Proton Nuclear Magnetic Resonance Investigation of the Mechanism of Flavin C-4a Adduct Formation Induced by Oxidized Nicotinamide Adenine Dinucleotide Binding to Monoalkylated Pig Heart Lipoamide Dehydrogenase†

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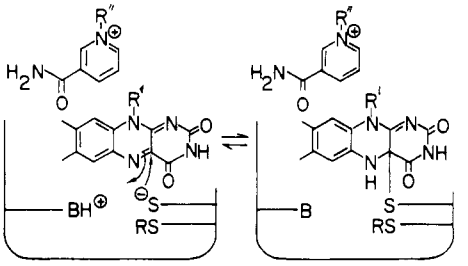
ABSTRACT: The active center thiol of monoalkylated pig heart lipoamide dehydrogenase, EHR, is induced to form an adduct to the enzyme-bound flavin adenine dinucleotide (FAD) at the C-4a position upon binding oxidized nicotinamide adenine dinucleotide (NAD^+) [Thorpe, C., & Williams, C. H., Jr. (1976) *J. Biol. Chem. 251*, 7726–7728]. In light of hypotheses on covalent electron transfer between pyridine nucleotide and flavin, the induction of the thiol–flavin C-4a adduct by NAD^+ is reasonably envisioned as involving a covalent bond between the modified flavin and the NAD^+. The double-resonance proton nuclear magnetic resonance technique of cross saturation was used to probe the existence of covalent bond formation between the modified flavin of EHR and its inducer molecule, NAD^+. Cross-saturation of the free NAD^+ signals was not observed even though the spin–lattice relaxation time of NAD^+ and the rate of exchange between free NAD^+ and NAD^+ bound to EHR were well within the limits required for cross-saturation. We conclude that a noncovalent interaction between NAD^+ and FAD induces the formation of the thiol–flavin C-4a covalent adduct in EHR. A model by which NAD^+ binding induces nucleophilic attack by the nascent thiolate of EHR is discussed.

The nascent thiols of two-electron-reduced lipoamide dehydrogenase, EH_2,[1] exhibit widely different reactivities toward iodoacetamide enabling preparation of a monoalkylated derivative, EHR, in which the cysteine closest to the amino terminus is predominately alkylated (Thorpe & Williams, 1976a). The addition of NAD^+ to EHR causes a rapid and reversible spectral change interpreted as the formation of a covalent adduct between the unmodified active site thiol and the C-4a position of the flavin (Scheme I) (Thorpe & Williams, 1976b). Upon saturation with NAD^+, the amount of flavin adduct and bound NAD^+ is half of the total concentration of FAD (Thorpe & Williams, 1976b). This stoichiometry can be accounted for by the dimeric structure of EHR, wherein the binding of one NAD^+ molecule to an EHR dimer induces formation of the flavin adduct in one subunit and promotes changes in the other subunit that preclude tight binding of a second molecule of NAD^+ (Thorpe & Williams, 1976b, 1981).

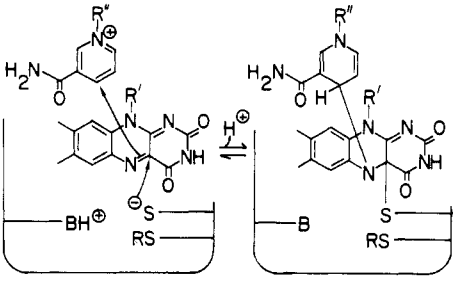
The requirement of NAD^+ binding for the formation of the flavin adduct in EHR, which we will refer to as E4a, suggests that the pyridine nucleotide induces the transfer of electrons from the active site thiols to the flavin moiety. Induction of the flavin adduct in EHR upon the binding of NAD^+ may be due only to noncovalent forces (Scheme I) between NAD^+ and EHR (e.g., a conformational change in EHR). Alternatively, the flavin adduct in EHR may involve a covalent bond between NAD^+ and the N-5 position of the FAD of EHR (Scheme II). Knappe observed a covalent adduct between NADH and oxidized FAD, which exhibits a single absorbance maximum at 354 nm and is thus very similar to the spectrum of the flavin adduct induced by NAD^+ binding to EHR [discussion following Blankenhorn (1977)]. Proposals of covalent electron transfer between pyridine nucleotide and flavin have been advanced by Hamilton (1971) and Hemmerich (1976).

