JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

Probing a Complex of Cytochrome *c* and Cardiolipin by Magnetic Circular Dichroism Spectroscopy: Implications for the Initial Events in Apoptosis

Justin M. Bradley,[†] Gary Silkstone,[‡] Michael T. Wilson,[‡] Myles R. Cheesman,*[,†] and Julea N. Butt*[,†]

[†]Centre for Molecular and Structural Biochemistry, School of Chemistry and School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, Norfolk NR4 7TJ, U.K.

[‡]Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, U.K.

Supporting Information

ABSTRACT: Oxidation of cardiolipin (CL) by its complex with cytochrome *c* (cyt *c*) plays a crucial role in triggering apoptosis. Through a combination of magnetic circular dichroism spectroscopy and potentiometric titrations, we show that both the ferric and ferrous forms of the heme group of a CL:cyt *c* complex exist as multiple conformers at a physiologically relevant pH of 7.4. For the ferric state, these conformers are His/Lys- and His/OH^--ligated. The ferrous state is predominantly high-spin and, most likely, His/−. Interconversion of the ferric and ferrous conformers is described by a single midpoint potential of −80 ± 9 mV vs SHE. These results suggest that CL oxidation in mitochondria could occur by the reaction of molecular oxygen with the ferrous CL:cyt *c* complex in addition to the well-described reaction of peroxides with the ferric form.

Heme is a versatile cofactor that can facilitate long-range electron transfer or form part of a catalytic center. The functionality is determined in large part by the ligation, oxidation, and spin states accessible to the heme iron. These parameters are clearly interrelated, and there is much interest in elucidating the means by which they are tuned for function in a given protein. Eukaryotic cytochromes *c* (cyt *c*) are at the heart of such discussions.[1−4] These widely available proteins contain a single protoheme covalently linked to the peptide chain via two thioether bonds. Located in the intermembrane space of mitochondria, the native form of cyt *c* participates in respiration by transferring electrons from the cytochrome bc_1 complex to cytochrome *c* oxidase by redox cycling between the ferric and ferrous states of the His/Met-coordinated heme.

In the native state of cyt *c*, the heme is low-spin and the ferric/ferrous transition has a midpoint potential (E_m) in the range +200 to +350 mV.[4] However, although water-soluble, cyt *c* is a peripheral membrane protein that can interact both with its reaction partners and proteolipid membranes to produce altered ligation and redox properties.[5−11] A case in point is the perturbation of cyt *c* induced when it binds cardiolipin (CL), a negatively charged mitochondrial phospholipid. Binding of cyt *c* to CL inhibits electron transfer from the cytochrome bc_1 complex to cytochrome *c* oxidase and results in a marked increase in peroxidase activity; these events are key in promoting the permeability of the outer mitochondrial membrane in what is widely accepted to be the "point of no return" in programmed cell death (apoptosis).[8−10,12]

The change of function induced by CL binding to cyt *c* must be underpinned by changes in the heme axial ligation and possibly an altered E_m. The complex of ferri-cyt *c* with CL (CL:ferri-cyt *c*) has been reported to contain varying amounts of high- and low-spin heme conformers.[13−15] Resonance Raman spectroscopy has shown that the low-spin conformers have two nitrogenous ligands variously assigned as His/His or His/Lys.[12,15] Recent NMR measurements have ruled out major structural rearrangement upon CL binding,[16] and hence, comparison to the native cyt *c* structure[17] lends support to the His/Lys assignment. Either His/His or His/Lys ligation would be expected to result in a significant drop in E_m of the ferric/ferrous couple when methionine sulfur is replaced by nitrogen.[3] However, CL:cyt *c* has been reported to display both reversible and irreversible redox transitions at potentials both above and below that of native cyt *c*.[6,10] Thus, the present descriptions of CL:cyt *c* are ambiguous.

Magnetic circular dichroism (MCD) spectroscopy in conjunction with in situ potentiometric control is ideally suited to address these discrepancies.[18,19] The pattern of bands in the UV-vis (250−800 nm) MCD spectrum can be used to deduce the spin and oxidation state(s) of the heme when poised at any given electrochemical potential.[20] Furthermore, the energy of the charge-transfer transition in the near-IR (nIR, 800−2000 nm) MCD spectrum of low-spin ferric heme is diagnostic of the chemical nature of the axial ligands.[21] The MCD spectrum of ferric horse heart cyt *c* at pH 7.4 displays a peak at 1750 nm with a vibrational sideband centered at 1500 nm that is diagnostic of the His/Met-ligated native state (Figure 1).[22−24] When CL:cyt *c* is formed upon addition of a 30-fold excess of CL to minimize any residual unbound cyt *c*,[25] there is no evidence of a peak at 1750 nm. Instead, peaks with maxima at 1470 and 1130 nm report the presence of at least two low-spin conformers in the complex (Figure 1).

