A Requirement for Matrix Processing Peptidase but Not for Mitochondrial Chaperonin in the Covalent Attachment of FAD to the Yeast Succinate Dehydrogenase Flavoprotein*

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Succinate dehydrogenase (EC 1.3.99.1) in the yeast Saccharomyces cerevisiae is a mitochondrial heterotetramer containing a flavoprotein subunit with an 8-N(3)-histidyl-linked FAD cofactor. The covalent linkage of the FAD is necessary for activity. We have developed an in vitro assay that measures the flavinylation of the flavoprotein precursor in mitochondrial matrix fractions. Flavoprotein modification does not depend on translocation across a membrane, but it does require proteolytic processing by the mitochondrial processing peptidase prior to flavin attachment. Since ATP depletion of the nonsedimentable, outer mitochondrial membrane matrix fractions inhibits flavoprotein modification, at least one additional matrix protein component appears to be required. Having previously suggested that the flavoprotein begins folding before FAD attachment occurs, we tested whether the mitochondrial chaperonin, heat shock protein 60, might be necessary. Co-immunoprecipitation of the flavoprotein and the chaperonin demonstrate that the proteins do indeed interact. However, immunodepletion of the chaperonin from matrix fractions does not inhibit FAD attachment. Nonprotein components are also required for flavoprotein modification. In addition to ATP, effector molecules such as succinate, fumarate, or malate also stimulate modification. Together, these results suggest that FAD addition is an early event in succinate dehydrogenase assembly.

The assembly of many mitochondrial proteins can depend on the activities of special proteins such as the mitochondrial processing peptidase (Arretz et al., 1991; Glick et al., 1992a) and the chaperonin, heat-shock protein 60 (hsp60)† (Stuart et al., 1994). The processing peptidase removes cleavable presequences, a step that for some proteins must occur before cofactor insertion or assembly into multisubunit complexes can proceed (Nicholson et al., 1989; Graham et al., 1993). hsp60 mediates the folding and assembly of imported proteins in an ATP-dependent reaction (Georgopoulos and Welch, 1993; Becker and Craig, 1994). Both mitochondrial processing peptidase and hsp60 are indispensable for mitochondrial biogenesis, as they are both essential for viability in Saccharomyces cerevisiae (Baker and Schatz, 1991).

We are using the S. cerevisiae succinate dehydrogenase (SDH) or complex II as a model system for studying the covalent attachment of flavins to the Fp subunit. Both SDH and fumarate reductase (FRD) are essential for viability in S. cerevisiae, the Fp, the Ip, and the membrane subunits are encoded by nuclear genes, SDH1, SDH2, SDH3, and SDH4, respectively (Lombardo et al., 1990; Chapman et al., 1992; Robinson and Lemire, 1992; Bullis and Lemire, 1994; Daignan-Fornier et al., 1994). The Fp contains the SDH active site and the FAD cofactor in an 8-N(3)-histidyl-FAD linkage. The small number of subunits and the availability of the SDH genes for manipulation make the yeast SDH an attractive and practicable system for examining cofactor insertion and the assembly of mitochondrial complexes.

Covalent cofactor attachment to mitochondrial and nonmitochondrial proteins can be mediated by special enzymes. For example, heme is attached to cytochromes c and c₅₅ by heme lyses (Nargang et al., 1985; Ackrell et al., 1992; Hederstedt and Ohnishi, 1992). In S. cerevisiae, the Fp, the Ip, and the membrane subunits are encoded by the nuclear genes, SDH1, SDH2, SDH3, and SDH4, respectively (Lombardo et al., 1990; Chapman et al., 1992; Robinson and Lemire, 1992; Bullis and Lemire, 1994; Daignan-Fornier et al., 1994). The Fp contains the SDH active site and the FAD cofactor in an 8-N(3)-histidyl-FAD linkage. The small number of subunits and the availability of the SDH genes for manipulation make the yeast SDH an attractive and practicable system for examining cofactor insertion and the assembly of mitochondrial complexes.

