Biochimica et Biophysica Acta, 936 (1988) 61–66
Elsevier

BBA 42857

Resonance Raman spectroscopy indicates a lysine as the sixth iron ligand in cytochrome *f*

Dan J. Davis, Melinda K. Frame and Dale A. Johnson

University of Arkansas, Fayetteville, AR (U.S.A.)

(Received 10 June 1988)

Key words: Cytochrome *f*; Raman spectroscopy; Heme iron ligand

The resonance Raman spectrum of turnip cytochrome *f* is similar to that of other *c*-type cytochromes with the exception of a single band at 1532 cm^{-1} which is shifted to lower frequency relative to its position (1542–1545 cm^{-1}) in other *c*-type cytochromes. Comparison of the frequency of this band with that in alkylated cytochrome *c* at high pH suggests that the sixth heme iron ligand in cytochrome *f* is a deprotonated lysine amino group rather than a methionine sulfur. Comparison of the amino-acid sequences of cytochromes *f* and c_1 suggests lysine-145 as a likely candidate for the sixth heme iron ligand in cytochrome *f*.

Introduction

Cytochrome *f* is an integral membrane protein associated with the photosynthetic electron transport chain of the inner chloroplast membrane [1–3]. It is a component of the cytochrome b_6/f complex which has similarities to the cytochrome b/c_1 complex present in the membranes of mitochondria and photosynthetic bacteria [4,5]. In their respective electron transport chains, cytochromes *f* and c_1 function to reduce small peripheral proteins, with cytochrome *f* serving as the reductant for plastocyanin (or a small cytochrome c-553 in some algae and cyanobacteria), an acidic protein, and cytochrome c_1 as the reductant for cytochrome *c*, a basic protein. Amino-acid sequences are now known for six cytochromes *f* [6–11] and five cytochromes c_1 [12–16].

Since the early 1970s, resonance Raman spectroscopy has provided an increasingly useful tool for examination of a number of features of heme proteins such as oxidation state, spin state, and axial ligation [17–19]. A resonance Raman spectrum was presented in 1975 [20] for a 'cytochrome *f*' from *Spirulina platensis*, a cyanobacterium. Based on the similarity between its resonance Raman spectra and that of mitochondrial cytochrome *c*, it was suggested that the fifth and sixth ligands to the heme iron are histidine and methionine [20]. However, it now seems clear from the description of the protein and its preparation [20,21] that the protein used was not a true cytochrome *f*, but rather cytochrome *c*-553. For this reason, we will refer to this protein as 'cytochrome *f* (*c*-553)'. Such a misidentification is not surprising, as the distinction between the small, peripheral cytochrome *c*-553 and the membrane-associated cytochrome *f* was not clearly made in such systems until the late 1970's [22,23] and literature reports involving cytochrome *c*-553 often referred to it as cytochrome *f* into the 1980's. We now report on the resonance Raman spectrum of an authentic cytochrome *f*, showing it to be different from that previously reported for mitochondrial cytochrome *c* and the 'cytochrome *f* (*c*-553)'.

Correspondence: D.J. Davis, Dept. of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701, U.S.A.

Materials and Methods

The monometric cytochrome *f* was purified from turnip greens by a method similar to that described by Gray [24]. The protein had a UV-visible absorption spectrum identical to that reported by Gray for charlock cytochrome *f* [24]. Analysis by SDS gel electrophoresis and gel permeation chromatography indicated it to be a monomeric cytochrome *f* with a molecular weight of 29000.

The turnip cytochrome *f* was concentrated to 0.1 mM using Amicon Centriprep centrifugation concentration cells with a molecular weight cut-off of 10000. The protein was in a 40 mM sodium phosphate, (pH 7.5) buffer. A crystal of dithionite was added to assure complete reduction. After filtration through a 45 μm Millipore filter, a 50 μl sample was placed in a glass capillary tube and the resonance Raman spectrum was recorded using the 514.5 and 488 nm lines from a Coherent Radiation Innova 90 Ar^+ ion laser. Spectra were collected using a Spex Triplemate Spectrograph (90° scattering geometry) and a Tracor Northern TN-6500 Rapid Scan Spectrometer system with a TN-6144 intensified diode array detector. Laser power at the sample was 15–20 mW. Spectra were digitally corrected for scattering from the buffer and dark current. The samples were illuminated for 15–30 s and accumulations of 20–30 spectra were averaged to improve the signal-to-noise ratio. The Raman shift scale was calibrated using indene as a standard [36]. Spectra were plotted using the Grapher™ software package (Golden Software).

