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A ^{1}H-NMR LONGITUDINAL RELAXATION STUDY OF THE INTERACTION BETWEEN CYTOCHROME *c* AND CYTOCHROME *c* OXIDASE

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The interaction between the oxidized forms of cytochrome *c* and cytochrome *c* oxidase (EC 1.9.3.1) has been investigated by ^{1}H-NMR longitudinal relaxation measurements. It is found that relaxation of methyl groups on the heme ring of cytochrome *c* markedly deviates from a simple exponential behavior in the presence of small amounts of cytochrome oxidase. A comparison with the relaxation behavior of cytochrome *c* modified by 4-carboxy-3,5-dinitrophenyl at Lys-13 shows that the oxidase induces a conformation in native cytochrome *c* that is closely related to that of the derivative. It is suggested that this change in conformation consists of a rupture of the salt bridge between Lys-13 and Glu-90 and a concomitant perturbation of the methionine ligand.

Introduction

Despite the fact that cytochrome *c* is one of the most thoroughly investigated proteins with respect to its functional role, i.e., acting as electron carrier between reductase and oxidase in the mitochondrion, no reports so far have been able to implicate which groups in the protein mediate the transport of electrons to and from its redox center. Much evidence has accumulated over the years showing that the interaction between cytochrome *c* and cytochrome oxidase primarily is electrostatic in nature. This is borne out by observations that the activity is strongly dependent upon ionic strength [1] and that chemical modification of cytochrome *c*, predominantly of lysine residues, produces altered binding properties and activities [2–5].

The findings of Ferguson-Miller et al. [5] suggest, on the basis of activity measurements using cytochrome *c* singly modified at several lysine residues, that the upper half of the solvent-exposed heme edge could be the actual site of electron transfer. This picture is consistent with NMR results showing that the maximal spin density is found on pyrrole rings II and IV in the heme ring of cytochrome *c* [6]. Subsequent studies of the interaction between cytochrome *c* reductase [7], cytochrome *c* peroxidase [8] and sulfite oxidase [9] show that the binding domains on the cytochrome *c* molecule for these proteins to a large extent overlap with that of cytochrome oxidase [5]. Also, small inorganic molecules, such as ferricyanide [10], bind to cytochrome *c* at the solvent-exposed heme edge. Thus, it seems very likely that the same pathway for electron transfer is utilized irrespective of the electron donor or acceptor.

In the present investigation ^{1}H-NMR longitudinal relaxation measurements were used to monitor the interaction between the oxidized forms of cytochrome *c* and cytochrome oxidase. It is found that binding of the oxidase to cytochrome *c* induces a

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conformation change in the latter protein so that its relaxation behavior closely resembles that of cytochrome *c* modified by CDNP at Lys-13. These results suggest that the Lys-13–Glu-90 salt bridge becomes disrupted as a consequence of binding to the oxidase and that this feature and a concomitant adjustment of the methionine coordination [11] may serve as a kinetic control mechanism.

Materials and Methods

Detergent-solubilized cytochrome oxidase was prepared from bovine heart mitochondria according to the method of Rosén [12] or Van Buuren [13]. Horse heart cytochrome *c* was prepared and further purified by ion-exchange chromatography as described in Ref. 14. Cytochrome *c* singly modified to 4-carboxy-3,5-dinitrophenyl (CDNP) lysyl derivatives were prepared as described in Ref. 15. Protein concentrations were determined from the difference in absorbance between the reduced and oxidized forms of cytochrome oxidase and cytochrome *c* using extinction coefficients of 24 $mM^{-1} \cdot cm^{-1}$ at 605 nm and 21 $mM^{-1} \cdot cm^{-1}$ at 550 nm, respectively. The pH is given as the meter reading and is designated pH*.

Measurements of the ^{1}H-NMR longitudinal relaxation was made with a Bruker WH 270 MHz spectrometer employing a $(180\text{-}t\text{-}90)_n$ pulse sequence. The two most downfield heme methyl resonances were recorded separately from the bulk of the protein resonances using a shorter fixed delay time. In cases where the relaxation rates could be described by a simple exponential behavior the estimated error in the T_1 values is 7%. Cytochrome *c* concentrations ranged from 4 to 6 mM and 500–1000 scans were collected for each delay time. Chemical shifts are quoted as positive downfield relative to 2,2-dimethyl-2-silapentane-5-sulfonate.

