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RELATION BETWEEN CONFORMATIONS AND ACTIVITIES OF LIPOAMIDE DEHYDROGENASE

I. RELATION BETWEEN DIAPHORASE AND LIPOAMIDE DEHYDROGENASE ACTIVITIES UPON BINDING OF FAD BY THE APOENZYME

J. F. KALSE AND C. VEEGER
Department of Biochemistry, Agricultural University, Wageningen (The Netherlands)

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SUMMARY

1. The apoenzyme of lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) can be prepared by acid $(NH_4)_2SO_4$ treatment. This apoenzyme is able to bind 1 mole FAD per 48 000 g protein. For this process $\Delta H = -8300$ cal·mole^{-1} and $\Delta S = -4$ e.u. The activities obtained are dependent on the temperature employed. At 0–5°, no return of the activity with lipoate is observed but the activity with 2,6-dichlorophenol indophenol (DCIP) is increased to a level 20-fold higher than that of the holoenzyme. The results indicate that at least two different conformations are involved in this temperature range. At 5–25° the activity with lipoate returns in a bimolecular reaction with respect to protein concentration, at the expense of the activity with DCIP. The activation energy is 21 000 cal·mole^{-1}.

2. The apoenzyme of the enzyme modified by Cu^{2+} prepared in a similar way is also able to bind FAD. However in this case, the DCIP activity returns to its original level. Treatment of the normal apoenzyme with Cu^{2+} leads to a total loss of the FAD-binding capacity.

3. Freezing and thawing at low ionic strength leads to an enzyme which shows stimulated activity with DCIP and diminished activity with lipoate; bovine serum albumin, $(NH_4)_2SO_4$ and EDTA protect against these changes. Incubation at 25° reverses this modification. Freezing and thawing induces a reversible shift in the flavin absorption from 455 mμ to 452 mμ; the maximum of the fluorescence emission shifts 2 mμ to the blue side of the spectrum.

INTRODUCTION

Flavoproteins show a heterogeneous behavior with respect to the removal of the prosthetic group. Some of them are well known for the easy removal of their flavins

Abbreviation: DCIP, 2,6-dichlorophenol indophenol.

under relatively mild conditions, *i.e.* dialysis against diluted acids, buffers or ammonium sulphate precipitation at acid pH, and their ability to recombine with their original prosthetic group. Examples are D-amino acid oxidase[1], glucose oxidase[2] and NADPH cytochrome *c* reductase[3], all of which contain FAD as prosthetic group. There are also FMN-containing enzymes belonging to this class, such as the old yellow enzyme[4], NADH cytochrome *c* reductase[5] and glycolic acid oxidase[6].

Recombination of the apoenzyme with flavin compounds different from the native one has been carried out in several cases, *e.g.*, for old yellow enzyme where FAD (ref. 7) or riboflavin[8] can replace the FMN moiety, and results in a complex which is nearly as catalytically active. HORECKER found with NADPH cytochrome *c* reductase that replacement of FMN by FAD resulted in a more active complex.

On the other hand, enzymes such as succinate dehydrogenase[9], xanthine oxidase[10] or L-lactate cytochrome *c* reductase[11] withstand all attempts to remove the prosthetic group without denaturing the apoenzyme.

To release the FAD from succinate dehydrogenase, GREEN, MÜ AND KOHOUT[12] found that tryptic digestion was required, because all conventional methods of deproteinization failed. Although it seems that some metalloflavoproteins are not very prone to release their prosthetic group and non-metalloflavoproteins are, one may not generalize. D-Lactate cytochrome *c* reductase from yeast, a metalloflavoprotein containing zinc[13], and nitrate reductase from Neurospora, containing molybdenum[14], split off their prosthetic groups readily. FAD from the metal-free enzyme L-amino acid oxidase cannot be removed without denaturation of the apoenzyme.

Until recently, the same opinion existed about lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) isolated from pig heart. Attempts to remove the FAD from the enzyme failed. WILLIAMS[15] succeeded in separating FAD from lipoamide dehydrogenase of *Escherichia coli*, but had limited success in re-adding FAD to reconstitute the original activity. The aims of this study have been to elucidate the conditions under which the FAD moiety could be split off without denaturing the apoenzyme of pig heart lipoamide dehydrogenase and the effect of temperature change on the recombination. The binding of FAD derivatives by the apoenzyme is dealt with in another paper[16]. The association–dissociation phenomenon connected with this recombination is described in a third paper[17]. Preliminary data of this study have been given[18–20].