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The mechanism of covalent FAD attachment for the Fp subunit of complex II has not been elucidated. Interestingly, 6-hydroxy-o-nicotine oxidase, a bacterial flavoprotein, autokinetically attaches a covalent FAD (Brandsh and Bichler, 1991). The modification is independent of ATP and is stimulated by effector molecules such as glyceral 3-phosphate (Brandsh and Bichler, 1989; Brandsh and Bichler, 1991). Autokinetically flavinylated has also been suggested for bovine monoamine oxidase and for Pseudomonas putida p-cresol hydroxylase (Weyer et al., 1990; Kim et al., 1994). Whether the formation of covalent protein-FAD bonds is autokinetically for all flavoproteins remains to be documented.
identification (Lang et al., 1991), suggesting that protein conformation may be the signal for FAD attachment. Second, autophagy-dependent flavinylation of the 6-hydroxy-N-nicotine oxidase is conformation-dependent (Brandsch and Bichler, 1992; Brandsch et al., 1993). Third, a number of observations suggest that FAD attachment to the SDH Fp follows partial folding of the Fp (Robinson and Lemire, 1996). These include increased FAD attachment to the Fp upon expression of the lp subunit in vivo and a dependence of Fp modification on the addition of substrate or substrate-like molecules. Both observations imply that folding is important because interactions with the lp or with substrate are specific. Finally, carboxyl-terminal truncations of the Fp, which should not affect flavinylation if the reaction proceeded with unfolded Fp as substrate, completely block modification (Robinson and Lemire, 1996).

Fp modification can be monitored after import into isolated mitochondria (Robinson and Lemire, 1996). To further investigate the mechanism of Fp modification, we have developed a flavinylation system consisting of in vitro translated Fp precursor and a mitochondrial matrix fraction and assayed for flavin attachment by immunoprecipitation with an anti-FAD serum. Our results indicate that Fp translocation is not linked to FAD addition. Flavinylation is stimulated by the presence of Krebs cycle intermediates such as succinate and malate, is ATP-dependent, and N-ethylmaleimide- or proteinase-sensitive, suggesting that other proteins are involved. Interestingly, proteolytic processing of the presequence is mandatory for FAD attachment to the Fp. Finally, we show that the Fp interacts with hsp60, although the absence of hsp60 does not prevent its modification.

MATERIALS AND METHODS

Media, Strains, and Plasmids—E. coli and yeast strains and their growth media have been described previously (Robinson et al., 1994). The plasmid, pJS1905, encodes the wild-type and a flavinylation-incompetent His-90—Ser Fp, respectively (Robinson and Lemire, 1992; Robinson et al., 1994). The plasmid, pIT7mFp, encodes an Fp subunit lacking its presequence in the vector, pBlueScript II KS—(Stratagene, La Jolla, CA). By a polymerase chain reaction, a methionyl codon was introduced in-frame immediately preceding the Gin-29 codon; Gin-29 corresponds to the amino-terminal residue of the processed Fp precursor (Bullis and Lemire, 1994). The plasmid pITLSC encodes a chimeric matrix-targeted precursor, LS, composed of a mitochondrial targeting sequence fused to the large subunit of ribulose bisphosphate carboxylase/oxygenase (Rospert et al., 1994). The SDH2 open reading frame was amplified by a polymerase chain reaction and placed downstream of the T7 promoter to produce the plasmid pSDH839.

Preparation of Matrix Fractions—Matrix fractions were prepared from mitoplasts (J asuri, 1991) suspended at 10 mg/ml protein in Flavinylation Buffer (20 mM Hepes-KOH, pH 7.4, 5 mM ATP, 50 mM MgCl2, 50 mM MnCl2, 50 mM ZnCl2, 50 mM FAD, 10 mM succinate, 10 mM fumarate, 10 mM glutathione, 0.1 mM EDTA, 0.005% bromphenol blue, 25% glycerol, 5% β-mercaptoethanol, pH 6.8) for 8 min. Beads were removed by centrifugation and re-extracted with IPLB as above, and the supernatants were combined.

Immunodepletion of Matrix Fractions—60 μl of matrix fraction at a protein concentration of 6 mg/ml was added to 80 μl of anti-A-Sepharose beads that had been preincubated with 30 μl of either anti-native hsp60 serum or preimmune serum. The slurry was incubated at 4 °C for 2 h with rocking, the beads were spun down with a brief centrifugation, and the supernatant was collected. The protein A-Sepharose beads were rinsed with 10 μl of Flavinylation Buffer, which was added to the first supernatant fraction. The immunodepleted matrix fractions (10 μl) were removed and analyzed with anti-hsp60 serum by Western blotting. An additional aliquot of ATP to 5 mM was added to the remaining immunodepleted matrix fraction. Rabbit reticulocyte lysate (10 μl) that had been incubated with mRNA encoding the Fp precursor was added immediately, and the FAD attachment assays were performed as described above.

