Studies on the Assembly of Complex II in Yeast Mitochondria Using Chimeric Human/Yeast Genes for the Iron–Sulfur Protein Subunit†

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Received August 10, 1993; Revised Manuscript Received October 25, 1993®

ABSTRACT: A series of chimeric human/yeast IP genes were constructed in order to investigate domains of the iron–sulfur protein (IP) that are important for assembly and/or activity of complex II of the electron transport system in *Saccharomyces cerevisiae*. These genes were expressed in a respiration-deficient yeast mutant in which the endogenous IP gene had been disrupted. Substitutions at the N-terminus were tolerable. Substituting the region covering the first iron–sulfur center [2Fe–2S] had no effect on assembly, while activity decreased 2–5-fold. The addition of seven amino acids from the human peptide, including four charged residues, at the C-terminus did not perturb either assembly or activity. A region between the first and second cysteine clusters was identified which when substituted caused a complete failure in the assembly of complex II. It includes a 15 amino acid stretch which shows the greatest variability between species. Larger substitutions including this segment failed as well. Exchanging the region between the second and third cysteine clusters making up the [4Fe–4S] and [3Fe–4S] centers enabled transformants to grow on nonfermentable carbon sources, yet no SDH activity was observed *in vitro*. The IP and FP proteins accumulate to wild-type levels in these mutants. We speculate that the lack of observed activity is due to the lability of iron–sulfur centers in isolated, broken mitochondria.

Succinate dehydrogenase (SDH) is a ubiquitous mitochondrial enzyme that catalyzes the conversion of succinate to fumarate. The electrons gained from this oxidation are passed to ubiquinone (coenzyme Q) of the electron transport chain. They are then transferred in a series of steps to molecular oxygen. SDH occupies a unique position in energy metabolism, since it serves as a direct link between the TCA cycle and the electron transport chain.

SDH consists of two nonidentical subunits with molecular masses of 70 and 27 kDa. The larger subunit contains the substrate binding site, has one covalently bound flavin (FAD), and is thus referred to as the flavoprotein (FP). The small subunit harbors three acid-labile iron–sulfur centers and is referred to as the iron–sulfur protein (IP) [for recent reviews see Singer and Johnson (1985), Ohnishi (1987), Ackrell et al. (1992), and Hatefi (1985)].

The IP/FP complex is bound to the matrix side of the inner mitochondrial membrane by a stable and noncovalent association with two small integral membrane proteins. These four subunits, which constitute complex II of the electron transport chain, are encoded by nuclear genes in eukaryotic cells (Ackrell et al., 1992; Hatefi, 1985; Hatefi et al., 1979). Thus, they are synthesized in the cytosol, imported into mitochondria, and processed to their mature form prior to or during assembly.

A sufficient number of amino acid sequences are available by now to establish that the FP and IP subunits are highly conserved in evolution. This is especially evident in critical regions of the polypeptides such as the cysteine-rich regions involved in the iron–sulfur clusters in the IP (Gould et al., 1989; Ackrell et al., 1992), and in the catalytic site and the FAD binding domain of the FP (Birch-Machin et al., 1992; Ackrell et al., 1992). The anchor polypeptides, on the other hand, show little sequence homology in the few species studied to date (Yu et al., 1992; Ackrell et al., 1992). Furthermore, their number is not conserved since they can be present as either one or two subunits (Ackrell et al., 1992).

Complex II is the simplest of the electron transport complexes and thus a good model system to study assembly; yet, limited information is available about this process in mitochondria. Little is known about assembly intermediates and the factor(s) involved, if any. Furthermore, the timing as well as the mechanism of addition of the various prosthetic groups is not understood.

A widely accepted model for the topology of complex II has the IP serving as the link between the integral membrane proteins and the FP subunit. It is not known what domains of the IP protein are involved in these interactions. In an attempt to address this question and the assembly problem, we performed in effect mass mutagenesis on the yeast IP protein. Segments of the yeast protein were systematically replaced with the corresponding human sequence. This was possible due to the significant homology between the two corresponding coding sequences. The results of these experiments are described in this paper.

EXPERIMENTAL PROCEDURES

Vectors and Cloned Genes. The cloning and characterization of the yeast IP gene and its expression from the CEN-ARS plasmid pRS315 (Sikorski & Hieter, 1989) have been described by us (Lombardo et al., 1990). The IP gene from the plasmid is used to complement a defective chromosomal IP gene (Lombardo & Scheffler, 1989). The vector carrying the IP gene which was previously designated pRSIP7 has been renamed pRS315IP in this paper. A human IP cDNA clone was isolated from a cDNA library using the partial clone described by Gould et al. (1989). Curiously, it was truncated in the same region encoding the signal sequence as the cDNA clone described by Kita et al. (1990).

† This research was supported by a grant from the U.S. Public Health Service to I.E.S. (GM33752). E.B. was supported by a grant from the UC Biotechnology Research and Education Program to I.E.S. (Participating Investigator).

