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Interaction of cytochrome *c* with liposomes: covalent labeling of externally bound protein by the fluorescent probe, azidonaphthalenedisulfonic acid, enclosed in the inner aqueous compartment of unilamellar vesicles

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The photoreactive fluorescent probe, 3-azidonaphthalene-2,7-disulfonic acid (ANDS) was encapsulated in the inner aqueous compartment of small unilamellar liposomes, prepared from egg phosphatidylcholine (PC) ± 20 mol% dihexadecylphosphate (DHP). After adding cytochrome *c* externally to a suspension of these vesicles, the probe was activated by ultraviolet irradiation, and the protein was separated from the lipids. When negatively charged (egg PC/DHP) vesicles at low ionic strength were used, which form an electrostatic complex with cytochrome *c*, the protein was labeled by ANDS. This process depended on irradiation time, and was inhibited by increasing the ionic strength of the medium. Labeling was not observed with isoelectric (egg PC) vesicles. These observations suggest that electrostatic binding of cytochrome *c* to the bilayer is accompanied by intramembrane penetration to such a depth that the protein can communicate with the inner membrane–water interface.

Introduction

It has long been established that cytochrome *c*, in addition to forming complexes with other proteins, can bind to lipid membranes. This binding is of an electrostatic nature, and seems to be essential to the physiological (electron-transfer) function of the protein in the mitochondria [1]. While there is no evidence for substantial intrusion of cytochrome *c* into the inner mitochondrial membrane in situ [1,2], experiments with model membranes have indicated varying degrees of penetration into the hydrocarbon region of the membrane following electrostatic binding [1–9]. In keeping with these observations, we have previously reported that cytochrome *c*, bound to negatively charged (egg PC/DHP) liposomes, can interact with the photogenerated triplet state of chlorophyll *a* (^{3}Chl) located in both the inner and outer bilayer shells [10].

In the present study we report on an attempt to find a direct proof for the intramembrane penetration of cytochrome *c* into liposome bilayers. We encapsulated a fluorescent label, ANDS, into the inner water space of both isoelectric and negatively charged vesicles and incubated them with externally added cytochrome *c*. It was presumed that if transmembrane diffusion or leakage of the

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Abbreviations: ANDS, 3-azido-2,7-naphthalenedisulfonic acid; ^{3}Chl, chlorophyll triplet state; DHP, dihexadecylphosphate; PC, phosphatidylcholine.

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probe could be ruled out, then labeling of the electrostatically bound protein would indicate its deep penetration into the bilayer, possibly reaching the inner lipid–water interface.

Materials and Methods

The source of chromatographically pure egg phosphatidylcholine was the same as reported earlier [11]. Dihexadecylphosphate was from Sigma Chemical Co., and was converted to the potassium salt with standard procedures. Oxidized horse-heart cytochrome *c* (type VI) was also from Sigma and was used without further purification. Reduced cytochrome *c* was prepared by adding sodium ascorbate to the protein in 5 mM phosphate buffer (pH 6.8) to give 10 mM final concentration. The 1 M ascorbate stock solution was prepared freshly and its pH adjusted to 6.8. The porphyrin ring of cytochrome *c* was opened up as follows. Sodium ascorbate was added to cytochrome *c* as described above, the solution was re-acidified by HCl and heated at 100 °C for 3 min. Methemoglobin and hemin were prepared as described in Refs. 12 and 13, respectively. As hemin is insoluble in water, pyridine hemochromogen was used as a heme analogue. The heme-pyridine complex was prepared by mixing equimolar amounts of hemin and pyridine in phosphate buffer. Catalase, lysozyme and urease were from Sigma and used without further purification.

3-Azidonaphthalene-2,7-disulfonic acid, a water-soluble fluorescent photoaffinity label, was purchased from Molecular Probes Inc. Its characteristics are discussed below. The rest of the materials were of the highest grade available.

