ELSEVIER

FEBS Letters 342 (1994) 217–220

FEBS 13862

FEBS LETTERS

Identification of axial ligands of cytochrome c_{552} from *Nitrosomonas europaea*

David M. Arciero[a], Qinyun Peng[b], Jim Peterson[b,*], Alan B. Hooper[a,*]

[a]*Department of Genetics and Cell Biology, University of Minnesota, St. Paul, MN 55108, USA*
[b]*Department of Chemistry, University of Alabama, Tuscaloosa, AL 35487-0336, USA*

Received 20 January 1994

Abstract

Cytochrome c_{552} from *Nitrosomonas europaea* was analyzed by visible, EPR and MCD spectroscopies. The visible and MCD data show that histidine and methionine are the axial ligands to the heme iron of the ferric protein. The EPR spectrum of the cytochrome shows an atypical highly axial low spin (HALS) type signal with g-values that make it difficult to identify the axial ligands. These results reinforce the value of near-infrared MCD spectroscopy for assigning ligands in ferric heme systems and point out the difficulties in using only EPR spectroscopy for the same purpose. The description of another *c*-cytochrome exhibiting a HALS-type EPR signal will eventually be helpful in explaining the physical basis for this unusual signal.

Key words: Cytochrome c_{552}; *Nitrosomonas europaea*; Axial ligand, 695 nm band; EPR; HALS signal; MCD, near-infrared

1. Introduction

Nitrosomonas europaea derives energy for growth by oxidizing ammonia to nitrite. The oxidation of hydroxylamine to nitrite is catalyzed by the enzyme hydroxylamine oxidoreductase (HAO; $NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^-$). Two of the electrons abstracted in the reaction are eventually transported to ammonia monoxygenase (AMO), the enzyme that catalyzes the first step in the oxidation of ammonia ($NH_3 + O_2 + 2e^- \rightarrow NH_2OH + H_2O$). The other two electrons are directed to the terminal oxidase. The monoheme cytochrome c_{552} may be the physiological electron donor to the oxidase.

Cytochrome c_{552} is approximately 10 kDa in size. The sequence of the first 47 residues of the N-terminal region [1] suggests that this cytochrome belongs to the family of small acidic cytochromes c_{551} (cytochromes *c7*), typified by cytochrome c_{551} from *Pseudomonas aeruginosa*. The percent identities are roughly 40–50% when the sequence is compared to other members of the family [2]. The N-terminal sequence included the heme-binding region of the cytochrome, confirming that histidine is present as the fifth ligand to the iron. The report also proposed that the sixth axial ligand to the Fe was a methionine residue based, apparently, on the presence in the cytochrome of a small peptide that contained a methionine residue surrounded by a cluster of prolines [1], a motif that is highly conserved in this class of cytochromes. However, identification of the sixth ligand to the heme in a *c*-cytochrome is more reliably determined by spectroscopic analysis rather than by comparison of an incomplete amino acid sequence to that of other members of a small family of related cytochromes. Nevertheless, little spectroscopic work has been reported for cytochrome c_{552} beyond a UV/visible absorption spectrum [3]. We report here a detailed spectroscopic analysis of cytochrome c_{552} that defines optical, EPR and MCD signatures. Methionine is firmly established as the sixth ligand to the heme of this cytochrome. Furthermore, the cytochrome is shown to exhibit a HALS-type EPR signal. Such signals are unusual, but are observed in a small number of very diverse cytochromes. Since the physical basis for a HALS EPR signal is not completely understood, the characterization of another cytochrome exhibiting this signal is helpful.

2. Experimental

Nitrosomonas europaea was grown as described previously [4]. Initial fractionations of cell-free extracts involving ammonium sulfate (AS) precipitation and gel filtration chromatography (Sephadex G-100) were also as described previously [5]. About 30% of the cytochrome c_{552} was found in the 80% AS pellet and the remainder in the 100% AS pellet. After fractionation of the 80% and 100% AS re-solubulized pellets on the gel filtration column, fractions containing cytochrome c_{552} were combined then further purified first on an octyl–Sepharose column essentially as described previously for cytochrome c_{553} peroxidase [5]; second on a DEAE–Sepharose column equilibrated with 10 mM KPO_4, pH 7.5, buffer and eluted with buffer + 50 mM KCl; third on a Sephadex G-50 column equilibrated with 50 mM KPO_4, pH 7.5, buffer

* Corresponding authors. [a]Fax: (1) (612) 625-5754.
[b]Fax: (1) (205) 348-9104.

