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RELATIONS BETWEEN CONFORMATIONS AND ACTIVITIES OF LIPOAMIDE DEHYDROGENASE

III. PROTEIN ASSOCIATION–DISSOCIATION AND THE INFLUENCE ON CATALYTIC PROPERTIES

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SUMMARY

1. The molecular weights of lipoamide dehydrogenase holoenzyme and its Cu^{2+}-modified form have been determined by gel filtration and ultracentrifugation to be twice those of the apoenzyme and the DCIP-active enzyme obtained by binding FAD to the apoenzyme at 0–5°, *i.e.*, 104 000 and 52 000, respectively.

2. Upon anaerobic reduction with excess NADH in 8 M urea, a protein could be isolated that was able to reconstitute the activity with oxidized lipoate.

3. The DCIP-active enzyme obtained upon freezing of the oxidized enzyme shows, under the present conditions, the original molecular weight, while upon anaerobic reduction with NADH, a DCIP-active enzyme is formed which is a monomer.

4. Association–dissociation phenomena are involved in the reversible conversion of activity with oxidized lipoate into DCIP activity. The evidence that association–dissociation of the protein occurs will be discussed in connection with proposed models for the structure of this enzyme.

INTRODUCTION

NADH:lipoamide oxidoreductase, EC 1.6.4.3, is found widely in nature and has been isolated from a number of animals and plants, *e.g.* pig heart [1–3], dog-fish liver[4], *Escherichia coli*[5,6], *Mycobacterium tuberculosis*[7], Saccharomyces species[8,9] and *Spinacea oleracea*[10,11]. Originally many preparations were found to have a high diaphorase or menadione activity[1,2,9] which can be lowered drastically by adding EDTA during the purification. Traces of metal ions, especially Cu^{2+}, are considered to be responsible for the modification of activity and spectrum[12,13]. The physiological function consists of catalyzing the oxidation of protein amide-linked dihydrolipoic acid by NAD^+, the lipoate having been reduced during the oxidative decarboxylation of pyruvate or α-oxoglutarate[5,14].

Abbreviation: DCIP, 2,6-dichlorophenol indophenol.

SAVAGE[2] reported a molecular weight of 81 000 for the pig heart enzyme based on sedimentation and diffusion coefficients. MASSEY, HOFMANN AND PALMER[15] calculated molecular weights on the basis of sedimentation and diffusion values or with the Archibald approach to equilibrium method, the values varying from 98 000 to 114 000. Approximately the same molecular weight has been determined for lipoamide dehydrogenase from other sources[8,11,16–18].

The enzyme contains 2 moles of FAD per 100 000 g of protein. A disulfide vicinal to the flavin was demonstrated to be involved in the catalysis as well as FAD itself[19–21]. The oxidized enzyme is stable in 6.5 M urea, but reduction under these conditions with NADH or dithionite denatures the enzyme. This results in a FAD-free protein with half the molecular weight (41 000–48 000). MASSEY, HOFMANN AND PALMER[15] proposed a model for the enzyme structure, the main features of which are two polypeptide chains held together by the active-center disulfide bridges close to the two FAD's. However the data presented in this study and the preceding one[22] do suggest an alternative model, namely a monomer–dimer system in equilibrium. Preliminary data of this study have been published[23].

MATERIALS AND METHODS

NAD^+ (98%), FAD, lipoate and ribonuclease were obtained from Sigma; NADH and alcohol dehydrogenase from Boehringer and Söhne; 2,6-dichlorophenol indophenol from British Drug House. Bovine serum albumin and ovalbumin were purchased from Kochlight; blue dextran 2000 and Sephadex G-200 from Pharmacia.

The native enzyme and the apoenzyme were prepared as described in the preceding paper[22] in which the methods for activity determination are also described. The absorbance ratio 280 mμ/455 mμ for the native enzyme varied between 5.4 and 6.0. Cu^{2+}-modified enzyme was prepared by incubating the EDTA-free enzyme with 8 atoms of Cu^{2+} per mole of FAD in a phosphate buffer (pH 7.2) at 0° for 3 h. This is a slight modification of the method reported by CASOLA, BRUMBY AND MASSEY[24].

