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Interaction of Ca^{2+} and H^+ with Heme A in Cytochrome Oxidase

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Abstract

Ca^{2+} ions shift the absorption spectrum of reduced cytochrome *a* in mitochondria by acting from the outside of the membrane. In isolated cytochrome oxidase the shift may be induced by either Ca^{2+} or H^+, the apparent pK varying between 6.20 and 5.75 depending on the state of cytochrome a_3. Studies of the Soret band show that Ca^{2+} also shifts the spectrum of ferrocytochrome a_3 in isolated oxidase in contrast to the situation in mitochondria or isolated oxidase reconstituted into liposomes. Model studies with reduced bis-imidazole heme A reveals an analogous spectral shift induced by Ca^{2+}. Esterification of the propionate carboxyls of heme A abolishes the spectral shift, suggesting that it is due to interaction of Ca^{2+} with these groups. When taken together with the data with intact mitochondria, this suggests that the propionate side chains of cytochrome *a* are accessible to Ca^{2+} and H^+ from the outside of the mitochondrial membrane. In the soluble enzyme both hemes *a* and a_3 are accessible. Thus heme *a* may be located near the outside of the inner membrane whereas heme a_3 experiences a different environment in which no Ca^{2+} shift occurs.

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) undergoes conformational changes associated with spectral shifts when isolated and tightly coupled mitochondria are "energized" by ATP [1–4]. The addition of ATP to anaerobic mitochondria in which energy-dependent redox changes are prevented causes a red shift in the spectrum of ferrous cytochrome aa_3 [1, 2, 4, 5]. A similar or identical shift is produced under conditions where cytochrome a_3 is oxidized and liganded to cyanide or reduced and liganded to CO or NO [4, 5], showing that the shift occurs in the spectrum of ferrocytochrome *a*. The ATP-induced shift is sensitive to uncouplers of oxidative phosphorylation and to oligomycin, and correlates with the ATP/ADP · P_i ratio rather than with the concentration of

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ATP [4, 6]. This indicates that the underlying change in configuration is in response to reversal of the pathway of oxidative phosphorylation.

We have previously reported [4, 6, 7] that Ca^{2+} ions mimic the energy-dependent spectral shift in ferrous cytochrome *a* both in uncoupled mitochondria and in isolated cytochrome oxidase. The Ca^{2+}- and energy-dependent spectral shifts are most likely due to a similar chemical event, having identical spectral properties and being nonadditive. However, the ATP-induced spectral shift is unlikely to be due to Ca^{2+} binding and was proposed to be the result of H^+ binding to cytochrome *a* upon mitochondrial energization. We also reported that the binding site for Ca^{2+} is located outside the permeability barrier of the mitochondrial membrane [7]. It was proposed that Ca^{2+} binding to cytochrome oxidase mimics the effect of energization by binding to an acidic group which is protonated in energized mitochondria and deprotonated under uncoupled conditions. This idea could be related to subsequent findings of cytochrome oxidase as a redox-linked proton pump (for a review, see [8]).

Materials and Methods

Cytochrome *c* oxidase was isolated according to Kuboyama et al. [9] with the following modifications. Beef-heart mitochondria [10] were used as starting material. The long 10–12 h incubation was omitted. Instead the beaker containing the suspended crude enzyme was allowed to warm up until a temperature of 14–16°C was reached (usually in 30 min). This resulted in precipitation of the remaining cytochrome *b*. The precipitate was subsequently removed by centrifugation at 40,000 *g* for 30 min at 4°C. The final purification steps using the detergent Emasol 1130 were also omitted. The purified enzyme was stored under liquid nitrogen.

The heme content was determined by reduction with dithionite using a millimolar extinction coefficient of 13.5 cm^{-1} for the reduced minus oxidized difference in absorption at 605 minus 630 nm. Protein was assayed by the method of Lowry et al. [11], using human serum albumin as standard. The heme/protein ratio of the cytochrome oxidase preparations varied between 9 and 11 nmol/mg protein. The polypeptide composition of this preparation has been published [12] and is comparable to preparations reported from other laboratories.

Heme A was isolated directly from a cytochrome oxidase preparation with 1% (v/v) concentrated HCl in acetone. The acetone–HCl mixture was evaporated to dryness under vacuum and the brown residue was dissolved in ethyl ether. The solution of heme A was stored at −25°C. A white precipitate formed slowly in these conditions and was discarded. The heme A content was

determined by the pyridine hemochromogen method [13] using a millimolar extinction coefficient of 27 cm^{-1} at the wavelength pair 587 minus 630 nm. The dimethylester–monomethylether derivative of heme A was prepared as described by Caughey et al. [14].

Cytochrome oxidase vesicles were prepared as described by Hinkle et al. [15] and rat-liver mitochondria were isolated by a conventional procedure [16].

