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Conformational Effects of Coenzyme Binding to Porcine Lactic Dehydrogenase

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Abstract. Changes induced on addition of the coenzyme, NADH or NAD^+, to porcine lactic dehydrogenase isoenzymes H_4 and M_4 have been studied by hydrodynamic and spectroscopic methods. As shown by ultracentrifugal analysis, the native subunit structure remains unchanged on holoenzyme formation; a ~5% increase of the sedimentation coefficient, parallelled by a slight decrease of the partial specific volume (< 1%) indicate a significant change in the native tertiary and/or quaternary structure of the enzymes, corroborating earlier calorimetric data (Hinz and Jaenicke, 1975). The binding constant for the enzyme from skeletal muslce (M_4) and NADH are found to be in agreement with K_D-values obtained from equilibrium dialysis, as well as spectroscopic and thermal titration experiments (8 μM). Far UV circular dichroism measurements do not show significant changes on ligand binding, indicating unchanged helicity or compensatory conformational effects. In the near UV, ligand binding is reflected by an extrinsic Cotton effect around 340 nm; in the range of aromatic absorption no changes are detectable.

The experimental results suggest that there are gross structural changes on coenzyme binding to lactic dehydrogenase which do not affect the intrinsic spectral properties normally applied to analyze transconformation reactions in protein molecules.

Key words: Circular dichroism – Coenzyme binding – Conformational analysis – Lactic dehydrogenase – Ultracentrifugation.

Introduction

The catalytic function of enzymes requires conformational flexibility in order to provide substrate binding and subsequent product release. In the case of oligomeric enzymes this flexibility may occur (1) at the level of specific residues performing translational or rotational motions, (2) at the level of the overall secondary structure or certain domains within the folded subunits, and (3) at the level of the quaternary structure in terms of an $R \rightleftharpoons T$ transition.

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There is wealth of experimental evidence proving one or the other of these alternative structural changes for a great variety of enzymes or proteins. The example presented in the following communication is of interest for several reasons: Lactic dehydrogenase has been the first oligomeric enzyme to be analyzed at high resolution with regard to the three-dimensional structure of its apo- and holoenzyme; evidence from this analysis proves significant structural changes to occur upon ligand binding [1]. This result has been corroborated by rapid kinetic experiments which suggested an isomerization reaction to be involved in coenzyme binding [2]. However, more recent studies [3] did not substantiate this result; instead a single step bimolecular reaction for the formation of the LDH · NADH binary complex was proposed, indicating that the expected transconformation occurs simultaneously with the binding process.

Calorimetric measurements have shown that coenzyme binding to the enzyme is accompanied by a high negative heat capacity change [4–6], in accordance with a tightening of the structure [7, 8]. On the other hand, previous spectroscopic experiments [3, 9, 10] did not indicate any significant structural effect, leaving us with the unique situation that there seem to be gross structural changes which do not affect the intrinsic spectral properties normally used to analyze transconformation reactions in protein molecules. The following experiments attempt to clarify this finding, making use of ultracentrifugation and circular dichroism measurements.

Materials and Methods

Lactic dehydrogenase from pig heart (LDH-H_4) and pig skeletal muscle (LDH-M_4), NAD^+, and NADH were obtained from Boehringer Mannheim, dithioerythritol from Calbiochem, Luzern. All other reagents were commercial preparations of A-grade purity (Merck, Darmstadt). The water used for preparing the buffer solutions was demineralized and quartz bidistilled. Stock solutions of the enzyme were prepared by centrifugation of the crystal suspension and subsequent dialysis at 4° C against 0.2 M phosphate buffer pH 7.0 in the presence of 1 mM dithioerythritol and 1 mM EDTA, or phosphate buffer pH 7.0, I = 0.1 M, in the presence of 10–100 mM 2-mercaptoethanol. Enzyme concentrations were calculated from $A_{280}^{0.1\%} = 1.4\ cm^2 \cdot mg^{-1}$ [11]. Molar concentrations refer to the subunit molecular weight of 35,000; the mean residue weight amounts to 113. The ratio of the absorption of the enzymes at 280 nm to that at 260 nm was 1.91 for LDH-H_4, and 1.98 for LDH-M_4; therefore charcoal treatment was considered unnecessary. Enzymatic activity was measured in phosphate buffer pH 7.0, I = 0.1 M, containing 1 mM dithioerythritol and 1 mM EDTA under standard conditions of the optical assay (0.74 mM pyruvate and 0.2 mM NADH) using a thermostated recording Eppendorf spectrophotometer. The specific activities of the two isoenzymes were 400 ± 20 (LDH-H_4) and 640 ± 30 IU/mg (LDH-M_4), respectively.

