Effect of Coenzymes on the Hydrogen-Deuterium Exchange of Chicken Heart Lactic Dehydrogenase as Measured by Infrared Spectrophotometry*

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ABSTRACT: The deuterium exchange of the peptide groups of lactic dehydrogenase from chicken heart has been studied at *p*H 6.9 in the absence or in the presence of various coenzymes. The exchange has been measured by the decrease in absorbancy of the Amide II band (1552 cm^{-1}). Native chicken heart lactic dehydrogenase has about 50% fast-exchangeable peptide hydrogens, about 15% slowly exchangeable, and about 35% hard-to-exchange peptide hydrogens. All the coenzymes tested decrease the hydrogen exchange of the native enzyme. The protection given by the reduced nicotinamide–adenine dinucleotide apparently consists in a decrease of the rate of exchange of the slowly exchangeable hydrogens, while the oxidized nicotinamide–adenine dinucleotide and the reduced and the oxidized 3-acetylpyridine–adenine dinucleotides appear to decrease the rate of exchange of the fast-exchangeable hydrogens. Sodium dodecylsulfate greatly enhances the hydrogen exchange of the enzyme. The coenzymes tested also partially counteract the increase in hydrogen exchange induced by sodium dodecylsulfate: the reduced coenzymes and the 3-acetylpyridine analogs exert a stronger protection than do the oxidized and natural coenzymes, respectively. The results are interpreted as suggesting conformational changes induced by the coenzyme of chicken heart lactic dehydrogenase.

It has been found that diphosphopyridine coenzymes and some of their analogs protect lactic dehydrogenase from inactivation by sodium dodecylsulfate (Di Sabato and Kaplan, 1964), urea, and lithium chloride [Di Sabato and Kaplan (1965)]. Analogous results have been obtained by Yonetani and Theorell (1962) with yeast alcohol dehydrogenase. More recently, Hvidt and Kägi (1963) found a decrease in the hydrogen exchange of yeast alcohol dehydrogenase upon binding of NAD^+ and NADH to the enzyme. These findings have been interpreted as due to conformational changes occurring in the dehydrogenase upon binding of the coenzymes and/or upon formation of ternary complexes with substrates.

In order to obtain further evidence for the existence of conformational changes in lactic dehydrogenases upon binding of the coenzymes, we undertook the study described in this paper. Its aim is to investigate the rate of hydrogen exchange of the enzyme alone and compare it with the rate of exchange of the enzyme in the presence of coenzyme. Furthermore, the hydrogen exchange of CHLDH[1] denatured with SDS and the action exerted by the coenzymes has been investigated. The idea behind this approach is that small conformational changes in the enzyme molecule should modify the rate of exchange of the peptide hydrogen with the solvent D_2O. The technique adopted in the present work is similar to that used by Hvidt and Kägi (1963) for yeast alcohol dehydrogenase. It consists in measuring the rate of exchange of the peptide hydrogen with the solvent D_2O by infrared spectroscopy. This method takes advantage of the decrease in absorbancy at 1552 cm^{-1} (Amide II band) concomitant to the deuteration of the —NH— of the peptide bond. Since the exchange rate varies with temperature and *p*H, all comparisons were made under identical conditions.

Experimental

CHLDH (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27), as used in previous studies, was obtained in form of ammonium sulfate paste as a gift from Dr. N. O. Kaplan, Brandeis University. The paste was dissolved in 0.1 M sodium phosphate buffer, *p*H 6.9, and dialyzed for 24 hours at 4° against about 1000 times its volume of the same buffer. Enzyme concentrations were measured from optical density at 280 mμ applying a molar extinction coefficient of 1.8×10^5. $AcPyAD^+$ and AcPyADH prepared according to the general procedure outlined by Kaplan and Stolzenbach (1957) were a kind gift from Dr. N. O. Kaplan, Brandeis University. NAD^+ and NADH were commercial preparations obtained from C. F. Boeringer und Soehne GMBH, Mannheim, Germany. SDS was obtained from E. I. du Pont de Nemours and recrystallized twice from ethanol. Deuterium oxide

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[1] Abbreviations used in this work: CHLDH, lactic dehydrogenase from chicken heart; $AcPyAD^+$, 3-acetylpyridine analog of NAD^+; AcPyADH, 3-acetylpyridine analog of NADH; SDS, sodium dodecylsulfate.

