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THE INFLUENCE OF pH ON THE EPR AND REDOX PROPERTIES OF CYTOCHROME *c* OXIDASE IN DETERGENT SOLUTION AND IN PHOSPHOLIPID VESICLES

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Summary

The EPR signals of oxidized and partially reduced cytochrome oxidase have been studied at pH 6.4, 7.4, and 8.4. Isolated cytochrome oxidase in both non-ionic detergent solution and in phospholipid vesicles has been used in reductive titrations with ferrocytochrome *c*.

The *g* values of the low- and high-field parts of the low-spin heme signal in oxidized cytochrome oxidase are shown to be pH dependent. In reductive titrations, low-spin heme signals at *g* 2.6 as well as rhombic and nearly axial high-spin heme signals are found at pH 8.4, while the only heme signals appearing at pH 6.4 are two nearly axial *g* 6 signals. This pH dependence is shifted in the vesicles.

The *g* 2.6 signals formed in titrations with ferrocytochrome *c* at pH 8.4 correspond maximally to 0.25—0.35 heme per functional unit (aa_3) of cytochrome oxidase in detergent solution and to 0.22 heme in vesicle oxidase. The total amount of high-spin heme signals at *g* 6 found in partially reduced enzyme is 0.45—0.6 at pH 6.4 and 0.1—0.2 at pH 8.4. In titrations of cytochrome oxidase in detergent solution the *g* 1.45 and *g* 2 signals disappear with fewer equivalents of ferrocytochrome *c* added at pH 8.4 compared to pH 6.4.

The results indicate that the environment of the hemes varies with the pH. One change is interpreted as cytochrome a_3 being converted from a high-spin to a low-spin form when the pH is increased. Possibly this transition is related to a change of a liganded H_2O to OH^- with a concomitant decrease of the redox potential. Oxidase in phosphatidylcholine vesicles is found to behave as if it experiences a pH, one unit lower than that of the medium.

Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) has been subjected to intense studies for several decades but the

mechanism of the electron transfer from the one-electron donor cytochrome *c* to the four-electron acceptor dioxygen is still largely unknown. Knowledge of the redox properties of the four metal ion centers in cytochrome oxidase provides a background for mechanistic studies. Since protons are involved in the formation of water by dioxygen, and may also be pumped across the mitochondrial membrane by cytochrome oxidase [1,2], the pH could be expected to influence the redox properties of the oxidase. Such influence has been found by several research groups both on isolated enzyme [3—5] and on cytochrome oxidase in submitochondrial particles [6—8].

We have studied both oxidized and partially reduced cytochrome oxidase with EPR spectroscopy which, in contrast to optical spectroscopy, offers the advantage that concentrations can be obtained without the knowledge of empirically determined extinction coefficients. Cytochrome oxidase has here been titrated with ferrocytochrome *c* at different pH values and the heme signals appearing at partial reduction were studied with respect to both signal shape and intensity.

In our earlier studies of cytochrome oxidase incorporated into phospholipid vesicles [9], we found that the EPR parameters of the partially reduced enzyme were different from those found for oxidase in detergent solution [10]. These studies have been extended to include the pH dependence of the redox components and their EPR signals. In addition, a comparison with oxidase in detergent solution has been made.

Materials and Methods

Cytochrome *c* oxidase was prepared from beef heart mitochondria either by the method of Rosén [11] or van Buuren [12] with an extra dialysis step to remove cholate and ammonium sulphate. The molecular activity of the enzyme at infinite cytochrome *c* concentration was 25—50 s^{-1} in 0.1 M potassium phosphate and 0.5% Tween 80 at pH 7.4. Cytochrome *c*, purchased from Sigma (horse heart cytochrome *c* type VI) was further purified by ion-exchange chromatography on Amberlite CG-50. Vesicle oxidase was prepared by a procedure including removal of detergent by dialysis and incorporation into vesicles by sonication [9]. For the preparation of anaerobic EPR samples as well as integration of EPR signals, see ref. 10. EPR spectra were recorded with Varian E-3 or E-9 spectrometers at 77 or 12 K.

Earlier EPR titrations of cytochrome oxidase at high pH were performed in Tris buffer [5,7]. We used potassium phosphate buffers with the same ionic strength in all experiments thereby avoiding the difficulties with Tris buffer reported in [13]. At pH 6.4 and 7.4 the phosphate concentration was 100 mM with 50 mM KCl added at the lower pH. At pH 8.4 the phosphate concentration was decreased to approx. 80 mM. In experiments in which the oxidase was not in a phospholipid environment 0.5% Tween 80 was added to the buffers. The change in pH of the enzyme solution was accomplished by dialysis against the proper buffer. When the enzyme was reduced anaerobically with ferrocytochrome *c*, the incubation time was 20 min at approx. 22°C.

