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Spectral shifts of cytochrome *c* oxidase induced by complexons

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Ca^{2+}-chelating agents, such as EDTA and ATP, are shown to bring about a rapid spectral response of the oxidized cytochrome *c* oxidase due to reversal of the Ca^{2+}-induced red shift of the γ- and α-absorption bands of the ferric enzyme. In addition, complexons are found to bring about Ca^{2+}-independent, slow irreversible spectral changes indicative of a conformational transition of cytochrome oxidase. 1 mol EDTA per mol enzyme is sufficient to produce the maximal effect even in the presence of excess Ca^{2+}, indicating high specificity of interaction. It is suggested that the conformation of cytochrome *c* oxidase may be regulated by the tightly bound 'non-redox' metal ions (Mg, Zn, Cu_x) known to be present in the enzyme. These ions might be involved in specific binding of physiological effectors with chelating properties, such as ATP.

Cytochrome-*c* oxidase; Conformational change; ATP; Spectral shift; Complexon; Metal ion binding

1. INTRODUCTION

Over a decade ago, Ca^{2+} was shown to bring about a red shift of the reduced cytochrome *c* oxidase absorption spectrum, which could be reversed by Ca^{2+} complexons [1–5]. Recently, we also showed a red shift to be induced by Ca^{2+} in the Soret and α-bands of the oxidized enzyme (Vygodina et al., unpublished; see also [2,6]). In the course of these studies we observed rapid reversal of the Ca^{2+}-induced spectral shift by EDTA to be accompanied by additional slow irreversible spectral changes. Subsequently, it became clear that EDTA and a number of other complexons bring about spectral transition of the oxidized 'resting' cytochrome *c* oxidase. A preliminary report on this effect has been published [6].

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2. MATERIALS AND METHODS

Cytochrome *c* oxidase was prepared from bovine heart mitochondria or Keilin-Hartree particles according to Fowler et al. [7] and Yonetani [8], respectively. The Fowler-type enzyme was further purified as described in [9].

Optical measurements were carried out in Aminco DW2a, Hitachi 557 or Shimadzu 3000 UV/Vis spectrophotometers in standard 10-mm rectangular quartz cells thermostatted at 25–27°C.

The basic incubation medium contained 50 mM Tris-HCl buffer (pH 8.0) and 0.5% Tween-80. About 10 μM adventitious Ca^{2+} was found to be present in the reaction mixture (experiment kindly carried out by Dr I. Afanasiev).

3. RESULTS

Addition of excess EDTA to Ca^{2+}-loaded ferric cytochrome oxidase brings about a biphasic spectral response in the Soret band. Initially, a symmetrical derivative-like difference spectrum is observed with λ_{max} = 423 nm, λ_{min} = 440 nm and $\Delta\epsilon_{440-423} \approx 6\ mM^{-1} \cdot cm^{-1}$ (fig.1a). This effect can be abolished by Ca^{2+}, and is due to reversal of the Ca^{2+}-induced red shift of the Soret peak of the ox-

