Synthesis and Properties of 8-CN-flavin Nucleotide Analogs and Studies with Flavoproteins*

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A high potential analog of riboflavin with a cyano function at the 8-position was synthesized by employing novel reaction conditions, starting from 8-amino-riboflavin. This was converted to the FAD level with FAD synthetase. The reduced 8-CN-riboflavin, unlike normal reduced flavin, has a distinctive absorption spectrum with two distinctive peaks in the near ultraviolet region. The oxidation-reduction potential of the new flavin was determined to be -50 mV, ~160 mV more positive than that of normal riboflavin. The 8-CN-riboflavin and 8-CN-FMN were found to be photoreactive and need to be protected from exposure to light. However such complications were not encountered with protein-bound flavins. The apoproteins of flavodoxin and Old Yellow Enzyme (OYE) were reconstituted with the 8-CN-FMN and apoDAAO was reconstituted with 8-CN-FAD. Spectral properties of the enzyme-bound neutral and anionic semiquinones were determined from these reconstituted proteins. In the case of 8-CN-FMN-OYE I, it was shown that the comproportionation reaction of a mixture of reduced and oxidized enzyme bound flavin is very rapid, compared with the same reaction with native protein, resulting in 100% thermodynamically stable anionic semiquinone. In the case of 8-CN-OYE I, it was shown that the rate of reduction of the enzyme bound flavin by NADPH is ~40 times faster, and the rate of reoxidation of reduced enzyme bound flavin by oxygen is an order of magnitude slower than with the normal FMN enzyme. This is in accord with the high oxidation-reduction potential of the flavin, which thermodynamically stabilizes the reduced enzyme.

A wide variety of redox transformations in biological systems are catalyzed by flavoenzymes. The oxidation-reduction potential of the flavin and the transfer of electrons by the semiunique or the fully reduced forms to the acceptor are among the most significant features of the chemistry of flavoprotein catalysis, which is in general controlled by the potential of the flavin. Hence it is possible to regulate and manipulate electron flow during catalysis by altering the redox potential of the flavin. Various structurally modified flavins with altered redox potentials have been synthesized previously and employed as mechanistic probes (1–4). While deazaflavin derivatives best represent the low potential probes, flavin analogs with various potentials have been synthesized previously and employed as mechanistic probes (1–4). 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‡ The abbreviations used are: OYE, Old Yellow Enzyme; HPLC, high performance liquid chromatography.
FAD with apoprotein in the presence of benzoate (23). Reconstitution of the apoproteins with 8-CN-flavins was accomplished by mixing 1.5-fold excess of the flavin with apoprotein and-incubating on ice for 3 h. Excess flavin was removed by a Centricon-30 microconcentrator (Amicon).

**Synthesis of 8-CN-riboflavin**—10 mg of 8-amino-riboflavin was suspended in 3 ml of water in a test tube. To this suspension, 6 μl HCl was added until a clear solution was obtained. This solution was cooled to 0 °C on ice, and three aliquots each of 40 μl of saturated sodium nitrite solution were added with continuous shaking of the test tube. After 5 min, 300 μl of saturated urea solution was added to destroy the excess sodium nitrite. The cold diazo salt solution was then added with a glass transfer pipette to a 10-ml saturated solution of NaCN + CuCN (70:30) in a 50-ml glass beaker with vigorous stirring at room temperature. After 20 min, the reaction mixture was loaded on a 20-cc C-18 Sep-Pak (Millipore, Milford, CT) cartridge. The cartridge was prewashed thoroughly with excess water, methanol, and again with water before loading the reaction mixture. The Sep-Pak cartridge was eluted with water followed by 5% acetonitrile to remove salts and a red band of unknown structure. Elution with 15% acetonitrile gave the 8-CN-riboflavin and with 20% acetonitrile gave 8-chlororiboflavin. Evaporation of the flavin solutions with a Speed Vac concentrator gave 6 mg of the 8-CN-riboflavin as a yellow powder.

**Conversion of 8-CN-riboflavin to FAD and FMN—**8-CN-riboflavin was converted to the FAD level with partially purified FAD synthetase from B. ammoniagenes by incubating the flavin in 0.002 μl potassium Pi, pH 7.5 at 25 °C, following the procedure of Spencer et al. (14). After 14 h, HPLC analysis of the incubation mixture showed 100% conversion to the FAD form. The reaction mixture was loaded on to the prewashed cartridge (as described in the case of 8-CN-riboflavin) 20-cc C-18 Sep-Pak cartridge and eluted with 100 ml of water to wash off salts and breakdown products from ATP. Elution with 5% acetonitrile in water gave pure flavin, which was concentrated on a Speed Vac concentrator to obtain 8-CN-FAD as a yellow powder. 8-CN-FMN was obtained by hydrolysis of the FAD in 0.05 μl potassium Pi, pH 7, with snake venom phosphodiesterase (Naja naja venom).

