The C-Terminus of the Succinate Dehydrogenase IP Peptide of *Saccharomyces cerevisiae* Is Significant for Assembly of Complex II†

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ABSTRACT: Site-directed mutagenesis was used to introduce mutations into the gene for the iron protein (IP) of succinate dehydrogenase (SDH) of *Saccharomyces cerevisiae*. Specifically, three mutations were examined which caused the synthesis of truncated IP peptides missing four, seven, or 17 amino acids from the C-terminus, respectively. The deletion of seven or more amino acids includes the loss of two lysine residues, which appear to have been highly conserved in evolution. While the deletion of four amino acids had no effect on the assembly of complex II and on its activity, the deletion including the two lysines abolished SDS activity completely and led to the failure of the imported IP peptide to be incorporated into a stable complex II or SDH complex. Replacement of one of the lysines by threonine had no effect, but replacement of both by threonine affected the specific activity of complex II but not its assembly and stability.

Succinate dehydrogenase (SDH) is an essential enzyme of the tricarboxylic acid (Krebs) cycle. SDH consists of two subunits, an iron protein subunit (IP) and a flavoprotein subunit (FP). In *Saccharomyces cerevisiae*, these peptides are 27 and 70 kDa, respectively. The enzyme is unique among those of the tricarboxylic acid cycle in that it is not free in the mitochrondrial matrix. Instead, SDH is bound to the mitochondrial inner membrane by a stable association with two small, integral membrane proteins. SDH may be dissociated from these two integral membrane proteins in vitro if purified mitochondrial membranes are treated with chaotropic ions (Hatefi et al., 1979). The IP/FP complex may subsequently be isolated, and it has been found to remain functional as a succinate dehydrogenase in the presence of artificial electron acceptors. The four peptides (the IP, the FP, and the two integral membrane proteins), together, constitute complex II of this pathway [for a review, see Hatefi et al. (1979), Hatefi (1985) and Singer and Johnson (1985)].

A widely accepted model for the topology of complex II has the IP subunit serving as the link between the integral membrane proteins of the inner membrane and the FP subunit, which extends into the matrix, based on earlier work by Merli et al. (1979) and Girdlestone et al. (1981). This arrangement would be consistent with the presumed electron flow within the complex (Hatefi et al., 1979). Recently, however, a different model for the bovine complex II has been proposed (Clarkson et al., 1991). Based on proteolytic digestions and immunochemical analyses, the alternate model has at least one large, hydrophobic domain of the FP inserted into the inner membrane. This model, however, has yet to explain the observation that SDH is easily isolated from membrane fractions by treatment with chaotropic ions.

All four of the peptides constituting complex II appear to be encoded by nuclear genes in eukaryotic cells. Thus, the precursor peptides are synthesized in the cytosol and must be imported into the mitochondria. Either concurrently with or following import, the peptides must be processed to their mature forms and assembled to form complex II. The biogenesis of a functional complex also requires the covalent attachment of a heme group to one of the two integral membrane proteins, the covalent attachment of a flavin cofactor to the FP, and the formation of three non-heme iron–sulfur clusters in the IP subunit (Tsukamoto et al., 1990).

In the case of *S. cerevisiae*, only the IP and the FP genes of complex II have been cloned thus far (Lombardo et al., 1990; Robinson et al., 1992) (Chapman and Boeke, personal communication). Comparison of the amino acid sequence data from the yeast IP with that of bovine has assisted in the identification of a prospective signal sequence (Lombardo et al., 1990). The sequence is approximately 20 amino acids in length and, if the yeast and bovine IP peptides are processed in a similar manner, it is undoubtedly cleaved off during maturation. The import and processing of the FP peptide are likely to be similar. On the other hand, the import and processing of the two integral membrane proteins may be more complicated. Elucidation of this pathway will have to await the cloning and sequencing of the corresponding genes.

Much remains to be learned regarding the fate of the different peptides once imported into mitochondria. Little is known of the order in which the subunits are assembled or of other proteins involved in complex II formation. Recent reports have suggested that heat shock-like proteins (i.e., chaperonins) may play a transient role in the assembly of similar multisubunit complexes (Ellis & Hemmingsen, 1989; Ostermann et al., 1989; Leustek et al., 1989; Rothman, 1989; Cheng et al., 1989). There is also a complete lack of understanding of the timing and mechanism of the conjugation of the flavin to the histidine residue of the FP and of the formation of the iron–sulfur clusters. The nature of the immediate donor in each of these steps is, likewise, completely obscure.

