Lipoamide Dehydrogenase from Pig Heart. Pyridine Nucleotide Induced Changes in Monoalkylated Two-Electron Reduced Enzyme†

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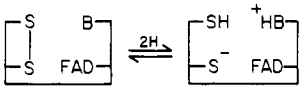
ABSTRACT: Two-electron reduced lipoamide dehydrogenase from pig heart reacted with iodoacetamide was monoalkylated almost exclusively in the nascent thiol nearer the amino terminus of the protein. The charge-transfer absorbance, maximal at 530 nm, characteristic of the two-electron reduced enzyme was lost as the alkylation proceeded, and the product had a spectrum similar to that of the oxidized enzyme. Treatment of the derivative with NAD^+ causes spectral changes which are consistent with the partial formation of an adduct at the C(4a) position of the flavin. The unmodified nascent thiol is the presumed substituent in this adduct. Only 1 mol of NAD^+ was bound per protein dimer [Thorpe, C., & Williams, C. H., Jr. (1976) *J. Biol. Chem. 251*, 3553–3557, 7726–7728]. We have measured the apparent dissociation constant for NAD^+ and the extent of adduct formation from pH 5.0 to 8.8. The reaction is 40–50% complete above pH 6.0 while the apparent K_D increases to a maximum of 370 μM at pH 6.8 and then declines to 35 μM at pH 8.8. The NAD^+ analogue 3-aminopyridine adenine dinucleotide (AAD^+) induces a spectral band at 580 nm in the monoalkylated derivative typical of charge-transfer complexes observed in lipoamide dehydrogenase. In these complexes thiolate is the donor and FAD is the acceptor. AAD^+ perturbs the spectrum of the native enzyme but does not produce a new band. Two molecules of AAD^+ are bound per monoalkylated protein dimer at sites which are equivalent or near equivalent. These data suggest that NAD^+ binding in one subunit induces adduct formation in that subunit and promotes changes in the other subunit which preclude tight binding of a second molecule of pyridine nucleotide. The interactions brought about by NAD^+ and AAD^+ involving the unmodified thiolate and the flavin make it reasonable that the same thiol, that is the one nearer the carboxyl terminus of the protein, is responsible for interaction with the flavin in the native enzyme.

The flavoprotein pig heart lipoamide dehydrogenase (reduced-NAD^+:lipoamide oxidoreductase, EC 1.6.4.3) catalyzes the reversible oxidation of dihydrolipoamide by NAD^+ via the half-reactions (Massey et al., 1960)[1]

$$E + Lip(SH)_2 \rightleftarrows EH_2 + LipS_2$$

$$EH_2 + NAD^+ \rightleftarrows E + NADH + H^+$$

In addition to FAD, the enzyme contains a redox-active cystine bridge which is reduced by $Lip(SH)_2$ to yield the catalytic intermediate EH_2 (Massey, 1963; Sanadi, 1963; Williams, 1976). The red color of this two-electron reduced form is thought to represent a charge-transfer complex between oxidized flavin as the acceptor and a thiolate anion donor, derived from reduction of the active-center disulfide bond (Kosower, 1966; Massey & Ghisla, 1974) (eq 1). Recent studies from



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this laboratory suggest the presence of an essential base in the active site of lipoamide dehydrogenase which stabilizes this thiolate residue at the EH_2 level (Matthews & Williams, 1976; Matthews et al., 1977).

The two thiol moieties, shown in eq 1, exhibit widely different reactivities toward iodoacetamide, allowing a monolabeled derivative of EH_2 to be prepared which is alkylated at the active-site cysteine residue closest to the amino terminus of the protein (Thorpe & Williams, 1976a). Alkylation disrupts the charge-transfer interaction in EH_2, and EHR exhibits a spectrum of oxidized bound flavin. This two-electron reduced derivative is air stable and inactive toward lipoamide substrates, since alkylation of a single residue prevents re-formation of the cystine bridge. However, several lines of evidence indicate that EHR retains a catalytically competent binding site for pyridine nucleotides; e.g., it has transhydrogenase activity at least as high as that of the native enzyme (Thorpe & Williams, 1976a).

We have recently reported that NAD^+ promotes a very rapid, reversible, partial bleaching of the flavin chromophore of EHR at 450 nm, with a concomitant rise at 380 nm (Thorpe & Williams, 1976b). These spectral changes are compatible with the formation of a C(4a)-thio-substituted dihydroflavin derivative. Model studies suggest that such an adduct is a likely intermediate in the internal redox reaction between cysteine and FAD which is required for completion of the pyridine nucleotide half-reaction (Hemmerich, 1968; Hamilton, 1971; Loechler & Hollocher, 1975; Yokoe & Bruice, 1975).

One unexpected aspect of our studies was that the spectral effects observed were associated with binding of 1 molecule of NAD^+ per 2 FAD molecules (Thorpe & Williams, 1976b). The native enzyme is a dimer of identical or near identical subunits each containing one FAD and one active center di-

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[1] Abbreviations used: E and EH_2, oxidized and two-electron reduced lipoamide dehydrogenase. The abbreviation EH_2R was previously used for the monolabeled derivative (Thorpe & Williams, 1976a,b). We have subsequently determined that alkylation of EH_2 is accompanied by release of a proton (see Materials and Methods), and thus EHR is a more appropriate abbreviation for this *S*-carboxamidomethyl derivative. $LipS_2$ and $Lip(SH)_2$ refer to oxidized and reduced lipoamide, respectively. Pyridine nucleotide analogues are $AcPyAD^+$, 3-acetylpyridine adenine dinucleotide; NHD^+, nicotinamide hypoxanthine dinucleotide; thio-NAD^+, thionicotinamide adenine dinucleotide; AAD^+, 3-aminopyridine adenine dinucleotide.

