Functional Significance of Conserved Histidines and Arginines in the 49-kDa Subunit of Mitochondrial Complex I*

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The respiratory chain of the inner membrane of mammalian mitochondria consists of four enzyme complexes and creates the transmembrane protonotive force used by ATP synthase to generate ATP. Complex I (NADH:ubiquinone oxidoreductase) is the largest and least understood of these multisubunit complexes. It oxidizes NADH to NAD⁺ and transfers electrons via FMN and up to eight iron-sulfur clusters to ubiquinone that is reduced to ubiquinol (1). Pumping of 4 H⁺/2 electrons across the inner mitochondrial membrane is coupled to this process. Since cluster N2 has been shown to couple magnetically with semiquinone (2), it is likely to be the immediate reductant for ubiquinone at the end of the electron transfer cascade formed by the iron-sulfur clusters of complex I.

In recent years we have established the obligate aerobic yeast *Yarrowia lipolytica* as a model to study structure and function of complex I (3). Based on a site-directed mutagenesis study we have proposed that the ubiquinone-reducing catalytic core of complex I has evolved from the active center of [NiFe] hydrogenases and residues at the interface between the 49-kDa and the PSST subunits (4, 5). The 49-kDa subunit is homologous to the large and the PSST subunit to the small subunit of bacterial water-soluble [NiFe] hydrogenases (6, 7) for which x-ray structures have been solved (8, 9). Of the three iron-sulfur clusters bound to the small subunit of these hydrogenases only the proximal one is retained as cluster N2 in the PSST subunit of complex I (10, 11). However, in all PSST subunits sequenced so far, only three of the four cysteines are left that ligate the proximal cluster in the small subunit of hydrogenases. A fourth cysteine is found immediately adjacent to the first of these three conserved cysteines in all known PSST sequences; for steric reasons, it has been generally considered unlikely that the resulting putative Cys-Cys-X₇-Cys-X₃-Cys motif ligates iron-sulfur cluster N2. However, this option has been put forward recently by Flemming et al. (12). The earlier idea that an acidic residue may serve as the fourth ligand of cluster N2 (13) could be ruled out by mutating every single conserved aspartate and glutamate in the PSST subunit (5). As cluster N2 is predicted to reside immediately at the interface between the two subunits, we have suggested that the missing fourth ligand may be provided by the adjacent 49-kDa subunit (4).

To explore this possibility and to gain further insight into structure-function relationships within the ubiquinone-reducing catalytic core of complex I, we have analyzed mutations of highly conserved histidines and arginines in the 49-kDa subunit of complex I (Fig. 1). The residues chosen for mutagenesis were predicted to be in the vicinity (within about 15 Å) of iron-sulfur cluster N2 based on the previously demonstrated conservation of the [NiFe] hydrogenase structural fold (4).

Materials and Methods

**Substrates and Inhibitors—**2-n-Decylubiquinone (DBQ),¹ deamino-NADH (dNADH), hexammine ruthenium(III) chloride (HAR), and rotenone were purchased from Sigma; hygromycin B was purchased from Invitrogen; and 2-decyl-4-quinazolinyl amine (DQA) was a generous gift from Aventis CropScience (Frankfurt am Main, Germany).

**Strains—** *Y. lipolytica* haploid strain ANCM1 (nucm::URA3, MatA, 30Hg2, ndh2, lys11–23, ural3–302, leu2–270, xpr2–322) was made by mating strain NK2.1 (nucm::URA3, MatA, ndh2, lys11–23, ural3–302, lys11–23, ura3–302, leu2–270, xpr2–322) with strain HAR, lys11–23, ndh2, ural3–302, leu2–270, xpr2–322.

The abbreviations used are: DBQ, 2-n-decylubiquinone; dNADH, deamino-NADH; HAR, hexammine ruthenium(III) chloride; DQA, 2-decyl-4-quinazolinyl amine; MOPS, 4-morpholinoopropanesulfonic acid; mW, milliwatt; mT, millitesla.

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Conserved His and Arg in 49-kDa Subunit of Complex I

RESULTS

Mutations of Histidine 226 Affect the Properties of Iron-Sulfur Cluster N2—His-226 is invariant in all known 49-kDa subunit sequences of complex I (Fig. 1). It corresponds to a highly conserved histidine in water-soluble [NiFe] hydogenases that forms a hydrogen bond to the proximal iron-sulfur cluster of the small subunit (8). His-226 was mutated into alanine and the potential iron-sulfur cluster ligands glutamine, cysteine, and methionine. As judged by blue native-PAGE (data not shown) and NADH:HYD activity (Table I), complex I was fully assembled to normal expression levels in all His-226 mutants.

