Resonance Raman spectroscopy of single crystals of bacterial cytochrome c_4

L. Sawyer and R.E. Hester+

Department of Applied Chemical Sciences, Napier College, Colinton Road, Edinburgh EH10 5DT, Scotland and *+Department of Chemistry, University of York, Heslington, York Y01 5DD, England*

Received 9 July 1984

The application of resonance Raman spectroscopy to single crystals of cytochrome c_4 from *Pseudomonas aeruginosa* is described. The technique is shown to be a suitable method of distinguishing between the oxidised and reduced forms of the protein. This is particularly useful when the crystal is to be used for X-ray diffraction data collection.

Cytochrome c_4 *Oxidation state Resonance Raman spectroscopy Single crystal spectroscopy*

1. INTRODUCTION

X-ray crystallography has furnished much of the framework on which our understanding of the relationship between the structure and function of protein molecules is based. Recent advances in the technique, notably the use of synchrotron radiation and area detectors [1,2], have allowed data for structures as large as whole virus particles to be collected efficiently [3,4]. The attainment of high-resolution data coupled with the improvement in the model structure obtained by least-squares refinement techniques [5,6] has resulted in models of proteins which are sufficiently accurate to allow detailed examination of the subtle movements which occur during binding of a substrate molecule [7–9] or change in oxidation state [10,11]. Although the binding of a substrate or inhibitor may lead to a dramatic change in conformation [12], often there is no clear indication of the occupation of the active site until X-ray diffraction data have been collected, processed and an electron density map calculated. This is the case even when the diffraction pattern shows small, visible differences between native and substrate-soaked crystals which can result from changes in mother liquor and are not necessarily associated with binding at the active site.

What is needed, therefore, is a quick method of examining the crystals before they are X-rayed to determine whether structural changes have taken place. Authors in [7] have observed the change in birefringence of a crystal as it reacts with substrate but a change does not occur in every case and, if the crystals are highly coloured, as is the case with haem proteins, then such a technique is inapplicable. The dichroism changes in haemoglobin accompanying oxygenation and reduction have been reported [13] and the colour change observed under the microscope when crystals of cytochrome c_{551} are reduced have been described [10]. Since the visible spectrum of a haem protein changes markedly on reduction, a micro-spectroscopic technique can also be used [14]. However, in our work the crystals for X-ray studies are sufficiently large that they appear black and consequently an alternative method was investigated.

We wish to report the use of resonance Raman (RR) spectroscopy to distinguish between the oxidised and reduced crystals of a dihaem, bacterial cytochrome c_4 (M_r 19600) before X-ray photographs were taken. Raman spectroscopy may be of wider application in this respect than has hitherto been generally appreciated.

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

2. EXPERIMENTAL

Cytochrome c_4 (E_m = 240, 300 mV) from *Pseudomonas aeruginosa* NCTC 10332 was prepared as described in [13] as modified in [16]. Crystals of the oxidised form were obtained as described [17] and transferred to a solution of 2 M ammonium sulphate–50 mM acetate buffer (pH 6.0) for storage. They are stable indefinitely between 4 and 22°C under these conditions.