RESULTS

Flavinylation Requires Mitochondrial Matrix Fractions—In vitro translated Fp precursor was prepared in rabbit reticulocyte lysate as described by the supplier (Promega Corp.) in the presence of radiolabeled methionine (Tran35S-label; ICN Bio- medicals). The anti-FAD and anti-Fp antisera have been described previously (Robinson et al., 1991; Robinson and Lemire, 1995). All protease inhibitors and other reagents are from Sigma.

Flavinylation Requiring Mitochondrial Matrix Fractions—Fp attachment to the Fp occurs during in vitro import into isolated mitochondria or mitoplasts (Robinson and Lemire, 1996). Investigation of the flavinylation mechanism with import experiments is limited to conditions that are compatible with mitochondrial import. Furthermore, since the inner membrane is a barrier that prevents full access to the matrix, the site of FAD attachment (Robinson and Lemire, 1996). To determine whether import and Fp modification are coupled and to remove the experimental limitations of maintaining import competent organelles, we developed an in vitro flavinylation system using matrix fractions.

We first tested whether mitochondrial integrity and import competence are necessary for flavinylation of the Fp to occur. Isolated mitochondria were solubilized with Triton X-100 in Flavinylation Buffer, which contains ATP, MgCl2, MnCl2, FAD, succinate, fumarate, and several protease inhibitors. We included ATP and magnesium because FAD attachment might be an energy-dependent reaction, succinate and fumarate because these molecules greatly stimulate flavinylation of the Fp in mitoplasts (Robinson and Lemire, 1996), and the metals ions, Zn2+ and Mn2+, because they are necessary for mitochondrial processing peptidase function. Fp precursor that had been translated in rabbit reticulocyte lysate was incubated with the solubilized mitochondria; precursor stability and processing were monitored by SDS gel electrophoresis and fluorography, while cofactor attachment was assayed by immunoprecipitation with the anti-FAD serum (Fig. 1). Not only are
Mitochondria that were at 5, 10, or 20 mg/ml (lanes 2-7) was incubated in Flavinylation Buffer without matrix (lane 1) was incubated 20 min at 30°C in Flavinylation Buffer without matrix fraction (lanes 2 and 6) or with matrix fractions at concentrations of 5 (lanes 3 and 7), 10 (lanes 4 and 8), or 20 mg/ml (lanes 5 and 9). After incubation, the samples were analyzed by SDS gel electrophoresis and fluorography to monitor Fp levels and processing (lanes 2-5) or by immunoprecipitation with the anti-FAD serum to assay for cofactor attachment (lanes 6-9). Flavinylation in the 10 mg/ml sample was normalized to 1, and other samples were compared with it.

We believe that the flavinylation activity associated with the detergent-solubilized mitochondria capable of proteolytically processing the Fp precursor (lane 1) to its mature size (lane 2), but they are also able to support flavinylation of a significant fraction of that mature Fp (lane 5).

To determine whether the flavinylating activity is located in the mitochondrial matrix or whether it is associated with a membrane, we fractionated mitoplasts. Mitoplasts suspended in Flavinylation Buffer were disrupted by freeze-thawing and sonication, and the membrane fraction was pelleted and solubilized with Triton X-100 in Flavinylation Buffer. The supernatant fraction is referred to as the matrix fraction. Flavinylation reactions containing in vitro translated Fp precursor were incubated with either the membrane (Fig. 1, lanes 3 and 6) or the matrix (lanes 4 and 7) fractions, and the extent of FAD attachment was determined. Both the membrane and the matrix fractions supported processing of the Fp precursor to the mature size (lanes 3 and 4, respectively). However, modification of the Fp with FAD was significantly more efficient in the matrix fraction (lane 7) than in the membrane fraction (lane 6). We believe that the flavinylating activity associated with the membrane fraction may reflect contamination by residual matrix components. The small apparent increase in the extent of flavinylation in reactions with matrix fractions over those with solubilized mitochondria could stem from competition for binding and precipitation by the anti-FAD serum by preassembled Fp present in mitochondrial membranes or from the presence of detergent. Flavinylation of the SDH Fp therefore requires matrix components.

