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RELATION BETWEEN CONFORMATIONS AND ACTIVITIES OF LIPOAMIDE DEHYDROGENASE

IV. APOENZYME STRUCTURE AND FLAVIN BINDING ASPECTS

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SUMMARY

1. The apoenzyme of lipoamide dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3) is thermolabile and urea-sensitive; the stability is also concentration-dependent. Upon prolonged incubation on ice the tertiary structure of concentrated apoenzyme (1 mg/ml) changes, though the flavin binding site is less affected. The apoenzyme partially withstands freezing for several weeks.

2. Compared with that of the holoenzyme the protein fluorescence is increased in the apoenzyme; the excitation spectrum shows two maxima at 284 nm and 290 nm. Resolution of the difference fluorescence emission spectra showed a tyrosine contribution in the 305-nm region and non-polar tryptophan emission around 330 nm.

3. The return of the lipoate activity is dependent on the apoenzyme concentration. Recombination systems with high apoenzyme concentrations (0.8 mg/ml) and excess of flavin develop a higher lipoate activity whilst the DCIP activity decreases faster than in the case of at lower apoenzyme concentrations (0.1 mg/ml). The dimerization is dependent on pH (7.2–7.5) and ionic strength (0.2 M). Urea, *p*-chloromercuribenzoate (PCMB) and L-cysteine disturb the recombination.

4. Flavin derivatives are able to bind with the apoenzyme. Flavin-8-bromoadenine dinucleotide (FBAD) partially restores the DCIP activity ($K_{ass} = 1.5 \cdot 10^4$ M^{-1}). FMN is a competitive inhibitor of FAD binding and does not restore any activity. FMN derivatives generally show a non-competitive inhibition pattern, with respect to restoration of the DCIP activity. 3(N)-adducts of FMN exert very little inhibition. 2-Derivatives, such as 2-thio-FMN and 2-NH-phenyl-FMN, are less effectively bound, as also is tetra-hydro-FMN. The inhibitors induce, like FAD, conformational changes in the protein, resulting in a time-dependent increase of the binding forces between apoenzyme and flavin.

5. Parts other than the isoalloxazine moiety of the flavin molecule, *viz.* the

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; FBAD, flavin-8-bromoadenine dinucleotide; nucleotides of flavin analogues were shown with the appropriate prefixes to the parent nucleotide; PCMB. *p*-chloromercuribenzoate.

adenine part and pyrophosphate, are also involved in the FAD binding: NADH, NAD^+, ADP, adenine, ATP and pyrophosphate inhibit the flavin binding. All nucleotides which inhibit the FAD binding affect the dimerization; only NAD^+ has a different behaviour in this respect since it promotes the return of the lipoate activity.

INTRODUCTION

The apoenzyme of lipoamide dehydrogenase (NADPH:lipoamide oxidoreductase, EC 1.6.4.3) can be prepared under aerobic as well as under anaerobic conditions[1,2]. It has a molecular weight of 52 000, half that of the holoenzyme. In previous studies[1-3] the recombination process in particular has been investigated. This process depends upon at least three reactions, *i.e.* (1) binding of the flavin molecule to the protein, which leads to opening of the intramolecular complex between the isoalloxazine ring and the adenine moiety of FAD and the formation of a 2,6-dichlorophenolindophenol (DCIP)-active monomer; (2) the transition to a complex which is less active towards DCIP and which can be trapped at low temperatures; (3) the temperature-dependent dimerization which determines the return of the lipoate activity.

It has been shown in our laboratory[4] that the apoenzyme used in our studies differs from that prepared with guanidine-HCl as described by BRADY AND BEYCHOK[5]. Recently STRITTMATTER[6] showed that several distinct conformations of cytochrome b_5 aporeductase exist. Evidence will be presented here that the apoenzyme and the DCIP-active enzyme of lipoamide dehydrogenase also exist in several forms. The recombination process is, in fact, a continous change in protein structure resulting in the dimeric holoenzyme. Binding studies of flavin derivatives and other molecules to the apoenzyme of lipoamide dehydrogenase demonstrate that interactions of the protein and the flavin are based on multiple binding forces, as has already been postulated by several authors[7-9] for the binding mechanism of FAD to flavoproteins.

METHODS

NAD^+, NADPH, $NADP^+$, AMP, 3,5-cyclic AMP, ADP, ATP, GDP, GTP, adenine, adenosine, lipoic acid and bovine serum albumin were obtained from Sigma. NADH was obtained from Boehringer and Söhne, 2,6-dichlorophenolindophenol (DCIP) from British Drug Houses. The FMN-derivatives were synthesized by HEMMERICH[10] (purity 90–99% except for 2-thio-FMN which had an 80% purity in freshly prepared solutions and did not contain FMN). The flavin–8-bromoadenine dinucleotide (FBAD) was synthesized by MCCORMICK[11]. *p*-Chloromercuribenzoate (PCMB) and urea solutions were freshly prepared daily, whereas flavin solutions were prepared once a week and stored in the dark at 0°.

The preparation of lipoamide dehydrogenase and of its apoenzyme has been described[1,12] in previous papers. The determination of the specific activities was carried out under the conditions mentioned earlier[1] using a Zeiss PMQII spectrophotometer and a Photovolt recorder. Activities are, in general, expressed in terms of per cent activity with respect to that of the pure holoenzyme, *e.g.* lipoate activity 20–25 μmoles$\cdot$mg$^{-1}\cdot$min^{-1} and DCIP activity 0.3–0.4 μmoles$\cdot$mg$^{-1}\cdot$min^{-1}. The

DCIP activity of the recombined enzyme decreases rapidly during the assay and this could be due to flavin dissociation. Fluorescence spectra were recorded with a Hitachi Perkin–Elmer MPF-2A fluorescence spectrophotometer. Spectra were corrected for the scatter effects of the solvent. Fluorescence polarization was measured with the modified Zeiss apparatus as described by KALSE AND VEEGER[1] and the K_{ass} values calculated according to the method of BAYLEY AND RADDA[13].