The initial velocities of oxidation of ferrocytochrome *c* (30 μM), measured at 550 nm in a Beckman Acta MIV spectrophotometer, were used for com-

parison of the activities of oxidase in different environments. In all cases the activities are based on the total concentration of cytochrome oxidase with no corrections made for the enzyme that may not be accessible to fast reduction in the case of vesicles.

Results

The heme signal in oxidized enzyme

Fig. 1 shows the effect of pH on the position of the low- and high-field peaks of the low-spin heme signal in oxidized cytochrome oxidase. Both in nonionic detergent and in phospholipid vesicles the resonance around g 3 shifts towards lower field and the one around g 1.45 towards higher field when the pH was increased.

The heme signals in partially reduced enzyme

In anaerobic titrations with ferrocytochrome c the shape of the high-spin heme signals was strongly dependent on the pH of the medium. Fig. 2 shows the high-spin signals obtained at pH 6.4, 7.4, and 8.4 in nonionic detergent and in phospholipid vesicles. The nearly axial g 6 signal appearing in vesicles at neutral pH [9] was also formed with the oxidase in nonionic detergent but at a lower pH. The rhombic type of high-spin signal appeared in vesicle oxidase when the pH was increased (Fig. 2b). In all samples where the rhombic g 6 signal was found the low-spin signal at g 2.6 also appeared. Thus, the g 2.6 signal was seen in nonionic detergent at neutral and alkaline pH but only at pH 8.4 in vesicles.

The shape of the heme signals at different degrees of reduction

The high pH type of g 6 signal consists of at least two rhombic and one nearly axial component [10]. Fig. 3 shows heme signals appearing in oxidase at different degrees of reduction. The shapes of the g 6 signals in vesicle oxidase at pH 7.4 (Fig. 3a) and the low and high pH types of g 6 signals of oxidase in detergent solution (Fig. 3b and c) are shown. At pH 7.4 in vesicles (Fig. 3a) and at pH 6.4 in detergent solution (Fig. 3b), the ratio between the signals with peaks at 0.1090 and 0.1065 T increased with the amount of ferrocytochrome c added. The relative amounts of the axial and rhombic g 6 components at high pH (Fig. 3c) changed in such a way that the axial signal increased as the reduction proceeded.

It has been shown [10,14] that the g 2.6 signal at low degrees of reduction consists of one component (g 2.59, 2.16, 1.86) which splits into two (g 2.61, 2.16, 1.86 and g 2.57, 2.16, 1.86) on further reduction. We found that the two signals have different saturation behaviour, the signal at g 2.57 being most easy to saturate. When the g 2.6 signals were recorded under non-saturating conditions (Fig. 3d) the ratio between the amplitudes of the high- and low-field parts increased with progressive reduction. We failed to find a simple correlation between the three high pH g 6 signals and the three g 2.6 signals.

