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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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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 *Escherichia coli* as a heterologous host. Using Mössbauer and EPR spectroscopy, we established that MCLK1 indeed belongs to this protein family. Biochemical analyses of the 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 protein 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, *Caenorhabditis elegans*, and mice (*1*−*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, *Mclk1*, increases life span (*6*, *7*) and improves biomarkers of aging (*6*). Interestingly, *clk-1* was the first longevity gene found to act in mitochondria (*8*).

CLK-1 is a small, membrane-associated protein that is located on the matrix side of the inner mitochondrial membrane (*8*, *9*, *10*). 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 (*11*). The authors also determined that CLK-1 functions as a monooxygenase to hydroxylate demethoxyubiquinone (2-methoxy-5-methyl-6-polyprenyl-1,4-benzoquinone) in the penultimate step of ubiquinone biosynthesis (*11*). 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 for embedding the protein into the membrane (*11*, *12*). The diiron center would most likely be housed within the four-helix bundle motif, a common structural element for this protein family (*13*).

A recent study of the metal-chelating agent clioquinol, 5-chloro-7-iodoquinolin-8-ol, which inhibits CLK-1, provided evidence that the enzyme contains iron (*14*). This result and the modeling work described above are taken as evidence that CLK-1 is a member of the carboxylate-bridged diiron family (*11*, *12*). However, there is to date no experimental proof for the presence of such a non-heme diiron active site in this enzyme. In this work, 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.

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 (Figure S1A of the Supporting Information) (*10*). 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 to improve the solubility of MCLK1 (*15*). With this strategy we obtained ∼25 mg, 290 nmol, per liter of soluble fusion protein after purification (Figure S1B of the Supporting Information). Fractions containing MCLK1 were yellow in color, and purified MCLK1 contained 2.62 ± 0.09 Fe atoms per protein. The UV−vis spectrum of the purified protein is displayed in Figure S2 of the Supporting Information. A feature at 340 nm ($\varepsilon_{340} \sim 5300\ M^{-1}\ cm^{-1}$) is present in the MCLK1 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 (*16*).

We then performed a reductive titration on MCLK1 using sodium dithionite and UV−vis spectroscopy (Figure 1). As expected, the A_{340} peak diminished upon successive additions of dithionite. Two electrons per MCLK1 molecule were required to fully reduce the protein (Figure 1, inset). Upon exposure of the fully reduced protein to air, the A_{340} peak was restored to ∼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 (Figure S2) (*17*, *18*).

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 and zero magnetic field (Figure S3 of the Supporting Information). In these samples, ∼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

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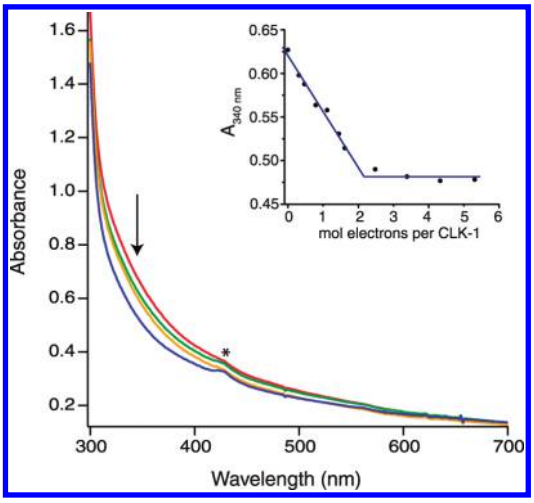

FIGURE 1: Reductive titration of 68 μM MCLK1 with 1 mM sodium dithionite in 25 mM MOPS (pH 7.5). The inset shows a decrease in absorbance at 340 nm upon successive additions of dithionite. The asterisk at ~420 nm designates a heme protein impurity.

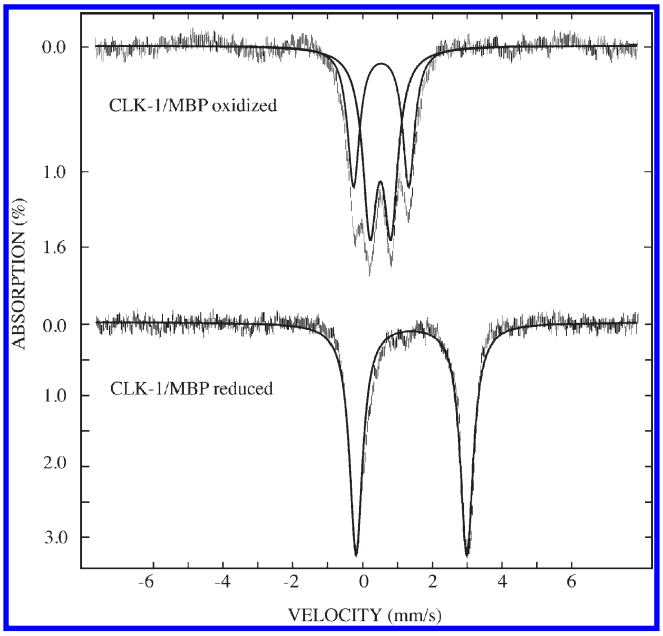

FIGURE 2: Mössbauer spectra of MCLK1 collected at 4.2 K and zero magnetic field. The top spectrum is that of oxidized MCLK1 showing the individual fits for the two iron(III) species, and the bottom spectrum is that of the sodium dithionite-reduced species. The contribution from adventitiously bound Fe was subtracted prior to fitting the data.