Sephadex G-200 columns were calibrated using alcohol dehydrogenase, ovalbumin, and bovine serum albumin as standard proteins. Void volumes were determined with blue dextran 2000. The effective pore radius, r, within the gel was calculated according to the procedure of ACKERS[25]. Stokes radii and frictional coefficients of lipoamide dehydrogenase were calculated from the elution volume as determined by activity measurements[26]. The buffer used for elution was a 0.05 M Tris–HCl buffer (pH 7.5) containing 0.1 M KCl and 0.3 mM EDTA, unless otherwise stated. The experiments were performed at low temperatures, 4° to 8°. Fractions of 3 ml were collected with an LKB fraction collector. The absorption pattern of the elution diagram was registered with a 8300A Uvicord II at 280 mμ. Fractions collected in the experiments with the apoenzyme were incubated with FAD for 1 h at room temperature (*cf.* ref. 22) before the activities with lipoate were measured. In the experiment with the recombined enzyme, the temperature of the column did not exceed 4°. The fractions were allowed to stand for 1 h at room temperature before measuring the activity.

Sedimentation and diffusion patterns were obtained using an M.S.E. analytical ultracentrifuge. Molecular weights were determined using the Svedberg relationship and the approach to the equilibrium method at different speeds of TRAUTMAN[28].

Preparations of apoenzyme were extensively dialyzed against 0.1 M or 0.16 M sodium phosphate buffer (pH 7.6) containing 0.3 mM EDTA.

RESULTS

Gel filtration with Sephadex G-200 columns

The results from the gel filtration experiments are given in Table I. The distribution coefficient for alcohol dehydrogenase was used to determine the effective pore radius of the Sephadex gel. The reliability of this value is supported by the Stokes radii which have been found for other proteins, *e.g.* bovine serum albumin and ovalbumin[27]. The r values were found to vary between 180 Å and 200 Å for different columns

TABLE I

GEL FILTRATION ON SEPHADEX G-200 COLUMNS

Column height, approx. 80 cm, ø, 12 mm. The buffer used for elution was 0.05 M Tris–HCl (pH 7.5) which contained 0.1 M KCl and 0.3 mM EDTA. For the apoenzyme, a 0.15 M sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA was used. Temperature was between 4 and 8°, except in the experiment with DCIP enzyme (2°). The r values of the columns were based on the K_d value determined for alcohol dehydrogenase and its known Stokes radius, 46 Å (refs. 25 and 26). The f/f_0 and $D_{20,w}$ values were calculated according to ref. 26. A mol. wt. of either 104 000, for native lipoamide dehydrogenase and Cu^{2+}-modified enzyme, or 52 000, for the apoenzyme and reconstituted DCIP enzyme (*cf.* Table III), was assumed in the calculation of f/f_0.

Preparation	*Number of experiments*	*Stokes radius (Å)*	f/f_0	$D_{20,w} \times 10^7$ $cm^2 \cdot sec^{-1}$
Lipoamide dehydrogenase	6	42	1.35	5.07
Cu^{2+}-modified enzyme	2	41	1.32	5.19
Apoenzyme	3	31	1.25	6.86
Reconstituted DCIP-enzyme	1	33	1.33	6.45

and at different temperatures. To calculate the other physical constants which are derived from the Stokes radius, one must assume that the molecule does not expand or shrink under these conditions. The data show that the apoenzyme differs considerably from the holoenzyme with respect to its physical properties. Though the distribution coefficient is more a function of the Stokes radius than of the molecular weight, the relation between $(K_d)^{1/3}$ and the $(\text{mol.wt.})^{1/2}$ is approximately valid. Using lactate dehydrogenase, alcohol dehydrogenase, bovine serum albumin, ovalbumin and ribonuclease for calibration of the column, the values for the molecular weights of lipoamide dehydrogenase and its apoenzyme were found to be approx. 110 000 and 62 500, respectively. More reliable values are obtained, however, by using the diffusion coefficient determined by gel filtration combined with the sedimentation coefficient (Table III).