**Cyanylation of 8-NH2-FAD**—To 500 μl of 8-NH2-FAD, 100 μl of 6 N HCl was added at ice temperature. Then two 20-μl aliquots of saturated NaNO2 were added with mixing. After 2 min, 100 μl of saturated urea solution was added. When the yellow was saturated with 500 μl of a saturated solution of 70:30 NaCN + CuCN solution was added into the d razo-FAD solution at room temperature. After 3–5 min, the solution was washed through a prewashed small Sep-Pak cartridge, and the salts were washed off with water. The flavin was then eluted with 5% acetonitrile. HPLC analysis of the 5% acetonitrile fraction showed both 8-CN-FAD (80%) and 8-CN-FMN (20%) when eluted with 80.01 μl potassium Pi, pH 6, and 20% methanol on a reverse phase C-18 column. Interestingly, no formation of 8-chloro-FAD or 8-chloro-FMN was observed.

**Enzyme Assays**—Old Yellow Enzyme activities were measured in 0.1 μ phosphate, pH 7.0 at 25 °C, in a stopped flow spectrophotometer (Kinetic Instruments, Ann Arbor, MI) either under anaerobic conditions (when cyclohexenone was employed as electron acceptor) or under controlled oxygen concentrations when oxygen was electron acceptor, monitoring both the consumption of NADPH at 340 nm and in the same experiments the level of flavin oxidation/reduction at 470 nm. The concentrations of both NADPH and the acceptor (cyclohexenone or O2) were varied systematically to determine true kcat and Km values.

**RESULTS AND DISCUSSION**

Attempts to convert the 8-diace-50 salts of flavins to 8-CN-flavins by the Sandmeyer reaction were unsuccessful. The choice of the cyanylating reagent has a critical effect on the course of the reaction (24, 25). For the 8-diazo-flavins, in our hands, either sodium cyanide or cuprous cyanide alone were ineffective. Lately several copper-cyano complexes of the kind Na2[Cu(CN)4]2−, K2(Cu(CN)4)2−, K2(Cu(CN)2NH2)2− were shown to have large advantages in cyanolation reactions affording high yields of nitriles (26). Before trying these complexes for the present cyanylation, we tried treating the diazoflavin with a saturated solution of an approximately 3:1 mixture NaCN + CuCN and found 8-CN-riboflavin in about 60% yield (Scheme 1). The 8-chlororiboflavin was obtained as a side product in about 20% yield, presumably because of the CuCl formed from CuCN and HCl. It was found that the ratio between NaCN and CuCN is crucial for both the reaction to occur as well as to obtain isolatable yields of the cyano flavin. When the CuCN ratio was increased, an insoluble solid was obtained with no isolation of any flavin.

The λmax of the UV-visible spectrum for 8-CN-riboflavin is slightly shifted from 445 nm for the normal flavin spectrum to 456 nm. The near-UV peak is shifted from 375 nm for the normal flavin to 338 nm for 8-CN-riboflavin (Fig. 1A). The cyanoflavins is fluorescent with an emission maximum at 530 nm and excitation maxima at 338 and 456 nm, identical with the absorption spectrum. Further characterization of the new flavin was made by recording the positive ion fast atom bombardment mass spectrum which showed M+1 at 388 (Fig. 1B) (molecular weight of the 8-CN-riboflavin is 387) and also by the 1H NMR spectrum (Fig. 1C). In the proton NMR spectrum, the electron-deficient nature of the flavin is well evident from the fact that the C6 and C9 protons are down-shifted and well separated due to the CN substitution in the benzene ring. The aromatic protons are at 8.0 and 8.4 ppm compared with 7.9 and 8.0 ppm in the normal flavin. The lone methyl group on the C7 is seen as a singlet at 2.6 ppm, down-shifted from 2.3 ppm in normal flavin. The rest of protons from the ribityl side chain are recorded between 3.6 and 4.8 ppm.

The 8-CN-riboflavin was converted to the FAD level by reacting with FAD synthetase from B. ammoniagenes in 100% yield without any complications. This again reaffirms the fact that the small cyano substitution meets the steric requirements of the 8-position of the flavin. The 8-CN-FMN was obtained by hydrolysis of the HPLC-pure 8-CN-FAD with the phosphodiesterase from snake venom. This reaction is accompanied by an increase in extinction for the FMM at 456 nm of 8.8% and with a 10.25-fold increase in fluorescence. The cyanylation reaction also worked with the 8-NH2-FAD, except that about 20% of the FAD got hydrolyzed to 8-CN-FMN because of the drastic acidic conditions of the diazotization reaction.

**Reduction of 8-CN-riboflavin**

It was found that the reduction of 8-CN-riboflavin to its dihydro form can be accomplished with several reducing agents such as dithiothreitol, dithionite, NaBH4, photochemically with EDTA as photodonor, NADPH, and NADH. The reduction is fully reversible with oxygen. However the spectrum for reduced 8-CN-riboflavin is different from that of normal flavin, as reported previously for 8-CN-7-nor-3-methyl-lumiflavin by Bruce et al. (13). Reduced 8-CN-riboflavin has two well defined peaks above the 300 nm region with maxima at 312 and 372 nm at pH 7 and at 316 and 362 nm under acidic conditions(Fig. 1A).

