Magnetic Circular Dichroism Studies on the Active-Site Flavin of Lipoamide Dehydrogenase†

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ABSTRACT: The magnetic circular dichroic (MCD) spectra of oxidized and reduced flavins are recorded in various solvents. They are shown to be sensitive to flavin environment. The MCD spectra of oxidized and reduced lipoamide dehydrogenase are reported. In the oxidized enzyme the sign of the *B* term associated with the 27 000-cm^{-1} band is reversed from free flavins. This is attributed to interaction of the disulfide with the short-axis dipole of FAD. The sign reversal is also present in a closely related disulfide enzyme, glutathione reductase, but absent in glucose oxidase. In the half-reduced enzyme, the appearance of an *A* term at 18 180 cm^{-1} is attributable to a charge-transfer complex with a thiolate anion as donor. Insensitivity of the term's energy and intensity to the redox state of flavin suggests that a protein residue may accept or stabilize the thiolate charge transfer.

The nature of the half-reduced EH_2[1] form of lipoamide dehydrogenase has been the subject of much debate. Initially, its red color was attributed to a flavin semiquinone, the second electron residing on a sulfur atom (Massey et al., 1960; Searls et al., 1961). Then a "strongly interacting biradical", approaching covalent linkage of flavin to sulfur, was considered (Palmer & Massey, 1968; Massey, 1963). Recently, the red color has been suggested to arise from charge transfer between a nascent thiolate anion and oxidized flavin (Searls et al., 1961; Massey & Ghisla, 1974).

The biological function of the enzyme is the reversible nicotinamide nucleotide linked dehydrogenation of dihydrolipoamide, which in both directions forms a mixed disulfide with one of the thiols arising from reduction of the enzyme (Williams, 1976; Matthews et al., 1977). Reduction of the enzyme gives rise to two new thiol residues, one of which forms the catalytic thiolate, while the other binds the lipoamide substrate. The FAD molecule is known to reside in a hydrophobic pocket of the enzyme (Ogasahara et al., 1976) within interaction distance of the disulfide (Williams, 1976).

The enzyme consists of two subunits of approximately 5.5 $\times 10^4$ daltons and contains one molecule of FAD per subunit (Massey, 1963). Flavin–flavin interaction has been considered by some authors (Tsai, 1973; Visser et al., 1970; van Muiswinkel-Voetberg & Veeger, 1973). The enzyme operates between oxidized (E_{ox}) and two-electron half-reduced (EH_2) forms in the normal catalytic cycle (Massey, 1963; Williams, 1976). Full reduction to the EH_4 level results in reduction of both the flavin cofactor and the active-site disulfide, on each subunit (Massey & Veeger, 1961). Reduction to the EH_2 level is accomplished by anaerobic addition of either reduced substrate, NADH or dihydrolipoamide, and overreduction to EH_4 does not normally occur at room temperature in the presence of an excess of either reductant (Massey & Veeger, 1961). Dithionite rapidly reduces the anaerobic enzyme to the EH_4 level (Matthews & Williams, 1976).

Glutathione reductase has been shown to be similar to lipoamide dehydrogenase in many ways (Williams, 1976). Both contain one molecule of FAD per subunit in a dimeric enzyme, have active center disulfides coupled to the flavin, show EH_2 spectra unattributable to either neutral or anionic flavin semiquinones, and react with disulfide substrates. The X-ray structure of glutathione reductase has been reported (Schulz et al., 1978), and indicates the absence of physical flavin–flavin interaction in this enzyme.

In the present work, we study MCD of an organic cofactor, FAD, in both the bound and unbound states. A preliminary study of the MCD of free oxidized flavins has appeared (Tollin, 1968). Subsequent work showed the sensitivity of the natural CD to flavin environment and conformation (Edmondson & Tollin, 1971), and it is of interest to extend this work to include MCD. In this paper, we report the MCD of the three biologically occurring flavins, riboflavin, FMN, and FAD, in various solvents. The effects on the FAD binding to lipoamide dehydrogenase in reduced and oxidized states are examined and compared with those to glutathione reductase and glucose oxidase. Active-site interactions are deduced from MCD studies.

Experimental Procedures

Chemicals. Riboflavin was obtained from Eastman Organic Chemicals. FAD (grade III), FMN (commercial grade), β-NADH (yeast, grade III), and β-NADPH (Σ grade) were all obtained from Sigma Chemical Co. Dihydrolipoamide was prepared from lipoamide (Sigma) following the method of Reed et al. (1958). All chemicals were used as obtained.

Enzymes. Lipoamide dehydrogenase (porcine heart) was obtained from Sigma Chemical Co. (type III) or Boehringer-Mannheim (Diaphorase, grade I). Glucose oxidase from *Aspergillus niger* was purchased from Worthington Biochemical Corp. Glutathione reductase (type II, crude) was from Sigma and was purified on a calcium phosphate gel cellulose column (2.8 × 15.5 cm) according to Williams et al. (1967). The gel was prepared by the method of Kosicki (1968). Similar purification of the lipoamide dehydrogenase preparations (Williams et al., 1967) yielded no detectable changes in the spectroscopic properties of the flavin chromophore or in the kinetic parameters of the enzyme.