NAD^+ and NADH were dissolved in the buffer; their concentration was determined spectrophotometrically, assuming absorption coefficients of $18.0 \cdot 10^6$ $M^{-1} \cdot cm^{-1}$ (at 260 nm) and $6.23 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ (at 340 nm), respectively [12]. For the coenzyme binding studies 1–200 μM solutions of the apoenzyme were titrated with neutralized 1–100 mM solutions of the coenzymes using syringe microburets

or microliter syringes. By this means, changes of enzyme concentration were kept insignificantly small during saturation; for 95% saturation the maximum dilution amounts to less than 3%.

Conformational analyses made use of circcular dichroism spectroscopy applying a Cary 61 CD-spectrograph, and a Roussel-Jouan Dichrographe II with high sensitivity equipment (10^{-6}/mm) and temperature control (Haake KT 33 thermostat and YSI Telethermometer, 20° C). For optical rotatory dispersion (ORD) a Cary 60 spectrograph was applied.

To characterize the gross structure and its change upon coenzyme binding, sedimentation velocity and equilibrium runs were performed in an analytical ultracentrifuge (Beckman Instruments, Model E) with Schlieren optics and high sensitivity photoelectric scanning system. Due to the high absorption of the coenzyme, maximum accuracy in the range of coenzyme saturation was provided by Schlieren runs using double sector centerpieces, 12 and 30 mm path length, and normal/wedge windows (± 1°), with the apo-enzyme as reference. Sedimentation coefficients ($s_{20,w}$) were calculated from log r vs t diagrams making use of a least square fit program.

Partial specific volumes were measured pycnometrically, and by using a digital density meter according to Kratky et al. [13] (DMA 02, A. Paar, Graz), thermostated at 20.00 ± 0.02° C, using coupled thermostates (Haake KT 33 and Colora Ultrathermostat NB-DB).

Results and Discussion

Sedimentation Analysis

As has been demonstrated by previous studies [14, 15], lactic dehydrogenase isoenzymes from various mammalian sources are characterized by the same molecular

Table 1. Sedimentation analysis of porcine lactic dehydrogenase in the absence and presence of coenzyme. Phosphate buffer pH 7.0, I = 0.1 M, 20° C[a]. Holoenzyme at > 95% saturation

Isoenzyme	Coenzyme	K_D (μM)	$s_{20,w}$ (S)[b]	$D_{20,w}$ (F)[b]	$\bar{M}_w$[c]
H_4	–	–	7.54	5.05	140,000
	NAD^+	320	7.80	5.08	141,600
	NADH	4.0	7.83	5.06	142.000
M_4	–	–	7.60	5.18	138,500
	NAD^+	500	7.79[d]	5.25	140,000
	NADH	7.4	7.86	5.22	144.000

[a] In the temperature range from 10° to 30° C the tightening is decreased: Δs for the M_4 isoenzyme at 10° C ~7%, 20° C ~5%, 31° C ~3%

[b] Ranges of error: s, 1%; D, 3%; $\bar{M}$, 2%

[c] Weight average molecular weight from sedimentation equilibrium (high speed meniscus depletion technique); the partial specific volume of both isoenzymes ($\bar{V}_2 = 0.740 \pm 0.002$; cf. [11]) shows a decrease of the order of ≦ 1% upon holoenzyme formation

[d] Addition of oxalate to form the ternary complex leads to a further increase of the sedimentation coefficient ($s_{20,w}$ = 8.00 S, in the presence of 12 mM oxalate + 4 mM NAD^+)