Quantitations of EPR signals in reductive titrations

The concentrations of the heme signals of cytochrome oxidase have been

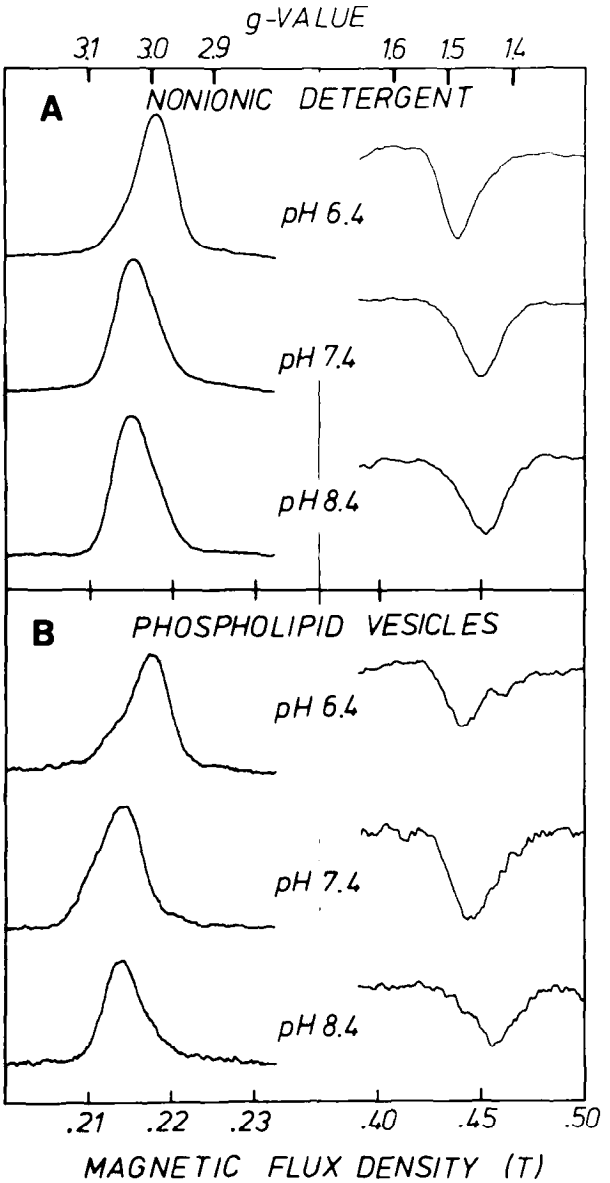

Fig. 1. EPR spectra of the low- and high-field parts of the low-spin heme signal in oxidized cytochrome oxidase at different pH values in 0.5% Tween 80 and in phospholipid vesicles. The buffers used are described in Materials and Methods, and the enzyme concentration was approx. 100 μM in Tween solution (A) and 30 μM in the vesicles (B). The *g* values obtained were in (A) 3.00 and 1.49, 3.03 and 1.45, and 3.03 and 1.45, at pH 6.4, 7.4 and 8.4, respectively, and in (B) 3.00 and 1.48, 3.01 and 1.47 and 3.04 and 1.42 at pH 6.4, 7.4 and 8.4, respectively. The spectra were recorded at microwave frequency 9.12 GHz, microwave power 2 mW, modulation amplitude 2 mT and temperature 12 K. The high-field signals are in all cases shown with 5 times higher magnification than the low-field signals.

estimated in anaerobic titrations with ferrocytochrome *c*. The results with the oxidase in nonionic detergent at pH 6.4 and 8.4 are shown in Fig. 4. The maximal amount of *g* 6 signal obtained at pH 6.4, corresponds to 0.46 heme