Results and Discussion

The resonance Raman spectrum of reduced turnip cytochrome *f* was recorded using 514.5 or 488 nm laser lines. The 514.5 nm line lies well within the β absorption band of the heme where resonance enhancement of Raman scattering would be expected and has been observed with other cytochromes [17–19]. The 488 nm line lies in the region between the Soret and β bands.

Preliminary resonance Raman spectra collected on turnip cytochrome *f* showed the presence of several peaks superimposed on a large broad envelope of background scattering or luminescence.

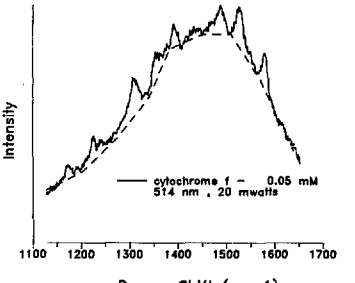


Fig. 1. Resonance Raman spectrum of turnip cytochrome *f*. The solid line represents the initial spectrum obtained as described in the text. The dashed line represents the estimated background scattering which was not removed by filtration through a Millipore filter.

Although the size of this envelope was not decreased by the inclusion of 0.1 M KI, its size was considerably diminished but not totally eliminated by passage of the sample through a 45 μm Millipore filter. The filter showed no evidence of retention of cytochrome *f* which might have resulted from protein aggregation at high initial protein concentration.

The resonance Raman spectrum of a filtered sample of turnip cytochrome *f* (about 50 μl) obtained with 514.5 nm excitation is shown in Fig. 1. The dashed line is an estimation of the background scattering which remained after filtration. Subtraction of this background scattering from the recorded spectrum results in the resonance Raman spectrum shown in Fig. 2. Peak positions were determined by taking the derivative of the top spectrum, a procedure which permits more accurate estimation of peak positions when a background of this type is present. When excited with 488 nm light, no resonance-enhanced Raman bands were observed.

The positions of the peaks in the resonance Raman spectrum are summarized in Table I. For comparison, peak positions for the resonance Raman spectra of mitochondrial cytochrome *c* [20], alkylated cytochrome *c* at pH 7.0 and 10.7 [20], 'cytochrome *f* (*c*-553)' [20], and cytochrome c_1

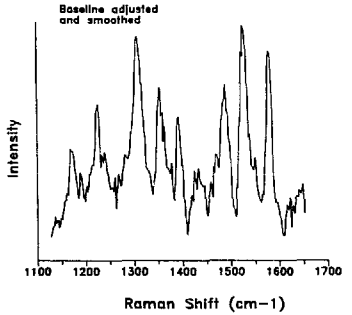


Fig. 2. Corrected and smoothed resonance Raman spectrum of cytochrome *f*. The background scattering indicated in Fig. 1 was digitally subtracted and smoothed using cubic spline fit.

[25] are also presented in Table I. With one exception, the positions of the resonance Raman peaks of turnip cytochrome *f* are similar to those of cytochrome *c*, alkylated cytochrome *c* (pH 7.0), and 'cytochrome *f* (*c*-553)'. The peak commonly used as an oxidation state marker [17–19] is located at 1361 cm^{-1}, consistent with the reduced state of the protein. The band characteristic of the spin state [17–19] located at 1581 cm^{-1} demonstrates a low-spin Fe(II) consistent with EPR studies of cytochrome *f* [26]. Polarization studies showed the bands at 1312 and 1581 cm^{-1} to be anomalously or inversely polarized (data not shown) as has been observed for other cytochromes [20,27].

The single peak which appears to be shifted in the resonance Raman spectrum of turnip cytochrome *f* relative to the other cytochromes shown is the 1532 cm^{-1} peak, which is 10–13 cm^{-1} lower than for the other cytochromes (Table I). This peak has been previously demonstrated to be sensitive to the nature of the sixth ligand to the heme iron [20]. Table I shows that a shift to similar frequency is observed when alkylated cytochrome *c* is shifted from pH 7.0 to 10.7. This pH shift has been related to the binding of a deprotonated lysine amino group as the sixth ligand to the heme iron [28,29].