Results and Discussion

The NMR spectrum of ferricytochrome *c* in Fig. 1 shows, in addition to the usual features of protein spectra, well resolved resonances both in the extreme high- and low-field parts due to the paramagnetic Fe^{3+} of the heme. Thus, protons located on or in the vicinity of the heme will

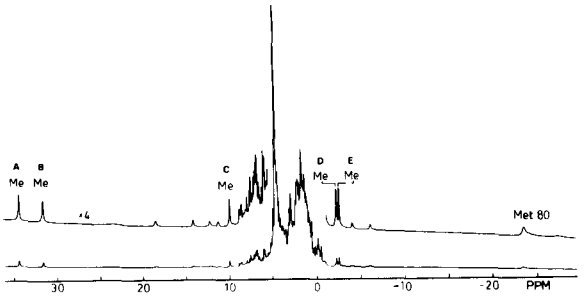

Fig. 1. ^{1}H-NMR spectrum of horse heart ferricytochrome *c* at 270 MHz in 0.1 M phosphate buffer, pH* 7.0 (298 K). The resonances labeled A–E were used in the longitudinal relaxation measurements.

experience large shifts which are of contact or pseudo-contact origin. The lettered resonances refer to the peaks used in the longitudinal relaxation measurements. Resonances A–C have been assigned to heme methyl groups and are located on pyrrole rings IV, II and III, respectively [6]. The peak labeled E arises from the Cys-14 thioether bridge methyl [6] while D has been assigned to Ile-75 or Ile-81 [16]. The positions of the methyl groups are shown in the schematic front view of cytochrome *c* in Fig. 2.

Ferricytochrome *c* exhibits a very wide range of T_1 values for the individual resonances. At 270 MHz these values vary from the extremely short T_1 of 2 ms for the CH_2 protons of Met-80, the sixth ligand of the heme iron, to approx. 1 s for methyl groups far from the paramagnetic center, e.g.,

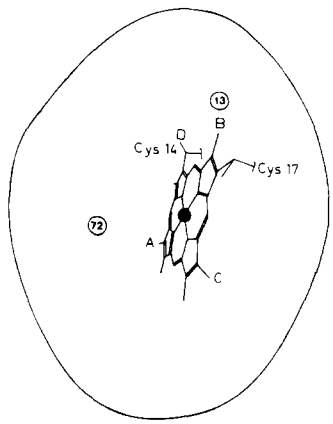

Fig. 2. Schematic respresentation of cytochrome *c* viewed at an angle slightly to the left from the front of the molecule. The lettered positions refer to the resonances in Fig. 1.

Met-65. The methyl resonances outside the main spectral region were chosen to monitor the effects of the oxidase where the very broad signals from this protein do not interfere.

Addition of cytochrome oxidase to a cytochrome *c* solution produces an overall broadening of the NMR spectrum and at a molar ratio of approx. 0.07 all resonances have experienced a 2-fold increase in their linewidths. The observed broadenings can only be brought about in a fast exchanging system, and assuming one rapidly exchanging site, the apparent molecular weight of the cytochrome *c*-cytochrome oxidase complex will be approximately twice of free cytochrome *c* at the above molar ratio. The resulting 2-fold increase in the rotational correlation time will thus produce linewidths twice as large as those of free cytochrome *c*. This situation is analogous to that observed for the cytochrome *c*-cytochrome *c* peroxidase complex [17]. Note also that at the ionic strength used in the present experiments only the low-affinity site on the oxidase molecule is expected to bind cytochrome *c* [18]. However, recent kinetic studies of the effects of anions on the cytochrome *c*-cytochrome oxidase system suggest that high ionic strength results either in a lower redox potential of bound cytochrome *c* at the high-affinity site, making it indistinguishable from the low-affinity site, or a perturbation of an equilibrium between two forms of bound cytochrome *c* in favor of a less active form [19]. Either interpretation ensures that our measurements reflect the binding of cytochrome *c* at the catalytically competent site(s) on the oxidase.