Difference spectra were recorded with an Aminco DW-2 split-beam spectrophotometer or with a DBS-1 (Johnson Research Foundation, Philadelphia) dual-wavelength scanning spectrophotometer, using glass cuvettes with a 1 cm light path. The wavelength pairs used in dual-wavelength spectrophotometry are specified in the figure captions. All experiments reported were performed at room temperature.

Reagents used were commercial products of the highest purity available. FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) was a kind gift from Dr. P. G. Heytler.

Results

Figure 1 shows the Ca^{2+}-induced spectral shift in reduced isolated cytochrome oxidase as determined anaerobically, or aerobically in the presence of KCN. In the former case the state of cytochrome oxidase hemes may be abbreviated $a^{2+}a_3{}^{2+}$, while the predominant state of the enzyme is $a^{2+}a_3{}^{3+} \cdot$ HCN in the latter case. In Fig. 1A it is seen that the Ca^{2+}-induced shift of the α-band is only little or not at all affected by the change in redox and ligand state of cytochrome a_3, as previously described for intact mitochondria [5, 7]. However, in contrast to the results with intact mitochondria, cyanide has a dramatic decreasing effect upon the extent of Ca^{2+} shift in the Soret region in the isolated enzyme as shown in Fig. 1B. When the shift extent (peak to trough) in the isolated enzyme is compared with that in intact mitochondria (see ref. 7), and related to the amount of cytochrome aa_3 present, it is found that the extinction coefficients are the same for intact mitochondria and KCN-treated oxidase (approx. 2–3 $mM^{-1} \cdot cm^{-1}$ based on the content of cytochrome aa_3). It is therefore clear that the Ca^{2+}-induced Soret shift is much larger in the isolated enzyme in the absence of cyanide as it is in mitochondria (with or without cyanide). This indicates that Ca^{2+} affects an additional component in the isolated enzyme which is not affected in mitochondria, the shift in this component being abolished by cyanide. As the spectral contributions of ferrous cytochromes *a* and a_3 are roughly equal in the Soret region (see, e.g., ref. 17), it seems plausible to conclude that Ca^{2+} shifts the spectrum of both cytochromes *a* and a_3 in the isolated enzyme, but

