Affinity Probing of Flavin Binding Sites. 1. Covalent Attachment of 8-(Methylsulfonyl)FAD to Pig Heart Lipoamide Dehydrogenase†

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ABSTRACT: 8-(Methylsulfonyl)FAD reacts with a single cysteine residue (Cys449) in pig apolipoamide dehydrogenase to generate a flavinylated enzyme containing covalently bound 8-(cysteinyl)FAD. Competitive behavior is observed in reconstitution reactions containing both FAD and 8-(methylsulfonyl)FAD. Covalently bound 8-(cysteinyl)FAD is shielded from solvent, as judged by spectral comparison with model 8-(alkylthio)-flavins in various solvents. Flavinylated lipoamide dehydrogenase is monomeric and catalytically inactive. Cys449 is located in the interface domain, near the active site histidine (His452). As shown previously, Cys449 is oxidized when native enzyme is treated with cupric ions. Cys449 is close to the isoalloxazine ring of FAD in native enzyme, as judged by alignment of the pig sequence with the structure of the homologous enzyme from *Azotobacter vinelandii*. The residue corresponding to Cys449 in *A. vinlandii* lipoamide dehydrogenase (Val447) is about 9 Å from the carbonyl oxygen at C(2) in the pyrimidine ring of FAD. Approximation of a substituent at position 8 in FAD with Cys449 requires a 180° flip of the isoalloxazine ring as compared with its orientation in the native structure. The different flavin orientation can explain the absence of dimerization and catalytic activity. Using the same method of apoenzyme preparation, noncovalent binding was observed with 8-chloroFAD, a less reactive flavin analogue. Relatively nonspecific covalent incorporation was observed with 8-chloroFAD when apoenzyme was prepared by an older method used in previous studies with this derivative [Moore, E. G., Cardemil, E., & Massey, V. (1978) *J. Biol. Chem. 253*, 6413–6422].

Many flavoenzymes can be reversibly converted to their corresponding apoproteins under relatively mild conditions, a feature which has prompted studies using flavin analogues to probe the active site environment and/or the mechanism of catalysis (Ghisla & Massey, 1986). Analogues with a methylsulfonyl ($MeSO_2$) substituent instead of a methyl group at position 8 exhibit reduction potentials 150 mV higher than normal flavin (Moore et al., 1979) and may find application as high-potential mechanistic probes. However, recent studies show that the methylsulfonyl group undergoes a facile nucleophilic displacement reaction with various thiols (Scheme 1), accompanied by dramatic changes in the flavin absorption spectrum (Raibekas et al., 1993). The reaction is not observed with other nucleophiles, suggesting that 8-$MeSO_2$-flavin might be useful as a chemically reactive probe for cysteine residues in flavin binding sites. An analogous reaction with thiols is observed with 8-halo-substituted flavins (Moore et al., 1978), but these derivatives are about three orders of magnitude less reactive.

8-$MeSO_2$-flavin has thus far been tested as a flavin site probe only with apoflavodoxin from *Clostridium beijerinckii MP*. Noncovalent flavin binding was observed with 8-$MeSO_2$-FMN[1] (Raibekas et al., 1993), as expected, since none of the protein's cysteine residues is near the flavin site (Burnett et al., 1974). A less reactive analogue, 8-chloroflavin, has been tested with many apoflavoenzymes. Covalent incorporation was observed only with pig heart lipoamide dehydrogenase (Moore et al., 1978), electron-transferring flavoproteins (Nuallain & Mayhew, 1987; Gorelick & Thorpe, 1986), and lysine N^6-hydroxylase (Macheroux et al., 1993). In each case, attachment probably involved cysteine but the precise residue(s) were not identified.

Pig lipoamide dehydrogenase, a member of the pyridine nucleotide–disulfide oxidoreductase family, contains two identical subunits [for a recent review, see Williams (1992)]. Each subunit contains 1 mol of FAD, seven cysteine residues, and an active site cystine (Matthews et al., 1974; Otulakowski & Robinson, 1987). Although the crystal structure of the pig enzyme is not known, similar structures have been found for several bacterial lipoamide dehydrogenases (Mattevi et al., 1991, 1992, 1993). Lipoamide dehydrogenase from *Azotobacter vinelandii* exhibits 47% sequence identity with the pig enzyme, and its structure is known at the highest resolution (2.2 Å) (Mattevi et al., 1991). In this paper we show that 8-$MeSO_2$FAD reacts with a single cysteine residue (Cys449) in pig heart apolipoamide dehydrogenase to yield enzyme containing covalently bound 8-(cysteinyl)FAD. In native enzyme, Cys449 is close to the isoalloxazine ring of FAD, as judged by alignment of the pig sequence with the *A. vinlandii* structure.

EXPERIMENTAL PROCEDURES

Materials. Highly purified pig heart lipoamide dehydrogenase was obtained from Biozyme. Modified trypsin was from Promega. Protein standards used for gel filtration chromatography, *N*-acetyl-L-cysteine, NADH, and 2,6-dichloroindophenol (DCIP) were from Sigma. Phenyl-Sepharose CL-4B was purchased from Pharmacia. Guanidine hydrochloride was from Heico. 8-Chlororiboflavin was a generous gift from Dr. John Lambooy.