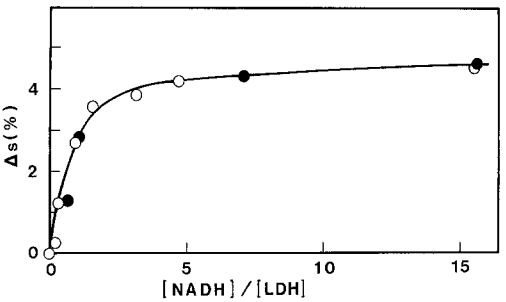


Fig. 1. Hydrodynamic titration of pig muscle lactic dehydrogenase with NADH. Sedimentation velocity at 30,000 and 44,770 rev/min. 0.2 M phosphate buffer pH 7.0 in the presence of 1 mM dithioerythritol and 1 mM EDTA (○), and phosphate buffer pH 7.0, I = 0.1 M, plus 10 mM 2-mercaptoethanol (●), 20° C. $\Delta s = 100 \times \frac{s_{20} - s_{20,\,\mathrm{apo}}}{s_{20,\,\mathrm{apo}}}$; $c_{LDH} = 0.1$ mM. The solid line is calculated on the assumption of a dissociation constant of 8 μM

weight and subunit structure. There is no indication for significant changes in the state of association upon coenzyme binding. However, the decreased accessibility of the enzyme towards proteolysis [16], as well as the slight change of the hydrodynamic parameters observed in preliminary earlier experiments [14, 15] point clearly to structural changes similar to those reported, e.g., for yeast glyceraldehyde-3-phosphate dehydrogenase [17].

Table 1 summarizes the result of sedimentation analyses for both isoenzymes. As is obvious from the comparison of the data both the sedimentation coefficient and diffusion coefficient show an increase when the coenzyme is added to the apoenzyme, while the partial specific volume is slightly decreased.

The magnitude of Δs exceeds the calculated increase in molecular weight of 1.6% resulting from the attachment of the four coenzyme molecules per tetramer. This estimate neglects the slight change in the diffusion coefficient which may not be significant because of the error limits of the determination of D in the ultracentrifuge (± 3%).

From the change of the hydrodynamic properties the conclusion may be drawn that the attachment of the coenzyme causes a definite change in the tertiary structure of the two isoenzymes under consideration.

This interpretation is corroborated by independent evidence from X-ray crystallography [1] and hydrogen-deuterium exchange [19]. Determining the dissociation constant of the enzyme-coenzyme complex from the "hydrodynamic titration" curve yields a K_D-value of 8 μM for the enzyme from pig muscle; the solid line in Figure 1 is calculated on the assumption of this value which is in agreement with the respective data obtained from equilibrium dialysis measurements and thermal titration [4], as well as fluorescence titration [18].

Aside from the given interpretation, the observed decrease in the frictional coefficient could reflect a diminution in dissymmetry, or a decrease in hydration. As mentioned, the transition to a more compact structure seems much the most likely alternative, taking into account the above mentioned X-ray and H-D exchange data.

Conformation

There are three different wavelength ranges where lactic dehydrogenase is expected to exhibit spectral changes in the process of coenzyme binding: firstly, transconformation of the backbone of the polypeptide chain will be reflected by changes in the far-UV circular dichroism spectrum at $190 < \lambda < 250$ nm; secondly, local isomerization processes in the neighbourhood of intrinsic chromophores of the enzyme will affect the absorption in the aromatic range ($250 < \lambda < 300$ nm) (cf. [14, 20]); finally extrinsic Cotton-effects in the range of absorption maxima of the ligand may be induced in the course of complex formation.

CD spectra in the respective wavelenght ranges are given in Figure 2. As becomes obvious from Figure 2a, the native apoenzymes show the typical CD spectra characteristic for a protein with a helix content of the order of 30% [21]. This value is in accordance with previous optical rotatory dispersion measurements [9], and high resolution X-ray data [1]. Adding increasing amounts of NADH does not affect the *far-UV* spectrum within the range of experimental error (3%). Similarly, no significant spectral changes could be detected for the binding of NAD^+. Since in this case the high K_D-values require rather high concentrations of the ligand, circular dichroism measurements are perturbed by a low signal/noise ratio in the far-UV.

