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Function of Conserved Acidic Residues in the PSST Homologue of Complex I (NADH:Ubiquinone Oxidoreductase) from *Yarrowia lipolytica**

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Proton-translocating NADH:ubiquinone oxidoreductase (complex I) is the largest and least understood enzyme of the respiratory chain. Complex I from bovine mitochondria consists of more than forty different polypeptides. Subunit PSST has been suggested to carry iron-sulfur center N-2 and has more recently been shown to be involved in inhibitor binding. Due to its pH-dependent midpoint potential, N-2 has been proposed to play a central role both in ubiquinone reduction and proton pumping. To obtain more insight into the functional role of PSST, we have analyzed site-directed mutants of conserved acidic residues in the PSST homologous subunit of the obligate aerobic yeast *Yarrowia lipolytica*. Mutations D136N and E140Q provided functional evidence that conserved acidic residues in PSST play a central role in the proton translocating mechanism of complex I and also in the interaction with the substrate ubiquinone. When Glu^{89}, the residue that has been suggested to be the fourth ligand of iron-sulfur center N-2 was changed to glutamine, alanine, or cysteine, the EPR spectrum revealed an unchanged amount of this redox center but was shifted and broadened in the g_z region. This indicates that Glu^{89} is not a ligand of N-2. The results are discussedin the light of structural similarities to the homologous [NiFe] hydrogenases.

Proton-translocating NADH:ubiquinone oxidoreductase (EC 1.6.99.3, complex I) is the first complex of the mitochondrial respiratory chain. It couples the transfer of two electrons from NADH to ubiquinone to the translocation of four protons across the inner mitochondrial membrane (1, 2).

In bovine heart, this enzyme is made up of 43 different subunits (3) with a molecular mass of nearly 1000 kDa. The homologous procaryotic complex I has a minimal number of 14 subunits with a total molecular mass of ~500 kDa (4). These 14 central subunits, seven of which are mitochondrially encoded in higher eucaryotes, are well conserved among species.

Electron microscopic studies of *Neurospora crassa* (5), *E. coli* (6), and bovine complex I (7) show that both the bacterial and the mitochondrial complexes are L shaped with an intrinsic membrane arm extending into the lipid bilayer and a peripheral arm protruding into the mitochondrial matrix. The seven central, nuclear-coded subunits are part of the peripheral arm (8, 9) and include the subunits that bear all known redox groups of complex I, namely one noncovalently bound FMN and at least six iron-sulfur centers (10). The membrane arm contains the seven hydrophobic, mitochondrially encoded subunits.

The mechanism that couples proton translocation across the membrane to electron transfer from NADH through FMN and several iron-sulfur centers onto ubiquinone is unknown. All postulated mechanisms are rather speculative (11). However, it seems likely that iron-sulfur center N-2 is the immediate electron donor to ubiquinone. The pH-dependent midpoint potential of N-2 might indicate that this redox center is directly involved in proton pumping (11–13). However, the location of iron-sulfur center N-2 is still controversial because two approaches to resolve this issue gave contradictory results. Site-directed mutagenesis in *E. coli* suggests the PSST homolog (14), and a similar study in *Rhodobacter capsulatus* (15) suggests the TYKY homolog as the most likely candidates to bind N-2.

These studies suffer from the fact that prokaryotic complex I tends to be rather unstable (16) and is down-regulated in deficient mutants from *E. coli,* the only bacterium that has an enzyme stable enough to be purified (17). Pure complex I from mitochondria of bovine heart and the fungus *Neurospora crassa* can be easily obtained in large quantities. However, genetic manipulation in these organisms is either impossible or rather difficult (18). Therefore, we have established the obligate aerobic yeast *Yarrowia lipolytica* as a model to study the structure and function of complex I. In contrast to brewer's yeast *Saccharomyces cerevisiae*, *Y. lipolytica* does contain complex I[1] and most of the powerful genetic tools that are routinely used in *S. cerevisiae* are available (19). Here we report the first application of this novel yeast genetic system to study the function of complex I.

To identify functionally important residues in the PSST homologue subunit of the *Y. lipolytica* complex I, we have introduced a series of single amino acid exchanges and replaced acidic residues that are strictly conserved among all known sequences. The altered protein complexes were analyzed for complete assembly in mitochondrial membranes and as purified enzymes. Functional changes were analyzed by enzyme kinetics, inhibitor sensitivity, and EPR spectroscopy.