Scheme I: Noncovalent Formation of Flavin Adduct[a]



[a] R = $-CH_2CONH_2$.

Scheme II: Covalent Formation of Flavin Adduct[a]



[a] R = $-CH_2CONH_2$.

† From the Veterans Administration Medical Center and the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48105. *Received January 13, 1983.* This research was supported by the Medical Research Service of the Veterans Administration, by Grant GM-21444 from the National Institute of General Medical Sciences, U.S. Public Health Service, and by Equipment Grant RR01077 from the Division of Research Resources of the National Institutes of Health to the Purdue University Biomedical Magnetic Resonance Laboratory, West Lafayette, IN. This paper was taken from a thesis submitted by M.E.O. to the Graduate School of the University of Michigan in partial fulfillment of the requirements for the Ph.D. degree.

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[1] Abbreviations: NAD^+, oxidized nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; EH_2, two-electron-reduced lipoamide dehydrogenase; EHR, two-electron-reduced lipoamide dehydrogenase in which the NH_2-terminal cysteine residue of the redox active dithiol has been alkylated wth iodoacetamide; E4a, EHR dimer in which one subunit has been induced by NAD^+ binding to form an FAD adduct presumably between the nascent thiol and the C-4a position of the FAD; NMR, nuclear magnetic resonance; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; pH*, the glass electrode reading of pH in deuterium oxide without correction for isotope effects.

The mechanism of electron transfer between pyridine nucleotide and flavin have been the subject of active debate. Therefore, we felt that it was important to determine the chemical state of NAD^+ in the E4a complex. Changes in hybridization of the pyridine ring are manifested in the ^1H NMR spectrum of the protons at C-2, C-4, C-5, and C-6 by large upfield shifts in their resonances. Thus, we have undertaken a ^1H NMR study to distinguish between the covalent and noncovalent mechanisms of flavin adduct induction in EHR by NAD^+. It should be noted that while the covalent adduct is pictured in Scheme II as involving the C-4 position of NAD^+, the techniques used in this study would detect a covalent adduct at any position leading to loss of aromaticity.

Materials and Methods

Pig heart lipoamide dehydrogenase was purchased from Miles Laboratories (Seravac Division) and further purified by calcium phosphate gel chromatography (Williams et al., 1967). NAD^+ was purchased from Sigma Chemical Co., and DSS and deuterium oxide (99.8% ^2H) were from Aldrich Chemical Co., Inc. EHR was prepared as described previously (Thorpe & Williams, 1976b), and its concentrations are expressed as the dimer.[2]

Deuterium Oxide Solutions of EHR and NAD^+. A ^2H_2O-containing buffer (99.8% ^2H enriched) of 0.1 M $Na_4P_2O_7$–0.3 mM Na_4EDTA–^2HCl, pH* 8.3 (buffer A), was made by lyophilizing $Na_4P_2O_7$ and Na_4EDTA from ^2H_2O, reconstituting the salts in ^2H_2O, and titrating to pH* 8.3 with 38% ^2HCl. EHR was precipitated in 530 g/L ammonium sulfate (80% saturated at 5 °C), collected by centrifugation, dissolved in buffer A, and clarified by centrifugation. The remaining ^1H_2O in the protein solution was exchanged into ^2H_2O by seven cycles of 2-fold concentration by ultrafiltration on a 43-mm Pellicon PTGC membrane (Millipore) followed by 2-fold dilution with buffer A, after which the solvent was 99.5% ^2H-enriched ^2H_2O as measured by integration of the HO^2H signal of a Varian T-60 NMR spectrum of the filtrate. The protein solution was concentrated by ultrafiltration to 0.5 mM EHR and was clarified by centrifugation. The sample was stored frozen in a desiccator for 36 h before use.

NAD^+ was lyophilized from ^2H_2O and was reconstituted in buffer A to give 100 mM NAD^+. The solution was clarified by filtration through a 0.45-μm membrane filter (Millipore).

Samples of NAD^+ containing EHR were made by mixing 26.5 μL of 100 mM NAD^+ in buffer A and 4 μL of 0.1 M DSS in ^2H_2O with 500 μL of 0.5 mM EHR in buffer A in a 5-mm glass NMR tube (Wilmad 528PP) immediately before insertion of the sample into the probe of the NMR spectrometer. Flavoprotein samples kept at 11 °C remained stable to spectral changes for over 10 h; nevertheless, fresh mixtures of NAD^+ and EHR were prepared for each experiment.

