OPTICAL ROTATORY DISPERSION OF FLAVIN NUCLEOTIDES*

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The growing importance of spectropolarimetry in studies of interactions of enzymes with prosthetic groups, coenzymes, coenzyme analogues, substrates and inhibitors has been reviewed recently (Ulmer and Vallee, 1965). While reports on the rotatory dispersion of enzyme complexes with diphosphopyridine nucleotide, pyridoxal, adenosine diphosphate and their analogues are on record (Ulmer and Vallee, 1961; Ulmer et al., 1961; Fasella and Hammes, 1964; Kägi and Li, 1965), spectropolarimetry has not been employed in the study of flavin-protein interaction. We wish to report here data on the optical rotatory dispersion (ORD) of flavin adenine dinucleotide (FAD) and alterations in the rotatory dispersion of this

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coenzyme on binding to diaphorase. These observations would appear to be pertinent both to the structure of FAD itself, and to the study of the interactions of substrates, flavins and apoenzymes by means analogous to those employed previously for other systems. A preliminary report has been made (Vallee, 1965).

EXPERIMENTAL

FAD and flavin mononucleotide (FMN) were obtained from Sigma Chemical Company. Diaphorase was obtained from the Boehringer Mannheim Corporation. All other chemicals were reagent grade.

pH was measured with a Radiometer pH meter equipped with a general purpose glass electrode. ORD was measured with a Cary Model 60 recording spectropolarimeter using an R-136 type photomultiplier to extend the useful wavelength range to 700 mμ. All polarimetric measurements were performed at 25° C. Specific rotations ([α]) and molar rotations ([M]) (Djerrasi, 1960) are not corrected for the refractive index of the solvent.

RESULTS

The ORD of FAD, FMN, and adenosine monophosphate (AMP) are shown in Fig. 1. AMP has a small, negative Cotton effect with an amplitude of about 1300° (Lamborg et al., 1965) at about 260 mμ, but anomalous rotatory dispersion is not evident at longer wavelengths. FMN exhibits a somewhat more complex pattern of rotatory dispersion with a crossover from positive to negative rotation at 350 mμ,

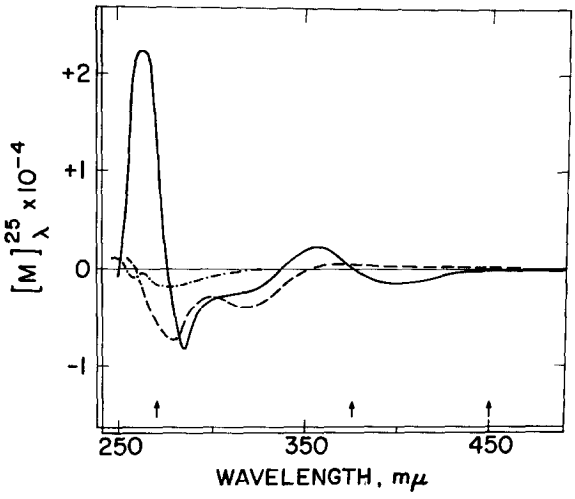

Figure 1. Optical rotatory dispersion of AMP (— ·—·), FMN (— —), and FAD (———), determined at pH 7 in 0.01 M phosphate buffer. The arrows indicate the absorption maxima of FAD.

a trough at 320 mμ which may be associated with two proximate, negative Cotton effects, and a crossover to positive rotation at approximately 255 mμ. The rotatory dispersion of FAD is strikingly more rich in detail than that of either FMN or AMP, the two moieties which constitute this coenzyme. There is a negative Cotton effect centered at 375 mμ, a complex region of negative rotation between 280 and 330 mμ which resembles that seen in FMN alone, and finally a large positive peak in the 260 mμ region.* In marked contrast, the rotatory dispersion of

* The present data on the rotatory dispersion of FAD are in substantial agreement with those of Dr. D. Wellner, to whom we are indebted for communicating his findings to us prior to publication.

equimolar mixtures of FMN and AMP is identical with the sum of the rotations of the components.