EXPERIMENTAL PROCEDURES

Materials—Taq DNA polymerase was from Sigma, cloned *Pfu* DNA polymerase, and *PfuTurbo* DNA polymerase were obtained from Stratagene. Restriction endonucleases and DNA-modifying enzymes were

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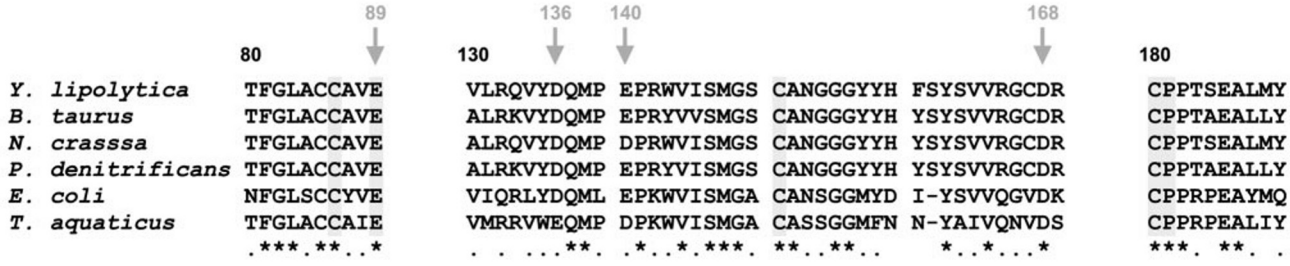

FIG. 1. **Site-directed mutagenesis of acidic residues in the PSST-homologue of *Y. lipolytica*.** The protein sequences from various organisms were aligned using the CLUSTAL program of the HUSAR 4.0 package, DKFZ Heidelberg, Germany. Identical amino acids are marked by *asterisks*, and similar residues are marked by *dots*. Residues forming the noncanonical binding motif that has been suggested to bind a Fe_4S_4 center are shaded in *gray*. Positions of point mutations in *Y. lipolytica* are marked with *arrows*.

from New England Biolabs, and T4 DNA ligase was from Life Technologies, Inc. Hybond N^+ membranes were obtained from Macherey and Nagel. The ABI Prism dye terminator cycle sequencing kit was purchased from Perkin-Elmer. NBQ[2] was synthesized according to the protocol of (20). *N*-methyl-*N*-(3, 4-dimethoxybenzyl)-4-(*p*-tert-butylphenoxy)benzamide (21) was a kind gift from H. Miyoshi, Kyoto University.

Strains—*Y. lipolytica* strains E129 (MatA, lys11–23, *ura3*-302, *leu2*-270, *xpr2*-322) and E150 (*Mat*B, *his*-1, *ura3*-302, *leu2*-270, *xpr2*-322) were a kind gift from Prof. C. Gaillardin, INRA, Paris, France. The diploid strain GB1 was produced by mating E129 and E150. *Y. lipolytica* genetic techniques were carried out according to Ref. 19.

Deletion of NUKM, the Y. lipolytica Homologue of PSST—The *NUKM* gene[1] was deleted by homologous recombination with a *LEU2*-marked deletion allele. A 3.5-kb genomic *Pst*I/*Pst*I fragment was subcloned into a pBluescript 228 SK(−) derivative from which part of the polylinker region had been deleted by digestion with *Kpn*I and *Hin*dIII, blunt ending using Klenow polymerase, and religation. A region of roughly 760 base pairs, encompassing nearly the complete *NUKM* open reading frame except for three amino acids at the 3′-end and about 140 base pairs of the 5′-flanking region were removed by digestion with *Hin*dIII and *Kpn*I and replaced with a 1.9-kb fragment carrying the complete *LEU2* gene from *Y. lipolytica*. The resulting 4.7-kb *Pst*I fragment in which the orientation of the *LEU2* gene was opposite to the original *NUKM* gene was used for transformation of diploid *Y. lipolytica* GB1 cells. 26 *LEU2* colonies were screened for homologous recombination at the chromosomal *NUKM* locus by polymerase chain reaction using combinations of inward primers derived from sequences outside the 4.7-kb *Pst*I fragment (nPSST/1 and nPSST/2) and outward primers derived from the *LEU2* sequence (ylleu2/us and ylleu2/ds) and by Southern blot analysis. Two heterozygous deletion mutant strains (PA1.1 and PA1.2) were identified.