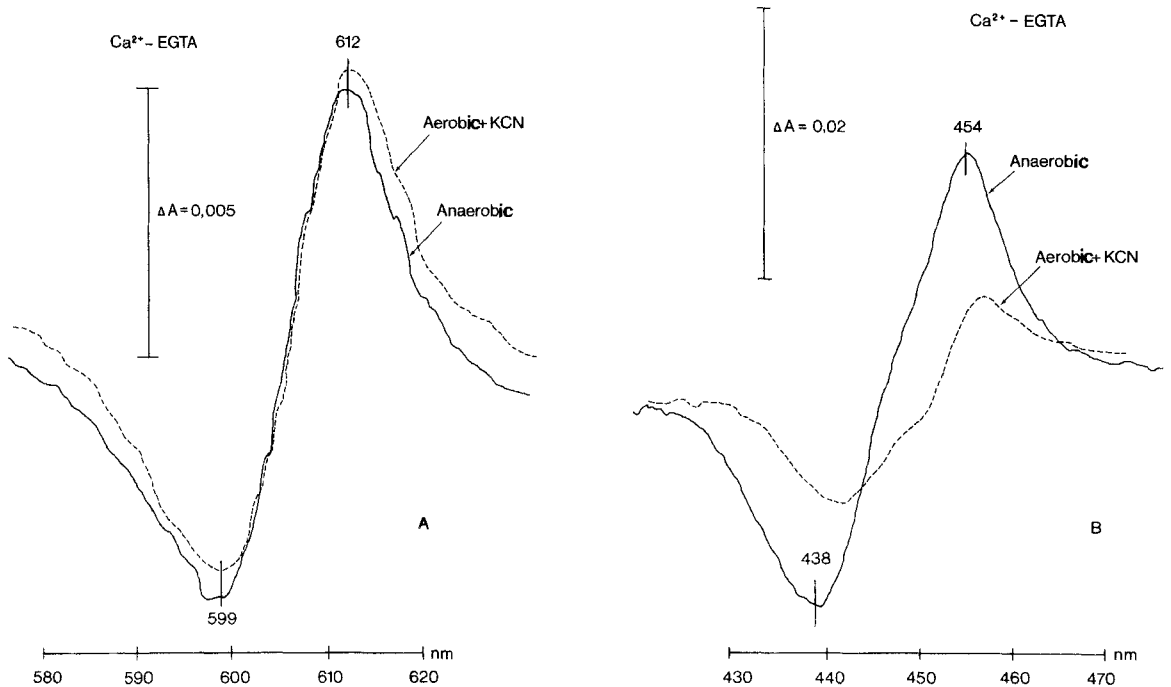

Fig. 1. Ca^{2+}-induced shift in the spectrum of ferrous isolated cytochrome oxidase in the presence and absence of cyanide. Isolated cytochrome oxidase was suspended in a medium consisting of 20 mM HEPES, 1% (v/v) Tween 80, and 40 μM EGTA, pH 7.8, to a concentration of 1.2 mg protein ml. In some experiments 4 mM KCN was added at this point (dotted lines). Cytochrome oxidase was reduced by 4 mM potassium ascorbate and 40 nM phenazine methosulfate (PMS). In the absence of cyanide (solid lines) anaerobiosis occurred quickly. The spectra of reduced anaerobic and reduced aerobic cyanide complex were recorded in the electrical memory of the spectrophotometer. Then 0.2 mM $CaCl_2$ was added and the respective difference spectra ("calcium minus no calcium") recorded in the α- and Soret bands (A and B), respectively.

is unable to shift the spectrum of ferrous a_3 in intact mitochondria. The cyanide sensitivity of the Ca^{2+}-induced shift in the isolated oxidase is thus simply due to the abolishment of the Soret band of a_3^{2+} when this heme is converted to the a_3^{3+} · HCN form. The KCN-sensitive Ca^{2+} shift in ferrous a_3 was abolished in cytochrome oxidase liposomes while the shift in cytochrome a was retained (not shown). Thus the shift in a_3 cannot be observed when the enzyme is membrane-bound.

Since the addition of cyanide has very little effect on the Ca^{2+}-induced shift in the α-region of the spectrum (Fig. 1A), it follows that the contribution of ferrocytochrome a_3 to the 605 nm band is minimal. This is in accordance with the "classical" interpretation of individual spectra of cytochromes a and a_3 [17, 18] and contradicts the suggestion that the two hemes would contribute equally to the 605 nm band [19]; see also [20].

The effect of pH on the Ca^{2+}-induced spectral shift is of some interest in view of the proposal [4, 7] that the energy-dependent shift in intact mitochondria may be the result of protonation rather than of Ca^{2+} binding. Figure 2 shows dual wavelength spectrophotometry of the isolated enzyme at two pH values. The upper traces show the redox changes measured at 605 minus 630

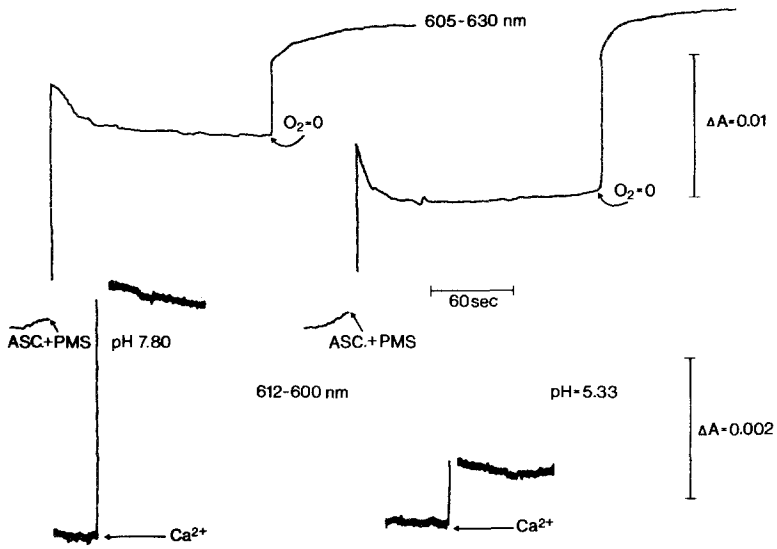

Fig. 2. pH dependence of the Ca^{2+}-induced spectral shift. Experimental conditions were the same as in Fig. 1. Reduction of the enzyme following addition of ascorbate and PMS was recorded at the wavelength pair 605 minus 630 nm at pH 7.80 and pH 5.33 (upper traces). After anaerobiosis, the wavelength pair was switched to 612 minus 600 nm to record the Ca^{2+}-induced shift on addition of 0.2 mM $CaCl_2$. Upward deflection of the traces signify an increase in absorption at the measuring wavelength (605 and 612 nm, respectively) with respect to absorption at the reference wavelength (630 and 600 nm).

