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Histidine 129 in the 75-kDa Subunit of Mitochondrial Complex I from *Yarrowia lipolytica* Is Not a Ligand for [Fe_4S_4] Cluster N5 but Is Required for Catalytic Activity*

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Antje Waletko, Klaus Zwicker, Albina Abdrakhmanova, Volker Zickermann, Ulrich Brandt, and Stefan Kerscher‡

From the Universität Frankfurt, Fachbereich Medizin, Institut für Biochemie I, F-60590 Frankfurt am Main, Germany

Respiratory chain complex I contains 8–9 iron-sulfur clusters. In several cases, the assignment of these clusters to subunits and binding motifs is still ambiguous. To test the proposed ligation of the tetranuclear iron-sulfur cluster N5 of respiratory chain complex I, we replaced the conserved histidine 129 in the 75-kDa subunit from *Yarrowia lipolytica* with alanine. In the mutant strain, reduced amounts of fully assembled but destabilized complex I could be detected. Deamino-NADH: ubiquinone oxidoreductase activity was abolished completely by the mutation. However, EPR spectroscopic analysis of mutant complex I exhibited an unchanged cluster N5 signal, excluding histidine 129 as a cluster N5 ligand.

Respiratory chain complex I links the transfer of two electrons from NADH to ubiquinone to the translocation of four protons across the respiratory membrane (1). Only low resolution structures of complex I are available, demonstrating that the mitochondrial enzyme is L-shaped and consists of a membrane arm and a peripheral arm (2–4). The reaction mechanism is largely unknown. Electrons from NADH are first transferred to FMN, non-covalently bound to the 51-kDa subunit, then to a series of iron-sulfur clusters in the peripheral arm, and finally to the ubiquinone reduction site which, as has been demonstrated by site-directed mutagenesis in the obligate aerobic yeast *Yarrowia lipolytica*, is partly contained in the 49-kDa subunit of the peripheral arm (5). Localization by electron microscopic single particle analysis of two anti-49-kDa subunit monoclonal antibody epitopes has provided evidence that this catalytic core of complex I is located ~70–80 Å away from the phospholipid bilayer (6). From these and other results, an indirect coupling mechanism between quinone reduction in the peripheral arm and proton pumping in the membrane arm seems likely (1, 7, 8).

Little information is available on the spatial arrangement of the other components of the electron transfer pathway. EPR[1]-detectable iron-sulfur clusters have been assigned to individual subunits (9) as follows: the 75-kDa subunit, containing the [Fe_2S_2] cluster N1b and the [Fe_4S_4] clusters N4 and N5; the 51-kDa subunit, containing [Fe_4S_4] cluster N3; the 24-kDa subunit, containing the [Fe_2S_2] cluster N1a; and the PSST subunit, containing the [Fe_4S_4] cluster N2. Two EPR-silent [Fe_4S_4] clusters, termed N6a and N6b, have been assigned to the ferredoxin-like subunit of *Neurospora crassa* complex I homologous to bovine TYKY (10). The tetranuclear iron-sulfur cluster N5 is only observed at very low temperatures and high microwave power. After its initial discovery in pigeon heart complex I (11), it has also been identified in bovine (9), *Rhodobacter spaeroides* (12), and *Y. lipolytica* (4, 13), but not in *N. crassa* (14) complex I. In *Y. lipolytica*, cluster N5 shows very fast spin relaxation with a half-saturation parameter of ~180 milliwatts at 5 K. The line width of the g_z signal (L_z) is in the range of 2.5–3 millitesla. Similar to observations in other model systems, the spin concentration of cluster N5 is much lower than that of the other EPR-detectable clusters (13).

The N-terminal part of the 75-kDa subunit contains three highly conserved iron-sulfur cluster motifs and is homologous to the N-terminal portion of iron-only hydrogenases (15). In the x-ray structure of the iron-only hydrogenase from CpI of *Clostridium pasteurianum*, these motifs ligate one [Fe_2S_2] and two [Fe_4S_4] iron-sulfur clusters (16). Recently, direct evidence for the presence of three iron-sulfur clusters in the heterologously expressed 75-kDa subunit from *Paracoccus denitrificans* has been presented (17). In the same study, the fast relaxing [Fe_4S_4] cluster N5 has been assigned to the second binding motif of the 75-kDa subunit, an unusual H*XXX*C*XX*C*XXXXX*C motif that contains three cysteines and a histidine. Secondary structure predictions using the PROF algorithm (26) suggest that this sequence element, both in hydrogenases and complex I from various organisms, forms a loop bounded by α-helices (Fig. 1). To test the proposed assignment of this sequence as a cluster N5 ligation motif in fully assembled complex I, we mutated the conserved histidine 129 of the *Y. lipolytica* 75-kDa subunit to alanine.

