Production of a Covalent Flavin Linkage in Lipoamide Dehydrogenase

REACTION WITH 8-Cl-FAD*

Edwin G. Moore, Emilio Cardemil,‡ and Vincent Massey
From The University of Michigan, Department of Biological Chemistry, Ann Arbor, Michigan 48109

A method is described for preparation of apolipoamide dehydrogenase which gives quantitative removal of FAD. Active holoenzyme can be reconstituted by incubation with FAD. Reconstitution of apoenzyme with 8-Cl-FAD results in the fixation of most of the flavin to the protein in a covalently bound form. The portion noncovalently bound was shown to be unmodified 8-Cl-FAD. The covalently bound flavin has an absorption spectrum quite different from that of 8-Cl-FAD. It has a single band in the visible with a maximum at 459 nm (extinction coefficient of 22 mmM{sup -1} cm{sup -1}) and a shoulder at 480 nm. Model reactions between 8-Cl-flavin (riboflavin or FAD) and organic thiols (thiophenol, β-mercaptoethanol, or N-acetylcyesteine) give products with spectra which are similar to that of FAD covalently bound to lipoamide dehydrogenase. The products of the model reactions have a single visible band with a maximum at 480 nm (extinction coefficient of 23.6 mmM{sup -1} cm{sup -1} to 28.4 mmM{sup -1} cm{sup -1}) and a shoulder at 460 nm. The products of the model reaction and the covalently bound FAD of lipoamide dehydrogenase appear to be the result of a nucleophilic attack on the carbon at position 8 of the flavin ring by a thiolate anion, displacing the chloride. Thus, the product of the model reaction is 8-(RS)-flavin, and the product of the reaction between 8-Cl-FAD and protein probably has a cysteinyl residue covalently attached at position 8 of FAD.

Reconstitution of apolipoamide dehydrogenase with 8-Cl-FAD gives two enzyme products which are fractionated by ammonium sulfate. Enzyme fractionating between 20% and 45% ammonium sulfate is monomeric and contains covalently bound FAD. Enzyme fractionating between 55% and 75% ammonium sulfate is dimeric and contains both covalently bound FAD and noncovalently bound 8-Cl-FAD. Both protein fractions contain one FAD per protein subunit and both are active with physiological substrates with $K_m$ values for NAD and dihydrolipoamide similar to those of native lipoamide dehydrogenase. The maximum turnover rates differ dramatically. Enzyme fractionating between 55% and 75% ammonium sulfate has a $V_{max}$ which is 61 times slower than native enzyme. Enzyme fractionating between 20% and 45% ammonium sulfate has a $V_{max}$ which is 7400 times slower than native enzyme. These slower rates are partially explainable by the oxidation-reduction potentials of the modified enzymes.

Both covalently bound FAD and noncovalently bound FAD appear to reside in the native flavin binding site of the enzyme. However, once dimerization of the protein has taken place, the noncovalently bound 8-Cl-FAD cannot be induced to form a covalent bond with the protein except under protein denaturing conditions. The implications of these findings are discussed.

Lipoamide dehydrogenase is the flavoprotein component of the α-ketoglutarate dehydrogenase and pyruvate dehydrogenase multienzyme complexes, catalyzing the terminal transfer of electrons from dihydrolipoamide to NAD (1). The flavoprotein was first isolated from the α-ketoglutarate dehydrogenase complex by Massey in 1960 (2) and subsequent investigations demonstrated that a protein disulfide is an essential oxidation-reduction center in the normal catalytic mechanisms (3, 4). FAD, the other essential oxidation-reduction center, is noncovalently bound to the protein.

Besides the active center disulfide, the native protein from pig hearts contains eight cysteine residues per subunit as free thiols (6). The sequences of seven of the cysteine containing peptides have been determined (6, 7). Six of the eight residues are believed to be near the active center of the enzyme (8). Several modifications of specific cysteine residues have been found. Treatment of the native enzyme with mercurials (8–10) gives two thiols which react rapidly with p-mercuribenzoate, two which react more slowly, and two to three which react still more slowly. Copper treatment oxidizes two specific cysteiny1 residues to a disulfide (10). Alkylation of the oxidized native enzyme by iodoacetamide results in the modification of two thiols other than the copper-modified thiols; one of the alkylated thiols lies in the NAD binding site (11, 12). However, since the three-dimensional structure of lipoamide dehydrogenase has not yet been determined, the proximity of the cysteine residues to the flavin is not known.

Yet certain thiols are important to the catalytic function of lipoamide dehydrogenase. Copper modification of the enzyme results in a 20-fold reduction in physiological activity while enhancing the diaphorase activity (reduction of dichloroindophenol from NADH) by 30-fold (10). Native lipoamide dehydrogenase normally functions between oxidized and two-electron-reduced enzyme (13). Copper-modified enzyme is more readily reducible to the four-electron-reduced enzyme (8).

The cysteinyl residues of the active center disulfide are integral components of the catalytic mechanism, transferring electrons from dihydrolipoamide to FAD (13). The two-electron-reduced enzyme has reducing equivalents partitioned between FAD and the two cysteinyl residues via a charge transfer interaction between thiolate anion and flavin (14, 15). A mechanism has recently been proposed to explain the transfer of reducing equivalents between flavin and active center disulfide, including the charge transfer interaction and

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‡ On leave from Laboratorio de Bioquímica Facultad de Ciencias Químicas, Universidad de Chile.

1 Unpublished results of G. Palmer and V. Massey quoted in Ref. 5.
the formation of a covalent bond between sulfur and position 8 of the flavin. The roles of other protein thiols are not well characterized.