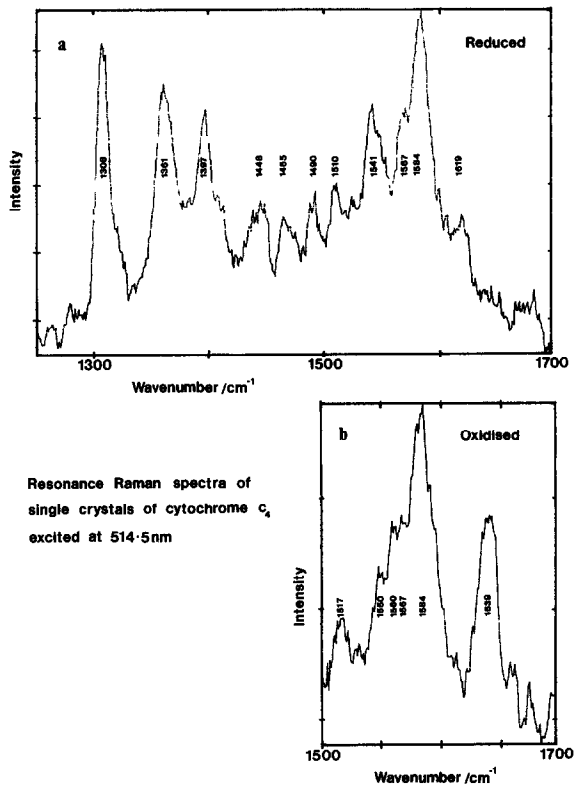
A wedge-shaped crystal of maximum dimensions 0.8 × 0.4 × 0.2 mm was transferred to a 2 ml portion of mother liquor which had been purged for 1 h with a stream of O_2-free nitrogen in a glovebox. This mother liquor was 5 mM in sodium ascorbate (Sigma, Poole) (E_0' = 60 mV) [18]. After 7 h soaking, no visible cracking had occurred and no change in the colour of the almost black crystal could be seen. The crystal was sealed under nitrogen in a 1 mm diameter, thin-walled glass capillary tube (Pantak, Windsor). In a parallel experiment, another crystal of similar size was soaked in 2 mM potassium ferricyanide (E_0' = 420 mV) [19] for the same time and sealed in a second capillary. This 'oxidised' crystal had two small satellites which became detached during mounting and were also sealed in the capillary a short distance from the main crystal. These served to optimise the conditions for the Raman measurements. Both capillaries were stored overnight at room temperature before the RR experiment.

The RR spectra were measured using a c.w. Ar^+ laser at 514.5 nm (Model CR4; Coherent Inc., Palo Alto, CA) together with a Spex Model 1403 Raman spectrometer, equipped with a UVISIR sample compartment, a cooled RCA 31034A-02 photomultiplier coupled to Spex photon-counting electronics and a SCAMP minicomputer for instrument control and data processing.

In order to position the crystal accurately in the laser beam, a stereo-microscope was arranged over the sample compartment, with great care being taken to avoid direct transmission of the laser beam into the microscope. In a preliminary experiment, the laser power measured at the crystal was approx. 12.5 mW and this instantly destroyed one of the satellite crystals. A neutral density filter (*A* 2.0) was used to reduce the power to about 0.125 mW and the second satellite crystal seemed quite stable when illuminated. Subsequently, the main 'oxidised' crystal was aligned in the laser beam such that the surface in contact with the glass wall of the capillary was facing the collection optics, thus maximising the RR signal strength.

The spectrum of the oxidised crystal was recorded from 1500–1700 cm^{-1} at a scan-speed of 1 $cm^{-1} \cdot s^{-1}$ with the slits adjusted to give a spectral resolution of around 10 cm^{-1}. Nineteen scans were accumulated and co-added before being subjected to a 7-point quadratic smoothing function, and then plotted. Having seen that the spectrum was broadly similar to what was expected, the spectrum of the 'reduced' (i.e., unoxidised) crystal was scanned 7 times between 1300–1700 cm^{-1} with the same instrument settings. The combined spectrum was smoothed as before.

X-ray photographs (2° oscillation) were recorded with Cu-K_α radiation for 15 h on each crystal some 72 h after the RR experiment. Both diffraction patterns extended to at least 0.18 nm resolution.



Resonance Raman spectra of single crystals of cytochrome c_4 excited at 514·5nm

Fig.1. Resonance Raman spectra of (a) reduced and (b) oxidised cytochrome from *Pseudomonas aeruginosa* excited at 514.5 nm. Details of the conditions used are in the text.