Proton NMR Spectroscopy. ^1H NMR spectroscopy was carried out on a 360-MHz Nicolet NT-360 spectrometer at the Purdue University Biochemical Magnetic Resonance Laboratory. The electromagnetic field was internally locked on the ^2H_2O signal of the solvent. The HO^2H signal of the solvent was decoupled with a 25-D gated decoupling frequency. NMR spectra were obtained by irradiating each sample at 11 °C every 3 s with single pulses (80° magnetization tip angle) until a total of 100–900 transients were collected and Fourier transformed. The chemical shifts of peaks in the NMR spectra are expressed as parts per million (ppm) from internal 0.8 mM DSS. NMR spectra of EHR containing 0.4–1.25 mM NAD^+ were collected on the Bruker WH-370 spectrometer at the University of Michigan under the same conditions as described above.

Spin–Lattice Relaxation Time (T_1). Measurements of T_1 were carried out by the spin-inversion recovery method (Dwek, 1973, pp 122–125) with a $180°-\tau_1-90°-\tau_2$ pulse sequence, where $\tau_2 = 5$ s and τ_1 was varied between 1 ms and 5 s. The free induction decay at each value of τ_1 was Fourier transformed after collecting eight transients. Approximate T_1 values for the NAD^+ pyridine ring protons in the presence of EHR were calculated as follows: $T_1 = \tau_{null}/\ln 2$, where τ_{null} is the time at which the intensities of all of the pyridine ring proton resonances were at or near zero.

Cross-Saturation. Cross-saturation was performed by using a 2-s gated pulse (H_2) immediately before sampling the spectrum. The 25-D H_2 saturation frequency was produced by a PTS-160 RF synthesizer (Program Test Source, Inc.). The radio-frequency output was modulated with a 100-Hz band of noise from an HP-3722A pseudorandom binary sequence bit shift noise generator (Hewlett-Packard) operated at a sequence of 31. Complete disappearance of proton signals beneath the noise-modulated H_2 band confirmed that the power of the H_2 gated pulse was sufficient to saturate proton resonances. A spectrum was obtained from 100 transients at each step as the H_2 frequency offset was varied sequentially in steps of 100 Hz (0.28 ppm) through the spectral region from 1.03 to 7.69 ppm.

Rate of Chemical Exchange. The rate of chemical exchange between NAD^+ bound to E4a and NAD^+ in free solution (k_{ex}) is described by eq 1. A solution containing E4a

$$\frac{1}{k_{ex}} = \frac{1}{k_{on}[L_f]} + \frac{1}{k_{off}} \quad (1)$$

was diluted 2-fold with buffer A at 11 °C in the stopped-flow apparatus designed and built by Dr. Shawn D. Black and Dr. David P. Ballou of the University of Michigan. The rate of appearance of unmodified FAD upon dissociation of NAD^+ from E4a was measured at 448 nm. The rate of approach to equilibrium, k_{obsd}, is described by eq 2 and 3, where $\bar{E}$ and $\bar{S}$

$$\ln (A_f - A_t) = -k_{obsd}t + \ln (A_f - A_0) \quad (2)$$

$$k_{obsd} = k_{on}(\bar{E} + \bar{S}) + k_{off} \quad (3)$$

are the new equilibrium concentrations of EHR and free NAD^+, respectively, A_f is the absorbance at the new equilibrium, A_t is the absorbance at time t, and A_0 is the initial absorbance. To calculate the values of k_{on} and k_{off}, the dissociation constant of NAD^+ from E4a was determined from spectral changes induced by additions of NAD^+ to EHR and substituted ($K_d = k_{off}/k_{on}$) into eq 3. k_{ex} was calculated from eq 1. The dissociation constant was measured under the conditions used in the NMR experiments. The equilibrium jump study was performed with 50 μM EHR and 250 μM NAD^+ (before 2-fold dilution) in buffer A. A rapid 2-fold dilution into a 2-cm path-length cell results in a total change at 448 nm of about 0.08 absorbance unit. The concentrations of enzyme and substrate used in the equilibrium jump experiment are not those used in the NMR experiments since for concentrations used in the NMR experiments (0.5 mM EHR and 5 mM NAD^+), the new equilibrium is reached within the dead time of the instrument.

