Identification of Lys79 as an Iron Ligand in One Form of Alkaline Yeast Iso-1-ferricytochrome c

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Received April 28, 1993

Ferricytochromes *c* have been known for over 50 years to exhibit five discrete pH-dependent conformational states.[1] The so-called alkaline conformation of ferricytochrome *c*, formed on raising the pH to alkaline values (pK_a = 8.5–9 depending on species), has received particular experimental attention.[2] Interest in this form of the protein has recently intensified as the result of evidence suggesting that cytochrome *c* may undergo related conformational changes upon binding to other electron-transfer proteins.[3] The alkaline isomer possesses a six-coordinate, low-spin heme iron that retains the native imidazole N_ϵ ligand of His18 while the sulfur atom of Met80 is replaced by a strong-field ligand, the identity of which has been disputed for 20 years.[2] EPR and MCD evidence suggests that an ϵ-amino group of one of the many lysyl residues in cytochrome *c* fulfills this role,[4] though studies of chemically-modified cytochromes have provided conflicting evidence concerning this possibility.[5,6] NMR studies have shown that the alkaline isomer of horse heart ferricytochrome *c* is not a single species, though the detailed molecular basis of this heterogeneity has not been explained.[7] We report that the alkaline form of yeast iso-1-ferricytochrome *c* is a mixture of two lysine-ligated conformers, and we demonstrate that Lys79 provides the sixth axial ligand in one of these alkaline forms.

Lys79 is a phylogenetically conserved residue in cytochromes *c*. On the basis of the crystallographic model of yeast iso-1-ferricytochrome *c*,[8] the replacement of Met80 by Lys79 as the sixth ligand to the iron would require a smaller conformational perturbation of the protein than would replacement by any of the other 16 lysyl residues present in the molecule. The role of this residue as the sixth iron ligand in the alkaline form of ferricytochrome *c* has been proposed by several workers, but it has never been demonstrated.[5] In the present study we have replaced Lys79 in yeast iso-1-cytochrome *c* with an alanyl residue (Lys79Ala variant) through the use of site-directed mutagenesis.[9] The variant protein and the "wild-type" cytochrome used in this work possessed a threonyl residue at position 102 instead of the normally occurring cysteine.[10] Both proteins were expressed in yeast and isolated as previously described.[11]

At neutral pH, the electronic spectrum of the Lys79Ala variant exhibits the maximum at 695 nm that is characteristic of methionine–iron ligation;[12] the presence of this band indicates that the heme crevice is intact in this protein. NMR and EPR spectra of this variant (pH 7) are also characteristic of native, wild-type ferricytochrome *c* (data not shown). Direct electrochemistry[11] of Lys79Ala, which has one fewer positive charge than the wild-type cytochrome, establishes that this variant has the same midpoint potential (292 ± 2 mV *vs* SHE (25 °C, pH 6.0, μ = 0.1 M)) as the wild-type protein (290 ± 2 mV).[11] This result is surprising in light of related studies of cytochrome b_5 variants in which elimination of surface charged groups produced systematic changes to the reduction potential of the protein.[13] With increasing pH, the 695-nm band in the electronic spectrum of Lys79Ala disappears with a single-proton transition (pK_a = 8.5 ± 0.03 (25 °C, μ = 0.1 M)) (data not shown) as observed for the wild-type protein,[14] indicating the displacement of the Met80.

The NMR[15] spectrum (p^2H 9.4) of wild-type yeast iso-1-ferricytochrome *c* (Figure 1) shows the presence of two alkaline conformers in equilibrium with the native form of the protein. The chemical shifts of the heme methyl resonances of both alkaline isomers are similar to those observed for cytochrome *f*, which has been shown to possess a low-spin heme iron with His–Lys ligation.[16] The ratio of the three forms present in the mixture is temperature dependent. Similar findings have been reported for horse heart ferricytochrome *c*.[7] In contrast, the replacement of Lys79 by an alanyl residue resulted in the abolishment of one set of heme methyl resonances and a general simplification of the NMR spectrum (Figure 1). The remaining set of heme methyl resonances exhibited the same chemical shifts as those of one of the alkaline forms of the wild-type protein. This result establishes that the alkaline form of yeast iso-1-ferricytochrome *c* is a mixture of two isomers and that Lys79 is the sixth axial ligand in one of these forms.[17]

The existence of two lysine-ligated conformers at alkaline pH, each lacking a Met ligand to the iron, explains why the Lys79Ala variant and other single lysine-modified cytochromes $c^{5c-d,6c}$ can give rise to a low-spin alkaline form and exhibit an apparently normal alkaline transition as determined by spectrophotometric

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(1) Theorell, H.; Åkesson, Å. *J. Am. Chem. Soc.* **1941**, *63*, 1804–1811.

