Biochimica et Biophysica Acta, 1016 (1990) 203–206
Elsevier

BBABIO 43197

EPR and redox potentiometric studies of cytochrome *c*-549 of *Anacystis nidulans*

Curtis W. Hoganson, Göran Lagenfelt and Lars-Erik Andréasson

Department of Biochemistry and Biophysics, Chalmers University of Technology and University of Göteborg, Göteborg (Sweden)

(Received 6 October 1989)

Key words: Cytochrome *c*-549; Low potential cytochrome; (*A. nidulans*)

Low potential cytochrome *c*-549 of *Anacystis nidulans* has been studied by EPR spectroscopy and redox potentiometry. The cytochrome clings to the photosynthetic membranes but can be solubilized by homogenization with 1 M NaCl. The cytochrome is low-spin and its *g* values of 3.04, 2.20 and 1.4 and the related ligand-field parameters are consistent with bis-histidine ligation of the heme iron. Cytochrome *c*-549 is approximately as abundant as cytochrome *b*-559 in the membranes. The midpoint potential of the extracted cytochrome varied from −280 to −314 mV, apparently depending on storage conditions, but was independent of pH between pH 6 and 8.

Introduction

A *c*-type cytochrome reducible by dithionite but not by ascorbate, containing one heme per polypeptide, was first observed in *Anacystis nidulans* [1–3] and has been observed in a number of blue-green and some other algae [4–7]. Its reduced form has, in addition to a prominent Soret band, a band with an absorption maximum between 548 and 550 nm. The cytochrome contains a single mesoheme [3,4] per 15.5 kDa polypeptide [7]. The cytochrome is an extrinsic membrane protein in *A. nidulans* [8] and other species, although it is readily extracted by water from cells obtained from some natural blooms [5]. It has an isoelectric point between 4 and 5 [7] so anion exchange chromatography is frequently used in its purification. A redox potential of −260 mV has been reported [3].

The function of this cytochrome is not yet known, although numerous suggestions have been made. Involvement in hydrogenase reactions [2], sulfide oxidation [5], nitrate reduction [7], respiration [4] and cyclic photophosphorylation [9] have all been suggested. Because cytochrome *c*-549 binds carbon monoxide [3], a ligand-binding function, such as a peroxidase, seemed possible. Cytochrome *c* peroxidase, for example, has a low potential *c*-type cytochrome [10]. Cytochrome *c*-549 was enriched in Photosystem II extracts of *Phormidium laminosum* [6], so a function related to Photosystem II was also considered possible.

To help understand the function of cytochrome *c*-549, the cytochrome from *A. nidulans* was characterized by electron paramagnetic resonance spectroscopy and by redox potentiometry. EPR is relevant, because most heme proteins that bind ligands have a 5-coordinate, high-spin iron in the absence of ligand, while most that function exclusively as electron transfer proteins have 6-coordinate, low-spin iron [11]. The results presented here show that cytochrome *c*-549 has a low-spin, six-coordinate iron. A short account of some of this work has been presented at the 8th International Congress on Photosynthesis (Stockholm, 1989).

Materials and methods

A. nidulans was grown aerobically and membranes and Photosystem II extracts were prepared as described previously [12]. For EPR measurements, membranes were suspended in 20 mM MES-NaOH (pH 6) 20% glycerol and Photosystem II extracts were suspended in 20 mM Mes-NaOH (pH 6)/20%/glycerol, 10 mM $CaCl_2$.

Partially purified cytochrome *c*-549 was prepared from the photosynthetic membranes, which were washed several times by suspending in 2 mM Mes-NaOH buffer (pH 6.0), followed by centrifugation. This solubilized over 95% of the phycocyanin but only traces of cyto-

Abbreviations: EPR, electron paramagnetic resonance; Mes, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

Correspondence: C.W. Hoganson, Department of Biochemistry and Biophysics, Chalmers University of Technology, S-412 96 Göteborg, Sweden.