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® Abstract published in *Advance ACS Abstracts,* December 1, 1993.

0006-2960/94/0433-159$04.50/0

Chimeric Constructs. Common restriction enzyme sites spanning the coding region of the human and the yeast IP genes were identified: *Pst*I, *Nco*I, *Afl*III, *Bsr*I, and *Bsp*H1. Yeast coding regions were removed from the pRS315IP construct and replaced with the human counterpart using standard cloning techniques. The series of chimeric constructs thus generated were designated pMS1–pMS10. They were verified by the appearance and/or disappearance of restriction enzyme sites, by the detection of the expected transcripts on Northern blots, and by the synthesis of immunoprecipitable proteins (see below).

Yeast Strains. An IP-deficient mutant, SC16$^-$ (phenotype *his$^-$ leu$^-$ sdh$^-$*), has been described by us (Lombardo & Scheffler, 1989). 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 as required (Sherman et al., 1982). All yeast transformations were performed by the lithium acetate method (Ito et al., 1983).

Northern Analysis. Northern analysis was carried out by standard methods (Maniatis et al., 1982; Ausubel et al., 1988) and as described by us (Lombardo et al., 1990, 1992).

Preparation of Mitochondria. Mitochondria were prepared by the glass beads method of Wills et al. (1986) in the presence of protease inhibitors (1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 μg/mL leupeptin, and 0.3 trypsin inhibitor unit/mL aprotinin) with the following modifications: The mitochondrial pellet was washed once with cell extraction buffer to remove cytoplasmic contaminants and resuspended in fresh buffer, followed by a low-speed spin (2000*g*, 3 min, 4 °C) to remove aggregates. Protein concentration was determined by the Coomassie protein assay method (Pierce).

Succinate Dehydrogenase Assays. The succinate–phenazine methosulfate (succinate–PMS) and the succinate–cytochrome *c* reductase assays were carried out as described by us previously (Soderberg et al., 1977; Schmidt et al., 1992). Activation of SDH by removing the residual bound competitive inhibitor, oxaloacetate, has been described (Ackrell et al., 1978).

Measurement of Oxygen Consumption. The capacity of whole cells or isolated mitochondria to respire was measured with a Clark oxygen electrode as described in more detail by a previous publication from our laboratory (DeFrancesco et al., 1975).

Anti-IP and FP Antisera. The generation of the anti-IP antiserum has been described (Schmidt et al., 1992). Similarly, the 3′ end of the FP coding sequence was cloned into the bacterial expression vector pET (Studier et al., 1990). Induction of the peptide in BL21 (DE3) was performed as directed. The induced peptide was prepared for injection into rabbits as described by us (Schmidt et al., 1992). A useful antiserum was obtained after the second boost. Antibodies against bovine IP were provided by Dr. Brian Ackrell, University of California, San Francisco.

^{35}S Labeling and Immunoprecipitation. Yeast cells were either grown continuously in glycerol minimal medium or grown in glucose minimal medium and shifted for 30–60 min to glycerol minimal medium. They were then labeled for 8 min with [^{35}S]methionine by the method of Yaffe (Smith & Yaffe, 1991) and chased for various times with cold methionine. IP and FP peptides were immunoprecipitated from labeled protein extracts using the anti-yeast FP antiserum described earlier and either the anti-yeast IP antiserum described by us (Schmidt et al., 1992) or the anti-bovine IP antibody provided by Dr. Ackrell.

Western Analysis. Western analysis was carried out by standard methods (Lombardo et al., 1990). IP and FP proteins were detected using alkaline phosphatase conjugated goat anti-rabbit IgG (Bio-Rad).

Restriction Enzymes, Isotopes, and Other Reagents. Restriction enzymes were purchased from Bethesda Research Laboratories or from New England Biolabs. They were used according to instructions provided by the suppliers. [^{35}S]-Methionine (specific activity ~1000 Ci/mmol) was purchased from ICN.

RESULTS

Construction of Chimeric Genes. Constructs pMS1-pMS10 were cloned by standard techniques (see Experimental Procedures). Restriction fragments of human cDNA were used to replace the corresponding yeast sequences, leaving the promoter, 5′ and 3′ untranslated regions, and the polyadenylation site intact. Figure 1 (top) shows the resulting human/yeast chimeric IP proteins, IP1–IP10. In all cases care was taken to preserve the yeast signal sequence for targeting into mitochondria and proper processing of the imported peptide.

Changes in the amino acid sequence that occurred as a result of the replacement are shown in Figure 1 (bottom). Conservative and nonconservative substitutions and changes in charged amino acids are emphasized. Regions which are thought to form iron–sulfur centers 1, 2, and 3, based on comparisons with bacterial ferrodoxins, are underlined (Ackrell et al., 1992). These cysteine-rich regions are highly conserved throughout evolution as shown in Figure 2. It should be noted that differences between the human and yeast sequences are clustered rather than being scattered throughout the protein. Furthermore, these same regions show the greatest variability among the known SDH IP sequences (see Figure 2).