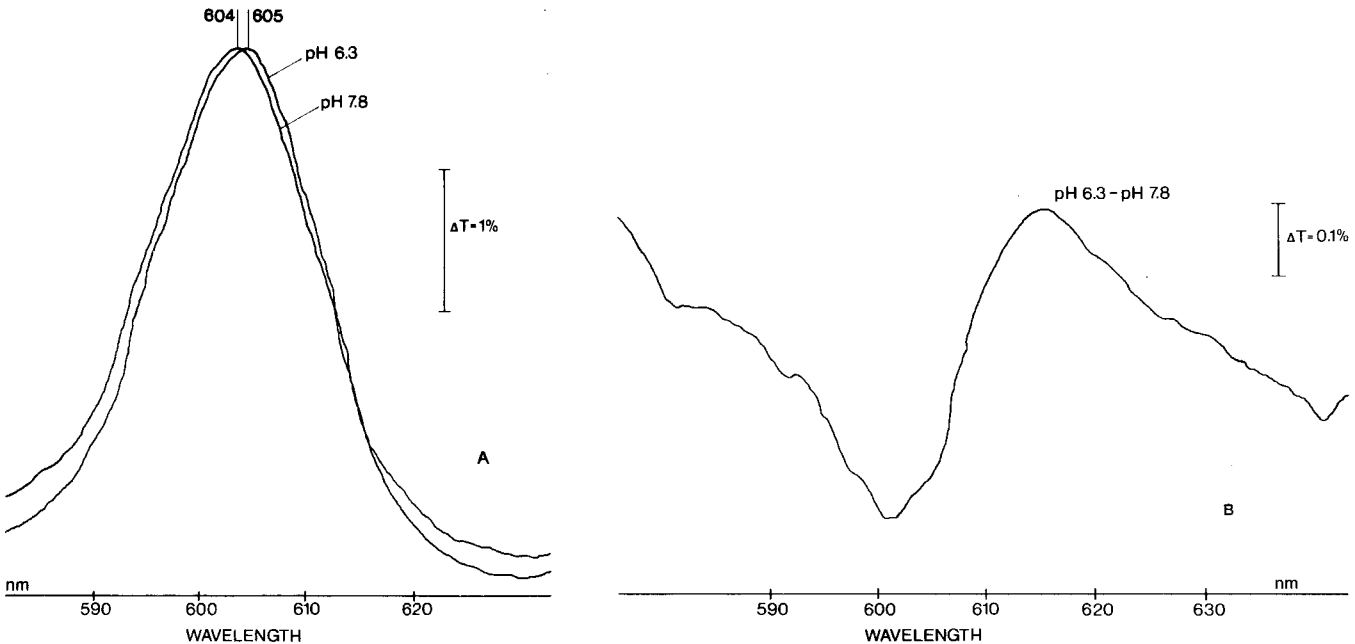

Fig. 3. Effect of pH on the absorption maximum of reduced minus oxidized cytochrome aa_3 in the α-region. Isolated cytochrome oxidase (0.9 mg protein) was suspended in 5 ml of medium containing 20 mM HEPES, 1% (v/v) Tween 80, and 0.2 mM EGTA, pH either 6.3 or 7.8. The enzyme solution was divided between two cuvettes (sample and reference). The sample cuvette was supplemented with 4 mM potassium ascorbate and 40 nM PMS. After anaerobiosis in the sample cuvette, the difference spectrum (sample *minus* reference) was recorded (A). Trace B shows the difference between the two spectra in A at pH 6.3 and 7.8 revealing the red shift caused by lowering the pH.

nm on respiration with ascorbate plus phenazine methosulfate (PMS). These traces are included to show that the overall spectral change of the 605 nm band from the aerobic to the anaerobic state is similar at pH 7.80 and 5.33 despite the difference in steady-state redox level. After anaerobiosis, the wavelength pair was changed to 612 minus 600 nm, which is appropriate for measurement of the Ca^{2+}-induced shift as seen from Fig. 1A. Figure 2 shows that the Ca^{2+}-induced shift is strongly pH-dependent, being diminished in extent at the lower pH.

Figure 3A shows the reduced minus oxidized 605 nm band of cytochrome oxidase at high and at low pH. It is clear that the band is shifted by approx. 1 nm to the red upon decreasing the pH from 7.8 to 6.3. The difference spectrum of this pH effect (Fig. 3B) is very similar if not identical to the shift induced by ATP in intact mitochondria (cf. ref. 7) and the Ca^{2+}-induced shift in the isolated enzyme (Fig. 1A). It seems likely therefore