Only mutation H226A had a pronounced effect on specific, inhibitor-sensitive ubiquinone reductase activity reducing it by 80%. Mutants H226Q and H226M had retained around 50% activity, and mutants from mutant H226M were only slightly less active than those from the parental strain. Hardly any changes were observed in the Michaelis-Menten constant $K_m$ of H226Q, the H226M and a minor hypersensitivity of mutant H226M and a minor resistance of mutant H226A toward the complex I inhibitors DQA and rotenone was observed. For mutants H226C and H226Q, the $I_{50}$ values for DQA and rotenone were unchanged (Table I). No increase of inhibitor-insensitive dNADH:DBQ oxidoreductase or dNADH oxidase activities was observed in membranes from the mutant strains (data not shown).

In EPR spectra recorded from both mitochondrial membranes and purified complex I, marked effects on iron-sulfur cluster N2, but not on the other EPR-detectable iron-sulfur clusters, resulted from all four mutations (Fig. 2). The quality of the spectra obtained with purified complex I was much higher because of markedly increased protein concentrations and the exclusion of interference from other paramagnetic components of the mitochondrial respiratory chain. However, the analysis of EPR spectra of mitochondrial membranes was critical to decide whether iron-sulfur clusters were lost during the purification procedure and allowed a direct comparison with the functional data summarized in Table I. Again, mutation H226A had the most drastic effect resulting in complete loss of characteristic iron-sulfur cluster N2 signals that were not even observed upon reduction of the sample with NADH/dithionite.
To exclude the possibility that the signal had disappeared due to a dramatic change in the spectroscopic properties of cluster N2, we scanned at different EPR settings (temperature, microwave power, and field ranges) but could not find any new paramagnetic species in mutant membranes or isolated enzyme complex (data not shown).

**Table I**

| Strain  | Complex I content<sup>a</sup> | Complex I activity<sup>b</sup> | $K_m$ of DBQ | $I_0$ | Degree of conservation |
|---------|-------------------------------|-------------------------------|------------|------|-------------------------|
|         | %                            | μM                           | nM         |      |                         |
| Parental| 1.0                          | 100                          | 13         | 600  |                         |
| H226A<sup>c</sup> | 1.0                          | 20                           | 18         | 770  | Invariant in complex I; highly conserved in water-soluble [NiFe] hydrogenases |
| H226Q   | 1.1                          | 56                           | 9          | 13   | 600                     |
| H226C   | 1.2                          | 43                           | 5          | 13   | 600                     |
| H226M   | 1.2                          | 80                           | 9          | 9    | 300                     |
| R141A<sup>c</sup> | 1.3                          | 17                           | 10         | 21   | 570                     |
| R141K   | 1.4                          | 45                           | 13         | 55   | 1500                    |
| R141M   | 1.1                          | 40                           | 11         | 11   | 500                     |
| H91A    | 1.2                          | <5                           |            |      | Invariant in complex I  |
| H91M    | 0.8                          | <5                           |            |      | Invariant in complex I  |
| H91R    | 0.8                          | <5                           |            |      | Invariant in complex I  |
| H95A    | 1.3                          | <5                           |            |      | Invariant in complex I  |
| H95M    | 1.2                          | <5                           |            |      | Invariant in complex I  |
| H95R    | 1.0                          | <5                           |            |      | Invariant in complex I  |
| R466A   | 0.5                          | 75                           | 11         | 35   | 750                     |
| R466M   | 0.7                          | 9                            |            |      | Invariant in complex I  |
| R466H   | 1.0                          | 85                           | 25         | 36   | 900                     |
| R466E   | 0.5                          | 53                           | 9          | 20   | 700                     |

<sup>a</sup> Complex I content was determined in at least two batches of mitochondrial membranes, and the mean value is expressed as relative specific NADH:HAR oxidoreductase activity that is not affected by the mutations (parental plasmid-complemented nucm:<ura3> deletion strain = 1.0).