Introduction and Expression of Chimeric Constructs in Yeast. Each construct of the series, pMS1–pMS10, and pRS315IP with the wild-type yeast IP gene was transfected into a yeast strain (SC16$^-$) in which the endogenous IP gene has been disrupted (Lombardo & Scheffler, 1989), to generate the strains yMS1–yMS10 and yMS11, respectively. Transformants were selected and routinely maintained on minimal glucose medium containing histidine to select for and maintain the single-copy plasmid.

Northern analysis identified, in each strain, the presence of the expected transcript from the IP gene carried on the vector (results not shown). We also verified that the mutations made do not affect the half-life and steady-state levels of the chimeric IP mRNAs relative to wild type (WT) (Lombardo et al., 1992; Creghino et al., submitted for publication).

Growth of Transformants on Nonfermentable Carbon Sources. If the chimeric IP proteins complement the IP-deficient parental cells (SC16$^-$), the transformants, unlike SC16$^-$, should be able to grow on a nonfermentable carbon source such as glycerol. Thus, cells were inoculated on SG (+histidine) plates and grown for several days at 30 °C. While mutants expressing IP1–IP3, IP5, and IP6 (yMS1–yMS3, yMS5, and yMS6) were able to grow on this nonfermentable carbon source, yMS7–yMS10 cells, which harbor the larger C-terminal substitutions, could not (Table 1). The smallest substitution which renders the strain incapable of growing on glycerol has been identified in the plasmid pMS4 (strain yMS4). It represents a 50 amino acid stretch between the

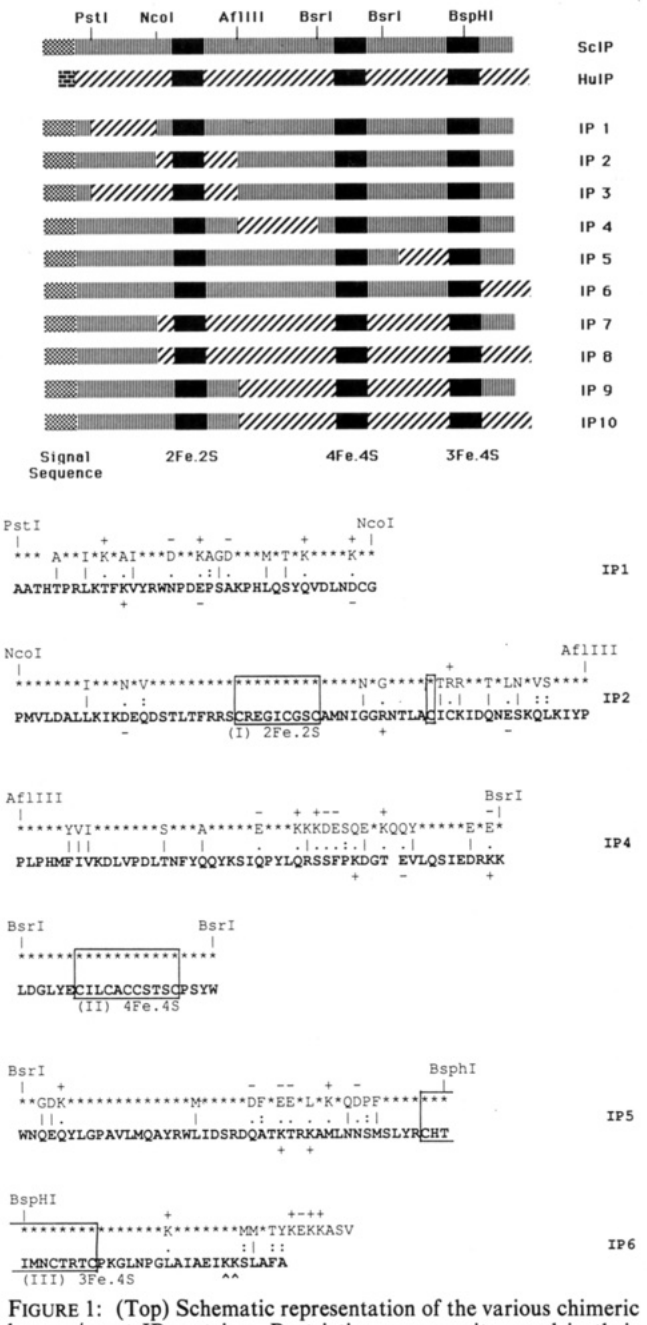

FIGURE 1: (Top) Schematic representation of the various chimeric human/yeast IP proteins. Restriction enzyme sites used in their construction are indicated. The three cysteine clusters are represented by solid boxes. SCIP = *Saccharomyces cerevisiae* iron–sulfur protein; HUIP = human protein. (Bottom) Changes in the amino acid sequence as a result of the mutagenesis are indicated. The yeast sequence is shown in boldface type. The three cysteine clusters are boxed. Conservative substitutions are indicated by lines, while nonconservative substitutions are represented by two dots. The loss or gain of a charged amino acid is represented by a single dot, and the charges are indicated. Arrowheads point to the two highly conserved lysines at the C-terminal end.

first and second cysteine cluster and includes a sequence of about 15 amino acids which vary greatly among all known IP sequences. The larger substitutions in the plasmids pMS7–pMS10 also contain this region, accounting for their failure to complement the defective IP yeast gene.