Site-directed reagents are powerful tools of protein chemistry which aid in elucidating the 3-dimensional structure. In this communication we report the binding of 8-Cl-FAD to apolipoamide dehydrogenase which results in covalent attachment of the flavin to the protein by a nucleophilic displacement of the chloride in position 8 of the flavin. The probable identity of the attacking nucleophile as a protein thiol is discussed.

EXPERIMENTAL PROCEDURES

Materials—Lipoamide dehydrogenase was isolated from pig hearts as previously described (13), or was purchased from Miles Laboratory, Inc. and further purified as described by Matthews et al. (6). Both preparations were physically and enzymatically identical. Enzyme concentrations are expressed on a per subunit basis of molecular weight 52,900 with one FAD per subunit (extinction coefficient at 455 nm of 11.3 M\(^{-1}\) cm\(^{-1}\) ) (17).

FAD (grade III) and riboflavin were purchased from Sigma; 8-Cl-riboflavin was synthesized by the method of Labenbo (18). The 8-Cl-riboflavin was purified by first crystallizing from absolute methanol and water. The dried crystals were redissolved in concentrated hydrochloric acid and treated with decolorizing charcoal. The charcoal was removed and the crystallized flavin was induced by addition of 5 volumes of hot water. Crystals were dried and stored, being protected from light at all stages. Synthesized 8-Cl-riboflavin had the following chemical composition (Spang Microanalytical Laboratories):

| 8-Cl-riboflavin  |
|------------------|
| Calculated: C 48.42 H 4.33 Cl 8.93 |
| Found: C 48.51 H 4.34 Cl 8.92 |

8-Cl-riboflavin was converted to 8-Cl-FAD by the partially purified FAD synthetase complex from E. coli ammonium acetate by the general procedures of Spencer et al. (19). 8-Cl-riboflavin was purified from residual 8-Cl-riboflavin by DE52 (Bio-Rad) and P2 (Whiseat) chromatography (cf Ref 19). The purity of the FAD was judged by thin layer chromatography on silica gel plates developed in 1-butanol/acetic acid/water, 12:3:5 (v/v). 8-Phenylthio-8-(N-acetylcycteine)lumiflavin, N-acetylcycteine lumiﬂavin derivatives, and 8-Cl-hydroxy-8-(N-acetylcycteine)lumiflavin were developed by reacting 8-Cl-riboflavin or 8-Cl-FAD with thiophenol (Aldrich), with N-acetylcycteine (Nutritional Biochemicals), or with β-mercaptoethanol (Matheson, Coleman, and Bell), respectively. The reaction was followed spectrophotometrically to completion. The FAD derivatives were purified by Sephadex G-15 chromatography. 8-Phenylthio-8-Cl-riboflavin, used for NMR for chemical composition determination, was purified by repeated crystallization from methanol/water solutions.

Guandine hydrochloride (ultrapure grade) and ammonium sulfate (special enzyme grade) were purchased from Schwarz/Mann. Sodium dithionite was purchased from J. T. Baker Chemical Co. Dithiothreitol (Sigma grade) was purchased from Sigma. All other reagents were of the best grade commercially available.

Methods—Apolipoamide dehydrogenase was prepared by a 2 min incubation of native lipoamide dehydrogenase with 5 mM guanidine in 0.1 M potassium phosphate, 0.3 mM EDTA, pH 7.6. The guanidine and FAD were removed from the apoprotein by passing over a Sephadex G-25 column. Typically, 10 mg to 15 mg of native lipoamide dehydrogenase in 1.0 ml of 0.1 M potassium phosphate, 0.3 mM EDTA, pH 7.6, on ice, was added to 1.0 ml of 0.1 M guanidine, 0.1 M potassium phosphate, 0.3 mM EDTA, pH 7.6, on ice, allowed to incubate for 2 min on ice, and applied to a Sephadex G-25 column (35.5 × 38 cm). The Sephadex G-25 column was equilibrated with 0.1 M potassium phosphate, 0.3 mM EDTA, pH 7.6, at 4°C. The eluate was collected in 5 ml fractions. The protein fractions were located by measuring optical absorbance at 280 nm, and were pooled for further experiments. The apoprotein is unstable above 15°C as previously reported (20). Even at lower temperatures, the protein often became turbid. The formation of turbidity could be reduced by addition of bovine plasma albumin to the apoprotein. Alternatively, incubation of the apoprotein at 25°C for 10 min resulted in some precipitation, especially when the initial protein concentrations were greater than 0.3 mg/mL. This heat treatment eliminated the slow subsequent formation of turbidity. The apoprotein which remained had properties which were indistinguishable from apoprotein which had not been heat-treated. Unless specifically stated, apoprotein was prepared in the absence of bovine plasma albumin and without heat treatment.

Dithionite titrations were performed anaerobically in all glass titration apparatus by the method of Burleigh et al. (21) as modified by Lambeth and Palmer (22). Solutions were made anaerobic by 10 cycles of vacuum followed by equilibration with prepurified nitrogen. Dithionite solutions were standardized with lumiflavin acetate using an extinction coefficient of 12.5 M\(^{-1}\) cm\(^{-1}\) at 445 nm for oxidized lumiflavin acetate.

The oxidation-reduction potential of free flavins was measured directly against a platinum electrode with a calomel electrode as a reference. The oxidation-reduction potential of the components of a Cary 118 or a Cary 17 spectrophotometer. The spectra in Fig. 8 were obtained with a computer-controlled digital spectrophotometer which uses the optical system of a Cary 118 or a Cary 17 spectrophotometer for the purposes of this interface between the spectrophotometer and the computer, and the data control program were obtained from On Line Instrument Systems (Athens, Georgia). The absolute absorption spectrum was recorded before and after each addition of dithionite to the solution. The raw data (301 points per spectrum at 1 nm intervals) were corrected for baseline and stored on magnetic tapes. Difference spectra were generated arithmetically from the stored data and corrected for dilution.