METHODS

NAD^+ (98%), FAD, FMN and lipoate were obtained from the Sigma Chemical Co.; NADH from Boehringer and Söhne; DCIP from the British Drug House, reduced lipoamide was a gift from Dr. H. Moed of Philips Duphar.

Lipoamide dehydrogenase from pig heart was prepared by a modification of published methods[21,22], by the addition of a second heat treatment (5 min at 70° under the same conditions[21]) followed by a $(NH_4)_2SO_4$ fractionation between 0.55 and 0.70 saturation.

The protein concentration of the enzyme was measured at 280 mμ with $A^{1\text{ cm}}_{1\text{mg/ml}} = 1$. The concentration of enzyme-bound flavin was calculated from the absorption at 455 mμ, assuming that the molar extinction coefficient of the enzymebound flavin is the same as that of the free FAD at 450 mμ, *i.e.* $11.3 \cdot 10^3$ cm^2/mmole (ref. 23).

Removal of the flavin was accomplished by precipitating the enzyme in the presence of 1.5 M KBr with a saturated $(NH_4)_2SO_4$ solution (pH 1.5 at 0–2°) according to the method of STRITTMATTER[24]. 6 mg of enzyme were dissolved in 2 ml of 1 M Tris–acetic acid buffer (pH 8.1) containing 3 M KBr and 1 mM EDTA. 2 ml of a saturated $(NH_4)_2SO_4$ solution (pH 1.5) were added over a 20-sec period; after 40 sec an additional 8 ml were added. The precipitated apoenzyme was centrifuged at 18 000 $\times$ g for 6 min. The yellow supernatant solution was decanted, after which the inside of the centrifuge tube was wiped with absorbant paper. The apoenzyme was dissolved in 1 ml of 1 M Tris buffer (pH 8.1), directly followed by 1 ml of 0.1 M phosphate buffer (pH 7.2) containing 3 mM EDTA to prevent denaturation, or by dissolving it in 0.3 M phosphate buffer (pH 7.6) + 3 mM EDTA and diluting this solution with 0.03 M phosphate buffer to a final phosphate concentration of 0.08–0.16 M. The solution was clarified by centrifugation.

The activity of the enzyme in the reaction with oxidized lipoate was determined according to the procedure of MASSEY AND VEEGER[25]. The method of SAVAGE[26] was used for the reaction with DCIP.

Fluorescence measurements were performed with a Zeiss spectrofluorimeter ZMF 4C using a 450-W Xenon lamp as a light source. Standardization of the measurements was achieved by referring to a Zeiss quartz standard, No. 8843. The spectra were corrected for slit width of the second monochromator and for phototube (IP 28) response. The excitation wavelength used was 395 mμ.

Fluorescence polarization measurements were performed with a modified Zeiss spectrofluorimeter. Behind the exit slit of the monochromator a cuvette holder was placed, containing a polarizer and an analyzer. The polarizer and analyzer are in a horizontal plane and make an angle of 90° with each other. In between them is the square cuvette of 1 cm. The polarizer and analyzer may rotate in their mounts between two stops that fix positions H and V. In the V position, the electric vector of the exciting light is perpendicular to the plane formed by the direction of propagation of the exciting light and the direction of observation. In position H the electric vector is in the plane. Before entering the detector, the polarized fluorescence light passes through an interchangeable filter; a Zeiss FL 56 filter was used. The polarization of the fluorescent light emitted at right angles to the direction of excitation is given by: $p = (I_V - I_H)/(I_V + I_H)$. I_V is the intensity of the component vibrating perpendicular to the plane determined by the direction of excitation and observation. I_H is the intensity of the component vibrating in the plane.

RESULTS

The effective removal of the flavin is very strongly dependent on the pH of the saturated ammonium sulphate solution. Furthermore the concentration of the enzyme is an important factor, the upper limit for effective removal of the FAD being 3 mg/ml. The residual activity of the apoenzyme with lipoate as electron acceptor might be considered as a criterion for the effectiveness of the removal of flavin. When protein concentrations higher than the critical 3 mg/ml are used, the residual activities vary from 20% up to 60% of those of the original enzyme. Fig. 1 shows the activity of the apoenzyme with lipoate expressed as percent of the activity of the holoenzyme, when precipitated with $(NH_4)_2SO_4$ solutions of various pH values. As can be seen from