Growth Rate of Yeast IP Mutants. When grown on a rich medium containing glucose, all transfected mutants have growth rates comparable to that of control cells (yMS11). Among cells that can be grown on glycerol, mutants expressing IP3 and IP5 show 20% and 50% decreases in growth rate, respectively. Measurements of oxygen consumption by whole cells were also performed. With cells grown in YPD (glucose) the difference between wild type and yMS5 cells was minor. However, when cells were grown in glycerol, the rate of respiration of yMS5 cells was approximately half that of the control cells, suggesting that respiration is the limiting rate in the growth of these cells.

Succinate Dehydrogenase Activity of Transformants with Chimeric IP Genes. Enzyme assays were performed with purified mitochondria isolated from cells grown in either glucose or glycerol (see Experimental Procedures). Two different assays were performed. Succinate–cytochrome *c* reductase requires intact complex II. The succinate–PMS reductase assay does not require membrane attachment. All reported activities were succinate-dependent and shown to be completely sensitive to malonate. As shown in Table 1, cells expressing chimeric proteins IP4 and IP7–IP10, which made them unable to grow on glycerol, had no measurable SDH activity by either assay. Mutants expressing IP1 and IP2 gave comparable specific activities to control cells when grown on YPG. However, when these cells were grown on YPD, a small reduction (2-fold or less) in the specific activity was observed for both assays.

Cells expressing IP3 with a substitution, covering the first iron–sulfur center region in the amino-terminal end, exhibited an approximately 5-fold reduction in cytochrome *c* reductase activity and a 2–3-fold reduction in PMS reductase activity, regardless of the conditions under which the cells were grown: with minimal mitochondria in glucose medium or with highly induced levels of mitochondria in glycerol medium. Lineweaver–Burk analysis indicated that the K_m for the substrate succinate had not been altered (results not shown). Furthermore, attempts to remove the residual bound competitive inhibitor oxaloacetate did not change the observed results.

Surprisingly, cells expressing IP5 (yMS5) with a 45 amino acid substitution between the second and third cysteine clusters had no measurable SDH activity *in vitro*, even though these mutants were able to grow on nonfermentable carbon sources such as glycerol, acetate, lactate, and ethanol. Neither the dissociation of oxaloacetate nor attempts to change the tertiary structure of complex II in mitochondria by varying the ionic strength restored any SDH activity.

As mentioned above, intact yMS5 cells are capable of respiration. However, even with freshly isolated mitochondria we could not detect any appreciable succinooxidase activity by measuring succinate-dependent oxygen consumption. In contrast, succinate clearly stimulated oxygen consumption in wild-type mitochondria, and this activity was completely sensitive to malonate.

Western Analysis. The steady-state levels of the IP and FP peptides in isolated mitochondria were determined by western analysis. Initially cells that can grow on glycerol were analyzed, since the synthesis of IP and FP (and other mitochondrial proteins) is derepressed, and therefore levels are easily quantitated by nonradioactive immunochemical staining. Figure 3 shows that cells expressing IP1, IP2, and IP6, (yMS1, yMS2, and yMS6) have equivalent levels of IP and FP compared to control cells. These mutants also have comparable levels of SDH activity (see Table 1). In the yMS3 strain the IP and FP levels are similar to those of control cells, yet this mutant shows a markedly reduced SDH activity (see Table 1). The IP and FP levels of yMS5 mutants are also similar to those of control cells even though no SDH activity is observed *in vitro*.

Pulse–Chase Experiments (yMS3 and yMS5 Mutants). Pulse–chase experiments were used to study the fate of the

