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The Aging-Associated Enzyme CLK-1 is a Member of the Carboxylate-Bridged Diiron Family of Proteins

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Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

ABSTRACT: The aging-associated enzyme CLK-1 is proposed to be a member of the carboxylate-bridged diiron family of proteins. To evaluate this hypothesis and characterize the protein, we expressed soluble mouse CLK-1 (MCLK1) in E. coli as a heterologous host. Using Mössbauer and EPR spectroscopy, we established that MCLK1 indeed belongs to this protein family. Biochemical analyses for in vitro activity of MCLK1 with quinone substrates revealed that NADH can serve directly as a reductant for catalytic activation of dioxygen and substrate oxidation by the enzyme, with no requirement for an additional reductase component. The direct reaction of NADH with a diiron-containing oxidase enzyme has not previously been encountered for any member of the protein superfamily.

The enzyme CLK-1 has emerged as a potential drug target to retard the rate of aging. CLK-1, which is necessary for ubiquinone biosynthesis in yeast, C. elegans, and mice (1, 2, 3), limits longevity. Loss-of-function mutations in the C. elegans clk-1 gene (4, 5), or the loss of one copy of the mouse orthologue, Melk1, increases lifespan (6, 7) and improves biomarkers of aging (6). Interestingly, clk-1 was the first longevity gene found to act in mitochondria (9).

CLK-1 is a small, membrane-associated protein that is located on the matrix side of the inner mitochondrial membrane (9, 11, 12). Inspection of the amino acid sequence for CLK-1 from a variety of organisms led to the hypothesis that this enzyme is a member of the carboxylate-bridged diiron protein family (13). The authors also determined that CLK-1 functions as a monooxygenase to hydroxylate de-methoxyubiquinone (2-methoxy-5-methyl-6-polyrenyl-1,4-benzoquinone) in the penultimate step of ubiquinone biosynthesis (13). Two parallel investigations independently used sequence homology to model the structure of CLK-1, both proposing the presence of a four-helix bundle with an additional helix to embed the protein into the membrane (13, 15). The diiron center would most likely be housed within the four-helix bundle motif, a common structural element for this protein family (17).

A recent study of the metal-chelating agent cliquotinol, 5-chloro-7-idoquinolin-8-ol, which inhibits CLK-1, provided evidence that the enzyme contains iron (19). This result and the modeling work described above are taken as evidence that CLK-1 is a member of the carboxylate-bridged diiron family (13, 15). However, there is to date no experimental proof for the presence of such a non-heme diiron active site in this enzyme. In the present rapid report we provide conclusive spectroscopic evidence that CLK-1 is indeed a carboxylate-bridged diiron enzyme and demonstrate that this enzyme turns over in the presence of the ubiquitous biological reductant NADH.

In order to provide sufficient quantities of soluble protein for spectroscopic characterization, we tested the expression level of soluble enzyme from a previously constructed plasmid containing the clk-1 gene from mouse (Fig. S1A in the Supporting Information) (12). Following purification, a small amount of soluble MCLK1 (~19 kDa) was obtained that did not contain iron. We therefore prepared a maltose binding protein (MBP) fusion both to increase the expression level and improve the solubility of MCLK1 (20). With this strategy we obtained ~25 mg, 290 nmol, per liter of soluble fusion protein after purification (Fig. S1B). Fractions containing MCLK1 were yellow in color and purified MCLK1 contained 2.62 ± 0.09 Fe per protein. The UV/vis spectrum of the purified protein is displayed in Fig. S2. A shoulder at 340 nm, ε_{340} ~ 5300 M^{-1}cm^{-1}, is present in the CLK-1 sample, but not in a MBP control. This absorption is characteristic of an oxo-bridged diiron(III) center, as previously established for several other well-characterized carboxylate-bridged diiron proteins and model compounds (21).