The availability of the IP and FP genes has now made it possible to begin addressing some of the questions raised above. Since complex II has the smallest number of subunits of all the complexes of the electron transport chain, it promises to become a good model system for studying some of the basic mechanisms of assembly of these complexes.

In the present study, we were led to a consideration of the role of the C-terminal portion of the IP peptide in the assembly

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of a functional SDH activity in yeast mitochondria. Two consecutive lysines near the terminal appear to be highly conserved in evolution (Lombardo et al., 1990; Keon et al., 1991). Furthermore, a potential role for such lysine residues in the interaction between SDH and the integral membrane proteins of complex II had been suggested some time ago by Yu and Yu (1981). The results with several mutants made by site-directed mutagenesis are described.

EXPERIMENTAL PROCEDURES

Yeast Strains. The parental yeast strain DL1 (*leu2 his3 ura3*) (van Loon et al., 1983) was obtained from Dr. M. Yaffe, Department of Biology, University of California, San Diego. An IP-deficient mutant derived from this strain has been described by us (Lombardo & Scheffler, 1989).

The parental yeast strain YH8 (*leu2 his3 ura3 trp1*) and the FP-deficient mutant KC122 were kindly provided by Dr. J. D. Boeke, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine.

Yeast cells were grown on YPD or YPG (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose or 3% glycerol, respectively), or on SD or SG (0.67% Difco yeast nitrogen base without amino acids, and 2% dextrose or 3% glycerol, respectively) with the appropriate supplements (Sherman et al., 1982). The continued presence of the plasmids was verified periodically by growth on selective media (Lombardo et al., 1990) or by Southern analysis.

All yeast transformations were performed by the lithium acetate method (Ito et al., 1983).

Vectors and Cloned Genes. The cloning and characterization of the yeast IP gene and the construction of pRS315 IP (previously designated pRS IP7) have been described by us (Lombardo et al., 1990).

Peptide Expression in Bacteria. The pET vector expression system (Studier et al., 1990) was kindly provided to us by Dr. F. W. Studier, Department of Biology, Brookhaven National Laboratory.

The vector pET IP was constructed by cloning the *Bgl*II–*Bam*HI restriction fragment (containing the 3′ end of the IP coding sequence) from pGEM3zf (–) carrying the IP *Pst*I–*Pst*I restriction fragment (Lombardo et al., 1990) into the *Bam*HI site of pET 3c. Following verification of this construct in the bacterial host HMS174, it was transferred to the host BL21 (DE3) for peptide expression. Induction of peptide expression was performed as directed.

Purification of the Peptide and Injection into Rabbits. The peptide was purified by polyacrylamide gel electrophoresis and extracted from the crushed gel by the method of Singer and Paradiso (1981).

Following lyophilization, the purified peptide was resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4) and mixed either with complete Freund's adjuvant (for the primary injection) or with incomplete Freund's adjuvant (for the subsequent boosts). For the primary injection and for the boosts, 1 mL of this mixture, containing approximately 50 μg of the recombinant peptide, was injected subcutaneously. Boosts were spaced 5 weeks apart. A useful antiserum was obtained 10 days after the second boost.

Western Analyses. Western analyses were carried out by standard methods (Lombardo et al., 1990). IP protein was detected using either [^{125}I]protein A (Amersham) or alkaline phosphatase conjugated goat anti-rabbit IgG (Bio-Rad).

Site-Directed Mutagenesis. A kit (the "Altered Sites in Vitro Mutagenesis System") was purchased from Promega Corp. for this purpose. Mutagenesis reactions and support protocols were performed as directed.

Custom phosphorylated oligonucleotides used in the mutagenesis reactions were synthesized by Promega Corporation.

Northern Analyses. Northern analyses were carried out by standard methods (Ausubel et al., 1988) and as described by us (Lombardo & Scheffler, 1989).