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sulfide bridge (Massey et al., 1962; Matthews et al., 1974). EHR is also dimeric, and all the available evidence shows that it is homogeneously labeled (Thorpe & Williams, 1976a,b). It therefore appeared that adduct formation was associated with the binding of one NAD^+ molecule per EHR dimer under the conditions employed. The object of the present work was to explore the interaction of pyridine nucleotides with EHR more fully, in our continuing efforts to define the chemical roles of the redox active cysteine residues in lipoamide dehydrogenase and in the closely related enzyme glutathione reductase (Arscott et al., 1981).

Materials and Methods

Biochemicals. NAD^+, $NADP^+$, $AcPyAD^+$, NHD^+, thio-NAD^+, ADP-ribose, 3-pyridinecarboxaldehyde adenine dinucleotide, and *N*-methylnicotinamide were obtained from Sigma. Enzyme was obtained from Miles Laboratories (Seravac Division). NAD^+ solutions were made up in glass-distilled water and standardized spectrophotometrically by conversion to NADH by using alcohol dehydrogenase (Dalziel, 1961). [*carbonyl*-^{14}C]NAD^+ was obtained from Amersham/Searle and its purity checked before and after enzymatic reduction, using chromatography on DEAE-cellulose paper (Whatman DE81) with 0.2 M NH_4HCO_3 as developing solvent (Silverstein, 1970).

AAD^+ was prepared essentially as described by Fisher et al. (1973). After the conversion of NAD^+ to AAD^+ had been stopped by neutralization, a small amount of radiolabeled NAD^+ was added to allow the separation of AAD^+ from unreacted NAD^+ on Dowex AG-1-X8 to be readily monitored. Complete separation was achieved by repeating this chromatographic step. The AAD^+ was recovered by lyophilization and freed from contaminating ammonium formate by gel filtration on a Sephadex G-10 column.

Spectrophotometric Measurements. Spectra were recorded on a Cary 118C spectrophotometer interfaced to a PDP-8/E computer (Williams et al., 1979). This arrangement greatly facilitates comparison of spectra and the generation of difference spectra. Fluorescence measurements at fixed wavelengths were made by using a Turner Model 111 fluorometer connected to a Varian A-25 recorder. Primary and secondary filters were Bausch and Lomb 444 38/2 band-pass and Turner 2A-12 sharp-cut, respectively. Fluorescence spectra were obtained by using an instrument designed and built by Dr. David Ballou, University of Michigan.

Unless otherwise stated, all experiments were performed at 15 °C in buffers containing 0.3 mM EDTA. Enzyme concentrations are expressed with respect to FAD content by using extinction coefficients of 11.3 mM^{-1} cm^{-1} at 455 nm for the native protein (Massey et al., 1962) and 11.1 mM^{-1} cm^{-1} at 448 nm for EHR (Thorpe & Williams, 1976a). The derivative was prepared as described previously (Thorpe & Williams, 1976b).

Ligand Binding. Binding of NAD^+ to EHR was estimated by the gel filtration method of Hummel & Dreyer (1962), with [*carbonyl*-^{14}C]NAD^+ as outlined earlier (Thorpe & Williams, 1976b). A 14 × 0.5 cm Sephadex G-25 superfine column was constructed from glass tubing sealed at both ends with serum caps pierced with short lengths of 20-gauge stainless steel tubing. The column was immersed in a water bath at 15 °C. A constant flow rate of about 6.1 mL/h was maintained by a Manostat Cassette pump. The enzyme (30–35 nmol) was applied in 0.1 mL of 100 mM phosphate, pH 7.6, and 2-min fractions were collected into scintillation vials for counting using Aquasol-2 cocktail (New England Nuclear) in a Packard Tri-Carb liquid scintillation spectrometer. The NAD^+ bound to EHR was estimated from the radioactivity associated with the enzyme peak (Hummel & Dreyer, 1962). A similar procedure was used to determine AAD^+ binding. Approximately 12 nmol of enzyme FAD was added in 0.1 mL of buffer containing the same concentration of AAD^+ used to equilibrate the column for that experiment. The concentration of AAD^+ in each 2-min fraction was determined at 331 nm [ϵ 3.09 mM^{-1} cm^{-1}; Fisher et al. (1973)] by using the microcell accessory to the Cary 118C instrument. The area of the trough following the enzyme peak was used to quantitate AAD^+ bound to EHR (Hummel & Dreyer, 1962).

Proton Release Studies. Anaerobic proton release experiments were performed by using the general techniques and apparatus described earlier (Matthews et al., 1977; Williams et al., 1979). A solution of oxidized native enzyme was dialyzed exhaustively against 50 mM NaCl and the pH adjusted to 7.4 by the careful addition of 10 mM NaOH. The enzyme (74 μM in 2.1 mL) was deoxygenated in the main space of an anaerobic cuvette apparatus; 40 μL of 22 mM iodoacetamide in the same buffer was contained in a side arm protected from light by black tape. The enzyme was titrated with dithionite in 50 mM NaCl until the concentration of EH_2 reached 62 μM; the spectrum was recorded. The dithionite syringe was replaced anaerobically by one containing phenol red in 50 mM NaCl and dye was added to the enzyme until the concentration reached 14 μM. Spectra were recorded immediately after addition and after a further 10 min to ensure that no oxygen had been introduced during these manipulations. The difference spectrum (EH_2 + dye) − (EH_2), generated by the computerized Cary 118C system (Williams et al., 1979), indicated that the visible spectrum of the dye was unaffected in the presence of the enzyme, and the ratio of the two absorption bands of the dye at 558 and 434 nm indicated that the pH of the solution before the addition of iodoacetamide was 7.1. Iodoacetamide was then tipped into the main space, and after completion of the alkylation (1 h), the new spectrum was recorded; the difference spectrum (EH_2 + dye) − (EHR + dye) was then generated. It differed from the difference spectrum of EH_2 − EHR by a prominent distortion in the 560-nm region due to an increased proportion of protonated dye after alkylation. The dye syringe was then replaced anaerobically by one containing 1.04 mM NaOH in 50 mM NaCl, and the enzyme solution was titrated until the shape of the difference spectrum (EH_2 + dye) − (EHR + dye) coincided with that of EH_2 − EHR (Matthews et al., 1977). From the volume of base required to neutralize the protons liberated and by use of the pK value of 7.9 for the ionization of EH_2 to EH^-, a value of 0.7 proton liberated per molecule of EH_2 alkylated was calculated. The phenol red indicator dye was added after dithionite titration of the enzyme because it was found that dithionite rapidly bleaches phenol red under the conditions employed.