RESULTS

Acidification of a holoenzyme preparation with a satd. $(NH_4)_2SO_4$ solution (pH 1.5) precipitates the apoenzyme. As this method is rather rough and is, in fact, based upon more or less reversible stages of protein denaturation, it is not surprising that the properties of different apoenzyme preparations may vary somewhat. For example, if the acid $(NH_4)_2SO_4$ solution is added quickly without complete and thorough mixing, the residual activity in the lipoate of the precipitate will be rather high (20–25%); in other preparations the lipoate activity will be low whilst the DCIP activity will be stimulated by a factor of 600–800%. Combined with the previous observation[1], that the FAD monomer has a high DCIP activity, this suggests that monomerization occurs before the FAD dissociation. In general, the residual activities of lipoate and DCIP, as compared with the activity of holoenzyme, are 4–8% and 150–200%, respectively. However, the less rigorously one treats the preparation, the better the lipoate activity returns.

Stability of the apoenzyme

The ability of the apoenzyme to recombine after ageing on ice for prolonged times is given in Table I. The main effect of standing concerns the return of the lipoate activity, *e.g.* the dimerization, although the maximum activity with DCIP also diminishes. Furthermore, the DCIP activity of an aged apoenzyme decreases less after elevating the temperature than in the case of a freshly made preparation. These

TABLE I

THE EFFECT OF AGEING ON THE RECOMBINATION PROPERTIES OF LIPOAMIDE DEHYDROGENASE APOENZYME

Apoenzyme (1 mg/ml) was stored on ice in 30 mM phosphate buffer (pH 7.2). At the times indicated samples of 0.1 mg were incubated in a volume of 0.3 ml containing 400 μM FAD and 30 mM sodium phosphate buffer (pH 7.2). After 5-min incubation on ice the DCIP activity was determined after which the samples were incubated at 20° and both the lipoate and the DCIP activity determined. In the control the rest activity was determined without preincubation with FAD.

Incubation time of apoenzyme (h)	*5 min on ice*	*1 h at 20°*	
	% DCIP activity	*% DCIP activity*	*% Lipoate activity*
Control	—	170	3
1	1240	340	66
50	1175	400	35
75	—	450	23
100	955	935	20
175	850	650	15

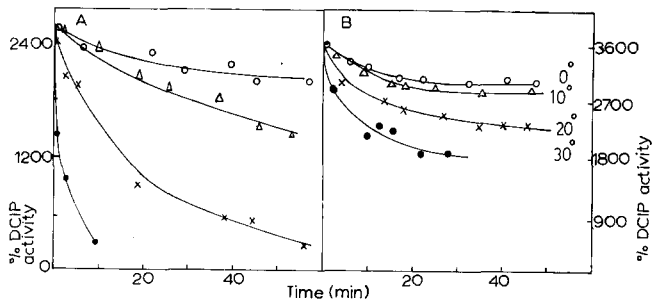


Fig. 1. A. Stability of lipoamide dehydrogenase apoenzyme under different conditions. Stability of apoenzyme incubated at different concentrations at 5° in 50 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA. A sample of the incubation mixture was added to the assay cuvette, to which 5 μM FAD was added and the DCIP reaction was measured at 25°. ○—○, 0.1 mg/ml; △—△, 0.05 mg/ml; ×—×, 0.1 mg/ml in the presence of 2 M urea; ●—●, 0.1 mg/ml in the presence of 3 M urea. B. The stability of the apoenzyme (0.4 mg/ml) at different incubation temperatures, and assayed under the conditions of A.

observations indicate that although the structure of the apoenzyme gradually alters, the FAD-binding site is much less affected. Sometimes a small increase (10–15%) in recombination ability with FAD during the first hour upon standing on ice in concentrated (1–2 mg/ml) solution is observed, as judged by fluorescence-polarization and DCIP-activity measurements. A process of refolding of a fraction of the apoenzyme, comparable with the observations made by STRITTMATTER[6], with cytochrome b_5 reductase, might be responsible for this phenomenon.

Dialysis of the apoenzyme for 2–3 h against 30 or 150 mM sodium phosphate buffers (pH 7.5) always results in apoenzyme preparations which are, upon the addition of FAD, 20–40% less active with lipoate and DCIP.

In contrast with the holoenzyme the apoenzyme is thermolabile (Fig. 1A). The inactivation, which is reflected in the loss of restoration of the DCIP activity, is temperature-dependent and promoted by low concentrations of urea. At 5° the half-time of inactivation at zero urea concentration is approx. 300 min whereas the values for apoenzyme incubated with 2 and 3 M urea are about 15 min and 2 min, respectively.

The inactivation is also dependent on the protein concentration. At 0.4 mg/ml the apoenzyme is, after an initial decline in restoration capacity, almost completely stable at 0–5° for at least 1 h (Fig. 1B). However, at 0.05 mg/ml the inactivation proceeds to an extent of 40% after 50 min standing at 5° (Fig. 1A).

The apoenzyme withstands freezing at −14° for several days. Prolonged freezing promotes irreversible structural changes in the apoprotein, reflected in a proportional lowering of the restoration of both the DCIP and lipoate activities. Apoenzyme which is frozen for 1 month after recombination with FAD, on ice, in the presence of urea (1–6 M concentration range), has lost all its activity in contrast to the control (apoenzyme *plus* FAD, frozen) which still shows considerable activity (60–70%).

The recombination process was studied mainly in sodium phosphate solutions. The pH profile is curved with an optimum value between pH 7.2 and 7.5; increase of ionic strength accelerates the pH-dependent return of the lipoate activity, but the maximum activities at low and at high ionic strength differ only slightly.

TABLE II

INFLUENCE OF UREA ON THE BINDING OF FAD

2.6 μM apoenzyme was incubated with a limited amount of FAD (1 μM) at 10° in 30 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA with or without pre-treatment with urea. The polarization of the flavin fluorescence was measured as previously described[1] with the exception that the excitation wavelength was 450 nm.