Site-directed Mutagenesis of the PSST Homologue of Complex I—Point mutations were created using the QuikChange site-directed mutagenesis kit from Stratagene, using as a template a 2.3-kb *Eco*RI/*Eco*RI fragment subcloned into the replicative vector pINA443 carrying the selection marker *URA*3 (in the following termed $pNUKM_{mut}$/*URA*3). Mutated plasmids were confirmed by DNA sequencing and used for transformation of the heterozygous deletion strain PA1.2. Haploid strains carrying the desired mutation were obtained by sporulation followed by random spore analysis. *LEU2*, *URA3* spores were tested for the absence of the genomic copy of *NUKM* by polymerase chain reaction or Southern blotting and the mutation was reconfirmed by sequencing of the entire open reading frame on the plasmid.

Analytical Methods—Protein was determined according to a modified Lowry protocol (22). Blue native polyacrylamide gel electrophoresis was as described in Ref. 23.

Preparation of Unsealed Mitochondrial Membranes—Unsealed mitochondrial membranes were prepared from haploid parental strain E150 and haploid mutant strains (*nukm::LEU2*, *ura3*, *leu2*, *lys,* $pNUKM_{mut}$/*URA*3) as described previously (24). Cells were grown in complete YPD medium to early stationary phase ($4–6 \times 10^8$ cells/ml), harvested by centrifugation for 10 min at 5000 × *g* (typical yield 30–35 g/liter wet weight), and resuspended in 150 ml of 400 mM sucrose, 20 mM Na^+/Mops, 1 mM EDTA, pH 7.2, at a concentration of 40–60 mg/ml total protein.

Purification of Complex I—NADH:ubiquinone oxidoreductase was purified by stepwise extraction of the mitochondrial membranes with dodecyl maltoside followed by ion exchange and size exclusion chromatography.[1]

EPR Spectroscopy—Low temperature EPR spectra were obtained on a Bruker ESP 300E spectrometer equipped with a liquid helium continuous flow cryostat, ESR 900 from Oxford Instruments. Samples were mixed with NADH in the EPR tube and frozen in liquid nitrogen after a 30-s reaction time. The protein concentration of purified complex I was 3–9 mg/ml.

Determination of Catalytic Activities—Steady state dNADH:NBQ activity was recorded in a Shimadzu UV-300 spectrophotometer as NADH oxidation ($\epsilon_{340-400\ nm}$ = 6.1 $mM^{-1}\ cm^{-1}$) using a thermostatted cuvette (30 °C). 100 μM dNADH and 50 μg/ml unsealed mitochondrial membranes were added to buffer containing 50 mM Tris/HCl, pH 7.4, and 2 mM KCN. The catalytic reaction was started by the addition of 60 μM NBQ (25). Complex I activity could be selectively measured with dNADH as a substrate, because as previously shown for other species (26), it is not oxidized by alternative NADH:ubiquinone oxidoreductases. Inhibitors were added to the cuvette prior to NBQ. Michaelis-Menten parameters were determined by varying the concentration of NBQ (1–100 μM). The pH dependence of the catalytic rate was measured in a multibuffer containing 10 mM each of Mes, Mops, Epps, Ches, and Caps and 2 mM NaN_3 to cover a pH range from 5.0 to 10.0. The buffer was adjusted to the appropriate pH by adding NaOH. Data were fitted according to the following equation (27),

$$Rate = C \times \frac{1}{1 + \frac{[H^+]}{K_A}} \times \frac{1}{1 + \frac{K_B}{[H^+]}} \qquad \text{(Eq. 1)}$$

where *Rate* is the observed catalytic rate in the presence of saturating substrate concentrations (100 μM dNADH, 60 μM NBQ), $[H^+]$ is the concentration of protons, K_A and K_B are the dissociation constants of two protonable groups A and B, and *C* is the optimal catalytic rate that would be observed if group A was deprotonated and group B was protonated in all enzyme molecules.

Detergent- and inhibitor-insensitive dNADH:HAR activity was measured using 200 μM dNADH and 2 mM HAR, 2 mM NaN_3 in 20 mM Na^+/Hepes, pH 8.0 at 30 °C (28). The reaction was started by the addition of 50 μg/ml unsealed mitochondrial membranes.

Kinetic data were analyzed using the Psiplot software package version 4.61 (Poly Software International). The numerical procedure used to fit experimental data was the Marquardt algorithm (29).