Fluorescence spectra were recorded with a ratio spectrophotometer constructed by Mr. Gordon Ford and Dr. David Ballou of this department. The instrument uses a xenon lamp source and gives expected excitation spectra for a series of dyes in the 250 nm to 650 nm wavelength range. The instrument was sensitive to detection of less than 2.7 nm lumiflavin acetate. NMR spectra were recorded with a Varian model T60 NMR spectrometer. The concentration of thiols was standardized by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (24). Protein concentrations were determined by the microbiuret method (25).

RESULTS AND DISCUSSION

Preparation and Properties of Apolipoamide Dehydrogenase—Replacement of flavins in flavoproteins with flavin derivatives for subsequent mechanistic studies requires apoprotein preparations which are free of all native flavin and which can be reconstituted to native enzyme. Removal of FAD by the 5 M guanidine procedure described under "Methods" yields apolipoamide dehydrogenase which is quantitatively and reproducibly free of FAD, unlike the previously published acid ammonium sulfate procedure of Kalse and Veeger (26) which leaves 5 to 10 % residual FAD under optimal conditions. Apoenzyme prepared by the 5 M guanidine procedure can be reconstituted with FAD and dithiothreitol to give a product which has a spectrum and which has physiological activity (Table I) identical to that of native enzyme (13). The extinction coefficient at 455 nm is 10.6 M\(^{-1}\) cm\(^{-1}\) based on protein content by biuret determination and is 11.3 M\(^{-1}\) cm\(^{-1}\) based on dithionite titration, compared with 11.3 M\(^{-1}\) cm\(^{-1}\) for native enzyme. However, only 30% to 40% of the apoprotein is reconstitutable. Most of the protein not reconstituted is readily removed by ammonium sulfate fractionation.

Apoprotein prepared by the 5 M guanidine procedure has physical properties similar to apoprotein prepared by the acid ammonium sulfate procedure. The tryptophan fluorescence of apolipoamide dehydrogenase has a maximum emission at 334 nm which extended at 280 nm similar to that previously reported (27) for the acid ammonium sulfate prepared apoprotein. Furthermore, when the apoprotein is subjected to guanidine treatment as shown in Fig 1, the emission maximum is shifted
Fig. 1 (left). Perturbation of tryptophan fluorescence of apo-
lipoamide dehydrogenase by guanidine. A stock solution of apo-
enzyme was prepared in 0.1 M potassium phosphate, 0.3 mM EDTA containing
varying concentrations of guanidine as follows: curve A, no guanidine;
curve B, 0.46 M guanidine; curve C, 0.96 M guanidine; curve D, 1.44
M guanidine; curve E, 2.90 M guanidine; and curve F, 2.58 M guanidine.
The final pH of each solution was maintained at pH 7.0. The tempera-
ture was 15°C. Excitation was at 280 nm. The emission spectra were
recorded immediately upon addition of guanidine. Upon removal of
the guanidine by dialysis, the emission maximum was shifted back to
334 nm.

Fig. 2 (center and right). Time course of activity recovery during
reconstitution of apolipoamide dehydrogenase. Apolipoamide dehy-
rogenase was prepared as described under “Methods” in the pres-
ence of bovine plasma albumin. The buffer was 0.1 M potassium
phosphate, 0.3 mM EDTA, pH 7.6. The temperature was 14°C. The concentration of FAD was 1.9 μM and the concentration of apo-
enzyme was 2 μM. Assay conditions were those of Massey et al. (13) using 0.16
mM dihydrolipoamide and 0.16 mM NAD. Percentage recovery of
native activity is based on the quantity of native enzyme used in the
apoenzyme preparation. A, the effect of dithiothreitol on reconsti-
tution using no dithiothreitol, [A] 0.9 mM dithiothreitol, [C] and 9 mM
dithiothreitol. ▲. Reconstitution with 9 mM dithiothreitol repre-
sents two different preparations of apoenzyme (closed and open triangles)
using the same preparation and reconstitution conditions. B, the
effect of prior incubation of apoenzyme with dithiothreitol before
addition of FAD. ○, apoenzyme was incubated with 9 mM dithioth-
reitol for 21 h at 14°C prior to addition of FAD. ▲, apoenzyme was
incubated with 9 mM dithiothreitol for 21 h at 14°C. The protein was
then dialyzed against four changes of 35 volumes each of 0.1 M
potassium phosphate, 0.3 mM EDTA, pH 7.6 at 3-h intervals. The
measured dithiothreitol concentration after dialysis was less than 5
μM. Reconstitution was initiated by addition of FAD, ○; or addition
of 9 mM dithiothreitol and FAD, ▲.

Table I
Steady state kinetics of dihydrolipoamide plus NAD-catalyzed
reaction under the same conditions as reported by Massey et al.
(13)

| Enzyme                  | K_M (mM) | kcat (mM/min) | V_max (μmol NADH/min/mg enzyme) |
|-------------------------|----------|---------------|--------------------------------|
| Native                  | 0.40     | 0.50          | 42,220                         |
| Native (commercially prepared) | 0.41 | 0.51          | 41,700                         |
| Reconstituted with FAD as in the legend to Fig. 1 | 0.38 | 0.41          | 34,500                         |
| AS I                    | 0.12     | 0.09          | 5.7                            |
| AS II                   | 0.12     | 0.16          | 600                            |
| Reconstituted with 8 phenylthio-
  lipoamide as in the legend to Fig. 12 | 0.26 | 0.33          | 140                            |

The abbreviations used are: ANS, 8-anilinonaphthalene-1-sulfonic
acid; AS I and AS II are the fractions from reconstitution of apo-
enzyme with 8-Cl-FAD which fractionate between 20% and 40% am-
onium sulfate and 55% and 78% ammonium sulfate, respectively.