Results

A titration of EHR with NAD^+ at pH 7.6 results in partial bleaching of the absorbance at 450 nm (Figure 1). These changes are similar to those observed at pH 8.3 (Thorpe & Williams, 1976b). In contrast to these marked effects, NAD^+ promotes very minor perturbations of the flavin chromophore in native oxidized lipoamide dehydrogenase (VanMuiswinkel-Voetberg & Veeger, 1973; Wilkinson & Williams, 1979). Double-reciprocal plots of $\Delta\epsilon_{448}$ and $\Delta\epsilon_{390}$ vs. NAD^+ concentration are linear, yielding an apparent K_D of 240 μM at both wavelengths (inset, Figure 1). The titration appears monophasic, with isosbestic points at 405, 375, and 326 nm. The dashed line in Figure 1 is extrapolated to infinite NAD^+ by

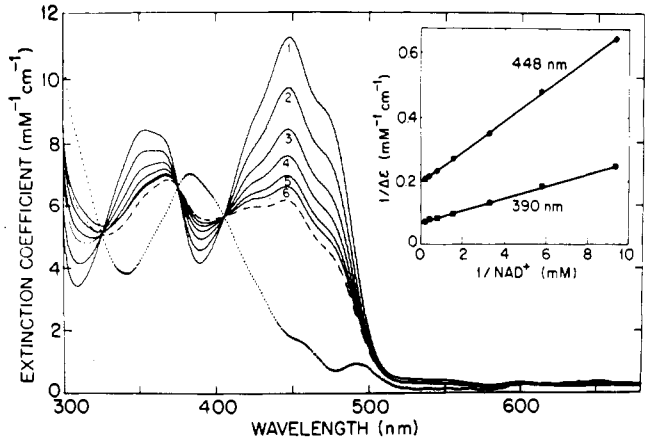


FIGURE 1: Spectral changes induced on addition of NAD^+ to EHR at pH 7.6. EHR (20.5 μM enzyme FAD) in 100 mM phosphate buffer, pH 7.6, at 15 °C (curve 1) was mixed with 0.11, 0.32, 0.67, 1.34, and 2.63 mM NAD^+ (curves 2–6, respectively). Equal concentrations of NAD^+ were added to the reference cuvette. Intermediate spectra have been omitted for clarity. The dashed line is the spectrum extrapolated to infinite NAD^+ by using the $1/\Delta\epsilon_{448}$ intercept obtained from a double reciprocal plot (see inset). The dotted line is an estimate of the spectrum of the modified flavin species after correction from the contribution of residual unmodified flavin (see text).

using the 448-nm intercept. This extrapolated spectrum exhibits about one-half of the original absorbance at 448 nm, but retains certain of the features of EHR (e.g., shoulders at 470 and 425 nm and a maximum at 448 nm). Under these conditions, NAD^+ only effects a partial conversion of the flavin into a species with increased 390-nm absorbance but lowered 450-nm absorbance. The dotted line shows an estimate of the absorption spectrum of this form (see later). Flavin fluorescence measurements provide additional evidence that NAD^+ promotes only partial modification of the flavin chromophore. In the absence of pyridine nucleotides, EHR is 75% as fluorescent as the native oxidized enzyme at pH 7.6. The emission spectrum, exciting at either 450 or 385 nm, is similar to that of E but displaced 6 nm to shorter wavelengths, reflecting corresponding differences in the absorption maxima of the native and modified flavoproteins. The flavin fluorescence of EHR is quenched 50% by saturating levels of NAD^+ at pH 7.6 (results not shown). No new features are observed in the residual fluorescence emission spectrum. In addition, the magnitude of the excitation spectrum is decreased by one-half at saturating NAD^+ concentrations without noticeable changes in the shape of the residual spectrum. These results suggest that approximately one-half of the original fluorophore remains at high levels of NAD^+, and that the species induced on NAD^+ binding is not appreciably fluorescent.

Titrations similar to Figure 1 were performed between pH 5.8 and 8.8, and the results are summarized in Figure 2. Little change in the extent of bleaching at infinite ligand is seen from pH 6 to 8.8. In contrast, the apparent K_D for NAD^+ shows an approximately 10-fold variation in this range, with a complex pH dependence. Binding is tightest at high pH (K_D of 35 μM at pH 8.8) and weakest around neutrality (K_D of about 370 μM). Below pH 5.8 the spectral changes are of the same form as those shown in Figure 1, but of decreased magnitude. Experiments performed at pH 7.6 established that neither the extent of bleaching nor the apparent K_D was dependent on the concentration of EHR used (over the range 15–100 μM enzyme flavin).

It was observed that at pH values at or above pH 8.3 time-dependent spectral changes occurred in the presence of

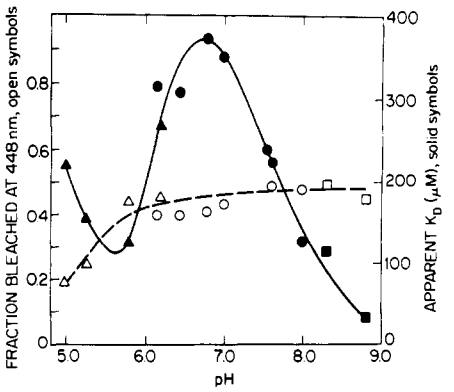


FIGURE 2: Extent of bleaching of EHR and apparent K_D for NAD^+ as functions of pH. NAD^+ titrations were performed at 15 °C in 50 mM citrate (Δ), 100 mM phosphate (O), and 50 mM pyrophosphate (□) buffers. Both the apparent K_D for NAD^+ (solid symbols) and the fraction bleached at 448 nm ($\Delta\epsilon_{extrapolated}/\epsilon_{initial}$, open symbols) were obtained from double-reciprocal plots of $\Delta\epsilon_{448}$ vs. NAD^+, as described for Figure 1.