Treatment	*Recombination time (min)*	*P*
(A) Control	2	0.206
	6	0.232
(B) 0.5 M urea added after 10-min incubation with FAD	16	0.220
(C) As (B) but with 2 M urea	6	0.156
	Several hours	0.134
(D) Apoenzyme preincubated with 2 M urea during 2 min; then FAD was added	6	0.080
	30	0.065

The urea sensitivity of the DCIP activity of the reconstituted enzyme has been mentioned before[2]. The apoenzyme itself is also very urea-sensitive, as demonstrated in Fig. 1A. However, some features need more attention. In Table II and Fig. 2A the influence of urea on the FAD binding, as measured by fluorescence polarization, has been surveyed. The apoenzyme incubated for a short time with urea, even in concentrations as low as 2 M, is not able to bind the FAD effectively, as can be concluded from the lowered polarization of fluorescence of the flavin. However, if the flavin is added prior to the urea addition, the polarization of the recombined enzyme decreases slightly and rather slowly. This means that the binding of the flavin quickly induces a protein conformational change which largely protects the flavin binding site itself against unfolding. Though the flavin–protein interaction is only

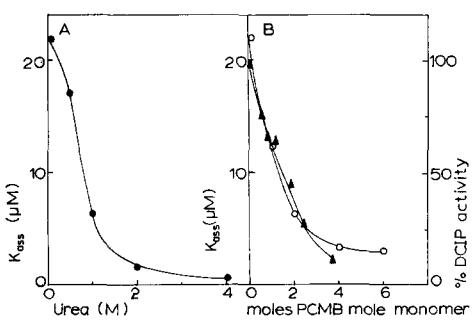


Fig. 2. A. Influence of urea on the binding of FAD. Apoenzyme (5 μM) and FAD (5 μM) were incubated at 10° in 30 mM sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA; the apoenzyme was pre-incubated for 5 min with various concentrations of urea. After 30-min recombination, the K_{ass} values were calculated from the fluorescence polarization as previously described[1]. B. Influence of PCMB on the binding of FAD. ○—○, as A with various PCMB concentrations relative to the monomer concentrations; ▲—▲, 6 μM apoenzyme pre-incubated for 30 min with the relative amounts of PCMB indicated. Samples were withdrawn and the DCIP activity determined in an assay cuvette to which 4 μM FAD was added.

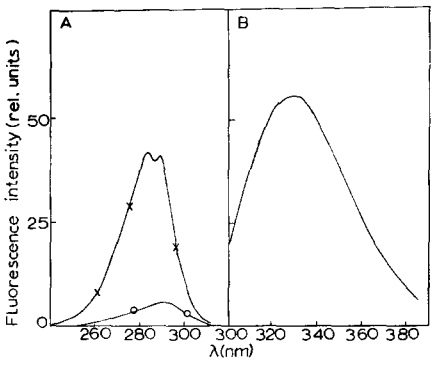
slightly affected, the function of the catalytic center is more easily disrupted as the activities with DCIP are strongly influenced. At urea concentration of 2 M this activity is inhibited to an extent of 70% (*cf.* ref. 2, Fig. 3), which indicates that the protein does undergo conformational changes. The results of Table II and Fig. 1 seem to be contradictory as the half-times are different. However, the techniques used are quite different. In the fluorescence polarization experiments the amount of FAD is limited. The stability experiments contain an excess of FAD, while during the measurements the samples are diluted in the assay which drastically lowers the urea concentration.

Addition of PCMB to the apoenzyme decreases the FAD-binding capacity. In Fig. 2B the K_{ass} value is given for flavin binding to the apoenzyme which is incubated with different amounts of PCMB. The influence of PCMB on the restoration of the DCIP activity is also shown. An amount of 1 mole PCMB per mole of apoenzyme incubated for approx. 30 min results in 35% inhibition of the DCIP activity while amounts of 2 and 4 moles PCMB per mole protein result in 60% and 90% inhibition, respectively. These values agree fairly well with the percentage of lipoate activity which returns upon elevating the temperature under these conditions. This behaviour is not very different from that of the holoenzyme. Binding of 1 and 2 moles phenylmercuric acetate per mole of enzyme gives almost the same loss of lipoate activity[14]; which has been ascribed by PALMER AND MASSEY[15] and VEEGER AND MASSEY[16] to two fast-reacting SH groups.

The addition of 50 μM L-cysteine to a recombination system without PCMB, containing 6.4 μM apoenzyme and 100 μM FAD at 20°, completely prevents the return of any lipoate activity (*cf.* ref. 17). On the other hand, addition of dithiothreitol does not stimulate the return of the lipoate activity, as has been stated by BRADY AND BEYCHOK[5].

Apoenzyme fluorescence

The apoenzyme shows a strongly enhanced protein fluorescence (280-nm excitation) as compared with the holoenzyme. At the emission maximum (330 nm) the increase is approx. 20-fold and the position of this maximum indicates a rather non-polar environment for the tryptophan groups[18]. In the holoenzyme the protein fluorescence is quenched, mainly due to the binding of the prosthetic group and the connected energy transfer to the flavin[4], although the influence of other factors such as protein conformational changes cannot be ruled out. The excitation spectrum (Fig. 3A) of the apoenzyme shows a double maximum, at 282–284 and 290 nm, whereas the holoenzyme shows only one peak at 287 nm. Lipoamide dehydrogenase contains 7 tyrosyl, 2 tryptophyl and 13 phenylalanyl residues per mole of flavin[19]. According to several authors[20–23], in protein fluorescence the contributions of the tyrosyl residues are, in general, small compared with those of the tryptophan residues. However, studies with the holoenzyme show[4] that the tyrosyl fluorescence contribution is relatively large with respect to that of the tryptophans. Difference fluorescence spectroscopy[24,25] shows that in the apoenzyme the contribution of the tyrosyl fluorescence compared with that of tryptophyl residues is relatively small. Urea (4 M), shifts the emission wavelength to 340–345 nm (*cf.* ref. 18) which indicates a change from apolar to more polar surroundings of the tryptophyl residues. Upon addition of urea the excitation spectrum is quenched; the 282–284-nm maximum is



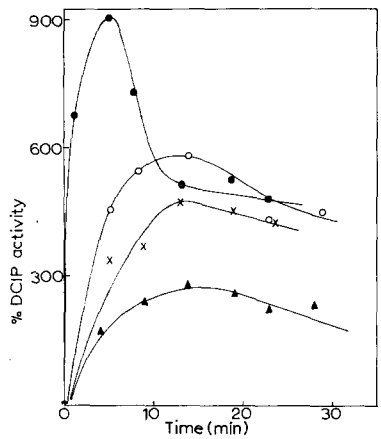


Fig. 3. A. Fluorescence of lipoamide dehydrogenase apoenzyme. Excitation spectrum in 30 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA. Emission wavelength, 330 nm; excitation slit, 4 nm; emission slit, 3 nm; ×—×, 0.04 mg/ml apoenzyme; ○—○, 0.33 mg/ml holoenzyme. B. Fluorescence emission spectrum of a 0.1 mg/ml apoenzyme solution; excitation wavelength, 280 nm; excitation and emission slits, 5 nm. Temp., 5°. The spectra are not corrected for lamp spectrum and photomultiplier sensitivity.