(99.8% pure, $d_4^{20} = 1.10518$) was obtained from Norsk Hydro, Norway.

CHLDH (200 μl), 1.1×10^{-4} M in 0.1 M sodium phosphate buffer, *p*H 6.9, was lyophilized according to the procedure used by Hvidt and Kägi (1963). The lyophilized samples were kept in a desiccator at room temperature for 1–14 days before use; no decrease of the enzymatic activity was detected during the period of storage. Samples of 0.1 M sodium phosphate buffer (200 μl), *p*H 6.9, were lyophilized using the same procedure as for the enzyme. Just before the experiment, 200 μl of D_2O (containing, when required, SDS and/or coenzyme) was added to a lyophilized sample of sodium phosphate buffer. The same solvent was used to dissolve a lyophilized sample of enzyme. Since *p*D values were not determined, acidity values were expressed as the *p*H of a corresponding solution in H_2O.

The starting time for each experiment was marked as the instant when the solvent made contact with the lyophilized enzyme. The solutions were rapidly inserted in a calcium fluoride cell, 0.1 mm path length; the cell containing the solvent (reference cell) was inserted in the I_0 beam, the cell containing the solvent plus the enzyme (experimental cell) was inserted in the I beam. The infrared spectra were obtained with a Perkin-Elmer Model 13 ratio-recording infrared spectrophotometer. All runs were made in constant I_0 mode. Usually, the decrease in absorbancy at 1552 cm^{-1} was followed at constant wavelength for the first 10 minutes; subsequently, complete spectra over the region 1760–1370 cm^{-1} were taken at suitable intervals of time. The times indicated in the text refer to the moment at which the maximum of the —NH— absorption band at 1552 cm^{-1} was passed. All runs were carried out in a constant-temperature room (21 ± 1°). However, the temperature of the cells increased somewhat when they were left in the beam of radiation. In order to minimize this effect, the cells were removed from the spectrophotometer between one run and the other. Care was also taken to run the spectra at the same intervals of time in the different experiments. The spectra were recorded on a linear transmittancy scale (T) and then transformed into absorbancy (OD) by means of the formula: $OD = -\log T$. The 100% transmittancy was adjusted, with no cells in the beam, at 95% of the full scale at 1760 cm^{-1}. In the interval 1760–1398 cm^{-1} this background line was constant within ±1% without cells and within ±2% with cells filled with D_2O. The decrease of the absorption at 1552 cm^{-1} was taken as a measure of the hydrogen exchange of the protein. The values of absorbancy at 1552 cm^{-1} were normalized on the values of absorbancy at 1658 cm^{-1} in order to correct for small differences in protein concentration between one experiment and the other.

The extinction coefficient of undeuterated peptide groups at 1552 cm^{-1} was assumed to be $k_{NH} = 182$ liters $mole^{-1}$ cm^{-1}. This value was estimated from the data reported by Hvidt (1963) for lysozyme, using linear interpolation to obtain the value for 0.143 M peptide bond concentration. The values of 0.143 M peptide groups were obtained from the concentration of CHLDH used in these experiments (1.1×10^{-4} M), assuming a molecular weight of 140,000 and 1300 peptide residues/mole of enzyme.[2]

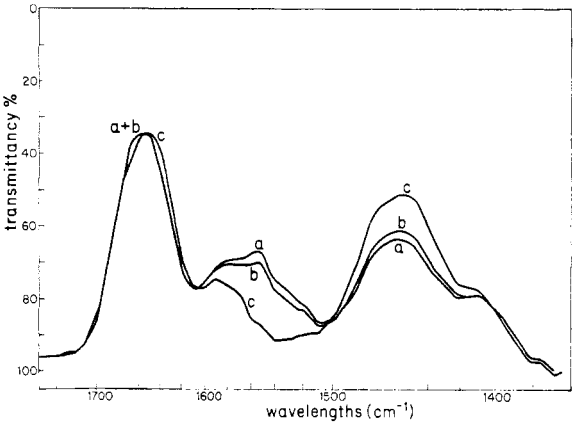

FIGURE 1: Infrared spectrum of CHLDH, 1.1×10^{-4} M. Medium: D_2O–0.1 M sodium phosphate buffer, *p*H 6.9. Curves: (a) at 42 minutes of exchange; (b) at 300 minutes of exchange; (c) at 300 minutes of exchange in the presence of 0.15 M SDS.