The pK of Reduced Cyanoriboflavin

The pK2 value of the reduced cyanoflavin was determined to be at pH 5.6 from the change in absorption spectrum with pH (Fig. 1A, inset). Hence the electronegative 8-CN-group de-
creases the pKₐ of the reduced flavin from the usual value (27) of −6.7 to 5.6 in accord with the electron-deficient nature of the flavin. The spectra of the neutral and anionic reduced forms are shown in Fig. 1A.

**Determination of Reduction-Oxidation Potential**

The reduction-oxidation potential for 8-CN-FAD was measured by using the xanthine/xanthine oxidase system and indigo...
tetrasulfonate as the reference dye (28). Reduction of the 8-CN-FAD with the xanthine/xanthine oxidase system proceeded with isosbestic points at 352 and 406 nm and the reduction of the dye with isosbestic points at 338 and 502 nm. These wavelengths were used to monitor the reduction of the components in a mixture of the two. A plot of log(ox/red) of the dye against log(ox/red) of the flavin gave a midpoint potential for 8-CN-FAD of \(-50\) mV, which is 158 mV more positive than that of normal flavin. This is in accord with the strongly electron withdrawing nature of the CN substituent, making it a more electron deficient system, shifting the potential to more positive values.

**Reaction with Sulfite**

Native flavin is known to form an N-5 adduct with sulfite, but the reaction is only half completed at saturating sulfite concentrations because of the high dissociation constant (~2.5 M) (29). The absorption spectrum of the flavin adduct is similar to that of reduced flavin with a new peak maximal at 320 nm. With a series of artificial flavins a good correlation was found to exist between the dissociation constant of the flavin-sulfite adduct and the two-electron redox potential of the flavin (29). The straight line obtained by plotting the logarithm of the dissociation constants of the complexes and the redox potentials of the flavins shows that the more positive the potential, the tighter the sulfite binding. It is reasonable that flavins with more positive potentials are the most electron deficient and prone for the addition of two electrons through adduct formation. As anticipated, the cyanoflavin formed a tight complex prone for the addition of two electrons through adduct formation (29), the oxidation-reduction potential at pH 7 would be predicted to be \(-50\) mV, in perfect agreement with direct measurement.

**Hydrolysis of 8-CN-riboflavin**

The hydrolysis of 8-CN-riboflavin in 0.5 M carbonate, pH 10.25, was studied. The spectral changes that were observed are similar to those of the cyanoisoalloxazine observed by Bruce et al. (13) and compatible with formation of a spiroydantoin (30). As is the case with the cyanoisoalloxazine derivative, repetitive spectral scans during the hydrolysis failed to provide any evidence for the 10a- or 4a-hydroxyl adducts, which were considered as possible intermediate species during hydrolysis.

**Photostability of 8-CN-flavin Derivatives**

It is well known that flavins are photoreactive and their spectroscopic and photochemical properties have been the subject of intense studies (31). It was found that 8-CN-riboflavin is highly photoreactive. When a sample of the flavin was left exposed to room light, it was transformed over a period of a few days into a stable product with absorbance maxima at 320 and 388 nm. A similar reaction was also observed with the 8-CN-FMN derivative. However when 8-CN-FAD was exposed under the same conditions, it was found to be extremely stable toward photoreaction. Only about 5% of the reaction was observed in the same time scale where 8-CN-riboflavin and 8-CN-FMN reacted completely. It is known that FAD forms an intramolecular complex between the adenine moiety and the isoalloxazine ring (32). The increased stability of FAD toward photolysis is attributed to this intramolecular stacking (33). The photoreaction involves the ribityl side chain, since 8-CN-lumiflavin is completely stable under the same conditions. The nature of the product and the chemistry of the photoreaction is under investigation and will be reported separately.

**Riboflavin-binding Protein**

Although this protein has no known catalytic activity, binding experiments of artificial flavins with this protein are useful in interpreting the structural characteristics of the flavin. Riboflavin binds to aporBP with a dissociation constant of 1.3 mM and shows considerable spectral shifts and resolution on binding (17). 8-CN-riboflavin showed similar spectral changes to those of normal flavin upon binding to aporBP. The flavin absorption peaks are red shifted from 340 and 454 nm to 344 and 470 nm with a decrease in the extinction for both peaks. The binding of 8-CN-riboflavin to aporBP resulted in the complete quenching of the fluorescence. The dissociation constant was calculated to be \(-0.27\) \(\mu M\) and by standardization of aporprotein with native riboflavin, the extinction coefficient of the 8-CN-riboflavin was determined as 10,400 M\(^{-1}\) cm\(^{-1}\). The 5-deazaflavin-catalyzed photoreduction (34) of 8-CN-riboflavin-RBP proceeded to the fully reduced form without stabilizing any semiquinone. The spectrum for the reduced protein-bound flavin is almost identical to that of the free reduced flavin except that the \(\lambda_{max}\) is slightly red shifted by 4 nm to 376 nm. The initial oxidized flavin spectrum is regained rapidly on admission of air.

