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A new spectral intermediate in cyanide binding with the oxidized cytochrome *c* oxidase

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Reaction of cyanide with oxidized cytochrome *c* oxidase at a low concentration of the ligand and pH >8 reveals an initial phase, not reported earlier, associated with a small blue shift of the absorption spectrum, which is followed by a conventional red shift of the heme a_3^{3+}. The initial blue shift resembles the spectral changes induced under the same conditions by low concentrations of azide and it is not observed in the presence of 0.3 mM azide. It is suggested that, similarly to NO, cyanide and HN_3 cannot only bind to heme a_3 but to Cu_B^{2+} as well, perturbing the spectrum of a_3^{3+} indirectly. A rapid binding to Cu_B^{2+} could provide the long-sought intermediate in the cyanide reaction with heme a_3^{3+}, the existence of which is implied by the Michaelis–Menten type kinetics of the latter process.

Cytochrome *c* oxidase; Cyanide; Cu_B; Ligand binding; Azide; Respiratory chain

1. INTRODUCTION

Cyanide reacts with oxidized cytochrome *c* oxidase (COX) to form a low-spin complex of heme a_3 bringing about a red-shift of the enzyme Soret band as in the case of other high-spin hemoproteins. However, the reaction has long been known to be more complex than simple ligand combination with heme a_3 iron. The two basic observations pertinent to the problem are: (i) the dependence of the cyanide binding rate constant upon the ligand concentration shows saturation behaviour, indicating that the process comprises at least two steps [1–5]; and (ii) under the conditions of catalytic turnover, inhibition of the enzymatic activity upon addition of cyanide develops much earlier than the spectral changes corresponding to heme a_3 ligation [6,7].

These observations point to the existence of an intermediate in cyanide combination with a_3^{3+}. The intermediate has been considered spectrally silent and therefore either not accumulating at any significant concentration or spectrally identical to the free enzyme [1–5].

It is well established that there are at least two ligand binding sites in the oxygen reducing centre of COX, namely, heme a_3 iron and Cu_B [8–11].

We earlier presented evidence for hydrazoic acid (HN_3) binding to Cu_B^{2+} with a high affinity as a reaction responsible for the inhibitory action of azide, the latter effect being well separated on a concentration scale from the binding of N_3^- at the heme site [12] (see also [13,14]). Notably, HN_3 binding at the high-affinity site is associated with a small blue shift of the enzyme's Soret band and with corresponding changes in the visible part of the absorption spectrum which closely resemble the perturbation of the optical characteristics of the ferric COX brought about by NO. The latter reagent definitely binds to Cu_B^{2+} in the oxidized enzyme [8,9].

It therefore seemed natural to propose that the initial 'spectrally silent' site of cyanide binding (actually, of HCN [3,5]) in COX, responsible for the rapid inhibition of the enzymatic activity, might also be Cu_B [15,16].

In this paper we show that under certain conditions, the red shift of the oxidized COX Soret band induced by cyanide is preceded by a small blue shift. A similar blue shift is brought about under these conditions by low concentrations of azide. The data favour the hypothesis [15,16] that Cu_B^{2+} is the initial binding site of cyanide.

2. MATERIALS AND METHODS

Cytochrome *c* oxidase was isolated from beef heart essentially according to Fowler et al. [17,18] (in Moscow), Yonetani [19] (in Kosice) or Kuboyama et al. [20] (in St. Catharines). Optical measurements were carried out in an Aminco-SLM 2000 or a Hitachi-557 dual-wavelength spectrophotometer (in Moscow) or in a Shimadzu-3000 instrument (in Kosice) operated in a dual-wavelength mode for the kinetic traces or in a double-beam mode for spectra recordings. A computer-interfaced single-beam Beckman DU-7 spectrophotometer was used in St. Catharines. KCN (analytical grade) was from Merck. Other chemicals were commercial products from Sigma, Serva, Merck and Fluka.