The only major difference between the two preparations of
apolipoamide dehydrogenase is that reconstitution to native
enzyme with apoprotein prepared by the 5 M guanidine
method requires dithiothreitol for maximum recovery,
whereas apoprotein prepared by the acid ammonium
sulfate method does not (26). The requirement of dithiothreitol
for reconstitution is shown by the rate of activity recovery in Fig.
2A. Upon addition of FAD alone, 6% of the initial native
activity was recovered. When FAD and dithiothreitol were
added, more than 30% of the initial native activity was
recovered. The specific activity of reconstituted enzyme was
the same as native enzyme. Thus, the incomplete recovery of
activity appears to be due to the presence of nonreconstitut-
ible protein. The time course of activity recovery is sigmoidal
(Fig. 2A) and the degree of sigmoidicity is dependent upon
the concentration of dithiothreitol added. When the apopro-
tein is incubated with 10 mM dithiothreitol for 21 h prior to
addition of FAD (Fig. 2B), the recovery pattern is exponential
and the half-time for activity recovery is shortened from 2.75
h without prior dithiothreitol incubation to 0.85 h when apo-
protein is incubated with dithiothreitol prior to addition of
FAD. Thus, dithiothreitol serves to modify the apoenzyme in
some specific way prior to activity recovery, presumably by
reduction of a disulfide which was oxidized during apoenzyme
preparation. Using dihydrolipoamide instead of dithiothreitol
leads to precipitation of the apoprotein rather than reconsti-
tution, suggesting that dithiothreitol does not reduce the
active center disulfide of the apoenzyme. Dithiothreitol is a
poor substrate for lipoamide dehydrogenase. A second role of
dithiothreitol is indicated in Fig. 2B. Apolipoamide dehydrogenase which was incubated with 10 mM dithiothreitol but no FAD for 21 h, was dialyzed against buffer to remove the dithiothreitol, and then added to FAD. The pattern of reconstitution still gives an exponential recovery of activity, however the amount of recoverable activity is reduced. When 10 mM dithiothreitol is added back to the dialyzed preparation, the time course of activity recovery is the same as that before dialysis. Dithiothreitol must therefore serve two roles in activity recovery.

Reconstitution of Apolipoamide Dehydrogenase with 8-Cl-FAD—8-Cl-FAD has a chemical composition the same as FAD except that the methyl group in position 8 of the isoalloxazine ring has been replaced by chlorine. The visible spectrum of 8-Cl-FAD (Fig. 3) is similar to that of FAD with maxima at 448 nm (extinction coefficient of 10.6 mM\(^{-1}\) cm\(^{-1}\)) and at 364 nm (extinction coefficient of 8.9 mM\(^{-1}\) cm\(^{-1}\)). When apolipoamide dehydrogenase is reconstituted with 8-Cl-FAD in the absence of dithiothreitol, an enzyme-bound flavin results which is spectrophotometrically quite different from the starting 8-Cl-FAD. The long wavelength transition of the product is shifted to longer wavelengths and the maximum extinction is enhanced, while the 360 nm band is substantially reduced. Addition of 8-Cl-FAD to native lipoamide dehydrogenase does not produce any spectrophotometric changes.

The product of reconstitution with 8-Cl-FAD was purified from unreacted apoenzyme by ammonium sulfate fractionation. Unreacted apoenzyme was found to precipitate between 0% and 20% ammonium sulfate giving a white insoluble precipitate. Further fractionation with ammonium sulfate yielded two yellow fractions; one precipitated between 20% and 45% ammonium sulfate and will be labeled AS I, the second precipitated between 55% and 75% ammonium sulfate (AS II) as does native lipoamide dehydrogenase. The two ammonium sulfate fractions were redissolved in buffer and dialyzed. The spectrum of each of the protein fractions is shown in Fig. 3. Each fraction has a spectrum which differs dramatically from that of 8-Cl-FAD. The visible band of AS I is red-shifted from that of 8-Cl-FAD with a maximum extinction at 459 nm. The 360 nm band is absent. The spectrum of AS II is intermediate between that of 8-Cl-FAD and that of AS I.

The nature of protein-bound flavin species is further elucidated by trichloroacetic acid precipitation of the protein.

When native lipoamide dehydrogenase is precipitated with trichloroacetic acid, the flavin is quantitatively released to the supernatant. A white protein precipitate is obtained which when redissolved in buffered guanidine gives a solution with only a protein spectrum (maximum extinction at 276 nm with no visible absorption). When AS I is precipitated with trichloroacetic acid, the supernatant is colorless. The redissolved protein precipitate contains all of the flavin. Trichloroacetic acid precipitation of AS II (Fig. 4) gives part of the flavin released to the supernatant and part of the flavin precipitated with the protein. Furthermore, the flavin released to the supernatant has a spectrum identical to that of 8-Cl-FAD, while the flavin in the protein precipitate of AS II has a spectrum similar to that of AS I flavin. Thus, while FAD in native lipoamide dehydrogenase is noncovalently bound and released by trichloroacetic acid denaturation, the FAD in AS I is covalently bound to the protein. AS II has approximately one-half of its FAD covalently bound to the protein.