**8-CN-FMN-Flavodoxin**

8-CN-FMN binds to apoflavodoxin with quenching of the fluorescence and with a dissociation constant of \(-0.45\) \(\mu M\). The binding of the flavin to the apoprotein results in 14% decrease in the extinction and the maxima of the absorption spectrum shift from 340 and 452 nm for the free flavin to 342 and 460 nm (\(\epsilon_{440} = 9.4\) m\(^2\) M\(^{-1}\) cm\(^{-1}\)) for the bound flavin. The extinction coefficient for 8-CN-FMN was determined as 11,000 M\(^{-1}\) cm\(^{-1}\) by standardizing the apoprotein with pure native FMN. Reduction of native flavodoxin by EDTA/light or with the xanthine/xanthine oxidase system proceeds to the fully reduced protein through the formation of neutral semiquinone (35). The same reduction process occurs with all flavodoxins substituted with artificial flavins, including 8-CN-FMN flavodoxin (Fig. 3). The blue neutral semiquinone shows maxima at 598 and 644 nm. Previous studies with native flavodoxin showed essentially
The coefficient of the cyano-FMN was determined as 11,000 M$^{-1}$ cm$^{-1}$.

Inset FMN-flavodoxin indicates neutral semiquinone; the solid line indicates the oxidized form of 8-CN-FMN-flavodoxin; the broken line to ensure rapid equilibration of reducing equivalents. The solid line $E$.

There is no adduct formation with sulfite in accordance with is retained with a shifted absorption band at 485 nm (Fig. 3).

Interestingly, 25% of the absorption at the 460 nm region 370 nm in the free flavin showed a bathochromic shift to 388 nm. Characteristic spectral shifts of the flavin spectrum found with the native FMN. The reduced flavoprotein showed two well resolved peaks in the absorption spectrum similar to the unbound reduced flavin. However the peak in the near-UV region shifted further to the lower wavelength region and the band at 370 nm in the free flavin showed a bathochromic shift to 388 nm. Interestingly, 25% of the absorption at the 460 nm region is retained with a shifted absorption band at 485 nm (Fig. 3).

There is no adduct formation with sulfite in accordance with studies of the native protein.

**Old Yellow Enzyme**

Old Yellow Enzyme is the first discovered flavoprotein and was isolated from brewers' bottom yeast. It is a mixture of homodimers and a heterodimer with one FMN per subunit, products of separate genes (38, 39). As the physiological role of this protein is yet to be determined, structural and chemical reactivity studies in the direction of elucidating its function are recommended. As the physiological role of this protein is yet to be determined, studies of the native protein (37).

**Reduction of 8-CN-FMN-OYE**—The reduction of 8-CN-FMN enzyme with the xanthine/xanthine oxidase system with benzylviologen as mediator was carried out at 25 °C in 100 mM potassium Pi, pH 7 (Fig. 5). It was found to proceed through the formation of the anionic semiquinone to the two-electron reduced form. The plot of $A_{270}$ versus $A_{410}$ suggest that ~98% of the semiquinone radical species was formed (Fig. 5, inset). The reduced 8-CN-FMN enzyme showed a peak at 360 nm, a blue shift of 10 nm from that of the free cyanoflavin. Photoreduction of the 8-CN-FMN enzyme in the presence of EDTA resulted in ~100% anionic semiquinone without any further reduction to the fully reduced form.

**Stabilization of Semiquinone**—With normal flavin semiquinone can be obtained either by one-electron reduction of the oxidized form or by one-electron oxidation of the reduced flavin. But in solution only about 2% of the total flavin is stabilized as
semiquinone at pH 7 (42). This is because of the fact that in the following equation, the equilibrium always favors the disproportionation (right to left) of the semiquinone.

\[
\text{Fl}_{\text{red}} + \text{Fl}_{\text{ox}} = 2 \text{Fl}^* \quad \text{(Eq. 1)}
\]

However, several apoproteins upon binding the flavin stabilize larger amounts of the semiquinone form through electrostatic interactions between the flavin and the protein (43). When native OYE I was reduced with ~0.5 equivalents of NADPH, a mixture of reduced and oxidized forms of the enzyme was obtained. This mixture underwent a slow comproportionation reaction over a period of 4 h to form ~60–65% stable anionic semiquinone, establishing the thermodynamic stabilization of the semiquinone by the protein. When this reaction was carried out with 8-CN-FMN enzyme under identical conditions, it resulted in ~100% formation of stable anionic semiquinone. The reaction was found to be very fast when compared with the native enzyme, being complete within a few minutes, with a rate constant of \(3 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) for this comproportionation reaction. These results show that the potential for the \(\text{EF}_{\text{FlSQ}}/\text{EF}_{\text{FlRed}}\) couple is much higher than that of the \(\text{EF}_{\text{FlSQ}}/\text{EF}_{\text{FlRed}}\) couple. The potential of the \(\text{EF}_{\text{FlSQ}}/\text{EF}_{\text{FlRed}}\) couple for 8CN-FMN OYE I was determined by equilibration \(\text{versus}\) toluidine blue using the xanthine/xanthine oxidase reduction method (28) and found to be 125 ± 3 mV. The potential of the second electron reduction, \(\text{EF}_{\text{FlRed}}/\text{EF}_{\text{FlRed}}\), was determined similarly by equilibration \(\text{versus}\) phenosafranin to be ~205 ± 13 mV. These values define the two electron potential of the 8-CN-FMN enzyme to be ~40 ± 18 mV, similar to that of the free flavin. A linear relationship is expected for the energy of the long wavelength transition of the charge transfer complex between oxidized flavin and phenolate anion and the first 1-electron reduction potential of the flavin (43). As can be seen in Fig. 6, the 115 nm red shift of the transition with \(p\)-chlorophenol fits well with the previously determined values with other artificial flavins and that with the 8-CN-FMN enzyme.