The peak at 1470 nm displayed by the CL:ferri-cyt *c* complex is diagnostic of low-spin heme having two nitrogenous ligands. His/His ligation typically gives rise to charge-transfer transitions between 1520 and 1600 nm, whereas His/Lys ligation such as found in ferri-cyt *c* at high pH (termed the alkaline form) gives

Received: September 28, 2011
Published: November 14, 2011

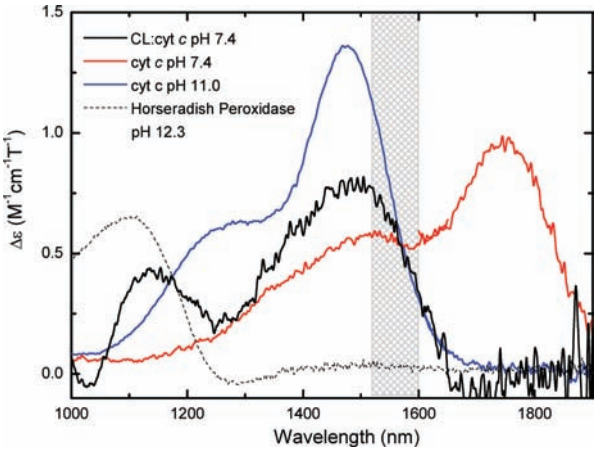

Figure 1. nIR MCD spectra at room temperature for CL:cyt *c* (black solid line), native cyt *c* (red line), and alkaline cyt *c* (blue line) and horseradish peroxidase (black broken line) at the indicated pH, measured at protein concentrations of 25, 100, 150, and 250 μM, respectively. The increased noise in the spectrum of CL:cyt *c* is a result of the lower sample concentration imposed by the limited solubility of CL. The shaded area represents the wavelength range spanned by the peak of charge-transfer bands arising from His/His-coordinated low-spin ferric heme.

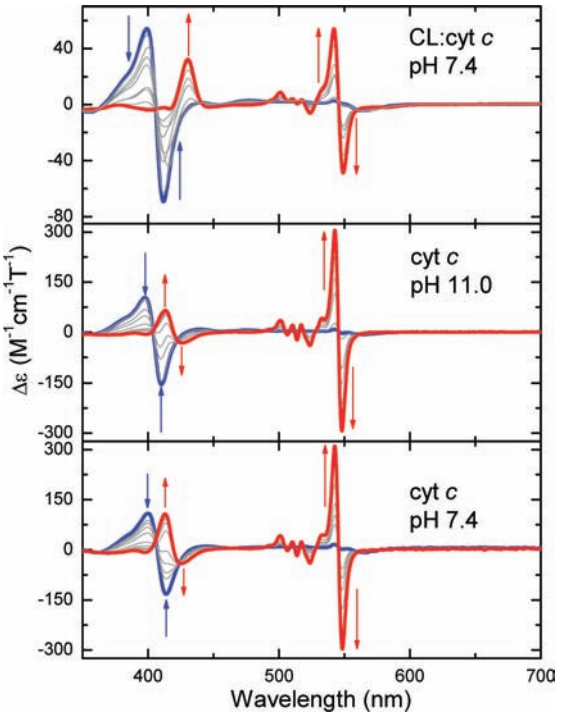

Figure 2. MCD monitored potentiometric titration of CL:cyt *c* and the alkaline and native forms of cyt *c* at the indicated pH. In each case the spectra of the fully oxidized (blue) and fully reduced (red) forms are highlighted and arrows indicate the direction of change of spectral intensity on lowering the potential.