Reaction of 8-Cl-flavin with Organic Thiols—8-Dimethylaminoflavin (32, 33) and the paraquinoid from of R-hydroxyflavin (32, 33) and the paraquinoid from of R-hydroxyflavin (32, 33) and the paraquinoid from of R-hydroxyflavin (32, 33) and the paraquinoid from of R-hydroxyflavin (32, 33) have spectral features resembling the spectrum of AS I. However, both differ from AS I in that each has a second peak near 310 nm, and each has a significantly greater maximum extinction than AS I. The phenolic form of 8-hydroxy-flavin has a maximum extinction at 435 nm. Additional evidence against the involvement of a protein hydroxyl or a protein amine function comes from model studies where ethylamine at pH 7.6 and phenol at pH 9.5 were added to 8-Cl-riboflavin giving results very different from the AS I spectrum. Addition of ethylamine resulted in loss of extinction at 450 nm and at 360 nm with the formation of a 315 nm band. Phenol gave similar results to ethylamine. When an organic thiol, such as β-mercaptoethanol, thiophenol, thioglycollic acid, or N-acetylcycteine, is added to 8-Cl-riboflavin or 8-Cl-FAD, the resulting spectrum is very similar to AS I (Fig. 5).
The spectrum of the product of 8-Cl-FAD with thiophenol has a maximum extinction at 478 nm (extinction coefficient of 23.6 $\text{m}^{-1} \text{cm}^{-1}$) and a shoulder at 459 nm (extinction coefficient of 22.3 $\text{m}^{-1} \text{cm}^{-1}$). The extinction coefficients of the S-phenylthio-FAD derivatives were determined by two methods. By the first method the extinction coefficient was determined by dithionite titration, assuming a two-electron reduction of flavin (see below). By the second method the extinction coefficient was determined by the stoichiometry of the 8-Cl-FAD reaction with thiophenol, assuming 1 mol of thiophenol reacts with 1 mol of 8-Cl-FAD. Both methods were in agreement. No peak at 310 nm was observable when any of the above thiols with 8-Cl-flavin are quantitatively similar to the spectrum of 8-methylthio-riboflavin (36). The spectra of the products of the reaction of organic thiols with 8-Cl-flavin are quantitatively similar to the spectrum of 8-methylthio-riboflavin. The spectral similarity of AS I and of products of the reaction of organic thiols with 8-Cl-flavin indicates that AS I must be a result of the reaction between a protein thiol and 8-Cl-FAD.

The specific nature of the reaction of thiols with 8-Cl-FAD was characterized by using the above model reactions. The product of the reaction between thiophenol and 8-Cl-riboflavin was three times crystallized from aqueous solution by addition of methanol. The spectrum of the product was unchanged by repeated crystallization. The chemical composition of 8-Cl-riboflavin and of the product of thiophenol and 8-Cl-riboflavin were consistent with a stoichiometric replacement of chloride by sulfur; 8-Cl-riboflavin was 8.92% (w/w) chloride (6.82% theoretical). An NMR spectrum of the product of 8-Cl-riboflavin and thiophenol was consistent with the replacement of chloride by thiol. The product of the reaction between 8-Cl-riboflavin and thiophenol is 8-phenylthio-riboflavin. A likely mechanism for the formation of 8-phenylthio-riboflavin is the nucleophilic displacement of chloride by a thiolate anion.

This mechanistic prediction is confirmed by the reactivity of 8-mercaptoethanol, of thiophenol in aqueous buffer, and of thiophenol in 10% ethanol and buffer with 8-Cl-riboflavin as shown in Fig. 7. The reaction with each of the thiols was first...
order in 8-Cl-riboflavin and first order in thiol concentration. A plot of the second order rate constants as a function of pH reflects the pK of the organic thiol. The pK of β-mercaptoethanol calculated from data in Fig. 7 was 9.37 (reported values of 9.4 to 9.7 (37)), and the pK value for thiophenol is 6.9 (reported value of 6.5 (37)). The pK of thiophenol is known to be alkaline shifted by the addition of alcohol (37). The observed value in 10% ethanol, 10% dimethylformamide (Fig. 7) is 7.1. Hence, the reaction between an organic thiol and 8-Cl-riboflavin proceeds by a nucleophilic displacement of chloride by attack of a thiolate anion. The flavin which is covalently bound to the protein in AS I and in AS II is presumably produced in a similar fashion by a nucleophilic displacement of chloride from 8-Cl-FAD by a protein thiol yielding 8-cysteinyl-FAD lipoamide dehydrogenase.

Properties of AS I and of AS II—The extinction coefficient of AS I was determined by dithionite titration. Hence, a knowledge of the number of electrons involved in the reduction is required. Reduction of the native enzyme is a four-electron process with two electrons required to reduce FAD and two electrons to reduce the active center disulfide (13). Fig. 8a gives a reduction of AS I in the presence of methyl viologen (oxidation-reduction potential of −440 mV (38)) which was added to indicate the reduction endpoint of the flavoprotein. The flavin is reduced first (Fig. 8a, curves I through 4) giving isobestic points at 391 nm and 525 nm. The fully reduced AS I spectrum (dashed line in Fig. 8a) was obtained by computer fit, assuming curve 4 in Fig. 8a to be a composite of fully reduced flavin and partially reduced methylviologen. The spectrum of fully reduced AS I is typical of the spectra of fully reduced flavins. A total of 12.8 nmol of dithionite was required to reduce AS I shown in figure 8a. Thus, assuming AS I reduction to be a two-electron process, an extinction coefficient of 21.5 mM⁻¹ cm⁻¹ at 459 nm is calculated.