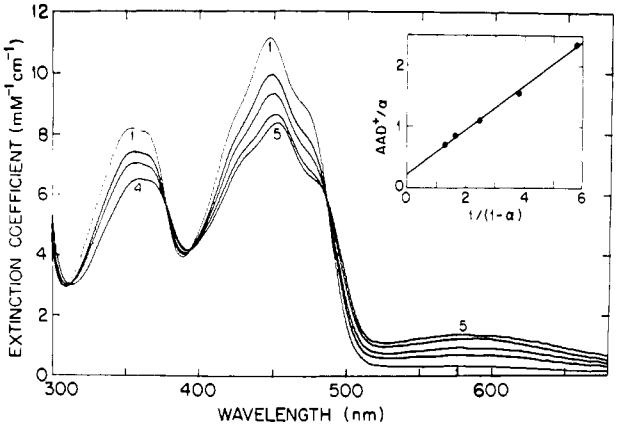


FIGURE 3: Perturbation of the visible spectrum of EHR at pH 7.6 induced by AAD^+. EHR, 22 μM in 100 mM phosphate buffer, pH 7.6, at 15 °C (curve 1), was mixed to give 33 μM, 65 μM, 194 μM, and 1 mM AAD^+ (curves 2–5, respectively). Equivalent amounts of AAD^+ were added to the reference cuvette. Intermediate spectra have been omitted for clarity. The inset shows a Stockell plot where α is $\Delta\epsilon_{448}$ observed at a given AAD^+ level divided by the maximal $\Delta\epsilon_{448}$ value extrapolated from double-reciprocal plots.

high concentrations of NAD^+. These changes did not complicate the spectral titrations summarized in Figure 2. For example, the single addition of 10 mM NAD^+ to EHR at pH 8.8 effects a slightly greater degree of bleaching ($\Delta\epsilon_{448}$ = 6.0 mM^{-1} cm^{-1} instead of 5.15 mM^{-1} cm^{-1} attained by extrapolation of titration data; data not shown), followed by the slow reappearance of an unresolved flavin spectrum. Gel filtration of the reaction mixture confirmed that release of FAD from the enzyme had occurred. Loss of flavin is accelerated both by higher pH and higher NAD^+ concentration. This effect has not been investigated further.

The NAD^+ analogue 3-aminopyridine adenine dinucleotide has been used in a recent study of lipoamide dehydrogenase from *Escherichia coli* (Wilkinson & Williams, 1979). Similar spectral perturbations accompany AAD^+ binding to native oxidized pig heart lipoamide dehydrogenase (data not shown). Similar spectral changes are induced on NAD^+ binding to the oxidized enzymes (VanMuiswinkel-Voetberg & Veeger, 1973; Wilkinson & Williams, 1979), but are approximately 5-fold smaller. Figure 3 shows that titration of EHR with AAD^+ produces spectral changes clearly different from those induced by NAD^+ at the same pH (Figure 1). A pronounced red shift and decreased in the main absorption band are accompanied

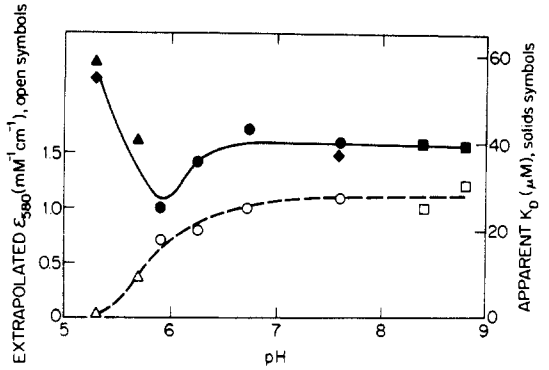


FIGURE 4: Variation of long-wavelength absorption of EHR and apparent K_D for AAD^+ as functions of pH. AAD^+ titrations were performed at 15 °C in 50 mM citrate (Δ), 100 mM phosphate (○), and 50 mM pyrophosphate (□) buffers, as described for Figure 5. Solid symbols represent apparent K_D values; open symbols are the ϵ_{580} values extrapolated to infinite AAD^+ concentration using double-reciprocal plots. K_D values obtained by monitoring titrations by flavin fluorescence quenching at pH 5.3 and 7.6 are indicated by ♦ (see the text).

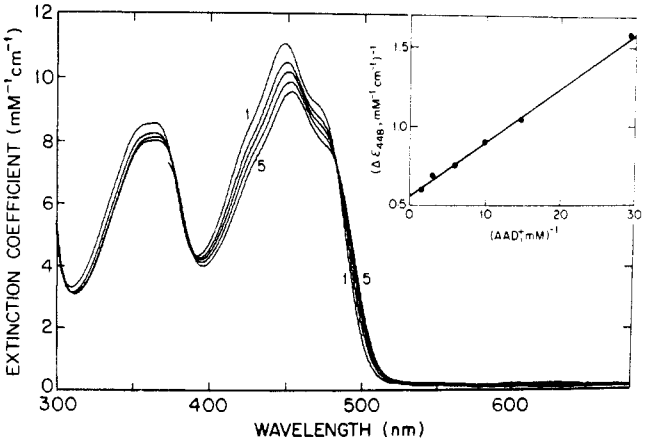


FIGURE 5: AAD^+ titration of EHR at pH 5.3. Curve 1: 14.7 μM enzyme FAD in 0.7 mL of 50 mM citrate buffer, pH 5.3, at 15 °C. Curves 2–5 are plus 34, 68, 169 and 664 μM AAD^+, respectively. Equivalent amounts of AAD^+ were added to the reference cuvette. The inset shows a plot of $(\Delta\epsilon_{448})^{-1}$ vs. $[AAD^+]^{-1}$.

by the appearance of a broad long wavelength band centered at 580 nm. Isosbestic points are observed at 378, 395, and 485 nm. A Stockell plot, using the 448 nm data (see inset), indicates that AAD^+ binds under these conditions with an apparent K_D of 37 μM. The intercept on the AAD^+/α axis indicates a stoichiometry of 1.1 molecules of analogue bound per FAD (i.e., 2.2 AAD^+/dimer) at saturation. This is in marked contrast to the 1.2 molecules of NAD^+ bound/dimer determined at pH 8.8 (Thorpe & Williams, 1976b; see below).

