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The assignment of the 655 nm spectral band of cytochrome oxidase

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The spectral characteristics of the '655 nm' band of cytochrome oxidase were found to be affected by ligands of the binuclear centre, including formate and chloride, and by the resting/pulsed transition. The band titrated with near $n=1$ characteristics at a midpoint of about 400 mV, in contrast to haem a_3, which exhibits strong redox interaction and a titration range at significantly lower potential. Thus, although the total reduced-oxidised difference spectrum of haem a_3 shows a trough at about 655 nm, this characteristic is absent in the low potential region. The 655 nm feature may arise from a charge transfer band of ferric high-spin haem a_3, which is modulated by the redox state of Cu_B, as suggested by Beinert et al. [(1976) Biochim. Biophys. Acta 423, 339–355].

Cytochrome oxidase; Binuclear centre; 655 nm band; Midpoint potential

1. INTRODUCTION

A high-spin ferric haem, such as haem a_3 in resting, oxidised cytochrome oxidase, is expected to exhibit charge transfer bands in the 450–500 nm and 600–650 nm regions involving the axial ligands [1,2]. On adding cyanide to oxidised aa_3, converting haem a_3 to a ferric low-spin form, the difference spectrum shows troughs centring at about 500 and 650 nm, while the band seen in the absolute spectrum at about 655 nm is abolished. Addition of formate to the oxidised enzyme, forming a ferric high-spin complex on the other hand does not abolish the 655 nm band. On the basis of these ligand effects, Wrigglesworth et al. [3] inferred that the band is associated specifically with high-spin haem a_3. Such a band had earlier been found in high-spin model compounds of haem a_3 by Carter and Palmer [2]. However, it was observed by Beinert et al. [4] that this spectral band appeared to bear a reciprocal relationship to the rhombic, high-spin EPR signal at $g=6$, normally seen only on reduction of Cu_B or when the antiferromagnetic spin coupling between the two metals of the binuclear centre is broken. It was therefore suggested that the band arises from a state involving interaction of ferric haem a_3 with the oxidised copper. The axial ligand of high-spin haem a_3 involved in the charge transfer might therefore be a bridging ligand with Cu_B, or another haem a_3 ligand whose interaction with haem a_3 is affected by the redox state of Cu_B.

The present work is an attempt to throw further light on this matter by observing the disappearance of the 655 nm band as the binuclear centre is reduced.

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2. MATERIALS AND METHODS

Bovine heart cytochrome oxidase was prepared by a variation of the Kuboyama method [5] developed by Dr John Moody at the Glynn Research Institute (details to be published separately). This method yields a preparation exhibiting rapid, nearly monophasic cyanide binding and fast internal electron transfer between the primary acceptor and haem a_3. The resting enzyme sample used in Fig. 1, also a Kuboyama-type preparation, was kindly supplied by Dr John Wrigglesworth of King's College, London. Cytochromes *c* (Type VI) and *f* (C 9022), catalase (C-100) and phenazine methosulphate were all supplied by Sigma, Poole, and riboflavin by BDH, Poole.

Experiments were carried out in a standard 1 cm cuvette fitted with a glass stirring flea and a capillary-bored glass stopper. This was inserted after filling the cuvette so as to exclude any gas phase, while permitting anaerobic additions by means of fine syringe needles inserted through the capillary. The top of the cuvette was enclosed by a cap continuously flushed with oxygen-free nitrogen. Exclusion of the gas phase was found to be essential, since even after repeated evacuation and refilling with oxygen-free Ar, slight uptake of oxygen into solution occurred. In the presence of partly-reduced enzyme, this led to formation of species showing absorption maxima at 607 and 580 nm, presumably the 2-electron reduced peroxy (compound C), and 3-electron reduced oxy-ferryl forms, respectively [6], from which the 655 nm band appears to be absent. The cuvette was mounted in a single-beam spectrophotometer constructed in this laboratory and fitted with a xenon flash unit to enable the sample to be photochemically reduced [7].