The procedures described in the following were carried out in dim red light. ANDS was encapsulated in small unilamellar liposomes by a combination of ethanol injection and sonication [11] as follows. 10 μmoles egg PC ± 2.5 μmol (20 mol%) DHP were dissolved in 25 μl ethanol and injected with a microliter syringe into 0.5 ml of 10 mM ANDS in phosphate buffer. The buffer was whirled by a vortex-mixer during injection and for approx. 1 min afterwards. The liposome suspension was subsequently sonicated for 5 min in a bath sonicator (Branson Model B-3), and unencapsulated ANDS separated from the vesicles by column chromatography on a Sephadex G-50 (18 × 0.8 cm) column. Preequilibration of the column and elution was done with the same phosphate buffer. The vesicles eluting with the void volume were pooled, and the volume adjusted (to 2.5 ml) to give 4 mM final egg PC concentration. The separation was checked each time by recording the ultraviolet spectrum of the fractions (the lipids and ANDS have maxima at 210 and 255 nm, respectively). The spectra were obtained on a Cary 15 spectrophotometer (with an Olis 3820 modification interfaced to a Northstar computer).

Subsequently, 0.3 ml of vesicles was transferred to the fluorimeter cuvette and deoxygenated by bubbling Ar through the suspension for 4–5 min. Cytochrome *c* was added to the vesicles from a 3 mM stock solution to give 0.1 mM final concentration, and the Ar flushing further continued for 1 min. The cuvette was then sealed with paraffin film and the system was irradiated by ultraviolet light from a distance of 2–3 cm by a long-wavelength (365 nm), high-energy ultraviolet lamp (Black-Ray B-100A, Ultraviolet Products Inc.). Unless otherwise indicated, the irradiation time was 5 min. The radiation dose after 5 min irradiation, as measured by ferrioxalate actinometry [14], was $3.9 \cdot 10^3$ J/m^{-2}.

After ultraviolet irradiation, 10 mM sodium deoxycholate was added (still under anaerobic conditions) to solubilize the vesicles [15], and the system was immediately applied to a carboxymethyl cellulose column (0.5 × 1 cm, packed in a Pasteur pipet), to isolate the protein. The column was preequilibrated with phosphate buffer, and the non-protein bound ANDS, together with the lipids, were eluted with the same buffer containing 10 mM sodium deoxycholate. Cytochrome *c*, bound on top of the column, was eluted with 1.2 ml of 0.15 M NaCl/5 mM phosphate (pH 6.8).

The uncorrected (technical) emission spectra [16] of the 1.2 ml protein eluates were recorded in a Perkin-Elmer MPF-2A spectrofluorimeter with parameters as given in the figure legend.

Results

Fig. 1a shows that cytochrome *c* labeled with ANDS has a fluorescence emission maximum at

