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RELATION BETWEEN CONFORMATIONS AND ACTIVITIES OF LIPOAMIDE DEHYDROGENASE

II. SOME ASPECTS OF RECOMBINATION WITH FAD ANALOGUES

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SUMMARY

1. The binding of 3-methylFAD and 3-carboxymethylFAD to lipoamide dehydrogenase apoprotein was studied. The methyl derivative is bound quite effectively in a process very similar to that of the binding of FAD. The binding is accompanied by a large increase of the activity with DCIP at all temperatures, followed by a decline at temperatures above 5°. During the decline the activity with oxidized lipoate increases. With the carboxymethyl analogue only a small increase of DCIP activity is observed. The association constants for these derivatives are smaller than for FAD.

2. The pH-dependent fluorescence of the derivatives is very similar to that of FAD, indicating that they are also internally complexed.

3. The sedimentation pattern of an enzyme containing 3-methylFAD indicates that the activity with oxidized lipoate is as with the FAD-holoenzyme connected with the dimer.

INTRODUCTION

The recombination of the apoenzyme of lipoamide dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3) with FAD was studied and some of the characteristics of this process delineated in the preceding paper[1]. These included changes in polarization and intensity of fluorescence as well as activities on both an artificial substrate, DCIP, and the more natural substrate, lipoic acid. One of the features was the almost instantaneous binding of most of the FAD, as can be deduced from a prompt increase in polarization. To obtain more detailed information about the sequence of the reactions following the attachment of the flavin, recombination with analogues of FAD has now been examined. The 3-methyl- and 3-carboxymethylFAD, compounds alkylated at the 3-imino position in the isoalloxazine system of the flavin-adenine dinucleotide, were used for this purpose.

Abbreviation: DCIP, 2,6-dichlorophenol indophenol.

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MATERIALS AND METHODS

Chemicals

Reagents were the same as mentioned before[1]. The 3-methyl-, 3-carboxymethyl- and 2-morpholinoFAD was synthesized *via* their mono phosphate esters[2,3] by condensation with 5-phosphoromorpholidate[4] and in part was a gift from Prof. P. HEMMERICH (Konstanz).

The enzyme and the apoenzyme were prepared and activity measurements performed as mentioned previously[1] using the same definitions for calculating specific activities.

Recombination

Recombination of apoenzyme with FAD or its derivatives was performed mostly in 0.13 M sodium phosphate buffer (pH 7.6) which contained 0.3 mM EDTA. Samples were withdrawn at the temperatures and times indicated to measure activities.

Fluorescence polarization

The technique and apparatus used were essentially the same as those described before[1]. However, as the emission intensity and polarization of fluorescence of the FAD analogues bound to the reconstituted enzyme were unknown, the method of BAYLEY AND RADDA[5] or the one described by LAURENCE[6] could only be used for the calculation of the fraction of bound analogue by assuming that these values were the same as for FAD bound to the holoenzyme. The vertical and horizontal vectors of intensity were corrected for contributions from the apoenzyme itself. The fraction of flavin which is bound enables one to calculate the association constants. The number of binding sites was calculated using the equation derived by KLOTZ[7].

Absorption spectrum

The absorption spectrum of the 3-methylFAD-modified enzyme was measured against that amount of the original holoenzyme which accounted for the residual

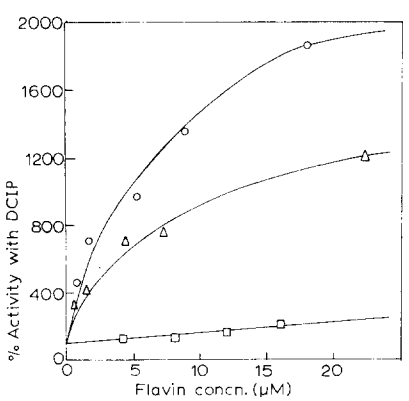


Fig. 1. Effect of coenzyme concentration on the restoration of diaphorase activity. The solutions contained varying amounts of FAD (○), 3-methylFAD (△), or 3-carboxymethylFAD (□) with 5 μM apoenzyme, 0.3 mM EDTA and 0.13 M sodium phosphate buffer (pH 7.6). After 5 min incubation at 0°, samples were withdrawn and activities measured with DCIP.

activity with lipoate of the apoenzyme. The spectrum was measured at 10° with a Cary 14 recording spectrophotometer.