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; 8-$MeSO_2$FAD, 8-(methylsulfonyl)FAD; 8-$MeSO_2$-FMN, 8-(methylsulfonyl)FMN; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; DCIP, 2,6-dichloroindophenol.

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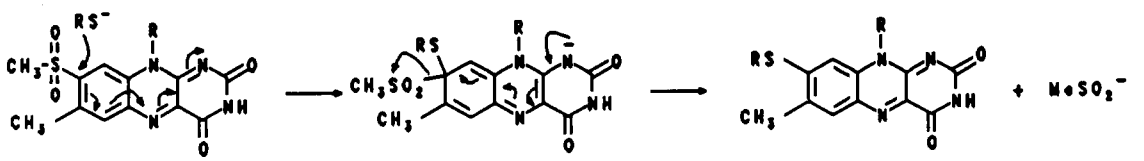
Scheme 1



Chromatography. Analysis of proteolytic fragments and purification of target peptides were performed using a Rainin gradient HPLC system equipped with a Vydac 218TP54 (C_{18}, 5-μm, 46 × 250 mm) reversed-phase column (The Separation Group) or a PolySULFOETHYL Aspartamide SCX (46 × 200 mm) HPLC column (The Nest Group). Analytical protein gel filtration studies were performed using the same system equipped with a Superose 12 column (Pharmacia). Sweet potato β-amylase, yeast alcohol dehydrogenase, pig heart lipoamide dehydrogenase, bovine serum albumin, and horse heart cytochrome *c* were used as standards. Blue dextran was used to determine the void volume. Chromatography was conducted at room temperature using 0.05 M potassium phosphate buffer, pH 7.0, containing 0.15 M sodium chloride and 0.5 mM EDTA.

Spectroscopy. Absorption spectra were recorded using a Perkin-Elmer Lambda 3B or Lambda 2S spectrophotometer. The concentration of native lipoamide dehydrogenase was calculated based on FAD absorbance at 455 nm (ϵ_{455} = 11 500 M^{-1} cm^{-1}) (Massey et al., 1962). The concentration of apolipoamide dehydrogenase was calculated on the basis of protein absorbance at 280 nm (ϵ_{280} = 40 000 M^{-1} cm^{-1}) (Van Berkel et al., 1991).

To determine the content of covalently bound 8-(cysteinyl)-FAD after reconstitution with 8-$MeSO_2$FAD, protein concentration was determined on the basis of the absorbance of the reconstituted enzyme at 280 nm after correcting for the flavin contribution. Flavin was determined using the extinction coefficient determined for the enzyme at 476 nm (ϵ_{476} = 21 900 M^{-1} cm^{-1}). The flavin contribution at 280 nm was estimated on the basis of the absorbance of free 8-(*N*-acetylcysteinyl)-FAD (ϵ_{280} = 18 900 M^{-1} cm^{-1}). 8-(*N*-Acetylcysteinyl)FAD was prepared by reacting 8-$MeSO_2$FAD (22.8 μM) with *N*-acetylcysteine (2.00 mM) in 0.1 M potassium phosphate buffer, pH 7.0, for 30 min at 25 °C. Its extinction coefficient at 280 nm was determined on the basis of a previously determined value at 480 nm (ϵ_{480} = 25 200 M^{-1} cm^{-1}) (Moore et al., 1978).

Alternatively, enzyme reconstituted with 8-$MeSO_2$FAD was precipitated by addition of 5% TCA. The protein pellet was redissolved in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 5 M guanidine hydrochloride. Protein was estimated as above. The flavin content was determined using the extinction coefficient reported for free 8-(*N*-acetylcysteinyl)FAD (ϵ_{480} = 25 200 M^{-1} cm^{-1}) (Moore et al., 1978). Similar results were obtained by either method.

The flavin content in enzyme reconstituted with 8-chloroFAD was determined after precipitating the protein with 5% TCA. Noncovalently bound 8-chloroFAD was released into the supernatant and estimated using an extinction coefficient reported for the free flavin (ϵ_{448} = 10 600 M^{-1} cm^{-1}) (Moore et al., 1978). The flavin contribution at 280 nm was estimated based on an extinction coefficient determined for free 8-chloroFAD (ϵ_{280} = 17 500 M^{-1} cm^{-1}). Covalently bound 8-(cysteinyl)FAD in the protein pellet was analyzed as described for enzyme reconstituted with 8-$MeSO_2$-FAD.