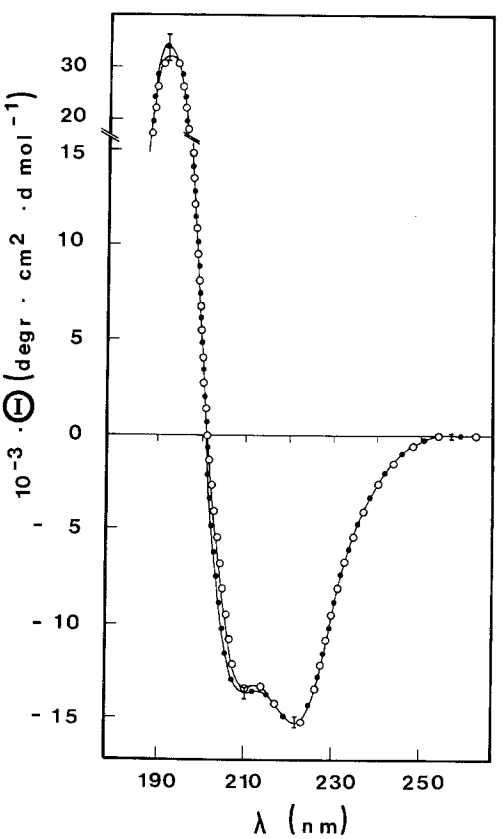
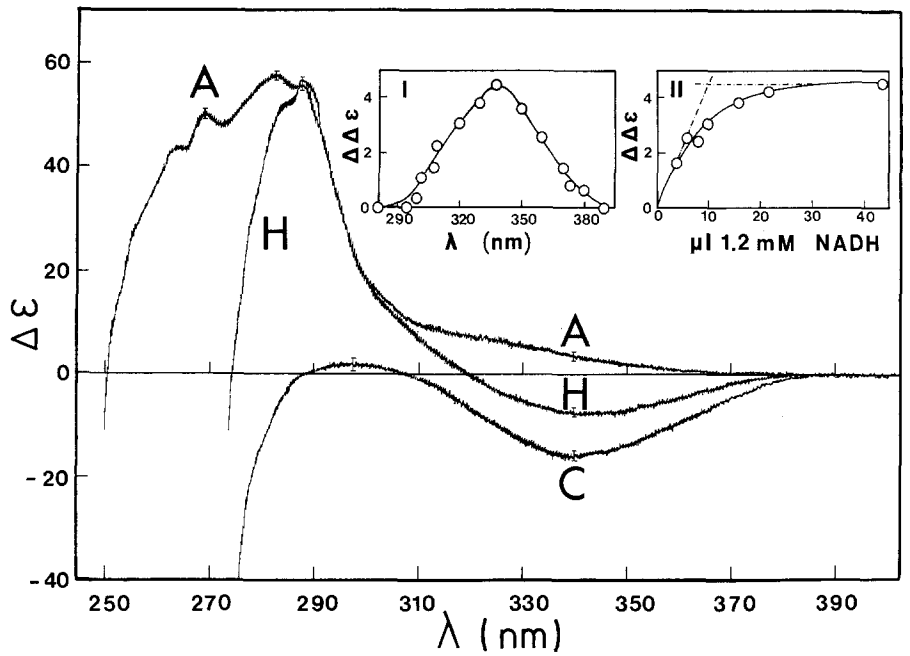


Fig. 2. Circular dichroism spectra of lactic dehydrogenase from pig muscle (**a, b**) and pig heart (**c**) in the absence and presence of coenzyme. Phosphate buffer pH 7.0, I = 0.1 M; 20° C. Spectra either corrected for the ellipticity of the coenzyme, or tandem spectra (comparing the ellipticities of the separated and mixed components). c_{LDH} = 10–50 µM, c_{NADH} = 1.5 mM

a. Far UV CD spectra of LDH-M_4. Apoenzyme (○) and holoenzyme. (●), >95% saturated with NADH. Pathlength 0.01 and 1.0 mm. The isoenzyme from heart muscle shows a closely similar profile with no detectable differences for the apo- and holoenzyme