The assumption of a two-electron reduction is confirmed by an oxidation-reduction titration of AS I as shown in Fig. 8b. Since most proteins and their bound prosthetic groups do not directly communicate with electrodes, a solution of AS I and riboflavin was used for the determination. Titration of this mixture with dithionite would give a partitioning of reducing equivalents between riboflavin and AS I depending upon the oxidation-reduction potential of AS I (E_{1/2}^\text{AS I}) and of riboflavin (E_{1/2}^\text{Flav}) and depending upon the number of electrons required for reducing each n_{AS I} and n_{Flav}, respectively. The relation describing the partitioning is:

\[
\ln \left( \frac{c_{\text{Flav}}}{c_{\text{AS I}}} \right) = \left( E_{1/2}^\text{Flav} - E_{1/2}^\text{AS I} \right) \frac{n_{AS I}}{RT} + \frac{n_{Flav}}{n_{AS I}} \ln \left( \frac{c_{\text{Flav}}}{c_{\text{AS I}}} \right) \tag{1}
\]

where c_{\text{Flav}}, c_{\text{AS I}}, E_{1/2}^\text{Flav}, and E_{1/2}^\text{AS I} are the concentrations of oxidized and of reduced AS I and riboflavin, respectively. F is the faraday, R is the gas constant, and T is the absolute temperature.Titration of the AS I-riboflavin mixture is shown in Fig. 8b as difference spectra of the observed spectrum after addition of dithionite minus the fully reduced spectrum of the mixture. Inspection of the difference spectra shows a migrating isobestic point which is initially at 385 nm and is shifted in the final stages of titration to 340 nm. When riboflavin alone is titrated to reduce an isobestic point at 334 nm is observed, whereas AS I has an isobestic at 391 nm (Fig. 8a).

Thus, in the AS I-riboflavin mixture reducing equivalents must first be taken up by AS I suggesting that the oxidation-reduction potential of AS I is more positive than riboflavin. Riboflavin has an oxidation-reduction potential of −207 mV and an n of 1.97 (reported value of −210 mV and 2, respectively (38)). The value of E_{1/2}^\text{Flav} and of n_{AS I} can be calculated directly from Equation 1 and the data in Fig. 8b, since the difference spectra are proportional to the sum of c_{\text{Flav}}/c_{\text{AS I}} and c_{\text{Flav}}/c_{\text{AS I}}. Using the difference spectrum of AS I obtained from Fig. 8a, and the difference spectrum of riboflavin, obtained from a separate titration, the spectra in Fig. 8b were computer-fitted to the individual difference spectra of AS I and of riboflavin to give the composition of c_{\text{Flav}} and of c_{\text{AS I}} at each stage of titration in Fig. 8b. The residuals after fitting were better than 0.01 A units for each of the curves. Knowledge of the total concentrations of riboflavin and of AS I allow calculation of the data according to Equation 1 plotting ln(c_{\text{Flav}}/c_{\text{AS I}}) versus ln(c_{\text{Flav}}/c_{\text{AS I}}) as given in the inset in Fig. 8b. The calculated value of E_{1/2}^\text{AS I} is −177 mV and of n_{AS I} is 1.86. Thus, reduction of AS I is a two-electron process and the extinction coefficient determined in Fig. 8, A and B is 21.5 mM⁻¹ cm⁻¹ and 22.0 mM⁻¹ cm⁻¹, respectively, at 459 nm. The results in Fig. 8a also indicate that dithionite can only reduce the flavin and not the active center disulfide since methylviologen reduction begins immediately after flavin reduction is completed. Furthermore, the oxidation-reduction potential of the active center disulfide...
must be more negative than that of covalently bound flavin since no reducing equivalents are transferred from the flavin to the disulfide as evidenced by $n_{AS}$ of 1.86. While catalytic turnover studies (see below) indicate that the active center disulfide and covalently bound flavin in AS I are kinetically competent, the above potentiometric measurements show that the oxidation-reduction potential of the active center disulfide must be at least 50 mV more negative than -177 mV in agreement with the native enzyme studies of Matthews and Williams (19) where the oxidation-reduction potential was -280 mV for $E/\text{EH}_2$ and -346 mV for $E\text{H}_2/\text{EH}_2$ at pH 7.0.

The oxidation-reduction properties of riboflavin, of 8-Cl-FAD, of 8-phenylthio-FAD, and of 8-(N-acetylcysteinyl)-FAD were determined by direct potentiometric titration using a standardized platinum-calomel electrode. The values of the oxidation-reduction potentials and the values of the number of electrons involved in the reduction process are given in Table II.

An extinction coefficient for AS II can be calculated based on the relative amounts of covalently and noncovalently bound flavin found from trichloroacetic acid precipitation (Fig. 4). The noncovalently bound flavin was shown to be 8-Cl-FAD by reaction of the neutralized trichloroacetic acid supernatant with thiophenol. The result was 8-phenylthio-FAD. Using 26 mm$^{-1}$ cm$^{-1}$ at 480 nm for the extinction coefficient of the redissolved trichloroacetic acid precipitate of AS II (see below) and using 10.6 mm$^{-1}$ cm$^{-1}$ at 448 nm for the extinction coefficient of the trichloroacetic acid supernatant of AS II, the relative amount of covalently bound and of noncovalently bound flavin was nearly equivalent in AS II reconstituted at pH 7.6 in eight preparations. An apparent extinction coefficient of 17 mm$^{-1}$ cm$^{-1}$ is calculated for AS II.

The flavin content of AS I and of AS II was calculated by protein determination by the microbiuret method (25). Comparing the concentration of flavin to the concentration of protein gave a value of 0.75 flavin per 52,000 daltons of protein for AS I and a value of 0.8 flavin per 52,000 daltons of protein for AS II. The molecular weights of AS I and of AS II were determined on a Sephadex G-150 column (1.9 cm x 55 cm) using native lipoamide dehydrogenase, bovine serum albumin (monomer and dimer), and myoglobin as standards. The exclusion molecular weight of the column was 200,000 daltons using blue dextran. From these determinations, AS I is a monomer (54,000 daltons). AS II is a dimer (90,000 daltons) analogous to the native enzyme (17). Hence, the AS I flavoprotein is monomeric, suggesting that the AS II dimer is a heterogeneous dimer with one subunit containing a covalently bound FAD and the other subunit containing a noncovalently bound FAD.