Figure 4 shows the pH dependence of the apparent K_D for AAD^+ binding to EHR and of the extinction changes at 580 and 448 nm extrapolated to infinite ligand. The binding affinity for AAD^+ is rather constant over the pH range 6.5–8.8 whereas the K_D apparent for NAD^+ varies widely in this region (Figure 2). It can also be seen from Figure 4 that the extent of long-wavelength absorbance observed drops sharply below pH 6.0. At pH 5.3 the character of the spectral changes obtained (Figure 5) resembles those shown by the native oxidized enzyme, consisting of a perturbation of the flavin chromophore without significant formation of the long-wavelength band.

As a check on the stoichiometries of pyridine nucleotide binding reported above, the binding of AAD^+ and NAD^+ to EHR was studied directly by the gel filtration method of Hummel & Dreyer (1962). Both sets of experiments were

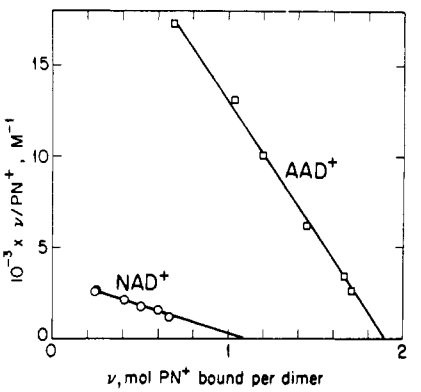


FIGURE 6: Direct determination of binding of NAD^+ and AAD^+ to EHR. Binding was evaluated at pH 7.6 in 100 mM phosphate buffer, 15 °C, by the gel filtration method (see Materials and Methods). The results are plotted as a Scatchard plot.

Table I: Interaction of Oxidized Pyridine Nucleotide Analogues with EHR

pyridine nucleotide	substituent	pH	spectral change[a]
NAD^+	$-CONH_2$	8.8	bleaching (35 μM)
thio-NAD^+	$-CSNH_2$	8.8	bleaching (80 μM)
NHD^+	$-CONH_2$	8.8	bleaching (1 mM)
$AcPyAD^+$	$-COCH_3$	8.8	none observed (2 mM)[b]
pyridinecarboxaldehyde adenine dinucleotide	-CHO	8.8	none observed (1.5 mM)[b]
$NADP^+$	$-CONH_2$	8.8	none observed at 3.4 mM
N-methylnicotinamide	$-CONH_2$	8.8	none observed at 6 mM
N-methylnicotinamide + ADP-ribose	$-CONH_2$	8.8	none observed at 6 and 1 mM, respectively
AAD^+	$-NH_2$	8.8	long-wavelength band (40 μM)
		5.3	perturbation (58 μM)

[a] K_D values in parentheses. [b] Approximate K_D values obtained in competition experiments with AAD^+.

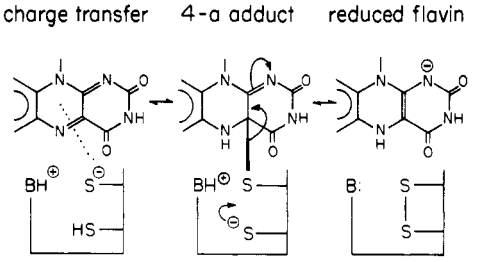
performed at pH 7.6 by using the same batch of enzyme, and the results are plotted in Figure 6. In agreement with the spectral data, 2 molecules of AAD^+ are bound per dimer with an apparent K_D of 35 μM. There is no evidence for two classes of binding sites on EHR, of unequal affinities for AAD^+, from either the optical titrations or the Scatchard plot (Figures 3 and 6). In contrast, NAD^+ binds to approximately 1.1 sites per dimer with a K_D apparent of 170 μM, in fair agreement with the value of 240 μM obtained in Figure 1. The discrepancy may reflect the limitations of the gel filtration method in measuring comparatively weak binding, and the sensitivity of the K_D to pH in the region of pH 7.6. Good agreement between the two methods was observed at pH 8.8 (Thorpe & Williams, 1976b).

Table I summarizes the spectral changes induced by various NAD^+ analogues on EHR. Thio-NAD^+ and NHD^+ produce spectral changes very similar to those shown in Figure 1. Both pyridinecarboxaldehyde- and acetylpyridine adenine dinucleotides bind weakly to EHR, as judged by competition experiments with AAD^+, but fail to perturb the flavin chromophore significantly.

Discussion

This work demonstrates the widely differing responses of EHR to NAD^+ and its analogue AAD^+. In summary, NAD^+ binds to EHR with a stoichiometry of 1/dimer (at pH 7.6 and at pH 8.8), whereas 2 molecules of AAD^+ are bound. In

Scheme I



contrast to AAD^+, NAD^+ binding is strongly pH dependent over the range 6.5–8.8. NAD^+ induces a partial bleaching of the flavin chromophore, with no long-wavelength absorption; AAD^+ binding leads to a pH-dependent long-wavelength band with concomitant perturbation of the flavin chromophore.