MCD Spectra. MCD spectra were recorded as previously described (Langford et al., 1980). Spectrometer gain and time constants were optimized by trial, and base-line correction was applied. A field strength of 47 kG was used throughout. No correction for natural CD has been applied. Most intense

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[1] Abbreviations used: EH_2 and EH_4, the two- and four-electron reduced species of the flavoproteins (per subunit); MCD, magnetic circular dichroism; FAD, flavin adenine dinucleotide.

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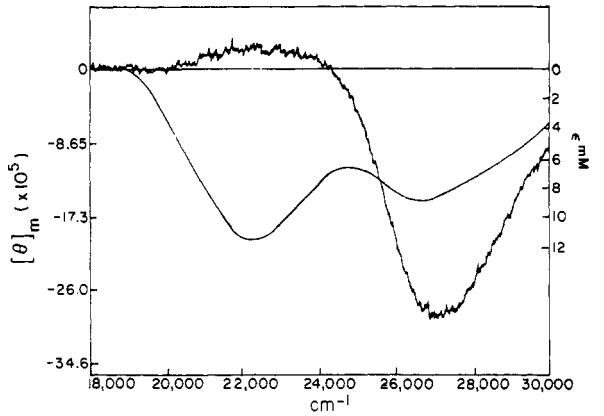

FIGURE 1: MCD (rough line) and absorption (inverted, smooth line) spectra of 3.0×10^{-5} M FAD in deionized water.

natural CD below 30 000 cm^{-1} is associated with the 27 000-cm^{-1} absorption, where it accounts for approximately 10% of the observed MCD at 47 kG. The machine is calibrated to an aqueous $CoSO_4$ standard, with $[\theta]_m = -0.062$ deg·cm^2·mol^{-1}·G^{-1} at 19 600 cm^{-1} (Holmquist et al., 1975). An anaerobic condition was maintained for recording MCD spectra of reduced enzymes. Absorption spectra characteristic of these enzymes were taken before and after MCD runs. Absorption spectra were obtained on a Cary 14 spectrometer.

Results

Free Flavins. Figure 1 shows the MCD and absorption spectra of FAD in water (pH 5.8) for the two lowest energy $\pi^* \leftarrow \pi$ transitions. The spectra for FMN and riboflavin are qualitatively similar, showing a weak, positive MCD in the low-energy band with a strong negative MCD for the second transition. Some resolution of both bands is seen. Following the notation of Edmondson & Tollin (1971), the three observable bands of the low-energy transition are designated I, II, and III for the 0–0 transition with its first (0 + 1000 cm^{-1}) and second (0 + 1000 + 1500 cm^{-1}) curve-resolved vibronic levels. The next transition (ca. 27 000 cm^{-1}) consists of bands IV (0–0) and V (0 + 1000 cm^{-1}). The first transition of the free flavins in water shows the greatest molar ellipticity in bands II and III, while the second transition shows its greatest in band V, consistent with the magnitudes of solvent shifts (Tollin, 1968). The spectra of these three flavins in 0.010 M potassium phosphate buffer, pH 7.0, are similar to each other but differ from the corresponding spectra in water. In this medium, the lowest energy transition displays a negative MCD and an increased molar ellipticity under the fundamental bands I and IV. In both solvents, riboflavin displays a higher molar ellipticity than FMN and FAD in bands I–III. In methanol, all three flavins retain a negative MCD for both low-energy transitions. The molar ellipticity of the first (low energy) transition has increased relative to that of the second for all analogues in the less polar solvent. The MCD of FAD corresponding to each of bands I–III has become better resolved.

Lipoamide Dehydrogenase. The MCD spectrum of the two lowest energy transitions of FAD in lipoamide dehydrogenase is shown in Figure 2. Bands I–III retain the resolution characteristic of FAD in a less polar environment, in both absorption and MCD spectra, and also retain a negative MCD. In contrast to free flavins in any solvent, the MCD of the second absorption in the enzyme becomes positive. The absorption bands are more fully resolved, and the MCD results predominantly from the 0–0 band IV.

Upon full anaerobic reduction of the enzyme with dithionite, the EH_4 form is produced. The resultant flavin hydroquinone

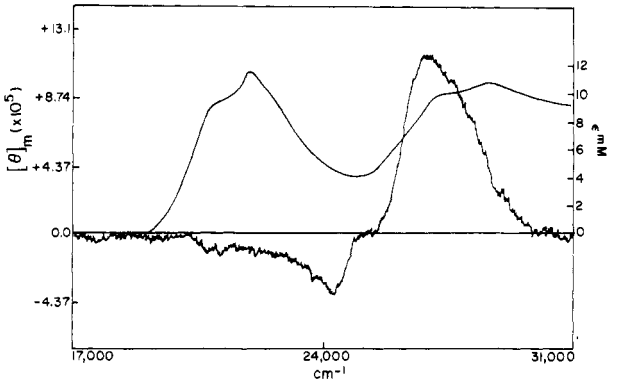

FIGURE 2: MCD (rough line) and absorption (smooth line) of 6.0 × 10^{-5} M lipoamide dehydrogenase in phosphate buffer (0.010 M, pH 7.0).