rise to peaks between 1470 and 1520 nm (Figure 1).[26,27] Thus, some of the CL:ferri-cyt *c* contains His/Lys-ligated heme. The second peak, with a maximum at 1130 nm, is comparable to that displayed by alkaline horseradish peroxidase and diagnostic of low-spin ferric heme with His/OH^- ligation.[28] The X-band electron paramagnetic resonance (EPR) spectrum of the CL complex (see the Supporting Information) has features diagnostic of two low-spin species contributing to an asymmetric feature at g_z = 3.0 and having distinct resonances at g_y = 2.3 and 2.1, although confident assignment of the g_x values was precluded by the low concentration of cyt *c* imposed by the limited solubility of CL. The His/OH^--ligated heme of alkaline horseradish peroxidase[28] has g_z = 2.96, g_y = 2.09, and g_x = 1.63. Examples of His/Lys-ligated heme are rare.[26,29] They may give rise to symmetric features with g_z = 3.5, but in alkaline cyt *c* there is a significant fraction with g_z = 3.3. More generally, features with g_z = 3.0 and g_y = 2.3 are typical of heme with two nitrogenous ligands. Thus, the EPR spectrum of CL:ferri-cyt *c* is consistent with the assignment of axial heme ligands deduced by nIR MCD spectroscopy. Approximately equal populations of the His/Lys and His/OH^- conformers are suggested by comparison of the nIR MCD intensities of CL:ferri-cyt *c* and the alkaline forms of cyt *c* and horseradish peroxidase (Figure 1).

The MCD spectrum of CL:ferri-cyt *c* in the UV-vis region is compared to that of the native and alkaline forms of ferri-cyt *c* in Figure 2 (blue lines). All three spectra contain features typical of low-spin ferric heme, with a prominent bisignate feature (having positive and negative signs) at ~400 nm and a weaker band having a Gaussian profile and negative sign centered at ~560 nm. The intensities and widths of these features reflect the distinct environment of the heme in each form of the protein. Significantly CL:cyt *c* displays no features between 600 and 700 nm that would indicate high-spin ferric heme or features between 300 and 600 nm that would be indicative of ferrous heme formed by autoreduction. Thus, combining the nIR and UV−vis MCD results, we conclude that CL binding to native ferri-cyt *c* at pH 7.4 results in solely low-spin conformers in which the methionine has been displaced by lysine or hydroxide.

Addition of a 5-fold excess of sodium ascorbate to CL:cyt *c* resulted in negligible spectral change, whereas the native protein was clearly reduced in a parallel experiment (data not shown). This demonstrated a significant lowering of the midpoint potential of CL:cyt *c* that was quantified by potentiometric titration monitored by visible-region MCD spectroscopy. Equilibrating the complex at potentials below 0 mV resulted in loss of the features typical of low-spin ferric heme (Figures 2 and 3). This was accompanied by the appearance of a positively signed feature at 430 nm diagnostic of high-spin ferrous heme and a series of sharp bisignate features between 500 and 560 nm typical of low-spin ferrous heme. When the complex was poised below −200 mV, identical spectra were obtained whose features were solely due to ferrous heme.

CL:ferro-cyt *c* was returned to a low-spin ferric state upon oxidation, and the nIR-MCD and electronic absorbance spectra were indistinguishable in both form and intensity from those obtained immediately after complex formation (data not shown). For all wavelengths, the variation of the MCD intensity with potential was independent of whether the sample was being reduced or oxidized and was well-described by the Nernst equation for a single-electron (n = 1) process with E_m = −80 ± 9 mV (Figure 3). This can be compared with E_m = +280 ± 9 and −60 ± 5 mV defined by the low-spin ferric/ferrous transitions displayed by native and alkaline cyt *c*, respectively (Figures 2 and 3). These are values in good agreement with previous observations,[4,18,30,31] validating our experimental strategy. For alkaline cyt *c*, this describes the transition from ferric His/Lys to ferrous His/Met.

dx.doi.org/10.1021/ja209144h |*J. Am. Chem. Soc.* 2011, 133, 19676–19679

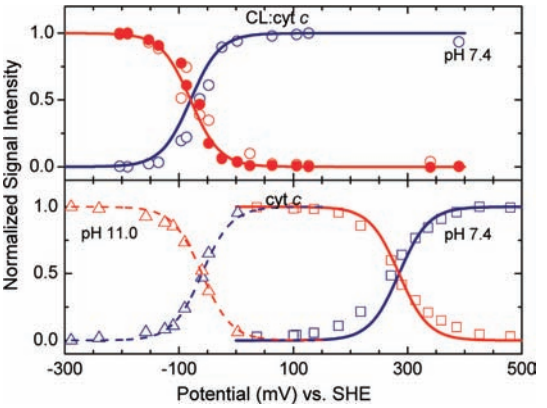

Figure 3. Potential dependence of the MCD intensities of CL:cyt *c* and native and alkaline cyt *c*, as indicated. The MCD intensity at 430 nm from high-spin Fe(II) (filled red circles), the peak-to-trough intensity from 544 to 550 nm from low-spin Fe(II) (open red symbols), and the peak-to-trough intensity from 398 to 406 nm from low-spin Fe(III) (blue symbols) are shown. Lines illustrate Nernstian behavior for a single redox site with $n = 1$ and the midpoint potentials detailed in the text.