0005-2728/90/$03.50 © 1990 Elsevier Science Publishers B.V. (Biomedical Division)

chrome. The membranes were next washed several times with 1 M NaCl and homogenized by hand to solubilize cytochrome *c*-549 and residual phycocyanin. This solution was dialyzed against 1 mM Tris-HCl (pH 7.5), centrifuged and applied to a DEAE-cellulose column, preequilibrated with the same buffer. Both the cytochrome and phycocyanin bound to the column. The column was eluted with an increasing potassium phosphate gradient from 40 mM (pH 6.2) to 200 mM (pH 6.0). The bulk of the phycocyanin eluted before cytochrome *c*-549. A yellow substance remained on the column. Peak cytochrome fractions were used for EPR and redox potentiometry. For EPR, the cytochrome was concentrated by ultrafiltration and transferred to 400 mM sucrose/15 mM NaCl/5 mM $MgCl_2$/20 mM Mes-NaOH (pH 6.3).

Absorption spectra were recorded with a Shimadzu UV-3000 spectrophotometer. Redox titrations were performed at 26 ± 2°C as described by Dutton [13] with a combination platinum electrode- Ag|AgCl reference electrode. Anthraquinone 2-sulfonate (Fluka), Saphranine O (Merck) and Neutral Red (Merck) were used as redox mediators. The solutions were buffered with Mes-NaOH (pH 6.0), Pipes-NaOH (pH 7.0) or Tris-HCl (pH 8.0) at concentrations of 100 mM. Titrations were made both oxidatively and reductively, by addition of air, $K_3Fe(CN)_6$ or $Na_2S_2O_4$. Depending on the mediators present, the reduction state of the cytochrome was monitored at either 417 nm or 549 nm, often by measuring the second derivative spectrum, which lessens interference due to the mediators. Calibration of the electrode was performed with a 10 mM ferri-ferrocyanide solution with 10 mM Tris-HCl buffer (pH 7.0) [14]. Titration of anthraquinone 2-sulfonate and Saphranine O gave values within 8 mV of the reported redox potentials (−225 mV and −289 mV, respectively, at pH 7 [15]). All potentials quoted are relative to the standard hydrogen electrode. For use as a reductant, anthraquinol 2-sulfonate was prepared by titrating an aqueous solution of anthraquinone 2-sulfonate with dithionite in the anaerobic cuvette.

EPR spectra were recorded with a Bruker ER 200 D-SRC X-band spectrometer equipped with a standard TE102 rectangular cavity and an Oxford Instruments ESR-10 helium flow cryostat. Samples were prepared by adding to the suspension in the EPR tube sufficient reductant to give a final concentration of 5 mM to accomplish a selective reduction of the different cytochromes (see Results). After mixing, N_2 was blown over the sample, the tubes were sealed with parafilm and incubated for 15 min on ice, and then frozen in liquid N_2. For EPR samples of membranes, 50 μM each of the following mediators were also present: 2-hydroxy-1,4-naphthoquinone (Fluka), 2,5-dihydroxy-*p*-benzoquinone (EGA-Chemie), anthraquinone 2,6-disulfonate (Fluka) and anthraquinone 2-sulfonate.

Results

Cytochrome *c*-549 was invariably found in the photosynthetic membranes of aerobically grown *A. nidulans*. The mixture of cytosolic proteins released during osmotic-shock rupture of the cells also usually contained cytochrome *c*-549, although much less than was present in the membranes. Absorption spectra of cytochromes from both sources are similar to that reported by Holton and Myers [2] (data not shown).

EPR spectra of cytochrome *c*-549 were obtained (Fig. 1.) The partially purified cytochrome sample contained no additional cytochromes, so the spectrum of an air-oxidized sample was recorded and is displayed (after subtracting signals due to the empty cavity and cryostat). In addition, the spectra of the membrane-bound cytochrome and of the cytochrome present in Photosystem II extracts were determined as difference spectra between samples reduced with anthraquinol 2-sulfonate and with dithionite, since preliminary redox titrations had indicated that pre-reduced anthraquinone 2-sulfonate could be used to reduce all the cytochromes present, including cytochromes *b*-559, *b*-563 and *f*, except cytochrome *c*-549.