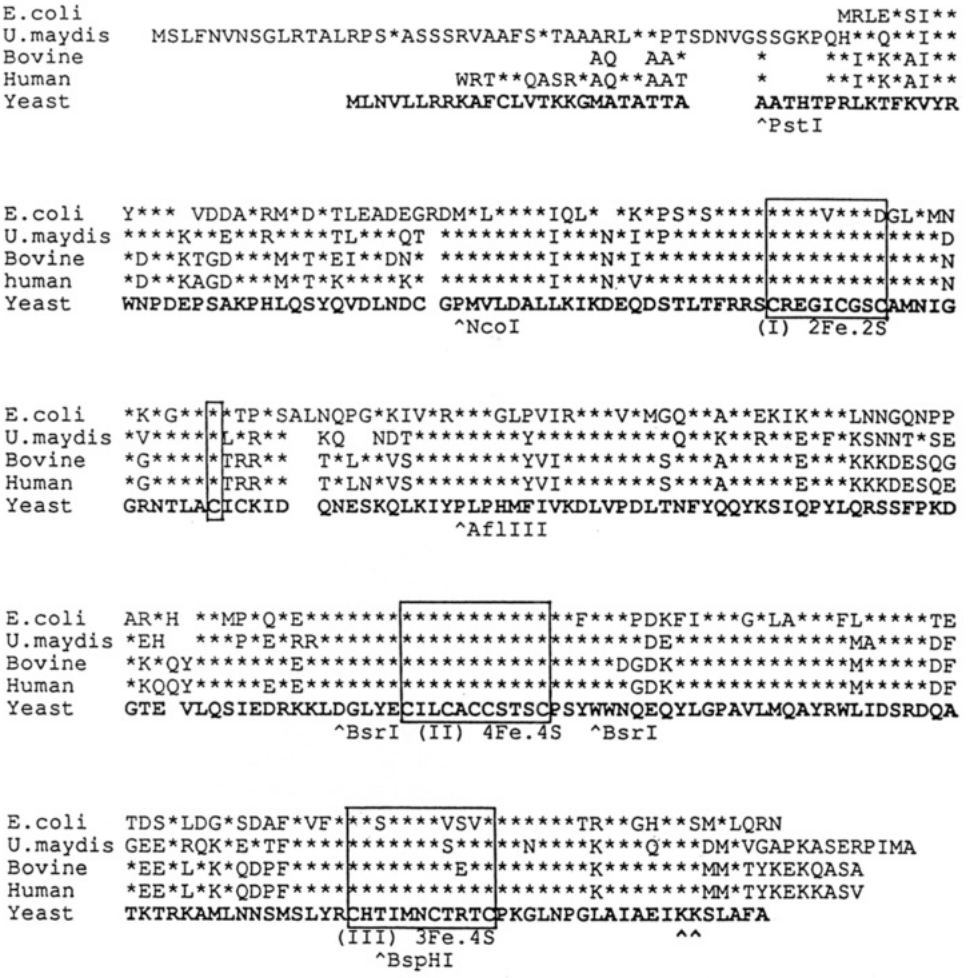

FIGURE 2: Alignment of the SDH IP sequences from various species. Yeast IP (Lombardo et al., 1990), human IP (Kita et al., 1990), bovine IP (Yao et al., 1987), *U. maydis* IP (Broomfield & Hargreaves, 1992), and *E. coli* IP (Darlison & Guest, 1984) sequences are compared. The yeast sequence is shown in boldface type. The three cysteine clusters are boxed. Identical amino acids are represented by asterisks. Restriction enzyme sites are superimposed on the yeast peptide sequence.

Table 1: SDH Activity and Ability To Grow on Nonfermentable Carbon Source[a]

yeast cells	IP protein expressed	growth on glycerol	% SDH activity			
			YPG		YPD	
			cyt *c*	PMS	cyt *c*	PMS
yMS1	IP1	+	84 ± 15	113 ± 7	52 ± 12	76 ± 12
yMS2	IP2	+	85 ± 10	100 ± 16	56 ± 15	66 ± 15
yMS3	IP3	+	21 ± 11	34 ± 13	19 ± 6	42 ± 10
yMS4	IP4	−				
yMS5	IP5	+				
yMS6	IP6	+	80 ± 7	84 ± 10	70	72
yMS7	IP7	−				
yMS8	IP8	−				
yMS9	IP9	−				
yMS10	IP10	−				
yMS11	WT IP	+	100	100	100	100

[a] SDH activities of cells grown in either YPD or YPG are indicated. Two different assays were performed: the cytochrome *c* and the PMS–DCPIP reductase assays. The reported activities are normalized with respect to control cells, yMS11. The results of at least six independent experiments are shown. yMS6 mutants grown in YPD were only assayed twice.

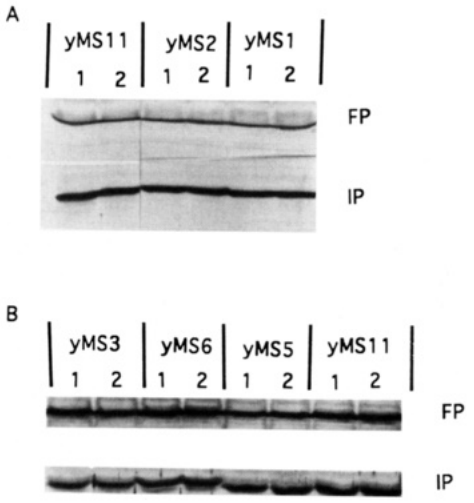

FIGURE 3: Western analysis of mutants that can grow on glycerol. Cells were grown on YPG and the mitochondria were isolated as described in Experimental Procedures. Proteins were fractionated by electrophoresis, blotted, and probed with anti-IP and anti-FP antisera directed against the yeast proteins. The results of two independent mitochondrial preparations are shown for each mutant.