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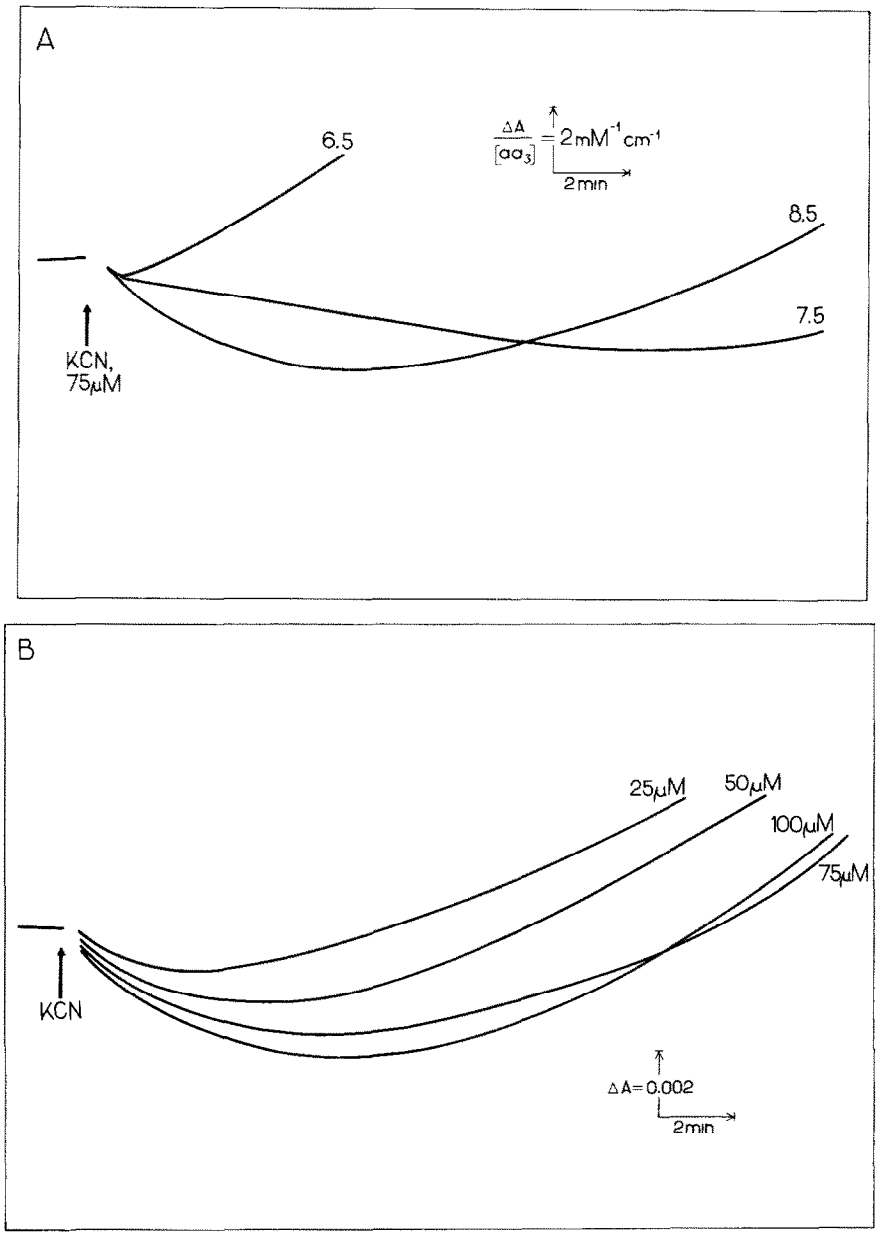

Fig. 1. Kinetics of cyanide-induced absorption changes in the Soret band of COX. (A) pH-dependence. 1 μM COX in the basic medium with MES (pH 6.5), HEPES (pH 7.5) or Tris (pH 8.5). KCN concentration, 75 μM. T = 25°C. (B) KCN-concentration dependence. 2 μM COX in the basic medium with Tris, pH 8.5. The KCN concentration is indicated in the figure (in μM). The absorbance changes were monitored at 434 *minus* 411 nm.

The basic reaction medium included a 50 mM pH-buffer (HEPES, Tris or MES) with a pK value closest to the pH desired, 0.5% Tween 80 (or 0.05% lauryl maltoside) and 100–200 μM ferricyanide.

3. RESULTS

We have routinely observed that when ferric COX reacts with cyanide at very low concentrations of the ligand, the initial difference spectra often look anomalous compared to the subsequent series of derivative-shaped curves with a clear isobestic point at approximately 425 nm, typical of the enzyme γ-band red shift.

At slow reaction rates, kinetic traces at 433 *minus* 411 nm often show a significant lag phase. These effects become very pronounced with some preparations of the enzyme, usually those with a red-shifted Soret band (424–425 nm), and especially in the vesicle-reconstituted cytochrome oxidase.

