# OPTICAL ACTIVITY AND CONFORMATION STUDIES OF TWO PREPARATIONS OF PIG HEART LIPOYL DEHYDROGENASE[1]

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Received June 4, 1968

In connection with our studies of the conformation of the subunits of the multienzyme $\alpha$-keto acid dehydrogenase complexes derived from E. Coli (Koike et al., 1960) and pig heart (Hayakawa et al., 1966), we are examining alterations in conformation of pig-heart lipoyl dehydrogenase wrought by reversible dissociation of FAD from the enzyme. The physical properties of two samples of lipoyl dehydrogenase (pig heart) prepared by different methods, i.e., Massey et al. (1960) and Savage (1957), are difficult to reconcile. There is a history of conflicting data, the most notable of which are the differences in molecular weight ($\lesssim$70,000 for the Savage preparation; 100,000 for that of Massey) and FAD content (Savage: 1 FAD per molecule of enzyme; Massey: 2 FAD per molecule of enzyme). We have studied the circular dichroism (CD), absorption spectra, enzyme activities and reactivity with different thiols of these two preparations. The CD spectra of the apo-lipoyl dehydrogenases derived from these samples

[1] Supported in part by grants from the National Institutes of Health, U.S. Public Health Service (GM 10576), and the National Science Foundation (NSF GB 7128).

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are markedly different, and it is shown that the different enzyme activities of the reconstituted samples are due to different extent of recombination with FAD.

## MATERIALS AND METHODS

The circular dichroism was measured on two instruments, a Cary 60 spectrophotometer and a Jouan Dichrographe with modifications, described by Beychok (1967). Absorption spectra were measured on a Cary 14 Spectrophotometer. These last two instruments were cooled ($0^{\circ}$ - $5^{\circ}$ C) with a Lauda K-2/R circulator. In the Cary instrument jacketed cells were used for cooling.

Samples of enzyme (diaphorase, lipoamide dehydrogenase, lipoamide oxidoreductase and lipoyl dehydrogenase) were obtained from the Boehringer Mannheim Corp., New York; from Sigma Chemical Co., St. Louis, Mo.; and from Gallard-Schlesinger Mfg. Corp., Carle Place, L.I., N.Y. The first of these companies (Boehringer) isolates its material according to the procedure of Savage (1957). The last two companies are supplied by Seravac Laboratories, Maidenhead, England, which follows the procedure of Massey et al. (1960). FAD was obtained from Sigma Chemical Co.

### Preparation of Apoenzyme.

The procedure for removal of FAD will be described more fully elsewhere. In brief, the enzyme is placed in a dialysis sac at a concentration of approximately 3 mg/ml and dialyzed against 1.5 M guanidine hydrochloride in 0.02 M potassium phosphate buffer, pH 7.6, for 2 days with several changes of external solution. When all enzyme activity is lost, the protein is dialyzed against 0.03 M phosphate buffer at pH 7.6 for 8 hours. Approximately half of the protein precipitates upon removal of guanidine,

and only the supernate was used for the studies reported here. EDTA at $10^{-4}$ M is maintained in all solutions, and extra care is given to the dialysis sacs by soaking them in glass distilled water with $10^{-3}$ M EDTA for several days before use.

## RESULTS AND DISCUSSION

The circular dichroism of both enzyme preparations is essentially identical in the region 400 - 200 mμ (Fig. 1). The UV and CD spectra show characteristically large changes (not shown in the figure), particularly in the visible region, when NADH or dihydrolipoic acid is added to lipoyl dehydrogenase. However, when we looked for differences in behavior of the two enzyme preparations toward the two substrates mentioned, none were found, in that both materials showed identical behavior. When each sample was exposed to thiol (mercaptoethanol, dithiothreitol), no significant variations were observed. There are very small changes in the CD spectra when the temperature of the enzyme is raised from $0^{\circ}$ C to room temperature, but the two preparations again showed the same behavior. This uniformity of behavior in the native enzyme contrasts sharply with the findings for the apoenzymes (Fig. 1). For apoenzyme derived from both preparations, the changes in CD spectra consequent to FAD removal suggest marked alterations in secondary structure. However, the changes observed in the apoprotein from the Savage preparation are considerably greater than those measured for the apoprotein of the Massey enzyme. The optical activity in the 222 mμ suggests that the Savage preparation is more highly disordered than the Massey material upon removal of FAD. Furthermore, this conclusion is strongly supported by curve resolution of the interval 240 - 200 mμ. The diminution of intensity in the negative 222 mμ band appears to be associated

predominantly with the CD bands generated by $\alpha$-helical segments.

Apo-lipoyl dehydrogenase recombines with FAD in the presence of dithiothreitol (V. Massey, private communication). Essentially all of the