Figs. 3 and 4 show the longitudinal relaxation of the methyl groups labeled A–E in Fig. 1. With no oxidase present the relaxation can be described by single T_1 values. On addition of cytochrome oxidase the relaxation of resonances A and C–E show a marked deviation from a linear dependence (for experimental conditions, see legend to Fig. 3). Fig. 3A also shows that this effect increases with increasing amounts of oxidase, which is true also for resonances C–E (not shown). The straight lines with oxidase present have been displaced approximately parallel to the ones without oxidase in order to facilitate comparison. Minor effects were also seen on the relaxation behavior of some of the aromatic resonances. However, these effects

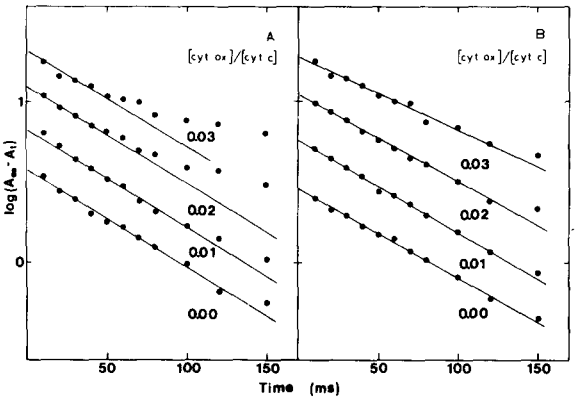

Fig. 3. Semilogarithmic plots of the longitudinal relaxation of the two most downfield resonances in the spectrum of cytochrome *c* with increasing amounts of cytochrome oxidase. The proteins were in 0.1 M phosphate buffer and 0.5% (v/v) Tween 80, pH* 7.0. The temperature was 298 K. The letters A and B refer to the resonances similarly labeled in Fig. 1.

are most likely due to the relaxation of the very broad signals from the oxidase.

Longitudinal relaxation of methyl groups in proteins cannot always be described by a simple exponential behavior. This fact can mostly be attributed to cross-relaxation [20], the transfer of magnetic energy by mutual spin flips between nearby protons. This effect is highly dependent on motion and one obvious reason for the observed behavior could be that in increasing τ_c, these effects become important within the cytochrome *c* molecule itself [20]. To check this possibility we increased the concentration of cytochrome *c* or serum albumin was added (this protein does not interact with cytochrome *c* in any specific way) to a point where the observed line broadening was approximately equal to that produced by 2%

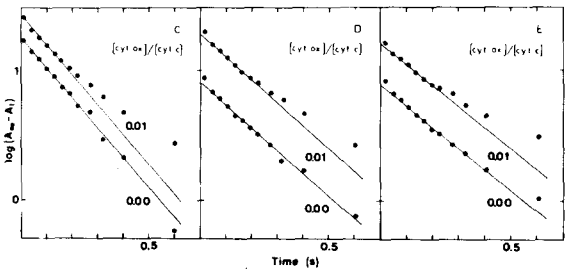

Fig. 4. Semilogarithmic plots of the longitudinal relaxation of resonances C–E in the cytochrome *c* spectrum (see Fig. 1). For experimental conditions, see legend to Fig. 3.

oxidase. The relaxation rates were somewhat slower in both cases, as expected, but could still be described by single T_1 values. Thus, we conclude that the nonexponential behavior results as a consequence of the specific interaction with cytochrome oxidase. It should also be mentioned that no effects on the relaxation behavior were discernable when either phosphate or Tween 80 or both were present in the cytochrome *c* solution.

An explanation for the observed relaxation behavior is offered by the results in Fig. 5. The two upper curves show the longitudinal relaxation of heme methyl groups A and B, respectively, for cytochrome *c* modified by CDNP at Lys-13 while the two lower curves show the relaxation behavior for the same methyl groups in the Lys-72 derivative. Pronounced deviations from linearity were found also for the other heme substituents in cytochrome *c* modified at Lys-13. We found no substantial effects on the relaxation rates or linearity in either derivative in the presence of approx. 1% cytochrome oxidase under the same conditions as those described in the legend to Fig. 3. The modification at these specific sites thus effectively reduces the binding of the oxidase to cytochrome *c*. This is also seen in the fact that the relaxation of the *meta* protons of the CDNP moiety are only marginally perturbed. These findings are consistent with binding data from gel filtration experiments [5].

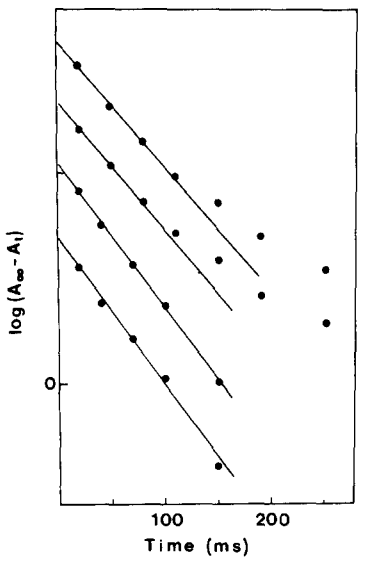

Fig. 5. Semilogarithmic plots of the longitudinal relaxation of resonances A and B for cytochrome *c* modified by CDNP at Lys-13 (the two upper curves) and Lys-72 (the two lower curves), respectively. The measurements were performed in unbuffered solutions at pH* 7.0 and 300 K.