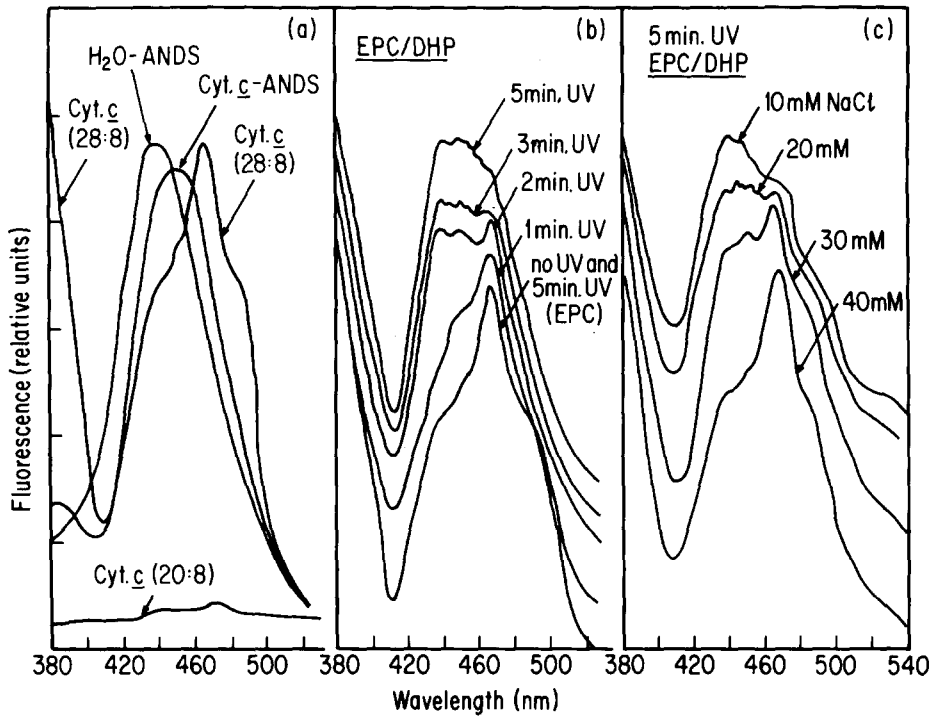

Fig. 1. Changes in the emission spectrum of cytochrome *c* (Cyt. *c*) after covalent labeling with free or liposome-encapsulated ANDS. (a) The spectrum of 0.1 mM cytochrome *c* in 5 mM phosphate buffer (pH 6.8) was recorded with excitation and emission bandwidths as indicated (in nm). The protein was then irradiated with ultraviolet (UV) for 5 min in the presence of 0.2 mM ANDS, and subsequently separated by ion-exchange chromatography from ANDS-labeled H_2O molecules (H_2O-ANDS). The spectra of the isolated, ANDS-labeled cytochrome *c* (Cyt *c*-ANDS) and that of H_2O-ANDS were recorded with excitation and emission bandwidths of 20:8 and 16:8, respectively. Excitation was at 344 nm. Other experimental details are in the Materials and Methods section. (b) Cytochrome *c* was incubated with ANDS-containing egg PC/DHP liposomes in 5 mM phosphate (pH 6.8), the system irradiated by ultraviolet for the indicated times, the protein isolated and the emission spectra recorded. Other experimental details are in the Materials and Methods section. The emission bandwidth was 8 nm, excitation was at 344 nm with 28 nm bandwidth. The originally overlaying curves were vertically shifted for easier comprehension. The bottom curve represents the spectrum of cytochrome *c* incubated with either egg PC/DHP vesicles without activation of ANDS with ultraviolet, or with egg PC vesicles and irradiated with ultraviolet for 5 min. Typical spectra from two identical experiments are presented. (c) Cytochrome *c* was incubated with ANDS-containing egg PC/DHP liposomes in the presence of the indicated concentrations of NaCl. The activation time was 5 min in each case. The originally overlaying curves were vertically shifted. Other experimental details are similar to panel (b).

450–455 nm, whereas the non-protein bound product of ANDS radiolysis (H_2O-ANDS) fluoresces at 440 nm. Non-labeled cytochrome *c* also shows a weak fluorescence between 440 and 480 nm, which at high excitation energy is observed to be an irregular band with a maximum at 470 nm. In an effort to identify the origin of this intrinsic fluorescence of cytochrome *c*, we examined other heme and non-heme compounds. Methemoglobin, catalase, as well as the water-soluble hemin derivative, pyridine hemochromogen, display similar fluorescence, whereas non-heme proteins (lysozyme and urease) do not (data not shown). These findings suggest that the fluorescence in question is related to the heme groups. Reduction of cytochrome *c* by sodium ascorbate has no influence on this fluorescence, but oxidative splitting of the porphyrin macrocycle increases it by several orders of magnitude (data not shown). Consequently, the small intrinsic fluorescence of cytochrome *c* is probably due to trace amounts of bile pigments in the source preparation. The re-

sults shown later indicate that this contamination is still present in cytochrome *c* after ion-exchange chromatography, implying that it is bound to the protein. The necessity for understanding the nature of this relatively minor fluorescence band lies in the fact that the measurements described herein were carried out under conditions in which the changes attributed to ANDS-labeling are superimposed upon it.