**Steady State Turnover of 8-CN-FMN-OYE with \(\beta\)-NADPH and Oxygen—Enzyme-monitored turnover experiments were carried out by using a stopped flow spectrophotometer. A known concentration of enzyme (6.2 \(\mu\)M) in 100 mM potassium Pi, pH 7, was reacted with a range of concentrations of NADPH in the same buffer, equilibrated with different concentrations of oxygen at 25 °C. The concentrations of oxygen used were 189, 256, 433, and 743 \(\mu\)M. The reaction was followed at both 470 nm, from approach to steady state until final reoxidation by excess oxygen, and also at 340 nm (Fig. 7).

The rate constant for the reductive-half reaction was obtained by following the initial reduction of the enzyme bound flavin at 470 nm. A rate constant of 245 s\(^{-1}\) was obtained from a double-reciprocal plot of \(k_\text{obs}\) \(\text{versus}\) NADPH concentration (results not shown). The \(k_\text{obs}\) values for this phase were found to be independent of the oxygen concentration used, indicating that \(k_\text{obs} \approx k_\text{red}\). The value of \(k_\text{red}\) for native OYE I by NADPH was determined as 5.9 s\(^{-1}\). Thus the reduction of 8-CN-FMN-OYE I by NADPH occurs about 40 times faster than with the recombinant enzyme. This is consistent with the high oxidation-reduction potential of the flavin, although the rate enhancement is smaller than might be expected from the large increase in thermodynamic driving force associated with the ~160 mV increase in redox potential. The electron-withdrawing nature of the cyano substituent might be expected to destabilize the oxidized isosalloxazine and stabilize the reduced (anionic) flavin, leading to an earlier transition state with less development of negative charge and a correspondingly relatively small effect on the reduction rate constant.

The rate constant for the oxidation of reduced enzyme by oxygen was estimated from the final part of turnover traces such as shown in Fig. 7, which show that flavin reoxidation occurs only on complete oxidation of NADPH. The final phase of these traces fit perfectly to a single exponential process. The values of \(k_\text{obs}\) obtained from such analyses were independent of the initial NADPH concentration and were directly proportional to the oxygen concentration, yielding a second order rate constant of \(3.8 \times 10^8 \text{ M}^{-1} \text{s}^{-1}\). Thus the reduction of oxygen is an order of a magnitude slower than with normal enzyme, in general accord with the high redox potential of the CN-flavin. From the model studies of Bruce and colleagues (44) the primary step in the reaction of reduced flavin with \(O_2\) is a one-electron oxidation to form the neutral flavin semiquinone and \(O_2^-\). Although we have measured the midpoint potential of the 8-CN-FMN enzyme oxidized/semiquinone couple, this is not the relevant potential for prediction of the thermodynamic driving force in the reaction with \(O_2\), since

\[P_e, \text{pH 7}, \text{was reacted with a range of concentrations of NADPH} \]

\[\text{in the same buffer, equilibrated with different concentrations} \]

\[\text{of oxygen at 25 °C. The concentrations of oxygen used were} \]

\[189, 256, 433, \text{and} 743 \mu M. \text{The reaction was followed at both} \]

\[470 \text{nm, from approach to steady state until final reoxidation by} \]

\[\text{excess oxygen, and also at} 340 \text{nm (Fig. 7).} \]

\[\text{The rate constant for the reductive-half reaction was} \]

\[\text{obtained by following the initial reduction of the enzyme bound} \]

\[\text{flavin at 470 nm. A rate constant of} 245 \text{ s}^{-1} \text{was obtained} \]

\[\text{from a double-reciprocal plot of} k_\text{obs} \text{versus NADPH} \]

\[\text{concentration (results not shown). The} k_\text{obs} \text{values for this} \]

\[\text{phase were found to be independent of the oxygen} \]

\[\text{concentration used, indicating that} k_\text{obs} \approx k_\text{red}. \text{The} \]

\[\text{value of} k_\text{red} \text{for native OYE I by NADPH was} \]

\[\text{determined as} 5.9 \text{ s}^{-1}. \text{Thus the reduction of} 8-CN-FMN-\]

\[\text{OYE I by NADPH occurs about 40 times faster than with the} \]

\[\text{recombinant enzyme. This is consistent with the high oxidation-} \]

\[\text{reduction potential of the flavin, although the rate} \]

\[\text{enhancement is smaller than might be expected from the large} \]

\[\text{increase in thermodynamic driving force associated with the} \]

\[160 \text{ mV increase in redox potential. The electron-withdraw} \]

\[\text{ing nature of the cyano substituent might be expected to de} \]

\[\text{stabilize the oxidized isosalloxazine and stabilize the reduced} \]

\[\text{(anionic) flavin, leading to an earlier transition state with less} \]