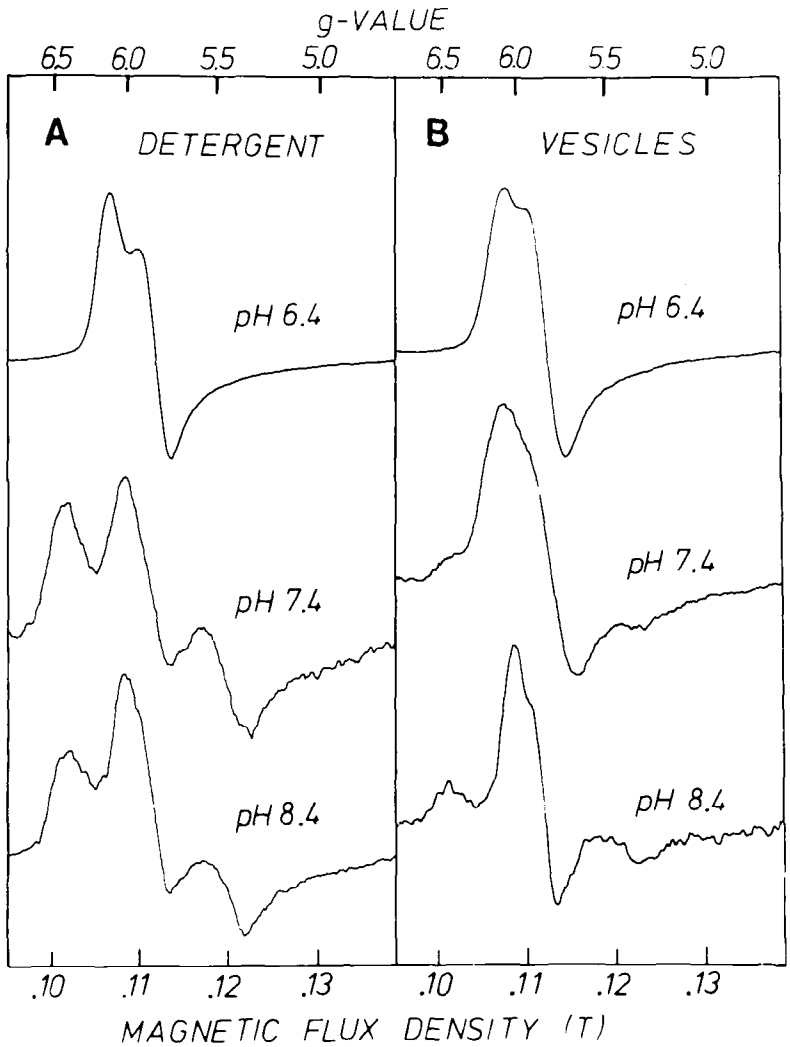

Fig. 2. EPR spectra of the high-spin heme signals of partially reduced cytochrome oxidase at different pH in 0.5% Tween 80 and in phospholipid vesicles. Approx. 2 equivalents of ferrocytochrome *c* were added anaerobically. In (A) the oxidase in Tween 80 is shown and in (B) the vesicle oxidase. Both enzyme concentrations and the conditions used for the recording of the spectra were the same as in Fig. 1.

per functional unit, which is significantly larger than the 0.23 heme found earlier at neutral pH [10] (see Discussion). An intense *g* 2.6 signal is also formed at pH 8.4, equivalent to maximally 0.25 heme per functional unit. The amount of *g* 6 heme at pH 8.4 is 0.18 per cytochrome aa_3, which means that at this pH and at half reduction the *g* 2.6 signal has become the major heme signal. While, in our many titrations of van Buuren preparations [12] with ferrocytochrome *c* at pH 7.4, the maximal intensity of the *g* 6 and *g* 2.6 signals on partial reduction has varied somewhat, the *g* 6 signals have always been the larger ones. Titrations of an oxidase sample prepared by the method of Rosén [11] gave *g* 6 and *g* 2.6 signals corresponding to the following maximal amounts of heme: 0.58 and 0.0 at pH 6.4, 0.13 and 0.22 at pH 7.4, and 0.12

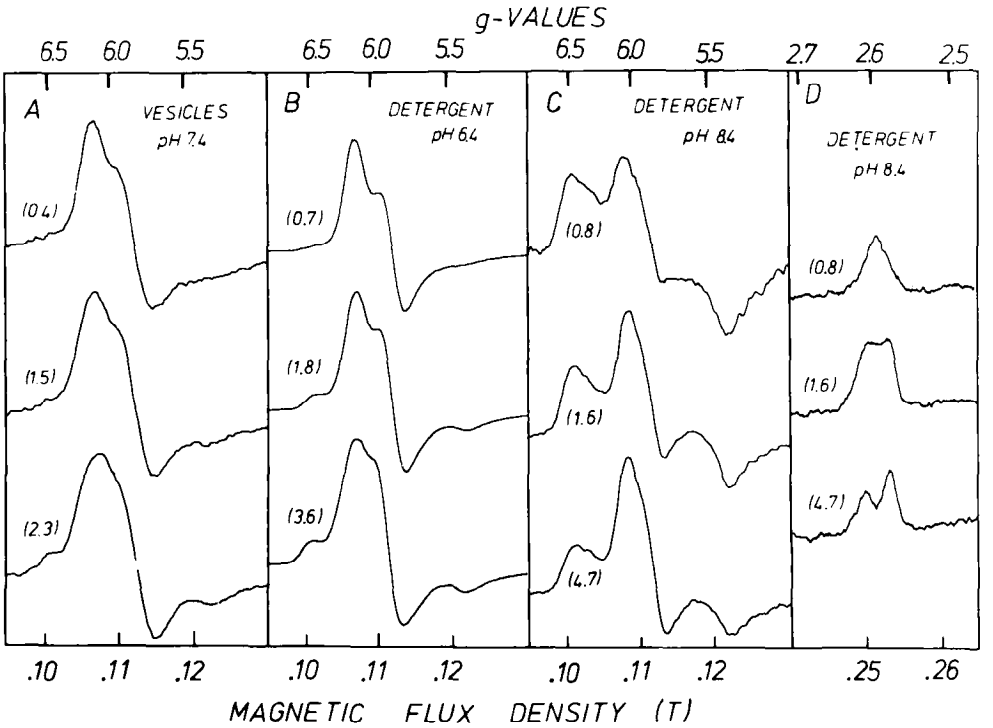

Fig. 3. EPR spectra of the low- and high-spin heme signals appearing at varying degrees of reduction of cytochrome oxidase in different milieus. A, B and C show the high-spin heme signal of vesicle oxidase at pH 7.4, of oxidase in Tween 80 at pH 6.4 and of oxidase in Tween 80 at pH 8.4, respectively. D shows the low-spin signal of oxidase in Tween 80 at pH 8.4. The numbers within parentheses show the amount of ferrocytochrome *c* added per cytochrome aa_3. The buffers, concentration of cytochrome oxidase and recording conditions were the same as in Fig. 1 except that in D the microwave power was 0.2 mW and the modulation amplitude 1 mT.