Fig. 1b shows the changes in the fluorescence of cytochrome *c* as a function of ultraviolet irradiation time in the presence of ANDS-containing egg PC or egg PC/DHP liposomes. In the case of egg PC vesicles the impurity-related fluorescence spectrum does not change even after 5 min irradiation, whereas incubation of cytochrome *c* with egg PC/DHP liposomes followed by ultraviolet irradiation leads to the development of a shoulder on the short-wavelength side of this band. This spectral change shows a clear progression with irradiation time in the 1–5 min time interval, and results after 5 min irradiation in a fluorescence band whose maximum around 450–455 nm is similar to that of the ANDS-cytochrome *c* complex (cf. Fig. 1a). The acquired fluorescence is, however, not so intense as to mask completely the impurity-related fluorescence band at 470 nm, which remains evident in these spectra. These observations suggest that ANDS inside the vesicles is able to come into direct contact with (some of) the externally bound cytochrome *c* molecules, and upon ultraviolet activation, covalently labels them. Relating the fluorescence intensity obtained upon transmembrane labeling of cytochrome *c* to that obtained when an identical amount of protein was labeled by free ANDS (cf. Fig. 1a) gives a ratio of about 1 : 30.

The lack of labeling of the protein in the case of isoelectric vesicles suggests that electrostatic complex formation between cytochrome *c* and the vesicles is a precondition for this reaction. There are, however, well-known structural differences between charged and uncharged liposomes, whose influence on the labeling process cannot be excluded a priori. Since it is known that the stability of the electrostatic complex between cytochrome *c* and negatively charged membranes decreases with increasing ionic strength in the suspension medium [1], we tested the influence of NaCl on the labeling process for the case of egg PC/DHP liposomes. Fig. 1c shows that increasing the NaCl concentration in the medium from 10 to 40 mM progressively inhibits the changes of protein fluorescence caused by ultraviolet irradiation, providing support for the essential role of complex formation in the observed labeling process. This salt effect was not due to osmotic changes, since 40 mM NaCl provided complete inhibition of labeling, irrespective of whether it was added to preformed vesicles, or whether they were prepared and chromatographed in the presence of 40 mM NaCl (data not shown).

Attempts were made to amplify the ANDS-related fluorescence signal by increasing the concentration of encapsulated ANDS from 10 to 40 mM. This modification, however, led to leakage of the probe, as shown by labeling of cytochrome *c* by ANDS even in the case of egg PC vesicles, and by the lack of inhibitory effect of NaCl in the case of egg PC/DHP liposomes (data not shown). In another approach, the 5 mM phosphate buffer was replaced by 50 mM betaine (pH 6.8), a medium which has been reported to provide the most favourable conditions for the interaction of cytochrome *c* with ^{3}Chl in negatively charged liposome membranes [10]. In this case, however, no protein labeling was detected when cytochrome *c* was added to egg PC/DHP vesicles containing 10 mM ANDS and irradiated for 5 min (data not shown). We attribute this observation to competitive inhibition of protein-labeling by the relatively high concentration of betaine molecules.

Discussion

There is much controversy in the literature concerning the issue of cytochrome *c* penetration into model membranes. Several studies suggest that following electrostatic binding of cytochrome *c* to negatively charged monolayer [2–4] or bilayer membranes [5–9], a secondary hydrophobic interaction between the acyl chains of fatty acids and the apolar part(s) of the protein results in deep intercalation of the latter into the hydrocarbon region of the membrane. Numerous other studies, however, are contradictory to significant hydrophobic interaction and penetration of cytochrome *c* into bilayer membranes [17–24]. The lack of

consensus is probably a consequence of the large number of factors that influence the interaction of cytochrome *c* with model membranes, such as the phospholipid composition and phase state (fluidity) of the membrane [1,2], as well as the oxidation state of cytochrome *c* [1,25]. The significance of clarifying this question lies primarily in understanding the relationship of cytochrome *c* to the inner mitochondrial membrane, and ultimately the mechanism of mitochondrial electron transfer.