Based on the frequency of the 1532 cm^{-1} peak, we would suggest that the normal sixth heme iron ligand in cytochrome *f* is also a lysine amino group, rather than a methionine. This is consistent with the lack of a 695 nm absorption band in the spectrum of oxidized cytochrome *f* [24,26,30]. The 695 band which is present in the spectra of oxidized cytochrome *c* [31] and cytochrome c_1 [32,33] is thought to be characterised of a heme iron with methionine as the sixth ligand. It is also consistent with the magnetic circular dichroism data of

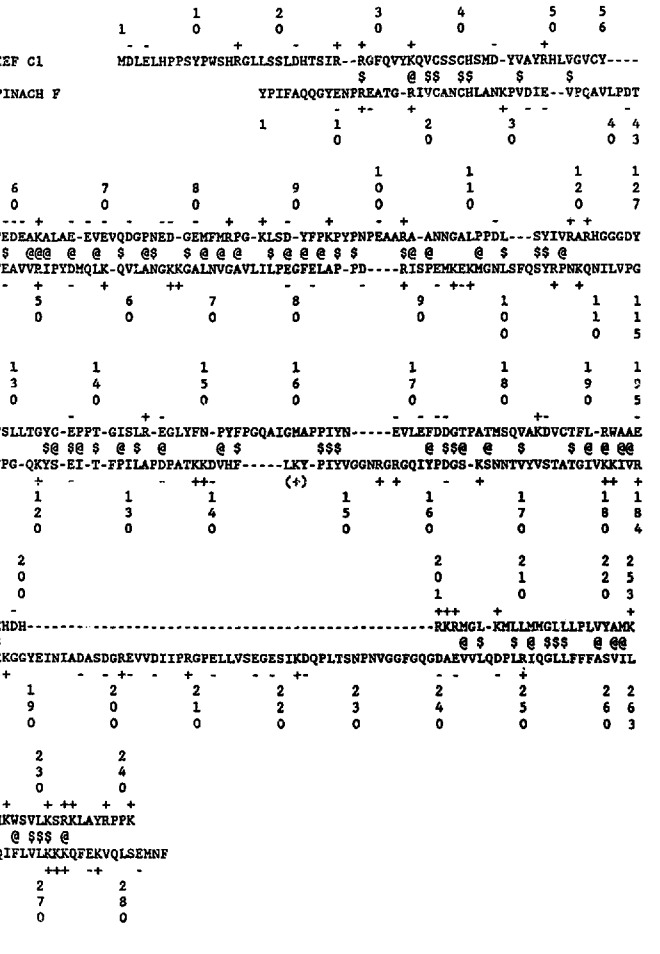
TABLE I

RAMAN SHIFTS (cm^{-1}) FOR CYTOCHROMES

Cytochrome *f* (This work)	Cytochrome *c* [20]	Alkylated cytochrome *c* (pH 7.0) [20]	Alkylated cytochrome *c* (pH 10.7) [20]	*S. platensis* cytochrome *f* (*c*-553) [20]	Cytochrome c_1 [25]
(1616) [a]	1621	1623	n.r. [b]	1622	1625
1581	1584	1585	1583	1586	1588
1532	1545	1542	1533	1545	1540
1490	1493	1492	1489	1493	1495
1398	1401	1399	1398	1400	1400
1361	1363	1361	1359	1361	1363
1312	1313	1311	1310	1312	1315
1226	1228	1228	1225	1229	1232
1173	1174	1172	1172	1176	1176
(1121) [a]	1130	1127	1124	1131	n.r. [b]

[a] The exact position of this peak is difficult to determine from the spectrum shown in Fig. 2 due to the signal-to-noise ratio. The value given should be considered only as an approximation.

[b] Not reported.


BEEF C1
SPINACH F
-TEDEAKALAE-EVEVQDGPNED-GEMFMRPG-KLSD-YFPKPYPNPEAARA-ANNGALPPDL---SYIVRARHGGGDY
VFEAVVRIPYDMQLK-QVLANGKKGALNVGAVLILPEGFELAP-PD----RISPEMKEKMGNLSFQSYRPNKQNILVPG
VFSLLTGYG-EPPT-GISLR-EGLYFN-PYFPGQAIGMAPPIYN-----EVLEFDDGTPATMSQVAKDVGTFL-RWAAE
PVPG-QKYS-EI-T-FPILAPDPATKKDVHF-----LKY-PIYVGGNRGRGQIYPDGS-KSNNTVYVSTATGIVKKIVR
PEHDH----------RKRMGL-KMLLMMGLLLPLVYAMK
KEKGGYEINIADASDGREVVDIIPRGPELLVSEGESIKDQPLTSNPNVGGFGQGDAEVVLQDPLRIQGLLFFFASVIL
RHKWSVLKSRKLAYRPPK
AQIFLVLKKKQFEKVQLSEMNF