Succinate Dehydrogenase Assays. The succinate-PMS reductase assay was carried out as described by us (Soderberg et al., 1977).

The succinate–cytochrome *c* reductase assay was carried out by standard methods (Sottocasa et al., 1967) with the following modifications: final [cytochrome *c*] = 0.8 mg/mL and final [NaCN] = 2 mM. For the Lineweaver–Burke analyses, variable amounts of succinate were used, while the cytochrome *c* concentration was constant at 0.4 mg/mL.

^{35}S Labeling and Immunoprecipitating. Yeast proteins were labeled in vivo for 8 min with [^{35}S]methionine by the method of Yaffe (1991). Cells were grown in YPD and shifted to YPG for 30 min prior to labeling.

IP peptides were immunoprecipitated from labeled protein extracts using the antiserum described earlier and protein A–Sepharose beads (CL-4B, purchased from Sigma) by standard methods (Ausubel et al., 1988).

Following fractionation by polyacrylamide gel electrophoresis in the presence of 5% β-mercaptoethanol, immunoprecipitated materials were visualized by autoradiography.

Other Methods. DNA sequencing was performed using the Sequenase kit from United States Biochemical Corp.

Mitochondria were purified by the glass bead method of Wills et al. (1986). Mitochondria were prepared either in the absence or presence of protease inhibitors (1 mM PMSF, 0.5 μg/mL leupeptin, and 0.3 TIU/mL aprotinin).

In the fractionation of yeast into a cytosolic and mitochondrial component, the method for mitochondria preparation described above was employed. The supernatant from the final differential pelleting of the mitochondria contains the cytosolic component.

Restriction Enzymes, Isotopes, and Other Reagents. Restriction enzymes were purchased from Bethesda Research Laboratories or from New England Biolabs. They were used according to the instructions provided by the suppliers.

[α-^{32}P]dCTP (specific activity 3000 Ci/mmol) was purchased from Amersham Corp.

All other chemicals were of the highest grade available.

RESULTS

Development and Testing of an Antiserum against the Yeast SDH IP. The fragment of the IP gene encoding the C-terminal portion (146 amino acids) of the mature protein was cloned into a pET vector (Studier et al., 1990) (see Experimental Procedures). With the recombinant pET vector maintained in a suitable bacterial host, induction of the vector's promoter resulted in the expression of large quantities of a peptide (16 kDa) identical in primary sequence to the C-terminal portion of the yeast IP. Following purification, the peptide was used to immunize rabbits. The antiserum obtained from these rabbits was tested on Western blots and found to recognize not only the recombinant peptide produced in bacteria but also a yeast mitochondrial protein of 27 kDa, the expected molecular mass of the SDH IP (Figure 1). The same antiserum did not react significantly with any protein from hamster mitochondria (not shown), contrary to our expectations based on the extensive homology in the amino acid sequences of the yeast and mammalian IPs (Lombardo et al.,

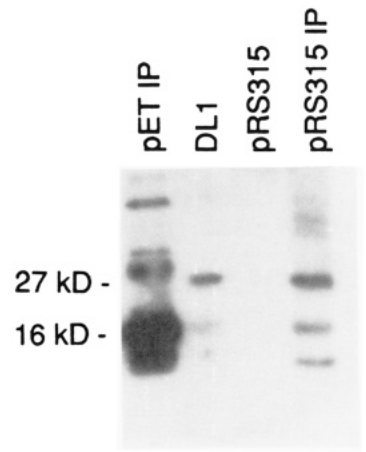

FIGURE 1: Western analysis of induced bacteria and yeast mitochondria. A polyclonal antiserum produced against the C-terminal portion of the yeast IP peptide was used to probe a blot of protein extracts fractionated by gel electrophoresis. (Lanes) pET IP, 20 μg of induced BL21 (DE3) bacteria carrying the plasmid pET IP; DL1, 50 μg of mitochondria from yeast strain DL1; pRS315, 50 μg of mitochondria from the IP-deficient yeast strain carrying the vector pRS315; pRS315 IP, 50 μg of mitochondria from the IP-deficient yeast strain carrying the vector pRS315 IP. Yeast cells were grown in YPD. We believe that the two smaller bands observed with DL1 and pRS315IP are degradation products of the 27-kDa peptide.

