Covalent Attachment of FAD Derivatives to a Fusion Protein Consisting of 6-Hydroxy-\(d\)-nicotine Oxidase and a Mitochondrial Presequence

**FOLDING, ENZYME ACTIVITY, AND IMPORT OF THE MODIFIED PROTEIN INTO YEAST MITOCHONDRIA**

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Autoflavinylation of 6-hydroxy-\(d\)-nicotine oxidase (6-HDNO) was successfully employed to modify the protein covalently with FAD derivatives. The model compounds \(N^6\)-(2-aminoethyl)-FAD and \(N^6\)-(6-carboxyhexyl)-FAD were spontaneously bound to a fusion protein consisting of the mitochondrial targeting sequence of *Neurospora crassa* F$_0$-ATPase subunit 9 (Su9) attached to 6-HDNO. When translated in the rabbit reticulocyte lysate, Su9–6-HDNO was in the trypsin-sensitive apoenzyme form. When translated in the presence of flavins it adopted a trypsin-resistant conformation characteristic of the 6-HDNO holoenzyme. With flavin derivatives, Su9–6-HDNO exhibited approximately 50% of the 6-HDNO activity observed with FAD.

The covalently modified Su9–6-HDNO was imported into *Saccharomyces cerevisiae* mitochondria with an efficiency equal to that of the apoenzyme. Apparently the increase in size and charge of the FAD moiety did not hamper translocation across the mitochondrial membranes. Yeast mutant secl–2 mitochondria deficient in mtHsp70 unfoldase activity imported the flavinylated Su9–6-HDNO protein. In mutant secl–3 mitochondria deficient in both mtHsp70 unfoldase and translocase activity Su9–6-HDNO was trapped as translocation intermediate; the Su9 presequence was passed to the matrix where it was proteolytically cleaved by the mitochondrial process peptidase (MPP); the translocation-arrested 6-HDNO moiety adopted a trypsin-sensitive conformation. Our results indicate that unfolding of the FAD-stabilized flavin-binding domain of 6-HDNO in passage through the mitochondrial general insertion pore does not require the activity of mtHsp70.

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6-Hydroxy-\(d\)-nicotine oxidase (6-HDNO\(\dagger\)) belongs to a group of flavoenzymes with covalently attached FAD (for a review, see Ref. 1). Formation of the histidyl(\(N^2\)-(8\(\alpha\))-FAD bond proceeds autocatalytically and depends on a flavinylatable function of the protein (2, 3). The autocatalytic nature of the process opens the possibility to bind FAD derivatives to the active site His\(\dagger\) of the enzyme in a spontaneous reaction. One aim of the present work was to test this assumption with *in vitro* synthesized apoenzyme, \(N^6\)-(2-aminoethyl)-FAD, and \(N^6\)-(6-carboxyhexyl)-FAD (4) and to establish how these substitutions affect folding and enzyme activity of the protein. A second aim was to use the protein-bound flavin derivatives as probes in an attempt to determine whether the increase in size and charge of the FAD moiety affects mitochondrial import of 6-HDNO supplied with a mitochondrial targeting sequence. Incorporation of FAD into the flavin-binding domain of 6-HDNO induces a conformational change in the trypsin-sensitive apoenzyme that renders the holoenzyme trypsin-resistant (5). Based on this observation, we used 6-HDNO as a model flavoenzyme to approach a third aspect in this work, namely whether the FAD-stabilized trypsin-resistant conformation of holo-6-HDNO requires functionally active mtHsp70 for its unfolding during mitochondrial import. To address this question, we took advantage of the recently described *Saccharomyces cerevisiae* mitochondrial mutants secl–2 with a defect in the unfoldase activity of mtHsp70 and secl–3 deficient in both mtHsp70 unfoldase and translocase activity (6, 7). We have shown previously that an N-terminal fusion of 6-HDNO with the mitochondrial targeting sequence of dimethylglycine dehydrogenase is efficiently imported into rat liver mitochondria (8). However, the rat mitochondrial targeting sequence did not support import of 6-HDNO into yeast mitochondria. Therefore, an N-terminal fusion of 6-HDNO to the mitochondrial presequence of *Neurospora crassa* F$_0$-ATPase subunit 9 (8) was constructed.