Ultracentrifugation

The sedimentation coefficients for the apoenzyme and the enzyme reconstituted on ice are given in Table II. Ultracentrifugation studies are rather limited due to the low protein concentrations of the apoenzyme preparations (2.5–3 mg/ml), which are

TABLE II

SEDIMENTATION COEFFICIENTS OF THE APOENZYME OF LIPOAMIDE DEHYDROGENASE AND THE ENZYME RECOMBINED ON ICE

The apoenzyme and the reconstituted enzyme were extensively dialyzed against 0.1 M or 0.15 M sodium phosphate buffers which contained 0.3 mM EDTA (pH 7.2). Rotor speed, 55 000 rev./min, temperature as indicated.

Preparation	*Protein concentration (mg/ml)*	*Temperature (°)*	$s_{20,w}$
Apoenzyme	1.2	12	4.0
	2.3	7.8	4.4
	2.6	19.5	4.2
Reconstituted DCIP enzyme	2.6	3	3.8
	2.6	6.8	3.7

due to limited solubility. The sedimentation coefficients at this concentration are much lower compared to the value of the holoenzyme. The values for the reconstituted DCIP-enzyme are very similar to those of the apoenzyme and suggest approximately the same dimensions. On the basis of diffusion coefficients as obtained with Sephadex G-200 and with the analytical ultracentrifuge, estimations of the molecular weight of apoenzyme and the DCIP-enzyme have been made. They are compared in Table III with values for holoenzyme and Cu^{2+}-modified lipoamide dehydrogenase.

The data clearly indicate a mol.wt. of approx. 52 000 for both the apoenzyme and the DCIP-enzyme. Additional evidence comes from the Trautman plot for the apoenzyme which is shown in Fig. 1. The non-linearity of this plot is partially due to residual holoenzyme left in the apoenzyme preparation. The mol.wt. determined by this method was calculated to be 54 400, again half that of the holoenzyme.

TABLE III

MOLECULAR WEIGHTS OF LIPOAMIDE DEHYDROGENASE HOLOENZYME, Cu^{2+}-MODIFIED ENZYME, APOENZYME AND DCIP-ENZYME

The values for diffusion coefficients and sedimentation coefficients are also given; for the apoenzyme and the DCIP-enzyme, the same buffer and the same temperature were used for both diffusion and sedimentation (see Table II). $\bar{v}$ is assumed to be 0.73. $D_{20,w}$ for Sephadex G-200 was calculated according to ref. 26; $D_{20,w}$ ultracentrifuge was calculated according to ref. 29.

Preparation	$D_{20,w} \times 10^7$ ($cm^2 \cdot sec^{-1}$) *Sephadex G-200*	$D_{20,w} \times 10^7$ ($cm^2 \cdot sec^{-1}$) *ultracentrifuge*	$s_{20,w}$	$M_{s,D}$ *D-Sephadex*	$M_{s,D}$ *D-ultracentrifuge*
Holoenzyme	5.07	–	5.8	102 800	
	—	4.63*	5.3*		102 800*
	–	6.08**	5.7**		84 200**
Cu^{2+}-modified enzyme	5.19	—	5.8	105 600	
Apoenzyme	6.86	6.92***	4.2	55 000	54 500
Reconstituted DCIP-enzyme	6.45	—	3.8	44 300	

* ref. 15.
** ref. 2.
*** Value still uncertain.

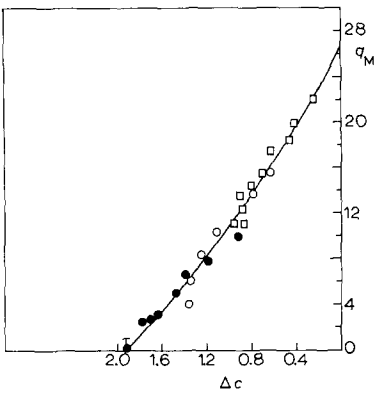


Fig. 1. Trautman plot of the apoenzyme of lipoamide dehydrogenase at 4°. 0.1 M sodium phosphate buffer (pH 7.6) which contained 0.3 mM EDTA was used. Rotor speeds, □—□, 21 000 rev./min; ○—○, 30 700 rev./min; ●—●, 40 500 rev./min. The protein concentration was 2.0 mg/ml; activities with oxidized lipoate and with DCIP were 3% and 25%, respectively.