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                                    ⇓⇓
---SLYRCHTIMNCTRTCPKGLNPGLAIAEIKKSLAFA
---...........E.........K.......MMATYKEKQASA
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FIGURE 2: Comparison of the amino acid sequences of the yeast and bovine SDH IP mature peptides at the C-terminus. The sequence for yeast is presented on the top. Differences observed in the sequence for bovine are indicated directly underneath. Critical lysines discussed in the text are indicated by arrows.

1990). However, it had been noted that the yeast and human IPs differ significantly at their C-termini: while nearly identical up to two adjacent lysines, the yeast and the human protein have five and 12 terminal amino acids, respectively, which differ completely (Figure 2).

Site-directed mutagenesis was employed to introduce amber mutations in the IP coding sequence carried out by the recombinant pET vector. Three pET vector IP amber mutants were created and verified by sequencing: pET IPA4, pET IPA7, and pET IPA17, which upon induction in bacteria, resulted in the expression of truncated IP peptides missing four, seven, or 17 amino acids, respectively. Each of the truncated peptides was still recognized by the antiserum (results not shown).

Introduction and Expression of IP Amber Mutants in Yeast. The mutated regions of the three pET IPA vectors were used to replace the corresponding regions in the fully intact IP gene in the nonintegrating shuttle vector pRS313 IP (Sikorski & Hieter, 1989) to create pRS315 IPA4, pRS315 IPA7, and pRS315 IPA17. After cloning and verification of these constructs in bacteria, they were transferred to a yeast strain in which the endogenous IP gene had been stably disrupted (phenotype *leu⁻his⁻sdh⁻*) (Lombardo & Scheffler, 1989). Transformants were selected and routinely maintained in SD medium containing histidine.

Northern analysis identified, in each strain, the presence of the transcript from the IP gene carried on the vector (results not shown). The IP probe consistently detected two transcripts: a 1.3 kb transcript from the IP gene in the vector and a 2.3 kb transcript from the disrupted IP gene in the chromosome (Lombardo & Scheffler, 1989). It is important to stress that the amber mutations do not alter the steady-state level of the mRNAs relative to wild type.

Table I

strain	succinate dehydrogenase activities[a]	
	PMS assay	cytochrome *c* assay
DL1 (host–wild type)	1.32	0.83
pRS315 IP (wild type)	1.00	1.00
pRS315 IPA4 (amber)	0.63	0.83
pRS315 IPA7 (amber)	0.00	0.01
pRS315 IPA17 (amber)	0.0	0.0
pRS315 IP6	0.96	1.05
pRS315 IP67	0.29	0.38

[a] Activities were normalized with respect to the mutant host cells carrying the wild-type gene on a single-copy plasmid.

Succinate Dehydrogenase Activity of Yeast IP Amber Mutants. SDH assays were performed with purified mitochondria from each of the IP amber mutant yeast strains. Initially, mitochondria from cells grown in YPD medium (high glucose) were assayed by the succinate–PMS reductase assay (see Experimental Procedures). As shown in Table I, a truncation of four amino acids from the C-terminus of the IP had a small effect on the observed activity (pRS315 IPA4), whereas a loss of seven or 17 amino acids completely abolished the activity (pRS315 IPA7 and IPA17). The same observation was made when cells were grown in glucose but shifted to glucose-free medium (YPG) for 2.5 h prior to the isolation of the mitochondria. This protocol leads to the well-known release from glucose repression and the induction of significantly higher levels of SDH activity (Fraenkel, 1982). However, even under these conditions no activity was found in the cells transfected with the two longer deletions.

To verify that the above results were not restricted to the particular assay used, the experiments were repeated with a second SDH assay: the succinate–cytochrome *c* reductase assay (see Experimental Procedures). This assay makes use of an exogenous cytochrome *c* as the ultimate electron acceptor (and chromophore) and is therefore more stringent, requiring an intact complex II. Again, the truncation of seven or 17 amino acids from the C-terminus of the IP was found to abolish the observed activity completely (Table I). The same observation was made when the cells were shifted to glucose-free medium (YPG) for 2.5 h prior to mitochondria isolation.