**Kinetics of Formation of Covalently Bound FAD—**Two possible mechanisms are proposed to account for the formation of AS I and AS II as seen in Scheme 1.

$$
\begin{align*}
K & \quad E\text{-SH} + 8\text{-Cl-FAD} \rightleftharpoons E\text{-SH} \cdot 8\text{-Cl-FAD} \\
E\text{-SH} \cdot 8\text{-Cl-FAD} & \rightleftharpoons E\text{-SH} \cdot 8\text{-Cl-FAD} \\
2E\text{-SH} \cdot 8\text{-Cl-FAD} & \rightleftharpoons E\text{-SH} \cdot 8\text{-Cl-FAD} \rightleftharpoons E\text{-SH} \cdot 8\text{-Cl-FAD} \\
(E\text{-SH} \cdot 8\text{-Cl-FAD})_2 & \rightleftharpoons (E\text{-SH} \cdot 8\text{-Cl-FAD}) \cdot (E\text{-SH} \cdot 8\text{-Cl-FAD}) \\
(E\text{-SH} \cdot 8\text{-Cl-FAD})_2 \rightleftharpoons (E\text{-SH} \cdot 8\text{-Cl-FAD}) \cdot (E\text{-SH} \cdot 8\text{-Cl-FAD}) \\
\end{align*}
$$

**Scheme 1A**

Both Scheme 1A and Scheme 1B involve the formation of monomeric E-S-FAD which is AS I. The schemes differ in the mechanism of AS II, (E-SH $\cdot$ 8-Cl-FAD) (E-SH $\cdot$ 8-Cl-FAD) $\cdots$, formation. In Scheme 1A a homogeneous dimer is formed with each subunit containing noncovalently bound 8-Cl-FAD. One of the 8-Cl-FAD molecules subsequently undergoes reaction to give a heterogeneous dimer of covalently and noncovalently bound FAD. Scheme 1B gives heterogeneous dimer directly with subsequent protein conformational change to give AS II.

The time course of covalent bond formation is spectrophotometrically observable due to the large extinction changes at 480 nm (Fig. 3). The nonexponential time course for two different enzyme concentrations, shown in Fig. 9, are best fitted by Scheme 1B. Since variations in the concentration of 8-Cl-FAD from 6 $\mu$m to 24 $\mu$m did not alter the apparent rate of covalent bond formation, the binding of 8-Cl-FAD to apoenzyme was assumed to be a rapid equilibrium. The apparent rate of formation was influenced by the total enzyme concentration. The value of the rate of covalent bond formation ($k_3$), the value of the rate of conformational change of the dimer ($k_3$), and the value of the equilibrium dimerization constant ($D$) were obtained by nonlinear least squares fits to the time course in Fig. 9. Values obtained at 4.1 $\mu$m and at 5.86 $\mu$m apoenzyme were 8.0 $\times$ 10$^{-5}$ min$^{-1}$ and 1.2 $\times$ 10$^{-5}$ min$^{-1}$ for $k_3$, 1.2 $\times$ 10$^{-5}$ min$^{-1}$ and 1.1 $\times$ 10$^{-5}$ min$^{-1}$ for $k_3$, and 4.1 $\times$ 10$^{-7}$ M and 4.2 $\times$ 10$^{-7}$ M for $D$, respectively. The solid lines in Fig. 9 are calculated from Scheme 1B with the fitted values. The value of the conformational change ($k_3$) is four times faster than Scheme 1A.

![Fig. 9 (left). Kinetics of covalent bond formation.](http://www.jbc.org/)

![Fig. 10 (right). Perturbation of the protein fluorescence of apolipoamide dehydrogenase, of native lipoamide dehydrogenase, and of AS II by guanidine.](http://www.jbc.org/)
smaller than the rate of reconstitution of FAD with apoprotein of lipoamide dehydrogenase shown in Fig. 2B. The fitted values of the dimerization constant are in agreement with previously reported values (30). At equilibrium, the concentration of covalently bound FAD formed was 2.2 μM, 2.75 μM, 2.56 μM, and 3.7 μM from starting total apoenzyme concentrations of 3.03 μM, 4.1 μM, 5.86 μM, and 6.86 μM, respectively.

The plausibility of Scheme 1B was substantiated by an alternative approach. Since the covalent bond formation occurs via a nucleophilic displacement mechanism, the protonated thiol will be a much poorer nucleophile. Hence, reconstitution with 8-Cl-FAD at a lower pH should yield an AS II material with much less covalently bound flavin. Apolipoamide dehydrogenase, which had been treated with dithiothreitol at pH 7.6 for 20 h at 14.5°C, was extensively dialyzed against 0.05 M potassium phosphate, 0.05 M sodium citrate, 0.3 M EDTA, pH 5.85 to remove the dithiothreitol and to lower the pH. The apoprotein was then incubated with 8-Cl-FAD for 15 h at 14.5°C. The major portion of the reconstituted protein fractionated between 45% and 60% ammonium sulfate and had a spectrum with a maximum absorption at 452 nm and with a broadened second peak (maximum absorption at 350 nm). The enzyme reconstituted with 8-Cl-FAD at pH 5.85 more closely resembled the spectrum of native enzyme than did the AS II protein reconstituted with 8-Cl-FAD at pH 7.6. By trichloroacetic acid precipitation, 87% of the flavin was noncovalently bound. The enzyme reconstituted at pH 5.85 provides a means of distinguishing between Scheme 1A and Scheme 1B, since Scheme 1A predicts that upon raising the pH to 7.6 covalent bond formation will occur, whereas Scheme 1B predicts that no covalent bond formation will occur upon raising the pH. Incubation at room temperature for as long a time as 2.5 days produced no covalent bond formation as judged spectrophotometrically and by trichloroacetic acid precipitation. Thus, covalent bond formation must occur prior to dimerization as in Scheme 1B. The inability of covalent bond formation to occur in the dimer may be explained by a change in the conformation of the protein upon dimerization as has been previously suggested (39). All attempts to convert noncovalently bound 8-Cl-FAD to covalently bound FAD in pH 5.85 or in pH 5.85 reconstituted protein have failed, except under denaturing conditions in 2 M guanidine.