Siedow et al. [26] which suggested that a lysine might be the sixth heme iron ligand in cytochrome *f*. Further evidence that methionine cannot be the sixth iron ligand in cytochrome *f* comes from comparison of the six available amino-acid sequences [6–11]. The only conserved methionine residue is located at position 283 in the amino-acid sequence. This position is in the region exposed to the outer surface of the membrane, while the heme binding domain is located on the inner surface of the membrane [6].

In an attempt to predict regions on cytochrome *f* and c_1 which might be involved in binding of plastocyanin and cytochrome *c*, respectively, a possible alignment of cytochrome *f* and c_1 amino-acid sequences has been presented [34]. The proposed alignment showed 17% homology between the two proteins based on strictly conserved positions and 30% if conservative replacements were considered as identical. Three regions were detected which were negatively charged in cytochrome c_1 but positively charged in cytochrome *f* with the change in charge distribution being suggested to account for the binding of either positively charged cytochrome *c* or negatively charged plastocyanin [34].

Fig. 3 shows a modified form of this alignment in which the sequence of bovine heart cytochrome c_1 and spinach cytochrome *f* are compared. (The sequence of turnip cytochrome *f* has not yet been determined.) The homology between the two proteins shown is about 32% when conservative replacements are considered identical. As cytochrome c_1 contains methionine as the sixth iron ligand [32,33] and the methionine at position 160 is the only conserved methionine in the five cytochrome c_1 sequences known to date [12–16], it seems reasonable to assume that this residue must be the sixth ligand in cytochrome c_1. (The recent studies of Mukai et al. [35] would appear to discount the suggestion that a cysteine, rather than a histidine, is in fifth ligand in cytochrome c_1 [33]. In addition, the only cysteine residues which are conserved in cytochrome c_1 sequences [12–16] are those involved in covalent linkage of the heme to the protein.) The proposed alignment brings a lysine at position 145 in the sequence of cytochrome *f* into alignment with the methionine at position 160 in cytochrome c_1. Although the sequences of the two proteins do not show a high degree of homology in this region, it is noted that the phenylalanine at position 143 and the tripeptide, proline-isoleucine-tyrosine, at positions 147–149 of cytochrome *f* are aligned with identical residues in cytochrome c_1. We would thus propose that the lysine at position 145 in cytochrome *f* is a strong candidate for the sixth heme iron ligand in cytochrome *f*.

For a lysine to serve as a ligand to the heme iron, it must be in the deprotonated state, even at pH 7.0. This would require a considerable shift to lower pK_a for the lysine group serving as the ligand. One environmental effect which might produce such a shift would be placing the lysine in a hydrophobic environment where the deprotonated state would be stabilized. Of the 20 lysines present in the cytochrome *f* sequence shown in Fig. 3 only the lysine at position 145 appears to be located in a highly hydrophobic region of the sequence with two hydrophobic residues preceding it and five following it. The lysine at position 145 in the sequence of cytochrome *f* is thus in an environment which might allow it to be easily deprotonated such hat it might serve as the sixth heme iron ligand.

Acknowledgements

The authors wish to thank Ms. Bridgitte Donohue for her assistance in the preparation of cytochrome *f*. This research was supported by grants from the USDA Competitive Grants Program (87-CRCR-1-2310) and the Department of Defense-University Research Instrumentation Program (N00014-85-G-0002).

Fig. 3. Comparison of amino-acid sequences of bovine heart cytochrome c_1 and spinach cytochrome *f*. The sequences of bovine heart cytochrome c_1 and spinach cytochrome *f* are from 12 and 8, respectively. The numbering system above the sequences refers to cytochrome c_1 and that below the sequences to cytochrome *f*. Charged R-groups are indicated by + and − signs above and below the sequences for the respective proteins. Areas of homology are indicated between the two sequences with a $, indicating an exact match of the aligned amino acids, and an @, indicating conservative replacements.