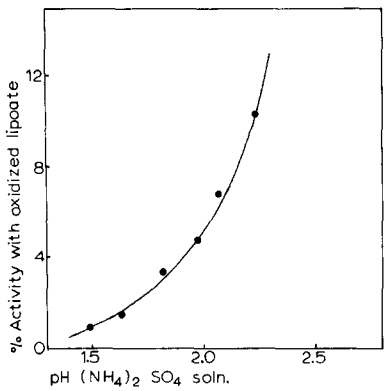

Fig. 1. The influence of pH on the dissociation of FAD from lipoamide dehydrogenase. Portions of 3 mg of enzyme were treated at 0° with saturated $(NH_4)_2SO_4$ solutions of various pH values, as described under METHODS. The apoenzyme was dissolved in 1 ml 0.17 M phosphate buffer containing 1 mM EDTA (pH 7.6) and tested for activity with oxidized lipoate as described under METHODS.

Fig. 1, the optimum pH for removal of the prosthetic group is 1.4 to 1.5. Lower pH values are not useful, since the loss of apoenzyme by denaturation becomes too high.

Measurements of the amount of FAD bound to the apoenzyme and the amount extracted in the supernatant after the low pH treatment reveal that more FAD is bound to it than is expected from the residual activity.

This amount comes into agreement with the activity with lipoate after incubating the apoenzyme with substrate, just by keeping it on ice for a prolonged period or after a shorter period at room temperature. The increase is 2–3-fold (Table I). The activity with DCIP is totally unpredictable; it can vary from 80% to 300% of the original activity, independent of the remaining activity of the apoenzyme with lipoate.

The apoenzyme is rather labile and easily denatures on freezing and thawing or on being kept at room temperature for a prolonged period. Although the apoenzyme

TABLE I

RESIDUAL ACTIVITY OF LIPOAMIDE DEHYDROGENASE APOPROTEIN UNDER DIFFERENT CONDITIONS

8 mg of apoenzyme were incubated at the temperature indicated for 90 min in a total volume of 1.4 ml containing 120 μmoles of phosphate buffer (pH 7.2), 400 μmoles of Tris buffer (pH 8.1) and 0.4 μmole of EDTA.

	% Specific activity oxidized lipoate
Apoenzyme	0.3
Apoenzyme + 4 μmoles reduced lipoamide, on ice	0.7
Apoenzyme + 4 μmoles lipoate, on ice	0.7
Apoenzyme, at room temperature	0.7
Apoenzyme one night on ice	0.7
Apoenzyme + 0.1 μmole FAD, on ice	7.0
Apoenzyme + 0.1 μmole FAD at room temperature	64.0

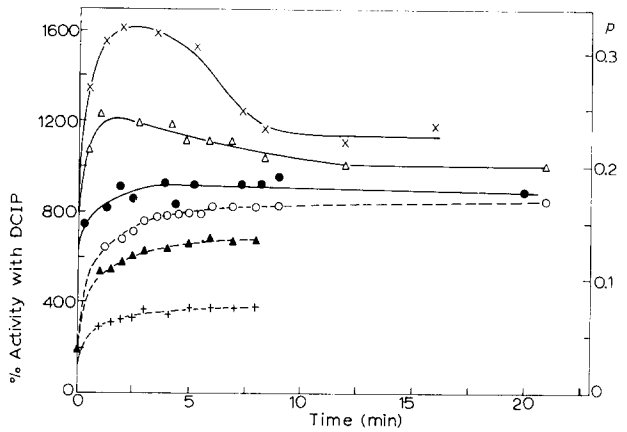

Fig. 2. The binding of FAD to lipoamide dehydrogenase apoprotein at 5°. The cuvettes contained in a volume of 2.1 ml 0.05 M phosphate buffer *plus* 1 mM EDTA (pH 7.6), apoenzyme 4.3 μM and the following amounts of FAD: 12.9 μM FAD; × × ×, DCIP activity; + + +, polarization; 4.3 μM FAD; △△△, DCIP activity; ▲▲▲, polarization; 2.2 μM FAD; ●●●, DCIP activity; ○○○, polarization. Samples were withdrawn at different times and the DCIP activity determined.

contains some residual FAD (less than 5%), its spectrum only shows the protein absorption band at 280 mμ. The concentration of the apoenzyme can be measured at 280 mμ taking $A^{1\text{ cm}}_{1\text{mg/ml}} = 0.80$, a value obtained by comparing the absorbance with the protein content found by the biuret method[27]. The molarity of the apoenzyme can be calculated by assuming a molecular weight of 50 000 (ref. 17).