We further examined the dependence of the flavinylation reaction on matrix components by preparing matrix fractions with different protein concentrations (Fig. 2). Fp precursor (lane 1) was incubated in Flavinylation Buffer without matrix fraction (lanes 2 and 6) or with matrix fractions prepared from mitochondria that were at 5, 10, or 20 mg/ml (lanes 3-5 and 7-9, respectively). Proteolytic processing (lanes 2-5) and FAD attachment (lanes 6-9) occur only in the presence of matrix fraction, demonstrating that these activities are not attributable to the reticulocyte lysate or to the Flavinylation Buffer. Furthermore, the extent of modification is directly proportional to the concentration of the fraction used. In all further experiments, we used matrix fractions prepared from mitochondria at 10 mg/ml.

In earlier work, we noted that the flavinylating of imported Fp precursor could be greatly enhanced by certain citric acid cycle intermediates such as succinate, fumarate, or malate, but not by others such as citrate or oxaloacetate or by glycerol-3-phosphate, a stimulator of 6-hydroxy-D-nicotine oxidase FAD attachment (Robertson and Lemire, 1996). The inability of the latter three compounds to stimulate FAD attachment in mitoplasts might be due to their slower uptake. We reassessed the efficacies of these molecules in flavinylating reactions performed with matrix fractions (Fig. 3). In the absence of additives, very little holo-Fp could be immunoprecipitated with anti-FAD serum (lane 2). Succinate, fumarate, and malate greatly increased the amount of holo-Fp detected (lanes 3, 4, and 6, respectively). Flavinylated attachment was only slightly or not at all stimulated by the competitive inhibitors, malonate and oxaloacetate (lanes 5 and 7, respectively), or with citrate and glycerol 3-phosphate (lanes 8 and 9, respectively). The His-90 → Ser Fp, which cannot be modified (Robertson et al., 1994), was also incubated with matrix fraction in the presence of succinate (lane 10). Only trace amounts of the His-90 → Ser Fp are immunoprecipitated with the anti-FAD serum, demonstrating the serum's specificity for the cofactor. These results demonstrate that FAD attachment in matrix fractions is also greatly stimulated by the same Krebs cycle intermediates that stimulate in intact organelles.

The requirement for ATP in Fp flavinylation was examined (Fig. 4). We increased the amount of ATP present in the matrix fractions by either supplementing the flavinylating reaction with additional ATP after the reaction had proceeded for 10 min (lanes 3 and 7) or by including an ATP-regenerating system (lanes 4 and 8). Neither of these changes stimulated flavinylation over untreated matrix fraction (lanes 2 and 6). Another sample was pretreated with apyrase to hydrolyze both ATP and ADP to AMP (Glick, 1991). ATP depletion reduced processing of the precursor (lane 5) and cofactor attachment by about 6-fold (lane 9). In this experiment, Fp recovery was low, but this was not reproducible (lane 5); the percentage of Fp flavinylated was not affected by recovery. Addition of either NADH or the ionophore, valinomycin, did not affect the amount of Fp modified (data not shown). Although not necessarily directly involved in the reaction, ATP is required for in vitro FAD attachment.

The Ip subunit stimulates FAD attachment to the Fp subunit under respiratory conditions in vivo (Robertson and Lemire, 1996). To test whether the same is true in vitro, flavinylating reactions were supplemented with in vitro translated Ip. The extents of Fp processing or modification are not significantly changed by the presence of the Ip subunit (not shown).

One of the most interesting questions about the covalent FAD attachment to flavoproteins is whether the reaction is catalyzed by an enzyme or whether an autocatalytic mechanism is employed. To determine whether a matrix protein is responsible for Fp modification activity, matrix fractions were
Flavinylation requires ATP. Untreated matrix fractions (lanes 2 and 6), fractions to which had been added an additional aliquot of 5 mM ATP after the first 10 min of incubation (lanes 3 and 7), or an ATP-regenerating system consisting of pyruvate kinase and phosphoenolpyruvate (lanes 4 and 8), or fractions pretreated with apyrase (20 units/ml for 10 min at 30 °C; lanes 5 and 9), were incubated with in vitro translated Fp precursor (lane 1). A portion of each sample was analyzed by SDS gel electrophoresis and fluorography (lanes 2-5) or by immunoprecipitation with the anti-FAD serum (lanes 6-9), and the ratios of flavinylated to total Fp were calculated. The level of flavinylated in the untreated sample was normalized to 100%.