Further evidence that the molecular weight of apoenzyme is different from that of the native holoenzyme comes from incubating apoenzyme with FAD at 25°. This restores the lipoate activity and results in a sedimentation peak with a sedimentation coefficient identical to that of the holoenzyme itself.

Monomerization under anaerobic conditions

MASSEY, HOFMANN AND PALMER[15] reported denaturation of lipoamide dehydrogenase if kept under anaerobic conditions in a 0.03 M sodium phosphate buffer which contained 6.5 M urea and excess NADH. Anaerobic denaturation under influence of NADH and dithionite resulted, as in the former case, in a flavin-free protein. In both cases the molecular weight proved to be approximately half that of the holoenzyme. As the apoenzyme is more easily soluble at high ionic strength, 3 mg of native lipoamide dehydrogenase were dissolved in 0.15 M sodium phosphate buffer (pH 7.2) which

TABLE IV

APOENZYME FORMED ON ICE UNDER ANAEROBIC CONDITIONS IN 6.5 M UREA

The percentage of the original activities with lipoate and DCIP are given. The experiment was carried out as described in the text.

	Oxidized lipoate act.	*DCIP act.*
Expt. 1		
Holoenzyme	100	100
Column fraction	8	24
Recombination during 6 min on ice with FAD	5	267
Expt. 2		
Holoenzyme	100	100
Column fraction	1	not determined
Recombination during 4.5 h on ice with FAD	0.1	136
30 min at 25°	22	not determined

TABLE V

INFLUENCE OF FREEZING ON REDUCED LIPOAMIDE DEHYDROGENASE

Holoenzyme (0.27 mg/ml) anaerobically frozen in 0.03 M phosphate buffer (pH 7.2) in the presence of a 10-fold molar excess of NADH for 5 days. Activities were determined immediately after thawing and again after incubating for 30 min at 25° and expressed as percent activity of the original holoenzyme.

	+ EDTA, mM 0.3 mM		*+EDTA, 0.3 mM trace NAD nucleosidase*		*−EDTA*		*−EDTA trace NAD nucleosidase*	
	DCIP	*Oxidized lipoate*	*DCIP*	*Oxidized lipoate*	*DCIP*	*Oxidized lipoate*	*DCIP*	*Oxidized lipoate*
After thawing	1600	17	1950	21	2150	21	2100	16
30 min at 25°	480	36	580	49	670	47	590	54

contained 0.3 mM EDTA and 6.5 M urea. The enzyme was made anaerobic in a Thunberg cell after which an excess of NADH was added. The solution was kept under anaerobic conditions at 7° for 30 min. After opening the Thunberg tube, the solution was poured immediately over a Sephadex G-25 column (10 cm height) which was equilibrated with 0.15 M sodium phosphate buffer (pH 7.2) and 0.3 mM EDTA. On the Sephadex G-25 column, 2 fluorescent bands separated: one containing the holoenzyme and apoenzyme in the void volume, the second one containing FAD which had been liberated. The activities before and after FAD addition are shown in Table IV.

The return of a relatively high DCIP activity on ice after adding FAD as well as the increase in lipoate dehydrogenase activity after elevating the temperature are phenomena characteristic of the presence of apoenzyme, *e.g.* monomerization (*cf.* ref. 22). Monomerization coupled with an increase in DCIP activity also takes place under anaerobic conditions upon addition of NADH. It is known from previous studies with this enzyme (*cf.* refs. 3 and 20) that anaerobic addition of NADH to lipoamide dehydrogenase results in the formation of a semiquinone by the uptake of two reducing equivalents, with a very small fraction of totally (4-equivalent) reduced enzyme present at 25°. The latter fraction increases by lowering the temperature to 0°; concomitantly, the activity of the preparation with DCIP increases.