SSDI 0014-5793(94)00247-S

containing 0.2 M KCl; and fourth, if necessary, on another DEAE–Sepharose column equilibrated with 2 mM KPO_4, pH 6.2, buffer and eluted isocratically with buffer containing 10 mM KCl.

Optical spectra were obtained on a Hewlett Packard HP8452A diode array spectrophotometer. EPR spectra were recorded with a Varian E109 spectrometer equipped with an Oxford Instruments ESR-10 liquid helium flow cryostat. Methods for temperature measurement and g-value calibrations have been described previously [6]. MCD spectra were recorded using an Aviv Associates 41DS circular dichroism spectrometer and Cryomagnetics Inc. cryomagnet. A typical spectrum is actually a difference spectrum in which data recorded with the applied field in the reverse direction is subtracted from data recorded with the applied field in the forward direction, the result then being divided by two. In this manner, contributions arising from natural circular dichroism are subtracted from the spectrum. For MCD work in the near infrared region, samples were prepared in deuterated buffers. Reported values of pD are actually pH meter readings + 0.4 in accordance with the suggestion of Glascoe and Long [7]. In order to obtain optical quality glasses upon freezing, aqueous samples were diluted in deuterated glycerol (50% v/v). Deuterated reagents were obtained from Cambridge Isotope Laboratories (Woburn, MA).

3. Results

The visible absorption spectrum between 450 and 750 nm for ferric cytochrome c_{552} is shown in Fig. 1. Like many c-cytochromes with methionine ligation, a weak band is observed near 695 nm. The molar absorptivity at 695 nm was estimated to be 970 $M^{-1} \cdot cm^{-1}$. This is in the same range as the molar absorptivity values reported for horse heart cytochrome c (850 $M^{-1} \cdot cm^{-1}$ [8]) or *Pseudomonas* cytochrome c_{551} (800 $M^{-1} \cdot cm^{-1}$ [9]).

The near-infrared MCD spectra of ferric cytochrome c_{552} at pD 6.1 and 9.6 are shown in Fig. 2. The two traces are identical in shape with the exception that the more alkaline sample gave a signal of slightly lower intensity. The low energy maximum at 1,800 nm is clearly indicative of histidine/methionine coordination [10,11]. The negative trough at approximately 705 nm is additionally suggestive of methionine coordination, but is more like the feature observed in *Escherichia coli* cytochrome b_{562} [12] and *P. aeruginosa* cytochrome c_{551} (J. Peterson, unpublished observation) than in horse heart cytochrome c [13]. The intensity of the 1,800 nm maximum (340 $M^{-1} \cdot cm^{-1}$ at 4.2 K and 5.0 T) is similar to that observed for *P. aeruginosa* cytochrome c_{551} [11]. This is approximately twice that observed for horse heart cytochrome c but about as expected for a system displaying a HALS-type EPR signal [11,14].

The EPR spectrum of ferric cytochrome c_{552} is shown in Fig. 3. A HALS-type (highly axial low spin) EPR signal was observed. Values for g_z and g_y were determined to be 3.34 and 1.768, respectively. Since g_x could not be observed, it was calculated to be 1.311 using $g_x^2 + g_y^2 + g_z^2 = 16$ [15]. Using the equations of Taylor (V/λ) and axial (Δ/λ) crystal field parameters are calculated to be 1.13 and 7.00. This gives $V/\Delta = 0.16$ and $a^2 + b^2 + c^2 = 1.001$, values which fall within acceptable ranges as the analysis demands [16]. The crystal field parameters are quite different, however, from those for cytochrome c_{551} from *P. aeruginosa* (V/λ and Δ/λ are 1.28 and 3.45, respectively [10]), another c-cytochrome with His/Met ligation which exhibits a HALS-type EPR spectrum.