Treated in several ways that either inactivate proteins or remove small molecules (Fig. 5). Boiled matrix fractions are incompetent for flavinylayion. Treatment with N-ethylmaleimide, a sulfhydryl-modifying reagent, or digestion with proteinase K also inhibit FAD attachment, thus implying that a protein component is involved in Fp flavinylation. Addition of ovalbumin, which might nonspecifically stabilize the Fp in protease-treated matrix fractions, does not restore FAD attachment activity, suggesting that protease treatment removes at least one specific protein. Depletion of small molecules from the matrix fraction with a Sephadex G-25 spin column results in an almost complete loss of flavinylayion and a reduction of processing activity. Readdition of ATP, Mg\(^{2+}\), succinate, fumarate, Zn\(^{2+}\), Mn\(^{2+}\), and FAD can partially restore FAD attachment activity to the depleted lysate. Collectively, these data strongly argue that the modifying activity found in the matrix lysate is a protein component, although small molecules such as divalent cations, ATP, and FAD are also needed.

Proteolytic Processing Is Required for FAD Attachment—Cofactor attachment to the mature but not the precursor form of the Fp is detected both in vivo and in vitro (Robinson and Lemire, 1996), although the fraction of Fp present in the precursor form is usually quite small unless import or processing are inhibited. Hence, mitochondrial processing peptidase, a mitochondrial matrix metalloprotease, is a candidate for the matrix protein component required for Fp modification (Arretz et al., 1991). We treated matrix fractions with EDTA in the presence or absence of ATP to inactivate the mitochondrial processing peptidase. We reasoned that the mitochondrial processing peptidase might not be inactivated in the presence of ATP since hsp60 might stabilize it. The peptidase's solubility and function are intimately connected to the hsp60 function (Glick et al., 1992b; Hallberg et al., 1993). Matrix fractions were prepared in Flavinylation Buffer without ATP and divalent cations, treated with EDTA for 10 min to chelate endogenous Zn\(^{2+}\), Mn\(^{2+}\), and Mg\(^{2+}\) ions, and then resupplied with one or more of the cations. As a control, Fp precursor (Fig. 6, lane 1) was incubated with a matrix fraction that had been prepared in the normal way (lane 2). This resulted in substantial processing to the mature size (lane 2) and flavinylation of the mature Fp (lane 6). Fractions treated with EDTA in the presence of added ATP showed similar levels of Fp processing (lane 3) but reduced levels of flavinylation (lane 7). Fractions treated in the absence of ATP and resupplied with all three divalent cations were able to process (lane 4) and inefficiently flavinylate the Fp (lane 8). Only in fractions treated in the absence of ATP and resupplied with Mg\(^{2+}\) was a significant increase in the levels of unprocessed Fp seen (lane 5). When the flavinylation products of this reaction were analyzed, only the mature-sized Fp was immunoprecipitated with the anti-FAD serum (lane 9). Even though substantial Fp precursor remains at the end of the flavinylation reaction (lane 5), it has not been flavinylated (lane 9), suggesting that the precursor is incompetent for FAD attachment.

Since the presequence appears to inhibit cofactor attachment, we engineered an amino-terminally truncated Fp that does not contain a presequence, called pseudomature Fp (mFp). The wild-type and the pseudomature Fps were translated (Fig. 7, lanes 1 and 2, respectively), incubated with the matrix fractions (lanes 3 and 4) and subjected to immunoprecipitations with the anti-FAD serum (lanes 5 and 6). Although the pseudomature Fp is efficiently translated and is not degraded by the matrix fraction, cofactor attachment is undetectable. Therefore, FAD attachment requires the proteolytically processed Fp.

hsdp60 Binds Fp—The requirement for proteolytic processing of the Fp prior to flavinylation could explain the need for a matrix protein component, but it does not account for an ATP
Covalent Flavin Attachment to Yeast Complex II

During the biogenesis of SDH and fumarate reductase enzymes, a covalent FAD cofactor is added to the flavoprotein subunit. Aside from the site of covalent attachment, the Fp interacts with the FAD in tight noncovalent binding (Blaut et al., 1989; Robinson et al., 1994). The covalent linkage of the cofactor is necessary to permit succinate oxidation (Blaut et al., 1989; Robinson et al., 1994). The covalent linkage of the cofactor is necessary to modify the redox midpoint potentials of the enzymes and to permit succinate oxidation (Blaut et al., 1989; Robinson et al., 1994). We have been studying the role of the covalent FAD and the mechanism by which this unusual cofactor is linked to the yeast SDH. We developed an assay for FAD addition that relies on an anti-FAD serum for immunoprecipitation of the modified protein following in vivo or in vitro import of the Fp precursor into mitochondria (Robinson and Lemire, 1996). In this work, we extended our assay system to include flavinylation reactions (Fig. 10, lanes 6 and 7), and we tested whether Fp modification requires hsp60, FAD attachment reactions were performed with matrix fractions immunodepleted of hsp60. Western blot analysis with anti-hsp60 serum confirms that hsp60 has been quantitatively removed from the matrix fraction by the immunodepletion (Fig. 9, lane 2) but is still present in normal amounts in the sample immunodepleted with the preimmune serum (data not shown). Immunodepleted matrix fractions were tested in flavinylation reactions (Fig. 10, lanes 6 and 7). The extents of Fp modification in the two fractions were approximately the same, demonstrating that the removal of hsp60 does not inhibit FAD attachment. Thus, hsp60 is not required for Fp modification.