Translation and Mitochondrial Import of Truncated IP Peptides. Synthesis and import of the truncated IP peptides were verified by precipitating mitochondrial and cytosolic fractions derived from [^{35}S]methionine pulse labeled cells using the antiserum discussed earlier. Immunoprecipitated material was then fractionated by polyacrylamide gel electrophoresis and analyzed by autoradiography (Figure 3). A significantly greater fraction of the labeled Ip was found to be associated with the mitochondrial fraction in all cases. This experiment was repeated several times, and the results indicated that all the truncated peptides are imported into the mitochondria. Furthermore, these peptides are synthesized in comparable amounts relative to wild type as judged by the intensity of the signals obtained. The short deletions in the IPA4 and IPA7 peptides are not detectable, but the longer deletion in IPA17 is clearly seen.

The small amount of IP found in the cytosol is probably due to contaminating mitochondria, since it corresponds in size to the mitochondrial peptide. Cytosolic peptide not imported would have been larger, since the Ip peptide has a leader sequence of about 20 amino acids which is presumably cleaved upon import into the mitochondria.

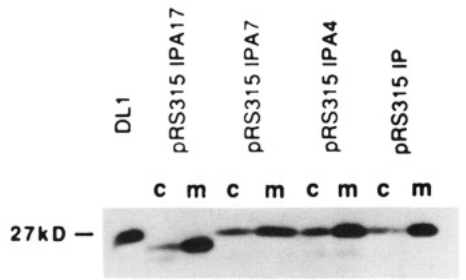

FIGURE 3: Immunoprecipitation of IP peptides from IP amber mutants after pulse labeling with [^{35}S]methionine. Anti-IP antiserum was used to immunoprecipitate total protein from the parental strain DL1 or from mitochondrial (m) or cytosolic (c) protein fractions derived from SDH-deficient yeast cells carrying the indicated plasmids with wild-type or mutant IP genes. Immunoprecipitates were fractionated by electrophoresis and analyzed by autoradiography. Cells were grown in YPD and shifted to YPG for 30 min prior to labeling with [^{35}S]methionine. Equal numbers of cells were labeled and fractionated into mitochondria and cytosol.

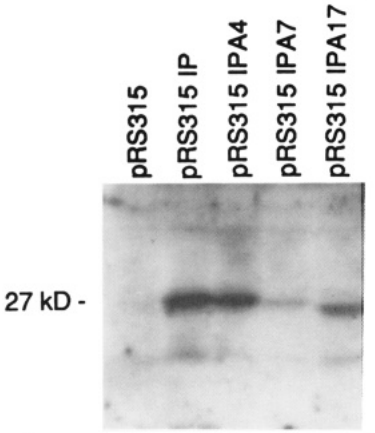

FIGURE 4: Western analysis of IP amber mutants. Total proteins from the purified mitochondria of the IP-deficient yeast strain carrying the vector indicated were fractionated by electrophoresis, blotted, and probed with the anti-IP antiserum. Each lane was loaded with 100 μg of total protein. Cells were grown in YPD and mitochondria were purified in the presence of protease inhibitors.

In an attempt to accumulate a cytosolic IP precursor, wild-type cells were labeled in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a compound known to interfere with the import of protein into mitochondria (Schatz & Butow, 1983; Eilers & Schatz, 1988). A wide range of CCCP concentrations were tested, and no significant amount of labeled immunoprecipitable material was ever observed. On the basis of these results, we concluded that a free, cytosolic IP precursor must be very short lived, even when it is not imported.

The results presented in this section show that all of the truncated IP peptides are synthesized, imported, and processed in yeast mitochondria. This observation is not surprising, since the region of the IP gene which had been mutated would not be expected to play any role in either of these processes.

