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Detection of a near infra-red absorption band of ferrohaem a_3 in cytochrome *c* oxidase

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We have detected weak absorption bands in the near infra-red region of reduced mammalian cytochrome *c* oxidase, analogous to those that we have recently reported to be present in the bacterial cytochrome *o* (Ingledew, W.J., Bacon, M. and Rich, P.R. (1992) FEBS Lett. 305, 167–170). The major band is centred at 784 nm and has an $\varepsilon_{\mathrm{mM}^{-1}\cdot\mathrm{cm}^{-1}}$ of around 0.1. It is shifted to 760 nm in the carbon monoxide compound and is absent in the reduced cyanide complex. We attribute it to a charge transfer band of ferrohaem a_3, equivalent to the 'band III' or 'conformational band' of haemoglobin.

Cytochrome *c* oxidase; Carbon monoxide; Cyanide; Haem a_3; Spectroscopy

1. INTRODUCTION

The near infra-red spectrum of oxidised mitochondrial cytochrome *c* oxidase is dominated by the absorption band of the type I $\mathrm{Cu_A}$ centre. This feature appears in a reduced *minus* oxidised spectrum as a broad trough in the 750–950 nm region and masks any weaker underlying features that might be present. Haem groups are well known to exhibit weak features in this region [2] and we have recently demonstrated such bands in cytochrome *o* from *E. coli* [1], an enzyme which shows many features in common with cytochrome *c* oxidase but which lacks the $\mathrm{Cu_A}$ centre [3]. These bands have been interpreted to arise from charge transfer transitions between the iron and porphyrin of the haem [2,4]. They can give new information on ligand state and local changes around the haem group and for this reason have been termed the 'conformational' bands by Iizuka et al. [5]. Hence, they are useful as probes of local changes within the binuclear centre of cytochrome *o*.

We have now detected equivalent absorption bands in cytochrome *c* oxidase in the absolute spectrum of the reduced enzyme. We also show, as was established with cytochrome *o* [1], that ligands of reduced haem a_3 can radically alter their positions and intensities.

2. MATERIALS AND METHODS

Cytochrome *c* oxidase from beef heart was prepared by a modification of the method of Kuboyama et al., as previously described [6] and was stored at 77 K until required. Near-IR scanning spectroscopy was performed with a single beam spectrophotometer built in-house, incorporating a near-IR ruled grating (1 micron blaze; 600 lines/mm) and a photodiode (1 cm^2 active area) detector.

The same machinery was used for room temperature flash photolysis of the carbon monoxide adduct [7]. The flash was provided by a xenon flashlamp (E.G. & G. FXP852) connected to a 15 μF capacitor charged to 1000 V, i.e. 7.5 J/flash. The flash had a duration of 2 μs at half maximal intensity. Two fibre-optic bundles (1 cm diameter) placed about 1 cm from the arc (length 3 mm) were used to collect the light and guide it, perpendicular to the measuring beam, to opposite sides of a 10 mm measuring beam pathlength cuvette. The optical pathlength of the actinic light was 1 cm or 0.3 cm, dependent on sample size. The flash was approximately 50% saturating for photolysis under the conditions used. In order to minimise flash artefacts, the photomultiplier detector was protected with WG360 and OG550 Schott glass cut-on filters and either a 600 ± 20 nm visible interference filter (visible region) or an RG715 Schott glass cut-on filter (near-IR region).

3. RESULTS

3.1. *Absolute spectra of cytochrome c oxidase*

The absolute absorption spectra of forms of cytochrome *c* oxidase are shown in Fig. 1. The top figure shows the well-known visible/near-IR features at low resolution for comparison, and the bottom part shows the same spectra in the near-IR region after expansion × 100.