Preparation of Apolipoamide Dehydrogenase and Reconstitution with 8-MethylsulfonylFAD. The apoenzyme was prepared by the phenyl-Sepharose column chromatography method described by van Berkel et al. (1991) except the final step, elution of the apoenzyme from the column, was performed at room temperature. The apoenzyme was stored at −20 °C in 0.1 M potassium phosphate buffer, pH 7.0, containing 50% ethylene glycol and 0.5 mM EDTA (buffer A). 8-$MeSO_2$-FAD was prepared as described by Raibekas et al. (1993). For reconstitution, apoenzyme (1–2 × 10^{-5} M) in buffer A was incubated with a 4-fold excess of 8-$MeSO_2$FAD for 18–23 h at 4 °C. As will be noted, some reconstitution reactions were conducted under anaerobic conditions using reduced 8-$MeSO_2$FAD in place of the oxidized flavin. In these experiments, 8-$MeSO_2$FAD was reduced with excess (≈100-fold) NADH or sodium dithionite prior to mixing with apoenzyme. For control experiments with normal FAD, the reduced flavin was generated with dithionite. Unbound flavin was removed by passing the sample through a Sephadex G-25 gel filtration column equilibrated with aerobic 0.1 M potassium phosphate buffer, pH 7.0, containing 0.15 M sodium chloride and 0.5 mM EDTA. The sample was then concentrated using an Amicon ultrafiltration cell (PM-10 membrane).

Isolation of an 8-(Cysteinyl)FAD-Containing Peptide from Lipoamide Dehydrogenase. The reconstitution procedure described above was used to prepare samples for proteolytic digestion except that the Sephadex G-25 column buffer was changed to 0.1 M Tris-HCl, pH 8.0, containing 2 mM calcium chloride and 2 M urea (digestion buffer). For digestion, samples were mixed with modified trypsin at an enzyme to substrate (lipoamide dehydrogenase) ratio of 1:2.5 (w/w) and incubated for 18 h at 37 °C. The digest was applied to a Vydac C_{18} reversed-phase HPLC column. Elution was conducted using a linear gradient of acetonitrile (5–70%) in the presence of 0.1% trifluoroacetic acid (TFA). The 8-(cysteinyl)FAD-containing peptide fraction was identified by its absorbance at 480 nm, dried *in vacuo*, and redissolved in 50–100 μL of 5 mM potassium phosphate buffer, pH 3.0, containing 25% acetonitrile. The sample was then applied to a PolySULFOETHYL Aspartamide SCX HPLC column and eluted with a linear gradient of potassium chloride (0–0.125 M) in 5 mM potassium phosphate buffer, pH 3.0, containing 25% acetonitrile. For desalting, the purified 8-(cysteinyl)-FAD-containing peptide was reapplied to a Vydac C_{18} reversed-phase column, eluted as described above, dried *in vacuo*, and then stored at −20 °C.

Amino Acid Sequence Analysis. Purified peptides (50–100 pmol) were sequenced by Edman degradation on an Applied Biosystems 477A protein sequencer equipped with an Applied Biosystems 120A Analyzer at the Laboratory for Macromolecular Analysis at the Albert Einstein College of Medicine, Bronx, NY.

Catalytic Assays. NADH-DCIP reductase activity was measured at 25 °C under conditions (44 mM potassium phosphate buffer, pH 7.2, containing 2.0 × 10^{-4} M NADH, 4.0 × 10^{-4} M DCIP, 0.08% bovine serum albumin, and 1.2 × 10^{-3} EDTA) similar to those described by Casola et al. (1966).

Reconstitution of Apolipoamide Dehydrogenase with 8-ChloroFAD. 8-Chlororiboflavin was converted to 8-chlo-

roFAD with partially purified flavokinase/FAD synthase from *Brevibacterium ammoniagenes* (Hausinger et al., 1986).

In one set of experiments, apoenzyme was prepared by the same phenyl-Sepharose column chromatography method used in studies with 8-$MeSO_2$FAD. Conditions for reconstitution with 8-chloroFAD were modified to approximate those used by Moore et al. (1978). Apoenzyme (1.2×10^{-5} M) was incubated with a 5-fold excess of 8-chloroFAD in 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 50% ethylene glycol for 49 h at 14.5 °C. Unbound flavin was removed by gel filtration using a Sephadex G-25 column equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl and 0.5 mM EDTA. The sample was concentrated using an Amicon ultrafiltration cell.

In second set of experiments, apoenzyme was prepared by the guanidine hydrochloride method used by Moore et al. (1978). Apoenzyme (2.5×10^{-5} M) was incubated with a 3-fold excess of 8-chloroFAD for 21 h at 4 °C in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.3 mM EDTA. The sample was then treated with 5% TCA. The protein precipitate was first washed with 1% TCA and then redissolved in digestion buffer and mixed with modified trypsin at an enzyme to substrate ratio of 1:2.5. After incubation for 14 h at 37 °C, the proteolytic digest was analyzed using the Vydac C_{18} reversed-phase HPLC column procedure described above.

RESULTS

Covalent Flavinylation of Lipoamide Dehydrogenase. Apolipoamide dehydrogenase was prepared by a hydrophobic column chromatography method (van Berkel et al., 1991). The absorption spectrum observed after reconstitution of the apoenzyme with 8-$MeSO_2$FAD exhibits a single broad band in the visible region with a maximum at 476 nm plus a very pronounced shoulder around 458 nm (Figure 1A, curve 1). Denaturation of the enzyme with guanidine hydrochloride caused an overall increase in absorbance plus a shift of the maximum to 480 nm, a feature which makes the shoulder at 458 nm less pronounced (Figure 1A, curve 2). The spectrum observed after denaturation is virtually identical to that reported for 8-(*N*-acetylcysteinyl)FAD (λ_{max} = 480 nm, with a shoulder at 459 nm) (Moore et al., 1978) and readily distinguished from the spectrum of free 8-$MeSO_2$FAD (Figure 1A, curve 3) (λ_{max} = 450, 333 nm).