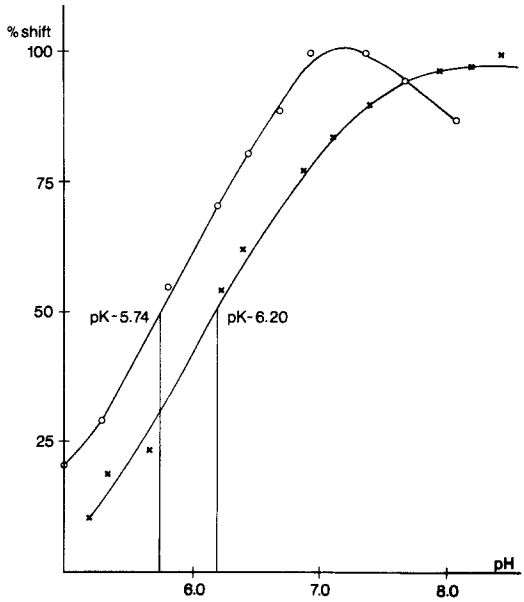

Fig. 4. Effect of pH on the extent of Ca^{2+}-induced spectral shift in the presence and absence of cyanide. Isolated cytochrome oxidase (0.9 mg protein/ml) was suspended and reduced by ascorbate plus PMS as described in the legends to Figs. 1 and 2 at various pH values in the presence or absence of potassium cyanide. The extent of Ca^{2+}-induced spectral shift in the α-band was measured as described in the legend to Fig. 2, and plotted as a function of medium pH. x, anaerobic without cyanide; O, aerobic with cyanide.

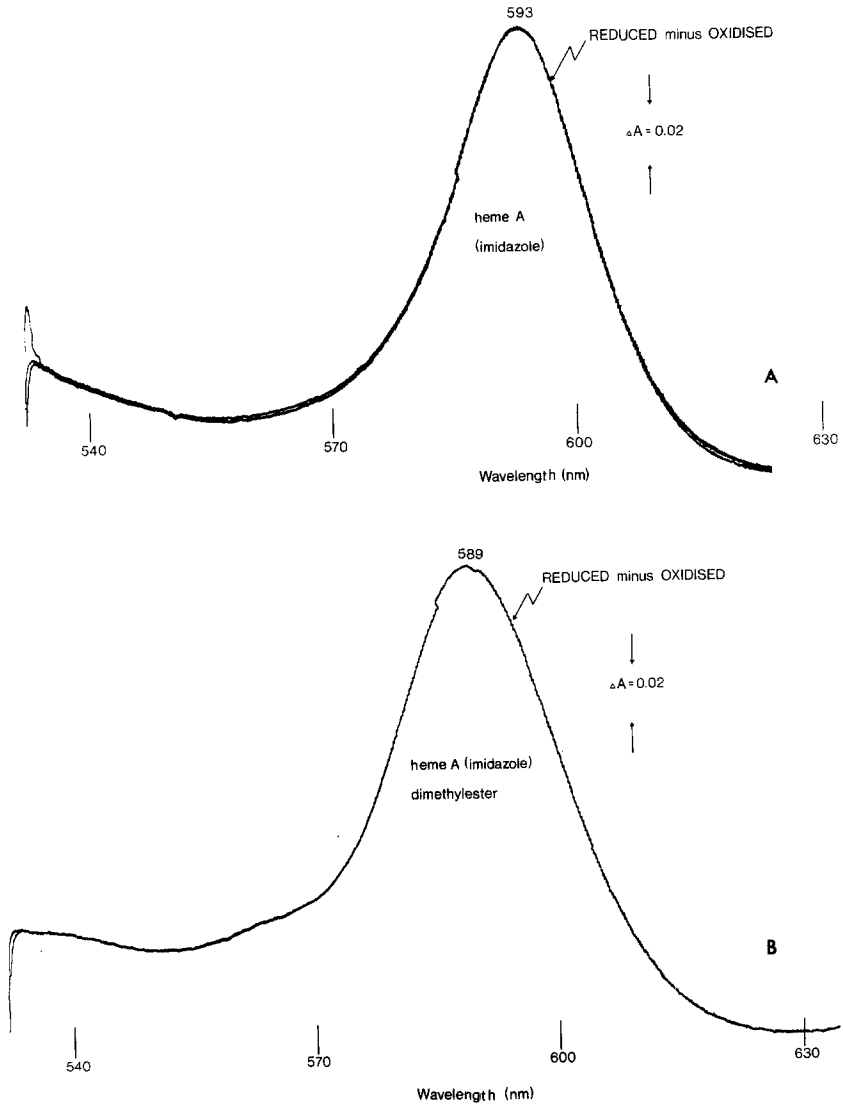

Fig. 5. Reduced minus oxidized difference spectra of bis-imidazole heme A complexes; the effect of Ca^{2+}. A 50-μl portion of the ethyl ether solution of heme A or methylated heme A (see Materials and Methods) was added to a cuvette containing 2 ml of 50 mM imidazole-HCl buffer, pH 7.4, 20% (v/v), ethanol, and 0.5 mM EDTA. The ethyl ether was evaporated under a stream of N_2 and the oxidized α-band was recorded into the electrical memory of the spectrophotometer. Then a few grains of solid sodium dithionite was added and the reduced minus oxidized difference spectrum was recorded using 650 nm as reference wavelength. Traces A and B show these difference spectra for the untreated and methylated heme A complexes, respectively. Following this, the reduced spectrum of each complex was recorded into the spectrophotometer memory, followed by addition of 1.0 mM $CaCl_2$. The Ca^{2+}-induced spectral change (reduced heme A + Ca^{2+} *minus* reduced heme A) was recorded in C for the bis-imidazole heme A, and in D for the bis-imidazole heme A dimethyl ester. Upward deflection in the latter spectra with respect to the baseline corresponds to a Ca^{2+}-induced increase of absorption.