**FIG. 6. Proteolytic processing is required for FAD attachment.** In vitro translated Fp precursor (lane 1) was incubated with matrix fractions that had been prepared in ATP and divalent cation-free Flavinylation Buffer supplemented with 50 ,uM MgCl₂ and treated in the following ways: untreated (lanes 2 and 6); incubated with 0.5 mM EDTA for 10 min in the presence of 5 mM ATP followed by the addition of 0.1 mM MnCl₂ and 0.6 mM ZnCl₂ (lanes 3 and 7); incubated with 0.5 mM EDTA for 10 min in the absence of ATP followed by the addition of 0.6 mM MnCl₂ and 0.6 mM ZnCl₂ (lanes 4 and 8); or incubated with 0.5 mM EDTA for 10 min in the absence of ATP followed by the addition of 0.6 mM MgCl₂ (lanes 5 and 9). Samples were analyzed for protein content and processing (lanes 2-5) and the amount of FAD attachment (lanes 6-9).

**FIG. 7. Pseudomature Fp is not flavinylated.** Wild-type (pFP and WT) or pseudomature (mFP) Fp were translated in reticulocyte lysate (lanes 1 and 2, respectively), incubated with matrix fractions, and analyzed by SDS gel electrophoresis and fluorography (lanes 3 and 4), and by immunoprecipitation with the anti-FAD serum (lanes 5 and 6). requirement. We have suggested that FAD attachment is a folded Fp molecule (Robinson and Lemire, 1996). For this reason, we investigated whether the mitochondrial chaperonin, hsp60, which assists in the ATP-dependent folding of proteins, has a role in Fp flavinylation (Becker and Craig, 1994). We first needed to determine whether the Fp interacts with hsp60. Co-immunoprecipitation with specific anti-hsp60 antibodies is a reliable method of monitoring association with hsp60 (Rospert et al., 1994). The fusion protein, LS, which contains a matrix targeting signal fused to the large subunit of ribulose bisphosphate carboxylase/oxygenase, was used as a control for hsp60 binding (Rospert et al., 1994). In vitro translated LS or Fp precursor proteins were incubated in matrix fractions for 15 min before the addition of apyrase to hydrolyze ATP, and inhibited the release of bound proteins from hsp60. Samples were divided, and the proteins were analyzed by denaturing gel electrophoresis and fluorography (Fig. 8, lanes 3-7) or by immunoprecipitations with anti-native hsp60 serum to determine hsp60 association (lanes 8-12). As a control to show that the anti-hsp60 serum does not recognize the Fp, a sample consisting of Fp in Flavinylation Buffer without matrix fraction was also subjected to the immunoprecipitation protocol (lane 12). Both the LS and Fp precursors were efficiently translated (lanes 1 and 2, respectively), proteolytically processed to mature forms (lanes 3 and 5, respectively), and co-immunoprecipitated with hsp60 (lanes 8 and 10, respectively). In the absence of matrix fraction, the Fp was neither proteolytically processed (lane 7) nor immunoprecipitated with the anti-native hsp60 serum (lane 12). The fractions of LS and Fp bound to hsp60 under these conditions are 15 and 43%, respectively. The addition of FAD to the matrix fractions did not affect Fp processing (lane 6), Fp association with hsp60 (lane 11), or results with the LS precursor (lanes 4 and 9). These results demonstrate that the Fp interacts with mitochondrial hsp60.