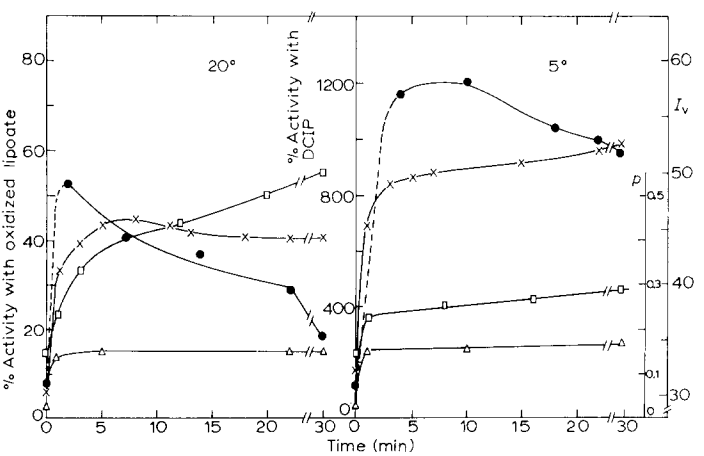

Fig. 3. The effect of temperature and time on the development of the activity with oxidized lipoate upon the addition of FAD to the apoenzyme of lipoamide dehydrogenase. The cuvettes contained in a volume of 2.5 ml: 0.05 M phosphate buffer *plus* 1 mM EDTA (pH 7.6), 8 μM apoprotein and 16 μM FAD. Samples were withdrawn from the cuvettes at the times indicated and the activities of the enzyme determined. ●●●, DCIP-activity; □□□, activity with oxidized lipoate; △△△, fluorescence polarization (p); × × ×, vertical component (I_v) of polarized light in arbitrary values. The fluorescence and fluorescence polarization values at zero time are those of the added free FAD.

The reverse reaction, *i.e.* the recombination of FAD with the apoenzyme is rather complicated and depends on factors such as pH, temperature and ionic strength. Reproducible results are only obtained in 0.08–0.16 M phosphate buffer (pH 7.6). Since the apoenzyme is half the molecular weight of the holoenzyme[17], an association of the protein must also occur in the course of this process.

The binding process was followed by measuring the polarization of the fluorescence, the intensity of I_V and the activities with DCIP and lipoate. Figs. 2 and 3 give the results obtained at 5° and 20°. Upon the addition of FAD, the activity with DCIP increases rapidly, usually reading 90% of its maximum activity within 1 min. The maximum activity is obtained in 2–3 min. The activity with DCIP is dependent on the FAD concentration and on the temperature. With saturating concentrations of FAD, activities 20 times higher than that of the holoenzyme can be obtained (see Fig. 1 of ref. 16), provided that the recombination is carried out between 0 and 5°. At higher temperatures lower DCIP activities are found, even when measured immediately after mixing. At 5° a decline in the DCIP activity is also observed, unaccompanied by increased activity with lipoate.

The polarization of the fluorescence shows that, in general, 1 min after mixing 70% of the maximum amount of FAD has reacted with the apoenzyme; the maximum polarization is found after 5 min. Lipoamide dehydrogenase shows an increased fluorescence compared with that of free FAD (ref. 28). At 20° the maximum value of I_V is found after 5–7 min, this is followed by a small decline, which does not change the polarization. This decline can be observed at temperatures higher than 10°. At 5° it takes much longer to obtain the maximum value of I_V; it gradually increases without changing the polarization.

Fig. 4 and Table II show that the binding of FAD to the apoenzyme is a temperature-dependent process. 1 mole of FAD is bound per 48 000 g of protein, which amounts to a 1:1 ratio. The binding is an exothermic process (Fig. 5). Assuming ideal solutions of protein and FAD, $\Delta H = -8300$ cal·mole^{-1}, whereas $\Delta S = -4$ e.u.

A significant return of the activity with lipoate occurs at temperatures higher than 10°. At 0° almost no increase of this activity is found. The rate of increase is

TABLE II

TEMPERATURE-DEPENDENT VARIATION OF THE ASSOCIATION CONSTANT OF FAD FOR THE DCIP-ACTIVE ENZYME

The fraction of bound FAD, was determined by two methods. Method A: from the polarization at the time of maximum DCIP activity, using the formula of BAYLEY AND RADDA[29] and assuming that the emission intensity and polarization of the FAD bound to the DCIP-active enzyme have the same values as those of the holoenzyme ($p = 0.380$), with $p = 0.030$ for the free FAD. Method B: taking 20 as the ratio of the activities of the DCIP enzyme and the holoenzyme. A correction was made for the amount of activity with lipoate present.