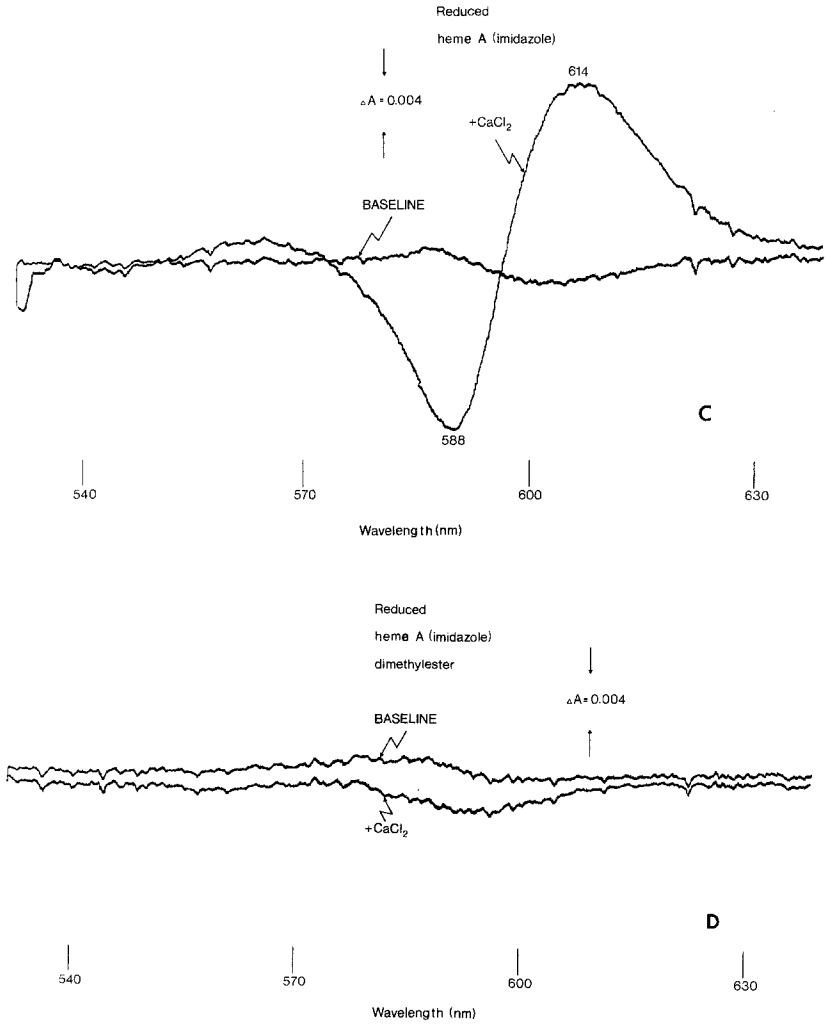

Fig. 5. Continued.

that the diminution of the Ca^{2+} shift at low pH (Fig. 2) is simply due to H^+ having already shifted the band by an analogous mechanism.

From these experiments we conclude that both Ca^{2+} and H^+ are able to induce a configurational change in cytochrome *a* that results in a 1–2 nm red shift of the 605 nm band. pH titrations (see Fig. 4) suggest that the apparent p*K* of the acidic group that binds Ca^{2+} and H^+ is of the order of 6.

The specificity of Ca^{2+} and H^+ in causing this effect is quite unique. From a long series of cations tested, only Mn^{2+} causes a slight reduction in the extent of the Ca^{2+} shift, but induces no spectral shift by itself. Cations tested in this respect included La^{3+}, Eu^{3+}, Mg^{2+}, Sr^{2+}, Zn^{2+}, Ni^{2+}, Ba^{2+}, Li^+, K^+, and Na^+, none of which were able to influence the Ca^{2+}-induced shift or produce a shift by their own. Also the polycation protamine sulfate had no effect. These findings suggest that the Ca^{2+}- and H^+-induced spectral shift is a specific effect. Nonspecific surface charge effects can readily be excluded.