All three spectra show peaks characteristic of a low-spin heme. In particular, a peak near $g = 3$ that may be assigned as the g_z value of the cytochrome appears in all three spectra. The two more biochemically resolved samples also exhibit g_y peaks of the low-spin heme at $g = 2.2$. In addition, a weak feature at $g = 1.4$ in the spectra of the partially purified cytochrome (data not shown) has been tentatively assigned as the g_x peak of the cytochrome. The principal g values for the three

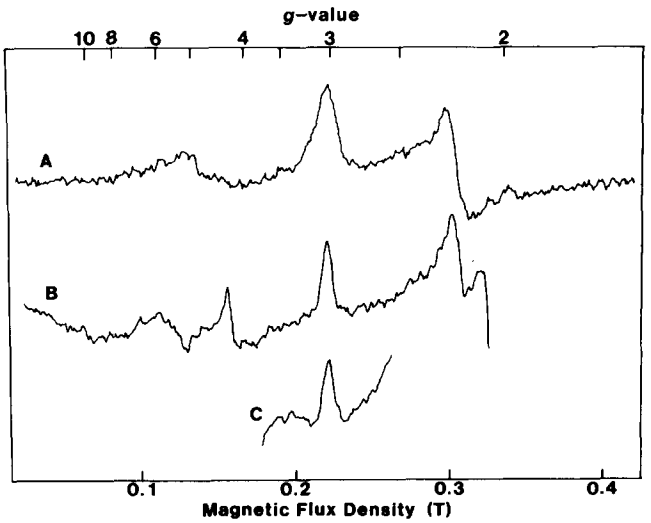

Fig. 1. EPR spectra of cytochrome *c*-549 of *A. nidulans*. Samples were: (A) membrane cytochrome, partially purified, (B) a detergent PSII extract, and (C) unfractionated thylakoid membranes. Spectra were obtained at 16 K with 2 mW microwave power and 32 G field modulation at 100 kHz.

samples (Table I) are very similar to each other, suggesting that the isolation procedure has not greatly altered the conformation of the isolated cytochrome. The peaks of the NaCl-solubilized cytochrome, however, are about twice as broad as the peaks of the other samples, which suggests that the isolated cytochrome may exist in a number of slightly different conformations. Cytochrome *b*-559, which also has peaks near $g = 3$, is chemically reduced in these samples, as well as in membrane samples reduced with hydroquinone or ascorbate, and so does not contribute to the spectra shown.

The g values of low-spin cytochromes permit calculation of ligand field splitting parameters and these can often be used to identify the heme iron axial ligands [16]. The ligand field parameters of cytochrome *c*-549, calculated according to Refs. 17,18 are given in Table I, along with parameters of several cytochromes with known bis-histidine ligation: cytochrome *b*-559 of Photosystem II [19,20], cytochrome b_5 [21] and cytochrome c_3 [22,23]. Because the ligand field parameters of cytochrome *c*-549 are intermediate between those of the reference cytochromes, we conclude that it also has bis-histidine ligation. This was recently suggested by Cohn et al. [24] from an examination of the primary structure of cytochrome *c*-550 from *Microcystis aeruginosa*. Methionine ligation has already been ruled out by the absence of an absorbance peak at 695 nm from the spectrum of the oxidized cytochrome [5].

EPR spectra were also recorded of membrane samples, redox-poised with either ascorbate or dithionite, in the presence of 5 mM of CN^-, N_3^- or F^- (not shown). No differences were observed between these spectra and those obtained in the absence of ligands. These results are consistent with the conclusion of Holton and Myers [3] that the cytochrome does not react with these anions.

TABLE I

Principal g values of cytochrome c-549 and several reference cytochromes along with ligand field parameters

Axial and rhombic fields are given in terms of the spin-orbit coupling constant and were calculated according to references [17,18]. n.d., not determined. In the second group of cytochromes each has two histidine ligands. The g_x value of high-potential cytochrome *b*-559 is that calculated in Ref. 20.

Sample/cytochrome	g_z	g_y	g_x	Axial field	Rhombic field	Ref.
NaCl-solubilized	3.04	2.20	1.4	3.26	1.61	
PS II extract	3.05	2.19	n.d.			
Membranes	3.04	n.d.	n.d.			
High-pot. *b*-559	3.08	2.16	(1.36)	3.35	1.52	19
Low-pot. *b*-559	2.94	2.27	1.54	3.36	1.92	20
b_5	3.05	2.22	1.41	3.22	1.62	21
c_3 (No. 4)	2.93	2.26	1.51	3.26	1.88	22
c_3 (No. 2)	3.05	2.24	1.34	2.86	1.56	22