newly synthesized chimeric peptides in an effort to distinguish between the rate of incorporation into a stable complex and the rate of turnover. Mutants with compromised SDH activities were analyzed and compared to the wild-type control. yMS3, yMS5, and control cells (yMS11) grown in glycerol minimal medium were pulse-labeled with [^{35}S]methionine and chased in the presence of excess cold methionine. A mixture of anti-yeast IP and anti-yeast FP antisera was used to precipitate both peptides simultaneously from total, solubilized cell extracts (see Experimental Procedures). Figure 4 shows no detectable FP degradation during the 4-h chase period for all cells analyzed. It should be noted that the FP signal is

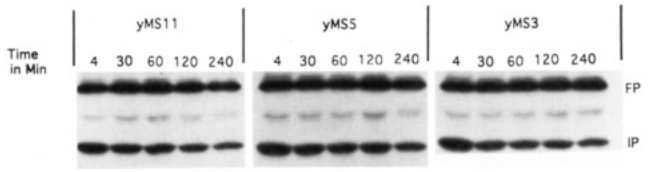

FIGURE 4: Pulse–chase experiments for mutants with compromised SDH activities. Control cells (yMS11) and yMS3 and yMS5 grown in glycerol were pulse-labeled with [^{35}S]methionine and chased for the times indicated with cold methionine. IP and FP proteins were immunoprecipitated using the appropriate antisera directed against the yeast proteins. The precipitated polypeptides were fractionated by electrophoresis and analyzed by autoradiography.

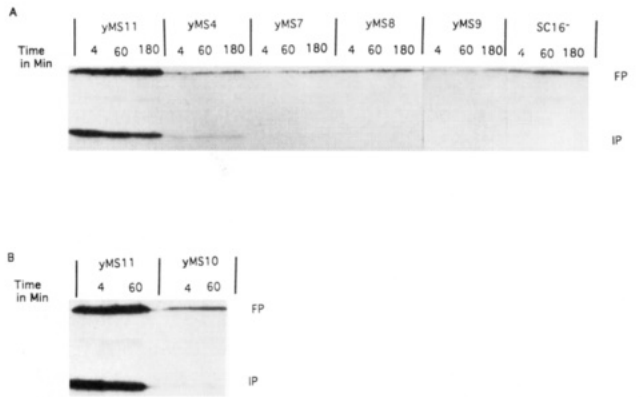

FIGURE 5: Pulse–chase experiments for mutants unable to grow on glycerol. Cells were grown in glucose, shifted for 45 min to glycerol and pulse-labeled with [^{35}S]methionine. They were chased for the indicated times with cold methionine. IP and FP proteins were immunoprecipitated and analyzed as described in the caption for Figure 4. (A) and (B) refer to the various mutants analyzed. yMS11 = control cells.

comparable between mutant and control cells. The IP peptide, on the other hand, is subject to some degradation, the extent of which varies between experiments by a factor of 2 or less and is comparable between mutants and control cells. We interpret this finding to mean that the IP peptide may be produced in a slight excess and that unassembled peptides are subsequently degraded.

As in previous experiments (Schmidt et al., 1992), we were unable to detect the cytoplasmic precursor in any of these experiments. All detectable peptides were associated with mitochondria and fully processed to their mature form at the end of the 8-min pulse.

Analysis of yMS4 and yMS7–yMS10 Mutants. Mutants expressing IP4 and IP7–IP10 are not able to grow on glycerol and thus are unable to induce SDH to levels necessary for visualization by the western analysis performed. The question remained whether assembly was taking place *in vivo* into an inactive complex II. In other words, is it possible to dissociate the assembly process from enzyme activity? Pulse–chase and immunoprecipitation experiments were used to address this question. We assume that if assembly is taking place, IP and FP peptides should be protected and persist at control levels during the chase. On the other hand, if there is no assembly, these proteins will be degraded during the chase by a mitochondrial matrix protease.

The behavior of the FP was initially monitored because our antibody against the yeast IP does not detect the human IP and therefore it may not recognize chimeric IPs where the antigenic end has been replaced with human sequences (IP7–IP10). Results shown in Figure 5 indicate that this polypeptide is significantly reduced in the mutants analyzed compared to control cells. The degradation must be very rapid, since steady-

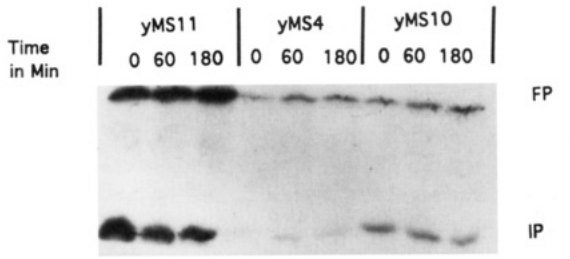

FIGURE 6: yMS4 and yMS10 mutants were analyzed by pulse–chase and immunoprecipitation experiments as described in Figure 5. However, this time anti-bovine IP antibodies were used in conjunction with the anti-yeast FP antiserum. Control cells (yMS11) immunoprecipitated with antisera directed against the yeast IP and FP proteins are shown.

state levels have apparently been reached at the end of the 8-min labeling period. A very small amount of FP peptide seems to persist throughout the chase, but it is not clear whether this material is part of a small amount of inactive complex II, or some other aggregate, for example, with one of the mitochondrial chaperonins (Georgopoulos, 1992; Craig, 1993), or an inactive IP/FP complex.