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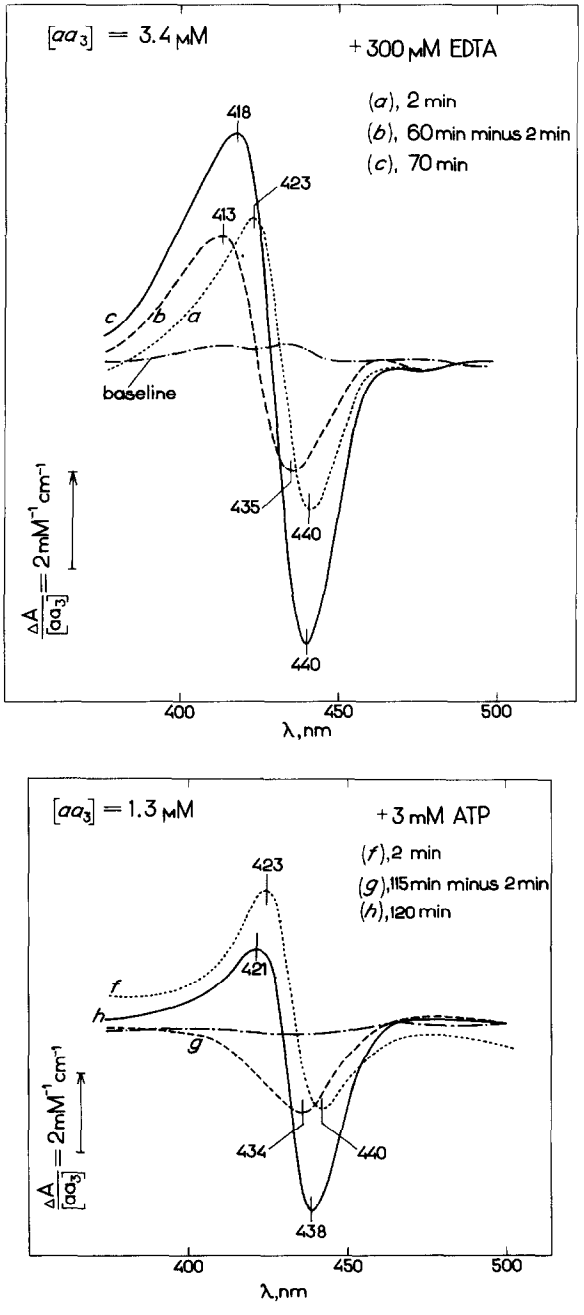

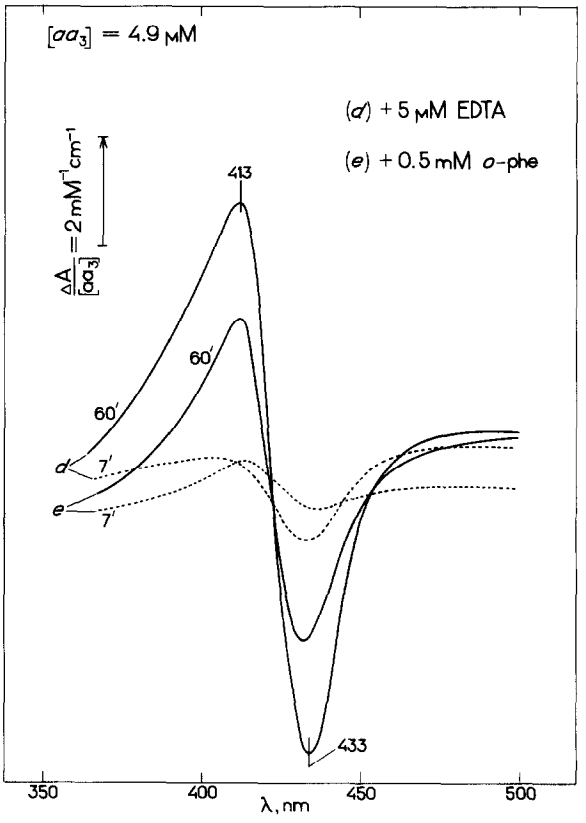

Fig.1. Spectral shifts in the Soret band of the oxidized cytochrome *c* oxidase induced by complexons. Sample and reference spectrophotometric cells contained Fowler-type cytochrome *c* oxidase in the basic medium supplemented with 150 μM $CaCl_2$. The following additions to the sample were made: (a–c) 300 μM EDTA; difference spectra recorded 2 min (a) or 70 min (c) after addition; (b) difference spectrum of the slow phase, i.e. (c) minus (a). (d,e) 5 μM EDTA (d) or 500 μM *o*-phenanthroline (e); spectra recorded 7 min (broken lines) or 60 min (unbroken lines) after additions. (f–h) 3 mM ATP; spectra recorded 2 min (f) or 120 min (h) after addition; (g) difference spectrum of the slow phase, i.e. (h) minus (f).

idized enzyme (to be published). Upon subsequent incubation, additional slow spectral changes develop ($t_{1/2} \approx 30$–40 min [6]) which approximately double the amplitude of the overall difference spectrum and change its lineshape (fig.1c). A more or less symmetrical spectrum of the slow phase of the EDTA-induced optical changes with $\lambda_{max} \approx$ 413 nm, λ_{min} = 433–435 nm and an isosbestic point at ~424 nm (fig.1b) is indicative of a blue shift of the Soret peak, as confirmed by recordings of absolute spectra (not shown).