Data	*Method*	K_{ass} ($\times 10^{-5}$)			
		0°	5°	10°	20°
Fig. 2	B		3.7		
Fig. 3	A		4.2		2.1
Fig. 3	B		3.8		1.5
Fig. 4	A			3.2	2.2
Fig. 1 (ref. 16)	B	5.3			

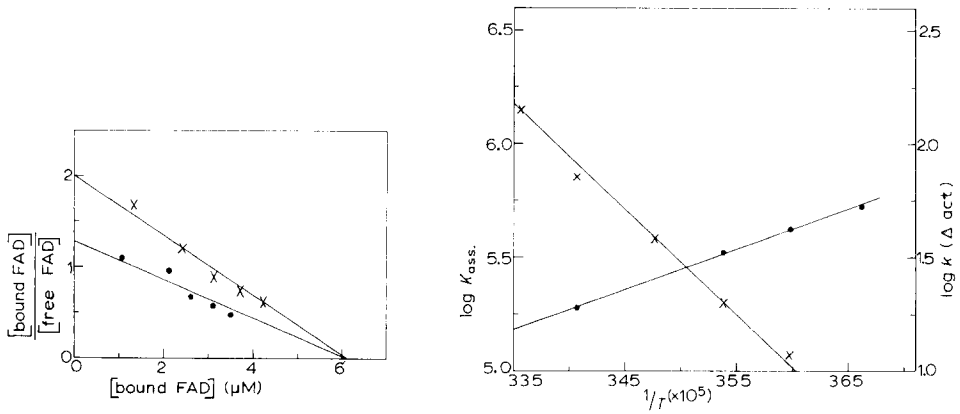

Fig. 4. SCATCHARD[30] plot of the equilibrium between apoenzyme and FAD. The cuvettes contained in 2 ml: 0.05 M phosphate buffer *plus* 1 mM EDTA (pH 7.6), 6.4 μM apoenzyme and the following concentrations of FAD: 2.2 μM; 4.4 μM; 6.6 μM; 8.8 μM; 11.0 μM. The polarization of fluorescence was determined at the temperature indicated at the time of maximum DCIP activity and the equilibrium constant calculated by method A of Table II. ●●●, 10°; × × ×, 20°.

Fig. 5. Variation with temperature of the binding of FAD to apoenzyme and the increase of activity with oxidized lipoate. ●●●, van 't Hoff plot of the data given in Table II; × × ×, Arrhenius plot of the second order rate constants calculated from the experiment given in Fig. 6.

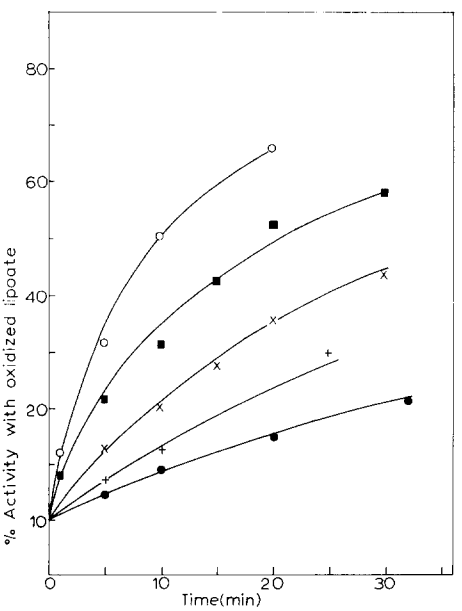

Fig. 6. Kinetics of the return of the activity with oxidized lipoate after the addition of FAD to the apoenzyme of lipoamide dehydrogenase to 1.4 ml of 0.09 M phosphate buffer (pH 7.6), containing 0.3 mM EDTA and 14 μM apoenzyme, incubated at the temperature indicated. 100 μM FAD were added. Samples were withdrawn at the times indicated and the activity with oxidized lipoate measured as described under METHODS. From the experimental points the second order reaction constant was calculated, after correcting for the amount of activity with oxidized lipoate present at zero time. The solid lines are computed from the average values of the reaction constants. Experimental values: ○○○, 25°; ■■■, 20°; × × ×, 15°; + + +, 10°; ●●●, 5°.

dependent on the protein concentration; at 25° at concentrations higher than 1 mg/ml, more than 90% of the original activity is restored within 30 min. There appears to be a direct correlation between the amount of activity with DCIP at 0–5° and the amount of activity with lipoate restored at 25°.