MBP under identical conditions. Protein-bound iron(II) was detected in samples of purified MBP using a ferrozine-based colorimetric assay (*19*). When we subtracted the contribution of the impurity from the Mössbauer spectrum, the resulting parameters agreed with those in related carboxylate-bridged diiron proteins (Figure 2). A summary of Mössbauer parameters for MCLK1 and related members of the family is presented in Table 1.

The oxidized MCLK1 sample (Figure 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 (*17*, *20*, *21*, *22*). Work on diiron model

Table 1: Mössbauer Parameters for Carboxylate-Bridged Diiron Enzymes[a]

	$Fe^{III}-Fe^{III}$		$Fe^{II}-Fe^{II}$		
protein	δ, mm/s	ΔE_Q, mm/s	δ, mm/s	ΔE_Q, mm/s	ref
MCLK1	0.49	0.62	1.39	3.18	this work
	0.54	1.59			
AlkB	0.55	1.70	1.1	3.2	*20*
	0.51	1.13			
MMOH	0.50	0.87	1.3	3.14	*21*
	0.51	1.16			
Δ^9D	0.50	0.74	1.30	3.04	*17*
	0.53	1.54	1.30	3.36	
PAP	0.54	−1.85	1.24	2.68	*23*

[a]Abbreviations: AlkB, alkane ω-hydroxylase; MMOH, soluble methane monooxygenase; RNR-R2, ribonucleotide reductase; Δ^9D, stearoyl-CoA Δ^9-desaturase; PAP, purple acid phosphatase.

compounds has established that (μ-oxo)diiron(III) centers typically have a ΔE_Q of $\geq$1 mm/s, whereas μ-hydroxo-bridged centers have a ΔE_Q of $\leq$1 mm/s (*16*). It is therefore possible that MCLK1 exists as a mixture of μ-oxo and μ-hydroxo diiron(III) active sites at pH 7.0.

Reduction of the diiron(III) center to diiron(II) in MCLK1 afforded one major species having δ = 1.20 mm/s and ΔE_Q = 3.15 mm/s (Figure 2, bottom). These parameters also agree with those for the reduced, diiron(II) state of several other carboxylate-bridged diiron proteins (Table 1). In addition to the Mössbauer parameters, another diagnostic feature of ferromagnetically coupled high-spin diiron(II) centers is the presence of an EPR signal near g = 16 (*13*, *24*). We therefore collected perpendicular mode EPR data on the reduced protein and observed a signal at $g \approx 16$ (Figure S4 of the Supporting Information). This result provides further evidence of the presence of a carboxylate-bridged diiron center in MCLK1.

We next examined the potential for CLK-1 to function as a diiron monooxygenase that utilizes dioxygen to catalyze the hydroxylation of demethoxyubiquinone. For such an activity, a reductant would be required to reduce the diiron(III) resting state to the diiron(II) form of the protein. The origin of such reducing equivalents for CLK-1 in vivo is not known. We therefore evaluated several possible biological reductants that are present at reasonable concentrations in mitochondria, including ascorbate, *p*-hydroquinone as a ubiquinol mimic, and NADH (*25*−*27*). When the enzyme was treated with sodium ascorbate or *p*-hydroquinone in the presence of dioxygen, there was no indication that the reductant could be oxidized, as monitored by UV−vis spectroscopy. When aerobic solutions of MCLK1 were treated with NADH, however, this reductant was consumed at a rate of 0.87 μM min^{-1} at pH 7.5. With NADPH under identical conditions, no oxidation was detected, which indicates that MCLK1 preferentially reacts with NADH.

To test the ability of our soluble MCLK1 construct to oxidize substrates, we used, as a model of demethoxyubiquinone-9, 2-methoxy-5-methyl-1,4-benzoquinone (DMQ), because of its greater water solubility compared to that of the native substrate. Addition of 200 μM DMQ to a mixture of CLK-1 (3.4 μM) and NADH (150 μM) increased the rate of NADH consumption from 0.87 $\pm$ 0.21 to 13.7 $\pm$ 1.4 μM min^{-1} (Figure S5 of the Supporting Information). GC−MS experiments revealed that the molecular mass of the product is 16 mass units larger than that of DMQ, consistent with substrate hydroxylation (m/z 168). When an $^{18}O_2$ atmosphere 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 (Figure S6 of the Supporting Information). 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 cytochromes P450 (*28*) and Δ^9 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 nonenzymatically (*30*), a control in which DMQ and NADH were allowed to interact showed little NADH consumption [$< 1\ \mu M\ min^{-1}$ (Figure 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 the best of 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 about 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 the transfer of hydride from NADH (*33*). This type of mechanism is promoted by hydrogen bonding interactions (*34*). In MCLK1, a possible reduction mechanism might involve the transfer of an electron to one iron center, followed by the transfer of a proton 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 *Escherichia 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.

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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.

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