The relaxation properties of the methyl resonances in cytochrome *c* when interacting with the oxidase are thus similar to the case where Lys-13 is modified by CDNP. The noticeable difference is the methyl group labeled B, which exhibits linear relaxation curves in the presence of oxidase. This methyl group is, however, located at the interaction surface [5] and may thus experience additional relaxation effects from the oxidase molecule. In such a case it is not surprising that the resulting relaxation curves are nearly linear.

It has been shown that a dynamic equilibrium between two different conformers of the native form of cytochrome *c* exists [21] and that this equilibrium involves the Lys-13–Glu-90 salt bridge [11]. The data in Figs. 3–5 suggest that binding of cytochrome *c* to the oxidase induces a conformation with relaxation characteristics similar to those of the Lys-13 derivative in which the Lys-13–Glu-90 salt bridge is broken. Recent kinetic studies on the interaction between singly modified lysine derivatives of cytochrome *c* and ferricyanide may be taken to indicate that the salt bridge also in this case breaks during the complex formation [10].

Charge complementarity between the highly conserved lysine residues at the binding domain on cytochrome *c* and corresponding negatively charged residues on its natural redox partners is expected to dominate the interactive forces. Indeed, hypothetical models of complexes between cytochrome *c* and cytochrome b_5 [22] and cytochrome *c* and cytochrome peroxidase [23] indicate that such complementarity exists. In the latter case, modification of carboxyl groups on the peroxidase in the presence of cross-linked cytochrome *c* showed that four aspartate residues located within the hypothetical interaction surface were shielded from chemical modification [24]. Furthermore, if an arylazido group is attached to Lys-13 on cytochrome *c* it cross-links with the peroxidase at a region containing two of the four aspartate residues [25]. It is therefore reasonable to assume that Lys-13 plays a significant role also in the interaction between cytochrome *c* and the oxidase and

that this interaction may involve a conformation change of this lysine residue in order to form a new salt bridge with a negatively charged group on the oxidase.

Addition of oxidase to a cytochrome *c* solution induces small shifts on the heme resonances (except resonance B) and the methyl resonance of the methionine ligand. In the presence of 3% oxidase, shifts of the order of 0.05 and 0.1 ppm are found for the heme resonances and the Met-80 methyl resonance, respectively. These shifts are in the same direction as those found for the Lys-13 derivative relative to native cytochrome *c*, suggesting that the -S-CH_3 position of Met-80 (in the heme plane) is slightly perturbed in the presence of oxidase [11]. No significant chemical shift changes were found in the aromatic region of the NMR spectrum of ferricytochrome *c* when oxidase was added. Such effects may have been obscured by the increased linewidths of these resonances. However, numerous small shifts could be seen on the resonances stemming from the ϵ-CH_2 protons of several lysine residues, reinforcing previous conclusions [1,5] that the interaction between the two proteins primarily is of an electrostatic nature.

It should be emphasized that the complex mentioned above is in the oxidized inhibitory form. This fact suggests one mechanism whereby the electron flow from cytochrome *c* to the oxidase may be regulated. Assuming that the reduced form of cytochrome *c* preferentially binds to the oxidase with the Lys-13–Glu-90 salt bridge intact then, after the electron has been delivered, the rupture of this bridge and the simultaneous perturbation of the methionine ligand should provide the cytochrome *c* molecule with a conformation that makes a reversed electron flow energetically less favorable. Such a conformation change would produce slightly altered energy levels which would be expected to effect the electron-transfer rate if an electron-transfer mechanism involving vibronically coupled tunneling is applicable to the cytochrome *c*-cytochrome oxidase case [26].

That Lys-13 on cytochrome *c* is involved in an electrostatic interaction with a negatively charged group on the oxidase is borne out by recent results by Bisson et al. [27]. They found that the Lys-13–arylazido derivative of cytochrome *c* cross-linked at or close to His-161 in subunit II of bovine heart cytochrome oxidase. The conserved Asp-158 is in the proximity of the cross-linking site and represents a likely candidate for the interaction with unmodified Lys-13 on cytochrome *c*.

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