Titrations were performed using 'fast' oxidase (see above) pretreated with catalase at room temperature for 2–3 h to remove small fractions of peroxy and ferryl forms that otherwise tended to be present (A.J. Moody, unpublished observation). The medium contained 0.1 M potassium phosphate, pH 7.5, 1 mM EDTA, 75 μM riboflavin with 2 μM PMS, 5 μM ferricytochrome *c* and 3 μM ferricytochrome *f*, with 0.2 mM ferricyanide to ensure complete oxidation. After bubbling for 2 min with oxygen-free nitrogen the cuvette was stoppered, and the oxygen-depleted oxidase subsequently added to a final concentration of 3 μM through the capillary. After 5 min equilibration, during which the α-band of the oxidase declined slightly, presumably owing to oxidation of some singly-electronated molecules, stepwise flash photoreduction was carried out, scanning the 530–700 nm range at 0.5 nm intervals after each reduction step,

allowing at least 2 min for equilibration. Further equilibration up to 15 min caused little change provided that oxygen was totally excluded, as above. Redox potential was measured with reference to cytochromes *c* and *f*. Cytochrome *c* was measured at 550–556.5 nm, cytochrome *f* at 554–545.5, haem *a* at 604 − (588 + 620)/2 nm and the 655 nm band at 660 − (636 + 684)/2 nm.

3. RESULTS AND DISCUSSION

From the outset of our attempts to titrate the '655 nm' band, and by comparison of our spectra with previously published examples, it was clear that the position of the band maximum was somewhat variable. In particular it was found that samples of oxidase in which a significant proportion was in a resting form showed a broader, red-shifted band compared with the pulsed form. In the example shown in Fig. 1, a 5 nm blue shift to 658 nm with a slight decrease in absorbance occurred on pulsing with oxygen. Fast enzyme (see Materials and Methods) was found to be indistinguishable from pulsed in this spectral region, with little change on pulsing (not shown), although after aging, with several cycles of freezing and thawing, these fast preparations also acquired resting characteristics, both in terms of cyanide binding kinetics (J. Moody, unpublished data) and red-shift of this spectral band (R.M., not shown). A rapid change of the fast, 658 nm form to a resting-type spectrum also resulted from treatment with 50 mM formate, and similarly with 50 mM chloride, although in the latter case the transition was very much slower, taking about 30 min at pH 6.5, and again with the band maximum shifted to 663 nm as with the resting enzyme shown in Fig. 1. It was in order to carry out the titration as far as possible in the absence of such band-shifts, that the titrations were performed using the reasonably-stable fast enzyme preparations.

This spectral blue-shift of the resting enzyme after reduction and oxygen pulsing subsequently reverts slowly over a period of hours and appears to provide an explanation of the slow 'loss and gain of small features at 640 and 665 nm, respectively' in the pulsed-resting difference spectra observed by Armstrong et al. [8] following reoxidation of the reduced enzyme.

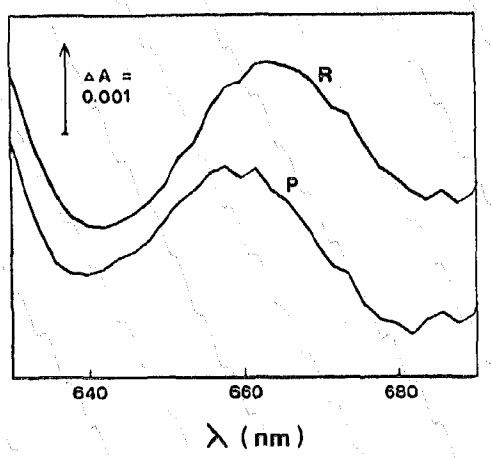

Fig. 1. Blue-shift of the '655 nm' band of resting oxidase following oxygen pulsing. Absolute spectra referred to a linear baseline drawn through the 620 and 740 nm points. R = 2.6 μM resting oxidase; P = the same sample after photoreduction, 20 min anaerobic incubation and finally aeration. Spectrum 5 min after aeration.

If the band were merely an indicator of ferric high-spin haem a_3, one would expect the titration to parallel that of haem a_3. The redox titration curves of haems *a* and a_3 are most clearly distinguished by MCD. At pH 7.4, these are closely similar, both showing strong negative redox interaction with another component of similar potential [9], and with the midpoints of the high and low potential waves at about 340 and 240 mV, respectively. Cu_B is generally believed to titrate over the same region [10–12], showing redox interaction with haem *a* [12]. If, as well as a ferric, high-spin state of haem a_3, oxidation of Cu_B is also required for appearance of the 655 nm band, one would expect the 655 nm band titration to occur over a narrower potential range, and with a significantly higher midpoint than the high potential waves of haem a_3 and Cu_B. For example, if the high potential midpoints of both components were at 340 mV, and neglecting the haem *a* interaction, a titration curve for the 655 nm band with a midpoint of 363 mV and a slightly narrower potential range than a standard $n = 1$ Nernst curve would be predicted.