RESULTS

Sequence Properties of the NUKM Gene Product—N-terminal sequencing by Edman degradation identified SAPAGT as the first six amino acids of the mature protein.[1] Thus, 27 amino acids represent the mitochondrial import presequence, and the molecular mass of the mature protein is 20.3 kDa. Fig. 1 shows a partial sequence alignment of the PSST homologue from *Y. lipolytica* with bovine, fungal, and bacterial proteins. As only one of the two adjacent conserved cysteins in positions 85 and 86 could serve as a ligand for sterical reasons the motif C*XX*E-$(X)_{60}$–C-$(X)_{29}$–CP has been suggested to bind iron-sulfur center

[2] The abbreviations used are: NBQ, *n*-nonylubiquinone; kb, kilobase; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Epps, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; Ches, 2-*N*-(cyclohexylamino)ethanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; HAR, hexammineruthenium(III)-chloride; DQA, 2-decyl-4-quinazolinyl amine.

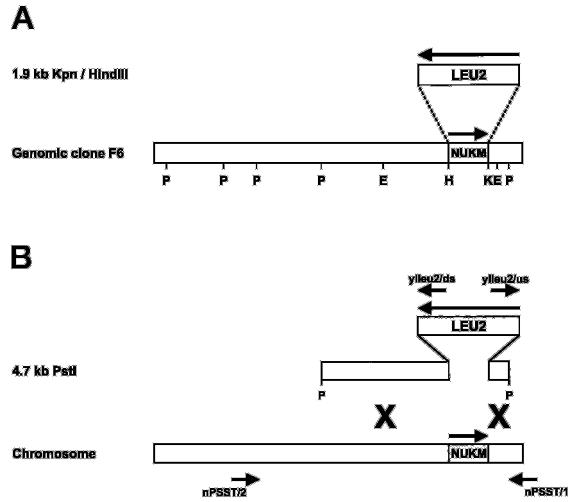

FIG. 2. **Construction of the *nukm::LEU2* deletion allele (*A*) and deletion of the genomic copy by homologous recombination (*B*).** The *NUKM* open reading frame is shaded in *dark gray*. Polymerase chain reaction primer pairs ylleu2/us, nPSST/1 and ylleu2/ds, nPSST/2 were used to screen *LEU2* colonies for marker insertion by homologous recombination. Restriction sites: *E*, *Eco*RI; *H*, *Hin*dIII; *K*, *Kpn*I; *P*, *Pst*I.

N-2 (30).

Deletion and Site-directed Mutagenesis of NUKM—The reading frame of the *NUKM* gene was deleted by homologous recombination with a *LEU2* marked deletion allele (Fig. 2) in the diploid *Y. lipolytica* strain GB1. Replicative plasmids carrying site-directed mutations (Table I), which altered four conserved acidic residues, including the putative glutamic acid ligand for the iron-sulfur center N-2 (Glu^{89} in *Y. lipolytica* PSST) were used for transformation of the diploid deletion mutant. Haploid strains carrying only the mutated copy of *NUKM* on the plasmid were obtained through sporulation and subsequent random spore analysis.

Complex I Content and Characterization of Mitochondrial Membranes from Y. lipolytica—Specific dNADH:HAR activities of *Y. lipolytica* mitochondrial membranes were in the same range for all seven strains (Table I). Among the representative membrane preparations listed in Table I, only the membranes from mutants E89C and E89A that were prepared in parallel batches exhibited a 45% higher activity. As the nonphysiological dNADH:HAR activity only depends on a functional 51-kDa subunit (31), this demonstrated that the complex I content of the membranes was not dramatically altered by the mutations. The observed differences in HAR activity were obviously because of variations in growth conditions and quality of the membranes. Loading unsealed mitochondrial membranes from all mutant strains corresponding to equal amounts of total protein on blue native polyacrylamide electrophoresis gels confirmed that complex I was fully assembled and expressed at comparable levels in the parental and all mutant strains (data not shown).