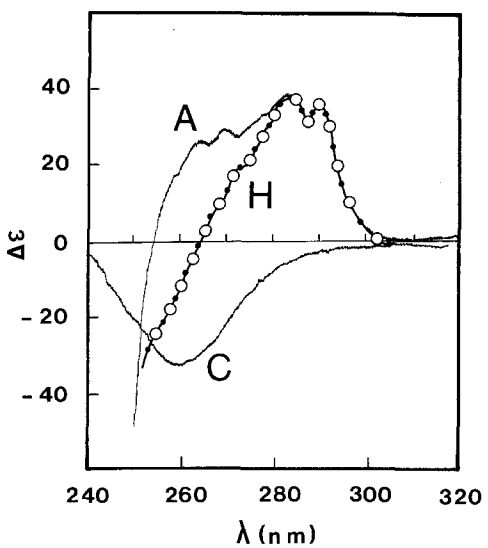


Fig. 2b. Near UV CD spectra of LDH-M_4 and NADH. Tandem cuvettes with 1 mm pathlength in each compartment.

A, apoenzyme (c = 125 µM) vs buffer;

C, coenzyme (c_{NADH} = 3 mM) vs buffer;

H, holoenzyme (mixture of A + C). *Insert I:* Extrinsic Cotton effect in the range of NADH absorption: Calculated as difference spectrum: H minus tandem (apo LDH/NADH). *Insert II:* CD-titration of LDH-M_4 with NADH as monitored by the maximum amplitude at λ = 340 nm. c_{LDH} = 5 µM, c_{NADH} = 1.2 mM. From the equivalent concentrations (144 µM for LDH and 147 µM for NADH) the number of binding sites is found to be 1.0 NADH per 36,000 subunit. K_D is estimated to be 5 µM

c. Near UV CD spectra of LDH-H_4 and NADH. Tandem cuvettes with 5 mm pathlength.

A, apoenzyme (c = 88 µM) vs buffer;

C, coenzyme (c_{NADH} = 1.2 mM) vs buffer;

H, tandem A/C (○), and mixture A + C (●)