MATERIALS AND METHODS

The *Y. lipolytica* deletion strain *nuam*Δ (ura3–302, *leu2*-270, *lys11*-23, *nuam*::*URA3*, *NUGM*-Htg2, *NDH2i*, *MatB*), in which a 1.7-kb ClaI/KpnI fragment corresponding to codons 34–598 of the *NUAM* gene encoding the 728-amino acid precursor of the 75-kDa subunit of complex I is replaced with the *Y. lipolytica URA3* gene (1.6 kb) oriented in opposite direction to the original *NUAM* open reading frame, was constructed by the double homologous recombination strategy published previously (18). A wild type and a PCR-generated H129A mutant version of the *NUAM* gene were then subcloned as 5.2-kb SalI fragments (after an internal SalI site in the *NUAM* open reading frame had been mutagenized to GTTGAC) into the replicative plasmid pUB4 (19) and transformed into strain *nuam*Δ, resulting in the strains *nuam*Δ, pNUAM-wild type (parental) and *nuam*Δ, pNUAM-H129A (mutant). The presence of the point mutation in large scale cultures of strain *nuam*Δ, pNUAM-H129A was verified by the isolation of total DNA from 5-ml aliquots and the direct sequencing of PCR products.

Complex I catalytic activities in *Y. lipolytica* mitochondrial mem-

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‡ To whom correspondence should be addressed. Tel.: 49-6301-6943; Fax: 49-6301-6970; E-mail: kerscher@zbc.kgu.de.

[1] The abbreviations used are: EPR, electron paramagnetic resonance; BN, blue native; HAR, hexa-ammine-ruthenium(III)-chloride.

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Cp_Hyd      SDAVNEKIKSRISQLLDIHEFKCGPCNRRENCEFLKLVIKYK   117
Yl_75kDa    TERVKQARENVMEMMLQNHPLDCPVCDQGGECDLQDQSMRYG   152
Bt_75kDa    SEKTKKAREGVMEFLLANHPLDCPICDQGGECDLQDQSMMFG   147
Pd_Nqo3     SPMVKKAREGVMEFLLINHPLDCPICDQGGECDLQDQAMAYG   129

C.I         :  .*:***.***::* *******:*************:* :*
All         :  .::  :. :. :*  * :.*  *::  :*:: .  : :
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FIG. 1. **Alignment of the cluster FS4C ligation domain in the iron-only hydrogenase from *C. pasteurianum* (*Cp*) with the putative cluster N5 ligation domains in the 75-kDa subunit of complex I from *Y. lipolytica* (*Yl*), *B. taurus* (*Bt*), and *P. denitrificans* (*Pd*).** The histidine and the three cysteine residues that ligate the hydrogenase cluster FS4C and the corresponding fully conserved residues in complex I are printed in *white* on *black*. α-Helical regions flanking the hydrogenase FS4C loop and the predicted α-helical regions in the 75-kDa subunits are shaded in *dark gray*; residues that are predicted to be in random coil conformation but have at least 30% α-helix probability are shaded in *light gray*. Secondary structure predictions were made using the PROF program (26) at www.aber.ac.uk/~phiwww/prof/. Identical (*), strongly (:), and weakly (.) homologous amino acids are indicated for the alignment of the three complex I sequences (*C.I*) and for all sequences (*All*).

branes were measured as deamino-NADH:*n*-decylubiquinone (DBQ) or NADH:HAR catalytic rates as described (20). A BN-PAGE (21) of mitochondrial membranes was performed using 4–16% gradient gels. A His-tagged complex I was isolated by the extraction of mitochondrial membranes with dodecyl maltoside followed by Ni^{2+} affinity and size exclusion chromatography as published previously (22). 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. 150-μl samples were reduced with 1 mM NADH.

