kaplan, 1964). Suffice it to state that they are practically the same for the iodinated enzyme.

It is well known that cysteine can react with iodine (Fraenkel-Conrat, 1959); it has also been demonstrated that the blocking with mercurial reagents of the four faster and homogeneously reacting sulfhydryl groups

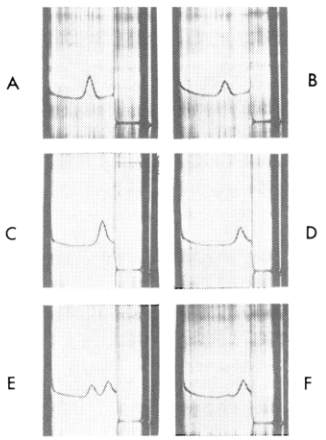

FIGURE 4: Ultracentrifugal patterns of CHLDH and iodinated CHLDH with and without SDS and DPNH. A, CHLDH; B, iodinated CHLDH; C, CHLDH + SDS; D, iodinated CHLDH + SDS; E, CHLDH + SDS + DPNH; F, iodinated CHLDH + SDS + DPNH. Medium, 0.05 M sodium phosphate buffer, pH 8.22; CHLDH, 2.1×10^{-5} M; DPNH, 1×10^{-3} M; runs performed at 20–21° and at 59,780 rpm; pictures taken 32 min after the ultracentrifuge had reached full speed; direction of sedimentation, from right to left.

of CHLDH causes practically complete inactivation of the enzyme (Di Sabato and Kaplan, 1963). In order to demonstrate that the inactivation of CHLDH upon iodination was not due to reaction of iodine with the sulfhydryl groups, we carried out a PCMB titration of the 3–4 faster reacting sulfhydryl groups of the iodinated enzyme. The data reported in Table III clearly show that the rate of binding of PCMB to iodinated CHLDH is, within experimental error, the same as for noniodinated enzyme. It was therefore concluded that iodine does not appreciably react with the sulfhydryl groups of CHLDH, under the adopted experimental conditions.

Reactions of iodine have also been described with histidine, tryptophan, and methionine (Koshland *et al.*, 1963). Histidine is converted into iodohistidine which is destroyed in substantial amounts when undergoing acid hydrolysis prior to amino acid analysis; tryptophan and methionine are oxidized, the latter to methionine sulfoxide. In Table IV are shown the histidine, tryptophan, and methionine contents of some samples of iodinated CHLDH; the differences found between iodinated and noniodinated samples are not larger for the more inactivated samples of iodinated CHLDH and they probably are within the limits of experimental error. It should be pointed out here that the methionine content has been determined in alkali-hydrolyzed samples in order to avoid the reversion of methionine sulfoxide to methionine (Ray and Koshland, 1962; Floyd *et al.*, 1963).

In these experiments the histidine and methionine contents were referred to the lysine and leucine con-

TABLE III: Binding of PCMB to CHLDH at Different Stages of Inactivation due to Iodination.[a]

Activity Lost upon Iodination (%)	Moles of PCMB Bound/Mole of Enzyme after	
	60 min	180 min
0	1.5	3.5
27	1.5	3.4
0	1.2[b]	2.9[b]
36	1.1[b]	2.8[b]
0	1.6	3.7
59	1.4	3.3
0	1.5	3.5
64	1.6	3.3
0	1.5	3.5
95	1.4	3.2

[a] Medium, 0.1 M sodium phosphate buffer, pH 6.9; CHLDH, 6×10^{-6} M; PCMB, 6×10^{-5} M; temperature, 25° unless otherwise indicated. [b] At 21°.

TABLE IV: Histidine, Tryptophan, and Methionine Content of Iodinated CHLDH.[a]

Activity Lost (%)	Histidine[b]	Tryptophan[c]	Methionine[d]
0	31[e] (30)[f]	24 (23)[f]	24[g] (25)[f]
27	31	...	...
36	30	...	...
59	...	23	...
64	...	23	...
76	31	...	...
87	...	...	24[g]
89	...	22	...
95	...	23	...

[a] Reported values are averages of 2–5 runs and are approximated to the nearest integer. [b] Values referred to lysine set to 100 residues/mole of enzyme (*cf.* Pesce *et al.*, 1964); 24-hr hydrolysis in 6 N HCl at 110°. [c] Measured by the method of Goodwin and Morton (1946). [d] Values referred to leucine set to 149 residues/mole of enzyme (*cf.* Pesce *et al.*, 1964); 16–24-hr alkali hydrolysis at 110° in 15% KOH according to Neumann *et al.* (1962). [e] Value unchanged after 48-hr hydrolysis. [f] Values found by Pesce *et al.* (1964). [g] From two analyses in two different samples.