TABLE VI

SEDIMENTATION COEFFICIENTS OF LIPOAMIDE DEHYDROGENASE UNDER DIFFERENT CONDITIONS

Holoenzyme (4–5 mg/ml) anaerobically frozen in 0.03 M phosphate buffer (pH 7.2) and $3 \cdot 10^{-4}$ M EDTA with a 3-fold molar excess of NADH and the additions given in the table. After thawing the samples were oxidized by oxygen.

	Addition	*Temperature (°)*	$s_{t,c}$	$s_{20,w}$
Lipoamide dehydrogenase (4–5 mg/ml) + NADH	trace NAD nucleosidase	1.7	3.5–1.9	6.1–3.3
	none	8.3	4.1–2.3	5.7–3.2
	sodium arsenite (10^{-2} M) + trace NAD nucleosidase	5.7	3.6–2.2	5.5–3.4
	none	5.9	4.0–1.8	6.1–2.8
	NAD^+ ($2 \cdot 10^{-3}$ M)	7	3.9–2.2	5.8–3.3

In Table V are summarized the results obtained upon freezing holoenzyme with a 10-fold molar excess of NADH under nitrogen for 5 days. Under these conditions the DCIP activities are much higher than those obtained at 0° under reducing conditions. The preparations have lost all colour during the freezing process and are practically non-fluorescent. As can be concluded from Table V, the differences in activities between the different treatments are only slight.

It is important to know if the four-electron reduced state is coupled to a monomer structure and permits the dimer to dissociate to give an enzyme comparable to the DCIP-active monomer, or if, on the other hand, it remains a dimer but with another conformation resulting in a high activity with DCIP, comparable to that of the Cu^{2+}-modified enzyme. Therefore, higher protein concentrations (5 mg/ml) were also frozen under anaerobic conditions with an excess of NADH. After reoxidation on ice, the preparations were analyzed with the analytical ultracentrifuge. The sedimentation