<sup>b</sup> Complex I activity was determined in at least two batches of mitochondrial membranes, and the mean value is given as inhibitor-sensitive dNADH:DBQ oxidoreductase activity relative to plasmid-complemented strain and normalized for complex I content (100% = 0.3 μmol min⁻¹ mg⁻¹).

<sup>c</sup> Data from Kashani-Poor et al. (4); included for comparison and completeness.

**Fig. 2.** EPR spectra from His-226 mutants. A, EPR spectra of mitochondrial membranes from His-226 mutants. Samples were reduced with NADH. The dashed line represents the field position of the $g_z$ signal of cluster N2 in the parental sample. B, EPR spectra of purified complex I from His-226 mutants reduced with NADH. The spectra show contributions from clusters N1, N2, N3, and N4 (26). The dashed line indicates the position of the $g_z$ signal of cluster N2 in the parental strain. The N2 $g_{xy}$ and the $g_z$ field positions in H226M are shifted to lower field values, and the signal has a reduced intensity. H226Q, H226C, and H226A show no shift but a very significant reduction of the N2 signal, which is comparable to the reduction of dNADH:DBQ activity.

$(E_h = -450$ mV; data not shown). To exclude the possibility that the signal had disappeared due to a dramatic change in the spectroscopic properties of cluster N2, we scanned at different EPR settings (temperature, microwave power, and field ranges) but could not find any new paramagnetic species in mutant membranes or isolated enzyme complex (data not shown).
Conserved His and Arg in 49-kDa Subunit of Complex I

Fig. 3. EPR spectra from Arg-141 mutants. A, EPR spectra of mitochondrial membranes from Arg-141 mutants. Samples were reduced with NADH. The dashed line represents the field position of the \( g_z \) signal of cluster N2 in the parental sample. B, EPR spectra of purified complex I from Arg-141 mutants reduced with NADH. The spectra show contributions from clusters N1, N2, N3, and N4 (26). The dashed line indicates the field position of the \( g_z \) signal of cluster N2 in the parental strain. Only in the R141K mutant a cluster N2 \( g_z \) signal is observable and is slightly shifted to lower field values.

shown). If the tetranuclear iron-sulfur cluster had been turned into a \( \text{Fe}_5\text{S}_5 \) cluster, it would have become paramagnetic in the oxidized state, resulting in a characteristic EPR spectrum, which was not observed (data not shown).

Similar results were obtained for mutants H226C and H226Q, but residual signals of cluster N2 corresponding to less than 10% of wild type level were observed in EPR spectra of purified complex I within the \( g_z \) and \( g_{xy} \) regions and of mitochondrial membranes within the \( g_{xy} \) region. In membrane preparations from mutant H226M, the \( g_z \) signal of cluster N2 was strongly overlapping with the \( g_z \) signal of cluster N1. Spectra of purified H226M complex I allowed a more detailed analysis of changes in the N2 EPR signals. The intensity was reduced by about one-third, and all components of the \( g \)-tensor, obtained by simulation (data not shown), were shifted to lower field values (N2 parental: \( g_z = 2.051, g_y = 1.926, \) and \( g_x = 1.918; \) N2 H226M: \( g_z = 2.054, g_y = 1.931, \) and \( g_x = 1.923).\)

Mutations of Arginine 141 Have Moderate Effects on Activity but Drastically Reduce Iron-Sulfur Cluster N2 EPR Signals—Arg-141 is invariant in all known 49-kDa subunits of complex I and large subunits of [NiFe] hydrogenases (Fig. 1). In the hydrogenase structure it is found in close vicinity to the proximal iron-sulfur cluster (8). We have changed this residue to alanine, lysine, and methionine. In all Arg-141 mutants complex I was assembled (data not shown), and complex I content in mitochondrial membranes tended to be even slightly higher than in the parental strain (see Table I).

In a pattern rather similar to the His-226 mutations, mitochondrial membranes from mutant R141A exhibited an inhibitor-sensitive dNADH:DBQ activity that was some 80% reduced, while the two more conservative exchanges R141K and R141M reduced activity only by about 50%. For different batches of mitochondrial membranes from mutant R141K ubiquinone oxidoreductase activity varied quite substantially between 30 and 70% of wild type activity; the value given in Table I is the average of the values from three membrane preparations. The \( K_m \) value for DBQ was not changed in any of the three mutants, but mutation R141K resulted in a 4–5-fold higher \( I_{50} \) for DQA and a 2–3-fold higher \( I_{50} \) for rotenone. Of the two other mutations only R141A seemed to have a subtle effect on DQA sensitivity. Again, no increase of inhibitor-insensitive dNADH:DBQ oxidoreductase or dNADH oxidase activities was observed in membranes from the mutant strains (data not shown).