3. RESULTS

The spectra obtained are shown in fig.1 with the wavenumber values of the most distinct peaks added. These show many of the features previously reported in the RR spectra of solutions of cytochrome *c* [20–22] measured with 1000-times greater laser power and a narrower bandwidth. The X-ray diffraction pattern obtained from the 'reduced' cytochrome crystal was compared with that of the oxidised form. The hexagonal cell parameters determined were:

	Oxidised (nm)	Reduced (nm)
$a = b$	6.24(1)	6.24(3)
c	17.42(1)	17.35(8)

4. DISCUSSION

In order to be sure that the observed differences do indeed represent the change from ferricytochrome to ferrocytochrome, it is necessary to consider the RR spectral assignments for haem proteins and other metallo porphyrins already reported [18–20]. The RR spectrum excited at 514.5 nm arises mostly from the in-plane bending mode of the methine C–H and the stretching modes of the C–C, C=C and C–N bonds since the $\pi - \pi^*$ transition of the porphyrin ring, which gives rise to the β-band ($\lambda_{max} = 523$ nm) in the visible spectrum of cytochrome *c*, is polarised in the plane of the ring and thus will selectively enhance these modes. Six of these bands, designated A–F, have been identified in [20] as distinguishing between oxidation and/or spin state in haem-containing molecules and the influence of the protein upon these bands has been found to be insignificant [21]. Since no change in spin state is involved in this case (high-potential cytochromes *c*, like c_4, are low spin in both oxidised and reduced forms), only 4 of the 6 need to be considered in detail. Owing to the random orientation of the crystals in the laser beam, the non-random nature of the haem orientation in the crystals and the polarisation-sensitive nature of some of the bands, only the depolarised resonance bands were considered reliable indicators of the oxidation state without performing a full series of experiments to determine the depolarisation ratio. In fig.1a, all of the bands which we observe correspond to bands reported inter alia in [20] and [22] for solutions of reduced cytochrome *c*, with the exception of the band at 1510 cm^{-1}. In particular the marker bands which we observe at 1361, 1541, 1490 and 1619 cm^{-1} correspond to bands A, B, E and F at 1363 (polarised), 1547 (depolarised), 1490 (polarised) and 1623 cm^{-1} (depolarised) for dithionite-reduced cytochrome *c* [20]. In contrast, when our spectrum for the 'reduced' form is compared with those of the oxidised protein (A, B, E and F reported at 1374, 1562, 1502 and 1636 cm^{-1} respectively), it is clear that our crystals are indeed in the reduced state. Fig.1b shows our spectrum of the crystal in oxidising conditions and peaks at 1560 and 1639 cm^{-1}, which correspond to the bands B and F already mentioned, are typical of ferricytochrome. It is the shift of the depolarised band, F, which gives the most convincing indication of the oxidation state of the Fe atom in the cytochrome c_4 crystals. A recent report of the RR spectrum excited at the Soret band, of the dihaem cytochrome c_{554} from *Nitrosomonas europaea* shows that there appears to be a 5-coordinate Fe atom in the oxidised form [23]. Our data for the oxidised crystal are insufficient to allow such a conclusion.

Inspection of the X-ray diffraction patterns showed that the two crystals were isomorphous and that small intensity changes might exist. However, as pointed out above, such changes are not necessarily indicative of the oxidation-state change.

We believe that this is the first application of RR spectroscopy for screening protein crystals before an X-ray diffraction experiment. In this respect, a haem protein, such as a cytochrome *c*, is an excellent choice because of the detailed spectral interpretation already available and the fact that the RR spectral data are specific to the haem part of the molecule. RR spectroscopy with haem-containing molecules has usually been done with solution samples at concentrations around 1 mM and with laser powers of some 100 mW. It seems from our present results that the increased sample concentration (~0.2 M) can sensibly be compensated by decreased laser power, provided that a suitable scattering geometry can be achieved through sample alignment. Probably a back-scattering sample illumination and light collection arrangement would further improve the results which were

obtained in this work with conventional 90° optics. However, we have shown that with the laser power used the crystal does not deteriorate and, if sufficient scans with a narrower spectral bandwidth were accumulated, a higher resolution spectrum could result. This would allow study of the partial reduction of the dihaem cytochrome c_4.