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Here we show that the fusion protein Su9–6-HDNO, when translated in the rabbit reticulocyte lysate (RL), spontaneously incorporated \(N^6\)-(2-aminoethyl)-FAD and \(N^6\)-(6-carboxyhexyl)-FAD and folded into a trypsin-resistant, enzymatically active conformation. The fusion protein with attached flavins was imported into yeast mitochondria where it was refolded in the matrix into the trypsin-resistant form; however, when imported as apoenzyme, that is without bound FAD, it was unable to adopt the mitochondrial matrix the trypsin-resistant conformation characteristic of the holoenzyme. The trypsin-resistant, flavin-stabilized fusion protein was imported into secl–2 mutant yeast mitochondria deficient in mtHsp70 unfoldase activity but was trapped as translocation intermediate in secl–3 mitochondria deficient in mtHsp70 unfoldase and translocase activity. The protein blocked in transit through the translocation channel became trypsin sensitive indicating that it was unfolded. Our results demonstrate that unfolding of the
flavin domain of 6-HDNO did not require the activity of mtHsp70.

**MATERIALS AND METHODS**

Chemicals and Biochemicals—All chemicals were of the highest quality available. Restriction endonucleases, DNA ligase, TqA DNA polymerase, and SP6 RNA-polymerase were from Boehringer (Mannheim, Germany). The rabbit reticulocyte lysate in vitro transcription-translation kit was from Promega (Madison, WI). The synthesis of N<sup>6</sup>-(2-aminomethyl)-FAD and N<sup>6</sup>-(6-carboxyhexyl)-FAD was performed as described in Ref. 4.

Construction of the Su9–6-HDNO Fusion Protein—The DNA sequence encoding the mitochondrial targeting amino acid sequence of the F<sub>0</sub>-ATPase of *N. crassa* (8) was amplified with the aid of the polymerase chain reaction using DNA primers with a recognition site for the restriction enzyme HindIII (primer A, 5′-GTAACCTAAGCT-TGAAAAA-3′; primer B, 5′-CCATGCGCTGGAACAGCTTGCAGAG-TAGGCG-3′). pDS5-Su9-dihydrofolate reductase (8) was used as the template DNA. The amplified DNA was restricted with HindIII, the DNA fragment inserted into pSP612–6-HDNO (3) linearized with HindIII by ligation with DNA ligase, and the recombinant plasmid DNA transformed into Escherichia coli cells by electroporation.

In Vitro Expression and Holoenzyme Formation of Su9–6-HDNO in the Rabbit RL—Transcription of the Su9–6-HDNO fusion protein mRNA was performed from the SP6 promoter situated on the vector plasmid with the aid of SP6 RNA polymerase. Transcription was coupled to translation in the rabbit RL system in the presence of [35S]Met at 30 °C for 2 h. Holoenzyme formation with covalent incorporation of flavins was performed in RL supplemented with either 10 μM FAD, N<sup>6</sup>-(2-aminomethyl) FAD, or N<sup>6</sup>-(6-carboxyhexyl)-FAD; following translation, the assays were incubated in the presence of 10 μM glycerol-3-P for an additional 60 min. The addition of glycerol-3-P to the transcription-translation assay stopped any further translation of the fusion protein during the 60-min incubation period. Folding of the 6-HDNO partner of the fusion protein into the protease-resistant conformation of the holoenzyme was tested by trypsin treatment (200 μg/ml) of the transcription-translation assay for 15 min at 0 °C. 6-HDNO activity of the Su9–6-HDNO fusion protein translated in the RL was determined according to Möhler et al. (9).

Isolation of *S. cerevisiae* mitochondria was performed as described previously (10, 11).

Protein Import into Isolated Mitochondria—Protein import into isolated mitochondria was performed in bovine serum albumin-containing buffer (250 mM sucrose, 3% (w/v) fatty acid-free bovine serum albumin, 80 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM MOPS/KOH, pH 7.2) including 2 mM ATP, 2 mM NADH, and 70 μg of mitochondrial protein/0.2 ml. Import reactions were performed at 25 °C for 20 min. Mitochondria were re-isolated by centrifugation at 15,000 × g for 10 min at 4 °C. Samples were treated with trypsin (200 μg/ml) for 15 min at 0 °C where indicated. Trypsin was inactivated by the addition of soybean trypsin inhibitor (4 mg/ml). After a washing step with buffer SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2), the pelletted mitochondria were analyzed by SDS-PAGE.