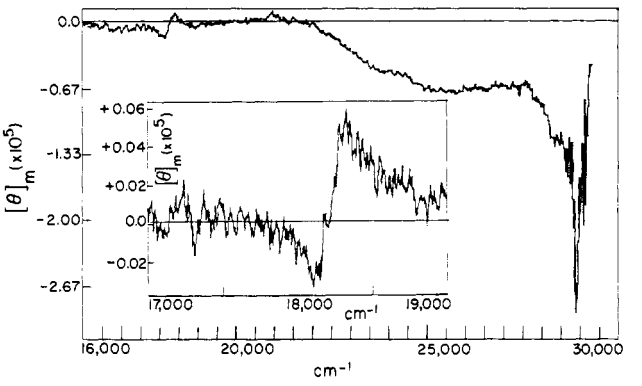

FIGURE 3: MCD spectrum of 1.1×10^{-4} M lipoamide dehydrogenase in phosphate buffer, anaerobically reduced to the EH_4 level with a pinch of dithionite as determined by the characteristic absorption spectra before and after the MCD recording, which was run under anaerobic conditions. Inset shows an expansion of the *A* term region recorded at increased instrumental gain.

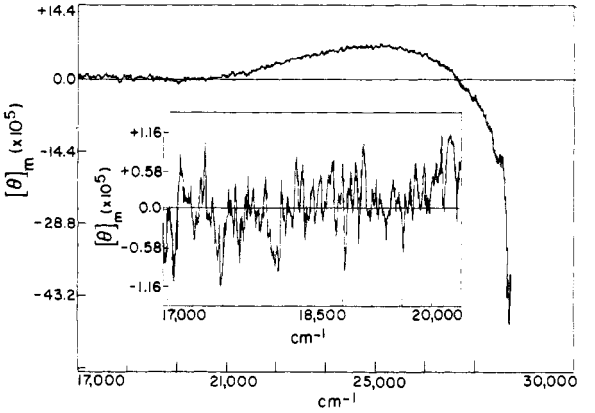

FIGURE 4: MCD spectrum of 3.0×10^{-5} M FAD in water, anaerobically reduced with a pinch of dithionite as determined by the characteristic absorption spectrum. Inset shows an expansion of the 17 000–20 000-cm^{-1} region recorded at the same increased gain as the inset of Figure 3.

is characterized by an ill-defined absorption beginning at about 21 000 cm^{-1}, rising to higher energy. This feature is present in the MCD spectrum (Figure 3) as a broad negative MCD. A corresponding feature is present when free FAD is reduced by dithionite (Figure 4). In addition, an *A* term at 18 180 cm^{-1} appears in the EH_4 MCD spectrum but is absent in that of free reduced FAD.

When the enzyme is reduced with either NADH or dihydrolipoamide in excess at room temperature, the EH_2 forms are obtained exclusively. In both cases (Figures 5 and 6) the intensity of the *B* terms associated with bands II and III of

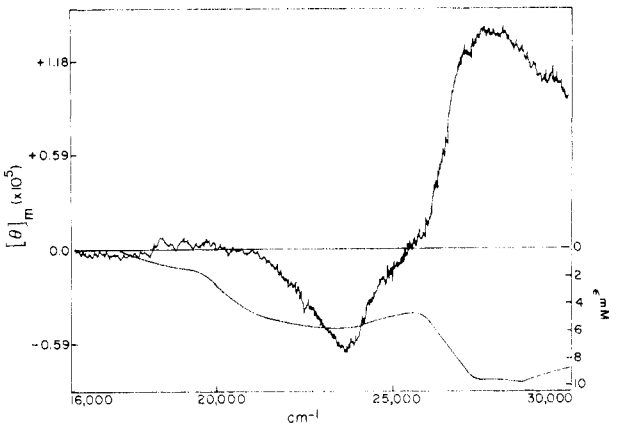

FIGURE 5: MCD (rough line) and absorption (inverted smooth line) spectra of 1.1×10^{-4} M lipoamide dehydrogenase in phosphate buffer, anaerobically reduced with a fivefold excess of NADH.

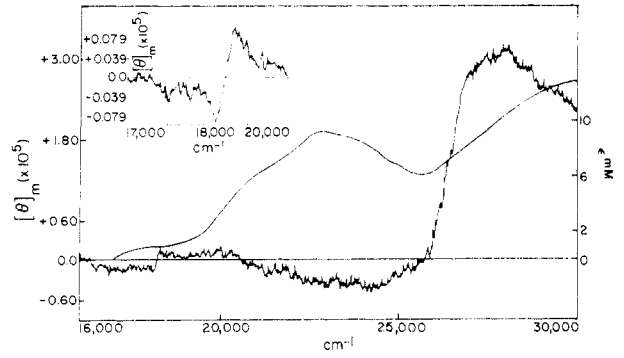

FIGURE 6: MCD (rough line) and absorption (smooth line) spectra of 1.1×10^{-4} lipoamide dehydrogenase in phosphate buffer, reduced anaerobically with a 10-fold excess of dihydrolipoamide. Inset shows an expansion of the 17 000–19 000-cm^{-1} region recorded at increased instrumental gain.