RESULTS

As a qualitative test for complex I assembly, mitochondrial membranes from strain *nuam*Δ, pNUAM-H129A were analyzed by BN-PAGE. As illustrated in Fig. 2, a fully assembled enzyme appeared to be present in the mutant strain, but the intensities of all subunit bands were strongly reduced compared with those in the parental strain. In contrast, electron transfer from NADH to the artificial acceptor HAR (23) was only slightly reduced in mitochondrial membranes from strain *nuam*Δ, pNUAM-H129A and still amounted to 74% of the parental strain value (Table I). This result could be explained by the presence of subcomplexes in the membranes from this mutant; because FMN bound to the 51-kDa subunit is sufficient to catalyze the non-physiological reaction, subcomplexes containing this subunit can show NADH:HAR activity. These subcomplexes may have escaped detection by BN-PAGE because of their instability. At any rate, it was clear that the H129A mutation had markedly reduced complex I stability. It should be noted that significant residual NADH:HAR activity was also found in the *nuam*Δ strain carrying the empty plasmid pUB4 (Table I), although no assembled complex I was detectable by BN-PAGE in this strain (not shown). We also mutated several cysteines in all three iron-sulfur binding motifs of the 75-kDa subunit to alanines, but in all cases we failed to detect complex I assembly or NADH:HAR activity above the level observed in strain *nuam*Δ, pUB4 (data not shown).

Specific deamino-NADH:*n*-decylubiquinone activity of membranes from strain *nuam*Δ, pNUAM-H129A was below detection level. This finding demonstrated that even the fully assembled fraction of complex I (Fig. 2) in the mitochondrial membranes lacked ubiquinone reductase activity. The mutant strain exhibited very low growth yields in complete media (14 g/liter wet weight, *i.e.* 4–6 times less than that commonly observed for the parental strain). This result is remarkable, as strain *nuam*Δ, which lacked most of the open reading frame for the 75-kDa subunit, grew normally. The reason for this growth phenotype is unknown.

Complex I from strain *nuam*Δ, pNUAM-H129A was purified by Ni^{2+} affinity and gel filtration chromatography (22). Mitochondrial membranes equivalent to 4.5 grams of total protein were used for a complex I preparation, which gave 1.5 mg of purified enzyme. This was <10% of the yield typically obtained from the parental strain, suggesting that a considerable portion of assembled complex I had dissociated during the purification procedure. This finding supports the notion that the mutation H129A had destabilized complex I.

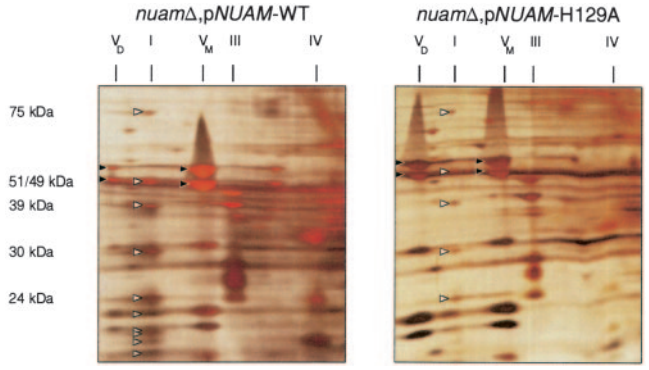

FIG. 2. **BN-PAGE analysis of mitochondrial membranes from strains *nuam*Δ, pNUAM and *nuam*Δ, pNUAM-H129A.** In the silver-stained gels, the positions of complexes V (V_D = dimer and V_M = monomer), I, III, and IV are indicated. Selected complex I subunits, including the 75-kDa subunit, are marked with *white arrowheads* and labeled according to their molecular masses. Several additional complex I subunits are marked with *gray*, and the α- and β-subunits of complex V are marked with *black arrowheads*. Differences in the amounts of the complex V dimer reflect subtle differences in effective protein-to-detergent ratios that do not affect the resolution of complex I.

EPR spectroscopy was performed with complex I from strain *nuam*Δ, pNUAM-H129A and from the parental strain. At a temperature of 40 K and a microwave power of 1 milliwatt, a single binuclear cluster called N1 could be detected (Fig. 3*A*). At a temperature of 12 K and a microwave power of 1 milliwatt, signals arising from clusters N1, N2, N3, and N4 could be seen (Fig. 3*B*). Cluster N5 signals were detected at 5 K and a microwave power of 100 milliwatts (Fig. 3*C*). Confirming previously published results (13), the g values of cluster N5 from wild-type *Y. lipolytica* complex I were $g_{z,y,x}$ = 2.062, 1.93, and ~1.89. The g_x and g_y signals of cluster N5 were difficult to assign, because they overlapped with the g_x and g_y signals of clusters N1 and even more with N4. Interference by the N4 signal also was much more severe, as this cluster was not completely power saturated under the EPR conditions used. However, the g_z signal of cluster N5 was clearly separated from any other EPR signal in isolated complex I from *Y. lipolytica*. The spectra shown in Fig. 3*C* clearly show that the cluster N5 g_z signals of both preparations were virtually identical in terms of peak intensity, peak width, and field position (Fig. 3*C*). This result indicated that cluster N5 was still present in complex I from strain *nuam*Δ, pNUAM-H129A and that its geometry was unaffected. Virtually identical results were obtained in two independent preparations of the mutant enzyme.