The absorbancy at 1658 cm^{-1} (Amide I band) for 1.1×10^{-4} M CHLDH was found to be 0.451, after correction for the background absorbancy at 1760 cm^{-1} (0.018 OD unit). From this value and from the assumed values of molecular weight and peptide concentration reported (*vide supra*), the extinction coefficient for peptide groups in the Amide I band, $k_{CO} = 315$ liters $mole^{-1}$ cm^{-1}, was calculated. This value is in agreement with the value of 353 reported by Hvidt (1963) for lysozyme.

In order to determine the background absorbancy at 1552 cm^{-1}, CHLDH was treated with 0.15 M SDS. After such a treatment, the absorbancy at 1552 cm^{-1} was 0.060 and it was unchanged after heating for 90 minutes at 80–82°. Since all previous experience (Hvidt and Linderstrøm-Lang, 1955; Hvidt, 1955; Wilcox, 1959; Hvidt and Kägi, 1963) indicates denatured proteins to exchange all hydrogens rapidly, we felt justified in regarding the value of 0.060 as the absorbancy of the fully deuterated protein and to use it as background for the Amide II band.

It should be pointed out that the absorbancy of the Amide I is unchanged at the concentrations of SDS used in this work (see Figure 1, curve C). This gave us confidence in considering the decrease of the Amide

[2] The values reported by Hvidt (1963) were obtained by extrapolation of the absorbancy of the Amide II band to zero time at a *p*H value where all peptide hydrogens exchange at a measurable rate. This estimate is supported by the agreement between the value obtained by Hvidt (1963) for the extrapolation to zero time of the Amide II/Amide I ratio (0.46) and the value obtained by Blout *et al.* (1961) for polypeptides and films of proteins (0.40–0.50).

II band as specifically due to an exchange of the protein hydrogen rather than to some effect of SDS on the infrared spectrum of the protein.

Results

Experiments in the Absence of Coenzymes. Figure 1 (curves a and b) shows the infrared spectrum at two different times of deuteration of CHLDH over the range 1760–1370 cm^{-1}. The band at 1658 cm^{-1} (Amide I band) corresponds to the C=O stretching frequency of the peptide bond; it is unchanged by the exchange process. The band at 1552 cm^{-1} (Amide II band) is due to a coupled CN vibration and NH deformation (Miyazawa *et al.*, 1958). It decreases as the exchange proceeds and —NH— is replaced by —ND—. Concomitantly, the band at 1458 cm^{-1} increases; this is caused partly by the formation of —ND— groups and partly by the DHO formed during the exchange reaction. Other bands at 1575 and 1414 cm^{-1} originate from the ionized carboxyl groups of the side chains of the protein and consequently they remain constant during the exchange process. Curve c of Figure 1 shows the infrared spectrum of 1.1×10^{-4} M CHLDH treated with 0.15 M SDS. It is evident that, while the absorbancy at 1552 cm^{-1} is strongly decreased, the Amide I band is not changed in height, but it is displaced by about 10 cm^{-1} toward higher wavelengths. This behavior, already observed by Hvidt and Kägi (1963), seems to be characteristic of the infrared spectrum of denatured proteins.