References

1 Bendall, D.S., Davenport, H.E. and Hill, R. (1971) Methods Enzymol. 23, 327–344.
2 Cramer, W.A. and Whitmarsh, J. (1977) Annu. Rev. Plant Physiol. 28, 133–72.
3 Bendall, D.S. (1982) Biochim. Biophys. Acta 683, 119–151.
4 Hauska, G., Hurt, E., Gabellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta 726, 97–133.
5 Barber, J. (1984) Trends Biochem. Sci. 9, 209–211.
6 Wiley, D.L., Auffret, A.D. and Gray, J.C. (1984) Cell 36, 555–562.
7 Willey, D.L., Howe, C.J., Auffret, A.D., Bowman, C.M., Dyer, T.A. and Gray, J.C. (1984) Mol. Gen. Genet. 194, 416–422.
8 Alt, J. and Herrmann, R.G. (1984) Curr. Genet. 8, 551–557.
9 Wu, N., Cote, J.C. and Wu, R. (1986) Gene 50, 271–278.
10 Tyagi, A.K. and Herrmann, R.G. (1986) Curr. Genet. 10, 481–486.
11 Ko, K. and Straus, N.A. (1987) Nucleic Acids Res. 15, 2391.
12 Wakabayashi, S., matsubara, H., Kim, C.H. and King, T.-E. (1982) J. Biol. Chem. 257, 9335–9344.
13 Sadler, I., Suda, K., Schatz, G., Kaudewitz, F. and Haid, A. (1984) EMBO J. 3, 2137–2143.
14 Gabellini, N. and Sebald, W. (1986) Eur. J. Biochem. 154, 569–579.
15 Romisch, J., Tropschug, M., Sebald, W. and Weiss, H. (1987) Eur. J. Biochem. 164, 111–115.
16 Nishikimi, M., Suzuki, H., Ohta, S., Sakurai, T., Shimomura, Y., Tanaka, M., Kagawa, Y. and Ozawa, T. (1987) Biochem. Biophys. Res. Commun. 145, 34–39.
17 Carey, P.R., (1982) Biochemical Applications of Raman and Resonance Raman Spectroscopies, pp. 99–153, Academic Press, New York.
18 Tu, A.T. (1982) Raman Spectroscopy in Biology: Principles and Applications, pp. 317–368, J. Wiley & Sons, New York.
19 Spiro, T.G. (1983) in Iron Porphyrins, Part II (Lever, A.B.P. and Gray, H.B., eds.), pp. 89–159, Addison-Wesley, Reading, MA.
20 Kitigawa, T., Kyogoku, Y., Iizuka, T., Ikeda-Saito, M. and Yamanaka, T. (1977) J. Biochem. 78, 719–728.
21 Yamanaka, T., Fukumori, Y. and Wada, K. (1978) Plant Cell Physiol. 19, 117–126.
22 Wood, P.M. (1977) Eur. J. Biochem. 72, 605–612.
23 Wood, P.M. (1978) Eur. J. Biochem. 87, 9–19.
24 Gray, J.C. (1978) Eur. J. Biochem. 82, 133–141.
25 Adar, F. and Erecinska, M. (1974) Arch. Biochem. Biophys. 165, 570–580.
26 Siedow, J.N., Vickery, L.E. and Palmer, G. (1980) Arch. Biochem. Biophys. 203, 101–107.
27 Strekas, T.C. and Spiro, T.G. (1972) Biochim. Biophys. Acta 278, 188–192.
28 Kihara, H., Hon-Nami, K. and Kitigawa, T. (1978) Biochim. Biophys. Acta 532, 337–346.
29 Smith, H.T. and Millett, F.S. (1980) Biochemistry 19, 1117–1120.
30 Ho, K.H. and Krogmann, D.W. (1980) J. Biol. Chem. 255, 3855–3861.
31 Dickerson, R.E. and Timkovich, R. (1975) in The Enzymes (Boyer, P.D., ed.), 3rd Edn., Vol. 11, pp. 397–547, Academic Press, New York.
32 Kaminsky, L.S., Chiang, Y.-L. and King, T.E. (1975) J. Biol. Chem. 250, 7280–7287.
33 Tervoort, M.J. and Van Gelder, B.F. (1983) Biochim. Biophys. Acta 722, 137–142.
34 Davis, D.J. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), 2, 573–576, Martinus Nijhoff, Dordrecht.
35 Mukai, K., Miyazaki, T., Wakabayashi, S., Kuramitsu, S. and Matsubara, H. (1985) J. Biochem. 1417–1425.
36 Hendra, P.J. and Loader, E.J. (1968) Chem. Ind. 718.