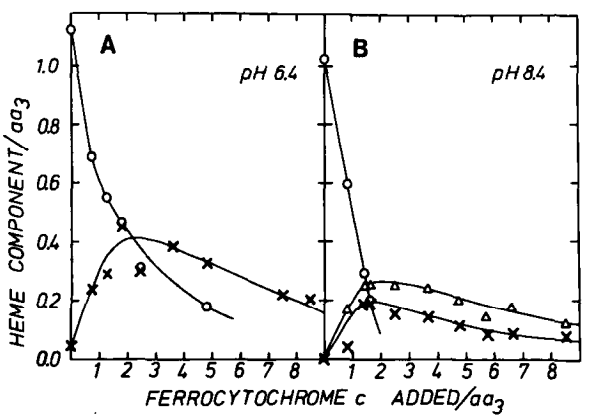

Fig. 4. Reductive titrations of cytochrome oxidase in Tween 80 followed by EPR spectroscopy at pH 6.4 and 8.4. The amounts of the different heme signals are plotted against the amount of ferrocytochrome *c* added, all normalized to the concentration of the functional unit of cytochrome oxidase. The buffers and concentrations of cytochrome oxidase were as described in Fig. 1. The pH was 6.4 and 8.4 in A and B, respectively. ○, represents the *g* 1.45 signal; ×, is the *g* 6 signal; and △, is the *g* 1.86 signal. The spectra were recorded with the same conditions as in Fig. 1 except for the *g* 1.86 signals which were recorded with microwave power 0.2 mW.

and 0.32 at pH 8.4. We have also titrated vesicle oxidase, but have had difficulties in attaining equilibrium. Even though it was shown by stopped-flow measurements [9] that only half of the cytochrome oxidase in vesicles was accessible to fast reduction by cytochrome *c*, it is probable that with the long incubation times used in this study most of the oxidase can be reduced. The *g* 6 signals at maximum amounted to 0.7 and 0.15 heme at pH 6.4 and 8.4, respectively. At pH 8.4 the *g* 2.6 signals corresponded maximally to 0.22 heme.

Fig. 4 shows the disappearance of the *g* 1.45 signal in titrations of the van Buuren preparation. At pH 6.4 more equivalents of ferrocytochrome *c* were needed to reduce the *g* 1.45 signal compared to the situation at pH 8.4 (see Discussion). More ferrocytochrome *c* was also needed for reduction of the copper signal at the lower pH (data not shown). Both signals showed the same pH dependence in the Rosén preparation. In titrations of the vesicle oxidase it was not possible to intergrate the *g* 1.45 signals, due to the low concentration of the enzyme (30 μM).

Activity measurements

The activity of both the vesicle and the detergent oxidase increased 2—3 times when the pH was decreased by 1 unit. The oxidase was about twice as active in vesicles as in detergent solution in the pH range investigated.

Discussion

The low-spin heme signal in the oxidized enzyme

Hartzell and Beinert [14] showed that the position of the *g* 3 signal in oxidized cytochrome oxidase is very sensitive to a number of factors, such as anions, ionic strength, gases and pH. We found that the *g* values of this heme signal were influenced by the buffer pH. Part of the shift between pH 6.4 and 7.4 may, however, be due to the presence of Cl^- at pH 6.4 [14].

High- and low-spin heme signals in partially reduced enzyme

In partially reduced enzyme the high-spin heme signals at *g* 6 changed from the nearly axial to the rhombic type as the pH was increased (Fig. 2) with concomitant loss of signal intensity. At the same time significant amounts of a low-spin heme signal (at *g* 2.6, 2.16, 1.86) appeared. With the assumption that the *g* 3 and *g* 6 signals originate from different hemes, the observations above imply that the *g* 6 signals and the *g* 2.6 signals are the low and high pH forms of the same species. pH dependent, high-to-low spin transitions are known to occur in other heme proteins, and have in ferrimyoglobin, for example, been interpreted as a change of the sixth ligand from H_2O to OH^- [15]. It is therefore striking that the *g* values observed here for cytochrome oxidase are very close to those of the low-spin form of ferrimyoglobin (*g* 2.57, 2.14, 1.84) [16]. The optical spectrum of oxidized cytochrome oxidase shows a shift of the γ-band to higher wavelength as the pH is increased (Rosén, S., unpublished results) which is also consistent with a high-to-low spin transition [17]. Magnetic circular dichroism [18] and magnetic susceptibility [19] measurements have been interpreted in terms of one high-spin and one low-spin heme in both the oxidized and reduced oxidase. These experiments were, however,

carried out at pH 7.4 and 7.0, respectively, and it would be of interest to test the suggested pH induced high-to-low spin transitions with these other methods.