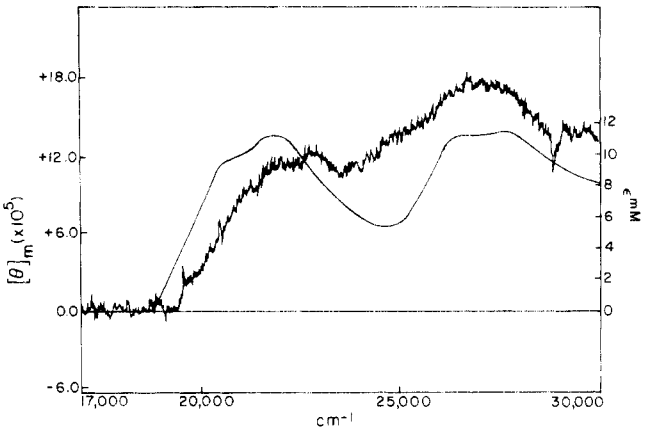

FIGURE 7: MCD (rough line) and absorption (smooth line) spectra of 2.08×10^{-5} M glutathione reductase in potassium phosphate buffer (0.010 M, pH 7.0).

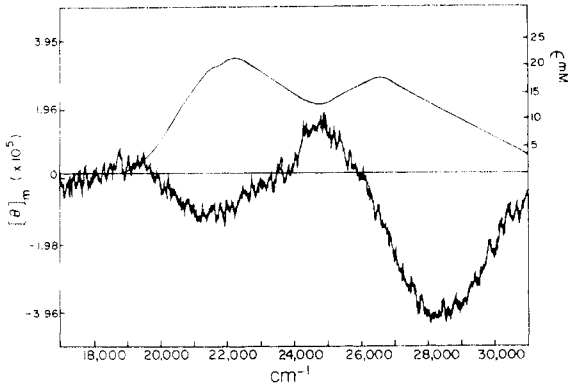

FIGURE 8: MCD (rough line) and absorption (smooth line) spectra of 2.86×10^{-5} M glucose oxidase in potassium phosphate buffer (0.10 M, pH 7.0).

the oxidized enzyme increases relative to that of band I, and the sign remains positive. The spectra differ slightly in the MCD, consistent with the spectroscopic (Brady & Beychok, 1971) and chemical (Huang & Brady, 1973) differences previously reported. In both cases the *A* term is present at 18 180 cm^{-1}. This feature is also present when the enzyme is titrated to the EH_2 level with dithionite. In these four cases where the *A* term appears, it is of nearly constant energy and intensity.

Glucose Oxidase and Glutathione Reductase. The MCD spectra of glutathione reductase and glucose oxidase are shown in Figures 7 and 8. The absorption spectrum of glucose oxidase at the low-energy band is slightly shifted hypsochromically, and the shoulder at 465 nm characteristic for the bound FAD is not as distinctively discernible as that of lipoamide dehydrogenase. The MCD spectrum of glucose oxidase differs drastically from that of lipoamide dehydrogenase in that the positive 27 000-cm^{-1} band shifts to negative at 28 300 cm^{-1}. By contrast, the absorption spectrum of glutathione reductase is bathochromically shifted from that of lipoamide dehydrogenase, and all MCD features are positive. Upon reduction of glutathione reductase by NADPH, an *A* term appears at 18 320 cm^{-1}, followed to high energy by a broad, featureless MCD. The *A* term is less intense than that found in lipoamide dehydrogenase.

Discussion

Previous work (Tollin, 1968; Edmondson & Tollin, 1971) has established natural circular dichroism as a sensitive probe of flavin environment and conformational changes, although correlation with absorption spectral features remains largely empirical. The isoalloxazine chromophore of the flavins is optically inactive, and any natural optical activity must therefore arise from interaction with the chiral ribityl side chain. The ribityl group is nonchromophoric in the visible and near-ultraviolet (UV) regions. Very little natural optical activity has been found to be associated with the 22 000-cm^{-1} flavin transition (Brady & Beychok, 1969).

As the isoalloxazine moiety has only reflection (C_s) symmetry, the MCD spectrum must be composed of B terms arising from magnetic field induced mixing of excited electronic states (Holmquist & Vallee, 1978; Schatz & McCaffery, 1970). The absorption spectrum below 30 000 cm^{-1} shows two major transitions, which have been shown to be $\pi^* \leftarrow \pi$ in nature (Fox et al., 1967; Song, 1971). These transitions display vibronic resolution, which is enhanced with decreasing solvent polarity (Harbury et al., 1959). A $\pi^* \leftarrow \pi$ transition is reported at 32 260 cm^{-1} (Tollin, 1968), but this and the higher energy transitions are obscured by the strong protein dichroisms in the flavoproteins. The free flavin MCD spectra reported here consist of a series of *B* terms lying under bands I–V of the absorption spectra.