The slow changes are not associated with Ca^{2+}

since the effect can be induced by (and saturated at) EDTA concentration equal to that of cytochrome oxidase in the presence of a large excess of Ca^{2+} (e.g. fig.1d). Moreover, similar slow spectral changes are brought about by *o*-phenanthroline (fig.1e [6]) which does not bind Ca^{2+} to any significant extent under these conditions; much more of the chelator is required to saturate the effect in this case (~0.5 mM). The effect of *o*-phenanthroline was prevented by low concentrations of EDTA and vice versa (not shown). The same slow blue shift of the Soret band was also induced by a number of other complexons including EGTA and nitrilotriacetate.

Slow Ca^{2+}-independent spectral changes induced by EDTA were also observed with a Yonetani-type enzyme (not shown).

It was obviously of interest whether ATP, a naturally occurring complexon reported to regulate cytochrome *c* oxidase [10–13], would exert effects similar to those of EDTA or *o*-phenanthroline.

At low (micromolar) concentrations ATP did not have any effects. When added to the Ca^{2+}-loaded enzyme, 3 mM ATP gives rise to a rapid spectral response analogous to that induced by excess EDTA with λ_{max} = 423 nm, λ_{min} = 440 nm and $\Delta\epsilon_{423-440}$ = 5–6 $mM^{-1}\cdot cm^{-1}$ (fig.1f). This response is fully prevented by EDTA added in excess over Ca^{2+} but not by *o*-phenanthroline, and is clearly due to reversal of the Ca^{2+}-induced red shift (cf. fig.1a). The same rapid spectral effect, although to a lesser extent, could also be observed in the absence of added Ca^{2+} (not shown). It is likely that a similar rapid response observed upon addition of ATP, inositol hexaphosphate or P_i to ferric cytochrome oxidase by Malatesta et al. [14] was also dominated by reversal of the adventitious Ca^{2+}-induced red shift rather than having been due to any specific effects of the compounds on the enzyme. According to our data, 10 μM Ca^{2+} present would be sufficient to account for the difference spectrum ($\Delta\epsilon_{420-438} \approx 2\ mM^{-1}\cdot cm^{-1}$) given in fig.4 of [14].

The initial Ca^{2+}-dependent spectral effect of ATP was usually followed by slow secondary optical changes (e.g. fig.1g) which varied in size and shape for different preparations and may represent a genuine ATP-specific conformational change of the enzyme. Significantly, ATP does not prevent