Stability of Truncated IP Peptides in Mitochondria. To investigate the stability and fate of truncated IP peptides in mitochondria, proteins from the purified mitochondria of each of the yeast clones were analyzed by a standard Western analysis (Figure 4). The technique measures steady-state levels of the peptide. Most of it would be expected to be incorporated into complex II. A single band at the expected molecular weight was seen for wild-type cells and for mutant cells carrying pRS315 IP and pRS315 IPA4. A significantly weaker signal was seen for mutant cells carrying pRS315 IPA7 and pRS315 IPA17, *and in several experiments these cells yielded no signal at all.* Since it had earlier been demonstrated that all truncated IP peptides are synthesized and imported into mitochondria at a normal rate, the lack of accumulation of IP peptides missing seven or more amino acids must be due to their rapid degradation by some as yet unspecified endogenous mitochondrial protease(s). It should be emphasized that, in contrast to the wild-type IP peptide or the IP peptide missing four amino acids, the IPs with larger truncations were detectable only when an assortment of protease inhibitors were included in the mitochondria isolation procedure (see Experimental Procedures). In a separate experiment, cells were treated with an inhibitor of protein synthesis for 60 min before the mitochondria were isolated for Western analysis: no signal was detectable in the cells transfected with the pRS315 IPA7 and IPA17 plasmids. This observation strengthens our conclusion that these peptides are rapidly turned over following import.

A similar result was observed when the cells were shifted to glucose-deficient medium for 2.5 h prior to the isolation of mitochondria. In the yeast strains transfected with pRS315 IP and IPA4, the expected de-repression of the IP gene produced an elevated amount of the peptide detected by the antiserum. A true steady state cannot be measured in the strains with pRS315 IPA7 or IPA17, since these cells remain respiration-deficient and require adequate glucose for growth.

It can also be pointed out that the signal detected on the Western blots (presumably due to the mature form of the IP) corresponds in size to the ^{35}S-labeled peptide immunoprecipitated from mitochondria (Figure 3).

IP Missense Mutants. The striking result from the data presented thus far is that the loss of four amino acids from the C-terminus of the SDH IP had little, if any, effect on the SDH activity, whereas the loss of only three additional amino acids had a profound effect on the activity, as well as on the steady-state level of the peptide in mitochondria. Sequence data indicate that the first two of these crucial three amino acids are lysines. The potential importance of these lysines in the peptide's stability and function is also suggested by the fact that they have been conserved during evolution (Figure 2).

We therefore sought to further elucidate the role of these two lysines by replacing either one, or both, with uncharged amino acids. Site-directed mutagenesis was used to modify the nucleotide sequence of the IP gene in PRS315 IP to change the lysine codons to codons for threonine. This modification would preserve the hydrophilic nature of the side chains in this region of the peptide but remove one or both of the positive charges. The resulting vectors were transferred into the IP-deficient yeast strain as described earlier. Purified mitochondria from these new strains were tested as before.

Replacing the lysine most proximal to the C-terminus had very little effect on the SDH activity as measured by both the succinate–PMS and succinate–cytochrome *c* reductase assays (pRS315IP6; Table I). It also did not appreciably alter the steady-state level of the IP peptide in mitochondria as determined by Western analysis (Figure 5A). In contrast, replacing both of the lysines with threonine caused a reduction in SDH activity to approximately one-third of the wild type's, as measured by both assays (pRS315IP67; Table I). The cause for the decrease in activity was further investigated by a Lineweaver–Burk analysis (results not shown). Identical results were obtained when the analysis was performed with mitochondria isolated from cells grown in media with a non-fermentable carbon source (de-repressed condition) or in media with glucose. The data suggest very clearly that the decrease

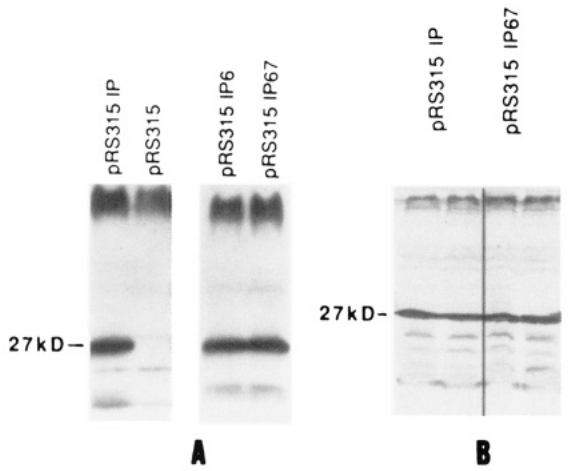

FIGURE 5: Western analysis of IP missense mutants. Total proteins from the purified mitochondria of the IP-deficient yeast strain carrying the vector indicated were fractionated by electrophoresis, blotted, and probed with the anti-IP antiserum. Protein–antibody complexes were detected using either [^{125}I]protein A (A) or alkaline phosphatase-conjugated goat anti-rabbit IgG (B). Each lane was loaded with 100 μg of total protein. Cells were grown in YPD (A) orYPG (B), and mitochondria were purified in the presence of protease inhibitors. pRS315IP6, one lysine substituted by threonine; pRS315IP67, both lysines substituted by threonine.