In Figure 2 (curve A) the corrected values for the absorbancy of the Amide II band are plotted versus time. Based on the value $k_{NH} = 182$ liters $mole^{-1}$ cm^{-1} (see Experimental), the initial absorbancy of the Amide II is calculated to be 0.26. The ordinate to the right in Figure 2 is based on this value and assumes a linear relationship between the decrease of the Amide II band and the number of exchanged peptide hydrogens. It seems that about 650 or half of the peptide hydrogens exchange rapidly, the following 200 exchange within 5 hours, while the remaining 450 hydrogens exchange very slowly. These data can be compared with those obtained by Hvidt and Kägi (1963) for yeast alcohol dehydrogenase: about 1120 fast-exchangeable hydrogens, 130 exchangeable at a measurable rate, and 150 hard to exchange. However, it should again be emphasized that exchange rates are strongly *p*H dependent and that these authors carried out the exchange at *p*H 8.0, while we worked at *p*H 6.9. For the sake of simplicity and emphasizing the arbitrariness of this nomenclature, in the following text the very fast exchangeable, those exchangeable at a measurable rate, and the hard-to-exchange peptide hydrogens will be called hydrogens of the n_1, n_2, and n_3 groups, respectively. It should be pointed out that this classification is made on a purely kinetic basis; in other words, no implication is made about the position of the different groups of hydrogens in the enzyme molecule. Moreover, this classification refers only to the hydrogens of the native enzyme.

Experiments in the Presence of Coenzymes. Figure 2 (curves B, C, D, and E) shows the time course of the hydrogen exchange of CHLDH in the presence of NADH, NAD^+, AcPyADH, and $AcPyAD^+$, respectively. The initial absorbancy in the presence of NADH is, within experimental error, the same as in the control (curve A), while the final value (5 hours of exchange) is somewhat higher (curve B).[3] It seems therefore that NADH does not effect the exchange of the peptide hydrogens of the n_1 group, but somewhat decreases the rate of exchange of those of the n_2 group. The curve in the presence of NAD^+ (curve C) starts higher than the control curve, and the vertical distance between the two curves remains practically unchanged. The most likely explanation of this phenomenon is a general decrease of the hydrogen-exchange rates in the presence of NAD^+, causing some hydrogens of the n_1 group to pass into the n_2 group and a similar number from the n_2 group to the n_3 group. Another possibility would be that some hydrogens of the n_1 group pass directly into the n_3 group, while the hydrogens of the n_2 group exchange at the same rate as in the absence of coenzyme. In the presence of AcPyADH and $AcPyAD^+$ (curves D and E) an even more pronounced decrease of the exchange rates is observed.

Experiments in the Presence of SDS. It has been shown by Di Sabato and Kaplan (1964) that the inactivation induced by SDS on CHLDH is largely prevented by NADH and AcPyADH; these findings were interpreted as indicative of conformational stabilization induced by the coenzymes on the enzyme molecule. In order to provide further evidence for this interpretation, the deuterium exchange of CHLDH treated with SDS has been studied both in the absence and in the presence of coenzymes. Two schemes have been adopted for these experiments: (a) The lyophilized sample of enzyme was dissolved in D_2O containing SDS and (when required) coenzyme (nonpredeuterated samples). (b) The lyophilized enzyme was dissolved in D_2O and allowed to exchange in a closed vessel at 21° for about 48 hours. After that a solution of SDS in D_2O with or without coenzyme was added (predeuterated samples). In these experiments the hydrogens of n_1 and n_2 groups have already exchanged with deuterium during the predeuteration period; therefore the observed decrease of the absorbancy of the Amide II band is due only to the exchange of the hydrogens belonging to the n_3 group (in the native enzyme). Figure 2 (curves F, G, H, I) shows the time course of the peptide hydrogen exchange (as measured by the decrease in absorbancy of the Amide II band and by number of peptide hydrogens exchanged) of CHLDH predeuterated and nonpredeuterated and treated with two different concentrations of SDS (0.022 and 0.044 M). It is evident that in all four cases SDS causes a

[3] In interpreting the curves reported in Figure 2, it should be pointed out that the deuterium-exchange method does not permit us to decide "which" peptide hydrogens are exchanged, but only the number of exchanged hydrogens; therefore the hydrogens exchanging in the presence of the coenzymes are not necessarily the "same" as in the control.

