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Spectroscopic studies of cytochrome c interaction with lipid membranes

SPECTROSCOPIC STUDIES OF CYTOCHROME C INTERACTION WITH LIPID MEMBRANES

The Coupling of Cytochrome c Function with Surface Absorption and Integration

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1 Introduction

Cytochrome *c* is a protein well suited to model studies on the association of peripheral proteins with lipid membranes. This is due to its basic character as well as to the visible absorption bands of the heme chromophore which is covalently linked to the protein backbone. Thus it is accessible to optical spectroscopic studies involving the chromophore. Cytochrome *c* participates in the electron transport in the repiratory chain of mitochondria. It has been demonstrated by Hildebrandt and Stockburger[1] that two conformational states of cytochrome *c*, I and II, coexist on electrode surfaces, as evidenced by resonance Raman spectroscopy, whereas only state I is present in the soluble protein. These two states have different redox potentials and thus are functionally distinct. The 2-state coexistence is also observed when cytochrome *c* binds to charged lipid membranes[2]. Furthermore, structural changes are indicated by changes in the amide hydrogen/deuteron exchange kinetics[3], suggesting tertiary structural changes of the protein upon membrane binding. They are accompanied by an overall destabilization of the protein structure leading to a decrease of the protein denaturation temperature by nearly 30° [4,5]. Therefore, cytochrome *c* can be considered as an example for a structure/function coupling with the properties of the surface substrate.
Cytochrome *c* is believed to bind predominantly by electrostatic means to charged surfaces, including charged lipid membranes. It has, however, become evident in recent years, that cytochrome *c* absorption is more complex and involves more than just a surface absorption step. The most dominant part of the binding free energy is provided by electrostatic contributions. Electrostatic interactions of cytochrome *c* with charged lipids can be adequately described by Gouy-Chapman double layer theory, if the ionic strength is above 40mM Na^+ [4]. At a lower ionic strength, the protein possibly inserts into the membrane, involving energetic contributions other than electrostatics. This is suggested by absorption experiments[3], electron spin resonance investigations[4] and recently by surface plasmon resonance studies[6].
In this paper we focus on the effect that the different modes of absorption have on the stucture of the protein, as well as on its function.

2. Materials and Methods

Horse heart cytochrome *c* (Sigma, St.Louis) and dioleoyl phosphatidylglycerol (DOPG) and dipalmitoyl phosphatidyglycerol (DPPG) (Avanti Polar Lipids, Birmingham) were dispersed in a buffer with 2-10 mM Hepes, 1mM EDTA and a pH/pD of 7.5. Details of the binding analysis and the evaluation of the 9 GHz electron spin resonance (ESR) spectra are found elsewhere[4]. Hydrogen exchange determinations by Fourier transform infrared (FTIR) spectroscopy were described by Heimburg and Marsh[3]. Calorimetry was performed on a Calorimetry Sciences Corp. (Provo, Utah) differential scanning calorimeter at a scan rate of 5°/hr. The analysis of the resonance Raman (RR) spectra was described by Hildebrandt et al. [7].

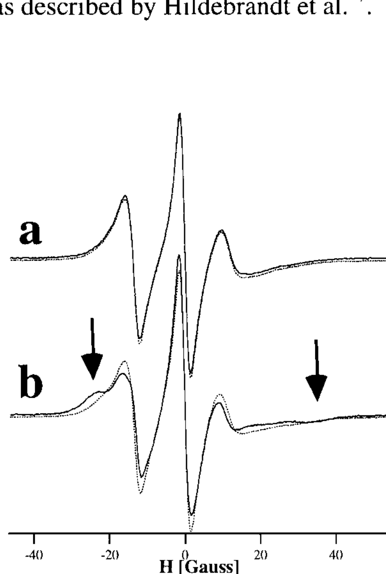

Figure 1
ESR scans of spin-labelled DOPG membranes a. in the absence of cytochrome c and b. in the presence of cytochrome c Solid and dotted lines represent an ionic strength of 4mM and 54mM, respectively. The arrow indicates the motionally restricted spectral component induced by protein binding at low ionic strength.