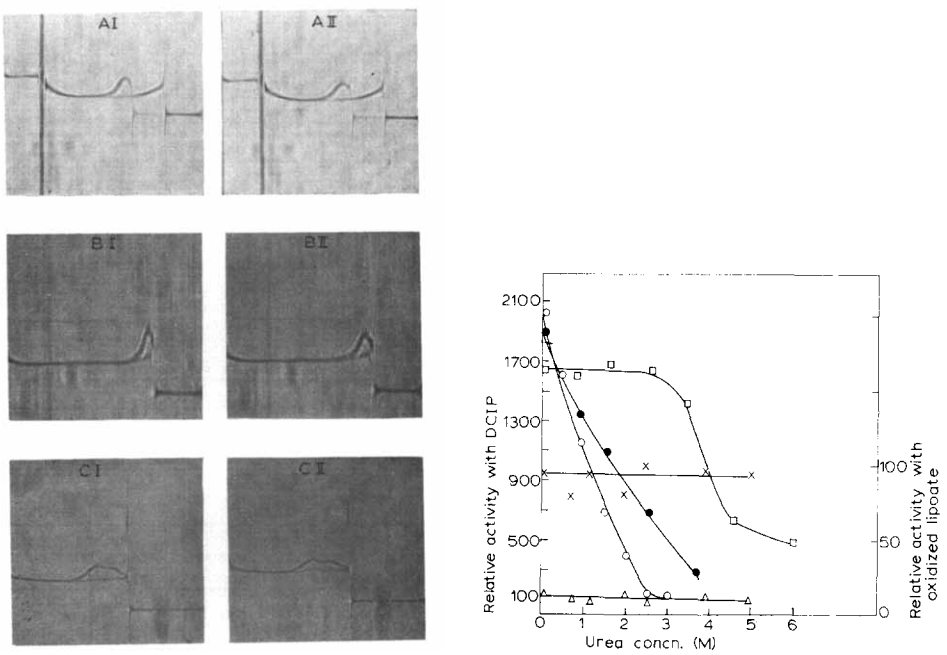


Fig. 2. Sedimentation patterns of lipoamide dehydrogenase apoenzyme, recombined DCIP active enzyme and anaerobically frozen enzyme. A. Apoenzyme (2.6 mg/ml) in 0.15 M sodium phosphate (pH 7.2) with 0.3 mM EDTA. Temperature, 19.2°; rotor speed, 58 400 rev./min; pictures taken at 13 min (I)and 17 min (II). B. Recombined DCIP-enzyme (2.6 mg/ml) in 0.10 M sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA. Temperature, 3°; rotor speed, 55 100 rev./min; pictures taken at 9 min (I) and 14 min (II). C. Anaerobically frozen enzyme (6 mg/ml) in 0.03 M sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA. Temperature, 8°; rotor speed, 49 600 rev./min; pictures taken at 48 min (I) and 60 min (II). Sedimentation is from right to left.

Fig. 3. Influence of urea on the different conformations of lipoamide dehydrogenase. The DCIP activities of the different forms of enzyme were measured, ○—○, DCIP-enzyme (monomer); ●—●, frozen enzyme; □--□, Cu^{2+}-modified enzyme; △—△, native holoenzyme (with DCIP). ×—×, activity with oxidized lipoate of native holoenzyme. The different enzymes were incubated with urea for 10 min on ice before samples were withdrawn and the activity measured as described under METHODS. The protein concentrations varied between 0.3 and 0.5 mg/ml. The reconstituted DCIP-active enzyme and the frozen enzyme were allowed to stand for 15 min on ice after thawing before use.

patterns showed two components in contrast to the oxidized or the oxidized frozen enzyme. This presumably represents a monomer–dimer equilibrium as judged from the observation that upon incubation at 25°, the DCIP activity, which is originally high, declines, accompanied by an increase of the activity with oxidized lipoate. In Table VI a survey of the results under different conditions is given.

Fig. 2 gives the sedimentation patterns of the frozen reduced enzyme, apoenzyme and reconstituted DCIP-active enzyme.

Sensitivity of the different enzyme conformations to urea

It is known that incubation of the normal oxidized lipoamide dehydrogenase with 6.5 M urea results in only a small decrease in activity with lipoate and with DCIP. The Cu^{2+}-modified enzyme, however, is less stable; a 10-min incubation with 4 M urea results in a 60% decrease of activity.

The apoenzyme itself is very sensitive to urea; a concentration of 1 M largely prevents recombination with FAD, as can be concluded from the small increase in fluorescence polarization and the small enhancement of activity with DCIP. This explains the relatively small restoration of DCIP activity after reductive urea treatment. The reconstituted DCIP-enzyme is also urea sensitive, though much less than the apoenzyme itself.

The frozen enzyme which has a high activity with DCIP behaves in a similar way to the DCIP-active enzyme. In Fig. 3 the results of urea treatment upon the lipoamide dehydrogenase forms are given.

DISCUSSION

The sedimentation coefficients for the holoenzyme and for the Cu^{2+}-modified enzyme are identical. An $s_{20,w}$ value of 5.8 S was calculated from experiments performed in 0.1 M sodium phosphate buffer (pH 7.2). Since the dependency of the sedimentation constants on protein concentration is very small for this enzyme, the discrepancy between this value and those reported previously[2,15] is rather small. The values described here are similar to that for the *E. coli* enzyme[17]. The differences in values for molecular weight reported in the literature are mainly due to differences in diffusion coefficients. SAVAGE found a $D_{20,w}$ value of $6.08 \cdot 10^{-7}$ cm$^2 \cdot$ sec^{-1} while MASSEY reported $4.63 \cdot 10^{-7}$ cm$^2 \cdot$ sec^{-1}.

The diffusion coefficients based on the present experiments with Sephadex G-200 are $5.07 \cdot 10^{-7}$ cm$^2 \cdot$ sec^{-1} and $5.19 \cdot 10^{-7}$ cm$^2 \cdot$ sec^{-1} for the holoenzyme and the Cu^{2+}-modified enzyme, respectively. These values are more in agreement with those of MASSEY and practically identical with the values reported for the *E. coli* enzyme.