Subfractionation of Mitochondria—Subfractionation of mitochondria was done by sonication at 100 watts for 3 × 30 s at 0 °C. The purity of the submitochondrial fractions was tested by Western blot with the aid of antibodies specific for a marker protein of the mitochondrial inner membrane.

RESULTS

Trypsin Resistance and Enzyme Activity of Su9–6-HDNO Expressed in the Rabbit RL—Fusion of the 66-amino acid long Su9 precursor to the N terminus of 6-HDNO resulted in a protein with an *M<sub>c</sub>* of 56,000. In *vitro* expression of the fusion protein in the rabbit RL gave rise to a main translation product and a slightly smaller molecular species which may arise from translation of a prematurely terminated RNA transcript (Fig. 1A, lane 1). Both 6-HDNO species were trypsin-sensitive (Fig. 1A, lane 2) indicating that they represented apoenzyme (5). When transcription-translation was performed in RL supplemented with 10 μM FAD and incubation continued in the presence of glycerol-3-P, a allosteric effector that stimulates flavinylation of 6-HDNO in *vitro* (2) trypsin digestion resulted in a protease-resistant species of the size of the native enzyme (Fig. 1A, lanes 3 and 4). Apparently trypsin removed the mito-

![Fig. 1. Trypsin resistance and enzyme activity of apo- and holo-Su9–6-HDNO translated in the RL. A. Su9–6-HDNO was translated in the presence of [35S]Met in the absence (lane 1) or presence of 10 μM FAD, followed by incubation with glycerol-3-P (G-3-P) (lane 3); one-half of each assay was trypsin digested (lanes 2 and 4). p indicates position of Su9–6-HDNO separated by SDS-PAGE on a 10% polyacrylamide gel; m, position of the trypsin-digested Su9–6-HDNO. B. 6-HDNO activity of the Su9–6-HDNO RL translation product synthesized in the presence or absence of FAD and glycerol-3-P as indicated in the figure.](image-url)
To this end [35S]Met-labeled Su9–6-HDNO was translated in the RL in the presence of the FAD derivatives followed by incubation with glycerol-3-P. Trypsin digestion of the translation assays revealed that similar to control assays performed with FAD, a protease-resistant 6-HDNO molecular species was formed (Fig. 4A). This processed trypsin-resistant protein corresponded to the 6-HDNO holoenzyme form and indicated that the flavin derivatives did not prevent folding of the enzyme into its native conformation. This assumption was confirmed by 6-HDNO activity measurements as shown in Fig. 4B. Both FAD derivatives supported enzyme activity levels 50–60% of that of the 6-HDNO holoenzyme generated with FAD. Enzyme activity was due to covalently attached FAD and FAD derivatives since noncovalently bound FAD results in enzyme activity with altered kinetic parameters (12).

Import of Su9–6-HDNO with Covalently Attached FAD Derivatives into Yeast Mitochondria—We asked whether the extension of the FAD moiety by the positively charged 2-aminoethyl or the negatively charged 6-carboxyhexyl groups affects the import of the covalently modified fusion protein into yeast mitochondria. [35S]Met-labeled Su9–6-HDNO with bound FAD derivatives was incubated with mitochondria and tested for import by trypsin digestion. As can be seen in Fig. 5A, both modified forms of the fusion protein were protected from digestion indicating they were intramitochondrially located. As in the case of holoenzyme and although different from apoenzyme, the imported and processed fusion proteins with bound FAD derivatives refolded in the mitochondrial matrix into a trypsin-resistant conformation (Fig. 5B). However, protease treatment resulted in a protein double band (Fig. 5B, lanes 2 and 4). The conformation of the refolded 6-HDNO carrying the attached N6-modified FAD derivatives apparently exposes in some of the protein molecules a trypsin recognition site that is shielded in the FAD-containing holoenzyme. At this site, a short peptide may have been removed from the protein.