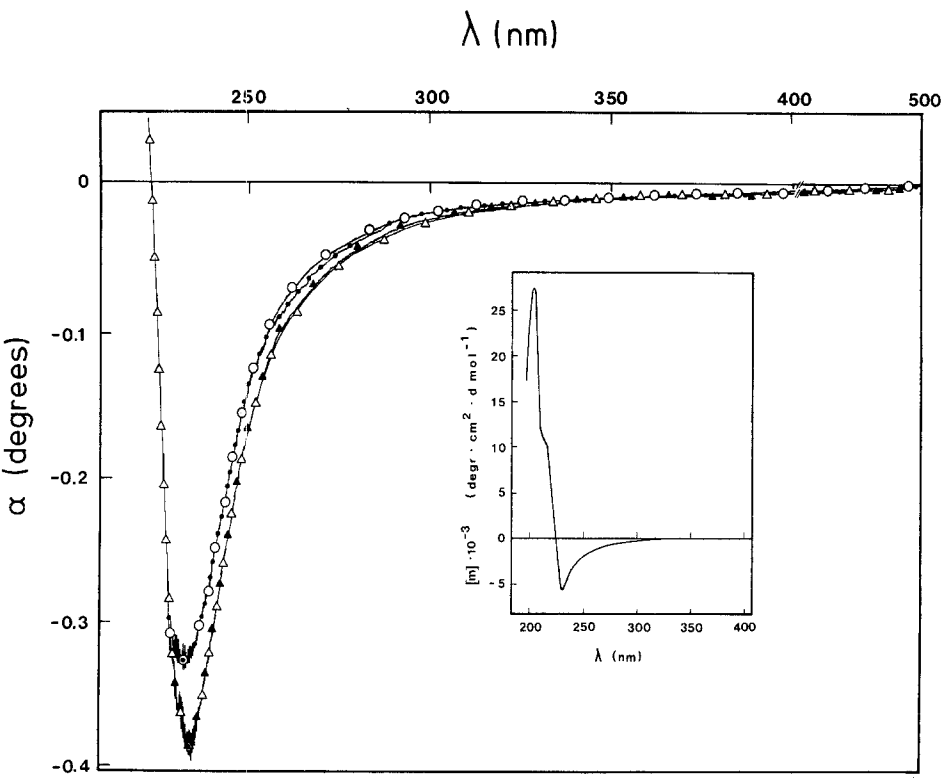


Fig. 3. Optical rotatory dispersion of lactic dehydrogenase from pig muscle (○, ●), and pig heart (△, ▲) in the absence and presence of coenzyme. Phosphate buffer pH 7.0, I = 0.1 M and 0.05 M K_2SO_4; 20° C. c_{LDH} = 15 μM (M_4), and 20 μM (H_4), respectively; c_{NAD} = 0.2–10 mM. Pathlength 0.01–10 mm. The apo- and holoenzymes are represented by open and closed symbols, respectively. *Insert:* Overall ORD spectrum of LDH-M_4 apoenzyme: c_{LDH} = 1 μM. For the anomalous dispersion in the aromatic region, cf. [14]

Therefore, ORD measurements were included which allow to detect structural changes in the wavelength range of plain dispersion at $\lambda > 230$ nm (Fig. 3). Again no significant changes are to be observed (cf. [3, 9]).

In the absorption range of the *aromatic chromophores,* spectral changes are detectable [15]. Blank experiments with the ligand alone prove, however, that the spectrum of the binary complex represents the superposition of the unchanged spectra of the enzyme and its coenzyme; using tandem cuvettes with the separated components in a first experiment, and the complex in a second one, shows that both spectra coincide in the wavelength range of aromatic absorption (Fig. 2b and 2c).

In the *NAD absorption* band a significant extrinsic dichroic absorption band is observed (Fig. 2b). The dissociation constant determined from the respective titration curve (Fig. 2b, insert) is in agreement with the previously mentioned figure (cf. Table 1). The latter result indicates that the enzyme brings about a change in the Cotton effect near the absorption maximum of the ligand molecule which may be tentatively attributed to the fixation of the open form of the coenzyme in the process of holoenzyme formation [1].

Conclusions

From the spectral results it is obvious that the binding of the coenzyme to lactic dehydrogenase is reflected only by an extrinsic Cotton effect, while intrinsic effects due to altered chromphore interactions or changes in the backbone conformation are not detectable, neither for NADH nor NAD^+.

Obviously, the movement of the α2G helix toward the coenzyme site [1] does not affect the helicity within a subunit, or compensatory effects are involved.

Similar considerations hold for the anomalous aromatic residues. There are two tyrosine residues which are engaged in coenzyme interactions [1, 22]: tyr 85 seems to participate only in the initial stages of coenzyme binding, while tyr 246 helps to position arg 101, which in the ternary complex forms an ion pair with the pyrophosphate moiety of the coenzyme [1]. In the binary complex no significant shifts of the chromophores seem to take place.

As mentioned before, considerable overall structural changes have been suggested by X-ray analysis. Direct evidence comes from the observation that soaking apoenzyme crystals with coenzyme causes shattering.

However, this effect seems to be caused by some initial conformational changes in the βD—αD loop (from aminoacid 97 to 120), which is involved in intermolecular crystal contacts rather than direct binary complex formation [1].

The observed changes of the hydrodynamic data indicate a thightening of the enzyme structure upon coenzyme binding, in accordance with the negative heat capacity change deduced from the calorimetric measurements. Whether changes of hydration play a major role cannot be decided. As shown by pH dependent measurements of K_D and ΔG [23] proton release or uptake do not contribute significantly to the observed effects.

The magnitude of the structural effects is less pronounced compared to the analogous effect in yeast glyceraldehyde-3-phosphate dehydrogenase [17]. In contrast to this enzyme lactic dehydrogenase does not show cooperative structural transitions upon coenzyme binding.

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