COMPLEXES OF CHICKEN HEART LACTIC DEHYDROGENASE WITH COENZYMES AND SUBSTRATES

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Incubation of chicken heart lactic dehydrogenase with DPN and pyruvate led to the formation of complexes in which the enzymatic activity was inhibited. These complexes were isolated over Sephadex and their spectral and chemical characteristics were investigated. Analogous complexes have been obtained with 3-acetyl pyridine DPN. A complex containing one molecule of coenzyme and one molecule of substrate per molecule of enzyme was isolated in the very early stages of incubation. Addition of sodium borohydride reduced the complexes. These reduced complexes were obtained with DPN and with a number of DPN analogs. Also these complexes were isolated over Sephadex and they also showed inhibition of the enzymatic activity. The possible implications of these findings from the enzymological point of view are discussed.

The formation of complexes of the type lactic dehydrogenase-oxidized coenzyme-pyruvate has been often postulated (Fromm, 1961; Fromm, 1963), however, no attempt has been made to isolate them. Lee et al.(1966) have obtained a complex lactic dehydrogenase-DPN-fluoropyruvate. The complex of lactic dehydrogenase with DPN and pyruvate has been studied by Gutfreund, et al.(1968). Some work on this problem seemed to be justified by the possibility that these complexes may help to elucidate the mechanism of action of lactic dehydrogenases. For instance, the active site(s) of these enzymes could be "labeled" and identified by means of these complexes. While work along this line is in progress in our laboratory, we present in this communication some aspects of the formation and isolation of complexes of lactic dehydrogenases with coenzymes and substrates.

Materials and Methods

The standard incubation mixture for the formation of the complexes was

the following: 1.2×10^{-5}M CHLDH,[1] 2.0×10^{-3}M coenzyme and 2.7×10^{-3}M pyruvate (or 1-C^{14}pyruvate) in 0.1 M sodium phosphate buffer, pH 6.90 at 21°. No appreciable differences were found by varying the incubation time between 20 minutes and 3 hours. The isolation of the complexes was carried out on a column of Sephadex G-25 (28 x 1.6 cm) eluted with 0.1M sodium phosphate buffer, pH 6.90. The protein was detected from its absorption at 280 mμ, the coenzyme from the absorbance at the peak or from ribose determination and the C^{14} substrate from radioactivity. In the experiments with sodium borohydride, two aliquots of 0.015 ml each of this compound were added 5 minutes apart to the mixture at the end of the incubation period.

Results and Discussion

Incubation of CHLDH with DPN and pyruvate led to the appearance of a spectrum having a peak at 323 mμ. The spectrum was corrected for the optical density at the respective wave lengths of the same amounts of CHLDH, DPN and pyruvate incubated separately in the same experimental conditions. No changes in optical density at 323 mμ were detected by incubating pyruvate and coenzyme alone.

Figure 1 shows the rate of appearance of the optical density at 323 mμ in a solution of CHLDH, DPN and pyruvate (triangles). It is evident that about one fourth of the total increase in optical density took place within 90-120 seconds. The optical density reached a plateau in 15-20 minutes. Concomitant with this increase in optical density was a loss in enzymatic activity. However, most of the enzymatic activity was recovered upon incubation of a diluted sample with 1.3×10^{-4}M DPNH in 0.1 M sodium phosphate buffer, pH 6.90. At pH 10.00 only 10 minutes were needed in order to recover 80-90% of the enzymatic activity. Substantial recoveries occurred simply by diluting the sample in either buffer.

[1] Abbreviations used: CHLDH: lactic dehydrogenase from chicken heart. APDPN: and APDPNH: oxidized and reduced 3-acetylpyridine DPN. DeAPDPN: Deamino 3-acetylpyridine DPN. PADPN: 3-pyridinealdehyde DPN. DePADPN: Deamino 3-acetylpyridine DPN. THDPN: 3-thionicotinamide DPN.

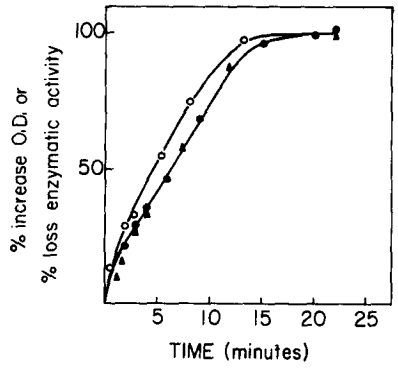

Fig. 1. Percentage increase in optical density at 320 and 340 mμ and percentage loss in enzymatic activity in function of time, upon incubation of CHLDH with DPN or APDPN and pyruvate. CHLDH: 0.73×10^{-5}M; DPN or APDPN: 2.0×10^{-3}M; pyruvate: 3.0×10^{-3}M, in 0.1 M sodium phosphate buffer, pH 6.90. Temperature of incubation: 21°. The values of optical density were blanked out for the absorbance of the single components of the mixture. The loss in enzymatic activity refers to the experiments with APDPN. ▲——▲ experiments with DPN; ●——● experiments with APDPN; O——O loss in enzymatic activity.