Our interest in the above problem is linked to previous attempts to use cytochrome *c* as a redox protein in biomimetic solar-energy conversion systems based on Chl-containing liposomes [10]. Cytochrome *c* added to negatively charged vesicles from the outside under low-ionic-strength conditions proved to be an effective quencher of ^{3}Chl, even of those which were localized mainly in the inner monolayer shell [10,26]. Quantitative considerations therefore suggested that the water-soluble cytochrome *c*, after binding to the vesicle surface, intrudes into the bilayer deep enough to reach the inner monolayer.

This study was undertaken in an attempt to find direct evidence for the above hypothesis. In principle, such proof would be obtained if the externally bound protein could be covalently labeled by a probe which is exclusively located in the inner monolayer of bilayer membranes. Such a probe, however, is not available. Fluorescence quenching experiments, using anthroylstearic acid as a label [18,19] appeared to be inconclusive in deciding the question, as a consequence of the high quenching efficiency of the protein (R_0, the distance at which the fluorescence of anthroylstearic is half-maximally quenched, is 4.9 nm [18], and because of the lack of exclusive localization of the probe in the inner membrane half. In our hands, the use of dansylphosphatidylethanolamine [23,24] also proved to be fruitless for the same reasons, coupled with a substantial inner filter effect of cytochrome *c* (Szebeni, J. and Tollin, G., unpublished data). The assumption was therefore made that an equally definitive answer to this question could be obtained if cytochrome *c* added externally to the vesicles could be labeled by a membrane-impermeant probe which is localized only in the inner aqueous compartment of the vesicles, and whose outward penetration does not exceed the inner monolayer. This requirement for a probe was best met by a recently developed fluorescent photoaffinity label, ANDS [27–30].

Photolysis of this aryl-azide leads to loss of nitrogen and formation of a highly reactive nitrene radical, which immediately inserts into carbon-hydrogen, oxygen-hydrogen or nitrogen-hydrogen bonds or adds to double bonds of neighboring molecules forming fluorescent products. Its favorable characteristics include long-wavelength fluorescence excitation (which does not not overlap with the excitation band of protein aromatic amino acids and is harmless to protein function), a short lifetime for the intermediate radical (10^{-7}–10^{-5} s) and non-specificity with respect to the moieties with which it reacts [28,29]. ANDS is completely water soluble and does not penetrate through intact biomembranes [27,28]. Its photolysis in water yields a fluorescent reaction product of the nitrene radical with water [28]. The fluorescence characteristics of ANDS-labeled products are sensitive to the polarity of the surrounding medium, thus providing information concerning the site of insertion.

The results reported above support the presumption that ANDS is able to contact directly cytochrome *c* across the membrane when the protein is electrostatically complexed to it. That the labeling required the electrostatic binding of the protein to the membrane, and was not merely due to leakage of the probe from the vesicles, was shown by the lack of labeling of (non-complexed) cytochrome *c* incubated with isoelectric liposomes, and by the inhibition of labeling upon dissociation of the negatively charged vesicle-cytochrome *c* complex with increasing ionic strength of the medium. It is notable that the dependence on salt concentration of the three processes (a) electrostatic complex dissociation [1,23], (b) ^{3}Chl quenching by cytochrome *c* [10] and (c) labeling of the protein by ANDS are very similar, indicating a strong correlation between them.

The lack of ANDS leakage from liposomes under the described conditions was also shown by the following observation. If the process of deoxygenation of the system with Ar was omitted, labeling of cytochrome *c* was observed irrespective of vesicle charge or NaCl concentration in the medium. At the same time, massive Schiff base

formation indicated lipid peroxidation in the system [30], due to the catalytic effect of cytochrome *c* on this process [31]. The observed labeling of the protein under these conditions is consistent with an increase in membrane permeability as a consequence of lipid peroxidation [32], as well as with oxygen-enhanced penetration of cytochrome *c* into the bilayer [33].

There was a remarkable correlation between the dependence of labeling of cytochrome *c* on irradiation time and the reported rate [29] of ANDS activation by ultraviolet irradiation. This observation further strengthens the lack of ANDS leakage, or in other words indicates that membrane diffusion is probably not a rate-limiting step in the labeling process.