Several independent methods, *viz.* gel filtration, sedimentation and diffusion, and approach to equilibrium centrifugation, give ample evidence for a molecular weight of the apoenzyme of approx. 52 000. This value is in fair agreement with the value MASSEY described for the inactive, urea-treated protein (*cf.* ref. 15). As has already been noted in this paper, it is possible to demonstrate the presence of apoenzyme under the anaerobic conditions used by MASSEY. One must take care to choose a buffer with a high ionic strength and not to expose the apoenzyme formed to the 6.5 M urea too long. Therefore, the apoenzyme can be made under both anaerobic and aerobic conditions (*cf.* ref. 22).

The present results indicate that the model described by MASSEY for lipoamide dehydrogenase in which two disulfide bridges are considered responsible for the association of the two polypeptide chains is rather unlikely. The consequence of such a model would be that one must assume either that the disulfide bridges must be opened to form sulfhydryl groups under aerobic acid conditions, which is very unlikely, or that an exchange must occur in which the interpeptide S–S bridges are replaced by two intrapeptide S–S bridges. If an interpeptide S–S bridge is replaced by an intrapeptide one by acid $(NH)_2SO_4$, it is difficult to understand why the apoenzyme does not show a molecular weight equal to that of the holoenzyme, since after preparation it is dissolved in neutral buffer in which a reversed exchange to the original interpeptide bridges could be expected. But even accepting this possibility one needs the binding of FAD to the apoenzyme *plus* elevated temperature for the sulphur exchange.

Although this mechanism cannot be completely excluded, it is, in our opinion, more logical to explain our results by a simpler mechanism, *i.e.*, the association, without sulphur exchange, of two molecules of DCIP-active monomer to the dimer which is active with oxidized lipoate.

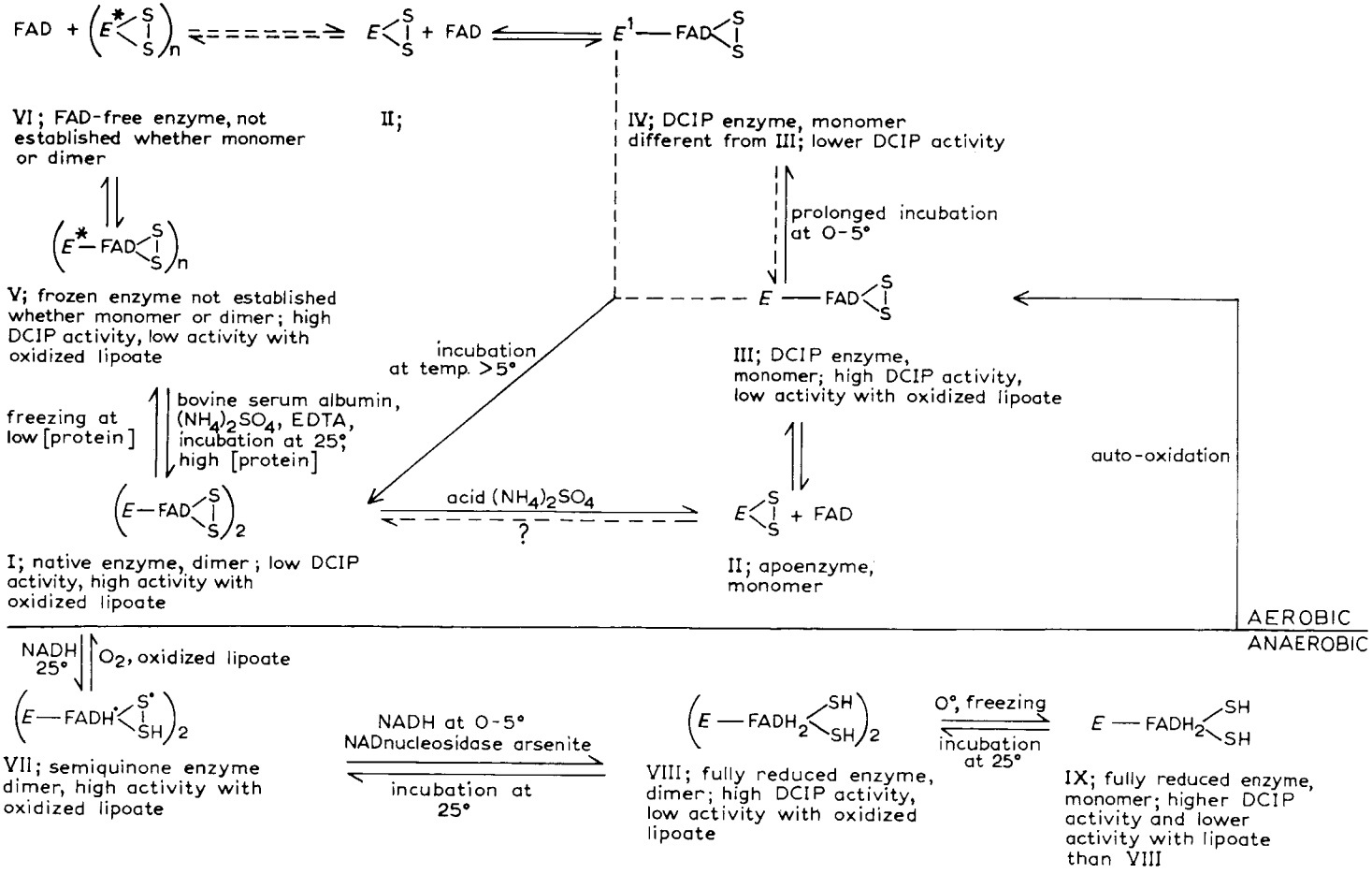
It accounts for the observed second order rate of return of the activity with oxidized lipoate (*cf.* ref. 22), which is much more difficult to envisage in the case of an exchange of S–S bridges. Further arguments can be found in Table VI in which it is shown that, in the presence of a large excess of arsenite, monomer is still present. In the case of an interpeptide S–S bridge this would be impossible, since arsenite would connect the reduced peptides. The most important argument in favor of an association is the observation that the FAD-containing monomer is very active with DCIP (ref. 22). This phenomenon cannot be explained at all by the original model of MASSEY, HOFMANN AND PALMER[15].