[2] In view of the half-the-sites nature of the NAD^+-induced formation of a flavin adduct in only one subunit of the EHR dimer (Thorpe & Williams, 1976b), enzyme concentrations are expressed in terms of EHR dimer. Specifically, 1 mol of enzyme represents 2 mol of FAD, in contrast to the established convention of reporting the molar concentration of enzyme in terms of single FAD sites (Williams, 1976). Thus, the millimolar extinction coefficient of EHR used in this study is 22.2 mM^{-1} cm^{-1}, twice that previously determined per FAD site in EHR (Thorpe & Williams, 1976a).

Table I: Values for Equilibrium Constants and Various Kinetic Constants for Binding of NAD^+ to EHR[a]

constant	value
K_d (μM)	85
k_{obsd} (s^{-1})[b]	167 ± 33
k_{on} (M^{-1} s^{-1})	8.07×10^5
k_{off} (s^{-1})	68.6
k_{ex} (s^{-1})	67.3

[a] Definitions of the constants shown are as follows: K_d, the dissociation constant of NAD^+ from E4a obtained from a Stockell plot of equilibrium data; k_{obsd}, the rate of approach to equilibrium from concentration jump experiments; k_{on} and k_{off}, calculated from the simultaneous solution of k_{obsd} and K_d; k_{ex}, the exchange rate of free and E4a-bound NAD, calculated from eq 1. [b] Average of 11 experiments.

Results

The aromaticity of the pyridine ring of NAD^+ will be lost in the case of NAD^+ that is covalently bound in the E4a complex. Disruption of the aromaticity in the pyridine ring is expected to cause a 2.0–6.0 ppm upfield change in the chemical shifts of the C-2, C-4, C-5, and C-6 ^1H resonances. In model compounds covalently modified in the pyridine ring, these signals have been shown to lie in the region from 1.8 to 7.4 ppm (Diekmann et al., 1964; Biellmann et al., 1979; Arnold et al., 1979). On the other hand, the same pyridine ring proton signals of NAD^+ bound noncovalently to E4a would be expected to resonate at frequencies between 8.1 and 9.4 ppm (Lee et al., 1973). Although a proton that resonates at a frequency greater than 2.0 ppm upfield from the free NAD^+ peaks will not be resolved from the absorption of the enzyme protons, the free pyridine ring signals can be used to probe the existence of covalently bound NAD^+ by employing the double-resonance technique of cross-saturation (Redfield & Gupta, 1971). In this technique, a saturating pulse of radio frequency is applied. During the process of chemical exchange between free NAD^+ and bound saturated NAD^+, the pyridine ring protons of the free NAD^+ molecules will also become saturated. Thus, the chemical exchange of unsaturated free NAD^+ with saturated NAD^+ in the E4a complex will result in the disappearance of the pyridine ring signals in the bulk of the free solution provided two conditions are met: first, the rate of ligand exchange must be slow relative to the NMR time scale ($k_{ex} < 2\pi\Delta\nu$); and second, the rate of ligand exchange must be fast relative to the rate of spin–lattice relaxation of the ligand nucleus, $1/T_1^{obsd}$. Hence, to determine if the cross-saturation technique could be used to probe the chemical nature of NAD^+ bound to E4a, the rate of chemical exchange between free NAD^+ and bound NAD^+ and the rate of relaxation of free NAD^+ in the presence of EHR were determined.

Rate of Chemical Exchange. The pyridine protons of an NAD^+ molecule that is covalently bound to E4a will absorb at frequencies greater than 2.0 ppm upfield from the corresponding protons on free NAD^+; the rate of exchange between covalently bound NAD^+ and free NAD^+ must be less than 4500 s^{-1} to be slow with respect to the NMR time scale; i.e., $k_{ex} < 2\pi(720\ s^{-1})$.