(2) Moore, G. R.; Pettigrew, G. W. *Cytochromes c. Evolutionary, Structural and Physicochemical Aspects*; Springer-Verlag: Berlin, 1990; pp 190–196.

(3) (a) Weber, C.; Michel, B.; Bosshard, H. R. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6687–6691. (b) Hildebrandt, P.; Heimburg, T.; Marsh, D.; Powell, G. L. *Biochemistry* **1990**, *29*, 1661–1668.

(4) (a) Brautigan, D. L.; Feinberg, B. A.; Hoffman, B. M.; Margoliash, E.; Peisach, J.; Blumberg, W. E. *J. Biol. Chem.* **1977**, *252*, 574–582. (b) Gadsby, P. M. A.; Peterson, J.; Foote, N.; Greenwood, C.; Thomson, A. J. *Biochem. J.* **1987**, *246*, 43–54. (c) Kobayashi, N.; Nozawa, T.; Hatano, M. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 919–920.

(5) (a) Wilgus, H.; Stellwagen, E. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 2892–2894. (b) Stellwagen, E.; Babul, J.; Wilgus, H. *Biochim. Biophys. Acta* **1975**, *405*, 115–121. (c) Smith, H. T.; Millet, F. *Biochemistry* **1980**, *19*, 1117–1120. (d) Looze, Y.; Polastro, E.; Deconinck, M.; Léonis, J. *Int. J. Peptide Prot. Res.* **1978**, *12*, 233–236. (e) Redfield, A. G.; Gupta, R. K. *Cold Spring Harbor Symp. Quant. Biol.* **1971**, *36*, 405–411. (f) Lambeth, D. O.; Campbell, K. L.; Zand, R.; Palmer, G. *J. Biol. Chem.* **1973**, *248*, 8130–8136. (g) Davis, L. A.; Schejter, A.; Hess, G. P. *J. Biol. Chem.* **1974**, *249*, 2624–2632.

(6) (a) Fanger, M. W.; Harbury, H. A. *Biochemistry* **1965**, *4*, 2541–2545. (b) Morton, R. A. *Can. J. Biochem.* **1973**, *51*, 465–471. (c) Osheroff, N.; Borden, D.; Koppenol, W. H.; Margoliash, E. *J. Biol. Chem.* **1980**, *255*, 1689–1697. (d) Pettigrew, G. W.; Aviram, I.; Schejter, A. *Biochem. Biophys. Res. Commun.* **1976**, *68*, 807–813. (e) Wallace, C. J. A. *Biochem. J.* **1984**, *217*, 601–604. (f) Bosshard, H. R. *J. Mol. Biol.* **1981**, *153*, 1125–1149.

(7) Hong, X.; Dixon, D. W. *FEBS Lett.* **1989**, *246*, 105–108.

(8) Berghuis, A. M.; Brayer, G. D. *J. Mol. Biol.* **1992**, *223*, 959–976.

(9) Inglis, S. C.; Guillemette, J. G.; Johnson, J. A.; Smith, M. *Protein Eng.* **1991**, *4*, 569–574.

(10) Cutler, R. L.; Pielak, G. J.; Mauk, A. G.; Smith, M. *Protein Eng.* **1987**, *1*, 95–99.

(11) Rafferty, S. P.; Pearce, L. L.; Barker, P. D.; Guillemette, J. G.; Kay, C. M.; Smith, M.; Mauk, A. G. *Biochemistry* **1990**, *29*, 9365–9369.

(12) Smith, D. W.; Williams, R. J. P. *Struct. Bonding* **1970**, *7*, 1–45.