DISCUSSION

We have replaced a strictly conserved histidine residue (His-129) in the 75-kDa subunit of *Y. lipolytica* complex I with alanine to test whether this residue is a ligand of iron-sulfur cluster N5, as has been proposed recently (17). As the EPR

TABLE I
NADH:HAR and dNADH:DBQ activities of mitochondrial membranes
The abbreviations used here that are not defined elsewhere are as follows: DBQ, n-decylubiquinone; dNADH, deamino-NADH; WT, wild type.

Strain	NADH:HAR activity		dNADH:DBQ activity	
	μmol min^{-1} mg^{-1}	Percent of activity	μmol min^{-1} mg^{-1}	Percent of activity
*Nuam*Δ,pNUAM-WT	0.84	100	0.290	100
*Nuam*Δ,pNUAM-H129A	0.62	74	<0.01	<3
*Nuam*Δ,pUB4	0.28	33	<0.01	<3

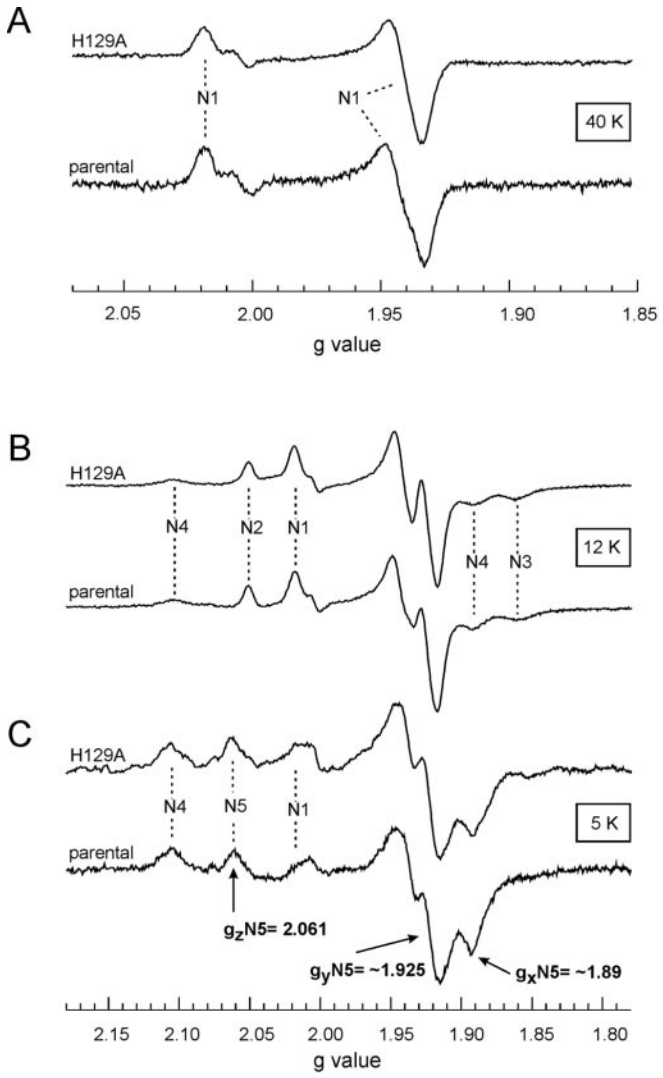

FIG. 3. **EPR spectra of purified complex I from mutant H129A (6.3 mg/ml) and parental strain (3.7 mg/ml).** A microwave frequency of 9.47 GHz and a modulation amplitude of 0.64 millitesla were used, as well as a spectrum at 40 K and a microwave power of 1 milliwatt (*A*), a spectrum at 12 K and a microwave power of 1 milliwatt (*B*), and a spectrum at 5 K and a microwave power of 100 milliwatt (*C*). Characteristic signal positions of individual clusters are indicated. The region between g = 1.9 and g =1.95 reflects contributions from all iron-sulfur clusters detectable under these conditions.