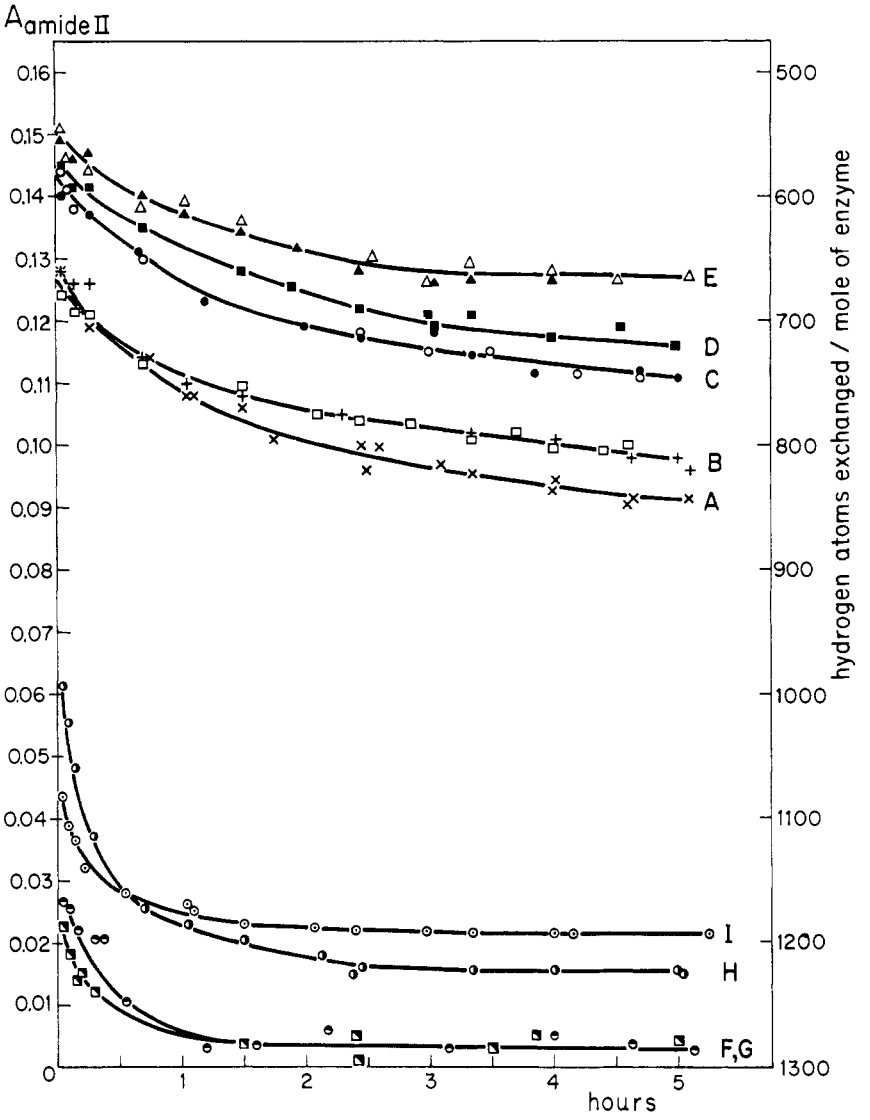

FIGURE 2: The action of SDS and coenzymes on the time course of the hydrogen exchange of CHLDH, 1.1×10^{-4} M, expressed as absorbancy of the Amide II band (left ordinates) and hydrogen atoms exchanged per mole of enzyme (right ordinates). Medium: D_2O–0.1 M sodium phosphate buffer, *p*H 6.9. All samples containing CHLDH, additions as follows: (A) none (×); (B) NADH, 7×10^{-4} M (□) and 33×10^{-4} M (+); (C) NAD^+, 87×10^{-4} M (⊖) and 46×10^{-4} M (●); (D) AcPyADH, 10×10^{-4} M (■); (E) $AcPyAD^+$, 80×10^{-4} M (△) and 40×10^{-4} M (▲); (F) SDS, 0.044 M (nonpredeuterated samples) (◓); (G) SCS, 0.044 M (predeuterated samples) (◥); (H) SDS, 0.022 M (nonpredeuterated samples) (◑); (I) SDS, 0.022 M (predeuterated samples) (⊙).

arge increase both in the rate and in the extent of exchange of the peptide hydrogens of CHLDH.