We then performed a reductive titration on MCLK1 using sodium dithionite and UV/vis spectroscopy, Fig. 1. As

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expected, the \(A_{340}\) peak diminished upon successive additions of dithionite. Two electrons per MCLK1 molecule were required to fully reduce the protein (Fig. 1, inset). Upon exposure of the fully reduced protein to air, the \(A_{340}\) peak was restored to \(\approx 95\%\) of its original intensity. This titration experiment provided the first convincing evidence that MCLK1 contains a diiron(III) site. Additional support for this conclusion is provided by the appearance of optical absorption bands at 345 and 450 nm, characteristic of carboxylate-bridged diiron protein azido adducts, upon addition of 2 M NaN\(_3\) to the oxidized enzyme (Fig. S2) (16, 22).

To further characterize the diiron center in MCLK1 we prepared \(^{57}\)Fe-labeled enzyme for Mössbauer spectroscopic analysis. We collected data on oxidized and reduced MCLK1 at 4.2 K at zero magnetic field (Fig. S3). In these samples, \(\approx 20\%\) of the iron was adventitiously bound to the enzyme, as indicated by the magnitude of a \(g = 4.3\) signal present in the EPR spectrum. In the oxidized sample the iron impurity had Mössbauer parameters of \(\delta = 0.48\) mm/s and \(\Delta E_q = 1.14\) mm/s, and in the reduced sample the values were \(\delta = 1.17\) mm/s and \(\Delta E_q = 3.10\) mm/s. That this extra iron is adventitiously bound was supported by a control expression of MBP under identical conditions. Protein-bound iron(II) was detected in samples of purified MBP using a ferrozine-based colorimetric assay (23). When we subtracted the contribution of the impurity from the Mössbauer spectrum, the resulting parameters agreed with those in related carboxylate-bridged diiron proteins (Fig. 2). A summary of Mössbauer parameters for MCLK1 and related members of the family is presented in Table 1.

The oxidized MCLK1 sample (Fig. 2, top) contained two species with isomer shifts typical of high-spin ferric species. The major difference between the two species was the magnitude of the quadrupole splitting parameters, 1.59 and 0.62 mm/s, present in relative abundances of 41.3% and 58.7%, respectively. A mixture of diiron(III) species is often observed in carboxylate-bridged diiron proteins (8, 10, 14, 16). Work on diiron model compounds has established that (μ-oxo)diiron(III) centers typically have \(\Delta E_q \geq 1\) mm/s, whereas μ-hydroxo-bridged centers have a \(\Delta E_q \leq 1\) mm/s (21). It is therefore possible that MCLK1 exists as a mixture of μ-oxo and μ-hydroxo diiron(III) active sites at pH 7.5.

Reduction of the diiron(III) center to diiron(II) in

![Figure 2. Mössbauer spectra of MCLK1 collected at 4.2 K in zero magnetic field. Top is a spectrum of oxidized MCLK1 showing the individual fits for the two iron(III) species and bottom is the sodium dithionite reduced species. The contribution from adventitiously bound Fe was subtracted prior to fitting the data.](image)