In a separate experiment, enzyme reconstituted with 8-$MeSO_2$FAD was precipitated with 5% TCA. No flavin was released into the supernatant. The protein pellet, redissolved in 6 M guanidine hydrochloride, exhibited an absorption spectrum similar to that observed for enzyme denatured directly with guanidine hydrochloride (data not shown).

The results show that 8-$MeSO_2$FAD had become covalently attached to the protein, probably via reaction with a cysteine residue (Scheme 1). An extinction coefficient for the flavin in the intact enzyme (ϵ_{476} = 21 900 M^{-1} cm^{-1}) was calculated using a value reported for free 8-(*N*-acetylcysteinyl)FAD (ϵ_{480} = 25 200 M^{-1} cm^{-1}) (Moore et al., 1978) and the data shown in Figure 1A. The extent of covalent incorporation varied from 0.67 to 0.76 mol of flavin per mol of protein in three separate preparations.

The Flavin Environment in Flavinylated Lipoamide Dehydrogenase. Studies with 8-(methylthio)riboflavin show that the spectral properties of flavins bearing an alkylthio substituent at position 8 are quite sensitive to solvent polarity. In water, 8-(methylthio)riboflavin exhibits a maximum at 474 nm and a shoulder around 454 nm. Addition of DMSO causes absorbance decreases at both wavelengths, but the effect is more pronounced at the longer wavelength. At intermediate DMSO concentrations (≈70%) a single broad peak is expected whereas in 100% DMSO the maximum is shifted to shorter wavelengths (λ_{max} = 452) with only a shoulder remaining at longer wavelengths (472 nm) (Figure 2).

The covalently bound, putative 8-(cysteinyl)FAD derivative in reconstituted lipoamide dehydrogenase is at least partially

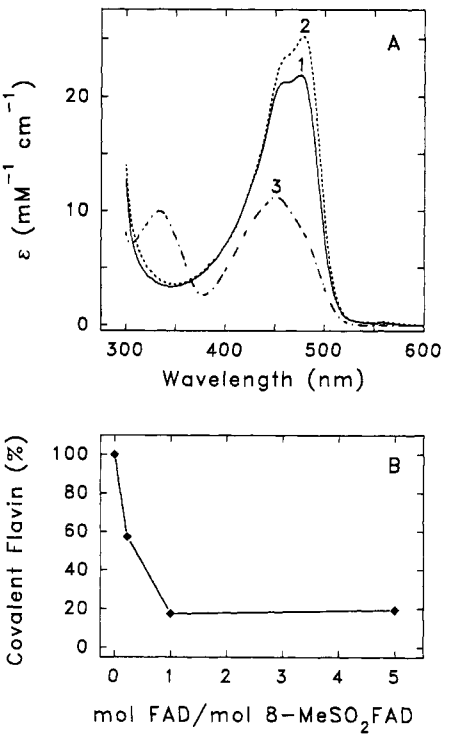


FIGURE 1: Reconstitution of apolipoamide dehydrogenase with 8-$MeSO_2$FAD. (Panel A) Spectral properties of the reconstituted enzyme. Curve 1 shows the absorption spectrum of a dilution of reconstituted enzyme in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.15 M sodium chloride and 0.5 mM EDTA at 25 °C. Curve 2 was recorded for a duplicate sample prepared in the same buffer containing 5.3 M guanidine hydrochloride. Curve 3 is the spectrum of free 8-$MeSO_2$FAD in 0.1 M potassium phosphate buffer, pH 7.5. (Panel B) Protection against covalent flavinylation in the presence of FAD. Reaction mixtures containing apolipoamide dehydrogenase (10 μM) in buffer A were incubated at 4 °C in the presence of 35 μM 8-$MeSO_2$FAD plus a variable amount of FAD. After 24 h, the protein was precipitated by addition of 5% TCA, and the extent of covalent flavin incorporation was determined as described under Experimental Procedures. Values are expressed relative to a control sample, incubated in the absence of FAD, which contained 0.71 mol of flavin per mol of protein (monomer).

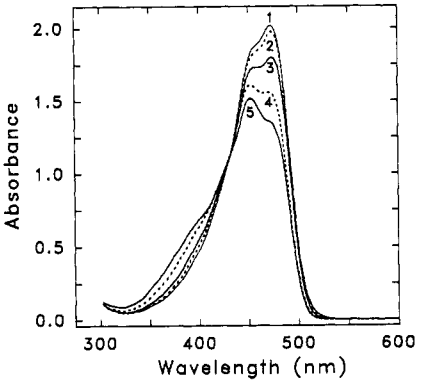


FIGURE 2: Effect of solvent on the spectral properties of 8-(methylthio)riboflavin. Curves 1–5 show spectra recorded at 14 °C for aqueous solutions containing 0, 40, 60, 80, and 100% (v/v) DMSO, respectively.

shielded from solvent, as judged by comparing its spectrum with data obtained for 8-(methylthio)riboflavin in various water/DMSO mixtures. FAD in native pig heart lipoamide dehydrogenase is also shielded from solvent, as judged by a visible absorption spectrum similar to the corresponding free flavin in nonpolar solvents (Williams, 1992) or by the crystal structure determined for the homologous *A. vinelandii* lipoamide dehydrogenase (Mattevi et al., 1991).