The dotted line in Figure 1 is an estimate of the spectrum of the species induced on NAD^+ binding. It was obtained (see Materials and Methods) assuming that the dashed line represents the binding of 1.1 NAD^+/dimer and that the spectral changes would increase proportionately until a stoichiometry of 2 NAD^+/dimer was reached and complete bleaching effected. This procedure is equivalent to subtracting the absorbance contribution of the residual fluorophore (see Results) from the spectrum extrapolated to infinite NAD^+. The dotted spectrum shows a single maximum at 384 nm with an extinction coefficient of 7.0 mM^{-1} cm^{-1}. It is similar to that obtained at pH 8.3 (Thorpe & Williams, 1976b) but has a somewhat lower extinction coefficient. The isosbestic points formed on either side of the 390-nm trough region of EHR reflect this change, occurring at 375 and 405 nm at pH 7.6, whereas at pH 8.3 they are at 371 and 408 nm. These spectra resemble those of substituted dihydroflavins carrying a carbon, oxygen, or sulfur function at the C(4a) position in appropriate solvent systems (Walker et al., 1970; Ghisla et al., 1973, 1974; Hevesi & Bruice, 1973; Kemal & Bruice, 1976) or introduced into various apoproteins (Scola-Nagelschneider et al., 1976; Ghisla et al., 1977). On the basis of these model studies, the intermediates observed during the interaction of reduced bacterial luciferase (Tu & Hastings, 1975), melilotate hydroxylase (Strickland & Massey, 1973), and *p*-hydroxybenzoate hydroxylase (Spector & Massey, 1972; Entsch et al., 1976) with O_2 have been assigned to C(4a)-peroxyflavins. We have, therefore, proposed that the NAD^+-induced bleaching of the 450-nm absorption of EHR reflects the formation of a similar adduct in which one of the redox active cysteine moieties is the 4a substituent (Thorpe & Williams, 1976b). This species had also been proposed as a catalytic intermediate in lipoamide dehydrogenase on the basis of model studies (Gascoigne & Radda, 1967; Loechler & Hollocher, 1976; Yokoe & Bruice, 1975).

Scheme I outlines a possible mechanism for the pyridine nucleotide half-reaction in the native enzyme. The "upper" thiolate attacks the C(4a) position of the isoalloxazine ring to generate the covalent adduct. Decomposition of this species, via nucleophilic attack from the "lower" thiol (as a thiolate), yields reduced flavin and regenerates the disulfide bond. In the final step (not shown), reoxidation of the flavin by enzyme-bound NAD^+ occurs. Although drawn as discrete steps for illustration, the process conceivably approaches a concerted reaction. This scheme incorporates the concept of a sequential flow of 2 reducing equiv, from active site dithiol to FAD to NAD^+ (Sanadi, 1963; Thorpe & Williams, 1976a). It can be seen that alkylation of the "lower" thiol could still allow covalent adduct formation but would prevent its breakdown, since the disulfide bond could not re-form to complete the internal redox reaction. Since adduct formation requires protonation of the N(5) position, the equilibrium is pH independent. The relatively constant degree of bleaching obtained from pH 6 to 8.8 at saturating NAD^+ levels is thus compatible with this.

Several lines of evidence suggest that the spectral changes observed with the monoalkylated derivative have catalytic significance for the native enzyme. The bleaching reaction is extremely rapid, being complete within 3 ms at pH 8.3, 15 °C (Thorpe & Williams, 1976b). In addition, those 3-substituted pyridine nucleotide analogues which fail to bleach EHR in the 450-nm region are also poor substrates in the $Lip(SH)_2$/pyridine nucleotide assay catalyzed by the native enzyme (Massey & Veeger, 1961). A third line of evidence comes from recent work on the crystal structure of glutathione reductase from human erythrocytes (Pai et al., 1978; Schulz et al., 1978).

Glutathione reductase and lipoamide dehydrogenase are very similar mechanistically and structurally (Massey & Williams, 1965; Williams, 1976; Williams et al., 1976). Indeed, the active-center disulfide sequences from both yeast (Jones & Williams, 1975) and erythrocyte (Krohne-Ehrich et al., 1977) glutathione reductases are highly homologous with those from *Escherichia coli* (Burleigh & Williams, 1972) and pig heart (Matthews et al., 1974; Brown & Perham, 1974) lipoamide dehydrogenases. The three-dimensional structure of erythrocyte glutathione reductase at 3-Å resolution indicates that the carboxyl-terminal redox-active cysteine residue is within bonding distance of the C(4a) position of the isoalloxazine nucleus (Schulz et al., 1978). This supports our previous suggestion that the carboxyl-terminal partner of the active-center dithiol pair in lipoamide dehydrogenase, participates in NAD^+-induced C(4a)-adduct formation (Thorpe & Williams, 1976b). The studies of Schulz et al. (1978) also show that structural elements from both subunits are used to form each of the two active centers of the dimer. In addition, there is close contact between the isoalloxazine ring of one subunit and the interface domain of the other subunit. These observations indicate the possibility of intersubunit communication between active-site regions. Our finding of negative cooperativity in NAD^+ binding to EHR suggests that such effects may occur in lipoamide dehydrogenase, under conditions where C(4a)-adduct formation is induced. The linearity of the Stockell and Scatchard plots for AAD^+ binding to EHR shows that the dimer has two pyridine nucleotide sites with similar or identical affinity for AAD^+. Thus, our working hypothesis is that NAD^+, on binding to one subunit of EHR, induces changes in that subunit which lead to covalent adduct formation and to a simultaneous lowering of the affinity of the second site for NAD^+.

The use of various pyridine nucleotide analogues (Table I) shows that the nature of the substituent at the 3-position of the pyridinium ring is crucial in determining the state of the flavin in the EHR–pyridine nucleotide complex. Although the bleaching reaction is still observed when the carboamide group is replaced by a thioamide moiety, substitution of the amide nitrogen by H or CH_3 results in no detectable spectral change, although both analogues bind to EHR. AAD^+, where the 3-position is occupied by an amino group, induces clearly different spectral changes, including formation of a long-wavelength absorption band. Although AAD^+ is formally an oxidized pyridine nucleotide, substitution of the 3-amino group confers additional electron density to the pyridinium ring, resulting in an analogue with some NADH character. AAD^+ is not enzymatically reducible, has an absorption maximum

at 331 nm, and is fluorescent (Anderson et al., 1959; Fischer et al., 1973).