In stark contrast to these rather normal functional properties, EPR spectroscopy revealed drastic effects specifically on iron-sulfur cluster N2 (Fig. 3); in membranes and purified complex I from mutants R141M and R141A, cluster N2 signals were undetectable. Again, this was not changed following reduction of membrane samples at a lower potential of about \(-450\) mV with NADH/dithionite (not shown). Scanning at different EPR settings or spectra of oxidized samples gave no indications for any newly formed paramagnetic species (data not shown). A small amount of cluster N2 in the order of 5% compared with wild type level was detectable in complex I from mutant R141K; the very weak \( g_z \) signal seemed to be shifted toward lower field values.

To further address the question of whether cluster N2 was in fact missing in the mutant membranes or whether the cluster had become just undetectable under standard EPR conditions, we considered the possibility that the mutations had turned the spin of the ground state of cluster N2 to \( S = 3/2 \). EPR signals from \( \text{Fe}_4\text{S}_4 \) clusters with \( S = 3/2 \) ground states should appear in the \( g \) region but are notoriously difficult to observe, particularly in the event of significant cluster hetero-
Conserved His and Arg in 49-kDa Subunit of Complex I

Mutations of Histidine 91 and Histidine 95 Result in Complete Loss of Catalytic Activity—Residues His-91 and His-95 are strictly conserved in all complex I 49-kDa subunit sequences but not in [NiFe] hydrogenases (see Fig. 1). Conservation of the structural fold between homologous subunits predicts both residues to be close enough to iron-sulfur cluster N2 to be candidate ligands. His-91 and His-95 were changed into alanine, methionine, and arginine. All His-91 and His-95 mutants contained normal or, in the case of H91M and H91R, only slightly reduced amounts of complex I (see Table I) that was assembled normally (data not shown). In mitochondrial membranes from all six mutant strains inhibitor-sensitive dNADH:DBQ activity was below the detection level of 5%. However, EPR spectra of all iron-sulfur clusters, including cluster N2, were indistinguishable from those of the parental strain (data not shown). This was also true for complex I from mutant H95A that was purified as a representative of this group of mutations.

Fig. 4. Wide range EPR spectra of isolated complex I from parental and mutant enzyme preparations. To detect possible EPR signals originating from $S = 3/2$ spin states two parental enzyme preparations, one highly pure (bottom) and another containing a small amount of cytochrome oxidase (COX) as a contaminant (top) were compared with mutant preparations R141A and R141M (same samples as in Fig. 3B). EPR signals from a $S = 3/2$ state, which should appear in the low field region between $g = 4$ and $g = 5.5$, could not be identified. The signals at $g = 6$ in the mutant samples were attributed to the high spin heme of cytochrome oxidase. At $g = 4.3$ signals from adventitious high spin Fe(III) were detectable in the region around $g = 2$ the $g_z$ signals of cluster N4 ($g_z = 2.10$), N5 ($g_z = 2.06$), and N1 ($g_z = 2.02$) could be identified. In this field range the EPR signals from cluster N2 are power-saturated completely. All samples had protein concentrations between 5 and 10 mg/ml and were reduced by NADH. EPR conditions were microwave frequency of 9.47 GHz, modulation amplitude of 1 mT, microwave power of 63 mW, and temperature of 4.3 K.

Mutations of Arginine 466 Change Functional Properties of Complex I—The C-terminal arginine 466 is also strictly conserved in complex I. In [NiFe] hydrogenases it corresponds to a strictly conserved histidine that is ligand to a Mg$^{2+}$ site and resides about 9 Å from the [NiFe] site and 14 Å from the proximal iron-sulfur cluster. Arg-466 was changed into alanine, methionine, histidine, and glutamate. Assembled complex I was found in membranes from all Arg-466 mutants (data not shown), but with the exception of mutant R466H, content was significantly reduced by 30–50%. As a strictly aerobic organism, Y. lipolytica does not regulate expression levels for complex I subunits. Hence diminished content usually indicates structural destabilization of the complex. In fact, reduced stability made it difficult to isolate sufficient amounts of protein for EPR spectra of purified complex I from these mutants. Still, inhibitor-sensitive, specific ubiquinone reductase activity was strongly reduced only in membranes from R466M, while the other mutants showed around 80% (R466A and R466H) or about 50% (R466E) of wild type activity (see Table I).