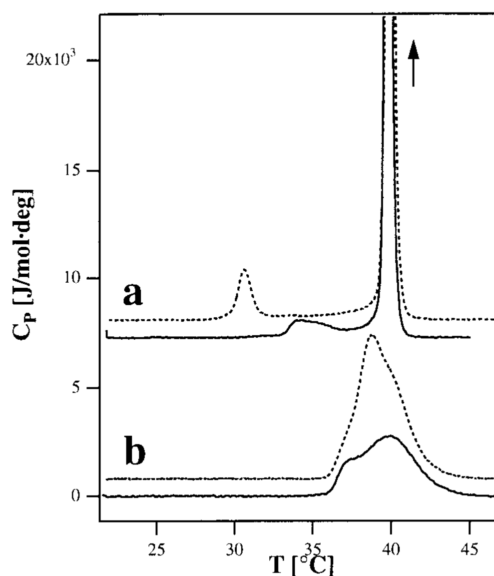

Figure 2
Calorimetric scans of DPPG membranes in the absence (a) and the presence (b) of cytochrome c, with DPPG:cyt.c=10:7.5 wt:wt (b). Solid lines represent 10mM Na+, dotted lines represent 50mM Na+.

3. Results and Discussion

The absorption of cytochrome *c* to anionic lipid membranes was investigated with ESR, calorimetry, FTIR and resonance Raman spectroscopy. Based on binding isotherms[4] the ionic strength conditions were chosen such that the degree of binding was known to be high.

The interaction of cytochrome *c* with dioleoyl phosphatidylglycerol (DOPG) membranes was studied by spin label ESR in the absence and the presence of excess cytochrome c at two different NaCl concentrations, 4mM and 54 mM, respectively. A spin label was attached to the C-14 position of the *sn*-2 chain of the lipid probe. At low ionic strength,

a motionally restricted component appears in the spectra, indicated by the arrows in Fig.1. This suggests an interaction of the protein with the chain segments in the central core of the bilayer. At 54mM NaCl, no such restricted spectral component was found although it is known from binding analysis[3] that the predominant part of the protein is membrane-bound (Fig.1). Thus, in the latter case, no integration into the membrane takes place, indicating a solely surface absorbed protein layer. This is consistent with the much higher binding capacity at low NaCl concentration[4] being due to an increase in the membrane area upon protein integration.

The differences of the absorption process at different ionic strengths is also evident in the calorimetric lipid melting profiles of DPPG membranes (Fig.2). A pure lipid dispersion was compared with a lipid/protein complex with a mixing ratio of 10:7.5 (wt:wt). Again conditions were chosen such that under all conditions complete binding of the added protein was assured. However, the melting profiles are different at 10mM NaCl from those at 40mM NaCl (Fig.2b). The heat capacity maximum at low ionic strength has a significantly smaller value than at 40mM and the overall lipid melting enthalpy is reduced nearly two-fold.

The binding to lipid surfaces at low ionic strength affects the amide hydrogen exchange rates of cytochrome *c*. In Fig.3 the exchange kinetics, monitored by FTIR spectral