\[\text{development of negative charge and a correspondingly relatively} \]

\[\text{small effect on the reduction rate constant.} \]

\[\text{The rate constant for the oxidation of reduced enzyme by} \]

\[\text{oxygen was estimated from the final part of turnover traces} \]

\[\text{such as shown in Fig. 7, which show that flavin reoxidation} \]

\[\text{occurs only on complete oxidation of NADPH. The final phase} \]

\[\text{of these traces fit perfectly to a single exponential process. The} \]

\[\text{values of} k_\text{obs} \text{obtained from such analyses were independent of} \]

\[\text{the initial NADPH concentration and were directly propor} \]

\[\text{tional to the oxygen concentration, yielding a second order rate} \]

\[\text{constant of} 3.8 \times 10^8 \text{ M}^{-1} \text{s}^{-1}. \text{Thus the reduction of oxygen is} \]

\[\text{an order of a magnitude slower than with normal enzyme, in general accord with the high redox} \]

\[\text{potential of the CN-flavin. From the model studies of Bruce and} \]

\[\text{colleagues (44) the primary step in the reaction of reduced flavin} \]

\[\text{with} O_2 \text{is a one-electron oxidation to form the neutral} \]

\[\text{flavin semiquinone and} O_2^-\). \text{Although we have measured the} \]

\[\text{midpoint potential of the 8-CN-FMN enzyme oxidized/semi} \]

\[\text{quione couple, this is not the relevant potential for prediction} \]

\[\text{of the thermodynamic driving force in the reaction with} O_2, \text{since} \]

\[3 \text{ B. J. Brown and V. Massey, unpublished results.} \]
8-CN-flavins and Flavoproteins

The reaction was also followed at 340 nm in the above experiments at varied concentrations of NADPH and oxygen. From the results (Fig. 7) it is clear that the disappearance of NADPH is essentially linear with time, indicating a low $K_m$ for NADPH. The plots of reciprocal of turnover numbers versus 1/[NADPH] showed parallel line kinetics, similar to the situation with native enzyme (38). However, the determination of kinetic constants was not possible, since the secondary plot of intercepts was almost directly proportional to the reciprocal of the oxygen concentration (results not shown). All of the results are thus consistent with a kinetic mechanism in which the reduction rate constant ($k_{cat}$) is high and the reaction rate constant with $O_2$ is low (45).

**Steady State Turnover of 8-CN-FMN-OYE with $\beta$-NADPH and Cyclohex-2-en-1-one**—It has been reported recently that cyclohexenone is an efficient electron acceptor wherein the carbon-carbon double bond is reduced to form cyclohexanone (39). Turnover experiments were carried out by reacting enzyme with mixtures of NADPH and cyclohexenone under anaerobic conditions in the stopped flow spectrophotometer. Oxidation of NADPH by the 8-CN-FMN reconstituted enzyme was monitored at 340 nm. It was found that the turnover numbers were independent of cyclohexenone concentration over the range of 50 $\mu$M to 1 mM. For 8-CN-FMN enzyme a turnover number of 7.5 min$^{-1}$ was obtained with a $K_m$ for NADPH of 0.2 $\mu$M, whereas the native OYE I has a turnover number$^{a}$ of $300$ min$^{-1}$. These results show that cyclohexenone is a poor electron acceptor for the 8-CN-FMN enzyme and the reoxidation of the reduced enzyme-bound cyanoflavin by cyclohexenone, unlike that of the native enzyme, is very slow, again a reflection of the high oxidation-reduction potential of the flavin. Attempts to measure directly the rate constant for oxidation of the reduced enzyme by cyclohexenone were unsuccessful, since the slowly produced oxidized enzyme reacted with remaining reduced enzyme to form anionic semiquinone (see earlier section). However, if the 8-CN-FMN enzyme functions like the normal enzyme, where it has been shown that the NADPH-cyclohexenone reductase reaction functions by a ping-pong kinetic mechanism, where $k_{cat} = k_{cat}^o k_{ox}(k_{red} + k_{ox})$, it may be concluded that $k_{cat}$ for cyclohexenone with the 8-CN-FMN enzyme = $k_{cat}^o$ i.e. 0.125 s$^{-1}$, an 870-fold decrease from the value of 109 s$^{-1}$ found for native enzyme. This large decrease in reaction rate constant compared with that with native enzyme is again consistent with the electron-withdrawing nature of the cyano substituent, which by stabilization of the anionic reduced flavin would make reduction of the olefinic bond of cyclohexenone thermodynamically less favorable. The magnitude of the decreased reactivity (≈400-fold) should be compared with the 40-fold increase in the reduction reaction rate constant with NADPH and implies a late transition state in the reaction.

**d-Amino Acid Oxidase**

The binding of 8-CN-FAD to apodAAO is accompanied by around 80% quenching of the fluorescence but by only small spectral shifts from free flavin to the bound flavin. The maxima of the free flavin shifted from 340 and 456 nm to 344 and 458 nm on binding to the apoprotein (Fig. 8). As in the case of the binding of native FAD, no vibronic resolution of peaks is observed on the binding of 8-CN-FAD. When the apodAAO was titrated with FAD in presence of benzoate, the titration plots suggest tight binding similar to that found with native FAD (23).