Import of Holo-Su9–6-HDNO Carrying FAD and FAD Derivatives into Yeast Mutant Mitochondria with Impaired mtHsp70 Functions—Two temperature-sensitive mtHsp70 mutants were employed. For ssc1–2, which is deficient in unfolding activity of mtHsp70, mitochondria import only completely unfolded precursor proteins; and for ssc1–3, impaired in both the unfolding as well as the translocating function of mtHsp70, mitochondria do not import precursor proteins into the mitochondrial matrix (6, 13). With the aid of these mutants, it should be possible to answer the following questions. 1) Is the FAD-binding domain of the 6-HDNO holoenzyme unfolded during import, or does it prevent translocation. Is unfolding of the domain dependent on a functional mtHsp70? 2) Does the increase in size and charge of the FAD moiety affect the efficiency of holoenzyme import with attached FAD derivatives into mitochondria with impaired mtHsp70 function?

The experimental results presented in Fig. 6A, demonstrate that holo-Su9–6-HDNO formed with FAD and N6-substituted FAD derivatives was imported into ssc1–2 mutant mitochondria (compare Fig. 6A, lanes 2, 4, and 6). Apparently translocation did not require the unfolding activity of mtHsp70. The increase in size and charge of the cofactor molecule did not seem to significantly influence the import of the flavinylated molecules. ssc1–3 mitochondria processed the precursor (Fig. 4A).
6-HDNO with \(N^6\)-substituted FAD

FIG. 5. Import of Su9–6-HDNO modified with FAD derivatives into yeast mitochondria. A, import of Su9–6-HDNO translated in RL supplemented with \(N^6\)-(2-aminoethyl)-FAD (AE-FAD, lane 1) or \(N^6\)-(6-carboxyhexyl)-FAD (C6-FAD, lane 3) followed by trypsin digestion (lanes 2 and 4, respectively). B, following import, the matrix fraction was isolated and trypsin digested as indicated in A. Shown is the autoradiography of the \([35S]\)Met labeled proteins separated by SDS-PAGE on a 10% polyacrylamide gel. \(p\), indicates position of Su9–6-HDNO separated by SDS-PAGE on a 10% polyacrylamide gel; \(m\), indicates position of the trypsin-digested Su9–6-HDNO.

6B, lanes 1, 3, and 5) but were unable to import the Su9–6-HDNO fusion proteins into the mitochondrial matrix as indicated by the accessibility of the protein to trypsin (Fig. 6B, lanes 2, 4, and 6). Processing of the precursor implies that the presequence was passed through the import pore and was accessible to the mitochondrial processing peptidase. The remaining polypeptide was sequenced as a membrane-spanning translocation intermediate in the import pore (14). Since it was sensitive to trypsin digestion, the protease-resistant native conformation of the holoenzyme must have been unfolded during the insertion of the fusion protein into the translocation pore.

A quantification of the processing efficiency of the different Su9–6-HDNO forms is presented in Table I. The data reveal that there was no significant difference in processing efficiency of apo-Su9–6-HDNO and Su9–6-HDNO stabilized by FAD, and \(N^6\)-substituted FAD derivatives into wild type and ssc1–2 mutant mitochondria. ssc1–3 mitochondria showed a somewhat reduced efficiency in processing when compared with wild type and ssc1–2 mitochondria. The strongest reduction in processing efficiency of about 50% of wild type and ssc1–2 mitochondria was observed with ssc1–3 mitochondria and Su9–6-HDNO with bound FAD. Processing of Su9–6-HDNO with bound \(N^6\)-(2-aminoethyl)-FAD and \(N^6\)-(6-carboxyhexyl)-FAD into ssc1–3 mitochondria proceeded with an efficiency similar to that of the apoenzyme.

DISCUSSION

The autocatalytic nature of the flavinylation reaction of 6-HDNO opens the possibility to bind flavin derivatives covalently to the active site of the enzyme. The results presented in this study demonstrate that \(N^6\)-substituted FAD derivatives may indeed be covalently attached to the protein. Since covalent attachment of FAD seems to proceed in other enzymes by autoflavinylation too (15–17), this approach of modifying proteins of this flavoenzyme group may have general applicability.