Presence of Covalently Bound and of Noncovalently Bound 8-Cl-FAD in the Native FAD Binding Site—The inability to convert noncovalently bound 8-Cl-FAD to covalently bound flavin in AS II raises the question of whether covalently bound flavin resides in the native FAD binding site. The first indication that covalently bound flavin and noncovalently bound 8-Cl-FAD lie in the native site comes from activity measurements given in Table I. Both AS I and AS II have physiological activity giving parallel Lineweaver-Burk plots as does native lipoamide dehydrogenase (13). The Km values for NAD and for dihydrolipoamide are slightly smaller than given by native enzyme or enzyme reconstituted with FAD. However, the maximum turnover rates differ drastically where AS II and AS I are 61-fold and 7400-fold slower than native enzyme, respectively. Slower rates might be expected on the basis of oxidation-reduction potentials (Table II) but such arguments do not explain the dramatic differences between AS I and AS II. The covalent bond between flavin and protein may hinder catalysis. In addition, the difference between AS I and AS II may be due to AS I being monomeric since monomeric native enzyme is much less active than the native dimer (41).

Measurement of the protein fluorescence perturbation in the presence of guanidine provides further evidence that the covalently bound flavin resides in the native FAD site. As previously noted, the tryptophan fluorescence of apolipoamide dehydrogenase is perturbed by guanidine denaturation. A measure of the perturbation of apoenzyme is given in Fig. 10 by a plot of the wavelength of maximum emission against guanidine concentration. Shifts in the apoenzyme emission spectrum begin at less than 0.5 M guanidine and are completed at less than 2 M guanidine. The conformation of native lipoamide dehydrogenase is thought to be stable to concentrations of guanidine less than 1.0 M (24), which is consistent with perturbation of tryptophan fluorescence of native enzyme in Fig. 10. Hence, the difference in the stability of native lipoamide dehydrogenase and its apoenzyme at less than 1.0 M guanidine must be due to stabilization of the protein conformation by FAD. Above 1.0 M guanidine, the maximum emission wavelength of native enzyme is shifted in an analogous manner to apoenzyme. Perturbation of the protein fluorescence of native enzyme is concurrent with the release of FAD as seen spectrophotometrically. The protein conformation of AS II is also protected at less than 1.0 M guanidine. The transition to denatured AS II protein requires higher guanidine concentrations (Fig. 10) than was found for native protein, suggesting that the covalently bound FAD of AS II stabilizes the protein against denaturation to a greater extent than does noncovalently bound FAD in native enzyme.

The spectrum of AS I and of AS II offer further evidence that both noncovalently bound and covalently bound FAD reside in the native FAD binding site. The visible spectrum of native lipoamide dehydrogenase is dramatically perturbed from that of free FAD. The 450 nm peak of FAD is shifted to 455 nm when bound to the protein, the 480 nm shoulder is broadened when bound to lipoamide dehydrogenase. These same features are observed when 8-(N-Acetylcysteinyl)-FAD is bound noncovalently to apolipoamide dehydrogenase, as found by reconstitution at pH 5.85. Covalently bound FAD in AS I exhibits spectral differences from its free analog, 8-(N-Acetylcysteinyl)-FAD. As I (Fig. 3) has a maximum extinction at 459 nm with a well defined shoulder at 480 nm. In all derivatives in which position 8 is protected from its free analog, 8-(N-Acetylcysteinyl)-FAD, has a maximum extinction at 480 nm (extinction coefficient of 26,400 M cm-1). In all derivatives in which position 8 is protected from its free analog, 8-(N-Acetylcysteinyl)-FAD, has a maximum extinction at 480 nm (extinction coefficient of 26,400 M cm-1). In all derivatives in which position 8 is protected from its free analog, 8-(N-Acetylcysteinyl)-FAD, has a maximum extinction at 480 nm (extinction coefficient of 26,400 M cm-1).
both covalently bound and noncovalently bound FAD derivatives bind to the native FAD binding site of the protein. However, the inability of noncovalently bound 8-Cl-FAD in AS II to react with the protein thiol poses a dilemma. This dilemma may be reconciled by proposing a conformational change in the protein upon dimerization as suggested by Veeger et al. (42) and reflected in the mechanism of AS I and AS II formation in Scheme 1B.

The reaction of 8-Cl-flavin with thiols provides a powerful tool for the investigation of protein thiols near position 8 of the flavin ring. The ease with which a strong nucleophile such as a thiolate anion undergoes this reaction is a further testament to the unusual electrophilicity of the 8 carbon of the isoalloxazine ring (43-45). Chloride at position 8 in 8-Cl-flavin merely provides a good leaving group. The unusual flavin spectrum which results from this reaction is readily distinguishable. Hence, this reaction provides a means of partially identifying the protein structure of lipoamide dehydrogenase and its proximity to the flavin ring. The identity of the cysteinyl residue which reacts with 8-Cl-FAD and the possible role this thiol might perform during normal enzyme catalysis are currently under investigation.

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