The visible/near-IR spectrum of oxidised cytochrome *c* oxidase (Fig. 1A) is dominated by the well-known absorption band of $\mathrm{Cu_A}$ centred around 830 nm. This band is lost on reduction (Fig. 1B) and very weak features can then be discerned instead at 710 and 784 nm. These bands lie on top of broad underlying absorptions and have extinction coefficients of around 0.1 $\mathrm{mM^{-1}\cdot cm^{-1}}$. Addition of carbon monoxide to the reduced enzyme causes a shift of the 784 nm band to shorter wavelengths so that it begins to merge with the

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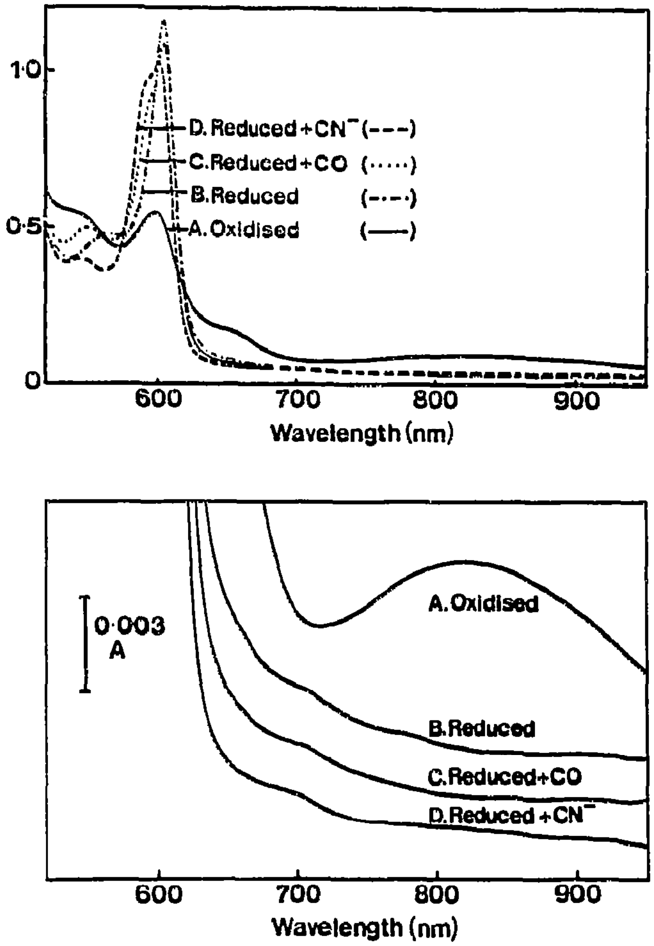

Fig. 1. Visible and near-IR spectra of cytochrome *c* oxidase. Cytochrome *c* oxidase was dissolved to a final concentration of 26 μM in 50 mM potassium phosphate, 2 mM EDTA and 0.05% lauryl maltoside at pH 7 after scanning a baseline of a buffer blank. The sample was scanned to give trace A. Dithionite added to form the fully reduced enzyme (trace B), followed by either saturating carbon monoxide (trace C) or potassium cyanide (trace D).

710 nm feature (Fig. 1C). Addition of cyanide to the reduced enzyme causes a loss of the 784 nm band and some change of the 710 nm band (Fig. 1D).

3.2. *Difference spectra*

The presence and behaviour of these bands is more easily seen in appropriate difference spectra. Because of the dominating effect of Cu_A on the oxidised spectrum, we were unable to study the features in the usual reduced *minus* oxidised difference spectra, as we had done with the analogous cytochrome *o* [1]. However, the [reduced-CO] *minus* [reduced] difference spectrum (Fig. 2A) clearly shows a trough at 784 nm and a shoulder at around 760 nm, consistent with a large blue-shift of the 784 nm band caused by CO binding. The [reduced-CN^-] *minus* [reduced] difference spectrum convincingly shows troughs at both 710 nm and 784 nm, consistent with a loss of the 784 nm band and a diminution of the 710 nm band.