Covalent Flavinylation of Lipoamide Dehydrogenase in the Presence of FAD. Preliminary evidence to determine whether attachment of 8-$MeSO_2$FAD occurs at the active site was sought by conducting reconstitution reactions in the presence of varying amounts of normal FAD plus a fixed amount of 8-$MeSO_2$FAD. As shown in Figure 1B, covalent labeling was decreased in the presence of FAD. Approximately 80% protection against covalent incorporation was observed at equimolar concentrations of FAD and 8-$MeSO_2$FAD. A 75% recovery of activity is observed upon reaction of FAD with apoenzyme prepared by the hydrophobic column chromatography method (van Berkel et al., 1991), a value which appears to correlate with the extent of protection observed with FAD against flavinylation.

Effect of Flavinylation on the Lipoamide Dehydrogenase Monomer/Dimer Equilibrium. Reconstitution of apolipoamide dehydrogenase with FAD has been shown to result in the conversion of the mainly monomeric apoenzyme into the homodimeric structure characteristic of native enzyme (Kalse & Veeger, 1968; van Berkel et al., 1991; Visser & Veeger, 1968b). Gel filtration studies showed that our preparations of apolipoamide dehydrogenase were largely monomeric with a small amount of dimer, similar to that reported by van Berkel et al. (1991). However, the gel filtration pattern observed for apoenzyme was unchanged after reconstitution with 8-$MeSO_2$-FAD (data not shown). Nearly complete dimerization was observed when the apoenzyme was reconstituted in the presence of equimolar amounts of FAD and 8-$MeSO_2$FAD, conditions which favor noncovalent incorporation of normal FAD, as described above.

Identification of the Covalent Attachment Site in Lipoamide Dehydrogenase. 8-$MeSO_2$FAD-reconstituted lipoamide dehydrogenase was digested with modified trypsin and then chromatographed on a Vydac C_{18} HPLC column. Figure 3 shows elution profiles obtained in duplicate analytical runs by monitoring absorbance at 214 or 480 nm. The digest contained a complex mixture of peptides, as judged by the 214-nm elution profile. The 480-nm profile suggested that the digest might contain a single flavin-containing peptide since nearly all of the 480-nm absorbance eluted in a single sharp peak. This flavin-containing peptide fraction was isolated in a preparative scale run and further purified on a PolySULFOETHYL Aspartamide SCX HPLC column, where again most (>90%) of the 480-nm absorbance eluted in a single peak (data not shown).

The amino acid sequence obtained for the purified peptide coincides with residues 448–460 in the sequence of pig lipoamide dehydrogenase, a region which contains a single cysteine (Figure 4). The results show that Cys449 in apolipoamide dehydrogenase has reacted with 8-$MeSO_2$FAD, generating a covalently attached 8-(cysteinyl)FAD derivative.

Does Flavinylated Lipoamide Dehydrogenase Exhibit Catalytic Activity? Incubation of FAD with apolipoamide dehydrogenase results in the initial formation of flavin-containing monomers which then dimerize in a second, temperature-dependent step. FAD-containing monomers exhibit NADH-dependent reduction of DCIP. NAD^+-dependent oxidation of reduced lipoamide is restored upon dimerization, as expected, since dimerization creates the binding site for thiol substrate (Kalse & Veeger, 1968; van Berkel et al., 1991; Visser & Veeger, 1968b). Since flavinylated enzyme remains monomeric, the enzyme was assayed only for NADH-DCIP reductase activity. Assays were performed after incubating an aliquot of apoenzyme with 8-$MeSO_2$FAD under standard reconstitution conditions and compared with results obtained for otherwise identical aliquots incubated without flavin or with FAD. No activity was detected with flavinylated enzyme after correcting for the residual activity observed with apoenzyme alone. The activity with FAD-reconstituted enzyme was 3.3-fold higher than native enzyme. Enhanced NADH-DCIP reductase activity has previously been observed for FAD-reconstituted enzyme.

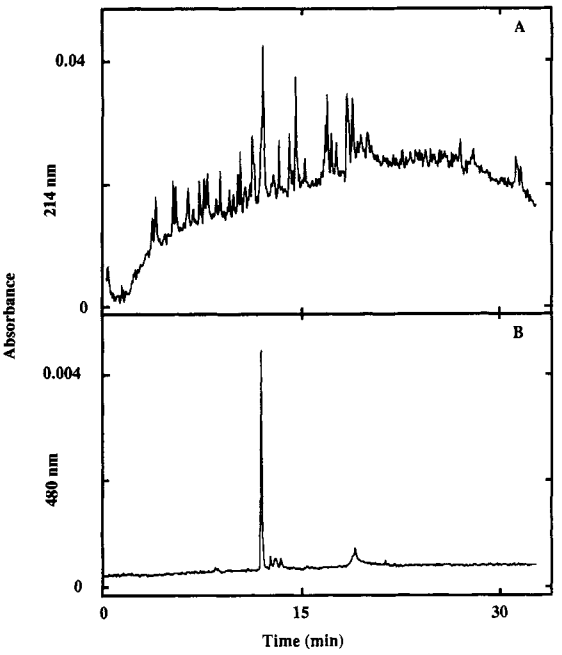


FIGURE 3: Reversed-phase chromatography of a tryptic digest of 8-$MeSO_2$FAD-reconstituted lipoamide dehydrogenase. The reconstituted enzyme was digested with modified trypsin, loaded onto a Vydac C_{18} reversed-phase column, and eluted with a gradient of acetonitrile in the presence of 0.1% TFA, as detailed under Experimental Procedures. Elution profiles obtained by monitoring absorbance at 214 and 480 nm are shown in panels A and B, respectively.