Ultracentrifugation

Sedimentation coefficients were obtained at 3 different temperatures in 0.03 M sodium phosphate buffer (pH 7.2) which contained 0.3 mM EDTA. The protein concentration was 3.3 mg/ml. The experiments were performed with an MSE analytical ultracentrifuge.

RESULTS

Restoration of activities

The effectiveness of 3-methyl- and 3-carboxymethylFAD relative to FAD in restoring the diaphorase activity can be seen from the data in Fig. 1. In comparison to activities with FAD, those obtained with the 3-methyl analogue are reasonably high, but those with the 3-carboxymethyl derivative are low. For example at 10 μM concentration, the 3-carboxymethylFAD shows only 5% of the activity restored by FAD while 60% is restored by the 3-methyl derivative. A similar experiment with

TABLE I

EXTENT OF RESTORATION OF DIAPHORASE AND LIPOATE DEHYDROGENASE ACTIVITIES WITH FAD AND ITS 3-ALKYL ANALOGUES

Solutions contained 13 μM each of flavin and apoenzyme, 0.3 mM EDTA and 0.13 M sodium phosphate buffer (pH 7.6). Activities with DCIP were measured after incubating 5 min at 0°; those with oxidized lipoate were measured after 30 min incubation at room temperature. Activities are expressed in comparison with the holoenzyme.

Flavin added	*Specific activities*	
	DCIP (%) act.	*Oxidized lipoate (%) act.*
None	168	9.9
FAD	1660	69.2
3-MethylFAD	1107	20.2
3-CarboxymethylFAD	778	10.6

2-morpholinoFAD showed this compound to be inactive*. In Table I are given the maximum values of the specific activities in the diaphorase and lipoate dehydrogenase reactions after incubation with FAD and its 3-substituted analogues.

As seen previously[1], the maximal diaphorase activity is obtained in the first minutes after incubating the apoenzyme with FAD on ice. Again both the 3-methyl- and the 3-carboxymethylFAD's partially restore the DCIP activity; both analogues are less coenzymatically active than FAD; the 3-carboxymethylFAD is least active.

The amount of activity with oxidized lipoate restored after raising the temperature indicates that the analogues are less able to replace FAD. The 3-methylFAD

* The 2-morpholinoFAD as well as 3-alkyl derivatives are essentially inactive with D-amino acid apoenzyme (ref. 2 and J. F. KOSTER, unpublished results).

restores only 15% of the activity obtained with the same amount of FAD; the 3-carboxymethylFAD is essentially inactive.

The recombination with the derivatives is strongly temperature dependent as is the case with FAD itself. Fig. 2 shows the results obtained at 2 different temperatures. Over the temperature range from 0° to 25°, DCIP activity increases practically linearly to a plateau in the presence of 3-carboxymethylFAD. The properties of 3-methylFAD are more in between those of FAD and the 3-carboxymethylFAD: the increase of the