To identify functionally relevant alterations in complex I, Michaelis-Menten parameters and pH dependence of dNADH:NBQ oxidoreductase activity and sensitivity toward several representative complex I inhibitors were determined. For membranes from the parental strain, a K_m value close to 14 μM was determined for the hydrophobic ubiquinone analog NBQ (Table I). The K_m value for NADH has been reported earlier (24) and was not monitored in this study as the NADH binding site is known to be located on the 51-kDa subunit (32, 33). The pH dependence of dNADH:NBQ activity in *Y. lipolytica* membranes was a bell-shaped curve with an optimum at around pH 7.6 (Fig. 3). Like for mitochondrial cytochrome bc_1 complex (27), this pH dependence could be simulated by assuming that

TABLE I
Characterization of mitochondrial membranes from Y. lipolytica

Strain	HAR		NBQ normalized V_{max} [a]	
	Activity	K_m (NBQ)		
	μmol min^{-1} mg^{-1}	μM	μmol min^{-1} mg^{-1}	%
Parental	1.1	14.1 ± 0.7	0.58 ± 0.02	100
D168N	1.1	21.1 ± 1.3	0.56 ± 0.02	97
D136N	1.1	17.6 ± 1.1	0.10 ± 0.02	17
E140Q	1.1	15.4 ± 0.9	0.27 ± 0.02	47
E89Q	1.1	9.3 ± 0.6	0.51 ± 0.02	88
E89C	1.6	17.8 ± 0.9	0.39 ± 0.02	67
E89A	1.6	18.6 ± 0.7	0.43 ± 0.02	74

[a] To account for variations of complex I content in different batches of mitochondrial membranes the dNADH:NBQ activities were normalized to dNADH:HAR activities that were not affected by the mutations and reflected the complex I content.

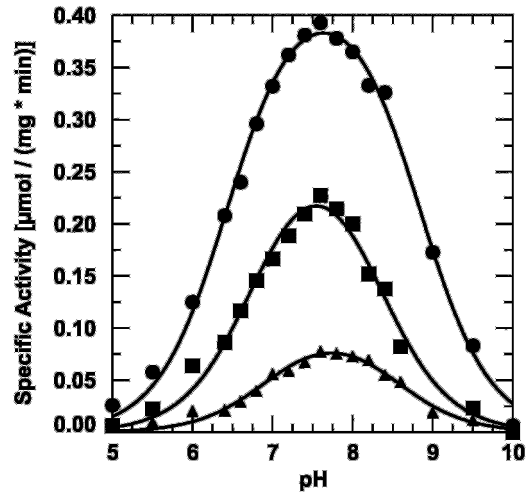

FIG. 3. **pH dependence of specific activity.** The steady-state rates of dNADH:NBQ oxidoreductase activity were measured covering a pH range from 5.0 to 10.0 using unsealed mitochondrial membranes (50 μg/ml total protein) at saturating substrate concentrations as described under "Experimental Procedures." The parameters used to plot the fitted curves are listed in Table II. ●, parental strain; ■, mutant strain E140Q; ▲, mutant strain D136N.

two protonable groups A and B control the steady-state activity of complex I and that the optimal catalytic rate C would be reached if group A was deprotonated and group B was protonated in all enzyme molecules (see Equation 1 under "Experimental Procedures"). For the parental strain, pK_A was 6.4 ± 0.1 and pK_B was 8.8 ± 0.1 (Table II).

Y. lipolytica complex I was efficiently inhibited (Table III) by the type A inhibitors piericidin A and DQA and the type B inhibitor rotenone (see Ref. 34 for the classification of complex I inhibitors). Whereas the I_{50} values for these three compounds were in the same range in *Y. lipolytica* membranes as for bovine heart submitochondrial particles (25), up to 10 μM of an efficient type C inhibitor, the capsaicin derivative *N*-methyl-*N*-(3, 4-dimethoxybenzyl)-4-(*p*-tert.-butylphenoxy)-benzamide (21), did not have any effect on the activity of complex I from *Y. lipolytica* (data not shown).

We also monitored the low temperature EPR spectra of isolated complex I reduced with NADH (Fig. 4) that have been discussed in detail elsewhere.[1]

Under these test criteria, characteristic alterations could be detected in all mutants analyzed here (see below) with the notable exception of D168N (Tables I and II), which exhibited virtually the same properties as the parental strain. This demonstrated that full complementation was achieved by the copy of the *NUKM* gene on the plasmid.

PSST Mutations D136N and E140Q Affect the Catalytic

Function of Complex I—The maximal dNADH:NBQ oxidoreductase activities at pH 7.4 for mitochondrial membranes from mutants D136N and E140Q were significantly decreased to about 20 and 50%, respectively, as compared with the parental strain, but the K_m for NBQ was only slightly affected (Table I). Mutations D136N and E140Q also resulted in a narrowed pH optimum for the steady-state activity (Table II and Fig. 3). pK_A was shifted up by about half a pK unit to 7.0 and 6.8, respectively, and pK_B was shifted down by about the same extent to 8.5 and 8.3, respectively (Table II). The I_{50} values for DQA (type A inhibitor) shown in Table III revealed for both mutations a slight but significant resistance, whereas a 4–5-fold hypersensitivity was observed for rotenone (type B inhibitor). Exchanging these two conserved acidic residues with the corresponding amide had no effect on the EPR spectra of the reduced iron-sulfur centers of complex I (data not shown).