Fig. 1A shows cyanide binding with resting Fowler-

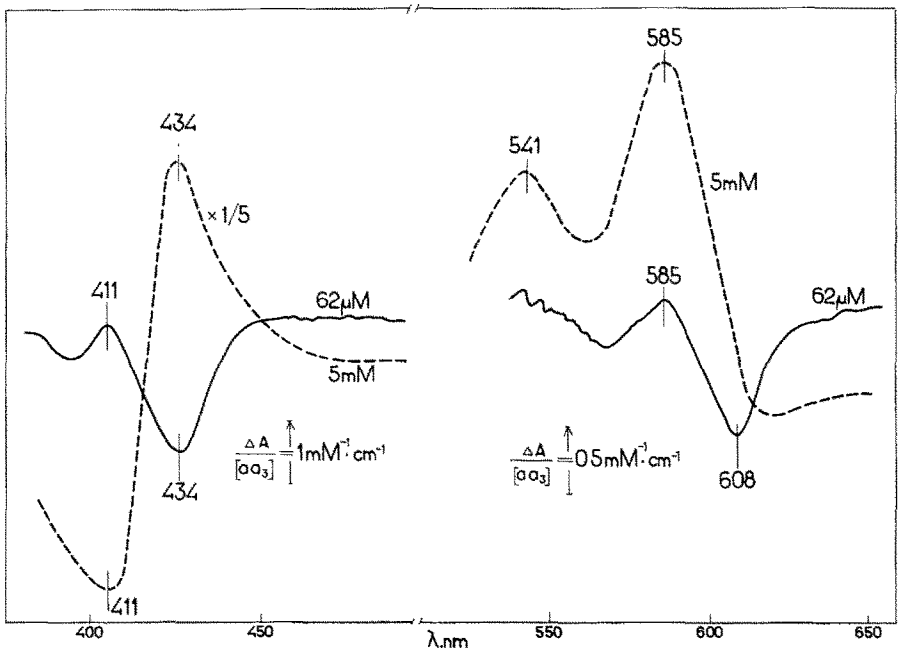

Fig. 2. Difference spectra of the cyanide induced absorption changes of COX. 2 μM COX in the Tris medium, pH 8.5. Solid lines, difference spectra recorded 2 min after addition of 62 μM KCN; dashed lines, difference spectra taken 10 min after the addition of 5 mM KCN in a separate experiment.

type COX at 75 μM ligand monitored in the Soret region. At an alkaline pH, the increase in absorbance at 434 *minus* 411 nm, typical of the heme a_3^{3+} ligation, is preceded by a distinct negative phase. This phase becomes less pronounced as the pH is lowered, almost disappearing below pH 7.

As shown in Fig. 1B, the negative phase of KCN-induced absorption change at 434 *minus* 411 nm increases when the ligand concentration is raised to approx. 100 μM. Upon further increase in the ligand concentration, the initial decrease gradually became masked by the second phase of the optical change, so that at the millimolar concentrations of KCN this phase was barely detectable (data not included).

The concentration dependence varied notably for different COX preparations (as does, incidentally, the apparent K_m value of the overall reaction with cyanide [5]). With some batches of enzyme a distinct initial negative phase could be resolved at a pH of 8.5 using KCN concentration as high as 1 mM (not shown).

The difference spectrum of the initial phase induced by low concentrations of cyanide at an alkaline pH is given in Fig. 2 (solid lines) and is quite different from the typical difference spectrum of the COX cyanide complex obtained following addition of 5 mM KCN to the enzyme (dashed line).

The spectral changes brought about by low cyanide concentrations are similar in size and shape to the spectral perturbation induced by low azide concentrations (Fig. 3). Accordingly, the addition of a low concentration of KCN to COX after azide does not bring about any further blue shift in the enzyme spectrum but immediately gives rise to the spectral changes typical of a_3^{3+}–CN complex formation (Fig. 3).

Similar observations have been made with several preparations of Yonetani- or Kuboyama-type preparations of COX, although in some batches of enzyme the negative phase of the KCN-induced changes in the Soret band is rather small in this case. The phase, however, increases to the level observed with the Fowler-type COX if the enzyme is pulsed or incorporated into liposomes (not shown)*. The variability of the results may correlate with the varying position of the enzyme γ-peak and with varying proportions of the 'slow' form of the enzyme in the several Yonetani- or Kuboyama-type preparations.

4. DISCUSSION

Our data may provide evidence for heterogeneity of the cyanide binding sites in the oxidized COX. Whereas conventional binding of cyanide to heme a_3^{3+} is slow ($k_v \approx 2\ M^{-1} \cdot s^{-1}$ for the 'fast' enzyme [1,5,21]) and gives rise to a substantial red shift of the Soret band associated

* The initial observations described in this work were subsequently confirmed and extended in collaborative research between P.N. and A.A.K. during a visit of A.A.K. to Brock University in July 1990. The results of these more detailed studies will be reported in a full-length paper.