Fig. 4. Influence of potassium halogenides on the recombination of lipoamide dehydrogenase apoenzyme with FAD. Apoenzyme (8 μM final concentration) was pre-incubated on ice in 30 mM phosphate buffer (pH 7.2) containing 0.3 mM EDTA. ●—●, no addition; ○—○, 60 mM KBr; ×—×, 60 mM KCl; ▲—▲, 60 mM KI. After 10 min FAD was added (10 μM final concentration) and samples withdrawn for measurement of the DCIP activity at the times indicated.

reduced to a shoulder at urea concentrations $\geqslant$ 4 M. The tyrosyl fluorescence contribution is affected by 4 M urea to the same extent as the tryptophan fluorescence.

The absorption spectrum of the apoenzyme shows a maximum at 277 nm with two shoulders at 282 and 292 nm. The maximum is probably due to tyrosine absorbance whilst it is likely that the shoulders belong to the tryptophan groups, although a contribution from S–S bridges cannot be excluded[26,27].

The apoenzyme fluorescence studied as a function of temperature declines between 12 and 17° faster than between 0 and 12° and above 17°. However, this process seems to be irreversible as upon lowering the temperature no return to the original emission level occurs.

Influence of halogen ions on the recombination

As KBr is used in the preparation procedure of the apoenzyme, its influence, if any, on the recombination process is of importance since the apoenzyme is not dialyzed before use. Fig. 4 shows the influence which potassium halogenides have on the recombination process on ice with respect to the DCIP activity. The order of magnitude in which the potassium salts are interfering with the level of the DCIP activity is KBr < KCl < KI. A second point of interest is the shift in the time necessary for the DCIP activity to reach its maximum value, from approx. 5–8 to 11–15 min.

Although variations in recombination patterns are observed between different preparations (*cf.* ref. 1, Fig. 3), the shifts in time required to reach maximum DCIP activity are always observed. Also the amount of decline in DCIP activity of the control at 15 min varies between different preparations (*cf.* Fig.7).

At a higher flavin concentration (60 μM) the difference in activity between the untreated and the salt-treated recombination systems disappears although there is still a distinct difference in the moment at which the maximum of the DCIP activity is reached. The return of the lipoate activity is diminished in the presence of low flavin concentrations but the DCIP activity remains high, which indicates that denaturation is not important. Furthermore none of the potassium halogenides (60 mM) interferes with the return of the lipoate activity when a saturating concentration (100 μM) of flavin is used.

TABLE III

THE INFLUENCE OF HALOGEN IONS ON THE FLUORESCENCE AND DCIP ACTIVITY OF A RECOMBINING SYSTEM

The apoenzyme was previously dialyzed at a concentration of 1.5 mg/ml against 50 mM sodium phosphate buffer containing 0.3 mM EDTA. 3.8 μM of apoenzyme was reacting at 10° with the same flavin concentration. The halogen ions were present in 60 mM concentration.

Halogenide	*Recombination time (min)*	*% DCIP activity after 5 min*	*P*	*P (free flavin)*
KBr	5	665	0.110	0.050
	30		0.120	
NaBr	5	405	0.110	0.045
	30		0.120	
LiBr	5	405	0.110	0.050
	30		0.120	
KI	5	175	0.100	0.050
	30		0.105	
NaI	5	155	0.095	0.055
	30		0.100	
KCl	5	500	0.080	0.030
	30		0.085	
NaCl	5	480	0.080	0.030
	30		0.095	
Control	5	780	0.090	0.030
	30		0.095	

In Table III the influence is summarized of several halogenides on the polarization of a recombination mixture as well as on its DCIP activity. Br^- and I^- are well-known fluorescence quenchers of free flavins (*cf.* ref. 28) and cause an increase in the flavin polarization as the life-time of the excited state declines. The polarization in the recombination systems is enlarged in the presence of Br^- whilst the DCIP activities are less than in the control. On the other hand, I^- causes a markedly reduced DCIP activity of the recombination systems but the polarization is slightly higher than in the control. Thus due to interaction of the flavin with the halogen ions, especially I^-, the flavin binding is partially prevented; nevertheless the polarization is increased and just coincidentally reaches a higher value than the control. The increase in fluorescence polarization is not due to binding of the flavin to an inactive form of the enzyme, since this cannot explain the observation that in the presence of halogen ions the maximum activity can be obtained at saturating concentrations of FAD.

Effect of protein concentration on the binding with FAD

In previous papers[1,2] evidence was presented that the return of the lipoate activity is due to a dimerization reaction as the apoenzyme has half the molecular weight of the holoenzyme, and that furthermore the return of this activity fits a second order reaction rate. The dimerization is promoted by increase of temperature, probably by an increase of hydrophobic interactions (*cf.* ref. 29). Fig. 5A demonstrates the return of the lipoate activity at 25° using different apoenzyme concentrations with excess of flavin. The influence of the protein concentration on the dimerization is clear; the lipoate activities in this experiment reach constant levels within 30 min. The activities with DCIP are still high at that time especially in the case of the lowest protein concentrations (Fig. 5B). It is therefore not very likely that the levelling off of the lipoate activity is caused by denaturation of unreacted apoenzyme but is rather due to an equilibrium between the lipoate-inactive monomer and -active dimer.

Assuming an equilibrium between these species and using the level of the lipoate activities to calculate the actual fractions of monomer and the fully active dimer, it is possible to calculate the association constants at the different protein concentrations. The association constants obtained vary with the protein concentration from $3.6 \cdot 10^6$ to $2.5 \cdot 10^5$ M^{-1} which suggests a more complicated system than a pure monomer–dimer equilibrium. One of the reasons for this discrepancy might be the changes of the apoenzyme properties upon extreme dilution (*cf.* apoenzyme stability).