(13) Rodgers, K. K.; Sligar, S. *J. Am. Chem. Soc.* **1991**, *113*, 9419–9421.

(14) Pearce, L. L.; Gärtner, A. L.; Smith, M.; Mauk, A. G. *Biochemistry* **1989**, *28*, 3152–3156.

(15) NMR spectra were recorded with a Bruker MSL-200 spectrometer at 200 MHz, equipped with a Bruker VT-1000 temperature controller ([protein] = 2 mM, 50 mM sodium phosphate (in D_2O), p^2H 9.4 at ambient temperature). The spectra were obtained by 8*K* points transform of 38.5-kHz spectral width after 2000–3000 pulses, using a superWEFT (Inubushi, T.; Becker, E. D. *J. Magn. Reson.* **1983**, *51*, 128–133) pulse sequence with a recycle delay of 220 ms. The spectral resolution was enhanced by multiplication of the free induction decay with a phase-shifted sine bell.

(16) 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.

(17) A more complex and less convincing interpretation of this result would involve the assumption that Lys79 is not the axial ligand in either of the two alkaline conformers but that replacement of Lys79 by an Ala residue prevents the conformational rearrangement leading to the formation of one of the alkaline forms.

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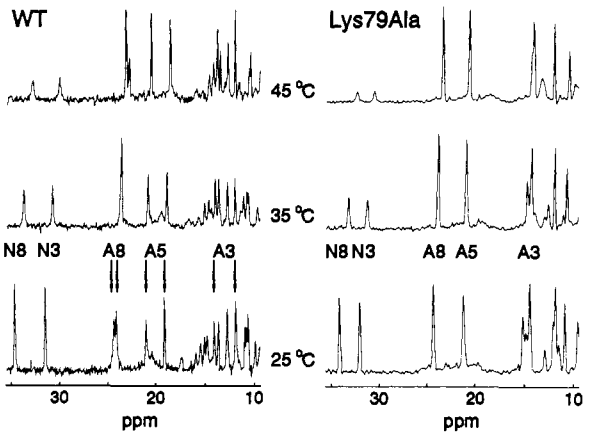

Figure 1. Downfield region of the NMR spectra of wild-type and Lys79Ala yeast iso-1-ferricytochrome *c* as a function of temperature. The assignments of the heme methyl resonances are indicated: N, native form; A, alkaline form. The numbers correspond to the number of the β-carbon atom of the porphyrin ring to which the methyl groups are attached. The assignments of the methyl resonances of the alkaline forms were obtained through magnetization-transfer measurements[21] at 50 °C, with a presaturation time of 0.5 s. In these experiments (data not shown), only saturation transfer between the methyl resonances of each of the alkaline isomers of the wild-type protein and the native form, but not between the two alkaline forms, could be detected.

titration. The same observation also explains previous results that apparently seemed to rule out the involvement of Lys79 in the alkaline form of cytochrome *c*.[6d-f] For example, it has been shown[6f] that the ϵ-NH_2 group of Lys79 in horse heart cytochrome *c* behaves as a freely accessible solvent-exposed amine at high pH. Since the alkaline form of cytochrome *c* is a mixture of two lysine-bound conformers in equilibrium with the native form, there will always be a fraction of either of these two lysyl residues behaving as a free amine at any pH.

The effects of replacing Lys79 with alanine on the EPR[18] spectrum of this cytochrome at alkaline pH are somewhat more difficult to interpret. At pH 10.5, multiple low-spin species are observed both in the wild-type protein and the Lys79Ala variant (Figure 2). The species with *g*-values of ~3.07 and ~2.24 can be assigned to the residual native form of the protein with histidine–methionine ligation to the heme iron.[4a-b] The other two low-spin forms in the wild-type protein with g_z-values of 3.56 and 3.33 and a g_y value of 2.06 are indicative of His–Fe–Lys ligation.[4a-b,16] Under identical conditions, the spectrum of Lys79Ala exhibits a major component with $g_z \sim 3.33$ and a minor component with $g_z \sim 3.56$. The observation that the signal at $g_z \sim 3.56$ in the spectrum of the Lys79Ala variant is diminished but not abolished can be understood if each of the two alkaline conformers gives rise to more than one EPR signal. The subconformations implied by this assumption are presumably in sufficiently rapid exchange at room temperature to escape detection by NMR spectroscopy. Related multiple heme pocket conformations have been observed in EPR freeze–quench experiments with methemoglobin.[19]