**Photoreduction of 8-CN-FAD-DAAO**—When the 8-CN-FAD-DAAO was irradiated with light in presence of EDTA, it formed $\sim100\%$ of anionic semiquinone (Fig. 8) with no further reduction to the two-electron reduced form on prolonged light irradiation. These results are similar to those found with native enzyme, where the anionic flavin semiquinone is also stabilized thermodynamically (35). The semiquinone has a sharp absorption maximum at 418 nm with an extinction coefficient of $20,000$ M$^{-1}$ cm$^{-1}$, somewhat lower than that found with SCN FNM OYE. However, when the photoreduction was carried out with the 8-CN-FAD enzyme-benzoate complex, the reaction resulted in essentially two-electron reduced enzyme with only $\sim5\%$ intermediate semiquinone. These results are consistent with the fact that the binding of benzoate modulates the oxidation-reduction potential of the protein-bound flavin in such a

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**Fig. 7.** Representative kinetic traces from turnover reactions of 8-CN-FMN-OYE with NADPH and oxygen. In this figure, 6.2 $\mu$m enzyme (concentration after mixing) in 0.1 M phosphate, pH 7.0, 25 °C was reacted with 55 $\mu$m NADPH and 560 $\mu$m oxygen (concentrations after mixing) in a stopped flow spectrophotometer. The reaction was monitored at 470 nm to follow changes in flavin oxidation-reduction state, and at 340 nm, to follow oxidation of NADPH. The absorbance changes at 470 nm are shown on two time scales, the overall reaction (broken line) over 70 s, and the approach to steady state (solid line) over a period of 70 ms. The absorbance trace at 340 nm (dotted line) is over a period of 70 s, and comparison with the 470 nm trace demonstrates clearly that the enzyme remains in the reduced state until all the NADPH is consumed. Reaction rate constants for reduction were obtained by single exponential fits to the traces of approach to steady state and $k_{ox}$ values for the reoxidation by similar single exponential fits to the final reoxidation phase at 470 nm. Turnover numbers were calculated from changes in absorbance at 340 nm.

**Fig. 8.** Absorption spectra for the different forms of 8-CN-FAD-DAAO. The solid line indicates the oxidized 8-CN-FAD-DAAO; the dotted line indicates the anionic semiquinone obtained by 3 min light irradiation of the oxidized form in 0.1 M sodium pyrophosphate buffer, pH 8.5; the broken line indicates the reduced enzyme obtained by the anaerobic reaction of the oxidized enzyme with 50 $\mu$m equivalents of d-alanine.
way that two-electron reduction is facilitated and making the formation of the anionic radical more difficult. It was shown that in case of the native protein, the redox potentials for the $E_{\text{FAD/FAD}_{\text{sq}}}$ and $E_{\text{FAD}_{\text{sq}}/E_{\text{FAD}}}$ couples are $-98$ and $-204$ mV for the holoenzyme and $-260$ and $-140$ mV for the benzoate complex (46). Although we have not attempted to determine the redox potentials of 8-CN-FAD DAAO, it is clear that a similar situation exists as with the native enzyme.

Reduction of 8-CN-FAD-DAAO with D-Alanine—When 8-CN-FAD-DAAO was reacted anaerobically with 50-fold excess D-alanine, the spectrum for the two-electron reduced enzyme was formed rapidly (Fig. 8). The reduced enzyme has a peak at 370 nm. On opening to air, the oxidized enzyme spectrum was regained quickly once turnover was complete.

**Benzate Binding**—Pronounced spectral changes were observed in the region of 480 and 520 nm upon binding of benzoate to the oxidized form of D-alanine, the spectrum for the two-electron reduced enzyme was formed rapidly (Fig. 8). The reduced enzyme has a peak at 370 nm. The oxidized enzyme spectrum for the holoenzyme and $E_{\text{FAD/FAD}_{\text{sq}}}$ and $E_{\text{FAD}_{\text{sq}}/E_{\text{FAD}}}$ couples are $-98$ and $-204$ mV for the holoenzyme and $-260$ and $-140$ mV for the benzoate complex (46). Although we have not attempted to determine the redox potentials of 8-CN-FAD DAAO, it is clear that a similar situation exists as with the native enzyme.

**REFERENCES**

Walsh, C., Fisher, J., Spencer, R., Graham, D. W., Ashton, W. T., Brown, J. E., Brown, R. D. & Rogers, E. F. (1978) Biochemistry 17, 1942–1951

Walsh, C. (1980) Acc. Chem. Res. 13, 148–155

Light, D. R., and Walsh, C. (1980) J. Biol. Chem. 255, 4264–4277

Giasha, S. & Massey, V. (1986) Biochem. J. 239, 1–12

Lambooy, J. P. (1971) Methods Enzymol. 18B, 437–444

Kasai, S., Sugimoto, K., Miura, R., Yamano, T., & Matsui, K. (1983) J. Biochem. (Tokyo) 93, 397–402