The binding of flavin analogues

It has been shown[3] that FAD and some of its derivatives, *viz.* 3-methyl-FAD and 3-carboxymethyl-FAD can be bound to the apoenzyme; only FAD and 3-

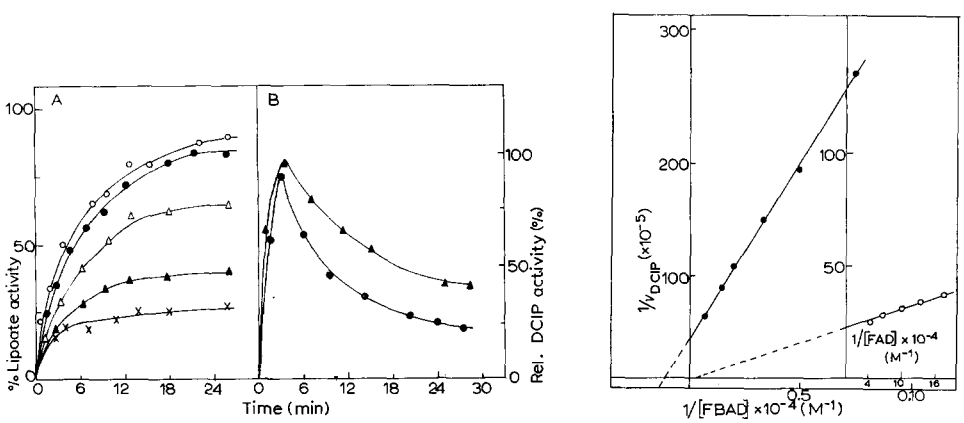


Fig. 5. A. The influence of the apoenzyme concentration on the return of the lipoate activity. The different protein concentrations were incubated with excess of FAD (100 μM) at 25° in 100 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA, and the activity was measured after different incubation times. ○—○, 0.83 mg/ml; ●—●, 0.42 mg/ml; △—△, 0.21 mg/ml; ▲—▲, 0.10 mg/ml; ×—×, 0.05 mg/ml. B. The influence of the apoenzyme concentration on the decrease of the DCIP activity. Conditions as in A. ▲—▲, 0.1 mg/ml; ●—●, 0.42 mg/ml.

Fig. 6. Lineweaver–Burk plot of the apoenzyme recombination with FBAD (●—●) and FAD (○—○). The apoenzyme (3.6 μM) was incubated on ice, in 30 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA, for 20 min, with the concentrations of flavin indicated, after which the DCIP activity was determined.

methyl-FAD are able to give a lipoate-active enzyme, but all compounds give DCIP-active complexes. It was thus of interest to study the binding of other substituted flavins, in order to obtain information about the different parts of the molecule involved.

FBAD, which is substituted in the adenine moiety, restores the DCIP activity upon incubation with the apoenzyme. The K_{ass} value for flavin binding of $1.5 \cdot 10^4$ M^{-1} after 20 min incubation on ice was calculated from the Lineweaver–Burk plot of the restored DCIP activities obtained at varying flavin concentrations (Fig. 6). A V_{max} value of 2400 was found, which is 55% of the V_{max} obtained with FAD. In contrast to the FAD-enzyme itself, the FBAD-containing enzyme shows in comparison with the free flavin compound a quenched flavin fluorescence. This cannot be due to differences in intramolecular complex formation of the free flavins, since the fluorescence spectra of FAD and FBAD have the same pH dependency[11]. It is possible that the bromo-group functions as a dynamic quencher of the flavin fluorescence, which explains the rather high value of the fluorescence polarization of apoenzyme-bound FBAD ($P = 0.4$; $\lambda_{exc} = 396$ nm) as calculated from standard calibration curves and the estimated K_{ass}; under the same conditions free FBAD shows $P =$ 0.02–0.025, slightly lower than FAD.

The bromo-compound does not restore any lipoate activity but interferes with the FAD-induced return of this activity. Addition at 25° of FBAD (60 μM) 2 min before that of FAD (30 μM) to 8 μM of apoenzyme, almost completely prevents the restoration of the lipoate activity. This behaviour will be discussed later, together with the FMN binding as this compound behaves similarly. It is of interest to note that FBAD restores the activity when added to D-amino acid oxidase apoenzyme (J. F. KOSTER AND C. VEEGER, unpublished results).

FMN can be bound by the lipoamide dehydrogenase apoenzyme, although it restores neither DCIP nor lipoate activity. Although the restoration of activities fails to occur, the binding of this compound can be demonstrated by equilibrium dialysis and by interference with the FAD–apoenzyme recombination process (Figs. 7 and 8). The spectrum of the oxidized enzyme does not show any shoulders on both sides of the 445-nm maximum. The amount of FMN bound was calculated to be 0.9 mole/52 000 g of apoenzyme. The enzyme is reduced very slowly by NADH (Fig. 9) and causes a slight increase in absorbance above 500 nm. The slow rate of reduction has no catalytic significance and might be due to photoreduction.

When FAD and FMN are added together to the apoenzyme, the DCIP activity when measured after 20-min incubation on ice does not significantly differ from that of the control with FAD alone or is even slightly higher. However, pre-incubation with FMN shows an inhibition of the restoration of the DCIP activity with respect to FAD (Figs. 7 and 8). The deviation from linearity of the Lineweaver–Burk plots in the presence of FMN is marked at the lower FAD concentrations. This is not observed in the presence of NADH, a compound competitively inhibiting the FAD binding. Although the last part of the FMN inhibition curve is drawn for competitive inhibition it is difficult to distinguish between competitive and non-competitive inhibition from these measurements at different times of incubation. It is also not possible to ascertain whether the V_{max} values found after different times of incubation with FAD are identical or different. The V_{max} values of different apoenzyme preparations are not always identical (*cf.* Figs. 7 and 8) but generally amount to 2500–

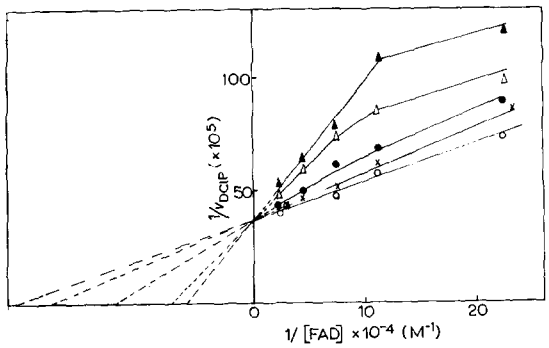


Fig. 7. Effect of different FMN concentrations on the inhibition of the FAD binding to the apoenzyme. Apoenzyme was pre-incubated for 30 min on ice in 30 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA, with FMN (90 μM) and incubated with the FAD concentrations given for 5 min (△—△) and 15 min (▲—▲). The apoenzyme was also pre-incubated with FMN (9 μM) followed by incubation with FAD for 15 min (●—●). Control with FAD after 5 min (○—○) and 15 min (×—×).