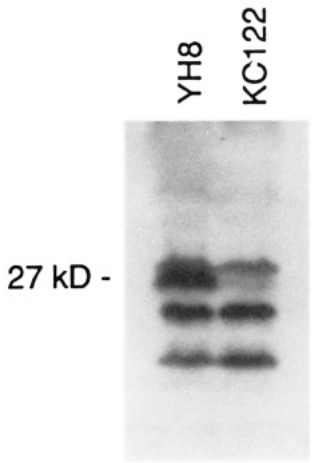

FIGURE 6: Western analysis of the FP-deficient yeast strain. Total proteins from the purified mitochondria of the parental yeast strain YH8 and the FP-deficient strain KC122 were fractionated by electrophoresis, blotted, and probed with the anti-IP antiserum. Each lane was loaded with 100 μg of total protein. Cells were grown in YPD and mitochondria were purified in the presence of protease inhibitors.

in activity was due to a change in v_{max} and not in the affinity of the complex II for the substrate succinate. Since succinate binds to the FP subunit, this result is perhaps not surprising. A decrease in the turnover rate of the reaction in mutant cells could result from a less active enzyme or from the presence of lower amounts of complex II. To distinguish between these possibilities, we examined the steady-state levels of the IP protein. Western analyses were performed on several independently purified mitochondrial preparations from cultures grown in either glucose or glycerol. These results indicate that the steady-state level of IP peptide had not been altered by the mutations (Figure 5A,B).

Stability/Accumulation of the IP in FP-Deficient Mitochondria. It had been previously observed that detectable levels of the FP subunit do not accumulate in the mitochondria of the IP-deficient yeast strain (A. Lombardo, unpublished observations). Recently, an FP-deficient yeast strain created by targeted gene disruption became available to us (see Experimental Procedures). Western analysis of purified mitochondria from this strain indicated that the IP subunit did not accumulate (Figure 6), presumably because in the absence of assembly it is rapidly degraded following import.

DISCUSSION

Much has been learned in the last decade about the targeting of protein precursors to mitochondria and their import and processing to the mature peptides (Attardi & Schatz, 1988; Pon et al., 1989; Pfanner & Neupert, 1987, 1990). Much less is known about the mechanism and pathway of assembly of many of these peptides into the complexes which make up the electron transport chain. Complex II, being the simplest of these complexes, may serve as a good model system for investigating some of the basic mechanisms of these processes.

The cloning of the gene of the IP subunit from yeast (Lombardo et al., 1990) and the isolation of a mutant in which this gene was disrupted (Lombardo & Scheffler, 1989) have permitted us to start an exploration of the role which specific amino acids and domains of this peptide play in the function and assembly of SDH and complex II. Our attention in this study was drawn to two lysine residues near the C-terminus, because they have been conserved in yeast, mammals (Kita et al., 1990; Yao et al., 1987), and fungi (Keon et al., 1991). The sequence up to the two lysines is highly conserved. Interestingly, a study by Yu and Yu (1981) has emphasized the potential involvement of some specific lysines in anchoring the SDH complex (IP and FP) to the inner membrane. Since the IP subunit is thought to interact directly with the two small membrane proteins of complex II, the focus was on the conserved lysines of the IP.

A truncation of four amino acids from the C-terminus of the yeast IP was found to have an insignificant effect on SDH activity and on the amount of this peptide detected in mitochondria at steady state. Since one of the SDH assays involved reduction of cytochrome *c*, complex II must have been assembled. In contrast, truncation of seven amino acids including the highly conserved lysine residues resulted in the complete loss of SDH activity. While the IP peptide was synthesized normally in the cytosol, imported into mitochondria, and proteolytically processed to its mature size, very little if any was retained in mitochondria in a stable form. The same observation was made for the case of a more severe truncation of 17 amino acids.