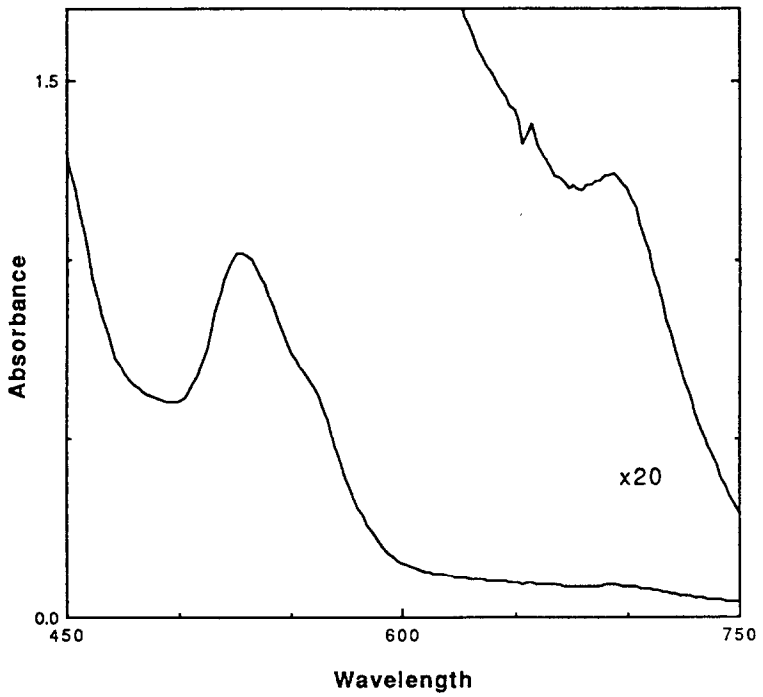

Fig. 1. Visible absorption spectra of oxidized cytochrome c_{552} in 50 mM KPO_4, pH 7.5.