Steady-state kinetics revealed a 2–3-fold increase in the $K_m$ for DBQ for mutant R466H, while this parameter was unaltered in mutants R466A and R466E. These three mutations also exhibited a small but significant resistance toward DQA and rotenone.
DISCUSSION

We have explored the function of conserved histidines and arginines of the 49-kDa subunit within the ubiquinone-reducing catalytic core of complex I. One of the specific aims of this study was to test whether these residues may act as the fourth ligand of tetranuclear iron-sulfur cluster N2, completing the set of three cysteine ligands located in the neighboring PSST subunit (21, 22). His-91 and His-95 can be excluded as ligands because even their replacement by alanine had no effect at all on the EPR spectra of cluster N2. Also Arg-466 seems an unlikely candidate because even mutation R466A did not completely abolish the EPR signature of cluster N2. The fact that the cluster was hardly detectable in mitochondrial membranes from three of the four Arg-466 mutants rather seemed to reflect a severe structural destabilization of complex I. Much more specific and remarkably parallel effects on cluster N2 were observed when residues His-226 and Arg-141 were exchanged (see Figs. 2 and 3). In both cases, cluster N2 was not detectable after substitution with a small hydrophobic alanine residue. On the other hand, the more conservative changes R141K and H226M gave rise to reduced but shifted EPR spectra. Other mutations in these positions resulted in dramatic reduction (H226C and H226Q) or virtually complete disappearance (R141M) of cluster N2 EPR signals. Based on these results one could consider both His-226 and Arg-141 as possible fourth ligands of cluster N2. However, the parallel pattern observed for mutagenesis of these two positions rather seemed to suggest that loss of the cluster N2 spectral signature reflected similar structural changes in its surroundings rather than an exchange or removal of its immediate ligand. These structural changes appeared to be local in nature, as, in contrast to mutations of Arg-466, no global destabilization of complex I or loss of residual cluster upon purification was observed. Thus, while we could not identify the fourth ligand of cluster N2, our data demonstrate a specific significance of Arg-141 and His-226 for the local environment of iron-sulfur cluster N2 and provide strong support to the structural model of the ubiquinone-reducing catalytic core of complex I (23).

Mutations of His-91 and His-95 did not provide any information on the ligation or properties of iron-sulfur cluster N2. However, our results still suggested a central functional role for these two residues; independent of the specific amino acid exchange made, all mutations in these two positions resulted in virtually complete loss of catalytic activity without affecting iron-sulfur cluster N2 or dramatically reducing stability of the complex. This behavior is strikingly similar to the results recently obtained for mutagenesis of two conserved aspartates (Asp-99 and Asp-115) in the PSST subunit (5). Based on the structures of homologous water-soluble [NiFe] hydrogenases, histidines 91 and 95 in the 49-kDa subunit and aspartates 99 and 115 in the PSST subunit are predicted to be located on opposing surfaces of the two proteins. Therefore, it is tempting to speculate that they might form specific ion pairs critically involved in the catalytic mechanism of complex I. It should also be noted that the two histidines reside in a loop connecting the first two strands of a predicted β-sheet of the 49-kDa subunit; recently, by electron microscopic single particle analysis of co-complexes of Y. lipolytica complex I and monoclonal antibody 49.2 we localized the second putative β-strand of this predicted β-sheet bound by glycines 96 and 106 near the end of the peripheral arm of complex I opposite to its connection to the membrane part (15). We consider the identification of two functionally deficient mutants in this part of complex I as another indication that critical parts of the catalytic machinery of complex I are clearly separated from the membrane domain.