The wild-type FP protein was also rapidly degraded in mutants expressing IP4. This chimeric protein contains the minimal human substitution which failed to complement IP-deficient mutants. Interestingly, the IP protein showed similar behavior. However, it is possible that our yeast IP antibody might not recognize this chimeric peptide.

Evidence that yMS4 and yMS7–yMS10 synthesize chimeric IP polypeptides was sought from immunoprecipitation experiments with anti-bovine IP antibodies. The human and bovine proteins are highly homologous; thus the bovine antibody was expected to detect the chimeric proteins with significant substitutions of human sequences. Again rapid degradation was observed (Figure 6). It is evident from results presented above that complex II is not assembled in these mutants.

DISCUSSION

Complex II, consisting of only four polypeptides, is the simplest of the mitochondrial electron transport complexes, yet several fundamental questions about its structure, topology, and biogenesis remain to be answered (Ackrell et al., 1992). The genes or cDNAs for several of the peptides have recently been cloned in eukaryotes (Kita et al., 1990; Lombardo et al., 1990; Robinson & Lemire, 1992; Birch-Machin et al., 1992; Broomfield & Hargreaves, 1992; Chapman et al., 1992), opening up new avenues for exploring these problems.

Sequence information revealed a high degree of homology (~70%) between the amino acid sequences of the human and yeast IP subunits (Figure 2). However, an initial attempt to complement a yeast IP mutation with a human/yeast chimeric protein (IP8), which has retained only the yeast signal sequence and the adjacent 38 amino acids, failed in restoring respiration or SDH activity. The possibility of testing other chimeric IP peptides consisting of human and yeast sequences offered an approach to ask some novel questions about the structure and assembly of complex II. The construction of the appropriate chimeric genes was greatly facilitated by the retention of significant homology within the coding region at the DNA level (Figure 1). In all these constructs the yeast signal peptide was maintained to ensure proper targeting into mitochondria and subsequent maturation by proteolytic cleavage. We also confirmed by Northern experiments that the chimeric genes

are expressed to yield mRNAs of the expected size and at steady-state levels in YPD and YPG which are comparable to the normal transcript (results not shown; Cereghino et al., submitted for publication).

We were especially interested to investigate whether the kinetics of assembly might be perturbed, whether assembly intermediates might be observed, and whether functional complexes could be formed at all. This in turn would help define regions on the IP which are important for the assembly/activity of complex II.

A series of systematic substitutions were analyzed. Chimeric peptides carrying human sequences near the N-terminal end, IP1 and IP2, corrected the respiration deficiency of IP$^-$ mutants. Furthermore, there was no effect on the assembly of complex II as determined by SDH activity measurements and the accumulation of IP and FP peptides to wild-type levels. Chimeric peptides IP3, encompassing regions covered by both IP1 and IP2, were tolerated; however, a significant reduction in activity was observed. Activity was measured using either PMS or cytochrome *c* as the substrate to be reduced. The reduction of cytochrome *c* requires the entire, membrane-associated complex II, and it appeared that the presence of the human sequence had a somewhat greater effect on the cytochrome *c* reductase activity (Table 1). The assembly of complex II does not appear to be affected in these mutants, since the rate of incorporation of IP and FP into a stable complex and their accumulation in mitochondria are comparable to those of the wild-type proteins.

Electron paramagnetic resonance (EPR) analysis of deletion mutants of the *Bacillus subtilis* complex II suggest that the IP N-terminal end interacts with the FP protein (Hederstedt et al., 1985; Aevarsson & Hederstedt, 1988). This conclusion is also inferred from bioenergetic considerations which suggest that the [2Fe–2S] center, located in the N-terminal end, may be the immediate acceptor of electrons from the reduced flavin moiety (Cammack, 1986). If the yeast complex follows the same topology, then the human N-terminal domain, in spite of a number of significant amino acid changes and altered charges (see Figure 1, bottom), appears to be able to interact with and accept electrons from the yeast flavoprotein. The rate of electron transport through this chimeric complex can, however, be affected, as is the case with the IP3 chimeric protein.

A recent solution of the structure of phthalate dioxygenase reductase (PDR) (Corell et al., 1992) may suggest how similar domains on two different polypeptides interact in the SDH complex. In PDR a single polypeptide folds into a domain for a flavin cofactor and a second domain which resembles the Cys-X_4-Cys-X_2-Cys ([2Fe–2S]) cluster in plant ferredoxins.