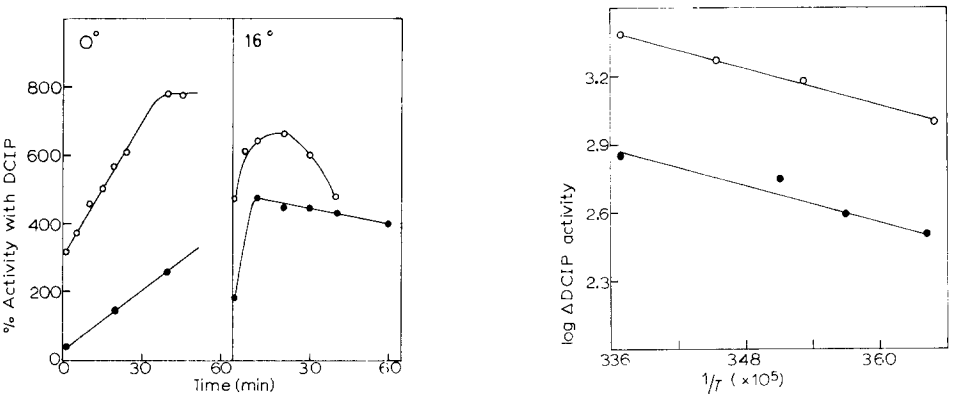


Fig. 2. Restoration of diaphorase activity as affected by time and temperature of incubation of apoenzyme with 3-methyl- (○) or 3-carboxymethylFAD (●). Solutions contained 14 μM each of flavin and apoenzyme, 0.3 mM EDTA and 0.13 M sodium phosphate buffer (pH 7.6).

Fig. 3. Arrhenius plots of the rates of restoration of DCIP activity with the 3-methyl-(○) and 3-carboxymethylFAD (●). Solutions contained 14 μM each of flavin and apoenzyme, 0.3 mM EDTA and 0.13 M sodium phosphate buffer (pH 7.6). Activities with DCIP were measured after 5 min at the given temperatures and corrected for the residual activity of the holoenzyme.

activity with DCIP is still rather linear at 0°, while at higher temperatures it shows similarities to the behavior of FAD (*cf.* ref. 1). With both the analogues, as with FAD, the DCIP activities reach a plateau followed by a decrease; the decline in activity is faster at higher temperatures.

Activation energies for the temperature-dependent increase of the DCIP activity upon recombination of apoenzyme with either 3-methyl- or 3-carboxymethylFAD,

TABLE II

ASSOCIATION CONSTANTS OF FAD AND ITS 3-ALKYL DERIVATIVES WITH LIPOAMIDE DEHYDROGENASE AT DIFFERENT TEMPERATURES

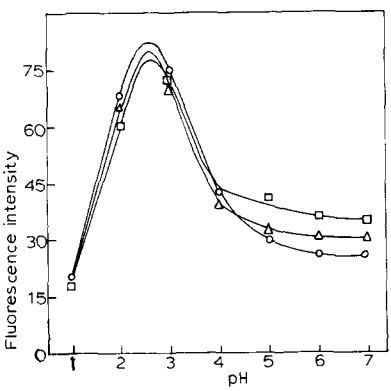
Solutions contained varying amounts of flavin with 1.9 μM apoenzyme, 0.3 mM EDTA and 0.13 M sodium phosphate buffer (pH 7.6) at the temperatures indicated. Fluorescence polarizations were measured and values for K_{ass} calculated as described in the text.

Flavin	$K_{ass} \times 10^{-5}$	
	10°	*20°*
FAD (Table II, ref. 1)	3.2	2.0
3-MethylFAD	1.1	1.1
3-CarboxymethylFAD	0.3	—

obtained from the slopes of the Arrhenius plots (Fig. 3), have approximately the same values (approx. 5500 cal·mole^{-1}). Similar values for the activation energies were obtained, when calculated from the DCIP activity after 1 min incubation.