The three naturally occurring flavins, riboflavin, FMN, and FAD, were investigated in water, neutral phosphate buffer, and methanol. In all cases, the 27 000-cm^{-1} transition gives rise to positive *B* terms (negative MCD). The MCD under the 22 200-cm^{-1} absorption is weak and changes sign from positive in water to negative in either buffered aqueous media or nonaqueous solvent. Tollin (1968) has found a similar sign inversion in the relatively weak natural CD of riboflavin in

this region. Resolution of the MCD of bands I–III is particularly marked in methanol, where the vibronic levels display narrowed line widths.

Michl (1978a–c), using a free perimeter electron model of cyclic π systems, has predicted that in general a ^1L_b transition will have a B term value of zero ($[\theta]_m = 0$), and a ^1L_a transition will have $B > 0$ ($[\theta]_m < 0$). The flavin transition at 22 200 cm^{-1} is long axis polarized and ^1L_b in character, while the next transition is ^1L_a (Penzer et al., 1970; Weimar & Neims, 1975). Thus, the observations of weak and negative molar ellipticities for the ^1L_b and ^1L_a transitions, respectively, are in agreement with the theoretical predictions.

The B term occurs when electronic states of the molecule can mix in the magnetic field. The expression for the B term is represented by an expansion of the basis function to include mixing of the ground state g and the excited state j with other molecular excited states k (Michl, 1978a). In general, only excited states are sufficiently close in energy to significantly perturb one another, and the major contribution comes from the term representing the mixing of the states k into j (Michl, 1978a). This dominating term is given by

$$B_{g\rightarrow j} = \sum_k \mathrm{Im}\{\langle j|\mu_z|k\rangle \cdot \langle g|\mathbf{m}_x|j\rangle \cdot \langle g|\mathbf{m}_y|k\rangle\}/\Delta E_{j,k} \quad (1)$$

where g and j are the wave functions representing the ground and excited states, respectively, of the transition considered. The summation is overall electronic excited states k which mix with j, and $\Delta E_{j,k}$ is the energy difference between j and a particular k. The μ_z and $\mathbf{m}_{x,y}$ are the magnetic and electric dipole operators, respectively.

Two possibilities exist to explain the sign reversal of the B term corresponding to the ^1L_a transition in the enzyme environment. The sign reversal is observed in glutathione reductase, where the X-ray structure (Schulz et al., 1978) reveals the absence of flavin–flavin interaction. If the flavins interact in a dimer complex, the two ^1L_a states could interact to give rise to an exciton splitting about the absorption maximum. In the coparallel or colinear limits, the intensity of one transition goes to zero, and B terms become of opposite sign (Hollebone & Stillman, 1978). Thus, we would expect sign reversal to be accompanied by a shift of the band center of the B term from the position in free flavin. Such an exciton splitting is not indicated by the spectra.

Secondly, the sign reversal could result through alterations to the higher energy perturbing k states in the enzyme environment. Such states are masked by the intense protein dichroism and are unobservable in the two enzyme systems studied here. The ^1L_a transition has been shown both experimentally and theoretically to be polarized at about 40° from the long-axis ^1L_b (Kurtin & Song, 1968), although more recent estimates (Sun et al., 1972) indicate that this angle may be as low as 20 ± 5°. Thus, the ^1L_a state is perturbed most effectively by in-plane short-axis polarized energy states k. The ^1L_b transition has effectively no short axis component, and the lack of such a component is consistent with retention of the negative MCD sign in native lipoamide dehydrogenase. The perturbation of the short-axis component of the ^1L_a transition is likely produced, therefore, by higher energy, short-axis polarized transitions of the flavin molecule. This necessitates a change in polarization of such transitions to account for the sign reversal in the enzyme.

Steiner & Michl have examined the B term behavior of anthracene and its aza analogues acridine and phenazine (Steiner & Michl, 1978). These systems are classified as soft chromophores, meaning that their B term signs are sensitive to substituent effects. These structures are analogous to the alloxazine system, which is also a soft chromophore. The perimeter model implies that the L transitions of these molecules have MCD signs sensitive to mesomeric substituent effects, dominated by substitution at the 9, 10, 1, and 3 positions of the tricyclic molecule. Both positions 3 and 10 of flavin are heteroatoms. In the flavins, the conjugated N(5) atom, which corresponds directly to N(9) of phenazine, is unsubstituted. There is good evidence that, in both lipoamide dehydrogenase (Thorpe & Williams, 1976) and glutathione reductase (Schulz et al., 1978), there is a disulfide within interacting distance of C(4a) and therefore the adjacent N(5). The presence of this electropositive group is therefore expected to affect the polarization of short-axis transitions and therefore a sign reversal of the B term of the ^1L_a transition. The absence of the active-site disulfide in glucose oxidase gives the negative band of noninteracting FAD as observed.