**Table 1.** Mössbauer Parameters for Carboxylate-Bridged Diiron Enzymes.

<table>
<thead>
<tr>
<th>Protein</th>
<th>(\delta_{\text{Fe}^{II}-\text{Fe}^{III}})</th>
<th>(\Delta E_q)</th>
<th>(\delta_{\text{Fe}^{I}-\text{Fe}^{IV}})</th>
<th>(\Delta E_q)</th>
<th>Ref.</th>
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<tr>
<td>MCLK1</td>
<td>0.49 mm/s</td>
<td>0.62 mm/s</td>
<td>1.39 mm/s</td>
<td>3.18 mm/s</td>
<td>This work</td>
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<tr>
<td></td>
<td>0.54 mm/s</td>
<td>1.59 mm/s</td>
<td>3.2 mm/s</td>
<td>6.2 mm/s</td>
<td></td>
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<tr>
<td>AlkB</td>
<td>0.55 mm/s</td>
<td>1.70 mm/s</td>
<td>1.1 mm/s</td>
<td>3.2 mm/s</td>
<td>(8)</td>
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<tr>
<td>MM0H</td>
<td>0.51 mm/s</td>
<td>1.13 mm/s</td>
<td>1.3 mm/s</td>
<td>3.14 mm/s</td>
<td>(10)</td>
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<tr>
<td>(\Delta D)</td>
<td>0.50 mm/s</td>
<td>0.74 mm/s</td>
<td>1.3 mm/s</td>
<td>3.04 mm/s</td>
<td>(16)</td>
</tr>
<tr>
<td>PAP</td>
<td>0.53 mm/s</td>
<td>1.54 mm/s</td>
<td>1.3 mm/s</td>
<td>3.36 mm/s</td>
<td>(18)</td>
</tr>
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Abbreviations: AlkB, alkane \(-\)hydroxylase; MM0H, soluble methane monoxygenase; RNK-R2, Ribonucleotide reductase; \(\Delta D\), stearoyl-CoA \(\Delta\)-desaturase; PAP, purple acid phosphatase.
phere was employed, the product had a mass 18 units greater than that of DMQ (100% incorporation, m/z = 170). These results support a monooxygenase mechanism in which the oxygen atom is derived from dioxygen rather than water. A similar rate enhancement was also observed for several other p-quinone substrates, including 1,4-naphthoquinone, 3-methoxy-1,4-naphthoquinone, and 1,4-benzoquinone (Fig. S6). The enhancement did not occur when NADPH was supplied as the reductant, however.

The increased rate of NADH consumption upon binding of 1,4-quinones establishes that MCLK1 is not simply acting as an NADH oxidase. This behavior may indicate either that a conformational change occurs as a result of substrate binding, or, in a manner similar to cytochrome P450s (28) and Δ3 desaturase (29), that substrate binding alters the electronic environment of the diiron site such that the redox potential of the metal centers is increased and reduction of the diiron(III) unit becomes more facile. Our work also supports the conclusion that NADH might directly reduce the diiron center in MCLK1. Experiments performed using apo-MCLK1 showed no oxidation of NADH or hydroxylation of p-quinones when they were present.

Because NADH is capable of reducing quinones non-enzymatically (30), a control in which DMQ and NADH were allowed to interact showed little NADH consumption (< 1 μM min⁻¹, Fig. S5). This result indicates that, under our experimental conditions, NADH does not directly reduce DMQ. It is also unlikely that MCLK1 utilizes NADH as a quinone reductase, because no reduced DMQ was detected by GC-MS (data not shown).

The reduction of a non-heme diiron center by NADH without the use of other cofactors is unprecedented. To our knowledge, the only other system that utilizes NADH to directly reduce an enzyme active site is a nitric oxide reductase, P450nor (31). In P450nor and flavoproteins (32), one typically thinks of NADH as a hydride donor; however, there has been some debate as to whether this chemistry proceeds by a one-step hydride transfer or multistep electron-proton-electron transfer mechanism. In synthetic model compounds, a multistep mechanism has been observed for hydride transfer from NADH (33). This type of mechanism is promoted by hydrogen bonding interactions (34). In MCLK1, a possible reduction mechanism might involve electron transfer to one iron center, followed by proton transfer to an active site water molecule or amino acid, concluding with transfer of the second electron to reduce the other iron atom. This process would result in a diiron(II) species for dioxygen binding and activation. A more detailed analysis of the MCLK1 reduction steps by NADH is in progress.

In summary, with sufficient quantities of soluble, recombinant MCLK1 in hand, we have provided definitive evidence to support the hypothesis that this enzyme contains a diiron active site, the spectroscopic parameters of which match with those of carboxylate-bridged diiron proteins. MCLK1 reacts directly with NADH but not NADPH, suggesting that NADH is its true biological reductant. The availability of a suitable E. coli expression system for MCLK1 suggests that this construct can be used for high-throughput screening of drug candidates for the enzyme, perhaps providing a means to uncover the involvement of CLK-1 in the progression of age-dependent neurodegenerative diseases.

**ACKNOWLEDGMENT**

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**Supporting Information Available:** Experimental procedures and Figures S1-S6. This material is available free of charge via the Internet at http://pubs.acs.org.

**REFERENCES**

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