The observation that the emission maximum of cytochrome-bound ANDS is slightly red-shifted relative to the water photolysis product of ANDS suggests that the label is inserted to the protein at a highly polar region [28], conceivably at the site of positively charged (lysyl-) residues.

The fluorescence changes that indicated the labeling of cytochrome *c* by ANDS were observed only at relatively wide excitation bandwidth and maximal sensitivity of the spectrofluorometer. Under these conditions, the specific fluorescence of the protein-ANDS complex overlaps with fluorescent impurities in the system, most probably due to protein-bound bile pigments. The failure to improve the signal-to-noise ratio by the described approaches points to the necessity of keeping the internal ANDS concentration low enough to avoid leakage, and to the requirement of using low buffer concentration to suppress the competition between the buffer and protein molecules for labeling. It is to be noted that the cytochrome *c* to egg PC/DHP mole ratio applied in the present experiments (i.e., 0.02) is about twice that which is necessary to cause complete binding site saturation in identical vesicles (0.013–0.009) [10]. Consequently, increasing the protein concentration would not be expected to increase the efficiency of labeling.

The fluorescence intensity of the ANDS-cytochrome *c* complex was approx. 30 times higher when the labeling was carried out by adding free ANDS to the protein, as compared to when it was done through the liposome membrane. This observation taken together with the fact that only half of the added cytochrome *c* was bound to the vesicles suggests that approx 1 out of 15 bound cytochrome *c* molecules was labeled by ANDS across the membrane. There is, however, substantial uncertainty in this calculation, due to the lack of information concerning the stoichiometry of protein labeling by ANDS under the two conditions. The low efficiency of cytochrome *c* labeling through the vesicle membrane may be due to the fact that only a small surface region of the protein molecule is accessible to labeling, as well as to the abundance of other molecules, e.g., lipids, also being labeled by the nitrene radicals.

The available data do not allow definitive conclusions to be reached regarding the extent of intramembrane penetration of ANDS. The highly charged (acid) nature of ANDS itself guarantees an expulsion from the (negatively charged) membrane. Taken together with the short lifetime and high reactivity of ultraviolet activated ANDS, it is likely that it does not significantly penetrate into the fatty-acid region of the inner monolayer, and much less likely into the outer monolayer. This assumption was supported in a preliminary experiment, using identical conditions as above, in which we analysed the amount of ANDS-labeled phospholipids after activation of the probe when present (a) only inside, or (b) both inside and outside the vesicles. The results suggested that internal ANDS does not label the outer monolayer (data not shown).

Though the lack of ANDS leakage from the vesicles suggests no major changes in the integrity of the bilayer, some structural alterations due to cytochrome binding and covalent modification of the head-group region of the membrane by ANDS molecules at the inner lipid-water interface cannot be ruled out, and may represent a contributing factor to the observed changes.

Finally, it should be mentioned that a recent study by Rietveld et al. [34] has particular relevance with respect to our results. These authors added apocytochrome *c* to trypsin-containing unilamellar egg PC/phosphatidylserine vesicles and showed that a part of the protein was digested by the enclosed enzyme. These findings were interpreted in terms of exposure of the protein to the internal vesicle medium. Several mechanisms have

been proposed to promote the insertion into and translocation of apocytochrome *c* across the bilayer, including (a) a shielding of the polar and charged amino acids due to aggregation of the protein on the vesicle surface and interaction with negative charges; (b) formation of non-bilayer phospholipid structures in the membrane; and (c) hydrophobic interaction between the N- and/or the C-terminal part of apocytochrome *c* with the acyl chains of fatty acids.

In summary, the present observations support our previous results and other experimental findings indicating that cytochrome *c*, electrostatically bound to negatively charged phospholipid bilayer membranes, can penetrate into the membrane to such depth that it communicates with the monolayer opposite to the binding site. The physiological and practical (solar energy conversion systems) implications of these results remain to be assessed.

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