It has been proposed[18] that the rate at which the lipoate activity returns in this process is linear with time. Fig. 6 shows the results of a closer examination of this process. The agreement between the computed and experimental data shows that the reaction is second order with respect to the protein concentration (in the presence of a saturating amount of FAD) at all temperatures investigated. No constant value for the first order rate constant could be calculated. The activation energy for this process is 21 000 cal · mole^{-1} (Fig. 5). A larger amount of apoenzyme was recombined with FAD at 20°. After recombination the newly formed enzyme was purified. The properties of the enzyme after purification are identical with those of the original enzyme with respect to its spectrum, its stability to heating up to 70° and the activities with lipoate and DCIP. The results are summarized in Table III.

TABLE III

PROPERTIES OF THE RECONSTITUTED LIPOAMIDE DEHYDROGENASE

Apoenzyme was prepared from 30 mg of native enzyme. The solution from which it was prepared contained 1.5 mg of native enzyme per ml. The apoenzyme was incubated with a saturating amount of FAD at 20° for 120 min. The incubation medium contained in a total volume of 30 ml: 6 mmoles of phosphate (pH 7.4), 10 μmoles of EDTA and 2 μmoles of FAD. After incubation the recombined enzyme was precipitated with $(NH_4)_2SO_4$, dialyzed 1 night against 5 l of 0.03 M phosphate buffer (pH 7.4) + 0.3 mM EDTA, centrifuged, passed through a calcium phosphate gel column[21,22], and fractionated with $((NH_4)_2SO_4$.

	Total protein (mg)	*% Specific activity oxidized lipoate*	*% Specific activity DCIP*
Native enzyme	30	100	100
Apoenzyme	28	3	80
Apoenzyme + FAD after 120 min incubation, precipitated and dissolved	27	82	420
After dialysis	26	87	400
After column	25	93	310
Fraction 45–75% saturated $(NH_4)_2SO_4$	24	97	240

The influence of Cu^{2+} on the recombination of FAD with the apoenzyme

Lipoamide dehydrogenase is modified by ions of heavy metals, especially cupric ions, as has been shown by VEEGER AND MASSEY[31]. It was found that treatment of the enzyme with traces of Cu^{2+} results in an almost inactive enzyme with respect to its physiological acceptor and a 20-fold increased activity with the artificial acceptor DCIP. This effect is catalytic and only reversible upon cysteine treatment[34].

The apoenzyme of the Cu^{2+}-modified enzyme can be prepared according to the same method as that used for the native enzyme. When it is incubated with FAD at 22°, the physiological activity does not return; only about 70% of the activity

TABLE IV

INFLUENCE OF Cu^{2+} ON THE BINDING OF FAD BY LIPOAMIDE DEHYDROGENASE APOENZYME

0.04 μmole of dialyzed enzyme was incubated at room temperature for 90 min in a total volume of 2 ml containing 0.2 μmole of Cu^{2+} and 75 μmoles of phosphate buffer (pH 6.3). After incubation 8 μmoles of EDTA were added, and the temperature was lowered to 0–2°. 1 mg of apoenzyme of either the modified or the native enzyme was incubated at room temperature in a total volume of 1 ml with 200 μmoles of phosphate buffer (pH 7.4), 0.3 μmole of EDTA and the additions indicated.

	% Specific activity oxidized lipoate	*% Specific activity DCIP*
Modified enzyme	4	1000
Apoenzyme of modified enzyme	0.6	100
Apoenzyme of modified enzyme + 0.1 μmole FAD	1.2	660
Apoenzyme of normal enzyme	0.8	80
Apoenzyme of normal enzyme + 0.1 μmole FAD	96	220
Apoenzyme of normal enzyme incubated with 5 atoms of Cu^{2+} and after removal of Cu^{2+} incubated with 0.1 μmole FAD	1	130

with DCIP returns. Upon recombination at 0°, higher DCIP activities are always obtained than are observed with the original Cu^{2+}-treated enzyme.

The spectral properties of the recombined modified enzyme are the same as those obtained after the original modification, that is having the maximum absorption at 451 mμ and weakened shoulders on both sides of it. On the other hand, incubation of the apoenzyme from the native enzyme with Cu^{2+} shows a different pattern. After the removal of the cations by means of EDTA, an inactive apoenzyme is obtained which is not able to recombine with FAD. The results are given in Table IV.