The rate of chemical exchange between NAD^+ bound to E4a and the NAD^+ in free solution was calculated by combining the K_d with an approach to equilibrium kinetic study as described under Materials and Methods. The dissociation constant shown in Table I for NAD^+ bound to E4a was determined from a Stockell plot (inset to Figure 1) of the equilibrium data shown in Figure 1. The intercept on the NAD^+/α axis indicates a stoichiometry of 0.528 molecules of NAD^+ bound per FAD (1.06 NAD^+ per EHR dimer) in agreement with the half-the-sites nature of NAD^+ bound to EHR determined by a different method (Thorpe & Williams, 1976b). The kinetic trace shown in Figure 2 is the time course of the absorbance changes at 448 nm following the rapid 2-fold dilution of a solution of E4a and is first order through two half-lives. The rate of chemical exchange, k_{ex}, between NAD^+ bound to E4a and NAD^+ in free solution was calculated from the concentration jump data and the dissociation constant. The values for the dissociation constant and the observed rate of approach to equilibrium, together with the k_{on}, k_{off}, and k_{ex} calculated from them, are given in Table I. The rate of chemical exchange between the free and bound forms of NAD^+ satisfies the condition of slow exchange with respect to the NMR time scale (67 s^{-1} < 4500 s^{-1}).

Spin–Lattice Relaxation. The T_1^{obsd} of free NAD^+ in the presence of EHR was measured under conditions identical with those used in the cross-saturation experiment (Materials and Methods) and was found to be 0.87 s. From the values of k_{ex} and T_1^{obsd}, the extent to which the cross-saturated solution of NAD^+ is relaxed during the process of chemical exchange with covalently bound NAD^+ under the conditions used in the cross-saturation experiment is calculated to be only 1.7% [$M_z = M^0(1 - 2e^{-\tau/T_1^{obsd}})$, where τ is the time constant for chemical exchange, M^0 is the initial magnetization, and M_z is the ligand

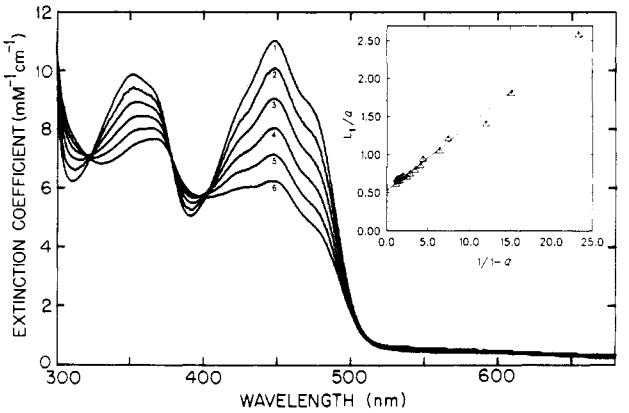

FIGURE 1: Spectral changes induced by additions of NAD^+ to EHR. 0.5 mM EHR in 0.1 M $Na_4P_2O_7$–0.3 mM Na_4EDTA–^2HCl, pH* 8.3, 11 °C (curve 1), was mixed with 0.131, 0.258, 0.422, 0.723, and 2.47 mM NAD^+ (curves 2–6, respectively). Intermediate spectra have been omitted for clarity. Enzyme was contained in a 0.2-cm pathlength quartz cuvette. The inset is a Stockell plot where α is ϵ_{448} observed at a given NAD^+ level divided by the maximal ϵ_{448} value extrapolated from a double-reciprocal plot. The line drawn through the data points is a linear-regression analysis of the data yielding K_d = 85 μM.

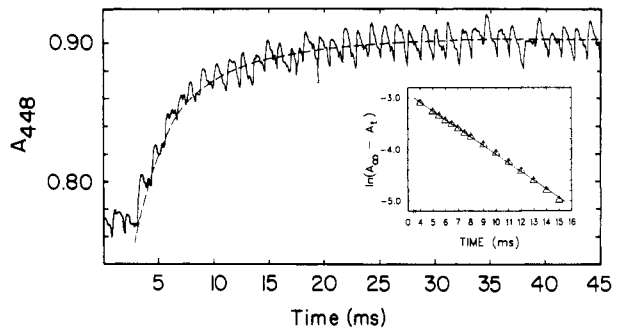

FIGURE 2: Kinetic trace of absorbance changes at 448 nm vs. time after mixing 0.1 M $Na_4P_2O_7$–0.3 mM Na_4EDTA–^2HCl, pH* 8.3, 11 °C, containing 50 μM EHR and 250 μM NAD^+ with an equal volume of buffer alone. The inset shows the log plot of the absorbance changes through four half-lives. The line drawn through the data points is a linear-regression analysis of the data yielding k_{obsd} = 169 s^{-1}.