Fluorescence changes

The fluorescence–pH profiles of FAD and its 3-alkyl derivatives are given in Fig. 4. As with FAD, both 3-carboxymethyl- and 3-methylFAD exhibit maxima for fluorescence intensity near pH 2.5. In all cases the fluorescence decreases toward neutral pH with a midpoint at pH 3.5 indicative of the pK_a for protonation of the 6-amino group of adenosine[8]. Relatively low fluorescence is seen from mildly acid to slithtly alkaline solutions wherein these flavins are intramolecularly complexed[9]. Thus the FAD analogues like FAD are presented as internally complexed species to the apoenzyme. The fact that the fluorescence characteristics of the derivatives are very



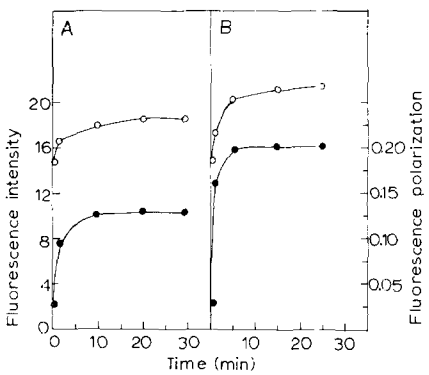


Fig. 4. Effect of pH on the fluorescence intensity of FAD and its 3-alkyl analogues. Solutions were 10 μM in FAD (○), 3-methylFAD (△), or 3-carboxymethylFAD (□) with 0.1 M buffers of KCl–HCl (pH 1); glycine–HCl (pH 2 and 3); sodium acetate (pH 4 and 5); sodium phosphate (pH 6 and 7).

Fig. 5. Change in intensity (○) and polarization (●) of fluorescence upon binding of FAD (B) and its 3-methyl analogue (A) to lipoamide dehydrogenase apoprotein. Solutions contained 2.5 μM each of flavin and apoenzyme, 0.3 mM EDTA and 0.13 M sodium phosphate buffer (pH 7.6) at 20°.

similar to those of FAD justifies the assumptions made for the calculation of the association constants, *i.e.* they show the same increase in fluorescence upon binding to the protein.

The patterns for change in polarization and intensity of fluorescence are given in Fig. 5 for the binding of the methylFAD at 20°. The polarization increases rapidly within the first minute; after this it keeps increasing, but much more slowly. The intensity also increases rapidly in a way grossly similar to the changes in polarization. The recombination with 3-methylFAD is very similar to that of FAD over the whole temperature range studied; however, the polarizations are somewhat less. The relatively small increase in polarization which characterizes the 3-carboxymethylFAD system, indicates that the affinity of this derivative for the protein is lower than those with FAD or the 3-methyl analogue. This low affinity of 3-carboxymethylFAD, in combi-

nation with a lower activity at saturating concentrations for the activity with DCIP, explains the shape of the saturation curve of Fig. 1.

The fluorescence polarizations as well as intensities of the free flavin-analogues were determined. The 3-carboxymethyl- and the 3-methyl FAD do not differ from FAD with respect to the polarization. All the p values found were between 0.028 and 0.032 and are practically temperature independent. A sample of apoenzyme (DCIP activity, 80%, activity with oxidized lipoate, less than 3%) was reconstituted with excess 3-methylFAD, precipitated with $(NH_4)_2SO_4$, and extensively dialyzed against 0.03 M sodium phosphate buffer (pH 7.2) which contained 0.3 mM EDTA. The polarization of fluorescence of the 3-methylFAD bound to this reconstituted enzyme was 0.37, determined at 10°. This is in fair agreement with values obtained for the FAD-containing lipoamide dehydrogenase (0.38). This reconstituted enzyme showed an activity with oxidized lipoate of 17% and a DCIP activity of 330%, indicating that the physiological activity is also partially restored. The value of 0.37 was used for the polarization of both the bound 3-methylFAD and 3-carboxymethylFAD in the calculations. The association constants are average values from at least 4 independent experiments using different flavin: apoenzyme ratios. By the method of KLOTZ[7] it was calculated that 1.0–1.2 moles flavin analogue was bound per 50 000 g of apoenzyme, a value which is close to the one obtained with FAD (*cf.* ref. 1).

A preliminary experiment with a small amount of purified 3-methyl FAD-containing enzyme shows that the absorption spectrum has maxima around 454 mμ and 350 mμ and minima around 410 mμ and 330 mμ. Because the solution was slightly turbid these values are not accurate. Since the spectrum shows a shoulder between 465 and 485 mμ, one can nevertheless conclude that this FAD analogue is bound in a way similar to FAD.