Although the Ca^{2+}-induced shift in cytochrome a_3 is abolished by cyanide in the isolated enzyme, cyanide nevertheless exerts an influence on the properties of the remaining shift in ferrocytochrome *a*. As shown in Fig. 4, in which data similar to those in Fig. 2 are plotted as percent spectral shift (of the 605 nm band) vs. pH, the presence of cyanide alters the apparent p*K* of the reactive group by nearly 0.5 pH units. Similar findings were also made in titrations of the shift in the Soret band (not shown). Thus the acidic group responsible for the spectral shift in ferrocytochrome *a* is affected by either the redox, the ligand, or the spin state of cytochrome a_3. This interesting finding is another example of heme/heme interaction between cytochromes *a* and a_3, which is a very prominent feature of cytochrome oxidase (see, e.g., refs. 20 and 21).

The spectral shift in reduced cytochrome oxidase induced by Ca^{2+} or by H^+ could easily be due to binding of these ions to an acidic site in the protein, followed by an indirect perturbation of the heme or its immediate environment mediated by the polypeptide framework. However, the effect could also be directly exerted on the heme, and if so, most likely through cation interaction with the ionized propionate carboxyl groups. To test this possibility we performed experiments with model heme A compounds.

As shown in Fig. 5C, calcium shifts the spectrum of reduced bis-imidazole heme A (λ_{max} = 593 nm, Fig. 5A), to the red, in good analogy with the effect of Ca^{2+} on cytochrome oxidase. Moreover, after methylation of the propionate carboxyls according to Caughey et al. [14], Ca^{2+} no longer perturbed the spectrum (Fig. 5D). Although the hydroxyl group in the farnesyl ethyl side chain of heme A is also methylated by this procedure, this hydroxyl is not likely to interact with Ca^{2+}. Moreover, the Ca^{2+}-induced shift was also observed with protoheme which lacks the farnesyl chain (not shown). We therefore conclude that the calcium shift with *bis*-imidazole heme A is

due to an interaction of this cation with the propionate carboxyls. In contrast to the case with isolated cytochrome oxidase, however, the shift in bis-imidazole heme A spectrum was also exerted by several other cations, bivalent cations being much more effective than monovalent cations. The shift was thus produced by, e.g., Mg^{2+}, Mn^{2+}, Ni^{2+}, and K^+ in contrast to the extraordinarily high cation specificity found with the isolated enzyme.

Discussion

Our studies provide an identification of the molecular events that underlie the Ca^{2+}- and H^+-induced shift in the spectrum of reduced cytochrome aa_3. The model studies with *bis*-imidazole heme A and its methylated derivative strongly indicate that Ca^{2+} and other bivalent cations shift the spectrum of the reduced heme through an interaction with the propionate carboxyl groups. It seems quite possible therefore that the Ca^{2+} and H^+ effect on cytochrome aa_3 is also exerted by this mechanism. This is supported by the finding that the apparent pK of the Ca^{2+}- and H^+-binding group is of the order of 5.8–6.2, this being precisely the pK range expected for porphyrin dicarboxylic acids [22].

In mitochondria, the spectrally shifted species can be unequivocally identified as ferrocytochrome *a* on the basis of the absence of a significant change in the spectral shift on drastic modification of the spectral properties of cytochrome a_3. The inability of Lindsay [23] to identify the spectrally shifted species as cytochrome *a* in redox titrations of ATP- and CO-supplemented mitochrondria was probably due to the absence of Ca^{2+}-chelating agents in their experiments. Under such conditions the spectral difference between cytochrome *a* in uncoupled and ATP-supplemented mitochondria is minimal or absent since the ferrocytochrome spectrum is equally red-shifted by Ca^{2+} in the uncoupled state as it is by "energization" in the ATP-supplemented coupled state.

Our previous finding that the Ca^{2+} shift in mitochondria is exerted from the outside of the mitochondria membrane [7] and does not require translocation of the cation across the permeability barrier provides a clue for the location of the cytochrome *a* heme in cytochrome oxidase *in situ*. Our data suggest, when taken together, that the heme of cytochrome *a* may be located near the outside (C side) of the inner mitochondrial membrane. This conclusion is strongly supported by the recent findings of Ohnishi et al. ([24] and personal communication) of magnetic interactions between impermeable paramagnetic ions with the heme of cytochrome *a* specifically under conditions where the former are added to the C side of the mitochondrial membrane. Although we may thus conclude that heme *a* must be near the C

side of the membrane, our data also suggest that the propionate carboxyls are not freely exposed to the external aqueous phase as the spectral shift shows high specificity toward Ca^{2+} and H^+ whereas free heme A is perturbed by a variety of bivalent (and by monovalent) cations. The reason for this high specificity is not understood, but may be due to a highly specific configuration of the propionate carboxyls *in situ* and/or location of these residues at the bottom of a channel or a well in the protein structure through which penetration is highly restricted.