To predict the nature of such changes, it will be convenient to use the molecular orbital description. In the unperturbed system, low-lying π^* orbitals on N(5) are available as acceptor orbitals for an excited electron. In free flavins, the transition dipole of the lowest energy short-axis polarized transition is most likely directed toward N(5) and away from the more electropositive alkane nitrogen atom at position 10. This model is in agreement with the higher spin density on N(5) found by Fox et al. (1967) for addition of an electron to the isoalloxazine system, and by Song (1968) for the lowest triplet state of flavin. However, π-type donation from the filled p orbitals of sulfur close to N(5) will increase the electron density of the N(5) π^* orbitals, since N(5) may act as a π acid. If this π charge transfer is sufficiently strong, the π^* orbital at N(5) can become electropositive with respect to N(10), effectively reversing the direction of the short-axis dipole and destabilizing the antibonding state. This reversal of sign of the short-axis component of the perturbing transition dipole will result in a reversal of the sign of the electric dipole matrix element on this short axis. This in turn reverses the sign of the MCD B term of the ^1L_a state (cf. eq 1). Furthermore, the destabilization of the perturbing state increases the value of $\Delta E_{j,k}$ and is the most likely cause of the two- to threefold decrease in molar ellipticity of the observed MCD in the enzyme. This argument is in qualitative agreement with the substituent effects deduced by Steiner & Michl (1978) and experimental observations with lipoamide dehydrogenase and glutathione reductase vs. glucose oxidase.

The appearance of an A term at about 18 200 cm^{-1} in all reduced enzyme species is of particular interest, since it is absent in the reduced free flavin. It is well established that both lipoamide dehydrogenase and glutathione reductase contain active center disulfides, which are reduced in addition to FAD in the fully reduced EH_4 form of the enzyme (Williams, 1976). Furthermore, both show similar EH_2 spectra. The initial assumption that the red EH_2 form of lipoamide dehydrogenase was flavin semiquinone in nature (Massey, 1963) has been abandoned in favor of structures which place two electrons on a nascent thiolate (Williams, 1976). In the extreme, a full thiolate anion has been suggested, the red color of EH_2 then arising from charge transfer with oxidized flavin as acceptor. The absence of any new bands attributable to C terms in the MCD of all EH_2 forms rules out any unpairing of electron density in the ground state of this species (Holmquist & Vallee, 1978). In addition, an A term centered under the 18 200-cm^{-1} shoulder of the absorption spectrum indicates unpairing of electron density in the excited state and is strongly supportive of triplet character in a charge-transfer complex. The singlet transition associated with this charge transfer gives

rise to a shoulder to the low-energy side of band I, but is devoid of magnetic field induced optical activity. The remaining features of the oxidized flavin spectra remain somewhat blue shifted.

That the *A* term remains at full intensity, when FAD is also reduced, in EH_4 produced by dithionite is at first puzzling. Reduced flavins are charge-transfer donors (Slifkin, 1971), and the degree of charge transfer would be expected to decrease upon reduction of the flavin. Furthermore, the *A* term remains at 18 180 cm^{-1}. The energy of a charge-transfer transition is dependent upon the electron affinity of the acceptor, which must clearly decrease when the flavin is reduced. The possibility that the thiolate anion is stabilized by charge-transfer complexation with a protein residue has not been previously considered, although, recently, stabilization of an EH_2 thiolate by an active center base has been discussed for lipoamide dehydrogenase from *Escherichia coli* (Wilkinson & Williams, 1979). A base at the active center has been proposed to be involved in the catalytic mechanism of the pig heart lipoamide dehydrogenase reaction. We have recently found evidence for such a residue to be an imidazole (Templeton & Tsai, 1979). An anionic dye, rose bengal, sensitizes histidine residues of lipoamide dehydrogenase to photooxidation. The photooxidized enzyme loses its dehydrogenase activity and the *A* term in the MCD spectrum of its reduced form. Therefore, the histidine residue must act to accept or stabilize the thiolate charge transfer.

Glutathione reductase is also shown to have a thiolate EH_2 form by MCD. In both instances the disulfide is viewed as being positioned in close proximity to C(4a) and therefore N(5) of the flavin ring, and this has been confirmed by X-ray structure for glutathione reductase (Schulz et al., 1978). Subsequent studies (Boggaram & Mannervik, 1978; Untucht-Grau et al., 1979) indicate the presence of a histidine residue (His-450) near the proximal nascent thiol of glutathione reductase. It has been proposed by those authors that a thiolate anion in the catalytic process is stabilized by the imidazolium cation. Thus, the stabilization of the thiolate charge-transfer complex by the active-site imidazole in lipoamide dehydrogenase is a possibility.

Summary

Resolution and strength of the molar ellipticity associated with the vibronic components of the flavin visible absorptions make MCD a sensitive probe of flavin environment. In addition, the sign of the MCD associated with the ^1L_b transition is sensitive to solvent effects.

The ^1L_a transition is sensitive to substituent effects at N(5), and the sign of the MCD associated with this transition may be used as an indicator of interactions at this site in the protein environment. This is exemplified with the flavoproteins lipoamide dehydrogenase and glutathione reductase, which both contain a disulfide in close proximity to N(5) vs. glucose oxidase.

On the basis of the MCD evidence, a structure for the half-reduced form of lipoamide dehydrogenase is suggested, which involves a thiolate anion charge-transfer complex stabilized by an active center imidazolium cation.