Such spectropolarimetric findings suggested interaction between the two ring systems of FAD by analogy to the interaction in adenylyl (3'-5') adenosine (vide infra), and led to investigations of varying solvent conditions to attempt to define the basis of this interaction. The rotatory dispersion of FAD remains unchanged as pH is lowered from 8 to 4. At more acidic pH's, however, the rotatory dispersion is altered; the Cotton effect centered at 375 mμ disappears and the positive rotation at 262 mμ is markedly diminished.

In strongly acidic solutions (pH 1), the ORD of FAD approximates quite closely the sum of the rotations of FMN and AMP. The molar rotation at 262 mμ, plotted as a function of pH, yields a titration curve with an inflection point at about pH 3.5 (Fig. 2), which coincides with the pK of the adenine amino group. Alteration of pH to strongly acidic values does not alter the rotatory dispersion of FMN, while the Cotton effect of AMP undergoes a red shift without a major alteration of rotatory strength.

The composition of the solvent also alters the characteristic optical rotatory dispersion of FAD. Concentrations of urea increasing to 8 M progressively diminish both the Cotton effects at 262 and 375 mμ in a manner similar to that noted at acidic pH's. Similar results are noted if the concentration of dioxane in the solution

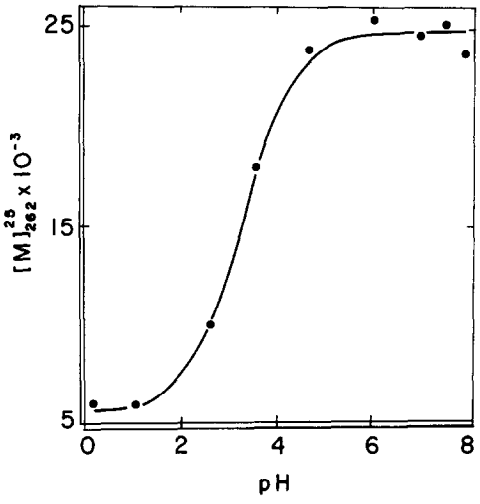

Figure 2. Abolition of the 262 mμ Cotton effect of FAD by acid. The molar rotation of FAD at 262 mμ is plotted as a function of the pH. Buffers employed were 0.1 M phosphate, pH 6-8, 0.1 M acetate pH 3-6, and HCl, pH less than 3.

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is increased from zero to 67% (v/v). Finally, a mixture of 0.01 M pH 6.8 phosphate buffer, benzyl alcohol and ethanol (33:22:45) reported by Bessey et al., (1949), to abolish the fluorescence quenching of FAD, also destroys the characteristic rotatory dispersion pattern of the molecule.

In view of previously demonstrated alterations in the rotatory dispersion of cofactors when bound to enzymes (Ulmer and Vallee, 1965), it was of interest to investigate the spectropolarimetric properties of one of the flavoproteins. The optical rotatory dispersion of the FAD enzyme, diaphorase, is shown in Fig. 3. A large positive Cotton effect at about 370 mμ is noted. Assuming plain rotation of the apoenzyme through this region, the

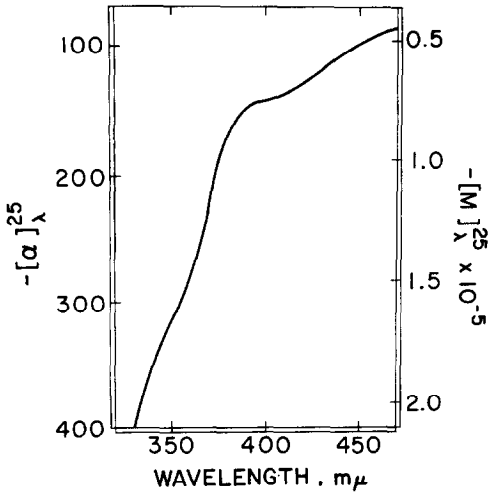

Figure 3. Optical rotatory dispersion of diaphorase, determined at pH 7 in 0.01 M phosphate buffer. Specific rotation is indicated on the left-hand ordinate, while the right-hand ordinate is shown in molar rotation for the FAD present, assuming one mole of FAD per 53,000 grams protein (Massey, 1963).