We made a puzzling observation for a number of mutants when we compared spectroscopic properties and catalytic activities; the pronounced effects on the EPR spectra of cluster N2 caused by mutagenesis of His-226, Arg-141, or Arg-466 were in stark contrast to comparably moderate reductions in inhibitor-sensitive ubiquinone reductase activities. In the three alanine mutations hardly any cluster N2 was detectable, but still around 20% (H226A and R141A) or even 75% (R466A) specific activity could be measured. This was most evident in the case of mutation R141M, where cluster N2 was not detectable but inhibitor-sensitive, specific dNADH:DBQ oxidoreductase activity was still 40%. We could rule out that these rates were due to an artificial reaction with decylubiquinone that does not occur with endogenous ubiquinone-9; inhibitor-insensitive residual rates were not increased in mutant membranes, no matter whether we measured dNADH oxidase or dNADH:DBQ oxidoreductase activity. The most obvious explanation for this apparent paradox would be that the redox center assumed to be the immediate electron donor for ubiquinone was still present.
in the mutant enzymes but could not be detected anymore by standard procedures. In principle, this could have been caused by two types of changes in the properties of iron-sulfur cluster N2. (i) The mutations could have altered the redox potential of the cluster to very negative values, preventing reduction by NADH or NADH/dithionite. However, this seems highly unlikely; the absence of even residual reduction in some mutants would correspond to midpoint potentials of below ~450 mV, reflecting a shift of at least ~300 mV. (ii) The mutations could have profoundly changed the magnetic properties of the cluster making it undetectable by the standard EPR conditions used; the option that the tetranuclear iron-sulfur cluster was turned into a Fe$_3$S$_4$ cluster by the mutations was ruled out by analyzing oxidized samples. Reduced samples were scanned at many different EPR settings (temperature, microwave power, and field ranges), but we could not find indications for a new paramagnetic species in the mutant membranes or isolated enzyme complexes. Even a wide range scan at very low temperature (4.3 K) and high microwave power (63 mW) to explore the possibility that the spin of the ground state of the cluster might have turned into $S = 3/2$ gave no hints at an altered state of the cluster. While in a strict sense, it is impossible to actually prove the absence of an iron-sulfur cluster by EPR spectroscopy, it still seemed rather unlikely to us that a conversion of the cluster N2 ground state into the unusual $S = 3/2$ species had been caused in a similar fashion by several mutations at different positions in the 49-kDa subunit. Therefore, we concluded from our extensive EPR spectroscopic analysis that some mutations of His-226 and Arg-141 probably had resulted in the loss of most or all of iron-sulfur cluster N2. It should be noted at this point that in previous studies exploring the ligation of iron-sulfur cluster N2 (10–12) only standard EPR conditions were applied, and the possible explanations for the disappearance of the typical EPR signature outlined above were not even considered. Therefore, the data and conclusions drawn from these studies have to be reconsidered critically in the light of the discussion presented here.

If in some of the mutants that still showed rather high specific catalytic activities the disappearance of the EPR spectrum of cluster N2 indeed reflected loss of this redox center, the question arises how this apparent paradox could be resolved. In fact, electron transfer theory offers a straightforward though at this point speculative explanation; in electron wires consisting of a series of iron-sulfur clusters or other components, distances between redox centers are typically around 5 Å (8, 9). This should be a good approximation for the iron-sulfur clusters of complex I as well. Estimating electron transfer rates using the “Moser-Dutton ruler” (24), this distance corresponds to a rate of at least $10^{6}$–$10^{7}$ s$^{-1}$ if no driving force is assumed. In a simple scenario, the electron would have to travel around 10 Å if one redox center in a chain of iron-sulfur clusters was removed. The estimated electron transfer rate would then drop by about two orders of magnitude but still would be in the range of $10^{5}$–$10^{6}$ s$^{-1}$. Comparison of these estimates with the steady-state turnover number of about $10^{2}$ s$^{-1}$ illustrates that removal of iron-sulfur cluster N2 does not necessarily limit steady-state turnover because electron transfer is still predicted to be much faster. A similar situation, although involving entirely different redox centers, has been analyzed recently by time-resolved absorption spectroscopy on the picosecond time scale in mutants of Escherichia coli DNA photolyase (25). Unfortunately, no methods are available so far to study electron transfer within complex I even in the submillisecond time range.

If, as suggested by our results, complex I can catalyze ubiquinone reduction at essentially normal rates even in the absence of cluster N2, this questions some of the central mechanistic functions that have been discussed for iron-sulfur cluster N2. The key issue that is now under investigation in our laboratory is whether the mutations discussed in this work interfere with the proton pump of complex I.

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Conserved His and Arg in 49-kDa Subunit of Complex I 21199

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