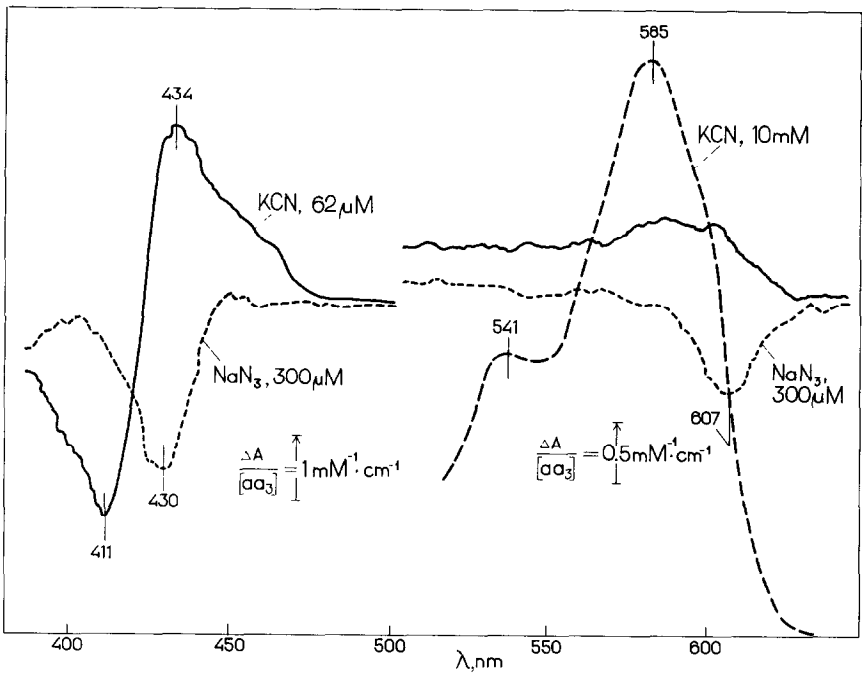

Fig. 3. Relationships between the effects of low concentrations of cyanide and azide. 2 μM COX in the Tris medium, pH 8.5. Dashed lines, spectral changes induced by 300 μM azide. Solid lines, the azide complex spectrum was recorded as a baseline. 62 μM KCN *was* subsequently added to the sample and the spectrum recorded immediately after the addition (cf. the solid line spectrum in Fig. 2). The spectrum induced by 10 mM KCN in the α-region is shown by a long-dashed line for comparison.

with a high- to low-spin transition of the heme iron, the new reaction phase described here is more rapid (k_v = 40–50 $M^{-1} \cdot s^{-1}$) and results in a small blue shift both in the γ- and α-regions.

With the Fowler-type preparation, the new phase of cyanide binding was most readily observed at low concentrations of the ligand (<200 μM) and an alkaline pH (8–8.5), conditions not frequently used in the past. This could explain why the initial phase was not observed earlier by other workers.

Presumably, at a low concentration of cyanide the heme ligation becomes so slow that it no longer obscures the reaction at the second site. Whether alkaline pH facilitates observation of the initial cyanide complex due to a deceleration of the second reaction phase [3–5], or whether it promotes binding at the alternative site cannot be decided on at present.

Notably, the cyanide reaction with the oxidized COX strikingly resembles that of azide. The difference lies in a much lower affinity of azide for the heme site ($K_d \approx 10^{-2}$ M [12–14]) so that binding at a second site, presumably Cu_B^{2+} [12], can be easily monitored at submillimolar concentrations of the ligand without interference from the heme ligation-associated spectral changes.

By analogy with azide, it would be tempting to identify the second site of cyanide binding as Cu_B^{2+}. Indeed, it has recently been found using IR-spectroscopy that cyanide can bind not only to heme a_3 but to Cu_B as well in the oxidised enzyme [22]. Unfortunately, these data were obtained at cyanide concentrations as high as 50–100 mM and it is consequently difficult to say whether they have any direct bearing upon the specific high-affinity interaction of this inhibitor with COX redox centres as studied by spectrophotometric and enzymatic activity assays.

It remains to be established whether the new phase of cyanide binding reflects a small spectral perturbation of the whole enzyme population or whether it is due to significant spectral changes but only in a small fraction of cytochrome oxidase. The initial phase of KCN binding to an admixture of singly reduced ('electronated') COX has been reported very recently [23]. However, the spectral changes in [23] appear to be in the direction opposite to those described in this work. Another possibility, compatible with the line-shape of the spectral changes, might consist in KCN or azide rapid binding to Cu_B inducing a decay of peroxi- state which can be present in a small proportion in the preparation of the oxidized enzyme.

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