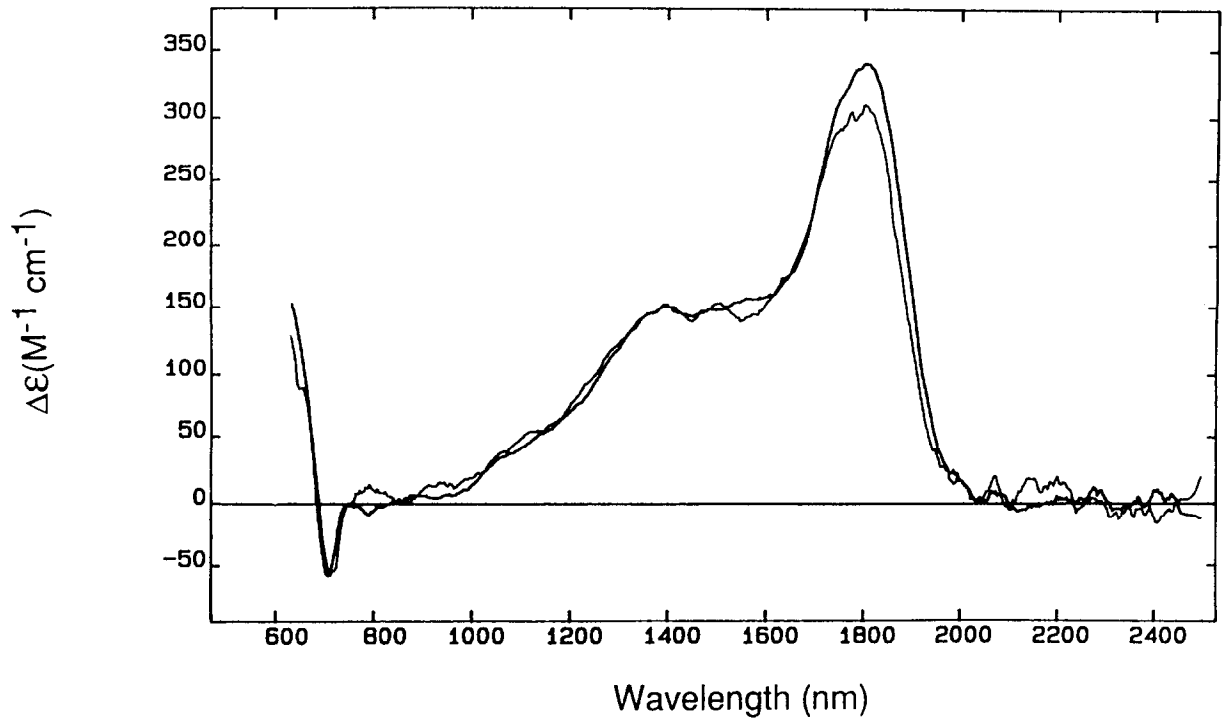

Fig. 2. Near-IR MCD spectra for oxidized cytochrome c_{552} at 4.0 K and 5.0 T in 50 mM MES, pD 6.1 (upper trace) and 50 mM CHES, pD 9.6 (lower trace). Both samples also contained deuterated glycerol, 50% (v/v). Path length = 0.3 mm.

4. Discussion

The wide range of EPR parameters now reported for ferric protoheme and c-type ferrihemes with His/Met axial coordination further supports the position that EPR spectroscopy alone is not a reliable indicator of axial ligand type [10,13,17,18]. In addition, while observation of a 695 nm band in the electronic absorption spectrum of a low spin ferric hemoprotein can be taken as strong evidence for the presence of a methionine ligand, this feature can be very difficult to detect and, therefore, difficult to distinguish from artifacts. It is the near-infrared MCD spectrum of Fig. 2 which provide the clearest evidence for His/Met coordination as well as the

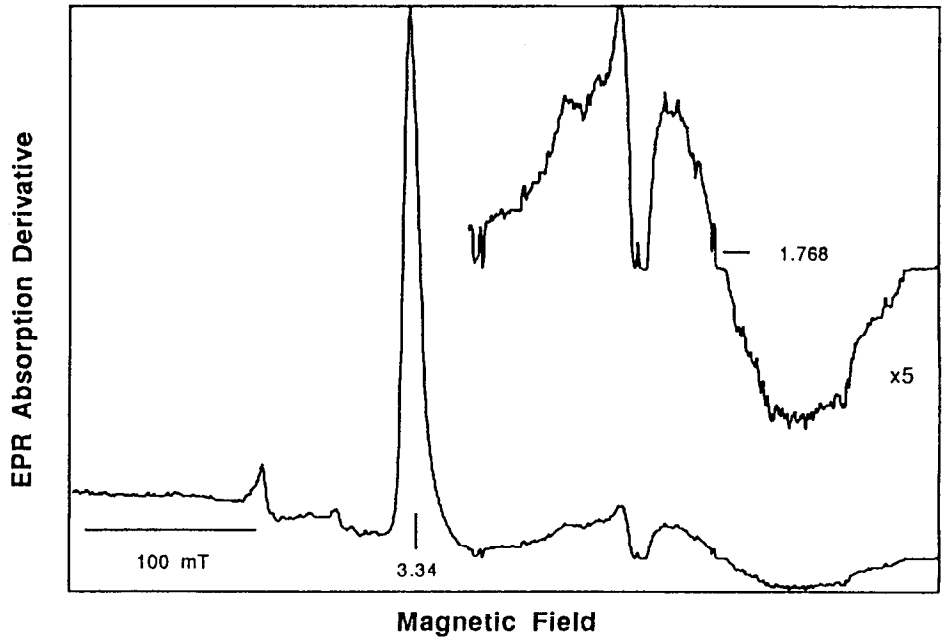

Fig. 3. EPR spectrum of oxidized cytochrome c_{552} in 50 mM KPO_4, pH 7.5. Conditions: microwave power, 1.88 mW; modulation amplitude, 10 Gauss; modulation frequancy, 100 kHz; microwave frequency, 9.215 GHz; temperature, 2.1 K.

absence of any pH-dependent ligand exchange processes in oxidized cytochrome c_{552}.