Two kinds of substitutions near the C-terminal of the IP subunit were tolerated and resulted in strains capable of growing on nonfermentable carbon sources. First, the substitution of the entire C-terminus, starting from within the last cysteine cluster, did not cause any measurable changes in the accumulation or activity of complex II (IP6). The substituted human sequence is almost completely identical up to the two consecutive lysine residues at the end of the yeast sequence. These two lysine residues are conserved in all published eukaryotic IP sequences (Figure 2) and in *Drosophila melanogaster* (Au & Scheffler, unpublished results). A deletion of seven amino acids including these two lysines from the yeast IP peptide leads to a complete failure of complex II formation (Schmidt et al., 1992). However, in the human/yeast chimeric IP6 neither the substitution of an internal lysine for leucine nor the addition of several charged amino acids at the C-terminus (Figure 1, bottom) had any detrimental consequences.

More puzzling results were observed with the second substitution near the C-terminal end (IP5, Figure 1). It involves a region between the second and third cysteine clusters. The resulting chimeric peptides contained several significant charge and side-chain differences in a narrow region, which has also diverged in other species (Figure 2). Complex II is assembled and accumulated to normal levels. It also must be active, since the strain yMS5 can grow on several nonfermentable carbon sources: glycerol, lactate, ethanol, and acetate. However, attempts to measure SDH activity *in vitro* were completely unsuccessful. We believe that the inactivation *in vitro* is due to the destruction of one or both iron–sulfur centers, possibly due to the loss of the labile sulfides which are an essential part of a functional IP subunit.

It is likely, based on the analogy with structurally characterized ferredoxins (Ackrell et al., 1992), that the cysteines of cluster I serve as ligands of the [2Fe–2S] center, but the [4Fe–4S] center is made from the first, second, and third cysteines of cluster II and the third cysteine of cluster III, while the first and second cysteines of cluster III and the fourth cysteine of cluster II serve as ligands for the [3Fe–4S] center. Further support for this interpretation comes from the study of *Escherichia coli* fumarate reductase and the [3Fe–4S] to [4Fe–4S] center conversion by site-directed mutagenesis (Manodori et al., 1992). Thus, the stability of the clusters is expected to depend on the secondary and tertiary structure of the IP subunit and hence on the primary sequence of the polypeptide. The sequence between cysteine clusters II and III may be especially critical.

In the future it may be of interest to determine the midpoint potentials of the Fe–S centers in these hybrid IP subunits to measure the effect of a changed cluster environment on the potential and hence on electron transport.

Since our initial attempt to complement an IP-deficient mutant with a chimeric protein (84% human in origin) failed, we were interested in the minimal incompatible region. It was localized to a segment between the first and second cysteine clusters (IP4, Figure 1) which is highly variable throughout evolution (Figure 2). Not surprisingly, larger substitutions (IP7–IP10) which include this region failed the complementation test as well. It remained to be established whether an inactive complex II can be assembled in these mutants. Labeling and pulse–chase experiments provided evidence against any appreciable assembly into a lasting complex. In fact, rapid degradation of both the chimeric IP and the wild-type FP was observed in all cases. Since the degradative rates are so high, one must conclude that, when possible, assembly is also an extremely rapid process, with no significant pools of mature precursors or intermediates present in the mitochondrial matrix.

We (Schmidt et al., 1992) and others (Robinson et al., 1991; Robinson & Lemire, 1992) have demonstrated that the IP and FP proteins do not accumulate in mutants that have deleted FP or IP genes, respectively. It is important to note that such rapid degradation in the absence of assembly has not been shown before for members of complex II in eukaryotic cells.

In summary, the substitution of human sequences for yeast sequences in the IP peptide of SDH/complex II can have one of three consequences. N-Terminal substitutions around the first cysteine cluster affect the activity but not the assembly of the complex (IP1, IP2, and IP3). A substitution of 45 amino acids between the second and third cysteine clusters

(IP5) yields a functional complex *in vivo*, which appears, however, to have unusually labile iron–sulfur centers when mitochondria are purified. A substitution of 50 amino acids in the middle of the peptide between the first and second cysteine clusters (IP4) prevents the formation of an active complex II. No SDH activity is observed, and both the IP and FP peptides are degraded as soon as they have entered the mitochondria.

The mutagenesis scheme employed in this study defined regions in the IP peptide which are important for the assembly/activity of complex II. Contact regions with the membrane anchor peptides and the FP subunit of SDH could not be established since no measurable assembly intermediates were observed. Future work with more limited mutagenesis restricted to these critical regions should shed more light on this problem.

ACKNOWLEDGMENT

We thank Dr. M. Yaffe (UCSD) and Dr. Y. Hatefi (Scripps Research Institute) for helpful criticism and advice.

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