3000% of the value for the holoenzyme. It is of interest to note that the amount of inhibition by FMN is dependent on the pre-incubation time and increases considerably. The K_{ass} values for FMN as calculated from these plots show an increase from $0.1 \cdot 10^5$–$0.3 \cdot 10^5$ M^{-1} after 30-min pre-incubation time to $2 \cdot 10^5$ M^{-1} after several hours of pre-incubation. The values obtained for binding of FAD are similar to those measured by means of fluorescence polarization (*cf.* ref. 1). The competition between both flavins indicates that the same protein-binding site is involved. Moreover, addition of different FMN concentrations give Lineweaver–Burk plots with approximately the same V_{max} value (Fig. 7) which underlines the competitive character.

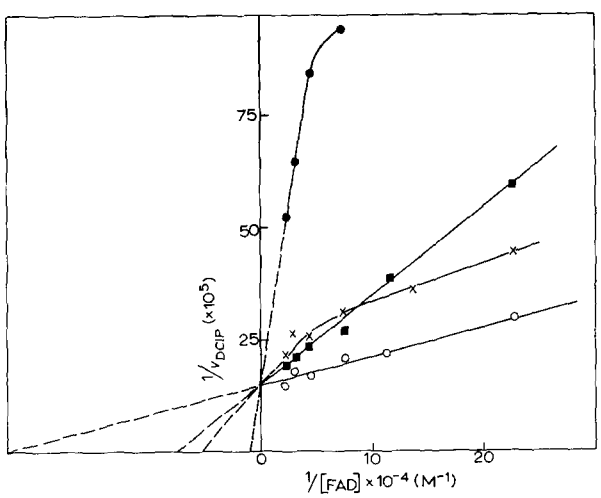


Fig. 8. Effect of different times of pre-incubation with FMN on the inhibition of FAD binding to the apoenzyme. The results are expressed in a Lineweaver–Burk plot. 5 μM of the pre-treated apoenzyme was incubated on ice, in 30 mM phosphate (pH 7.2) containing 0.3 mM EDTA, for 20 min with the FAD concentrations given, before the DCIP activity was determined. The apoenzyme was pre-treated on ice with FMN (95 μM) for 2 h (●—●); for 30 min (×—×); without addition (○—○) and with 1.1 mM NADH (■—■) for 2 min.

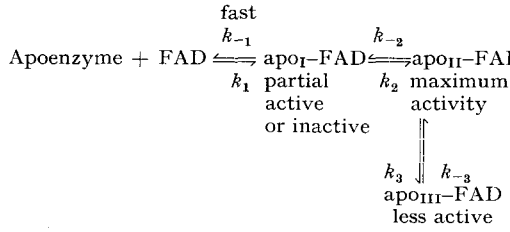
One might argue that the slow increase in K_{ass} is due to a slow reaction of FMN with the apoenzyme. However, in that case one would expect a slow dissociation of the FMN-apoenzyme complex, but upon incubation with an excess of FAD no increase in rate with time is found.

The increase of the flavin association, a phenomenon most likely due to protein conformational changes, is perceptible upon exchange of a non-specific flavin compound, such as FMN, for FAD. In order to demonstrate that the binding of FMN to the apoenzyme leads to a series of time-dependent conformational changes, the experiments given in Fig. 10 were performed. At different times FAD was added to a recombining apoenzyme–FMN system and the DCIP activities belonging to the FAD-containing enzyme were followed. It is clear that both the maximum velocity and the time taken to reach the maximum are dependent on the time of pre-incubation with FMN. The maximum activity is obtained always between 2 and 5 min after FAD addition; after pre-incubation with FMN for 30 min on ice the maximum appears almost immediately (within 1 min) after the addition of FAD and then declines with time, as has been found in three independent experiments. Longer periods of pre-incubation with FMN lead to much lower maximum values of the DCIP activity, although part of the flavin still exchanges very fast, as can be concluded from this rapid restoration of part of the DCIP activity.

Pre-incubation with FMN at higher temperatures lowers the amount of lipoate activity restored after FAD addition. The amount of lipoate activity restored is also dependent on the FMN concentration. Remarkable is the slow decline of the lipoate activity in the presence of FMN after the maximum lipoate activity is obtained.

Addition of FMN to the dimerizing apoenzyme–FAD system at 20° diminishes the restored lipoate activity, even after full activity has been reached. The dimerizing system becomes less sensitive to FMN upon prolonged incubation at 25° though even after 1 night a 15–20% decline of activity is observed. Similar effects have been found to occur in the presence of FBAD. The holoenzyme itself is not FMN-sensitive. Incubation on ice of low holoenzyme concentrations, less than 0.1 mg/ml, leads, due to dissociation of the enzyme, to a 60–70% inactivation of the lipoate activity[4] but addition of FMN does not affect this process.

The binding of FAD by apoenzyme, as followed by the rate of increase of flavin fluorescence[1], occurs in a few msecs. This was shown by preliminary experiments using the stopped-flow fluorescence technique*. Therefore we propose the following scheme for the recombination process:



Thus the association constant measured at the point of maximum development of apo$_{II}$–FAD is actually a combination of several rate constants. The Lineweaver–

* The stopped-flow experiments were kindly performed by Dr. J. F. Koster.

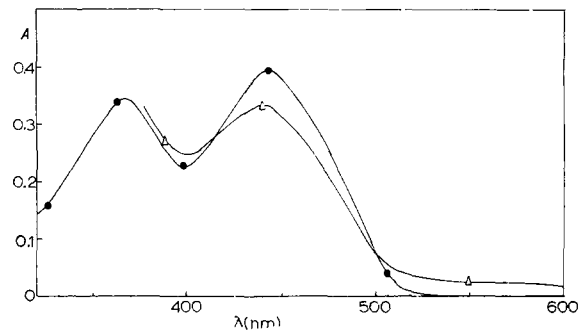


Fig. 9. Spectrum of lipoamide dehydrogenase apoenzyme–FMN complex. 1.5 ml apoenzyme (37 μM) was pre-incubated with FMN (500 μM) at 0° for 16 h and dialysed for 48 h against 7 ml 0.03 M sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA. Difference spectra were taken with a Cary-14 spectrophotometer at 4°. ●—●, spectrum of the oxidized enzyme; △—△, spectrum 90 min after the anaerobic addition of NADH (75 μM).

Burk plots of Fig. 8 were determined after 20-min incubation and thus after the apo$_{III}$–FAD complex is formed. It is possible that the binding of FMN to the apoenzyme occurs in a similar, though slower, process as schematically indicated.