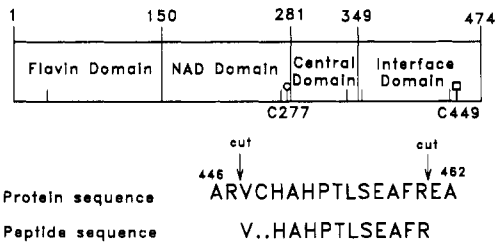


FIGURE 4: Identification of the 8-(cysteinyl)FAD covalent attachment site in lipoamide dehydrogenase. The diagram in the top part of the figure shows the domain structure of pig lipoamide dehydrogenase as deduced by amino acid sequence alignment with other lipoamide dehydrogenases with known structures (Jentoft et al., 1992). The positions of all seven cysteine residues (34, 271, 277, 338, 353, 442, 449) are marked, but only two are labeled. (The active site disulfide between C45 and C50 is not marked.) The bottom half of the figure shows the reported protein sequence of pig lipoamide dehydrogenase near C449 (Otulakowski & Robinson, 1987) (arrows indicate expected tryptic digestion sites) and the sequence determined for the flavinylated peptide. No amino acid derivative was detected in the second cycle of the peptide sequence analysis, as indicated by the space in the sequence.

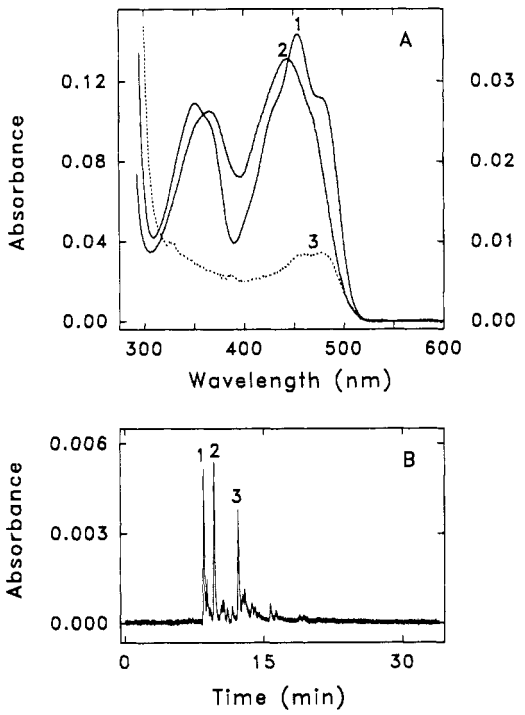


FIGURE 5: Reconstitution of apolipoamide dehydrogenase with 8-chloroFAD. (Panel A) The apoenzyme was prepared by the phenyl-Sepharose column chromatography method. Curve 1 was recorded for the intact reconstituted enzyme in 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl and 0.5 mM EDTA at 10 °C. Curve 2 is the spectrum of the supernatant obtained after precipitation of the protein with 5% TCA. Curves 1 and 2 are plotted according to the left-hand axis. Curve 3 shows the spectrum of the redissolved TCA pellet in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 5 M guanidine hydrochloride. Curve 3 is plotted according to the right-hand axis. (Panel B) Reversed-phase chromatography of a tryptic digest of 8-chloroFAD-reconstituted lipoamide dehydrogenase. Apoenzyme was prepared by the guanidine hydrochloride method. The digest was analyzed using the Vydac C_{18} reversed-phase HPLC method detailed under Experimental Procedures and in the legend to Figure 3. The plot shows the elution profile obtained by monitoring absorbance at 480 nm.

Rate enhancements from 1.3- to 17-fold have been reported and may depend on the apoenzyme preparation method and reconstitution conditions (Kalse & Veeger, 1968; van Berkel et al., 1991; Visser & Veeger, 1968b; Visser et al., 1968a).