The nature of the 580-nm band induced by AAD^+ is of considerable interest, especially since it resembles the long-wavelength band shown by EH_2–NAD^+ complexes of native pig heart lipoamide dehydrogenase (Veeger & Massey, 1963; Matthews et al., 1976). On the basis of studies with several flavoproteins, spectral changes of the type shown in Figure 3 have been explained by the formation of charge-transfer complexes between a suitable donor and oxidized flavin as the acceptor (Massey & Ghisla, 1974). If this assignment is accepted here, the question arises as to the identity of the charge-transfer donor. Two obvious candidates are either the 3-aminopyridinium ring system of the bound analogue or the free cysteine residue which participates in covalent adduct formation at the C(4a) position. A firm choice between these two possibilities cannot be made, but the available evidence favors the carboxyl-terminal cysteine as the donor. Thus, long-wavelength bands have never been observed in E–AAD^+ complexes of *E. coli* or pig heart lipoamide dehydrogenase over a range of pH values (e.g., see Figure 6; Wilkinson & Williams 1979). If AAD^+ were a suitable charge-transfer donor, long-wavelength bands would also be expected for these complexes. Rather, the spectral perturbations observed are very similar in form to those induced in EH_2 by NAD^+ (which could not itself be a charge-transfer donor to oxidized flavin). However, if the donor in EHR–AAD^+ is the carboxyl-terminal cysteine residue, the lack of a long-wavelength band in E–AAD^+ can be readily rationalized, since a disulfide bridge would have no donor ability. Further, the loss of long wavelength absorption of EHR-AAD^+ at low pH (Figure 5) can be explained by the protonation of the charge-transfer donor thiolate. The band is also lost upon addition of phenylmercuric acetate which could be reacting with the charge-transfer thiolate; the band returns upon addition of 2-mercaptoethanol. We tentatively conclude that AAD^+ binding changes the orientation of the carboxyl-terminal cysteine residue in EHR and/or sufficiently lowers its microscopic pK to allow a significant charge-transfer interaction with the isoalloxazine ring system.

The charge-transfer role of the equivalent cysteine residue in native yeast glutathione reductase and in a monoalkylated derivative is established in the accompanying paper (Arscott et al., 1981). Comparison of the properties of these two analogous derivatives provides additional insight into the chemical roles of the redox-active cysteines in these closely related flavoproteins.

References

Anderson, B. M., Ciotti, C. J., & Kaplan, N. O. (1959) *J. Biol. Chem. 234*, 1219–1225.

Arscott, L. D., Thorpe, C., & Williams, C. H., Jr. (1981) *Biochemistry* (following paper in this issue).

Brown, J. P., & Perham, R. N. (1974) *Biochem. J. 137*, 505–512.

Burleigh, B. D., Jr., & Williams, C. H., Jr. (1972) *J. Biol. Chem. 247*, 2077–2082.

Dalziel, K. (1961) *Biochem. J. 80*, 440–445.

Entsch, B., Ballou, D. P., & Massey, V. (1976) *J. Biol. Chem. 251*, 2550–2563.

Fischer, T. L., Vercelloti, S. V., & Anderson, B. M. (1973) *J. Biol. Chem. 248*, 4293–4299.

Gascoigne, I. M., & Radda, G. K. (1967) *Biochim. Biophys. Acta 131*, 498–507.

Ghisla, S., Hartmann, U., Hemmerich, P., & Muller, F. (1973) *Justus Liebigs Ann. Chem. 380*, 1388–1415.

Ghisla, S., Massey, V., Lhoste, J. M., & Mayhew, S. G. (1974) *Biochemistry 13*, 589–597.

Ghisla, S., Entsch, B., Massey, V., & Husein, M. (1977) *Eur. J. Biochem. 76*, 139–148.

Hamilton, G. A. (1971) *Prog. Bioorg. Chem. 1*, 83–157.

Hemmerich, P. (1968) *Proc. R. Soc. London, Ser. A 302*, 331–334.

Hevesi, L., & Bruice, T. C. (1973) *Biochemistry 12*, 290–297.

Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.

Jones, E. T., & Williams, C. H., Jr. (1975) *J. Biol. Chem. 250*, 3779–3784.

Kemal, C., & Bruice, T. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 995–999.

Kosower, E. M. (1966) in *Flavins and Flavoproteins* (Slater, E. C., Ed.) pp 1–148, Elsevier, Amsterdam.

Krohne-Ehrich, G., Schirmer, R. H., & Untucht-Grau, R. (1977) *Eur. J. Biochem. 80*, 65–71.

Loechler, E. L., & Hollocher, T. C. (1975) *J. Am. Chem. Soc. 97*, 3235–3237.

Massey, V. (1963) *Enzymes, 2nd Ed. 7*, 275–306.

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

Massey, V., & Williams, C. H., Jr. (1965) *J. Biol. Chem. 240*, 4470–4480.

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.

Massey, V., Hofmann, T., & Palmer, G. (1962) *J. Biol. Chem. 237*, 3820–3828.

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

Matthews, R. G., Arscott, L. D., & Williams, C. H., Jr. (1974) *Biochim. Biophys. Acta 370*, 26–38.

Matthews, R. G., Wilkinson, K. D., Ballou, D. P., & Williams, C. H., Jr. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 464–472, Elsevier, Amsterdam.

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

Pai, E. F., Schirmer, R. H., & Schulz, G. E. (1978) in *Mechanisms of Oxidizing Enzymes* (Singer, T. P., & Ondarza, R. N., Eds.) pp 17–21, Elsevier/North Holland, New York.

Sanadi, D. R. (1963) *Enzymes, 2nd Ed. 7*, 307–344.