Fig. 6 shows that the sedimentation patterns of reconstituted 3-methylFAD-containing lipoamide dehydrogenase maintain a very sharp peak which is asymmetrical

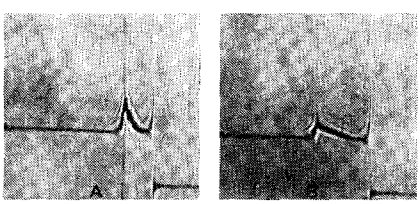


Fig. 6. Sedimentation of 3-methylFAD reconstituted lipoamide dehydrogenase in 0.03 M sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA. Rotor speed: 55 300 rev./min; temperature 12°. Photographs taken after 24 min (A) and 39 min (B). Sedimentation direction, from right to left.

and skewed to the side of the meniscus, which suggests heterogeneity. The rather low ionic strength and dissociation phenomena, as suggested by the high DCIP activity, may both be responsible for this. Sedimentation coefficients were calculated from the top of the peak. At 7°, 12° and 17° the respective values are 4.1, 4.9 and 5.2 S. An average $s_{20,w}$ value of 6.0 S can be calculated from these results. Extrapolation to zero protein concentration was not possible, as there was not enough material available.

DISCUSSION

The present finding that the apoenzyme of lipoamide dehydrogenase is able to recombine with FAD analogues is of real significance in studying flavin–protein interactions. Up to now, FAD-analogues have been tested for their coenzymatic functions almost exclusively on the apoenzyme of D-amino acid oxidase. However, the results now obtained emphasize that one cannot appraise coenzyme function on the basis of only one enzyme.

Replacement of FAD by the two 3-alkyl derivatives in the lipoamide dehydrogenase resulted in reasonably active complexes with the artificial substrate DCIP. As to physical properties, the 3-methyl- as well as the 3-carboxymethylFAD do not differ very much from FAD, *e.g.* the redox potentials are the same[10]. Spectra and fluorescence behavior are also practically the same. Apparently the equilibria between the internally quenched complexes of the 3-alkyl analogues and their open fluorescent forms, evidenced by fluorescence–pH profiles, are similar to that of FAD (ref. 10). This suggests that replacement of the 3-imino hydrogen of FAD by a substituent which is larger or negatively charged does not markedly influence the ability to form an intramolecular nonfluorescent complex.

The increase in fluorescence polarization upon addition of the 3-alkyl analogues, as with FAD, reflects the binding process. Moreover, since the unbound flavins are less fluorescent than when bound, the increase in their intensities must reflect an opening of their intramolecular complexes upon association with the enzyme. The analogues cannot fully replace FAD. This is not surprising as the association constants have been shown to be different. From the midpoints of the saturation curves (see Fig. 1) one can calculate the association constants only approximately, as the maximum obtainable velocities are uncertain. These are assumed for the DCIP activity to be 2000% and 1300% of the value of holoenzyme for the FAD and the 3-methyl derivative, respectively. However, the latter value is quite uncertain, since saturating concentrations of the methyl derivative were not applied. The fact that 3-substituted flavins are able to be bound to form an active enzyme indicates that the 3-position is not involved primarily in the binding to the protein as has been proposed previously[13] for other flavoproteins (*cf.* ref. 14).

Recently DE KOK, SPENCER AND WEBER[15] concluded from lifetime studies that upon binding FAD to lipoamide dehydrogenase, the complex between the isoalloxazine nucleus and the adenine moiety is broken. The fluorescence quenching of the bound flavin is probably due to another group in the vicinity of the flavin. The slow increase of the fluorescence intensity towards its maximum in comparison with the rapid initial phase (*cf.* ref. 1) indicates that a series of consecutive reactions is involved in this binding process.

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