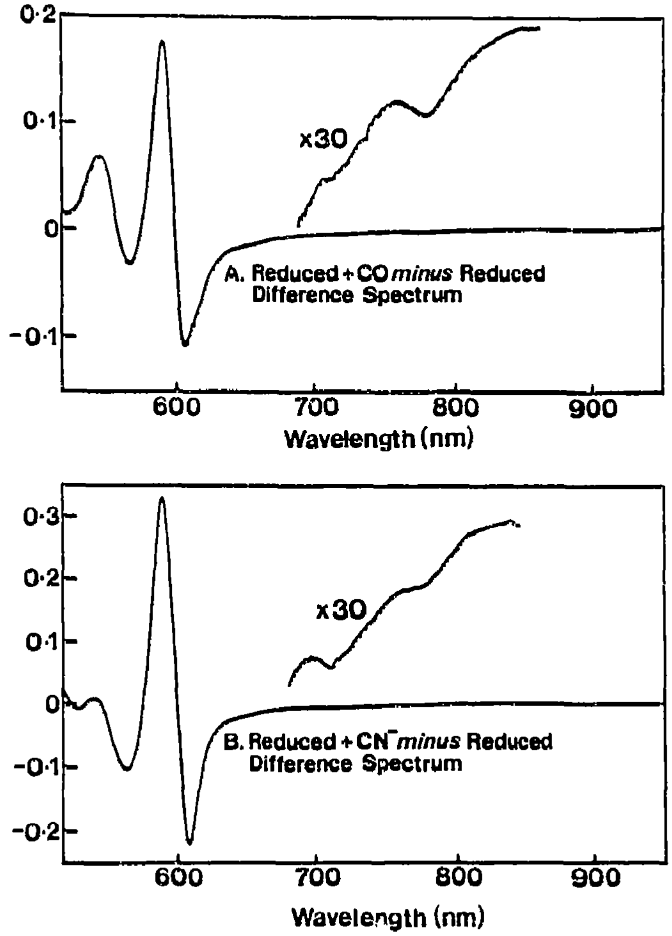

Fig. 2. Visible and near-IR difference spectra of cytochrome *c* oxidase. The data of Fig. 1 were used to derive difference spectra of (A) [reduced *plus* CO] *minus* [reduced] or (B) [reduced *plus* cyanide] *minus* [reduced].

3.3. *Flash photolysis of the carbon monoxide compound*

Further definitive detection of the 784 nm feature can be achieved by observation of optical changes caused by the transient photodissociation of carbon monoxide from the reduced haem by flash photolysis [7], even at room temperature (Fig. 3). Photodissociation of the CO results in a reversion of the band from 760 nm to 784 nm (Fig. 3, inset), as expected from Figs. 1 and 2. The kinetics of this transient measured at 784–0.5 (760 + 808) nm matched perfectly the more familiar visible region kinetic changes observed at 590–605 nm (Fig. 3) with a pseudo-first order rate constant of recombination at room temperature and 1 atmosphere of CO of 70 s^{-1} [7].