Analogous results were obtained with APDPN (closed circles in Figure 1). Loss of enzymatic activity was found also with this coenzyme (open circles). However, unlike in the experiments with DPN, only about 10% of the original enzymatic activity was recovered upon treatment with DPNH.

No losses in enzymatic activity occurred upon incubation of CHLDH with pyruvate alone or DPN or APDPN alone. Therefore, it was assumed that the spectral changes and the losses in enzymatic activity occurring upon incubation of CHLDH with pyruvate and DPN or APDPN were due to the formation of complexes of the type enzyme-coenzyme-substrate. Supporting this assumption was the observation that, if oxalate was used instead of pyruvate, very small spectral changes were detected. Oxalate is known to be a competitive inhibitor of the substrates of lactic dehydrogenases. (Novoa et al., 1959).

In the experiments designed to isolate the complexes, CHLDH was incubated with APDPN and 1-C^{14} pyruvate for about 30 minutes. At the end of the incubation, the mixture was passed through a column of Sephadex G-25. Figure 2 clearly shows that the protein (O.D. at 280 mu), the coenzyme (O.D.

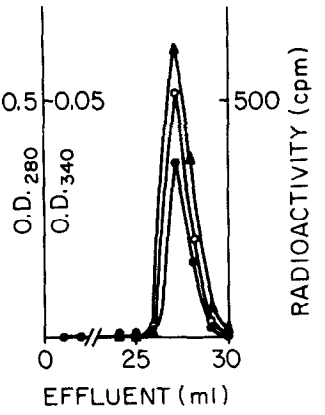

Fig. 2. Ghomatogram on Sephadex G-25 (28 x 1.6 cm) of the complex enzyme-APDPN-pyruvate. Left ordinates: optical density at 280 and 340 mμ . Right ordinates: radioactivity (counts per minute). For experimental conditions: see text. ●——● OD 280, O——O OD 340 ▲——▲ radioactivity.

Table 1

Stoichiometric composition, λ max and enzymatic activity of CHLDH-coenzyme-substrate complexes.

The incubation mixture was as follows: 1.2×10^{-5}M CHLDH, 2.0×10^{-3}M coenzyme, 2.7×10^{-3}M 1-C^{14} pyruvate in 0.1 M sodium phosphate buffer, pH 6.90, unless otherwise indicated. The samples were incubated for about 30 minutes, unless otherwise indicated, at 21° and were then passed through a column of Sephadex G-25 (28 x 1.6 cm).

Experiment	Coenzyme	Composition of complexes coenzyme[a] / enzyme	substrate[a] / enzyme	λ max	Loss enzymatic activity %
1	DPN	0.5	0.5	280,323	15
2	APDPN	1.8	1.4	280,340	50 48[b]
3	APDPN[c]	0.8	0.8	280,340	23
4	DPN[d]	1.7	2.4	280,330	27
5	APDPN[d]	2.2	3.3	280,350	95

a. Molar ratio. b. In this experiment, the diluted complex was incubated with 1 x 10^{-} M DPNH in 0.1 M sodium phosphate buffer, pH 6.90 for 10 minutes. c. sample incubated for 1-2 minutes. d. After the incubation, the sample was treated with two aliquots of 0.015 ml each of sodium borohydride, 5 minutes apart then it was passed through a Sephadex column.

at 340 mu) and 1-C^{14} pyruvate (radioactivity) emerged together from the column, indicating the formation of a complex enzyme-coenzyme-substrate.

Table 1 shows the stoichiometric composition and the loss in enzymatic activity of the isolated complexes. The complex with DPN (expt. 1) was composed of 0.5 molecules of coenzyme and 0.5 molecules of substrate per molecule of enzyme. It had peaks of absorption at 280 and 323 mμ. The low amount of coenzyme and substrate present in this complex probably derives from partial dissociation during passage through the column. This is expression of the relative lability of the complex with DPN, already noticed in the unresolved mixture. The complex enzyme-APDPN-pyruvate emerged from the Sephadex column with 1.8 molecules of coenzyme and 1.4 molecules of pyruvate per molecule of enzyme. The spectrum showed a peak at 340 mμ, like in the unresolved material. Assuming that all of the optical density at 340 mμ was due to the coenzyme bound to the enzyme, an ε_M of 1.0×10^4 was estimated for the 1:1 complex enzyme-APDPN. On the basis of this extinction coefficient, it was calculated that the complex present in the incubation mixture before passage through Sephadex had about 4.4 molecules of APDPN bound per molecule of enzyme. It is clear, therefore, that some dissociation of these complexes took place upon passage through the Sephadex column and that the initial complex (before passage through Sephadex) had about 1 molecule of coenzyme and 1 molecule of substrate per subunit of enzyme. On the other hand, even after passage through Sephadex, substantial amounts of coenzyme and substrate were still bound to the enzyme. It was also calculated that the complex formed within 90-120 seconds of incubation (cf. Figure 1) had about 1 molecule of coenzyme per molecule of enzyme. When this complex was isolated through Sephadex, it was found to be composed of about 0.8 molecules of coenzyme and 0.8 molecules of pyruvate per molecule of enzyme (expt. 3).