![Import of Su9–6-HDNO modified with FAD derivatives into yeast mitochondria](Image 5)

![Efficiency of Su9–6HDO processing by S. cerevisiae wild-type, ssc1–2, and ssc1–3 mitochondria](Image 6)

The crystal structure of 6-HDNO has not yet been solved. Therefore the position of the \(N^6\) amino group in the protein structure is not known. From the observations that folding of the enzyme into the trypsin-resistant conformation characteristic for the holoenzyme is not prevented in the presence of the \(N^6\)-(2-aminoethyl)-FAD and \(N^6\)-(6-carboxyhexyl)-FAD and that the FAD derivatives support 6-HDNO activity, we conclude that the extension at the \(N^6\) position and the additional positive or negative charge at this site do not interact with essential functional groups of the enzyme. As with other flavoproteins (18, 19), the adenine moiety of FAD may be exposed at the surface of 6-HDNO making this site suitable for the attachment of fluorescent groups or cross-linkers as molecular markers in the cellular interactions of the enzyme during compartmentation, folding by chaperones, and degrading by proteases (4). The reduced 6-HDNO activity with \(N^6\)-substituted FAD derivatives as compared with the FAD-containing enzyme is in line with the results of reconstitution experiments performed with apo-NADH oxidase and \(N^6\)-(2-aminoethyl)-FAD (4). This enzyme contains loosely bound FAD, which makes the preparation of apoenzyme convenient. The \(N^6\)-(2-aminoethyl)-FAD-reconstituted enzyme showed an increased \(K_m\) value, which

| Table I: Efficiency of Su9–6HDO processing by S. cerevisiae wild-type, ssc1–2, and ssc1–3 mitochondria |
|---------------------------------|---------------------------------|---------------------------------|
| Wild type | ssc1–2 | ssc1–3 |
| % | % | % |
| −FAD | 49 | 52 | 45 |
| +FAD | 58 | 63 | 30 |
| +AE-FAD | 50 | 50 | 40 |
| +C6-FAD | 50 | 50 | 42 |
may indicate a sterically somewhat hindered alignment of the N^6-(2-aminoethyl) moiety in the adenine part of the FAD-binding site of the protein (4).

Mitochondrial precursor proteins seem to be maintained in the cytosol in an import-competent loosely folded conformation by cellular chaperones. There have been specialized presequence-binding proteins described that inhibit folding of the precursor protein and participate in its targeting to the mitochondrial compartment (20). The 6-HDNO partner of the Su9–6-HDNO fusion, however, was folded and flavinylated in the RL. This observation confirms the finding that the mitochondrial presequence does not prevent the folding of 6-HDNO made previously with a fusion of the mitochondrial presequence of dimethylglycine dehydrogenase and 6-HDNO (3). A similar observation has been made with a fusion of the Su9 presequence and dihydrofolate reductase (21). The amino acid sequence joining the presequence and 6-HDNO may be flexible and allow folding of 6-HDNO. This may be different with some authentic mitochondrial precursor proteins. Why the rat mitochondrial presequence of dimethylglycine dehydrogenase did not support the import of the fusion protein into yeast mitochondria is unknown, but such a functional incompatibility between mammalian and yeast presequences has been observed before (22).

Import of mitochondrial precursor proteins requires an unfolded conformation although the extent of secondary structural elements preserved by various precursors during their passage into the organelle is not well established. There is indirect evidence suggesting an extended conformation of the polypeptides in transit (14). Unfolding as a prerequisite for import is supported by experimental data showing that stabilization of the native protein conformation prevents its translocation across the mitochondrial membrane (for a review, see Ref. 20). The driving force responsible for the translocation of the polypeptide through the import pore was associated with mtHsp70 to which an unfoldase and translocase deficient mtHsp70 demonstrate that unfolding of the protease-resistant 6-HDNO holoenzyme portion of the fusion protein takes place following insertion of the precursor into the import pore. This seems sufficient to induce the unfolding of the 6-HDNO fusion partner located outside the translocation channel. Recently it was shown with the aid of a purified outer mitochondrial membrane vesicle system that unfolding of the mature dihydrofolate reductase fused to the presequence of Su9 or cytochrome b_2 is accomplished by components of the outer membrane translocation pore (23). Binding to the trans site of the translocation pore was accompanied by the unfolding of parts of the mature protein immediately following the presequence. Such a mechanism may also account for the observed unfolding of the FAD-stabilized Su9–6-HDNO by mitochondria of the ssc1–3 mutant.

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