In Figure 3 are shown analogous plots for CHLDH in the same conditions as before, but in the presence of both coenzymes and SDS. Comparing these data with those of Figure 2 it appears that the exchange curves are displaced upward, indicating a decreased exchange rate of the hydrogens of both n_2 and n_3 groups. In the predeuterated experiments all peptide hydrogens of the n_1 and n_2 groups had exchanged before SDS and coenzymes were added, and the decreased exchange rates thus reflect a protective action of the coenzymes on the hydrogens of the n_3 group. The degree of protection exerted by the different coenzymes on the hydrogen exchange of CHLDH in the presence of SDS during a 5-hour incubation is shown in Table I. The protection exerted by AcPyADH has been set arbitrarily at equal to 100 and to this the protection exerted by the other coenzymes has been referred. It is evident that the reduced coenzymes protect more than the oxidized coenzymes and that the acetylpyridine analogs protect more than the natural coenzymes.

Discussion

The coenzymes and coenzyme analogs tested modify the rate of hydrogen exchange of CHLDH. In the

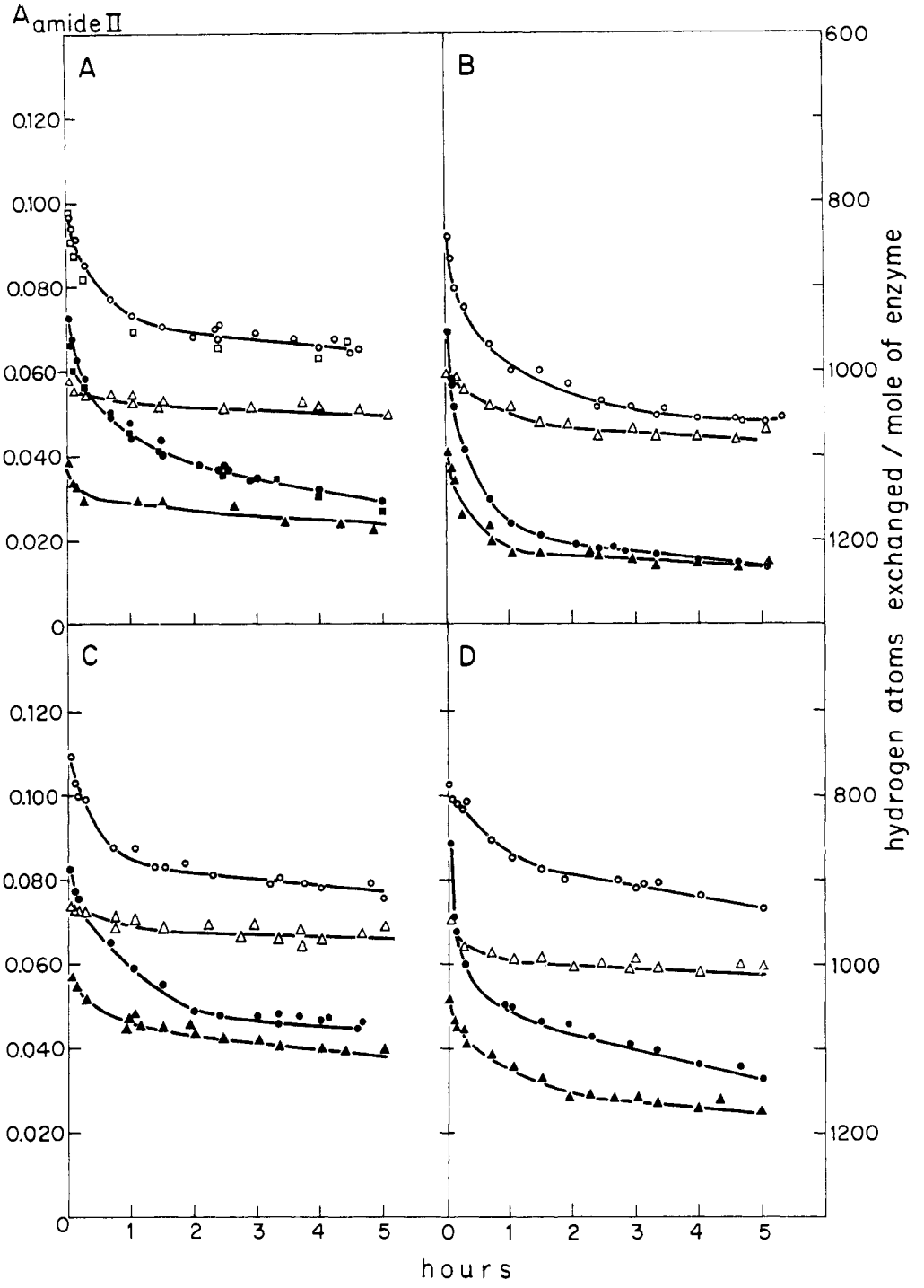

FIGURE 3: The action of SDS and coenzymes on the time course of the hydrogen exchange of CHLDH, 1.1×10^{-4} M, expressed as absorbancy of the Amide II band (left ordinates) and hydrogen atoms exchanged per mole of enzyme (right ordinates). Medium: D_2O–0.1 M sodium phosphate buffer, *p*H 6.9. (A) NADH, 15×10^{-4} M (squares) and 80×10^{-4} M (circles and triangles); (B) NAD^+, 90×10^{-4} M; (C) AcPyADH, 9×10^{-4} M; (D) $AcPyAD^+$, 90×10^{-4} M. Circles and squares, nonpredeuterated samples; triangles, predeuterated samples; open symbols, 0.022 M SDS; filled symbols, 0.044 M SDS.