Scheme I summarizes our results.

Evidence for the existence of conformations II, III and IV with their activities and molecular weights has been given in a previous paper[22] and does not require discussion here.

A few remarks should be made in connection with the molecular weights of conformations V and VI. No actual evidence indicates that freezing of the oxidized holoenzyme leads to a monomer with DCIP activity. However, the strong dependence of the formation of these forms on the protein concentration, the similar values of the association constants for FAD binding by the apoenzymes prepared by acid $(NH_4)_2SO_4$ and freezing, as well as the urea sensitivity, support this. As a counterargument one might use the results with Cu^{2+}-modified enzyme indicating that this protein is a dimer. However, it cannot be excluded that, at the very low protein concentrations of the assay (1–5 μg/ml), dissociation does occur.

The present results show that freezing causes monomerization under reducing conditions. This is accompanied by an increase in DCIP activity in comparison with reduction at 0° without freezing. It is possible that the DCIP activity of the enzyme reduced by NADH at 0° in the absence or presence of arsenite (*cf.* ref. 19) reflects the presence of monomer. Experiments in this laboratory have shown that the conversion of VII into VIII by lowering the temperature in the presence of an excess of NADH, as seen by the appearance of the FADH–NAD charge transfer band at 700 mμ (*cf.* ref. 12), is slow and takes several hours. Upon raising the temperature to 25°, the reverse



Scheme I. Relation between conformations and activities.

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reaction is much faster. This indicates that this process is directed by conformational changes rather than shifts in equilibrium.

Recently STEIN AND CZERLINSKI[30] have reported that addition of NADH to lipoamide dehydrogenase results in an S-shaped titration curve. Our own results (*cf.* ref. 15) have never shown such a relationship. It is possible that the presence of DCIP-active enzyme, like the frozen enzyme, is responsible for this. A gradual conversion at 25° into the native enzyme during titration can cause such a result, since it may be expected that the frozen enzyme, like the Cu^{2+}-modified enzyme, will show a spectral response at 450 and 530 mμ which is different from that of the native enzyme.

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