Glu^{89} Is Not a Ligand of Iron-Sulfur Center N-2—Exchanging Glu^{89}, the glutamate that has been proposed as the forth ligand of iron-sulfur center N-2, with three different amino acids had only moderate effects on complex I activity in mitochondrial membranes (Table I). Complex I from all three mutants showed a normal pH profile that could be fitted with the same p*K* values as the enzyme from the parental strain (Table II).

Whereas normal amounts of iron-sulfur center N-2 could be detected by EPR spectroscopy in all three mutant enzymes, a clear shift of the g_z signal to higher and of the g_{xy} signal to lower values (Fig. 4*A*) was observed. The 16 K spectra shown in Fig. 4*B* also revealed that in parallel to this shift the g_z line of N-2 was significantly broadened. The corresponding line widths (L_Z) are listed in Table IV. Moreover, the power saturation characteristics at 12 K of center N-2, expressed as half saturation parameters ($P_{½}$), were altered in all three Glu^{89} mutants (Table IV). Remarkably, the most conservative exchange E89Q had the most pronounced effect and was the only mutation that also caused a slight decrease of the K_m for ubiquinone (*cf.* Table II). In contrast, exchanging Glu^{89} for the potential iron-sulfur ligand cysteine and the small hydrophobic alanine caused much smaller but similar changes of the EPR parameters of center N-2 (Fig. 4 and Table IV). As replacing Glu^{89} by three different residues had only minor effects on the EPR spectra of the iron-sulfur center N-2, we conclude that it is not a ligand of this redox group.

TABLE II
pK values controlling dNADH:ubiquinone oxidoreductase activity
p*K* values were determined by fitting pH dependent activities to Equation 1.

Strain	pK_A	pK_B
Parental	6.4 ± 0.1	8.8 ± 0.1
D168N	6.4 ± 0.1	9.0 ± 0.1
D136N	7.0 ± 0.1	8.5 ± 0.1
E140Q	6.8 ± 0.1	8.3 ± 0.1
E89Q	6.4 ± 0.1	8.9 ± 0.1
E89C	6.4 ± 0.1	8.7 ± 0.1
E89A	6.3 ± 0.1	8.8 ± 0.1

TABLE III
Inhibition of complex I

Strain	I_{50}		
	Piericidin A	DQA	Rotenone
		nM	
Parental	3	20	590
D168N	3	14	660
D136N	5	48	100
E140Q	4	45	150
E89Q	2	11	620
E89C	8	14	630
E89A	5	13	590

DISCUSSION

With the work presented here we have established *Y. lipolytica* as a powerful yeast genetic model for the analysis of mitochondrial complex I. We have demonstrated efficient deletion of a gene for a nuclear coded subunit of complex I by homologous recombination using the *LEU2* marker gene and the introduction of site-directed mutations using a replicative plasmid.

PSST, the subunit studied here, is one out of five complex I subunits that are homologous to subunits of bacterial membrane-bound [NiFe] hydrogenases (35) (Fig. 5). Those two that are conserved between soluble (36, 37) and membrane-bound [NiFe] hydrogenases, and complex I may be regarded as the catalytic core of complex I. The large subunit of soluble [NiFe] hydrogenases harbors the binuclear [NiFe] active site and is homologous to the 49-kDa subunit of complex I, and the small iron-sulfur subunit is related to PSST. PSST can be labeled specifically with a photoreactive pyridaben derivative (38),