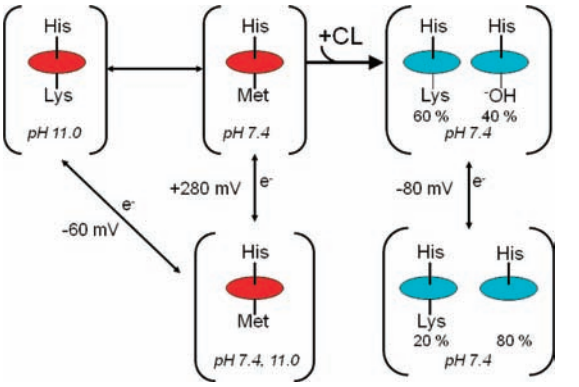

Figure 4. Changes in E_m and heme ligation of the (top) ferric and (bottom) ferrous states of cyt *c* induced by CL binding and increased pH. The blue and red disks represent the heme of cyt *c* in the presence and absence of CL, respectively.

The proportion of low- and high-spin conformers in CL:ferro-cyt *c* is best assessed using the peak-to-trough intensity of the low-spin feature, as the extinction coefficients of the high-spin bands are known to vary significantly.[32,33] Taking an average of the 544 to 550 nm intensities for the native ($618\ M^{-1}\ cm^{-1}\ T^{-1}$) and alkaline ($602\ M^{-1}\ cm^{-1}\ T^{-1}$) forms as relevant for describing the equivalent feature arising from the complex suggests that 20% of the heme is low-spin. The feature at 430 nm arising from high-spin ferrous heme then accounts for 80% of the sample, giving $\Delta\varepsilon_{430nm} \approx 40\ M^{-1}\ cm^{-1}\ T^{-1}$, a value that is not unreasonable for such species.[33] The MCD spectrum of the ferrous state cannot be used to identify the chemical nature of heme ligands in the same way as for the ferric state. However reduction of His/OH^--ligated heme is known to result in hydroxide dissociation, as in alkaline horseradish peroxidase,[34] making it most likely that reduction of this CL:ferri-cyt *c* conformer generates high-spin pentacoordinate ferrous heme. The remainder of the pentacoordinate heme could originate from reduction of the His/Lys conformer with protonation and dissociation of lysine.

The spectroscopic and electrochemical descriptions of CL:cyt *c* presented here are summarized in Figure 4, where they are compared to those of native and alkaline cyt *c*. Binding of CL induces a decrease of ~350 mV in the reduction potential of the ferric/ferrous couple, a drop that is similar to that induced by the alkaline transition. However, there are striking structural differences between CL:cyt *c* and the alkaline form. Raising the pH of native cyt *c* from neutral to pH 11 results solely in His/Lys ligation of the ferric heme iron. Upon reduction, the greater affinity of the ferrous iron for methionine results in a reversion to the native ligand set. In contrast, CL:cyt *c* exhibits mixed ligation in both the ferric and ferrous forms. CL:ferri-cyt *c* exists as His/Lys (60%)- and His/OH^- (40%)-ligated conformers. However in CL:ferro-cyt *c*, the major conformer (80%) contains high-spin heme iron, suggesting ligation solely by His18. Thus formation of CL:cyt *c* appears to inhibit the binding of methionine to the ferrous heme, resulting in a vacant coordination site.

When considering the nature of CL:cyt *c* that may be present in mitochondria, one should note that in liposomes and micelles with various CL contents the CL acyl chain(s) penetrate the globular protein structure and that binding is further stabilized by the electrostatic attraction between the CL phosphate groups and basic side chains of cyt *c*.[35,36] Although the molecular details of these interactions have yet to be resolved, in light of our results, it is of interest to reflect on the mechanism of CL oxidation by CL:cyt *c* that has attracted much attention for its contribution to apoptosis.[12] A Compound I oxo-ferryl intermediate has been proposed as key to this mechanism, and the well-described reaction between peroxides and CL:ferri-cyt *c* suggests that this intermediate may form in a mechanism analogous to that of the heme peroxidases.[37] In mitochondria, however, CL:cyt *c* will encounter glutathione ($E_m = -260$ mV vs SHE[38]), leading to the prediction of a significant population of the ferro complex. In this form, the heme is predominantly pentacoordinate and readily binds small exogenous ligands.[25] Thus, CL:ferro-cyt *c* is expected to react with molecular oxygen in such a way that addition of a further electron from glutathione or ubiquinol would provide an alternative route to Compound I analogous to that used by P450s. In both systems, modulation of the heme midpoint potential on binding of the organic substrate regulates catalysis. In P450s, the midpoint potential increases to allow access to the ferrous state, whereas the lowering of the cyt *c* midpoint potential induced by CL binding facilitates access to Compound I. Work is in progress to assess the reactivity of CL:cyt *c* with oxygen and peroxide in both the ferric and ferrous states.