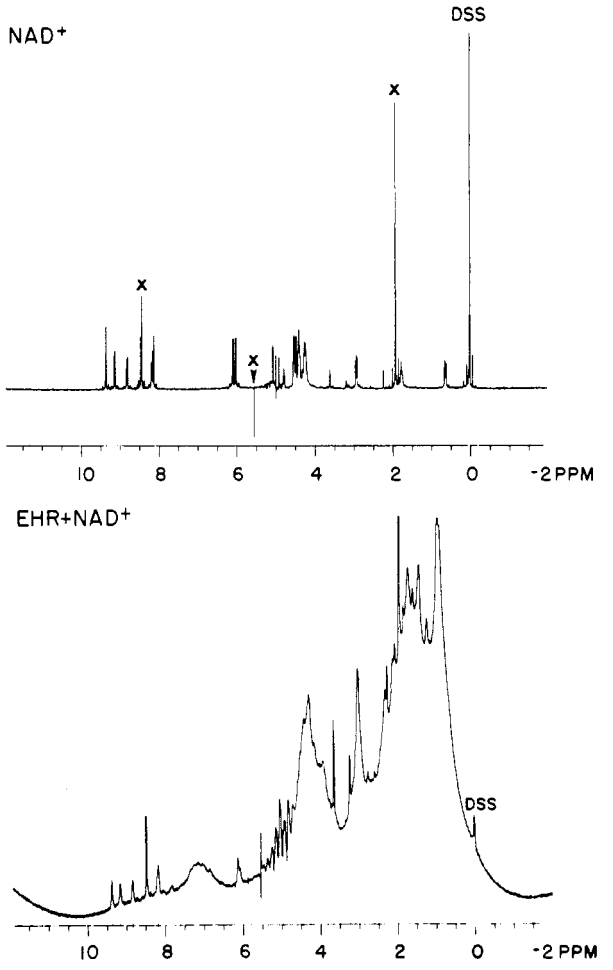

FIGURE 3: 360-MHz ^1H NMR spectra of 5.0 mM NAD^+ (top) and 5.0 mM NAD^+ in the presence of 0.5 mM EHR (bottom) in 0.1 M $Na_4P_2O_7$–0.3 mM Na_4EDTA–^2HCl, pH* 8.3, 11 °C. Chemical shifts are expressed in ppm from internal DSS. The symbol × in the top spectrum stands for a radio-frequency artifact and pertains to the bottom spectrum also.

magnetization after time τ (Dwek, 1973, pp 122–123)]. Thus, in the case of cross-saturation, 98.3% of the signal of the free NAD^+ pyridine ring protons will be lost.

Cross-Saturation. The double-resonance technique of cross-saturation can be applied to probe the existence of covalently bound NAD^+ in the E4a complex since the spin–lattice relaxation time of NAD^+ and the rate of exchange between free and bound NAD^+ are well within the limits required for cross-saturation. The NMR spectra of 5.0 mM NAD^+ in the presence and absence of 0.5 mM EHR are shown in Figure 3. The cross-saturation technique was performed by applying a pulse of saturating H_2 at frequencies expected for the resonance of covalently bound pyridine protons (10.0–8.0 ppm). Cross-saturation of the free pyridine protons was not observed.[3] The absence of detectable cross-saturation shows that NAD^+ is not covalently bound in the E4a complex under these conditions.

Discussion

The binding of NAD^+ to EHR induces the formation of E4a, a form of EHR that contains an FAD adduct (Thorpe & Williams, 1976b). Possible covalent participation of NAD^+ in the induction of E4a was studied by using the NMR cross-saturation technique. The results show that E4a is induced by noncovalent interactions between NAD^+ and EHR (Scheme I).