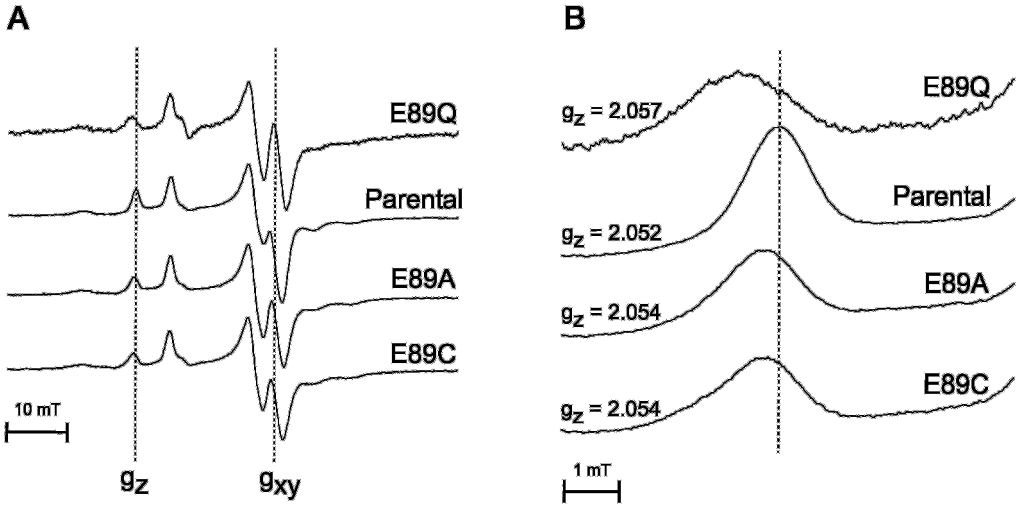

FIG. 4. **EPR spectra of purified complex I.** EPR spectra of purified complex I from *Y. lipolytica* strains carrying mutations of the putative N-2 ligand Glu^{89} are compared with spectra obtained with parental enzyme. Complex I was reduced with 6 mM NADH. *A*, EPR spectra of complex I obtained at 16 K; the g_z and g_{xy} signal positions for center N-2 are indicated; *B*, enlarged view of the g_z region for iron-sulfur center N-2. EPR conditions: microwave frequency, 9.48 GHz; microwave power, 2 milliwatts; modulation amplitude, 1 millitesla (*mT*).

whereas a piericidin A-resistant mutant of *R. capsulatus* was found to reside in the 49-kDa subunit (39).

In the present work, we have studied a series of site-directed mutations of acidic residues in the subunit of *Y. lipolytica* complex I homologous to PSST to gain insight into the proposed functional role of this critical subunit. Exchanging Asp^{136} with the corresponding amide resulted in fully assembled and stable complex I that exhibited specific functional alterations when compared with the parental enzyme, namely lowered catalytic activity, slight resistance to DQA and hypersensitivity to rotenone. Most notable was a pronounced narrowing of the pH profile for the steady-state activity that could be expressed as shifts of 0.3–0.6 units in two p*K* values fitted to an equation that quantitatively described the pH dependence of activity (Fig. 3). Qualitatively, mutation E140Q had the same but somewhat less pronounced effects. Global structural changes caused by the mutations could be ruled out, as the EPR spectra of all iron-sulfur centers and especially of center N-2 were not altered at all. The fact that removing either one of these two adjacent conserved acidic residues caused such well defined and similar changes seems to indicate that Asp^{136} and Glu^{140}, which are not in the vicinity of any residues previously proposed to be functionally important within PSST, play a specific role in the mechanism of complex I. Although the limited effect on the catalytic rate excludes that these two residues correspond to the two pK_A values controlling catalytic activity, it is tempting to speculate that they might contribute to a channel that transfers protons to or away from center N-2. The changed affinity for complex I inhibitors fits well with covalent labeling of this subunit with a derivative of pyridaben (38).

It is still controversial whether iron-sulfur center N-2 is located in subunit PSST or TYKY as removing the putative cysteine ligands by site-directed mutagenesis of TYKY in *R. capsulatus* (15) and PSST in *E. coli* (40) gave conflicting results. Subunit PSST lacks a canonical motif for Fe_4S_4 coordination, because only three of the four cysteine residues ligating the corresponding iron-sulfur center in the small subunits of [NiFe] hydrogenases (36, 37) are conserved. However, it has been speculated that the conserved glutamic acid in position 89 downstream of the first cysteine may represent the fourth ligand for center N-2 (30) (see Fig. 1). This option can be excluded by the data presented here. If one assumes that despite the rather weak sequence homology between the small subunit of [NiFe] hydrogenases and PSST, the overall folding of the protein was preserved during evolution; the residue corresponding to Glu^{89} in the structure of the hydrogenase from *Desulfovibrio fructosovorans* (36), or *Desulfovibrio gigas* (37) is found to be close but on the "wrong" side with respect to the location of a potential iron-sulfur center. In fact, based on this structural homology it is very difficult to identify conserved residues in PSST that could replace the cysteine missing in position 83 of the *Y. lipolytica* protein. As deduced from the hydrogenase fold, Asp^{168} is the conserved acidic residue that comes closest to the correct position near the putative iron-sulfur center in PSST. However, we could not find that replacing this residue by an asparagine had any effect on complex I and its EPR spectra. Thus, if N-2 were to be in PSST, the fourth ligand has yet to be identified.