Moore, E. G., Giasha, S., & Massey, V. (1979) J. Biol. Chem. 254, 8173–8178

Raibekas, A. A., Ray, A. J., & Jorns, M. S. (1990) Biochemistry 32, 4420–4429

Schoepfer, L. M., Massey, V., & Claisborne, A. (1981) J. Biol. Chem. 256, 7329–7337

Moore, E. G., Cardemil, E., & Massey, V. (1978) J. Biol. Chem. 253, 6413–6422

Raibekas, A. A. & Jorns, M. S. (1994) Biochemistry 33, 12566–12564

Bruice, T. C. (1976) Biochemistry 15, 1043–1053

Berenzovski, V. M., Tulchinskyas, L. S. & Polyakova, A. N. (1965) Zh. Obshch. Khim. 35, 673–677

Giasha, S. & Mayhew, S. G. (1976) Eur. J. Biochem. 63, 373–390

Becvar, J. & Palmer, G. (1980) Biochem. J. 190, 567–567

Mayhew, S. G. & Massey, V. (1969) J. Biol. Chem. 244, 794–802

Mayhew, S. G. (1971) Biochim. Biophys. Acta 235, 289–302

Saito, K., Thiele, D. J., Davison, M., Lockridge, O., & Massey, V. (1991) J. Biol. Chem. 266, 20720–20727

Abramowitz, A. S. & Massey, V. (1976) J. Biol. Chem. 251, 5232–5236

Abramowitz, A. S. & Massey, V. (1976) J. Biol. Chem. 251, 5237–5336

Mayhew, S. G., Curti, B., & Ganther, H. (1966) J. Biol. Chem. 241, 2547–2557

Hodgson, H. H. & Heyworth, F. (1949) J. Chem. Soc. 1311.

Suzuki, N., Arum, T., Kaneo, Y., Izawa, Y. & Tomioka, H. J. (1987) J. Chem. Soc. Perkin Trans 1, 445, and references therein

Yonezawa, N., Hino, T., Namie, T. & Katakai, R. (1996) Synth. Commun. 26, 1575–1578

Bruece, T. C. (1976) Prog. Bioorg. Chem. 1, 1–84

Massey, V. (1991) in Flavins and Flavoproteins 1990 (Curti, B., Ronchi, S., and Zenatti, G., eds) pp. 59–66, Walter de Gruyter, Berlin

Müller, F. & Massey, V. (1969) J. Biol. Chem. 244, 4007–4016

Dudley, K. H. & Hamer, M. (1967) J. Org. Chem. 32, 3049–3054

Heeles, P. F. (1992) in Chemistry and Biochemistry of Flavoenzymes (Muller, F., ed) Vol. 1, pp. 171–194, CRC Press, Inc., Boca Raton, FL

Weber, G. (1950) Biochem. J. 47, 114–121

Miles, D. W. & Urry, D. W. (1965) Biochemistry 4, 2791–2799

Massey, V. & Hemmerich, P. (1977) J. Biol. Chem. 252, 5612–5614

Massey, V. & Palmer, G. (1966) Biochemistry 5, 3181–3189

Mayhew, S. G. (1971) Biochim. Biophys. Acta 257, 287–288

Mayhew, V., Muller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, G. L., Mayhew, S. G., Matthews, R. G. & Fouts, G. P. (1969) J. Biol. Chem. 244, 3989–4006

Schroepfer, L. M. & Massey, V. (1990) in A Study of Enzymes (Kuby, S.A., ed) pp. 247–283, CRC Press Inc., Boca Raton, FL

Stott, K., Saito, K., Thiele, D. J. & Massey, V. (1993) J. Biol. Chem. 268, 6097–6106

Fox, K. M. & Karpplus, P. A. (1994) Structure 2, 1089–1105

Theorell, H. & Nygaard, A. P. (1954) J. Biol. Chem. 213, 1029–1047

Ehrenhauer, A., Muller, F. & Hemmerich, P. (1987) Eur. J. Biochem. 166, 286–293

Stewart, J. C. & Massey, V. (1985) J. Biol. Chem. 260, 13847–13857

Kemal, C., Chan, T. W. & Bruece, T. C. (1977) J. Am. Chem. Soc. 99, 7272–7286

Niino, Y. S., Charkrubarto, S., Brown, B. J., & Massey, V. (1995) J. Biol. Chem. 270, 1983–1991

Van den Bergh-Smeenk, S. & Sankovitch, M. T. (1985) J. Biol. Chem. 260, 3373–3379

Yang, K. & Ozawa, T. (1962) Biochem. Biophys. Acta 56, 413–419

Quay, S. & Massey, V. (1977) Biochemistry 16, 3348–3354

Curti, B., Ronchi, S. & Simonetta, M. P. (1992) in Chemistry and Biochemistry of Flavoenzymes (Muller, F., ed) Vol. II, pp. 69–94, CRC press, Boca Raton, FL

4 We were able to accomplish the reversal of OYE redox chemistry by reconstituting the apoprotein with 8-CN-FMN. The reduratase activity of the native enzyme toward α,β-unsaturated carbonyl compounds was changed so that the enzyme now acted preferentially to dehydrogenate the corresponding saturated carbonyl compounds, using molecular oxygen as acceptor (Y. V. S. N. Murthy and V. Massey, manuscript in preparation).