TABLE V: Optical Rotation of CHLDH and Iodinated CHLDH.[a]

λ (mμ)	−[α] of CHLDH	−[α] of Iodinated CHLDH[b]
578	30	32
546	34	36
436	63	65

[a] Medium, 0.05 M sodium phosphate buffer, pH 8.22; CHLDH, 5–7 × 10^{-5} M; temperature, 21°. Values are averages of determinations on three different samples. [b] Enzyme 87–95% inactivated.

tents, respectively; moreover, the amino acid analyses and the spectrophotometric determination of tryptophan were carried out on the same day and in exactly the same experimental conditions in the iodinated and noniodinated enzyme. We trust that, by so doing, we obtained an accuracy of 5% or better, necessary in order to detect the loss of 1–2 amino acid residues/mole of enzyme.

Although some of the experiments reported above point to the absence of denaturation in the molecule of iodinated CHLDH, the optical rotation of the iodinated and noniodinated enzyme has been measured at different wavelengths. The results of the experiments, reported in Table V, show no substantial differences between the iodinated and noniodinated enzyme.

Discussion

The spectrophotometric titration of native CHLDH shows that about 10 tyrosines are free to ionize between neutral pH and pH 11.3. On the other hand, we could confirm the data of Pesce *et al.* (1964) by finding that about 30 tyrosines titrate in the alkali-denatured enzyme. Furthermore about 20 tyrosines are back-titrated in the denatured enzyme between pH 11.3 and 9.2. Since CHLDH is denatured at pH values higher than 11.3, one has to conclude that *only* a part of the 30 tyrosyl residues titrate normally in CHLDH and that the remainder titrate *only* upon denaturation of the enzyme, and that as many as about $^2/_3$ of the total number of the tyrosyl residues of CHLDH are "buried" in the structure of the native enzyme.

The spectral modifications found in iodinated CHLDH clearly indicate a reaction of the tyrosyl residues with iodine. However, the amount of iodine added to CHLDH is 2–3 times larger than the theoretical value calculated from the amount of mono- and diiodotyrosine formed (*cf.* Table II and Figure 3.) This is probably due to the formation of iodoglycine from the reaction between iodine and the glycine of the buffer present in the medium. Strong support to this interpretation is given by the fact that a 0.001 M solution of I_2 containing 0.002 M KI is colorless in 0.4 M NaOH–glycine buffer, pH 9.45, while it is yellow in 0.2 M sodium carbonate–bicarbonate buffer of the same pH. A similar reaction between iodine and ammonia has been described by McAlpine (1952). Since the reaction between iodine and amino compounds is easily reversible (McAlpine, 1952; Hughes, 1957), one might visualize a competition between the glycine of the buffer and the tyrosines of the enzyme for iodine, which can explain the excess of reactant used in the iodination experiments. Although it is possible that the iodination of very few amino acid residues was not detected, the analysis of the cysteine, histidine, tryptophan, and methionine contents of iodinated CHLDH shows that these amino acids are not appreciably iodinated under the adopted experimental conditions. Therefore, the data obtained with iodinated CHLDH will be discussed in terms of the involvement of the tyrosyl residues in the mechanism of action of the enzyme. The evidence for such an involvement rests on the following points.

(1) DPNH and AcPyDPNH shift the pK of the titratable tyrosines by 0.45 and 0.65 pH unit, respectively, toward the alkaline side. It is possible that such shifts in pK might be due to the formation of hydrogen bonds between the tyrosyl residues of the enzyme and the coenzyme. The reason why AcPyDPNH causes a greater shift than DPNH is not clear at present, since the dissociation constants for the two coenzymes are very similar (McKay and Kaplan, 1964; Di Sabato, unpublished results). Obviously, the

reported pK values of the tyrosyl residues are only averages and should not be interpreted in the sense that *all* of the free tyrosines of CHLDH have the same pK or that the coenzymes induce a pK shift of *all* the tyrosyl residues. For instance, a satisfactory fitting to the experimental points for the ionization of the tyrosyl residues of CHLDH in the presence of DPNH (line B of Figure 1) has been obtained by assuming that one-half of the tyrosyl residues have unchanged pK (10.25) and one-half undergoes a pK shift of 0.9 pK unit. Also on the basis of the results of the iodination experiments, it appears more probable that only a few tyrosyl groups interact directly with the coenzyme.

(2) A linear relation exists between iodinated tyrosyl residues and loss of enzymatic activity. Although some error is involved in the determination of the number of iodinated tyrosyl residues, mainly due to the formation of both mono- and diiodotyrosine, it seems fair to state that about 4 diiodotyrosines and 1–2 monoiodotyrosines are formed in the fully inactivated enzyme. On the other hand, the use of radioactive iodine would not be useful for a direct measurement of the iodinated tyrosines, because it would not be possible to distinguish between mono- and diiodotyrosine. It has been demonstrated that CHLDH has four active sites (Di Sabato and Kaplan, 1963). The finding that 5–6 tyrosyl residues appear to be iodinated before the enzyme is fully inactivated could be due either to the fact that the extinction coefficients for the free mono- and diiodotyrosine are not exactly the same as for the two amino acid derivatives in the enzyme, or to the fact that the tyrosyl residues at the active site of the enzyme react with iodine at the same rate as 1–2 tyrosyl groups located elsewhere in the molecule.