Similarly, from the observed shift and broadening of the EPR line shape of iron-sulfur center N-2 by mutations in position Glu^{89} it does not follow that N-2 is in PSST as this could also be interpreted as an indirect effect on the redox-center residing in TYKY. The membrane-bound [NiFe] hydrogenase from *Methanosarcina barkeri* (35) contains homologues of both of these subunits (Fig. 5), which both carry iron-sulfur centers, one Fe_4S_4 center in the PSST and two Fe_4S_4 center in the TYKY homolog. Apparently, already acquisition of a subunit homologous to TYKY by these membrane-bound hydrogenase was accompanied by the loss of two of the iron-sulfur centers found in the PSST homologous subunit of soluble hydrogenases. Therefore, the two Fe_4S_4 centers in TYKY could have taken over the function of these iron-sulfur centers. This hypothetical arrangement is likely to be found in complex I and makes it very likely that TYKY and PSST are in close contact to each other. This would position the Fe_4S_4 centers in TYKY within a couple of angstroms to Glu^{89} in complex I and may also explain why studies with site-directed mutations in the more fragile bacterial enzymes lead to contradictory results.

Overall, our analysis of site-directed mutants of conserved acidic residues in the PSST subunit provides additional strong

TABLE IV
Characteristic features of the EPR spectra of center N-2 of mutant strains Glu^{89} in comparison with parental strain

Strain	g_z[a]	L_z[a]	$P_{½}$[b]
		millitesla	*milliwatt*
Parental	2.052	1.26	17.2 ± 1.1
E89Q	2.057	2.04	26.1 ± 3.9
E89C	2.054	1.65	11.9 ± 1.0
E89A	2.054	1.51	10.7 ± 0.4

[a] g_z and L_z values were taken from Fig. 4.
[b] $P_{½}$ values were determined at 12 K.

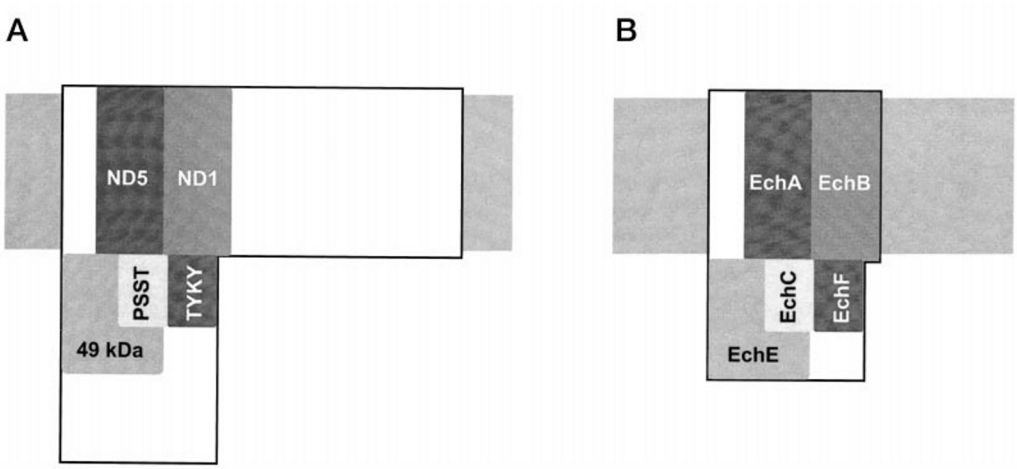

FIG. 5. **Homologous subunits from complex I and [NiFe] hydrogenases.** A schematic model for the arrangement of homologous subunits of (*A*) complex I and (*B*) membrane-bound [NiFe] hydrogenase from *M. barkeri* (35) is shown. EchE and the 49-kDa subunit are homologous to the large subunit, and EchC and PSST are homologous to the small subunit of soluble [NiFe] hydrogenases.

evidence that this subunit plays a critical role in the catalytic mechanism of complex I. However, there is still no definite answer whether it contains iron-sulfur center N-2. Because we could exclude Glu^{89} as the forth ligand for center N-2, models proposing that N-2 resides in PSST now have to solve the problem of the missing fourth ligand.

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