The interpretation that the *g* 2.6 signal appears by the dissociation of a proton from a heme-coordinated H_2O molecule presupposes that cytochrome a_3 is available to external ligands. This is consistent with its function as the dioxygen binding site.

As the *g* 3 and *g* 6 signals are ascribed to different hemes, it may be surprising that the changes of pH affect both the *g* values of one of the hemes (Fig. 1) and induces a high-to-low spin transformation in the other. This could depend on the presence of one titrating group in the vicinity of each heme but equally probable it is yet another manifestation of 'heme-heme' interaction [6,20,21].

Comparison of cytochrome oxidase in detergent solution and in phospholipid vesicles

The change in the *g* 6 signals when going from low pH to high pH (Fig. 2), the appearance of the *g* 2.6 signals and the shifts in the *g* 3 signal (Fig. 1) all show that the vesicle oxidase titrates at a higher pH than the detergent oxidase. The difference is between 1 and 2 pH units. Our activity measurements show that transfer of oxidase from detergent solution to vesicle enhances the activity to the same extent as lowering the pH by about 1 unit. This could mean that the local 'pH' at or in the phospholipid bilayer is lower than in the buffer bathing the vesicle or that the immediate environment of the titrating groups has been altered in vesicle oxidase. A third possibility is that phospholipids as well as a lower pH induce the same kind of conformational changes in the enzyme. Freezing effects, like those suggested in [22], are not likely as the low- and room-temperature data are consistent with each other.

In working with purified oxidase one must always make sure that the isolation procedure does not seriously alter the properties of the enzyme. One reason for examining the vesicle oxidase is to try to mimic the hydrophobic milieu of the mitochondria while still having the advantage of working with a pure enzyme. It is, therefore, of importance to note that the *g* 2.6 signal is also present in submitochondrial particles and that the amount of this signal decreases as the pH is descreased [7]. The mitochondrial *g* 6 signals consist of both rhombic and axial components at the pH studied (pH 7.0) [7], but it is not known if the signals change to the low pH type with a decrease in pH.

Redox titrations

One important result of our titrations is that the maximal amounts of *g* 2.6 signals are large enough to balance the decreasing intensity of the *g* 6 signals when the pH is increased. Even though other authors have under some conditions observed strong *g* 2.6 signals [14], the signals have generally been neglected at neutral pH [7,14].

The observation that less ferrocytochrome *c* was needed to reduced the *g* 1.45 signal when the pH was increased (Fig. 4, see also ref. 5) can be interpreted as a decrease in redox potentials of the other electron acceptors in the oxidase. This would actually be expected for cytochrome a_3, as a displacement of H_2O for OH^-, seen as a high-to-low spin transition, has been observed

to lead to a decrease in redox potential [15]. In a system with potential couplings of the type used for simulations of EPR data [21], the titration behaviour of cytochrome *a* is very sensitive to changes in the redox potential of cytochrome a_3. Earlier optical titrations [3,4,6] were interpreted mainly as a decrease in the redox potential of a high-potential heme as the pH was increased. However, the conditions used in these titrations deviated from the present in one or several aspects, e.g. type of reductant used, presence of redox mediators or the physical properties of the enzyme. In addition the type of data analysis performed differs.

Concluding remarks

It has long been known that the activity of isolated cytochrome oxidase increases, when the pH is decreased or when phospholipid is added to the enzyme solution [23]. We have found that both these treatments of cytochrome oxidase result in the same kind of changes in EPR signals. In the partially reduced enzyme these conditions cause a replacement of the rhombic high-spin heme signals with essentially axial signals and a disappearance of the low-spin heme signal at *g* 2.6. Also the *g* values of the heme signals in the oxidized enzyme are changed. A further study of these pH effects might yield clues to the role of protons in cytochrome oxidase reactions. In addition, experiments at low pH should be of interest as the EPR picture is simplified concomitantly with an increase in catalytic activity.

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