The use of a laser flash to initiate a reaction in a protein crystal has been described for myoglobin in the context of time-resolved diffraction measurements [24]. The experiment described in the present paper affords an excellent way of monitoring the state of a crystal throughout the time X-ray data are being collected. Not only are the oxidation states of haem proteins amenable to such observation but also the binding of any chromophoric substrate can be checked provided it has been previously well characterised.

ACKNOWLEDGEMENTS

We thank Mr R.B. Girling for his skillful experimental assistance and the SERC for financial support.

REFERENCES

[1] Greenhough, T.J. and Helliwell, J.R. (1983) Prog. Biophys. Mol. Biol. 41, 67–124.
[2] Helliwell, J.R. (1982) Nucl. Instrum. Methods 201, 153–174.
[3] Abad-Zapatero, C., Abdal-Meguid, S.S., Johnson, J.E., Leslie, A.G.W., Rayment, I., Rossmann, M.G., Suck, D. and Tsukihara, T. (1980) Nature 286, 33–39.
[4] Usha, R., Johnson, J.E., Moras, D., Thierry, J.C., Fourme, R. and Kahn, R. (1984) J. Appl. Crystallogr. 17, 147–153.
[5] Hendrickson, W.H. and Konnert, J.H. (1980) in: Biomolecular Structure Function, Conformation and Evolution (Srinivasan, R. ed.) vol. 1, pp. 43–57, Pergamon, Oxford.
[6] Isaacs, N.W. (1982) in: Computational Crystallography (Sayre, D. ed.) pp. 381–397, Clarendon, Oxford.
[7] James, M.N.G., Sielecki, A.R., Brayer, G.D., Delbaere, L.T.J. and Bauer, C.-A. J. Mol. Biol. 144, 43–88.
[8] James, M.N.G., Hsu, I.-N., Hofmann, T. and Sielecki, A.R. (1981) in: Structural Studies of Molecules of Biological Interest (Dodson, G. et al. eds) pp. 350–389, Clarendon, Oxford.
[9] Baldwin, J. and Chothia, C. (1979) J. Mol. Biol. 129, 175–220.
[10] Matsuura, Y., Takano, T. and Dickerson, R.E. (1982) J. Mol. Biol. 156, 389–409.
[11] Takano, T. and Dickerson, R.E. (1981) J. Mol. Biol. 153, 95–115.
[12] Anderson, C.M., Zucker, F.H. and Steitz, T.A. (1979) Science 204, 375–380.
[13] Perutz, M.F. (1953) Proc. Roy. Soc. B 141, 69–71.
[14] Day, P., Smith, D.W. and Williams, R.J.P. (1967) Biochemistry 6, 3747–3750.
[15] Ambler, R.P. and Wynn, M. (1972) Biochem. J. 131, 485–498.
[16] Coulson, A.F.W. and Oliver, R.K. (1979) Biochem. J. 181, 159–169.
[17] Sawyer, L., Jones, C.L., Damas, A.M.A., Gould, R.O., Harding, M.M. and Ambler, R.P. (1982) J. Mol. Biol. 153, 831–835.
[18] Clark, W.M. (1960) Oxidation-Reduction Potentials of Organic Systems, Williams and Wilkins, Baltimore, MD.
[19] O'Reilly, J.E. (1973) Biochim. Biophys. Acta 292, 509–515.
[20] Spiro, T.G. and Strekas, T.C. (1974) J. Am. Chem. Soc. 96, 338–345.
[21] Spiro, T.G. and Burke, J.M. (1976) J. Am. Chem. Soc. 98, 5482–5489.
[22] Cartling, B. (1983) Biophys. J. 43, 191–205.
[23] Andersson, K.K., Babcock, G.T. and Hooper, A.B. (1984) FEBS Lett. 170, 331–334.
[24] Bartunik, H.D. (1983) Nucl. Instrum. Methods 208, 523–533.