It is known that APDPNH at pH 1.0 shows a spectrum with a peak at 300 mμ. Treatment of the complex enzyme-APDPN-pyruvate with 1.0 N HCl (final concentration) caused precipitation of the protein while 95-97% of the radioactivity remained in the supernatant. The supernatant had a spectrum with a peak at 260 mμ , but no peak was evident in the 300 mμ region, indicating that the co-

enzyme was not present in the reduced form. It is probable, therefore, that the peak at 340 mμ of the complex enzyme-APDPN-substrate (323 mμ for the complex involving DPN) derives from the binding of the coenzyme to the enzyme or to the substrate. On the other hand, about 80% of the radioactivity of the supernatant was precipitated with 2.4 dinitrophenylhydrazine showing that the bulk of the substrate was recovered in the form of pyruvate.

In other experiments the formation and isolation of reduced complexes was attempted. The incubation of CHLDH with DPNH and 1-C^{14}lactate did not lead to the formation of complexes detectable spectrophotometrically. Morever, when the incubation mixture was passed through a Sephadex column, the protein emerged with essentially no radioactivity and with only small amounts of DPNH bound to it. Formation of reduced complexes was also attempted by treating the complexes CHLDH-oxidized coenzyme-pyruvate with sodium borohydride. The data relevant to the experiments with DPN and APDPN are shown in Table 1 (expt. 4 and 5). It is evident that the complexes treated with sodium borohydride had more coenzyme and substrate per molecule of enzyme than the complexes non-treated with sodium borohydride. Also the losses in enzymatic activity were more pronounced. Control experiments showed that treatment with sodium borohydride of CHLDH alone or CHLDH plus coenzyme or plus pyruvate did not cause appreciable decrease in enzymatic activity. Complexes analogous to those obtained with DPN or APDPN, were obtained using different coenzyme analogs. All these complexes showed a peak at 280 mμ and a peak in the visible range: at 330 mμ for DPN; at 350 mμ for APDPN, DeAPDPN, PADPN and DePADPN; at 385 mμ for THDPN.

Material having the spectrum of the acid-degradation products of 1,4 APDPNH appeared when 1N HCl (final concentration) was added to the complex formed with APDPN, and treated with sodium borohydride.[2] Moreover, the sub-

[2] It should be pointed out that the peak of absorption of the reduced complexes in the visible range is shifted by about 10 mμ from the peak of absorption of the free reduced coenzyme. It is very probable that these shifts derive from the binding of the coenzyme to the enzyme and/or the substrate.

strate present in the complex was not precipitated with 2,4 dinitrophenylhydrazine. Control experiments showed that treatment of pyruvate alone with sodium borohydride led to the formation of a compound which also was non-precipitable with 2,4 dinitrophenylhydrazine. On the other hand, pyruvate is known to be reduced by sodium borohydride (Chaikin and Brown, 1949).

It is still not clear why a stable reduced complex is not formed from the incubation of CHLDH with reduced coenzyme and lactate, while it is formed upon reduction of the oxidized complex with sodium borohydride. It should be mentioned that borates are probably formed in the reduction of pyruvate with sodium borohydride (Chaikin and Brown, 1949). It is also possible that sodium borohydride is responsible for the formation of relatively stable bond(s) between two or more of the components of the complex. At any rate, it is rather unlikely that sodium borohydride reduces a Schiff base between pyruvate and the enzyme, because of the fact that essentially all of the substrate was dissociated from the enzyme upon treatment of the reduced complex with 1.0 N HCl.

In conclusion, in this work oxidized and reduced complexes have been obtained with lactic dehydrogenase from chicken heart, substrate and coenzyme. At least some of these complexes were very stable and it is conceivable that they may be important from the kinetic point of view. In particular, the rapid formation of enzyme: coenzyme: substrate complexes in about 1:1:1 ratio and the concomitant loss in enzymatic activity suggest that they may be relevant to the phenomenon of the high substrate inhibition of lactic dehydrogenases (Hakalan *et al.*, 1956; Gutfreund *et al.*, 1968). It is also believed that these complexes may be of value in elucidating the mechanism of action of lactic dehydrogenases. For instance, the active site(s) of these enzymes may be "labeled" and identified by means of these complexes. Moreover, the "labeling" of the active site(s) of these enzymes may be used in the elucidation of the tridimensional structure of these enzyme by X-ray diffraction.

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References:

Chaikin, S. W. and Brown, W. G. (1949), J. Am. Chem. Soc. 71, 122.
Fromm, H. J. (1961), Biochim. Biophys. Acta 52, 199.
Fromm, H. J. (1963), J. Biol. Chem. 238, 2938.
Gutfreund, H., Cantwell, R. and McMurray, C. H. (1968), Biochim. J. 106, 683.
Hakalan, M. T., Glaid, A. J. and Schwert, G. W. (1956), J. Biol. Chem. 221, 191.
Lee, H. A., Cox, R. H., Smith, S. L. and Winer, A. D. (1966), Federation Proc. 25, 711.
Novoa, W. B., Winer, A. D., Glaid, A. J. and Schwert, G. W. (1959), J. Biol. Chem. 234, 1134.