References

Boggaram, V., & Mannervik, B. (1978) *Biochem. Biophys. Res. Commun. 83*, 558–564.

Brady, A. H., & Beychok, S. (1969) *J. Biol. Chem. 244*, 4634–4637.

Brady, A. H., & Beychok, S. (1971) *J. Biol. Chem. 246*, 5498–5503.

Edmondson, D. E., & Tollin, G. (1971) *Biochemistry 10*, 113–124.

Fox, J. L., Laberge, S. P., Nishimoto, K., & Forster, L. S. (1967) *Biochim. Biophys. Acta 136*, 544–555.

Harbury, H. A., LaNoue, K. F., Loach, P. A., & Amick, R. M. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 1708–1717.

Hollebone, B. R., & Stillman, M. J. (1978) *J. Chem. Soc., Faraday Trans. 2 74*, 2107–2127.

Holmquist, B., & Vallee, B. L. (1978) *Methods Enzymol. 49G*, 149–179.

Holmquist, B., Kaden, T. A., & Vallee, B. L. (1975) *Biochemistry 14*, 1454–1461.

Huang, G. C. L., & Brady, A. H. (1973) *Biochemistry 12*, 1093–1099.

Kosicki, G. W. (1968) *Biochemistry 7*, 4299–4303.

Kurtin, W. E., & Song, P.-S. (1968) *Photochem. Photobiol. 7*, 263–273.

Langford, C. H., Hollebone, B. R., & Vandernoot, T. (1980) in *Interfacial Photoprocesses* (Wrighton, M. S., Ed.) p 144, American Chemical Society, Washington, DC.

Massey, V. (1963) *Enzymes, 3rd Ed. 7*, 275–306.

Massey, V., & Veeger, C. (1961) *Biochim. Biophys. Acta 48*, 33–47.

Massey, V., & Ghisla, S. (1974) *Ann. N.Y. Acad. Sci. 227*, 446–465.

Massey, V., Gibson, Q. H., & Veeger, C. (1960) *Biochem. J. 77*, 341–351.

Matthews, R. G., & Williams, C. H., Jr. (1974) *Biochim. Biophys. Acta 370*, 39–48.

Matthews, R. G., & Williams, C. H., Jr. (1976) *J. Biol. Chem. 251*, 3956–3964.

Matthews, R. G., Ballou, D. P., Thorpe, C., & Williams, C. H., Jr. (1977) *J. Biol. Chem. 252*, 3199–3207.

Michl, J. (1978a) *J. Am. Chem. Soc. 100*, 6801–6811.

Michl, J. (1978b) *J. Am. Chem. Soc. 100*, 6812–6818.

Michl, J. (1978c) *J. Am. Chem. Soc. 100*, 6819–6824.

Ogasahara, K., Koike, K., Hamada, M., & Hiraoka, T. (1976) *J. Biochem. (Tokyo) 79*, 967–975.

Palmer, G., & Massey, V. (1968) in *Biological Oxidations* (Singer, T. P., Ed.) pp 263–300, Interscience, New York.

Penzer, G. R., Radda, G. K., Taylor, J. A., & Taylor, M. B. (1970) *Vitam. Horm. (N.Y.) 28*, 441–466.

Reed, L. J., Koike, M., Levitch, M. E., & Leach, F. R. (1958) *J. Biol. Chem. 232*, 143–158.

Schatz, P. N., & McCaffery, A. J. (1970) *Q. Rev. Chem. Soc. 23*, 552–584.

Schulz, G. E., Schirmer, G. H., Sachsenheimer, W., & Pai, E. F. (1978) *Nature (London) 273*, 120–124.

Searls, R. L., Peters, J. M., & Sanadi, D. R. (1961) *J. Biol. Chem. 236*, 2317–2322.

Slifkin, M. A. (1971) *Charge Transfer Interactions of Biomolecules*, Chapter 7, Academic Press, London.

Song, P.-S. (1968) *J. Phys. Chem. 72*, 536–542.

Song, P.-S. (1971) in *Flavins and Flavoproteins* (Kamin, H., Ed.) pp 37–61, University Park Press, Baltimore, MD.

Steiner, R. P., & Michl, J. (1978) *J. Am. Chem. Soc. 100*, 6861–6867.

Sun, M., Moore, T. A., & Song, P.-S. (1972) *J. Am. Chem. Soc. 94*, 1730–1740.

Templeton, D. M., & Tsai, C. S. (1979) *Biochem. Biophys. Res. Commun. 90*, 1085–1090.

Thorpe, C., & Williams, C. H., Jr. (1976) *J. Biol. Chem. 251*, 7726–7728.

Tollin, G. (1968) *Biochemistry 7*, 1720–1727.

Tsai, C. S. (1973) *Arch. Biochem. Biophys. 159*, 453–457.