Uncorrected titrations carried out as above showed a major disappearance of the 655 nm band centred at about 400 mV with a small secondary wave some 100 mV lower. A comparison of the spectral difference over the two titration regions showed that while the high potential wave developed a trough corresponding approximately to the band as seen in absolute spectra (Fig. 1), at lower potentials a very different broad trough was seen with a minimum at about 630 nm, similar to that expected from haem *a* reduction. The data were therefore reprocessed using a matrix deconvolution to correct for mutual interference of cytochromes *c*, *f*, *a* and a_3. Standard reduced-oxidised spectra of the first three components were easily obtained (haem *a* by selective reduction in aerobic, cyanide-inhibited enzyme). Haem a_3 was obtained by taking the slow phase of photoreduction of formate-treated fast enzyme and adding back the formate binding spectrum (see lower trace of Fig. 3). The spectrum thus obtained was similar to that found for the slow phase of dithionite reduction of resting enzyme (see Fig. 2 of [3]) and featured a trough at 658 nm.

As a first approximation, the trough at 658 nm was presumed to be an integral part of the spectrum of haem a_3. The titration using deconvoluted data was closely similar to that shown in Fig. 2A, where it is evident that the 604 nm peak shows only a small degree of haem *a* reduction at a potential where the 655 nm band is almost abolished. In fact, the 655 nm band scarcely changes below 330 mV, even though about 65% of the

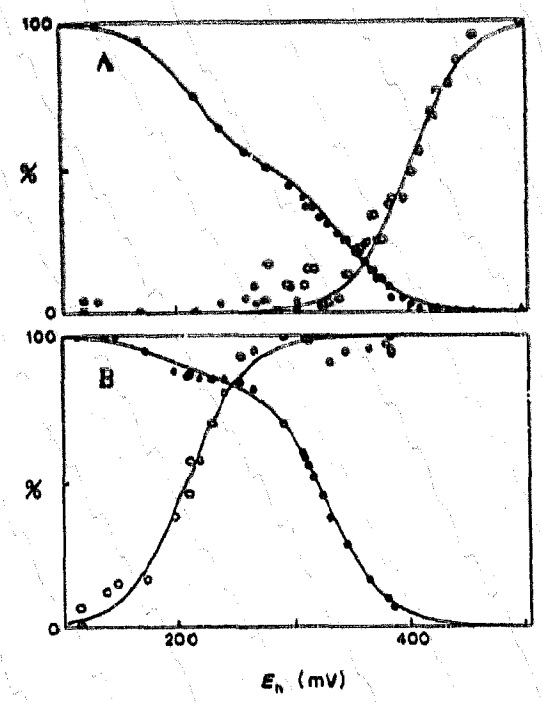

Fig. 2. Titration of 604 nm peak (filled symbols) and 655 nm band (open symbols). (A) Unliganded oxidase. The potentials are calculated assuming midpoints of 365 mV for cytochrome *f* and 255 mV for cytochrome *c*. Points below about 300 mV are based on *c* alone, those above 400 on *f*, with an overlapping region between. (B) 100 mM formate present, cytochrome *f* omitted. Single component $n = 1$ curves are fitted to the 660 nm data at 402 and 208 mV, and 2 component $n = 1$ curves to the 604 nm data at 345 (50%) and 215 mV and at 328 (85%) and 190 mV in (A) and (B), respectively.

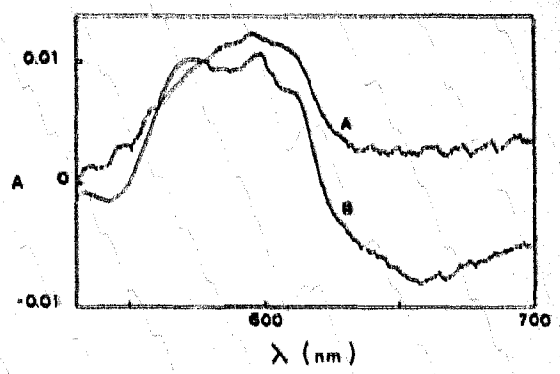

Fig. 3. Reduced-oxidised difference spectrum of the < 330 mV region of the titration of Fig. 2A (A) after subtracting standard spectra of cytochromes *a*, *c* and *f*. (B) reduced-oxidised difference spectrum of haem a_3 obtained from the slow phase of photoreduction in the presence of formate. After equilibration of the near-anaerobic oxidase solution in the usual way, 30 μl anaerobic 10 M potassium formate was added. When spectral changes due to formate binding appeared to be complete (about 10 min), the oxidase was reduced by a series of 30 flashes at about 2 flashes per second. Spectra were taken at 75 s (when haem *a* reduction was complete) and at intervals for about 30 min when haem a_3 reduction also appeared to be complete. Three further series of 5 flashes were given during this period to ensure continuing reduction. The difference spectrum of the slow phase was scaled up by 16% to correct for the calculated reduction of haem a_3 during the first 75 s, and finally the formate-binding spectrum was added back.