Previous studies, together with these studies on EHR, permit a tentative description of the mechanism by which noncovalent NAD^+ binding induces adduct formation between FAD and the nascent thiol of EHR. In native lipoamide dehydrogenase, the difference in oxidation–reduction potential between the dithiol and FAD in EH_2 is decreased upon binding NAD^+ as indicated by the NAD^+-induced shift in the wavelength maximum of the sulfur-to-flavin charge-transfer absorption band of EH_2 (Matthews et al., 1976, 1979). This conclusion is supported by model studies of biscoenzymes of the Nic_{ox}^+–$(CH_2)_n$–Fl_{ox} type (n = 2–4), which show that the oxidation–reduction potential of the flavin is raised (0.092 V) as the linker between the coenzymes is decreased from four to two methylene groups (Blankenhorn, 1975). It is well established that the electrophilicity of FAD analogues increases in proportion to their more positive values of oxidation–reduction potential (Muller & Massey, 1969; Gascoigne & Radda, 1967). Thus, it is possible that the NAD^+-induced formation of the flavin adduct in EHR and the NAD^+-induced shift in the maximum of the sulfur-to-flavin charge-transfer absorption band in EH_2 share a common explanation, that is, a specific interaction between the pyridine ring of NAD^+ and the isoalloxazine ring of FAD that increases the oxidation–reduction potential of the FAD. In the case of EH_2, the elevated oxidation–reduction potential of the FAD induces a shift in the thiolate-to-flavin charge-transfer absorbance maximum. In the case of EHR, the elevated oxidation–reduction potential of the FAD induces nucleophilic addition of the nascent thiol at the flavin C-4a position.

Acknowledgments

We are grateful to Dr. Jules Shafer for helpful discussions and to Dr. John Markley, Dr. Jerry Dallas, and Milo Westler of the Purdue University Biomedical Magnetic Resonance Laboratory for their help in acquainting us with the 360-MHz NMR spectrometer.

Registry No. Lipoamide dehydrogenase, 9001-18-7; NAD, 53-84-9; FAD, 146-14-5.

References

Arnold, L. J., Jr., Oppenheimer, N. J., Lee, C. Y., & Kaplan, N. O. (1979) *Biochemistry 18*, 2787–2793.

Biellmann, J. F., Lapinte, C., Haid, E., & Weimann, G. (1979) *Biochemistry 18*, 1212–1223.

Blankenhorn, G. (1975) *Eur. J. Biochem. 50*, 351–356.

Blankenhorn, G. (1977) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., Ed.) pp 185–205, de Gruyter, Berlin.

Diekmann, H., Englert, G., & Wallenfels, K. (1964) *Tetrahedron 20*, 281–291.

Dwek, R. A. (1973) *Nuclear Magnetic Resonance (NMR) in Biochemistry: Applications to Enzyme Systems*, Clarendon Press, Oxford, England.

[3] Collapse of the pyridine ring C-2 proton peak (9.35 ppm) of free NAD^+ was observed when the saturating H_2 was centered at 7.1 ppm. Accumulation of the free induction decay during irradiation by the saturating H_2 at 7.1 ppm showed a sharp band of noise in the Fourier-transformed spectrum that was centered over the collapsed pyridine C-2 proton peak. Movement of the saturating H_2 50 Hz downfield and repeating the above sequence resulted in a downfield shift of the sharp band of noise by 50 Hz accompanied by the full reappearance of the pyridine C-2 proton signal. Thus, collapse of the pyridine C-2 proton signal by application of the saturating H_2 at 7.1 ppm was the result of saturation by a harmonic frequency of the applied H_2 rather than cross-saturation by the applied H_2.

Gascoigne, I. M., & Radda, G. K. (1967) *Biochim. Biophys. Acta 131*, 498-507.
Hamilton, G. A. (1971) *Prog. Bioorg. Chem. 3*, 83-157.
Hemmerich, P. (1976) *Fortschr. Chem. Org. Naturst. 33*, 451-527.
Lee, C., Eichner, R. D., & Kaplan, N. O. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1593-1597.
Matthews, R. G., Wilkinson, K. D., Ballou, D. P., & Williams, Ch. H., Jr. (1976) in *Flavins and Flavoproteins, Proceedings of the International Symposium, 5th, 1975* (Singer, T. P., Ed.) pp 464-472, Elsevier Scientific Publishing Co., New York.
Matthews, R. G., Ballou, D. P., & Williams, C. H., Jr. (1979) *J. Biol. Chem. 254*, 4974-4981.
Muller, F., & Massey, V. (1969) *J. Biol. Chem. 244*, 4007-4016.
Redfield, A. G., & Gupta, R. K. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 405-411.
Thorpe, C., & Williams, C. H., Jr. (1976a) *J. Biol. Chem. 251*, 3553-3557.
Thorpe, C., & Williams, C. H., Jr. (1976b) *J. Biol. Chem. 251*, 7726-7728.
Thorpe, C., & Williams, C. H., Jr. (1981) *Biochemistry 20*, 1507-1513.
Williams, C. H., Jr. (1976) *Enzymes, 3rd Ed. 13*, 89-173.
Williams, C. H., Jr., Zanetti, G., Arscott, L. D., & McAllister, J. K. (1967) *J. Biol. Chem. 242*, 5226-5231.