Reconstitution of Apolipoamide Dehydrogenase with 8-ChloroFAD. Two enzyme forms were reported after reconstitution with 8-chloroFAD: monomeric enzyme containing only covalently bound flavin and dimeric enzyme containing equimolar amounts of covalently bound flavin and unmodified 8-chloroFAD (Moore et al., 1978). We sought to identify the cysteine residue(s) flavinylated by 8-chloroFAD for comparison with results obtained with 8-$MeSO_2$FAD. Apoenzyme was prepared by the phenyl-Sepharose chromatography method and then reconstituted with 8-chloroFAD under conditions similar to those used by Moore et al. Surprisingly, the absorption spectrum of the reconstituted enzyme exhibited two peaks (λ_{max} = 454, 351 nm) with shoulders at 480 and 430 nm (Figure 5A, curve 1), suggesting that 8-chloroFAD was bound noncovalently at a hydrophobic site. Noncovalent binding was confirmed by precipitation with TCA. 8-ChloroFAD was released into solution (0.98 mol of flavin/subunit) as judged by the absorption spectrum obtained for the TCA supernatant (λ_{max} = 448, 364 nm) (Figure 5A, curve 2). A very small amount of covalent flavin (0.04 mol of flavin/subunit) was detected when the TCA pellet was redissolved in buffer containing guanidine hydrochloride (Figure 5A, curve 3). Gel filtration studies with the intact protein showed that the reconstituted enzyme was fully dimeric.

To determine whether the results might be due to the different method of apoprotein preparation, reconstitution experiments with 8-chloroFAD were repeated using apoenzyme prepared by the guanidine hydrochloride method used by Moore et al. (1978). Reconstitution conditions were the same as used by Moore et al. except for a shorter incubation time (21 h versus 40 h) and a lower temperature (4 °C versus 14 °C). The lower temperature was chosen to avoid protein precipitation which occurred at 14 °C with this apoprotein preparation but not with material prepared by the phenyl-Sepharose method. Significant covalent flavin incorporation (1.1 mol of flavin/mol of monomer) was observed, as judged by TCA precipitation and analysis of the redissolved protein pellet which exhibited a single broad band at 478 nm with a shoulder at 461 nm in digestion buffer (data not shown). The redissolved protein was digested with modified trypsin and then analyzed by the same Vydac C_{18} reversed-phase HPLC column method described above. The elution profile obtained by monitoring absorbance at 480 nm revealed three major flavin-containing bands, labeled according to elution time (Figure 5B). The relative amounts of peptides 1, 2, and 3 was about 2:2:1. Peptide 3 elutes at the same position as the 8-(cysteinyl)FAD-containing peptide characterized in 8-$MeSO_2$FAD-labeled protein digests (see Figure 3).

DISCUSSION

Each of the two identical subunits in native lipoamide dehydrogenase contains one mol of FAD plus four domains which form a kind of bilayer, as judged by the crystal structure determined for *A. vinlandii* lipoamide dehydrogenase (Mattevi et al., 1991). Each of the enzyme's two active sites is located at the subunit interface, with residues contributed from both subunits. The largest domain (FAD) interacts with the other three domains (NAD, central, and interface domain) which lie approximately in a single plane. The isoalloxazine ring of FAD is largely sandwiched within the bilayer structure but the pyrimidine ring sticks out and interacts with the interface domain from the other subunit which contributes the active site base, His450 (*A. vinlandii*) or His452 (pig). In the *A. vinlandii* structure the carbonyl oxygen of His450 forms a hydrogen bond with the N(3)H in the pyrimidine ring of FAD.

8-$MeSO_2$FAD reacts with a single cysteine residue (Cys449) in pig apolipoamide dehydrogenase to generate a flavinylated enzyme containing covalently bound 8-(cysteinyl)FAD. Cys449 is located in the interface domain, near the active site histidine (His452). The residue corresponding to Cys449 in *A. vinlandii* lipoamide dehydrogenase (Val447) is about 9 Å from the carbonyl oxygen at C(2) in the pyrimidine ring of FAD (Figure 6). Approximation of a substituent at position 8 in FAD with Cys449 would require a 180° flip of the isoalloxazine ring as compared with its orientation in the native structure. This difference can explain the failure of flavinylated monomer to dimerize and the absence of catalytic activity. Although the orientation is different, the covalently bound flavin may overlap with the normal FAD binding site, as suggested by the competitive behavior observed in reconstitution reactions containing both FAD and 8-$MeSO_2$FAD. In the accompanying paper we show that 8-$MeSO_2$FAD reacts with a single cysteine residue in the flavin domain of *Escherichia coli* DNA photolyase to form a flavinylated protein that retains the ability to repair DNA and to bind its antenna chromophore (Raibekas & Jorns, 1994).

The 180° flip of the isoalloxazine ring in flavinylated lipoamide dehydrogenase is somewhat analogous to a phe-