Our previous proposal that the energy-dependent red shift in ferrous cytochrome *a* of intact mitochondria might be due to the binding of H^+ to cytochrome aa_3 finds support in the present work, where H^+ was indeed shown to compete with Ca^{2+} in producing the effect in isolated oxidase. The fact that the red shift is as extensive upon mitochondrial "energization" as it is with Ca^{2+} indicates, again on the basis of the data presented here, that the region in the vicinity of the propionate carboxyls of heme *a* is dramatically altered in the "high energy state." We have shown previously that this "energization" can also be accomplished in the presence of oligomycin by applying an electrical diffusion potential (with positive polarity in the C phase) across the mitochondrial membrane [6]. The spectral shift may thus be due to a field-induced change in the p*K* of the carboxylic group and/or to a field-induced accumulation of H^+ ions from the aqueous C phase in the vicinity of the carboxyls. These two alternatives are merely two ways of looking at the same kind of electrostatic effect. The extent of spectral shift on mitochondrial "energization" (more than 91% of the Ca^{2+}-induced shift; cf. ref. 7) suggests that the electrostatic effect corresponds to a change in p*K* or pH of at least two units. This is a large effect and deserves further study since it might indicate that a large fraction of the electrostatic potential drop across the mitochondrial membrane occurs across only a very short distance in the cytochrome oxidase protein. If this is a correct interpretation, it is indicative of the presence of ionic (protonic) channels in the protein. These possibilities clearly demonstrate that further detailed studies of the spectral shift could yield valuable information about the molecular mechanism of mitochondrial energy transduction.

It is interesting that Ca^{2+} causes a spectral shift also in ferrocytochrome a_3 in the isolated enzyme. However, this effect disappears upon organization of the enzyme in a phospholipid membrane. Thus Ca^{2+} shifts the spectrum of cytochrome *a* alone in mitochondria and in cytochrome oxidase proteoliposomes. This suggests that the environment of the propionates of hemes *a* and a_3 are different in the membrane-bound enzyme and that the incorporation of cytochrome oxidase into a phospholipid membrane affects the environment of heme a_3 in particular. This could be related with the known differences in reactivity of cytochrome a_3 in the isolated enzyme as compared to that bound to phospholipid membranes.

Except for the likely participation of the propionate carboxyl groups in the Ca^{2+}- or H^+-induced spectral shift, the mechanism of the red shift in the absorption spectrum is not fully understood. Two possible mechanisms, one general and one more specific, may be discussed here although final proof for neither is available at the present time.

Propionic acid carboxyls are generally considered to be rather well insulated electronically from the porphyrin nucleus so that substitutions have small effects on the optical spectra in contrast to the very large effects seen on substituting carbonyl or vinyl side chains (see, e.g., ref. 22). However, due to the negative charge of the ionized carboxylates and their relatively short distance from the pyrrole nitrogens of the porphyrin nucleus, an electrical field effect is exerted. When the electrical charges are neutralized by Ca^{2+} or by H^+, the electron density at the periphery of the porphyrin ring may be slightly increased. This would be expected to decrease the energy of the optical transitions, i.e., to yield a red shift in the spectra.

A more speculative but also more interesting proposal is made possible by the finding of Caughey et al. [25], who showed by NMR that there is an interaction between the methyl hydrogens of a methylated propionate with the nitrogenous axial ligand in isolated heme derivatives. This could mean that the propionate is mobile enough and sufficiently long to "flip up" from its usually depicted position below the edge of the porphyrin ring to make direct contact with an imidazole axial ligand. Preliminary trials with models suggest that this may indeed be feasible stereochemically.

It is suggested that the heme may take two configurations with respect to the propionate side chain. In the "strained" configuration the propionate is bent toward the axial imidazole ligand, forming a hydrogen bond with the imidazole nitrogen. This leads to a partial positive charge at the iron-bound imidazole nitrogen, which in turn could lead to mobilization of electrons of the iron's d_{xz} and d_{yz} orbitals into partial bonding of π-bond character to the imidazole. Since electrons in the two *d* orbitals communicate with electrons of the porphyrin ring, this is expected to lead to a blue-shifted absorption band.

The hydrogen bond may be broken by binding either Ca^{2+} or H^+ to the propionate carboxylate with consequent movement of the propionate to its conventionally depicted position below the porphyrin ring. Through a reversal of the effects suggested above, this is expected to lead to regaining of electrons by the porphyrin ring and a resultant red shift in the optical spectrum.

Although very speculative, this proposal provides an interesting mechanism for the redox-linked proton translocation catalyzed by cytochrome oxidase [26]. The large change in pK of the propionate carboxyl of cytochrome *a* and/or the large acidification in its vicinity on mitochondrial energization may be taken to suggest that this site is close to the output "channel" of the oxidase proton pump [8], or that it itself constitutes part of

the molecular machinery of this pump [26]. The extraordinary specificity of this site to H^+ and Ca^{2+} is consistent with this idea.

Acknowledgments

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