Purification of Acetylcholine Receptors from the Muscle of *Electrophorus electricus*†

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ABSTRACT: Muscle from the electric eel *Electrophorus electricus* contains acetylcholine receptors at 50 times the concentration of normal mammalian muscle and fully one-tenth the concentration of receptors in its electric organ tissue. Receptor is organized much more diffusely over the surface of *Electrophorus* muscle cells than is the case in normally innervated mammalian skeletal muscle. Receptor was purified from *Electrophorus* muscle by affinity chromatography on cobra toxin-agarose and found to contain subunits which correspond immunochemically to the α, β, γ, and δ subunits of receptor from electric organ tissue of *Torpedo californica*. Receptor purified from *Electrophorus* muscle appears virtually identical with receptor purified from *Electrophorus* electric organ tissue.

Acetylcholine receptors were first purified from the electric organs of the electric eel *Electrophorus electricus* 10 years ago (Biesecker, 1973; Karlin & Cowburn, 1973; Klett et al., 1973; Meunier et al., 1974; Chang, 1974; Lindstrom & Patrick, 1974), but we only recently checked skeletal muscle of *Electrophorus* for its content of receptor. Surprisingly, it contains much more receptor than does innervated mammalian muscle, and even more than denervated or fetal muscle.

Previously we (Lindstrom et al., 1980) found that acetylcholine receptors purified from electric organ tissues of *Electrophorus* were composed of four subunits corresponding to the α, β, γ, and δ subunits of receptor from the electric organ tissue of the electric ray *Torpedo californica* (Weil et al., 1974; Raftery et al., 1975). Subsequent determination of the N-terminal amino acid sequence of each of these subunits showed that they were present in the same $\alpha_2\beta\gamma\delta$ stoichiometry (Reynolds & Karlin, 1978; Lindstrom et al., 1979a,b; Raftery et al., 1980) observed in receptor from *Torpedo* and that, as in *Torpedo*, there was substantial amino acid sequence homology between the α, β, γ, and δ subunits of *Electrophorus* (Conti-Tronconi et al., 1982). These results indicated that these subunits evolved by duplication and reduplication of a primordial gene and that the subunit stoichiometry was established before the divergence of primitive vertebrates into cartilaginous and bony fish some 400 million years ago.

Electric organ tissue evolved from muscle tissues independently in several genuses (Bennett, 1970; Mellinger et al., 1978). The presence of a relatively high concentration of receptor in *Electrophorus* gave us a chance to purify significant amounts of receptor from two related tissues in one animal and compare their structures. This is an especially interesting question because acetylcholine receptors in muscle are known to change several of their properties asynchronously during development (Dennis et al., 1981) and because receptor from normally innervated muscle differs in properties like channel open time (Sakman, 1978) or reaction with some antibodies (Weinberg & Hall, 1979) from receptors in denervated or fetal tissue, yet has apparently similar subunit structure (Nathanson & Hall, 1979; Sumikawa et al., 1982). These studies of receptor from normal and denervated muscle were complicated by the fact that only tiny amounts of receptor were obtained, and this was so proteolytically degraded that all four types of receptor subunits could not be detected. It has only recently become possible to purify significant amounts of receptor from fetal bovine muscle with α, β, γ, and δ subunits immunochemically demonstrable (Einarson et al., 1982). Muscle from *Electrophorus* has 4 times the receptor concentration of fetal bovine muscle. In addition to their relevance to studies of changes in receptor during muscle development, comparative studies of receptor from electric organ and muscle are relevant to studies of changes in receptor during evolution and provide

† From the Salk Institute for Biological Studies, San Diego, California 92138. *Received December 30, 1982.* This work was supported by grants from the National Institutes of Health (NS11323 and NS116686), The Muscular Dystrophy Association, The McKnight Foundation, the Los Angeles and California Chapters of the Myasthenia Gravis Foundation, and the Office of Naval Research. L.W.S. is a Clayton Foundation investigator.

0006-2960/83/0422-3796$01.50/0