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

Scola-Nagelschneider, G., Brustlein, M., & Hemmerich, P. (1976) *Eur. J. Biochem. 69*, 305–314.

Silverstein, E. (1970) *Biochim. Biophys. Acta 215*, 205–206.

Spector, T., & Massey, V. (1972) *J. Biol. Chem. 247*, 7123–7127.

Strickland, S., & Massey, V. (1973) *J. Biol. Chem. 248*, 2953–2962.

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.

Tu, S.-C., & Hastings, J. W. (1975) *Biochemistry 14*, 1975–1979.

VanMuiswinkel-Voetberg, H., & Veeger, C. (1973) *Eur. J. Biochem. 33*, 285–291.

Veeger, C., & Massey, V. (1963) *Biochim. Biophys. Acta 67*, 679–681.

Walker, W. H., Hemmerich, P., & Massey, V. (1970) *Eur. J. Biochem. 13*, 258–266.
Wilkinson, K. D., & Williams, C. H., Jr. (1979) *J. Biol. Chem. 254*, 863–871.
Williams, C. H., Jr. (1976) *Enzymes, 3rd Ed. 13*, 89–173.
Williams, C. H., Jr., Arscott, L. D., & Jones, E. T. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 455–463, Elsevier, Amsterdam.
Williams, C. H., Jr., Thorpe, C., & Arscott, L. D. (1978) in *Mechanisms Oxidizing Enzymes* (Singer, T. P., & Ondarza, R. N., Eds.) pp 3–16, Elsevier/North Holland, New York.
Williams, C. H., Jr., Arscott, L. D., Matthews, R. G., Thorpe, C., & Wilkinson, K. D. (1979) *Methods Enzymol. 62*, 185–198.
Yokoe, I., & Bruice, T. C. (1975) *J. Am. Chem. Soc. 97*, 450–451.

Glutathione Reductase from Yeast. Differential Reactivity of the Nascent Thiols in Two-Electron Reduced Enzyme and Properties of a Monoalkylated Derivative†

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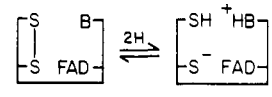
ABSTRACT: Two-electron reduced glutathione reductase from yeast reacted with iodoacetamide is alkylated almost exclusively in the nascent thiol nearer the amino terminus of the protein. The charge-transfer absorbance, maximal at 530 nm, characteristic of the two-electron reduced enzyme is not lost as the alkylation proceeds, and the product has a spectrum virtually identical with that of the two-electron reduced enzyme. This observation demonstrates that the thiol alkylated is not the charge-transfer-donor thiolate which interacts with the FAD. The spectrum of the monoalkylated derivative is stable in the presence of oxidized glutathione, indicating that the charge-transfer-donor thiol is not involved in interchange with the substrate in the native enzyme. Thus, the nascent thiols produced upon two-electron reduction of glutathione reductase have distinct functions, interchange with the substrate and interaction with the FAD. Treatment of the monoalkylated derivative with the apolar phenylmercuric acetate eliminates the charge-transfer interaction. The spectrum of the resulting species is similar to that of the oxidized enzyme but less resolved and blue shifted by 10 nm. The dependence on pH of the absorbance associated with the thiolate to FAD charge-transfer interaction in native two-electron reduced glutathione reductase is biphasic, with pK values at approximately 4.8 and 7.4. By analogy with glyceraldehyde-3-phosphate dehydrogenase and papain, these data indicate that the thiolate is stabilized by an adjacent basic residue. The pK 7.4 is associated with the titration of the base to give the ion pair, and the pK of 4.8 is associated with the titration of the thiolate. Unlike lipoamide dehydrogenase, glutathione reductase is sufficiently stable to allow titration with dithionite at pH 3.7. The spectrum at this pH is essentially the same as that of the monoalkylated derivative treated with phenylmercuric acetate. The changes with pH are completely reversible.

Glutathione reductase (EC 1.6.4.2) and lipoamide dehydrogenase (EC 1.6.4.3) are very similar flavoproteins, each catalyzing a specific pyridine nucleotide–disulfide oxidoreduction (Williams, 1976). Both structural and mechanistic similarities have been extensively documented; thus each contains an oxidation–reduction active cystine residue (Searls & Sanadi, 1960a,b; Massey & Veeger, 1960, 1961; Black & Hudson, 1961; Massey & Williams, 1965) which is located in a highly homologous section of the polypeptide chain (Jones & Williams, 1975). Two-electron reduction of either enzyme produces a spectrally characteristic red intermediate (Searls & Sanadi, 1960a,b; Massey & Veeger, 1960, 1961; Black & Hudson, 1961; Massey & Williams, 1965) which is a charge-transfer complex between a thiolate anion (as the donor) and oxidized flavin (as the acceptor) (Searls et al., 1961; Kosower, 1966; Massey & Ghisla, 1974). A base in the active site accepts the second proton (Matthews & Williams, 1976; Matthews et al., 1977). In catalysis, these enzymes cycle between the oxidized (E)[1] and the two-electron reduced (EH_2) states (Massey et al., 1960).



Catalysis by lipoamide dehydrogenase involves a sequential flow of two electron equivalents from dihydrolipoamide to the active center disulfide to FAD to NAD^+ (Searls & Sanadi, 1960a,b; Thorpe & Williams, 1976a,b). In glutathione reductase, this sequence is reversed, beginning with NADPH and ending with thiol–disulfide interchange between the ac-

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[1] Abbreviations used: TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; SE-Sephadex, sulfoethyl-Sephadex; E, EH_2, and EH_4 refer to the oxidized, two-electron, and four-electron reduced enzyme species, respectively; EHR, two-electron reduced alkylated derivative; PhHgOAc, phenylmercuric acetate; AAD^+, aminopyridine adenine dinucleotide; thio-NAD^+, thionicotinamide adenine dinucleotide; STI, soybean trypsin inhibitor; GSSG, oxidized glutathione.

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