Thompson and co-workers [10,11] have shown that the position of the lowest energy near-infrared charge transfer transition (E_{CT}) observed in low spin ferric hemes is indicative of axial ligand type. Moreover, there appears to be a linear correlation between E_{CT} and the crystal field parameters of the central ferric ion. Teixeira et al. [18] have pointed out that this could reasonably be expected to lead to the same kind of ambiguity in ligand assignment as noted for EPR 'truth diagrams'. Although this logic is correct, the analysis of Gadsby and Thompson [10] simply provides a convenient rationalization of results obtained from a large number of very diverse systems. Furthermore, it must be remembered that the electronic structure of these systems is poorly delineated. For example, in low spin ferric derivatives (which are nearly covalent) one does not actually observe transitions from pure π-type to pure d-type orbitals since considerable orbital mixing occurs. Consequently, while it may be surprising that the combined application of EPR and MCD spectroscopies to the problem of axial ferriheme ligand identification works as well as it does, it has proven to be entirely reliable. Specifically regarding the present work, all substantiated cases of His/Met coordination, for which near-infrared MCD data have been reported, exhibit E_{CT} in the range 1,740–1,860 nm. They are thus easily distinguished from ferric hemes with other combinations of axial ligation since these others yield substantially different E_{CT}'s.

Acknowledgements: This work was supported by grants NSF/DMB-9019687 (A.B.H.) and Biomedical Research Support Grant SO7RR07151-13 (J.P.).

References

[1] Miller, D.J. and Nicholas, D.J.D. (1986) Biochem. Int. 12, 167–172.
[2] Moore, G.R. and Pettigrew, G.W. (1990) Cytochromes *c*: Evolutionary, Structural and Physicochemical Aspects, Springer-Verlag, Berlin.
[3] Yamanaka, T. and Shinra, M. (1974) J. Biochem. (Tokyo) 75, 1265–1273.
[4] Logan, M.S.P. (1991) Hydroxylamine Oxidoreductase from *Nitrosomonas europaea*: Inhibition by Cyanide and the Suicide Substrates Alkyl and Aryl Hydrazines, Ph.D. Thesis, University of Minnesota.
[5] Arciero, D.M. and Hooper, A.B. (1994) J. Biol. Chem. 269, in press.
[6] Lipscomb, J.D. (1980) Biochemistry 19, 3590–3599.
[7] Glasoe, P.K. and Long, F.A. (1960) J. Phys. Chem. 64, 188–190.
[8] Schejter, A. and George, P. (1964) Biochemistry 3, 1045–1049.
[9] Vinogradov, S.N. (1970) Biopolymers 9, 507–509.
[10] Gadsby, P.M.A. and Thompson, A.J. (1990) J. Am. Chem. Soc. 112, 5003–5011.
[11] Cheeseman, M.R., Greenwood, C., and Thompson, A.J. (1991) Adv. Inorg. Chem. 36, 201–253.
[12] Moore, G.R., Williams, R.J.P., Peterson, J., Thompson, A.J., and Matthews, F.S. (1985) Biochim. Biophys. Acta 829, 83–96.
[13] Gadsby, P.M.A., Peterson, J., Foote, N., Greenwood, C., and Thompson, A.J. (1987) Biochem. J. 246, 43–54.
[14] Thompson, A.J. and Gadsby, P.M.A. (1990) J. Chem. Soc. Dalton Trans. 1921–1928.
[15] DeVries, S. and Albracht, S.P.J. (1979) Biochim. Biophys. Acta 546, 334–340.
[16] Taylor, C.P.S. (1977) Biochim. Biophys. Acta 491, 137–149.
[17] Rigby, S.E.J., Moore, G.R., Gray, J.C., Gadsby, P.M.A., George, S.J. and Thompson, A.J. (1988) Biochem. J. 256, 571–577.
[18] Teixeira, M., Campos, A.P., Aguiar, A.P., Costa, H.S., Santos, H., Turner, D.L. and Xavier, A.V. (1993) FEBS Lett. 317, 233–236.