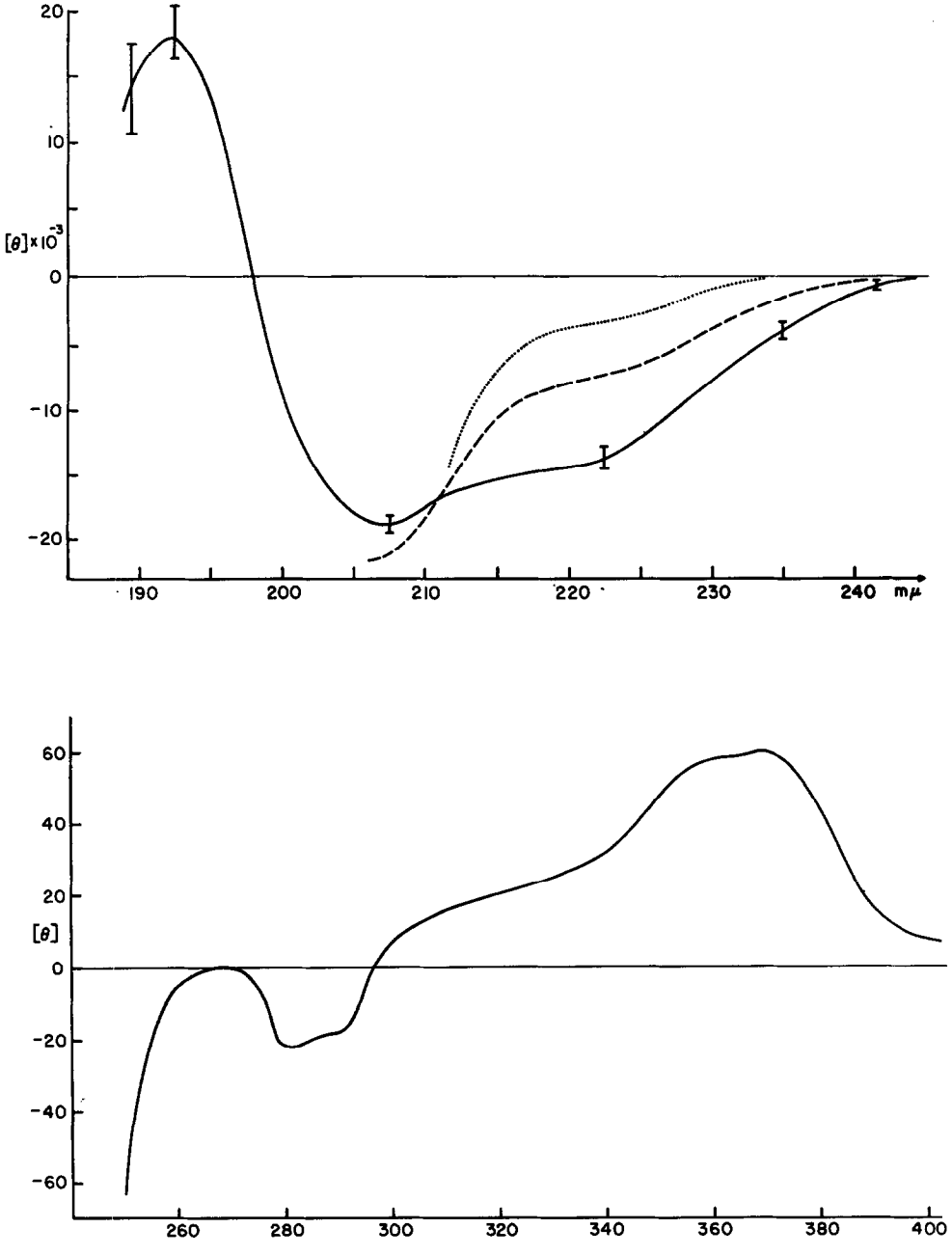


Fig. 1

Figure 1. Circular Dichroism Spectra of Lipoyl Dehydrogenase and Apo-Lipoyl Dehydrogenase. The units of $[\theta]$ are deg. $cm^2$/decimole, assuming a mean residue weight of 115.

Upper - Far UV region. Solid line (————): Native lipoyl dehydrogenase (Savage, N., Biochem. J. 67, 146 (1957); Massey, V. et al., Biochem. J. 77, 341 (1960)). Dashed line (— — — —): Apo-lipoyl dehydrogenase derived by treatment of the Massey preparation. Dotted line (·····): Apo-lipoyl dehydrogenase derived by treatment of the Savage preparation.

Lower - Near UV and visible region. Same legend as above. No circular dichroism bands could be detected in the apo-lipoyl dehydrogenase preparations at wavelengths longer than 260 mμ.

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soluble protein derived from the Massey preparation recombines stoichiometrically overnight (at 5° C) with FAD to give fully active enzyme. Under the same conditions only 50% of the enzyme activity is recovered in the apoenzyme obtained by treatment of the Savage preparation. We believe this finding to be due to the fact that only 50% of the apoenzyme recombines with FAD. UV spectra indicate that the 50% enzyme activity recovered upon recombination with FAD is due to a 50% recombination of FAD. This conclusion is further substantiated by the following result: The enzyme of both preparations is relatively stable at room temperature. This is not true for the apoenzyme. If not maintained at 0° - 10° C, the protein readily precipitates. Thus, if the apoenzyme derived from the Savage preparation is recombined with FAD in the presence of dithiothreitol, 50% of the protein can be precipitated by bringing the sample to room temperature. The solution is centrifuged and a white pellet obtained, indicating the absence of FAD in this fraction. On the other hand, the recombined Massey preparation shows the same stability as the native enzyme at room temperature.

It appears then from the CD data and from recombination studies that

lipoyl dehydrogenase, prepared by the Savage procedure, does not yield a homogeneous material. The removal of FAD from this preparation results in a more drastic reduction of the negative band around 220 mμ, twice that of the Massey diaphorase. The CD data are consistent with the interpretation that the Savage enzyme is unusually susceptible to denaturation in guanidine, and only half of the Savage apoenzyme recombines with FAD in the presence of dithiothreitol.

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