Influence of freezing and thawing on the enzyme

When the holoenzyme is dialyzed twice against a 3000-fold greater volume of

TABLE V

THE INFLUENCE OF FREEZING ON LIPOAMIDE DEHYDROGENASE

The dialyzed enzyme was frozen in portions of 1 ml containing 0.26 mg of enzyme, 15 μmoles of phosphate buffer (pH 7.2) and the additions as indicated.

	% Activity					
	After thawing		*2 h at room temp.*		*1 night at room temp.*	
	Oxidized lipoate	*DCIP*	*Oxidized lipoate*	*DCIP*	*Oxidized lipoate*	*DCIP*
Enzyme	12	1100	18	1000	75	400
Enzyme + 2% $(NH_4)_2SO_4$	80	550	85	500	96	140
Enzyme + 2% $(NH_4)_2SO_4$ + 1 μmole EDTA	100	100	100	100	100	100
Enzyme + 1 μmole EDTA	80	600	90	200	95	120
Enzyme + 2% bovine serum albumin	100	100	100	100	100	100

TABLE VI

DISSOCIATION OF FAD FROM FROZEN LIPOAMIDE DEHYDROGENASE

1 ml of enzyme solution (200 μM enzyme–FAD) was kept frozen for 24 h and immediately after thawing, dialyzed against 15 ml of 0.03 M phosphate buffer (pH 7.3) at the temperatures indicated. After 40 h dialysis, the concentration of FAD in the dialyzate was determined spectrofluorometrically.

Enzyme–FAD	*FAD in dialyzate*	*Temp.*	K_{ass} ($\times 10^{-5}$)
200 μM	0.95 μM	0°	7.1
200 μM	1.2 μM	25°	5.0*

* Due to the slow return of activity with lipoate, this value is too high.

0.03 M buffer (pH 7.4) to remove EDTA and $(NH_4)_2SO_4$, diluted, frozen at −15° and kept frozen for 14 h or longer, it undergoes a change resulting in high activity with DCIP and low activity with lipoate. Contrary to the effect of Cu^{2+} (*cf.* ref. 31), which results in the same change of the activities, this effect is reversible. After thawing and raising the temperature to 25°, the activity with DCIP decreases, while that with lipoate increases. The activity with lipoate is not fully restored but reaches a level of about 80%, as shown in Table V. These effects are independent of the way of freezing, *i.e.* rapid freezing in liquid N_2 leads to the same alterations as observed after slow freezing at −16°. On the other hand, these alterations are more pronounced after longer periods of freezing.

When the dialyzed enzyme is frozen for 16 h at −15°, thawed and then dialyzed immediately against buffer, contrary to the case with the holoenzyme FAD, the FAD of the frozen enzyme easily dissociates. By dialysis against a known amount of buffer, the FAD in the dialyzate was determined by fluorescence measurements and the association constant was calculated. The results are given in Table VI.

The frozen enzyme differs in other respects from the native enzyme. The maximum absorption shifted to a shorter wavelength, 452 mμ, analogous to that of the Cu^{2+}-modified enzyme. Upon heating there is a hypsochromic shift to the original maximum of 455 mμ. Similar shifts occur in the fluorescence spectrum, as shown in Fig. 7.

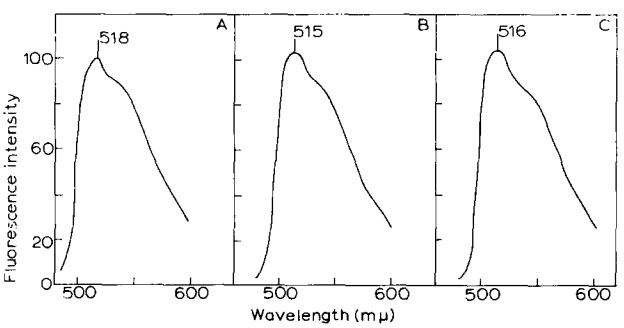

Fig. 7. Fluorescence emission spectra of native enzyme, Cu^{2+}-treated enzyme and frozen enzyme at 10°. Enzyme–flavin concentrations were 5 μM in 0.03 M phosphate buffer (pH 7.2) and 0.3 mM EDTA. Excitation wavelength, 396 mμ; exciting light, 18-mμ band. The spectra are corrected for the dispersion of the emission slit and the photomultiplier sensitivity. A, holoenzyme; B, Cu^{2+}-treated enzyme; C, frozen enzyme.