case of NADH the n_1 group appears unchanged, while the exchange rate of the n_2 group is significantly decreased. In the cases of NAD^+, AcPyADH, and $AcPyAD^+$, a considerable decrease in the n_1 group is observed followed by an unchanged or decreased rate of exchange of the n_2 group. If these differences in exchange rates are interpreted as owing to conformational changes in the enzyme molecules, our results indicate that CHLDH in the presence of coenzyme may undergo conformational changes during the oxidation and reduction of the coenzymes. The degree of protection observed with the different coenzymes is subject to some error, since small differences are involved; therefore only a semiquantitative interpretation is justified.

TABLE 1: Relative Protection Exerted over 5 Hours by Different Coenzymes on the Hydrogen Exchange of CHLDH in the Presence of SDS.[a]

	Nonpredeuterated Samples		Predeuterated Samples	
	0.022 M SDS	0.044 M SDS	0.022 M SDS	0.044 M SDS
AcPyADH	100	100	100	100
NADH	81	65	62	60
	81[b]	58[b]		
NAD^+	57	28	51	32
$AcPyAD^+$	94	74	80	64

[a] Protection exerted by AcPyADH arbitrarily set to 100. Medium: D_2O–0.1 M sodium phosphate buffer, *p*H 6.9; CHLDH, 1.1×10^{-4} M; AcPyADH, 9×10^{-4} M; NADH, 15×10^{-4} and 80×10^{-4} M; NAD^+ 90×10^{-4} M; $AcPyAD^+$, 90×10^{-4} M. [b] NADH, 15×10^{-4} M.

The experiments in the presence of SDS show a large increase of the hydrogen exchange of CHLDH. This is explained by the disruptive effect of SDS on the protein structure, thereby permitting the hydrogens of the peptide bonds to come in more direct contact with the solvent and therefore exchange better with D_2O.