(18) EPR spectra were obtained at X-band frequencies with a Bruker ESP 300E spectrometer equipped with an Oxford Instruments liquid helium cryostat and an Oxford Instruments ITC4 temperature controller. The protein solution (~3 mM) in distilled water was mixed with CAPS buffer (100 mM, pH 10.5) and glycerol in a 1:1:2 ratio and frozen immediately in liquid nitrogen. Experimental conditions: 10 K, 2.0-mW microwave power, 9.45-GHz microwave frequency, 100-kHz modulation frequency, and 1-mT modulation amplitude.

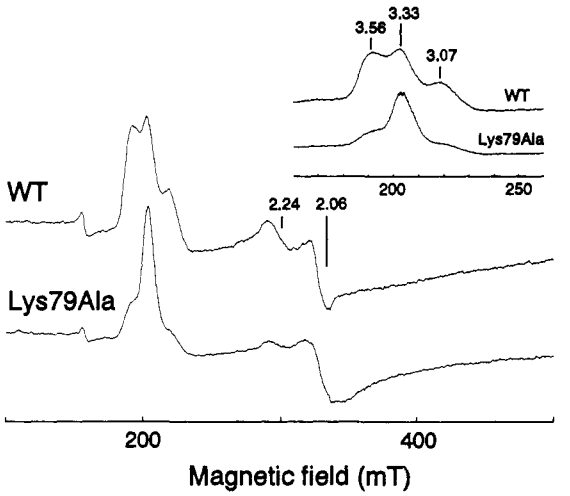

Figure 2. EPR spectra of wild-type and Lys79Ala yeast iso-1-ferricytochrome *c* (50 mM CAPS buffer, pH 10.5, 50% (v/v) glycerol). Inset: low-field region of the spectra. The g_y values are shown in the main panel, and the g_z values are shown in the inset.

As our recent determination of the reduction potential of alkaline cytochrome *c* failed to detect the presence of two electrochemically distinct alkaline species,[20] we conclude that the two alkaline forms of the wild-type protein have nearly equivalent midpoint reduction potentials.

No high-spin or thermally mixed-spin forms of the protein, characteristic of His–hydroxide ligation,[2] were detected in the EPR or NMR spectra of Lys79Ala at alkaline pH. This observation, combined with the fact that the two alkaline isomers of the wild-type protein are present in similar concentrations at room temperature (Figure 1), indicates that the free energies of the conformational changes leading to the formation of both alkaline forms are similar and are more favorable than the ligation of a hydroxide ion to the heme iron.

The identity of the new axial ligand to the heme iron in the second alkaline conformer of wild-type ferricytochrome *c* remains unknown though presumably this residue is located in the region between residues 66 and 104.[5a] Lys72, which also resides near the heme iron of native cytochrome *c*[8] and has also been discussed as a possible iron ligand in alkaline ferricytochrome *c*, is trimethylated in yeast cytochrome *c* and cannot bind to the heme iron. Therefore, the most likely candidates for this role at present are Lys73, -86, -87, or -89, although extensive conformational changes will be required to accommodate any of these residues.

Acknowledgment. We thank Federico Rosell for assistance in purification of the cytochrome variant. This work was supported by NIH Grant GM-33804 (to A.G.M.), by MRC of Canada Grant MT-1706 (to M.S.), and by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (to R.B.). The NMR spectrometer was supported by MRC Maintenance Grant ME-7826 (to Prof. P. R. Cullis), and the EPR spectrometer was funded by the Protein Engineering Network of Centres of Excellence.

(19) Levy, A.; Kuppusamy, P.; Rifkind, J. M. *Biochemistry* **1990**, *29*, 9311–9316.
(20) Barker, P. D.; Mauk, A. G. *J. Am. Chem. Soc.* **1992**, *114*, 3619–3624.
(21) Alger, J. R.; Shulman, R. G. *Q. Rev. Biophys.* **1984**, *17*, 83–124.