Does the association of the Fp with hsp60 precede or follow covalent cofactor attachment? We first addressed this question by determining whether Fp found in association with hsp60 is already flavinylated. Co-immunoprecipitations of the Fp with the anti-hsp60 serum were used to separate Fp molecules into hsp60-bound and free fractions. In order to mimic the immunoprecipitation of the bound fraction with the anti-hsp60 serum, Fp in the free fraction was immunoprecipitated with anti-Fp serum. Both Fp fractions were removed from the protein A-Sepharose beads of the first immunoprecipitations and subjected to a second immunoprecipitation with the anti-FAD serum to determine the levels of FAD attachment in each fraction. The hsp60-bound and free fractions contain equal proportions of holo-Fp when compared with the amount of Fp recovered after the first immunoprecipitation (not shown). Thus, both the apo- and holo-Fp forms are found in association with hsp60.

**FIG. 8. The Fp is co-immunoprecipitated with hsp60.** The LS protein and the Fp precursor were translated (lanes 1 and 2, respectively) and incubated in Flavinylation Buffer with (lanes 3-6, and 8-11) or without (lanes 7 and 12) matrix fraction. Each sample was incubated with 20 units/ml apyrase for 10 min at 30 °C. Where indicated, 50 μM FAD was added to the incubation mix. 20% of each sample was analyzed by SDS gel electrophoresis and fluorography (lanes 3-7). The remaining sample was analyzed by native immunoprecipitation with the anti-hsp60 serum (lanes 8-12). Note that after immunoprecipitation with the anti-hsp60 antibodies, LS migrates faster than usual on the gel because of co-migrating IgG heavy chains (Rospert et al., 1994).
the matrix where FAD attachment occurs. Therefore, membrane-impermeable reagents can be tested and experiments can be performed under conditions that are incompatible with the translocation of proteins across the mitochondrial inner membrane.

Matrix proteins are necessary for the flavinylation of the Fp subunit to proceed. The extent of Fp modification is directly proportional to the protein concentration of the matrix fraction (Fig. 2). Furthermore, treatment of matrix fractions by boiling or with N-ethylmaleimide, or digestion with proteinase K all inactivate the flavinylation activity (Fig. 5).

One protein component not involved in the flavinylation reactions in vitro is the Ip subunit. This is in contrast to Fp flavinylation in vivo (Robinson and Lemire, 1996). The yeast SDH Fp and Ip subunits are believed to interact with each other to form an assembly intermediate (Lombardo et al., 1990; Robinson et al., 1991; Schmidt et al., 1992). Similarly, the E. coli fumarate reductase subunits form an active heterodimer in the absence of the membrane subunits (Lemire et al., 1992).

The inability of the Ip to stimulate Fp flavinylation in matrix fractions may be because its iron-sulfur clusters are not assembled under the conditions used here, leaving the Ip in an inappropriate conformation. Alternatively, the Ip may need to interact with the membrane subunits, which are missing in these experiments, prior to its association with the Fp. Accordingly, when the membrane subunits are present, the E. coli fumarate reductase Ip assembles with them first and subsequently with the Fp (Latour and Weiner, 1988). Similarly, the Bacillus subtilis SDH Fp and Ip subunits will not form a heterodimer in the absence of the membrane subunit (Hederstedt and Rutberg, 1980; Hederstedt et al., 1982).

Mitochondrial processing peptidase, which removes the amino-terminal presequence from the Fp precursor, is required to allow cofactor addition to proceed. Similarly, cytochrome c1 must be proteolytically processed to its intermediate form by the mitochondrial processing peptidase before the heme cofactor can be attached (Nicholson et al., 1989). In our experiments, no modified precursor Fp is detectable even when the activity of the peptidase is inhibited, and a significant fraction of the added Fp remains as precursor (Fig. 6). It is unlikely that the lack of detection is because the anti-FAD serum does not recognize the modified precursor, since it detects covalently attached FAD in completely unrelated flavoproteins, even when the flavin is present in different linkages (Robinson and Lemire, 1995). The simplest explanation is that the presence prevents flavinylation by inhibiting Fp folding, which we suggest is essential for FAD attachment (Robinson and Lemire, 1996). It may do this by interacting with proteins such as the presequence binding factor (Murakami et al., 1988; 1992; Murakami and Mori, 1990) or the mitochondrial import stimulation factor (Hachiya et al., 1993) or by interacting with the remainder of the Fp molecule. By preventing folding, the presequence may delay in vivo flavinylation until after Fp import into mitochondria. Mitochondrial processing peptidase by removing the presequence may initiate folding and events leading to flavinylation. Whether other protein components in addition to mitochondrial processing peptidase are needed for flavinylation remains to be determined.