From the experiments carried out in the presence of SDS plus coenzymes two conclusions can be drawn: (a) The protection exerted by the coenzymes decreases in the order AcPyADH > $AcPyAD^+$ ~ NADH > NAD^+. This is the same order in which the four coenzymes protect CHLDH from inactivation by urea or LiCl (Di Sabato and Kaplan, 1965). Moreover, AcPyADH has been shown to be a better protector than NADH from the effects of SDS on CHLDH ($AcPyAD^+$ and NAD^+ have not been tested) (Di Sabato and Kaplan, 1964). The same order of effectiveness of the four coenzymes has been found for the protection of CHLDH and beef heart lactic dehydrogenase from inactivation caused by binding of *p*-mercuribenzoate to the sulfhydryl groups of the enzymes (Di Sabato and Kaplan, 1963). This protective effect of the coenzymes was interpreted as indicating binding of the coenzymes to the sulfhydryl groups of the enzymes. However, in the light of the present findings, the hypothesis may be formulated that the binding of *p*-mercuribenzoate to the enzymes is slower in the presence of the coenzymes because of a "masking" of the sulfhydryl groups due to conformational changes induced by the coenzymes. (b) The predeuteration experiments, in which the hydrogens of the n_1 and n_2 groups have been exchanged before SDS and coenzymes were added, show that the coenzymes strongly counteract the action of SDS on the hydrogens belonging to the n_3 group in the native enzyme.

As for the mechanism by which the coenzymes influence the hydrogen exchange of lactic dehydrogenase, three main hypotheses can be considered: (1) The conformation of the peptide chain in the protein molecule might be of different "tightness" of folding in different sections. If this is the case the hydrogen-exchange rates could reflect these structural differences. The coenzyme could then act on the protein molecule by increasing the tightness of some sections. The existence of conformational modifications in enzymes bound to coenzymes or substrates has already been postulated in several cases (Sekuzu *et al.*, 1957; Nozaki *et al.*, 1957; Yonetani and Theorell, 1962; Hvidt and Kägi, 1963; Di Sabato and Kaplan, 1964, 1965). Such conformational modifications induced by the binding of the coenzyme to the enzyme might be due to variations of electrostatic factors, breaking and/or formation of hydrogen bonds, or modifications of the water structure around the protein molecule.

(2) Linderstrøm-Lang and Schellman (1959) proposed the term "motility" to indicate continuous structural changes in parts of the protein molecule. If this aspect of the protein structure is predominant, the rate and extent of hydrogen exchange might be determined by the speed at which the protein fluctuates between exchanging and nonexchanging conformation. The coenzyme could modify the "motility" of the enzyme molecule by stabilizing some conformational forms which would be favored over the others with consequent modifications of the rate and extent of hydrogen exchange.

Both hypotheses (1) and (2) could be invoked to explain the action of the coenzymes on CHLDH in the presence of SDS. In this case one has to postulate that the coenzymes induce the formation of structural forms which are more resistant to the action of SDS than the forms existing in the absence of coenzymes.

(3) The possibility should be considered that the coenzymes decrease the hydrogen exchange of CHLDH simply by "shielding" some parts of the enzyme molecule from contact with the solvent. This possibility is unlikely, however, if one considers that the coenzymes protect up to 200 peptide hydrogens of the n_1 and n_2

groups (Figure 2)[4] and up to 200 of the n_3 group, as shown by the experiments in the presence of SDS and with predeuteration (compare Figure 2 and Figure 3). Therefore the coenzymes might decrease the rate of exchange in about one-third of the total number of peptide hydrogens. It is hard to visualize how a relatively small molecule like a coenzyme could "shield" the exchange of such a large number of peptide hydrogens unless some special structural features were present in the enzyme molecule. For instance, it could be that the decrease in rate of hydrogen exchange was owing to the fact that the coenzymes shielded an area of the molecule from which the denaturation by SDS or other agents had to start, and from there proceed to the rest of the molecule. This possibility has already been discussed elsewhere and, on the basis of experimental evidence, considered unlikely (Di Sabato and Kaplan, 1965).

In conclusion, the evidence presented indicates the existence of some changes in the conformation and/or "motility" of CHLDH caused by various coenzymes. These changes were relatively small in the native molecule; however, a more evident effect was observed when the coenzymes counteracted the denaturing effect of SDS. This is interpreted as due to the fact that the changes induced by the coenzymes on the protein conformation make it less susceptible to the action of SDS.

Acknowledgments

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[4] It should be pointed out that this number is probably an underestimate, because the hydrogens of the n_1 group might be exchanged at a slower rate in the presence of the coenzyme, but still at a rate too fast to be measured.

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