signature of cluster N5 was completely unchanged in complex I purified from the mutant strain, we could exclude this possibility. Our results are in contradiction with a recent study with a heterologously expressed 75-kDa subunit from *P. denitrificans* (17). From changes in the EPR spectrum of the iron-sulfur cluster N5 that were caused by a mutation changing the corresponding histidine to a cysteine, the authors concluded that the H*XXX*C*XX*C*XXXXX*C motif of the 75-kDa subunit binds cluster N5. The most obvious explanation for the results obtained with the *P. denitrificans* H106C mutant would be that structural changes had indirectly changed the geometry of cluster N5. Such EPR signal reduction, peak broadening, and shifting effects have been described, *e.g.* for point mutations affecting amino acids in the vicinity of cluster N2 of complex I from *Y. lipolytica* (5, 24, 25), and can be expected to be more pronounced when single subunits instead of completely assembled multi-subunit complexes are studied. In fact, significant instability of the mutant *P. dentrificans* 75-kDa subunit was noted (17). Although we also observed destabilization of *Y. lipolytica* complex I by the H129A mutation, it obviously did not affect the EPR spectra of the purified enzyme.

A more fundamental problem with complex I from *P. denitrificans* is that the g signal values of cluster N5, detected at 5 K, are very close to those of cluster N4, detected at 12 K (17). Distinction between these clusters relies on the assumption that the cluster N4 signals of *P. denitrificans* complex I are completely power saturated at 5 K and a microwave power of 100 milliwatt, which in our hands is clearly not the case for cluster N4 of *Bos taurus* and *Y. lipolytica* complex I. Therefore, although at least the g_z signals of clusters N4 and N5 are well separated in the latter two model organisms, the assignment of EPR signals to cluster N5 of *P. denitrificans* complex I is more difficult. It should be noted that the cluster N4 EPR signals were also found to be somewhat affected by the H106C mutation in *P. denitrificans* complex I (17). Thus, there is another possibility in regard to how the findings in *P. denitrificans* and *Y. lipolytica* could be reconciled. If the iron-sulfur cluster assignment within the 75-kDa subunit was revised, the mutation of the conserved histidine in *Y. lipolytica* may have resulted in the removal of a cluster N1b ligand, leaving the EPR signature of cluster N5 unchanged. The histidine to cysteine exchange in *P. denitrificans* as such may then have been EPR-silent, but it may have caused a structural alteration that affected the EPR patterns of both clusters N4 and N5.

However, because the study by Yano *et al.* (17) clearly demonstrates the presence of three iron-sulfur clusters in the heterologously expressed 75-kDa subunit of *P. denitrificans*, this proposal raises another problem. If all three motifs in the 75-kDa hold an iron-sulfur cluster, why did the H129A mutation not have any effect on the EPR spectra of *Y. lipolytica* complex I? Possible answers are that cluster N1b is either not present or undetectable by standard EPR spectroscopy in complex I from *Y. lipolytica*. Although complex I from bovine heart mitochondria and various other sources is known to contain two EPR detectable binuclear iron-sulfur clusters called N1a and N1b (9), only one binuclear iron-sulfur cluster (called N1) could be detected in complex I from *Y. lipolytica* to date. The stoichiometry of clusters N1/N2/N3/N4 was determined as 1:1:1:1 (13). As the N1 signal of *Y. lipolytica* complex I was not detectable in a subcomplex[2] including the 75-kDa but lacking the 24-kDa subunit, which in bovine heart complex I contains binuclear cluster N1a, it seems tempting to speculate that also in the *Y. lipolytica* enzyme the 75-kDa subunit may carry a second binuclear cluster that has, to date, escaped detection by

[2] V. Zickermann, K. Zwicker, M. Bostina, M. Radermacher, and U. Brandt, manuscript in preparation.

EPR. If the H*XXX*C*XX*C*XXXXX*C motif ligated this EPR silent cluster, it would have been removed by the H129A mutation, offering a straightforward explanation of why ubiquinone reductase activity was lost.

As a consequence, one has to conclude that in complex I the use of the iron-sulfur binding motifs has changed from iron-only hydrogenases. Either the histidine in question that is conserved in all known complex I sequences has acquired a different function in complex I, or the motif ligating a binuclear cluster in hydrogenase binds a tetranuclear cluster (N5) in complex I and *vice versa*.

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