Untucht-Grau, R., Schulz, G. E., & Schirmer, R. H. (1979) *FEBS Lett. 105*, 244–248.
van Muiswinkel-Voetberg, H., & Veeger, C. (1973) *Eur. J. Biochem. 33*, 271–278.
Visser, J., Voetberg, H., & Veeger, C. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., Ed.) pp 359–373, Springer-Verlag, West Berlin.
Visser, A. J. W. G., Grande, H. J., Muller, F., & Veeger, C. (1974) *Eur. J. Biochem. 45*, 99–107.
Weimar, W. R., & Neims, A. H. (1975) in *Riboflavin* (Rivlin, R. S., Ed.) pp 1–47, Plenum Press, New York.
Wilkinson, K. D., & Williams, C. H., Jr. (1979) *J. Biol. Chem. 254*, 852–862.
Williams, C. H., Jr. (1976) *Enzymes, 3rd Ed. 8c*, 90–174.
Williams, C. H., Jr., & Arscott, L. D. (1972) *Z. Naturforsch. B 27*, 1078–1080.
Williams, C. H., Jr., Zanetti, G., Arscott, L. D., & McAllister, J. K. (1967) *J. Biol. Chem. 242*, 5226–5231.

Small Circular Deoxyribonucleic Acid of *Drosophila Melanogaster*: Homologous Transcripts in the Nucleus and Cytoplasm†

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ABSTRACT: We have recently characterized small circular DNA of *Drosophila* cultured cells in terms of its average size, sequence complexity, and homology to intermediate repetitive DNA. We show here that transcripts homologous to small circular DNA are present in various RNA fractions. Nuclear poly(A+), nuclear poly(A−), and polysomal poly(A+) RNA drive 10, 7, and 20%, respectively, of in vitro labeled small circular DNA tracer into hybrid. Sequences complementary to small circular DNA are at least 10-fold more concentrated in nuclear poly(A+) RNA than in nuclear poly(A−) or polysomal poly(A+) RNA. We do not detect significant homology between poly(A−) cytoplasmic RNA and small circular DNA. Assuming that only the least complex component of small circular DNA is driven into hybrid and that transcription is asymmetric, we use the results obtained here and previously published data to calculate the sequence complexity and relative concentration of nuclear poly(A+), nuclear poly(A−), and polysomal poly(A+) RNA homologous to small circular DNA.

Heterogeneous small circular DNA is an intriguing class of molecules for which a number of genetic regulatory functions has been proposed. This type of DNA has been described in a wide variety of eukaryotic organisms, including *Neurospora*, *Euglena*, trypanosomes, yeast, tobacco, *Xenopus*, chickens, and boar, and in cell culture lines from monkey, mouse, and humans (Agsteribbe et al., 1972; Nass & Ben-Shaul, 1972; Ono et al., 1971; Billheimer & Avers, 1969; Wong & Wildman, 1972; Delap & Rush, 1970; Buongiorno-Nardelli et al., 1976; Hotta & Bassel, 1965; Smith & Vinograd, 1972; Delap et al., 1978).

We have previously described a number of properties of small circular DNA from cultured cells of *Drosophila melanogaster* (Stanfield & Helinski, 1976; Stanfield & Lengyel, 1979). This DNA, isolated as covalently closed circular molecules, is heterogeneous in contour length, with an average size of 3300 NTP.[1] It is localized predominantly, if not entirely, in the nucleus and has a buoyant density indistinguishable from that of the main band of nuclear DNA. The sequence complexity of the major component (82%) of the small circular DNA is 1.8×10^4 NTP. A majority (possibly all) of the small circular DNA is homologous to middle repetitive sequences of *Drosophila* chromosomal DNA. Thermal denaturation studies of hybridized small circular and chromosomal DNA demonstrate that there is ~2% mismatch between small circular DNA and its homologous chromosomal sequences.

In order to define the role(s) of small circular DNA within the cell, it is necessary to determine if small circular DNA, or genomic sequences homologous to it, is transcribed into RNA. Our studies described here demonstrate that transcripts homologous to small circular DNA are present in both nucleus and cytoplasm and that these transcripts are more concentrated in nuclear poly(A+) than in nuclear poly(A−) or polysomal poly(A+) RNA.

Experimental Procedures

Cell Fractionation and RNA Purification. Drosophila Schneider line 2 cells (Schneider, 1972) were collected and lysed, and the cytoplasmic fraction was prepared as described by Levis & Penman (1977), with the substitution of Mg^{2+} for Ca^{2+} in the wash and lysis buffers. Nuclei were freed of contaminating cytoplasm by sedimentation through mixed-detergent sucrose (0.64 M sucrose, 1% Tween-40, 0.5% sodium deoxycholate, and 0.25% diethyl pyrocarbonate, in 0.85 × lysis buffer) and then digested with DNase and ethanol precipitated (Levis & Penman, 1977).

Polysomal RNA was prepared by EDTA release from polysomes, a technique designed to minimize contamination of mRNA by hnRNA (Galau et al., 1974). Cytoplasm was prepared as described above, with the exceptions that emetine (25 μg/mL) was added to the cells a few seconds before

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[1] Abbreviations used: NTP, nucleotide pairs; NT, nucleotides; NaDodSO$_4$, sodium dodecyl sulfate.

0006-2960/80/0419-3873$01.00/0