It follows from this observation that the loss of seven or 17 amino acids from the C-terminus of the SDH IP dramatically impairs the proper assembly of this subunit with the others of complex II. If these truncated peptides were assembled in an inactive complex, they should have been detected in mitochondria by the Western analysis.

On the basis of the above results, it is possible to speculate that the two lysines, in particular, are essential for the successful assembly of the IP subunit into a stable, functional complex II.

Our observations may reflect part of a more general theme governing the assembly of mitochondrial subunits. It has recently been demonstrated that the conserved positively charged lysine residue at the C-terminal of subunit 8 is required for the efficient assembly of the membrane F_o sector of yeast mitochondrial ATP synthase (Grasso et al., 1991).

A dramatic loss of accumulation of IP was also observed in the mitochondria of an FP-deficient yeast strain. Moreover, it had been found that the FP subunit did not accumulate in mitochondria of an IP-deficient yeast strain (A. Lombardo,

unpublished results). On the basis of these observations, we conclude that, unless these subunits are assembled into a stabilizing complex, they are subject to rapid degradation by endogenous mitochondrial proteases.

The observed lack of stability of the IP and the FP peptides in the absence of the other must be considered in relation to possible models for the order of assembly. In the absence of the FP subunit, a normal IP subunit fails to be protected by interaction with the membrane proteins. Thus, membrane attachment of IP may not be an early, independent step in complex II assembly. Alternatively, the IP and FP subunits may assemble in the matrix prior to their membrane attachment. If this were the case, and if the conserved lysines of the IP were exclusively involved in this membrane attachment, one might anticipate the formation of a functional SDH complex in the mitochondrial matrix, as detected by the PMS reductase assay, in the mutant expressing an IP peptide truncated by seven or even 17 amino acids. No evidence in favor of its existence could be derived from SDH activity measurements or Western analysis.

Unfortunately, monitoring the presence and levels of the integral membrane proteins of complex II in these mutants is technically difficult due to the lack of antisera.

The observations with the mutants in which threonine was substituted for one or both lysines suggest that the model has to be more complicated. Substitution of threonine for one of these lysines was found to have no effect on the total activity of complex II or on the steady-state levels of IP in the mitochondria. Substitution of threonine for both of the lysines, however, was found to significantly effect activity, while not affecting complex II assembly. Thus the two lysines may not play an essential role in the assembly mechanism, but their replacement with uncharged amino acid side chains generates a less active enzyme. The kinetic analysis suggests that the change is in the turnover rate rather than in the affinity of the enzyme for the substrate succinate. Whether this is due to an altered interaction between the SDH complex and the membrane or due to conformational changes within the SDH complex remains to be explored. Detailed studies with the purified complex II or SDH will be required to resolve this issue.

The amino acid substitutions chosen may not have been sufficiently different to affect the overall nature of the domain of interest: hydrophilic amino acids were substituted for charged amino acids. In the truncated IP missing seven amino acids, which was completely nonfunctional, a negatively charged C-terminus was moved in place of the positively charged lysines. Obviously, more mutants in this region will have to be examined.

It is highly likely that the assembly of complex II will require an interaction of the imported peptides with chaperonins and scaffolding proteins (Ackerman & Tzagoloff, 1990a,b; Bowman et al., 1991; Flynn et al., 1989; Ellis & Hemmingsen, 1989; Ostermann et al., 1989; Leustek et al., 1989; Rothman, 1989; Kang et al., 1990). The C-terminal domain of the IP may also represent the recognition domain for such an interaction. A potentially interesting approach to distinguish the various possibilities would be to search for suppressor mutations which would permit the assembly of an active complex II even with the truncated IP peptides.

Future work with additional specific mutations introduced by site-directed mutagenesis will attempt to address some of the questions raised above and to delineate the domains of IP the peptide interacting with the membrane and the FP subunit. A parallel set of experiments is now also possible with the FP subunit, since the gene has recently been cloned.

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Registry No. SDH, 9002-02-2; Lys, 56-87-1; Thr, 72-19-5; succinate–coenzyme Q reductase, 9028-11-9.