That presequence cleavage is related to flavinylation is also demonstrated by the lack of modification of pseudomature Fp, which only differs from mature Fp by the addition of an amino-terminal methionine residue (Fig. 7). We propose that the pseudomature Fp is not modified because without a presequence to impede folding, it misfolds in the reticulocyte lysate and adopts a conformation incompatible with FAD attachment. A flavinylation-incompetent state has been observed with the 6-hydroxy-D-nicotine oxidase (Brandsch and Bichler, 1992; Brandsch et al., 1993). Curiously, the amino acid sequence predicts that the yeast SDH Fp is cleaved in two steps: first by the mitochondrial processing peptidase to an intermediate species, and then the remaining octapeptide is removed by the mitochondrial intermediate protease producing the mature sized Fp. Correspondingly, a yeast mitochondrial intermediate protease mutant has no SDH activity, suggesting that this protease is required to process at least one of the SDH subunits (Isaya et al., 1994). Perhaps flavinylation is restricted to the intermediate sized Fp and not to the mature or pseudomature Fps. However, we have never detected an intermediate Fp species and have not examined its role in modification.

Nonprotein components are also required for Fp flavinylation by matrix fractions. ATP may be required for cofactor activation as for biotin or lipoic acid (Schmidt et al., 1969; Gross and Wood, 1984), but this is unlikely because 6-hydroxy-D-nicotine oxidase flavinylation is independent of ATP (Brandsch and Bichler, 1991). Alternatively, the ATP may be required for Fp release from either cytosolic (Pfanner et al., 1990) or mitochondrial heat shock proteins (Cheng et al., 1989; Ostermann et al., 1989) that are necessary for protein folding. Certain Krebs cycle intermediates, especially succinate, fumarate, and malate, which are substrates for SDH, stimulate FAD attachment. Strangely, both oxaloacetate and malonate, which bind tightly to the enzyme active site located in the Fp, do not enhance FAD attachment. Perhaps the nascent active site has a different geometry from that of the fully assembled enzyme and does not recognize these molecules. Alternatively, FAD attachment may require a molecule that can also be oxidized or reduced (Decker, 1993). Clearly, the interconversion of K rebs cycle intermediates in the matrix fractions is not efficient enough to erase the different levels of stimulation we observed with individual intermediates. In contrast to our results with the yeast Fp, citrate and succinate were the most efficient stimulators of E. coli SDH Fp modification (Brandsch and Bichler, 1989). A better understanding of the mechanism of effector molecule stimulation may require a more purified system.

Our results suggest that FAD attachment is one of the earliest steps in the SDH Fp assembly pathway. After presequence cleavage, the Fp begins folding with the aid of substrate-like molecules and possibly ATP-dependent chaperones such as mitochondrial heat shock protein 70. FAD itself may act as a nucleation site for Fp folding as it does for the medium chain acyl-CoA dehydrogenase, a noncovalent flavoprotein (Sajio and Lehmann, 1989).
Tanaka, 1995). Since hsp60 is not required for Fp modification, it most likely interacts with the Fp following FAD attachment. Subsequent to these experiments, we determined that the rabbit reticulocyte lysate added to the matrix fractions contains sufficient FAD to support Fp modification (data not shown). Hence, our experiments do not address whether the Fp must associate with FAD noncovalently before it can be bound by hsp60 or if the chaperonin still recognizes the apo-Fp in the absence of FAD. Notably, the medium chain acyl-CoA dehydrogenase binds to hsp60 only when its flavin cofactor is present (Saijo and Tanaka, 1995). Since almost half of the SDH Fp present is bound by hsp60 (Fig. 8), this interaction is probably real and may reflect a role for hsp60 in assembling the Fp into the SDH holo-complex.

In summary, we have developed an in vitro flavinylation assay using mitochondrial matrix fractions. Translocation across a membrane is not a prerequisite for FAD attachment to the Fp. At least one matrix protein appears to participate in Fp flavinylation, the mitochondrial processing peptidase, which removes the presequence from the precursor. Curiously, although folding seems to be crucial for Fp modification, hsp60 is not required for cofactor attachment. The participation of mitochondrial processing peptidase in cofactor addition does not eliminate the possibility that bond formation is an autocatalytic process; rather, it may, as we believe, signify the need for an appropriate conformation before modification can proceed. Further insights into the mechanism of FAD addition may await the development of an assay with purified components.

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A Requirement for Matrix Processing Peptidase but Not for Mitochondrial Chaperonin in the Covalent Attachment of FAD to the Yeast Succinate Dehydrogenase Flavoprotein
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