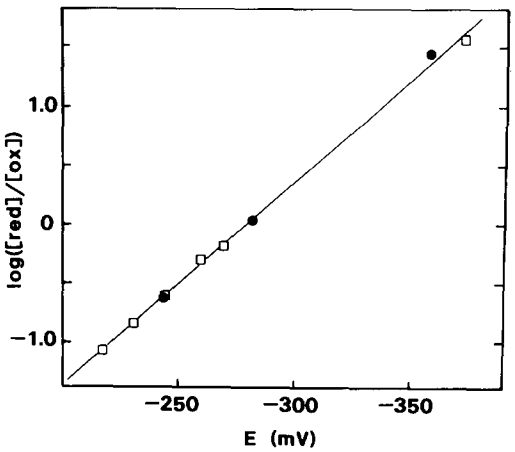

Fig. 2. Redox titration of cytochrome *c*-549 at pH 7.0 as described in the text. Cytochrome *c*-549 was used at about 0.5 μM. Mediators were used in the following concentrations: anthraquinone 2-sulfonate, 20 μM; Saphranine O, 20 μM; Neutral Red, 20 μM. The reduction state of the cytochrome was monitored at 417 nm using the second derivative function of the spectrometer. Open symbols, oxidative titration; filled symbols, reductive titration.

The similarity in EPR spectral parameters between cytochrome *c*-549 and cytochrome *b*-559 allows us to estimate their relative abundance in the membrane by comparing the areas of the g_z peaks. The relative abundances of cytochromes in the membrane may also be estimated from the magnitude of the absorbance changes in the spectral region from 540 to 570 nm that are induced by various reductants. For these calculations, we assume that the cytochromes have identical difference extinction coefficients at their absorbance maxima. These measurements (not shown) suggest that the ratio of cytochromes in the membranes is about 2–3 cytochrome *c*-549: 2 cytochrome *b*-559: 1–2 cytochrome *b*-563. These proportions are similar to those determined by Stewart and Bendall [6] and Matsuura and Fujita [25], except that the latter group detected only about half as much cytochrome *c*-549.

Redox potentiometric titrations were performed on the NaCl-solublized cytochrome. Typical data are shown in Fig. 2. All titrations in which adequate mediator concentrations were present gave n values between 0.9 and 1.1. The midpoint potentials at pH 7 of three samples stored under different conditions were -314 ± 2 mV, -294 ± 3 mV and -280 ± 3 mV (means and standard deviations of three, four and two measurements, respectively). Because a suitable assay of the cytochrome's function is lacking, it is not clear which of these values corresponds most closely to that of the native protein. One sample was examined also at pH 6 and 8 and its potential was found to be independent of pH. Thus, the midpoint potential of the cytochrome,

although mildly sensitive to storage conditions, is pH independent in the physiological range.

Discussion

The EPR spectra of cytochrome *c*-549 indicate a low-spin heme and suggest that the iron is six-coordinate with two axial histidine ligands. These data support an electron-transfer function for this cytochrome rather than a ligand-binding function. The ability of the reduced cytochrome to bind CO [3], however, would suggest five-coordinate iron. A similar situation holds for cytochrome c_3 which binds CO and has four low-potential, low-spin hemes [22], each with known bis-histidine ligation [23]. CO-binding can be reconciled to six-coordinate iron if, as according to Stellwagen [26], a cytochrome's low potential implies a heme group that is highly exposed to the surrounding aqueous solution. A high degree of exposure would allow external ligands ready access to the heme and could allow CO to replace the endogenous sixth ligand. The hemes of cytochrome c_3 are highly exposed to the solvent [23]. Extrapolating from the data for high-potential *c*-cytochromes [26] yields for cytochrome *c*-549, a figure of 40% for the surface area exposed to the aqueous solvent. The failure of anionic ligands to react with the oxidized cytochrome may be due to stronger bonds formed between the ferric iron and the endogenous ligands.

The midpoint potential of cytochrome *c*-549 measured here is similar to that determined for cytochrome *c*-551 of *Chromatium vinosum* (-299 mV), which has similar properties [27], but is somewhat lower than that reported by Holton and Myers [3] for *A. nidulans* (-260 ± 20 mV). They observed several peaks of cytochrome *c*-549 eluting from DEAE-cellulose columns and used for analysis a fraction that eluted before phycocyanin [2], whereas we have used a cytochrome fraction that elutes after phycocyanin. Unfortunately, the source of the apparent heterogeneity in the cytochrome, indicated also by the varying ease of its extraction from cells or membranes [5], remains unclear.