■ ASSOCIATED CONTENT

Supporting Information. Preparation of samples of cyt *c*, details of spectroscopy and potentiometry, the EPR spectrum of CL:cyt *c*, the MCD spectrum of CL:cyt *c* plotted on an expanded scale, and complete ref 8. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
m.cheesman@uea.ac.uk; j.butt@uea.ac.uk

■ ACKNOWLEDGMENT

We thank Profs. Andrew Thomson and Nick Le Brun for helpful discussions and the U.K. Biotechnology and Biological

Sciences Research Council (BB/G024758/1 and 83/B18695) and UEA for funding.

REFERENCES

(1) Battistuzzi, G.; Borsari, M.; Cowan, J. A.; Ranieri, A.; Sola, M. *J. Am. Chem. Soc.* **2002**, *124*, 5315–5324.
(2) Tezcan, F. A.; Winkler, J. R.; Gray, H. B. *J. Am. Chem. Soc.* **1998**, *120*, 13383–13388.
(3) Battistuzzi, G.; Borsari, M.; Sola, M.; Francia, F. *Biochemistry* **1997**, *36*, 16247–16258.
(4) Pettigrew, G. W.; Moore, G. R. *Cytochromes c: Evolutionary, Structural and Physiochemical Aspects*; Springer: Berlin, 1990.
(5) Wackerbarth, H.; Hildebrandt, P. *ChemPhysChem* **2003**, *4*, 714–724.
(6) Salamon, Z.; Tollin, G. *J. Bioenerg. Biomembr.* **1997**, *29*, 211–221.
(7) Petrovic, J.; Clark, R. A.; Yue, H. J.; Waldeck, D. H.; Bowden, E. F. *Langmuir* **2005**, *21*, 6308–6316.
(8) Kagan, V. E.; et al. *Nat. Chem. Biol.* **2005**, *1*, 223–232.
(9) Belikova, N. A.; Vladimirov, Y. A.; Osipov, A. N.; Kapralov, A. A.; Tyurin, V. A.; Potapovich, M. V.; Basova, L. V.; Peterson, J.; Kurnikov, I. V.; Kagan, V. E. *Biochemistry* **2006**, *45*, 4998–5009.
(10) Basova, L. V.; Kurnikov, I. V.; Wang, L.; Ritov, V. B.; Belikova, N. A.; Vlasova, I. I.; Pacheco, A. A.; Winnica, D. E.; Peterson, J.; Bayir, H.; Waldeck, D. H.; Kagan, V. E. *Biochemistry* **2007**, *46*, 3423–3434.
(11) Jing, W. G.; Liu, C. W.; Tang, J. L.; Wu, Z. Y.; Dong, S. J.; Wang, E. K. *Chin. J. Chem.* **2003**, *21*, 544–549.
(12) Kagan, V. E.; Bayir, H. A.; Belikova, N. A.; Kapralov, O.; Tyurina, Y. Y.; Tyurin, V. A.; Jiang, J. F.; Stoyanovsky, D. A.; Wipf, P.; Kochanek, P. M.; Greenberger, J. S.; Pitt, B.; Shvedova, A. A.; Borisenko, G. *Free Radical Biol. Med.* **2009**, *46*, 1439–1453.
(13) Nantes, I. L.; Zucchi, M. R.; Nascimento, O. R.; Faljoni-Alario, A. *J. Biol. Chem.* **2001**, *276*, 153–158.
(14) Zucchi, M. R.; Nascimento, O. R.; Faljoni-Alario, A.; Prieto, T.; Nantes, I. L. *Biochem. J.* **2003**, *370*, 671–678.
(15) Sinibaldi, F.; Howes, B. D.; Piro, M. C.; Polticelli, F.; Bombelli, C.; Ferri, T.; Coletta, M.; Smulevich, G.; Santucci, R. *J. Biol. Inorg. Chem.* **2010**, *15*, 689–700.