The results shown in Fig. 7 suggest that the K_{ass} value of FAD-binding diminishes slightly with time. We could not definitely prove that this is a real effect. One has to keep in mind that activity measurements are an indirect way to determine binding constants but the agreement with the values obtained from fluorescence polarization is rather good (*cf.* ref. 1). Pre-incubation with FMN for a short period leads to a process of FAD binding not distinct from the control or slightly higher in activity. However, after pre-incubation with FMN for 30 min the apoenzyme$_{II}$ con-

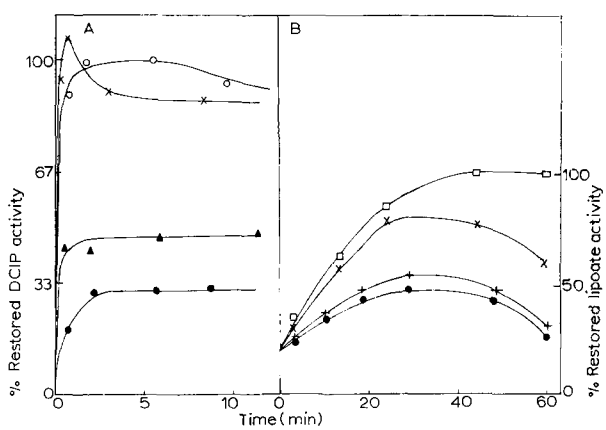


Fig. 10. A. The influence of FMN pre-incubation with lipoamide dehydrogenase apoenzyme on the restoration of activity with DCIP by FAD. Apoenzyme (12 μM) was incubated on ice with FMN (200 μM) in 0.03 M sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA. Samples were withdrawn at 5 (○—○), 30 (×—×), 90 (△—△) and 180 (○—○) min, incubated with 100 μM FAD on ice and the DCIP activity determined with time. B. Influence of FMN on the restoration of the activity with lipoate. Recombination of 9 μM apoenzyme of lipoamide dehydrogenase at 20° with 20 μM FAD in 0.03 M sodium phosphate buffer (pH 7.2) with 0.03 mM EDTA. □—□, apoenzyme not pre-incubated with FMN; ×—×, apoenzyme preincubated for 30 min with 10 μM FMN; +—+, with 50 μM FMN; ●—●, with 90 μM FMN.

formation is mainly present. The optimum DCIP activity is due to the rapid exchange, reached very quickly upon FAD addition.

The binding properties of other FMN analogues were also studied. None of the FMN derivatives restored any catalytic activity, neither did a mixture of AMP and FMN. Lineweaver–Burk plots (DCIP activity) for FAD binding with apoenzymes pre-incubated with 3(N)-methyl-FMN, 3(N)-carboxymethyl-FMN, 2-NH-hydroxyethyl-FMN and 2-(NH)-phenyl-FMN show non-competitive inhibition patterns, which are dependent on the pre-incubation time with the analogue, as in the case with FMN itself. The 3-substituted derivatives are better inhibitors than the 2-substituted compounds. Iso-FMN behaves similarly to FMN; the plots are not straight but show a break or are curved. 6,7,8,9-Tetrahydro-FMN shows a decreased affinity for the apoenzyme with respect to FMN, which suggest that multiple binding forces exist between the isoalloxazine moiety and the protein.

TABLE IV

INFLUENCE OF FMN, FMN DERIVATIVES AND OTHER NUCLEOTIDES ON THE RETURN OF THE LIPOATE ACTIVITY

The flavin derivatives were preincubated on ice in 100 μM concentration with 7.3 μM apoenzyme during 30 and 60 min after which 100 μM FAD was added while the temperature was raised to 20°. Lipoate activities were determined after 1 h incubation. Medium: phosphate buffer (pH 7.2) containing 0.3 mM EDTA. The nucleotides were incubated on ice in 100 μM concentration with 6.4 μM apoenzyme; after 30-min preincubation 100 μM FAD was added. The lipoate activity was determined after 30-min incubation at 20°.

	% Lipoate activity	
	30-min preincubation	*60-min preincubation*
Flavin derivative added		
FAD	100	100
FMN	62	43
Iso-FMN	68	50
Tetrahydro-FMN	81	67
3(N)-methyl-FMN	48	32
2-thio-FMN	92	65
2-NH-hydroxyethyl-FMN	66	38
2-NH-phenyl-FMN	102	70
3(N)-carboxymethyl-FAD	50	24
	% Lipoate activity	
Nucleotide added		
FAD	100	
ATP	74	
ADP	62	
AMP	98	
3′,5′-cyclic AMP	91	
Adenosine	84	
Adenine	72	
NAD^+	115	
$NADP^+$	94	
GTP	83	
GDP	74	
Pyrophosphate	63	

In Table IV the influence of these flavin derivatives on the dimerization reaction is summarized. 3(N)-methyl-FMN and 3(N)-carboxymethyl-FAD are even better inhibitors than FMN. The influence of the 2-substituted derivatives depends on the kind of substitutent. Substitution of sulphur for oxygen on position 2 (2-thio-FMN) decreases the inhibitory properties as compared with those of FMN; since the 2-thio-derivative was not completely pure, this conclusion has to be made with reservations.

NADH and NAD^+, pre-incubated with apoenzyme, show a weak competitive inhibition with respect to the FAD binding, as concluded from the lowered DCIP activity. K_{ass} for NADH and NAD^+ are $1 \cdot 10^3$ M^{-1} and $3 \cdot 10^2$ M^{-1}, respectively. The binding of NADH to the apoenzyme can also be followed by the increase in polarization of the NADH fluorescence upon working under anaerobic conditions. NADPH in the concentration tested (1 mM) did not interfere with the FAD binding, as judged on the basis of the DCIP activity.

The influence of ADP on the FAD binding results in a decreased polarization of the flavin fluorescence. Other nucleotides tested, such as GTP, GDP and ATP have no effect on the DCIP activity up to a concentration of 1 mM, but the compounds affect markedly the return of the lipoate activity, as does ADP.

The effect of NAD^+ on the lipoate activity is rather peculiar. It behaves competitively with respect to the FAD binding but stimulates the rate of the dimerization reaction at elevated temperature in concentrations up to 0.5 mM. Pyrophosphate, adenosine and adenine also affect negatively the return of the lipoate activity; $NADP^+$, NADPH, AMP and 3',5'-cyclic AMP have very little or no effect. The other substrate, lipoate, has a stimulating effect on the dimerization.