DISCUSSION

The evidence presented indicates that under controlled conditions the resolution of FAD from lipoamide dehydrogenase can take place without denaturation. Utmost care has to be taken in preparing the apoenzyme, to prevent its denaturing. In the course of our investigation it has been found that, when prepared with some residual activity with lipoate, the apoenzyme is more stable and the recombination more complete. This is in accordance with the findings of KOIKE, REED AND CARROL[32] with bacterial lipoamide dehydrogenase. These workers employed a very mild (pH 3.5) $(NH_4)_2SO_4$ treatment, which resulted in a loss on only 70% of the lipoate activity; on incubation with FAD, 95% of the activity could be restored. It is possible that upon drastic treatment with acid $(NH_4)_2SO_4$ the apoenzyme undergoes an additional conformational change and that this altered enzyme cannot recombine with FAD. The presence of an amount of FAD keeps the apoenzyme in the right conformation, probably by reversible association–dissociation of this prosthetic group. The presence of this residual FAD does not reflect itself in the activity with lipoate; most of the apoenzyme preparations, however, do show a 2–3-fold stimulated activity with DCIP. Upon incubation this artificial activity is converted into physiological activity by increasing the temperature or by adding substrate at low temperature. It seems that substrate and DCIP enzyme are able to form a complex which at low temperature accelerates the conversion into the lipoate-active enzyme, or prevents the denaturation of either the apoenzyme or the DCIP-active enzyme.

Similar phenomena have been found by STRITTMATTER[24]; changes in protein conformation are necessary for recombination of cytochrome b_5 reductase with its prosthetic group. On preparing the apoenzyme of lipoamide dehydrogenase from *E. coli*, WILLIAMS[15] found irreversible changes in the protein which preclude binding of the FAD. The enzyme from *E. coli* might differ in this respect from the enzyme from pig heart. On the other hand, it is possible that other conditions, such as a different ionic strength or a different temperature, give better results.

From the data presented here, it is clear that the formation of the holoenzyme from apoenzyme and FAD is not a one-step process. The data show that binding of FAD does not lead to drastic conformational changes in the protein. Binding mainly leads to a change in enthalpy rather than in entropy. At least two intermediates can be detected: a DCIP-active conformation (I) being a monomer, with an activity at least as high as that of the Cu^{2+}-treated enzyme[31], but with the difference that the latter is a dimer (*cf.* ref. 17). This conformation is only stable to a limited extent. The studies at 5° clearly show that the maximum activity is followed by a decline in activity, while at that stage the polarization of the fluorescence still increases, indicating that FAD is bound due to a consecutive reaction which displaces the equilibrium between apoenzyme and FAD. This second form (II) might be a non- or partially active conformation in equilibrium with the first form, for which it has not been established whether it dimerizes to the dimer (*cf.* ref. 17) active with oxidized lipoate.

An observation which is rather puzzling is the increase of I_V without change in polarization when working at 0–5°. This increase of I_V reflects the unfolding of FAD after binding has taken place (*cf.* ref. 33). Whether this unfolding leads to the formation of conformation (I), in other words is formed from an inactive FAD–apoenzyme complex, or reflects the formation of complex(II) is not clear. The latter explanation would

make an equilibrium between conformation (II) and free FAD a necessary condition. Some arguments in favor of the latter can be found in the work of CASOLA, BRUMBY AND MASSEY[34], who showed that the DCIP-active Cu^{2+}-treated enzyme easily loses FAD. This might indicate that DCIP-active conformations, which, according to the absorption spectra of the oxidized enzyme, have their FAD bound in a more polar environment (*cf.* ref. 18), easily lose FAD.

Although no direct evidence is yet available which would permit a more definite statement, it seems probable that, after freezing, an independent DCIP-active conformation is responsible for the increased DCIP activity and the decline of the activity with oxidized lipoate. The increase in DCIP activity is much smaller than the decline in activity with lipoate. On the other hand, it has been shown with the Cu^{2+}-modified enzyme[31] that this relation is dependent on the conditions employed, particularly the ionic strength. The decline in activity with oxidized lipoate upon Cu^{2+} treatment relative to the increase in DCIP activity is much larger at low than at high ionic strength. Ultracentrifuge experiments have provided no evidence that a monomer is present after freezing. Concerning the nature of this conversion one can only speculate. This process is not dependent on the method of freezing but on the time of exposure to freezing. This suggests that, at least with this enzyme and perhaps with other enzymes showing similar properties, interaction of the protein with the ice lattice leads to conformational changes. The presence of ions like $(NH_4)_2SO_4$, EDTA and also bovine serum albumin protect against such alterations.

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