Cha and Scheraga (1963) have demonstrated that the iodinated tyrosyl residues of ribonuclease undergo destruction when the protein is treated with performic acid prior to hydrolysis in 6 N HCl. A few attempts to apply this technique to iodinated CHLDH did not meet with success, apparently because a considerable number of noniodinated tyrosyl residues underwent destruction.

(3) DPNH protects CHLDH from inactivation by iodine. The limitation of this method due to the probable formation of an iodine–DPNH complex have already been discussed under Results. It should be added here that conformational modifications occurring in the enzyme upon binding of the coenzyme (Di Sabato and Kaplan, 1964; Di Sabato and Ottesen, 1965; Di Sabato and Kaplan, 1965) could contribute to lessen the binding of iodine in the presence of DPNH.

(4) DPNH does not protect iodinated CHLDH from the dissociating action of SDS. This finding, similar to that found by Di Sabato and Kaplan (1964) with PCMB-bound CHLDH, should be interpreted as the inability of DPNH to bind to the iodinated enzyme and points to the tyrosyl residues as forming part of the site of binding of the coenzyme. A less likely possibility is that iodination weakens the subunit interaction sufficiently to prevent the protection exerted by the coenzyme on the dissociation of the enzyme by SDS.

(5) The structure of the iodinated enzyme does not seem to be altered. The fact that the sedimentation coefficient as well as the specific optical rotation are unchanged upon iodination indicates that extensive denaturation does not take place in the molecule of the iodinated enzyme. The existence of denaturation is also made unlikely by the finding that the rate of binding of PCMB is unchanged upon iodination. If denaturation should occur, one would expect a difference in the rate of binding of PCMB between iodinated and noniodinated enzyme, owing to unfolding of the protein structure. Indeed it has been demonstrated (G. Di Sabato, A. Pesce, and N. O. Kaplan, unpublished results) that PCMB binds faster to denatured CHLDH than to the native enzyme.

In conclusion: while none of these points should be taken alone as conclusive evidence for the involvement of the tyrosyl groups of CHLDH in the mechanism of action of the enzyme, taken together they constitute a strong indication for such an involvement.

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Influence of Carbohydrates on Phosphorylase Structure and Activity. I. Activation by Preincubation with Glycogen*

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ABSTRACT: Preincubation of phosphorylase *a* at 20° with glycogen results in an approximately 6-fold increase in maximal velocity with glucose 1-phosphate with no apparent alteration in the Michaelis constant. Activation does not appear related to formation of a better primer. The dependence of the extent of activation with protein concentration, temperature, and ionic strength suggests that activation is related to enzyme dissociation and is consistent with the conversion of a less active tetrameric form of phosphorylase *a* or *b* to a more active dimeric species. Activation is not related to priming capacity of polysaccharide since amylopectin or its β-amylase limit dextrin, or hydrolyzed amylose, were found to be more efficient activators than glycogen. Although hydrolyzed amylose was found to be a competitive inhibitor with respect to glycogen in the activity test, activation by hydrolyzed amylose is not inhibited by glycogen, a weaker activator. These data show that activation by amylose is not a simple consequence of binding at the primer site and suggest that activation is a result of binding at an additional or activation site on the enzyme.

Although it has been clearly established that glycogen acts as a primer for the *in vitro* synthesis of glycogen by muscle phosphorylase (Cori and Cori, 1939), the exact consequences of binding of polysaccharide on enzymic structure and activity have not been fully realized. The interaction of phosphorylase with polysaccharide appears to involve the nonreducing ends of the primer (Brown and Cori, 1961), and this interaction, although not absolutely specific for glycogen, is favored by highly branched polysaccharides (Swanson and Cori, 1948). Ultracentrifugal studies of Madsen and Cori (1958) showed that the nonreducing ends of one molecule of corn glycogen can interact with as many as 33 enzyme molecules. Further investigation by Selinger and Schramm (1963) indicated that glycogen and phosphorylase can form an insoluble complex similar to the type formed by the reaction of an antigen with an antibody. Recent light-scattering measurements support the view that phosphorylase has several combining sites for glycogen (Lowry *et al.*, 1964). In the preparation of liver phosphorylase (Sutherland and Wosilait, 1956) and phosphorylase *b* kinase (Krebs *et al.*, 1964), a strong association between phosphorylase and glycogen was also observed; this interaction is no doubt of some physiological significance since this interaction has been found to depend in part on the nutritional status of the animal (Tata, 1964; Sie *et al.*, 1964).

Kinetic studies from this laboratory indicated that the dimeric form of phosphorylase *a* is more active than the tetrameric form of the enzyme and that glycogen blocked the conversion of the more active species to the less active form (Wang and Graves, 1964). The study of the consequences of this interaction on enzymic structure and activity of the various molecular forms of phosphorylase has been initiated. The results reported herein show that phosphorylase may be activated by preincubation with glycogen and that activation is related to enzyme dissociation.

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