DISCUSSION

The apoenzyme of lipoamide dehydrogenase has to be dissolved in buffer solutions of high ionic strength; removal of the flavin completely alters the solubility properties and promotes the hydrophobic character of the protein. Physical properties, such as molecular weight and frictional coefficients, are also changing[1,2]. The helix content increases from about 30% in the holoenzyme to about 60% in the apoenzyme, as found by VEEGER *et al.*[4], in contrast with the results of BRADY AND BEYCHOK[5]. Since the latter authors refer to a high degree of denaturation during the preparation of the enzyme, we suggest that their apoenzyme preparation has reached further stages of denaturation than our preparation. Several indications for subsequent alterations in the apoenzyme tertiary structure could explain the variations occurring in recombination ability. Supporting evidence for this comes from the effects of freezing, ageing and urea on the properties of the apoenzyme.

Unfolding occurs in the presence of urea, as concluded from the shift of the protein fluorescence emission maximum towards longer wavelengths. The position of the tyrosine fluorescence (304-nm emission maximum) is not influenced by urea; the difference spectra (280–292-nm excitation) indicate that the relative contribution of tyrosine fluorescence is hardly altered by 4 M urea. The tryptophan fluorescence is quenched by urea (*cf.* refs. 20, 21) whilst the difference between the spectra excited at 292 and 297 nm becomes less.

The quenching of the protein fluorescence which occurs upon addition of the

flavin to the apoenzyme occurs in a similar way to the increase in flavin fluorescence intensity and polarization, *i.e.* an initially rapid phase is followed by a slow and small further increase. The time dependence of the DCIP activity upon addition of flavin to apoenzyme (*cf.* ref. 1, Fig. 2) can be explained by either differences in V_{max} values of distinct species or a decrease in the value of the K_{ass} of FAD after a protein conformational change induced by the bound FAD itself. On the basis of our results we favour the latter possibility.

Undoubtedly, the bound flavin molecule plays the most important role in the stabilization of the tertiary protein structure. The slow time-dependent increase in the affinity of the apoenzyme for bound FMN and FMN analogues is an example of the KOSHLAND[30] induced fit theory. Upon binding the small flavin molecule introduces a series of protein conformational changes which lead to an increase in the binding forces of the nucleotide, ultimately leading to the original holoenzyme structure. These structural changes already concern the monomer, as the urea-stability experiments indicate. The flavin fluorescence polarization of a recombined apoenzyme–FAD system to which urea is added is higher than the value of the same mixture to which urea was added before addition of the flavin. Moreover, the influence of flavin concentration on the effects of the potassium halogenides indicates the importance of the flavin for protein conformational changes. A reasonable explanation for the deviation from linearity at low FAD concentrations, as observed in Fig. 8, is the existence of different apoenzyme–FMN species with different affinities for the flavin. Only at higher concentrations is FAD able to displace FMN in the complex with the highest affinity. Furthermore it must be kept in mind that the results show that the DCIP activity measured after 20-min incubation with FAD is a resultant of several processes. (*cf.* ref. 1). The decrease in FMN sensitivity of the lipoate activity with time suggests that even after reaching maximum activity the protein conformation undergoes small changes without much influence on the catalytic center. However, a fraction of the recombination mixture apparently maintains a more labile structure since the holoenzyme as isolated is not FMN-sensitive at all.

Restoration of the DCIP activity is bound to FAD and some of its derivatives, *e.g.* 3(N)-methyl-FAD, 3(N)-carboxymethyl-FAD and FBAD; substitution of a large group on position 2 does not restore activity (2-morpholino-FAD). Introduction of side-chains in the FMN nucleus gives derivatives which affect the FAD-binding non-competitively. 3(N)-Derivatives are fairly good inhibitors (*cf.* 3(N)-FAD derivatives); 3-methyl-FMN is even slightly better than FMN itself. Changes on position 2 of the nucleus affect the inhibitory properties to some extent. Iso-FMN and FMN have similar inhibitory action but the introduction of hydrogen in the nucleus (tetrahydro-FMN) decreases the inhibition. Unpublished observations show that the reduced flavin has a lower affinity for the apoenzyme as it partially dissociates from the holoenzyme if held in the cold under anaerobic conditions in the presence of NADH, with (*cf.* ref. 31) or without urea, followed by gel filtration or electrophoresis.

Flavin binding to several non-metallo apoenzymes appears to occur in different ways. In D-amino acid oxidase, which has its FAD loosely bound, neither 3(N)- nor 2-substituted derivatives restore any activity[32,33], whereas FBAD partially restores the activity. The binding of FMN is non-competitive with respect to FAD. Both the FMN and the AMP moiety are of importance for the FAD binding to the apoenzyme[34]. In the L-amino acid oxidase the flavin is firmly bound while upon substrate reduction

this enzyme is more stable[35], in contrast to lipoamide dehydrogenase[31] and glutathione reductase[36,37]. In the latter two enzymes the flavin binding shows similarities in the influence of FMN on the FAD binding. The holoenzyme of glutathione reductase is not FMN-sensitive but the reconstituted enzyme produced from apoenzyme and FAD is sensitive after reaching the maximum activity[38].

The importance of the polarity of the flavin surroundings for the catalytic properties was emphasized earlier[16]; a shift to 450 nm always results in a stimulated DCIP activity in lipoamide dehydrogenase. No exact information is yet available about the groups of the protein involved in the binding process. The quenching of the tryptophan fluorescence and the energy transfer between protein and flavin (*cf.* ref. 4) provides no indication for a direct tryptophan–flavin interaction as energy transfer can occur over large distances[39]. However, it is known that flavin–indole complexes are easily formed[40] by charge-transfer complex formation[41,42]; on the other hand complexes have only been found with the oxidized, neutral form of the isoalloxazine ring[43]. The primary interaction in flavin–indole complexes is envisaged between the rings themselves; the strength of the interaction should be relatively independent of changes in side chains but is dependent on modifications in the electronic structure of the ring system. These results are interesting, as they have similarities with our own observations; the 3-imino-position can be altered but ring modifications diminish the affinity of the protein for the flavin compound.

Recently DE KOK *et al.*[44] have suggested a sulphydryl group in the neighbourhoud of the flavin to be responsible for the dynamic quenching of the flavin fluorescence.

The refolding processes vary among different apoenzyme preparations. On the assumption that apoenzyme formation is a reversible denaturation process, one can expect to find some lesions in the protein structure which are only slowly and partially, or even not, restored. Another questionable point in this respect is the homogeneity of the apoenzyme population.

While this paper was in progress, SWOBODA[45] has found that interactions occur between glucose oxidase apoenzyme and adenine nucleotides, but not between the isoalloxazine moiety and the apoenzyme.

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