604 nm peak is formed between 330 and 100 mV. Assuming that the MCD data of Kojima and Palmer [9] and the 'neoclassical model' [13,14] are basically correct, the difference spectrum over the 100–320 mV region should contain a high-spin haem a_3 contribution that is free from the 655 nm feature. After subtraction of standard spectra of cytochromes *c*, *f* and *a*, we obtain the upper trace of Fig. 3, showing strong similarity to the high-spin haem a_3 spectrum obtained by the formate method (see above), except for the absence of the shoulder at about 570 nm, but completely lacking the 658 nm trough, which we therefore do not consider to be an intrinsic part of the spectrum of haem a_3. (The shoulder at 570 nm, and also that at about 610 nm are considered likely to be extraneous features, the latter possibly reflecting a band shift of haem *a* as haem a_3 is reduced, or slight peroxy compound formation.) Apparently, close to 65% of the haem a_3 as well as of haem *a* titrates in this 100–320 mV region. The spectral deconvolution was therefore repeated, but assuming the haem a_3 to titrate in parallel with haem *a*, and the 655 nm band to be an isolated spectral feature, with the result shown in Fig. 2A. The deconvolution matrix used is given in Table I.

The high midpoint of the 655 nm band is a little surprising. If oxidation of both haem a_3 and Cu_B is required for the band to appear, and using midpoint potentials of the high potential waves of haems *a* and a_3 of 340 mV, with −100 mV and −60 mV for the redox interaction energies of haem *a* with haem a_3 and Cu_B, respectively, the high potential midpoint of Cu_B would have to be at 400 mV to account for the observed 50% disappearance at 402 mV. This is higher than the generally accepted value of about 340 mV [15], but is more nearly consistent with the half-wave potential for appearance of the $g = 6$ EPR signal of 380 mV at pH 7.0, probably reflecting reduction of Cu_B, reported by Wilson et al. [16]. No account is taken in the above model of any possible redox interaction between haem a_3 and Cu_B, but since there is evidently little reduction of haem a_3 at 400 mV, the effect on the calculated Cu_B midpoint can be only of the order of 2 or 3 mV.

It was noted by Palmer (personal communication) that if, as this work implies, the separation of the midpoints of haem a_3 and Cu_B is of the order of 60 mV, one would expect that in the early stages of reduction, Cu_B would be selectively reduced. Over this range, the stoichiometry of the appearance of the $g = 6$ signal, i.e. moles of high-spin haem against number of electrons added to the enzyme, should be near 1 rather than about 0.2, as found [17]. However, there may be reasons, other than the oxidation state of Cu_B why a $g = 6$ signal from ferric haem a_3 may not be seen. For ex-

ample, not all of the haem a_3 may be high-spin. In addition, there may be changes in redox equilibrium between the metal centres on cooling to EPR temperatures.

Presumably, in the cyanide-liganded enzyme, the Cu_B midpoint is lower, i.e. around 340 mV [12], so that while a simple mutual interaction between the haems is observed in the unliganded state [9], on stabilising the oxidised state of haem a_3 with cyanide, a strongly distorted haem *a* titration is still found, now interacting with the nearly equipotential Cu_B. This interaction would be unobserved with the widely separated midpoints of the unliganded state.

The titration was repeated in the presence of formate, where the haem a_3 midpoint is lowered to about 220 mV [9]. The midpoint of the 655 nm band was also lowered (to 208 mV in Fig. 2B), implying, if the above interpretation is correct, that formate lowers the midpoint of Cu_B to well below this value. Difference spectra (not shown) reveal that the major wave of the titration at 604 nm is purely haem *a*, with haem a_3 titrating in the small low-potential wave. The data do not conform closely to a simple 2-component curve. The distortion around 250 mV is consistent with a slight reoxidation of haem *a* in this potential region, as already reported by Kojima and Palmer [9].

In conclusion, the data indicate that the appearance of the 655 nm band requires oxidation of both components of the binuclear centre, as suggested by Beinert et al. [4], presumably reflecting the influence of the redox state of Cu_B on a charge transfer band of haem a_3 and one of its ligands. The band titrates at a potential which implies that the midpoint of Cu_B at pH 7.5 is around 400 mV in the unliganded enzyme, and no higher than about 200 mV in the presence of 0.1 M formate.

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