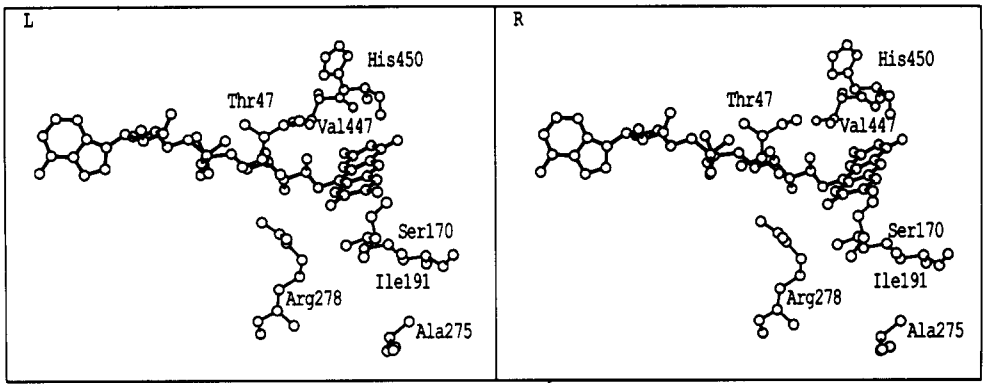


FIGURE 6: Flavin binding site in lipoamide dehydrogenase from *A. vinlandii*. Arg278, Ile191, Ser170 and Thr47, Ala275 (Cys227 in pig), and FAD are from the same subunit. The first four of these residues and two others (Val51, Gly52, omitted for clarity) are within van der Waals distance of the 8-methyl group in FAD. His450 and Val447 (Cys449 in pig) are from the interface domain of the other subunit. The figure was prepared using coordinates deposited in the Brookhaven data base and the program PLUTO.

nomenon observed upon noncovalent binding of heme derivatives to apomyoglobin or apocytochrome b_5. A mixture of two forms is observed after reconstitution with heme orientations differing by a 180° rotation about the α–γ axis (Lecomte et al., 1985; La Mar et al., 1978; Lee et al., 1990). The difference in orientation in myoglobin and cytochrome b_5 is of course less drastic than in lipoamide dehydrogenase since heme derivatives are less asymmetric molecules than flavin.

Cys449, identified as the flavinylation target in apoenzyme, is also subject to modification upon treatment of native lipoamide dehydrogenase with cupric ions. In this reaction, Cys449 and Cys442 are oxidized to a disulfide, accompanied by a decrease in NADH–lipoamide reductase activity but an increase in NADH–DCIP reductase activity (Casola et al., 1966; Matthews et al., 1974; Matthews & Williams, 1974; Thorpe & Williams, 1975).

The methyl group at position 8 in FAD bound to native lipoamide dehydrogenase is sandwiched, in part, between the FAD and NAD domains but also partly visible at the surface in a space-filling model. In the *A. vinelandii* structure, six residues from the FAD (Thr47, Val51, Gly52) and NAD (Arg278, Ileu191, Ser170) domains are clustered within van der Waals distance (<4 Å) of the 8-methyl group. Cys227 in pig lipoamide dehydrogenase (Ala275 in *A. vinlandii*) is about 8 Å from the 8-methyl group (Figure 6). The other five cysteine residues in pig lipoamide dehydrogenase are all 14 Å or farther from the flavin ring.

A 180° flip of the flavin ring would not be required for covalent attachment of 8-$MeSO_2$FAD at Cys227 in pig lipoamide dehydrogenase, although significant local conformational changes would probably be needed to permit access. Since nucleophilic displacement reactions with 8-$MeSO_2$-substituted flavin are observed only with the oxidized flavin, we reasoned that reconstitution of apolipoamide dehydrogenase with reduced 8-$MeSO_2$FAD might yield enzyme containing noncovalently bound flavin in a normal orientation. In this case, covalent attachment at Cys227 might occur upon flavin reoxidation. To test this hypothesis, reconstitution reactions with reduced 8-$MeSO_2$FAD were conducted under anaerobic conditions as described under Experimental Procedures. No flavin incorporation was detected in experiments where 8-$MeSO_2$FAD was reduced with NADH, and only a small amount (<10%) of covalent flavin was incorporated when dithionite was used as the reductant. In contrast, stoichiometric FAD binding was observed in control studies when apoenzyme was reconstituted with dithionite-reduced FAD.

That the 8-$MeSO_2$ substituent in 8-$MeSO_2$FAD appears to interfere with binding in the normal orientation was somewhat unexpected since 8-(phenylthio)FAD is readily bound to pig lipoamide dehydrogenase (Moore et al., 1978). The results suggest that, owing to steric crowding near the 8 position, it may be difficult to compensate and accommodate the greater bulk of the tetravalent sulfur in 8-$MeSO_2$FAD.

The studies with 8-$MeSO_2$FAD reported in this paper were conducted using apoprotein prepared using a phenyl-Sepharose column chromatography method. This procedure yields a stable apoprotein which gives good recovery of activity after reconstitution with FAD in a DTT-independent reaction (75%) (van Berkel et al., 1991). Moore et al. (1978) observed covalent incorporation with 8-chloroFAD using apoenzyme prepared by treating native enzyme with guanidine hydrochloride. This procedure yields an unstable apoprotein which gave poor recovery of activity upon reconstitution with FAD (6%). The yield could be improved in the presence of DTT (30%), but DTT was not included during reconstitution with 8-chloroFAD under conditions where covalent incorporation was observed. We sought to identify the cysteine residue(s) that reacts with 8-chloroFAD but found that only noncovalent binding occurred with apoenzyme prepared using the phenyl-Sepharose method. Covalent incorporation was observed using apoenzyme prepared by the guanidine hydrochloride method. However, preliminary analysis of a proteolytic digest revealed relatively nonspecific labeling with flavinylation at Cys449 estimated to account for less than 20% of the total incorporation. It would appear that covalent incorporation of the 10^3-fold less reactive 8-chloroflavin analogue becomes competitive only when the pathway leading to normal holoenzyme formation is blocked.

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