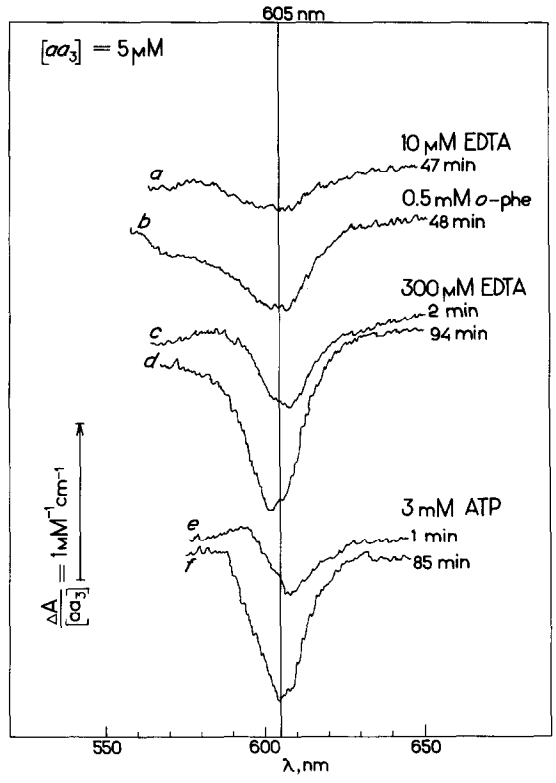

Fig.2. Spectral changes induced by complexons in the α-band of oxidized cytochrome *c* oxidase. Conditions as in fig.1, but $CaCl_2$ concentration 200 μM. Additions to the sample and incubation times are indicated in the figure.

slow optical changes induced by EDTA or *o*-phenanthroline. Hence, the effect of the nucleotide appears to differ from that of other complexons.

Fig.2 shows the spectral effects induced by chelators in the α-band region. EDTA at low concentrations and *o*-phenanthroline gave rise to slow, small and rather variable changes of extinction around 600 nm (e.g. fig.2a,b). On the other hand, an excess of EDTA and ATP rapidly brought about a well-defined trough at 607–608 nm due to reversal of the Ca^{2+}-induced red shift of the ferric enzyme α-band (fig.2c,e); the rapid phase was followed by slower optical changes leading to an increase in the trough and a distinct shift of the minimum to the blue (fig.2d,f).

4. DISCUSSION

At present we have not yet ascertained the molecular basis of the slow Ca^{2+}-independent ab-

sorption changes of the 'resting' cytochrome oxidase induced by EDTA, *o*-phenanthroline and a number of other chelators tested; the effect can be assigned provisionally to conformational changes of the enzyme. Interestingly, rather similar spectral changes occur spontaneously when the enzyme is allowed to stand at room temperature in a detergent solution (in preparation) so that the complexons may be considered as promoting a conformational transition inherent in the enzyme, rather than inducing additional structural perturbation. According to our preliminary observations, the slow absorption changes promoted by complexons may be related to the presence of reducing equivalents in the apparently oxidized enzyme (cf. [15,16]).

The effect of complexons appears to be rather specific as shown by the 1:1 stoichiometry for the action of EDTA. Accordingly, one can suggest the structure of cytochrome oxidase to be stabilized or regulated by a specifically bound heavy-metal ion. Two possibilities may then be concerned. First, EDTA and other chelators could extract this putative ion from the enzyme, entailing conformational rearrangement of the protein. Second, the ion could remain bound to the enzyme with some of its coordination valencies available to extrinsic ligands, thus serving as a specific binding site for complexons. The latter possibility is appealing, since cytochrome oxidase has been shown to contain tightly bound Mg, Zn and the so-called Cu_x ions which are not removed by prolonged dialysis vs EDTA [17–19] and it is tempting to suggest that at least one of these ions might be involved in the interaction of the enzyme with complexons.

Such an interaction could be of physiological significance. Of course, EDTA and *o*-phenanthroline are not normal metabolites, but ATP is, and, reportedly, this and some other nucleotides bind to cytochrome oxidase bringing about perturbation of the structure and enzymatic activity of the enzyme [10–13]. It is not unreasonable to suggest that slow Ca^{2+}-independent spectral transitions induced by ATP and other complexons, as observed here, are related to binding of the compounds at the regulatory sites. It is noteworthy that whereas the interaction of ATP with proteins usually requires divalent cations, Mg^{2+} has been shown to inhibit binding of the nucleotide with cytochrome *c* oxidase at the regulatory site [12], which accords with a suggestion of intrinsic metal ion(s) being involved in the binding. Thus, one of the thus far unknown functions of the tightly bound non-catalytic metal ions in cytochrome oxidase could consist of mediating the regulatory effects of nucleotides. Finally, the ability to react with chelators would render cytochrome oxidase susceptible to regulation not only by nucleotides but also by many other metabolites and drugs with complexing properties.

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