(16) Kapralov, A. A.; Yanamala, N.; Tyurina, Y. Y.; Castro, L.; Samhan-Arias, A.; Vladimirov, Y. A.; Maeda, A.; Weitz, A. A.; Peterson, J.; Mylnikov, D.; Demicheli, V.; Tortora, V.; Klein-Seetharaman, J.; Radi, R.; Kagan, V. E. *Biochim. Biophys. Acta* **2011**, *1808*, 2147–2155.
(17) Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585–595.
(18) Marritt, S. J.; van Wonderen, J. H.; Cheesman, M. R.; Butt, J. N. *Anal. Biochem.* **2006**, *359*, 79–83.
(19) Bradley, J. M.; Butt, J. N.; Cheesman, M. R. *Anal. Biochem.* **2011**, *419*, 110–116.
(20) Cheesman, M. R.; Greenwood, C.; Thomson, A. J. *Adv. Inorg. Chem.* **1991**, *36*, 201–255.
(21) Gadsby, P. M. A.; Thomson, A. J. *J. Am. Chem. Soc.* **1990**, *112*, 5003–5011.
(22) Sutherland, J.; Greenwood, C.; Peterson, J.; Thomson, A. J. *Biochem. J.* **1986**, *233*, 893–898.
(23) Cheesman, M. R.; Ferguson, S. J.; Moir, J. W. B.; Richardson, D. J.; Zumft, W. G.; Thomson, A. J. *Biochemistry* **1997**, *36*, 16267–16276.
(24) Rawlings, J.; Stephens, P. J.; Nafie, L. A.; Kamen, M. D. *Biochemistry* **1977**, *16*, 1725–1729.
(25) Kapetanaki, S. M.; Silkstone, G.; Husu, I.; Liebl, U.; Wilson, M. T.; Vos, M. H. *Biochemistry* **2009**, *48*, 1613–1619.
(26) Gadsby, P. M. A.; Peterson, J.; Foote, N.; Greenwood, C.; Thomson, A. J. *Biochem. J.* **1987**, *246*, 43–54.
(27) Ferrer, J. C.; Guillemette, J. G.; Bogumil, R.; Inglis, S. C.; Smith, M.; Mauk, A. G. *J. Am. Chem. Soc.* **1993**, *115*, 7507–7508.
(28) Foote, N.; Gadsby, P. M. A.; Berry, M. J.; Greenwood, C.; Thomson, A. J. *Biochem. J.* **1987**, *246*, 659–668.
(29) Rigby, S. E. J.; Moore, G. R.; Gray, J. C.; Gadsby, P. M. A.; George, S. J.; Thomson, A. J. *Biochem. J.* **1988**, *256*, 571–577.
(30) Armstrong, F. A.; Hill, H. A. O.; Walton, N. J. *Acc. Chem. Res.* **1988**, *21*, 407–413.
(31) Barker, P. D.; Mauk, A. G. *J. Am. Chem. Soc.* **1992**, *114*, 3619–3624.
(32) Pond, A. E.; Sono, M.; Elenkova, E. A.; Goodin, D. B.; English, A. M.; Dawson, J. H. *Biospectroscopy* **1999**, *5*, S42–S52.
(33) Hendriks, J. H. M.; Prior, L.; Baker, A. R.; Thomson, A. J.; Saraste, M.; Watmough, N. J. *Biochemistry* **2001**, *40*, 13361–13369.
(34) Oganesyan, V. S.; Sharonov, Y. A. *Spectrochim. Acta, Part A* **1997**, *53*, 433–449.
(35) Rytomaa, M.; Kinnunen, P. K. J. *J. Biol. Chem.* **1994**, *269*, 1770–1774.
(36) Kalanxhi, E.; Wallace, C. J. A. *Biochem. J.* **2007**, *407*, 179–187.
(37) Belikova, N. A.; Tyurina, Y. Y.; Borisenko, G.; Tyurin, V.; Arias, A. K. S.; Yanamala, N.; Furtmueller, P. G.; Klein-Seetharaman, J.; Obinger, C.; Kagan, V. E. *J. Am. Chem. Soc.* **2009**, *131*, 11288–11289.
(38) Millis, K. K.; Weaver, K. H.; Rabenstein, D. L. *J. Org. Chem.* **1993**, *58*, 4144–4146.