Cytochrome *c*-549 has been shown to catalyze in vitro Photosystem I-dependent cyclic photophosphorylation [9]. A Photosystem I function for cytochrome *c*-549 has also been suggested by a study in which the spectral distribution of light during growth altered the Photosystem I/Photosystem II ratio: cytochrome *c*-549 abundance paralleled that of Photosystem I and not Photosystem II [25]. This argues against a function related to Photosystem II, in spite of the cytochrome's presence in Photosystem II extracts. The midpoint potential of cytochrome *c*-549 is sufficiently low as to enable it to reduce cytochrome *b*-563 or plastoquinone, and is consistent with roles in cyclic photophosphorylation or in Photosystem I-dependent anoxygenic photosynthesis [28].

Acknowledgements

The authors thank T. Vänngård and R. Aasa for helpful discussion. This work was supported by a grant from the Swedish Natural Science Research Council. C.W.H. is the recipient of a fellowship from the American-Scandinavian Foundation.

References

1 Holton, R.W. and Myers, J. (1963) Science 142, 234.
2 Holton, R.W. and Myers, J. (1967) Biochim. Biophys. Acta 131, 362–374.
3 Holton, R.W. and Myers, J. (1967) Biochim. Biophys. Acta 131, 375–384.
4 Pulich, W. (1977) J. Phycol. 13, 40–45.
5 Ho, K.K., Ulrich, E.L., Krogmann, D.W. and Gomez-Lojero, C. (1979) Biochim. Biophys. Acta 545, 236–248.
6 Stewart, A.C. and Bendall, D.S. (1981) Biochem. J. 194, 877–887.
7 Alam, J., Sprinkle, J., Hermodson, M.A. and Krogmann, D.W. (1984) Biochim. Biophys. Acta 766, 317–321.
8 Bricker, T.M. and Sherman, L.A. (1984) Arch. Biochem. Biophys. 235, 204–211.
9 Kienzl, P.F. and Peschek, G.A. (1983) FEBS Lett. 162, 76–80.
10 Ellfolk, N., Rönnberg, M., Aasa, R., Andréasson, L.-E. and Vänngård, T. (1983) Biochim. Biophys. Acta 743, 23–30.
11 Wood, Paul M. (1984) Biochim. Biophys. Acta 768, 293–317.
12 Lagenfelt, G., Hansson, Ö. and Andréasson, L.-E. (1987) Acta Chem. Scand. B41, 123–125.
13 Dutton, P.L. (1978) Methods Enzymol. 54, 411–435.
14 O'Reilly, J.E. (1973) Biochim. Biophys. Acta 292, 509–515.
15 Fultz, M.L. and Durst, R.A. (1982) Anal. Chim. Acta 140, 1–18.
16 Blumberg, W.E. and Peisach, J. (1971) Adv. Chem. Ser. 100, 271–291.
17 Taylor, C.P.S. (1977) Biochim. Biophys. Acta 491, 137–149.
18 Bohan, T.L. (1977) J. Mag. Res. 26, 109–118.
19 Bergström, J. and Vänngård, T. (1982) Biochim. Biophys. Acta 682, 452–456.
20 Babcock, G.T., Widger, W.R., Cramer, W.A., Oertling, W.A. and Metz, J.G. (1985) Biochemistry 24, 3636–3645.
21 Ikeda, M., Lizuka, T., Takao, H. and Hagihara, B. (1974) Biochim. Biophys. Acta 336, 15–24.
22 Moura, I., Teixeira, M., Huynh, B.H., LeGall, J. and Moura, J.J.G. (1988) Eur. J. Biochem. 176, 365–369.
23 Haser, R., Pierrot, M., Frey, M., Payan, F., Astier, J.P., Bruschi, M. and LeGall, J. (1979) Nature 282, 806–810.
24 Cohn, C.L., Sprinkle, J.R., Alam, J., Hermodson, M., Meyer, T. and Krogmann, D.W. (1989) Arch. Biochem. Biophys. 270, 227–235.
25 Matsuura, K. and Fujita, Y. (1986) Plant Cell Physiol. 27, 685–691.
26 Stellwagen, E. (1978) Nature 275, 73–74.
27 Gray, G.O., Gaul, D.F. and Knaff, D.B. (1983) Arch. Biochem. Biophys. 222,78–86.
28 Padan, E. and Cohen, Y. (1982) in The Biology of Cyanobacteria (Carr, N.G. and Whitton, B.A., eds.), pp. 215–235, Blackwell Scientific Publications, Oxford.