4. DISCUSSION

Our experience with detection of the near-IR features

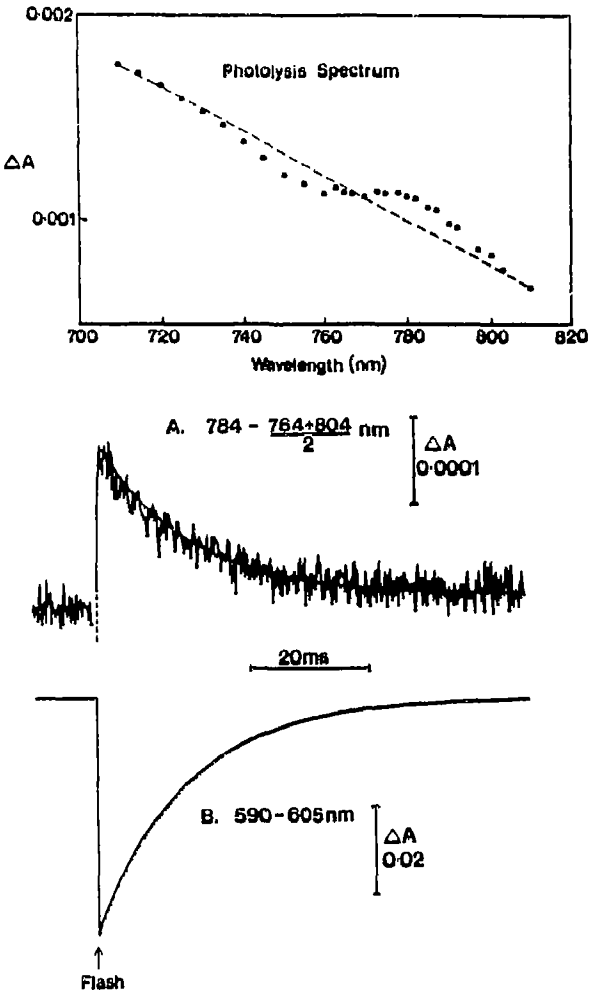

Fig. 3. Flash photolysis of the carbon monoxide adduct of fully reduced cytochrome *c* oxidase. Cytochrome *c* oxidase was dissolved to a final concentration of around 5 μM in carbon monoxide-saturated buffer of 50 mM potassium phosphate, 2 mM EDTA and 0.05% lauryl maltoside at pH 7. The sample was reduced with sodium dithionite and sealed against gas exchange. Flash photolysis data at different wavelengths were accumulated and computer-manipulated to produce the kinetic transients and photolysis spectra illustrated. The transients in both cases correspond to monophasic, first order decays with a rate constant of 70 s^{-1} in both cases. A simulated curve is plotted over the 784 nm band data for comparison. Data are the average of either 5 (590–605 nm transient) or 100 (near-IR transient) recordings per wavelength.

of *E. coli* cytochrome *o* [1] prompted us to reinvestigate whether cytochrome *c* oxidase itself exhibited such features. These we have now clearly detected, despite the interference from Cu_A in this enzyme. By analogy with our interpretation of the cytochrome *o* features, and by consideration of the literature on near-IR spectra of haem compounds and of myoglobin (Mb) and haemoglobin (Hb) in particular (see Discussion in [1]), it is clear that the 784 nm band is due to the reduced high-spin haem present in cytochrome *c* oxidase, i.e. haem a_3. The equivalent ~760 nm band in deoxyhaemoglobin has been assigned as a charge transfer transition between the porphyrin π system and the iron. The assignment is based on polarised absorption data, circular dichroic and magnetic circular dichroic studies [2,4]. Addition of CO and cyanide to deoxyMb or deoxyHb causes the compound to become low-spin with consequent loss of the 760 nm band. Cyanide and CO ligation to ferrous cytochrome aa_3 also causes the haem a_3 to become low-spin [8], and this is presumably the reason for the loss of the 784 nm signal. The absorbance at 760 nm of the CO compound may arise from the low-spin haem a_3 species by analogy with horse carboxyHb which has bands at 630 nm, 720 nm and 800 nm, attributed to (*d–d*) transitions of the low-spin haem [9]. The feature at around 710 nm in cytochrome *c* oxidase (Fig. 1) appears to be insensitive to carbon monoxide but may be modulated (but not removed) by cyanide. Whether this is a feature of haem a_3 or haem *a* requires further investigation.

The detection of near-IR features in both cytochrome *c* oxidase and cytochrome *o* is a further indication of the structural similarity of their binuclear centres. It is likely that the binuclear centre of both is responsible not only for the oxygen reduction chemistry, but also for the essential primary events of proton translocation [10]. Since these near-IR transitions of haemoproteins are sensitive to local environment and structure around the haem group, they provide a new sensitive probe for structural and mechanistic studies of the chemistry of oxygen reduction and proton translocation.

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REFERENCES

[1] Ingledew, W.J., Bacon, M. and Rich, P.R. (1992) FEBS Lett. 305, 167–170.
[2] Eaton, W.A., Hanson, L.K., Stephens, P.J., Sutherland, J.C. and Dunn, J.B.R. (1978) J. Am. Chem. Soc. 100, 4991–5003.
[3] Ingledew, W.J. and Bacon, M. (1991) Biochem. Soc. Trans. 19, 613–616.
[4] Chavez, M.D., Courtney, S.H., Chance, M.R., Kiula, D., Nocek, J., Hoffman, B.M., Friedman, J.M. and Ondrias, M.R. (1990) Biochemistry 29, 4844–4852.
[5] Iizuka, T., Yamamota, H. and Yonetani, T. (1974) Biochim. Biophys. Acta 371, 126–139.
[6] Moody, A.J., Cooper, C.E. and Rich, P.R. (1991) Biochim. Biophys. Acta 1059, 189–207.
[7] Sharrock, M. and Yonetani, T. (1977) Biochim. Biophys. Acta 462, 718–730.
[8] Price, B.D. and Brand, M.D. (1985) Biochem. Soc. Trans. 13, 695–696.
[9] Eaton, W.A. and Charney, E. (1969) J. Chem. Phys. 51, 4502–4505.
[10] Rich, P.R. (1992) Bioscience reports 11, in press.