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amplitude of this Cotton effect is estimated to be about 70° of specific rotation, corresponding to a molar rotation for the FAD present of approximately $37,000^{\circ}$, an enhancement of ten-fold when compared to the amplitude of the 375 mμ Cotton effect of FAD alone. The absorption band at 450 mμ remains optically inactive even when FAD is bound to this enzyme. Anomalous rotatory dispersion was not apparent at the 260 mμ absorption band, however, the strong rotation of the peptide backbone in this wavelength region might conceal a Cotton effect.

DISCUSSION

During the past decade ORD has become of increasing importance both for investigation of the steric relation-

ships of small organic molecules (Djerassi, 1960) and as a guide to the conformation of biological macromolecules (Urnes and Doty, 1961). More recently spectropolarimetry has been employed in the investigation of the interaction of coenzymes with proteins (Ulmer and Vallee, 1965) and the current studies are of particular interest in this regard.

A number of ring systems have been shown to possess anomalous rotatory dispersion at their absorption maximum. Thus, both AMP (Lamborg et al., 1965) and FMN appear to have small Cotton effects at the 260 mμ absorption band. Further, FMN exhibits Cotton effects which presumably arise from the ketonic absorption band at approximately 310 mμ, probably by a mechanism similar to that seen with the asymmetric carbonyl chromophore in the ketosteroids (Djerassi, 1960). In the bimolecular complex of FAD, both the 262 and the 375 mμ bands are optically active. The rotational strength of the 262 mμ transition is significantly greater than that observed for either FMN or AMP, while the 375 mμ absorption band exhibits anomalous rotatory dispersion only in FAD. Optical activity at the 450 mμ peak (Gascoigne and Radda, 1965) was not detected in the present study. Anomalous rotatory dispersion in the 280 to 330 mμ region is present in FAD as well as in FMN, supporting the assignment of this Cotton effect to the ketonic groups of the isoalloxazine ring system.

The interaction of the two ring systems of FAD most likely gives rise to the additional asymmetry resulting in

the complex rotatory dispersion of this molecule. Both fluorescence quenching (Weber, 1950) and hypochromism in absorption spectroscopy (Whitby, 1953) have previously suggested such an interaction between the adenine and isoalloxazine ring systems. At a pH which corresponds to the apparent pK of the amino group of the adenine moiety, the anomalous optical rotatory dispersion is altered. This might suggest either that this amino group is involved in hydrogen bonding or that the positive charge on the amino group in strong acid may disrupt hydrophobic interactions. Abolition of the anomalous optical rotatory dispersion of FAD by urea, dioxane, and a solvent system containing a large proportion of an aromatic alcohol supports the contention that the hydrophobic nature of the two ring systems may well contribute to the interaction. Thus, interaction between the two ring systems of FAD is reflected by marked alterations in optical rotatory dispersion, when compared to that of the component moieties of the coenzyme. These results are quite analogous to the alterations in the optical rotatory dispersion of adenylyl (3'-5') adenosine when compared to its components, 5' adenylic acid and adenosine (Warshaw et al., 1965), also interpreted to reflect interaction between two ring systems.

The alterations in the optical rotatory dispersion of FAD when part of a flavin enzyme are similar in kind to those previously demonstrated for the binding of other specific cofactors to their apoenzymes, and are the first indications

of the potential utility of extrinsic Cotton effects in the study of flavoproteins. The spectropolarimetric properties of those flavoproteins previously studied demonstrated that their anomalous rotatory dispersion arose from metal-enzyme interactions, since it was observed after removal of the flavin moieties (Handler, et al., 1965). However, in diaphorase, not known to contain metals (Massey, 1963), the anomalous rotation of the enzyme-coenzyme complex is opposite in sign and approximately ten times greater in amplitude than that of the coenzyme alone, results quite analogous to those previously demonstrated for the interaction of ADP with creatine phosphokinase (Kägi and Li, 1965).

Based on rapidly accumulating experience with other systems (Ulmer and Vallee, 1965) these data enhance the prediction that spectropolarimetry will prove important in further investigations of the flavin enzymes, much as it has been in the study of alcohol dehydrogenase (Ulmer and Vallee, 1965).

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