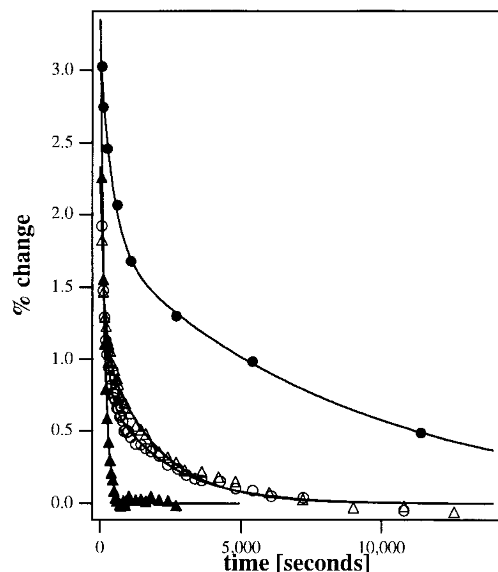

Figure 3

Deuteration profiles of cytochrome c on DOPG membranes (filled symbols) or in solution (open symbols), obtained at 10°C (circles) and 30°C (triangles), with an ionic strength of 4mM. Displayed is the area of the difference spectrum between a spectrum at time t and a reference spectrum, recorded after long times, originating from the spectral shift of the amide I band in on deuterion. The exchange characteristics are very different for the soluble and bound protein.

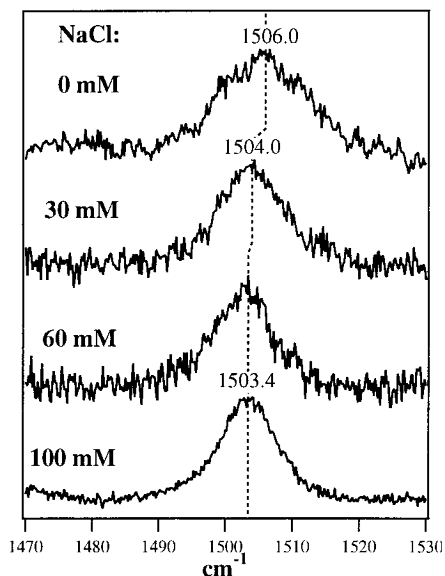

Figure 4

Resonance Raman spectra of the marker band region of cytochrome c bound to DOPG vesicles at different NaCl concentrations. The shift and the broadening of the line shape with decreasing salt concentration are indicative of a change in the redox potential (see Hildebrandt and Stockburger (1989)).

changes of the amide I band, is shown for two temperatures (circles: 10°C, triangles: 30°C) in the absence (open symbols) and the presence of DOPG vesicles (filled symbols). The exchange characteristics are quite different. At 30°C the membrane bound protein exchanges very fast (within a few minutes) whereas it takes several hours at 10°C. In the absence of membranes the exchange is hardly affected by temperature and fewer protons are exchanged. However, the protein secondary structure is not affected upon binding[2]. The large change in the exchange kinetics therefore suggests tertiary structural changes upon binding that expose amino acid residues to water that are buried in the unbound protein.

Resonance Raman studies on the heme chromophore have been shown to provide information on the spin state of the iron complex and to be a means to monitor the redox-potential of the protein[1]. In Fig.4 the RR spectra of cytochrome *c* absorbed to DOPG are given in the spectral region of the ν_3 marker band at ~1500 cm^{-1} (for details of the analysis see Hildebrandt et al.[7]). The protein concentration was chosen to be much smaller than the saturating value. Therefore, nearly all the protein is membrane bound. At 100mM and 60mM NaCl the spectra are roughly identical to those of the unbound protein[7], which is known to be predominantly in state I. At 30mM and 0mM NaCl spectral changes take place, shifting the spectrum by several wavenumbers, accompanied by a spectral broadening. The underlying features of the broadened band are described by several Lorentzian lines[7]. These changes are indicative of a shift in the equilibrium between the two functional states I and II of cytochrome *c*, being accompanied by a change in the redox potential[1]. These changes only take place under ionic strength conditions where the binding stoichiometry of the protein is increased.

4. Conclusions

It has been demonstrated that the modes of interaction of cytochrome *c* with DOPG and DPPG membranes are highly dependent on ionic strength, even though it has been assured that binding occurred under all conditions. Below 40mM NaCl concentration the protein integrates more closely with the membrane, coupled with changes in the ESR spectra, in calorimetric lipid melting profiles and melting enthalpy, and in the amide exchange kinetics. Resonance Raman spectroscopy shows that coincident with this process the state equilibrium of the heme and therefore the redox potential of the protein are also affected. This suggests that the more intimate association of cytochrome *c* at low ionic strength could be important for the functional control of the protein.

5. References

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