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The Invariant ARG^{91} Is Required for the Rupture of Liposomes by Cytochrome C

Esa K. J. Tuominen, Carmichael J. A. Wallace,* and Paavo K. J. Kinnunen[1]
*Department of Medical Chemistry, Institute of Biomedicine, University of Helsinki, P.O. Box 8 (Siltavuorenpenger 10 A), FIN-00014 Helsinki, Finland; and *Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7*

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Lipid binding properties of cytochrome c (cyt c) were investigated by using semisynthetic mutant protein having amino acid substitution on the evolutionary invariant residue Arg^{91}. We demonstrate here that the membrane binding properties of cyt c are dramatically altered by substituting norleucine for the invariant Arg^{91}. More specifically, while the binding of this mutant to liposomes per se is indistinguishable from the wild type protein, it completely lacks the ability of the native cyt c to rupture liposome bilayers. © 1997 Academic Press

Cytochrome c (cyt c) is an evolutionarily highly conserved peripheral membrane protein present in all organisms which depend on mitochondrial respiration. As a component of the respiratory chain this water soluble 13 kD protein shuttles electrons from cyt c reductase to cyt c oxidase, its heme moiety switching between ferro and ferri forms (1). Cyt c bears a net positive charge of +8 and binds avidly to membranes containing acidic phospholipids, with no major differences between the different negatively charged phospholipid species been reported sofar (2). Recently cyt c has been identified to have a novel function as a key component in apoptosis. Interestingly, the release of cyt c from mitochondria was shown to be rate limiting in the entry of a cell into the death programme (3,4).

In the inner mitochondrial membrane the cyt c binding site has been suggested to be provided by cardiolipin, either as such or as a complex with cyt c oxidase (5,6). Phosphatidylserine (PS) is the most abundant acidic phospholipid in the outer mitochondrial membrane as well as other cytoplasmic membranes and hence also provides probable binding sites for cyt c in these membranes following the release of this protein from mitochondria in apoptosis (7). Accordingly, molecular level elucidation of the determinants of cyt c-phospholipid interactions are warranted.

More than twenty residues out of a total of 104 in cyt c are invariant in all eukaryotic species, Arg^{91} being one of them (1). Most of the invariant residues of cyt c clearly have a role in maintaining protein structure, or are located in the area of the protein responsible for interactions of cyt c with its redox partners in the electron transfer chain (8,9). However, Arg^{91} is not located in the fore mentioned site, and the biological or structural reasons for its invariance have been the subject of speculation and experiment, since cyt c molecules with specific chemical modification on Arg^{91} function with >90% efficiency in the mitochondrial respiratory chain (10).

Stabilization of the structure of cyt c as well as ATP binding with a regulatory role in electron transfer have been proposed (8,11). Photoaffinity labeling of Lys^{86} or Lys^{87} in the vicinity of Arg^{91} with an 8-azido-ATP results in a significantly decreased electron transfer rate (12,13). There is as yet no direct evidence of an inhibitory effect of the noncovalent binding of ATP at the Arg^{91}-containing site on electron transfer rate, and it may be that the conservation of this residue is for alternative or additional roles. Equilibrium gel filtration revealed the semisynthetic analog in which Arg^{91} was replaced by norleucine to bind only about one third of the ATP bound by the native protein, whereas no differences in protein conformation were observed between this mutant and the wild type protein. In terms of their redox properties Nle^{91} mutant and the wild type cyt c were also practically indistinguishable (14). We demonstrate here that the membrane binding properties of cyt c are dramatically altered by substituting norleucine for the invariant Arg^{91}.

MATERIALS AND METHODS

Preparation of liposomes. Bovine brain phosphatidylserine (Avanti, Birmingham, AL, USA), 1-palmitoyl 2-oleyl phosphatidyl-

[1] Address correspondence to Paavo K. J. Kinnunen. Fax: 358-9-1918276. E-mail: Paavo.Kinnunen@Helsinki.Fi.

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choline (Avanti) and 1-palmitoyl-2-[10-(pyren1-yl)]decanoyl-*sn*-glycero-3-phosphocholine (K&V Bioware, Espoo, Finland) were dissolved in chloroform and mixed in this solvent to obtain the desired lipid compositions. The solvent was removed under a gentle stream of nitrogen and the lipid residue subsequently maintained under reduced pressure for at least 2 h. Dry lipids were hydrated in 20 mM Hepes, 0.1 mM EDTA, pH 7 at room temperature to yield one mM concentration. Unilamellar vesicles were obtained by extruding the lipid dispersion 19 times through a stack of two 100 nm poresize polycarbonate membranes (Nucleapore, Pleasanton, CA, USA) with a LiposoFast small-volume homogenizer (Avestin, Ottawa, Canada) (15). Minimal exposure of the fluorescent lipid to light was ensured throughout the procedure. Prior to use the liposome solutions were diluted to a final concentration of 25 μM total phospholipid.

Fluorescence measurements. The association of cyt c with liposomes was assessed as described previously by monitoring resonance energy transfer between the pyrene containing lipid (PPDPC) and the heme of cyt c (16). Cytochrome c (Sigma) and the semisynthetic analog were dissolved in water to yield concentrations of 25-100 μM. Detailed description of the synthesis and characterization of the Nle^{91} mutant will be reported elsewhere (14). According to their absorption spectra, both the wt cyt c and the mutant were completely in the oxidized state. Measurements were conducted with a Perkin-Elmer LS50B spectrofluorometer using 2.5 nm bandwidths for both excitation and emission, with the respective wavelengths of 344 nm and 398 nm selected by monochromators. Two mls of liposome solution were placed into a magnetically stirred four-window quartz cuvette in a holder thermostated at 25 °C with a circulating waterbath. Cyt c or the analog were added in 5 - 10 μl aliquots. The extent of quenching of measuring pyrene monomer fluorescence was then recorded, allowing for 2 min equilibration after each addition.

Light scattering measurements. Intensity of 90° light scattering at 500 nm was monitored using the spectrofluorometer with both excitation and emission bandwidths set to minimum. Intensity was measured continuously and aliquots of cyt c or the Nle^{91} mutant were added at constant time intervals of 4 min. Otherwise conditions were as in fluorescence measurements. Scattering due to the buffer solution and after the addition of cyt c in the absence of liposomes were neglible.

RESULTS

Binding of wt cyt c and the Nle^{91} mutant to large unilamellar phosphatidylcholine (PC) vesicles containing 20 mol % PS was measured by resonance energy transfer between a fluorescent labelled marker phospholipid and the heme moieties and revealed no differences in the membrane association of these two proteins (Fig. 1). However, resonance energy transfer only monitors proximity of cyt c to the fluorescent donor lipid. In order to obtain information on cyt c induced structural changes in liposomes we utilized the light scattering assay described by Nelsestuen and Lim (17). These measurements revealed a dramatic and unexpected difference in the nature of the interactions of the wild type and the mutant cyt c with liposomes (Fig. 2). More specifically, upon exceeding a threshold value of approx. 1:13 in protein:acidic phospholipid stoichiometry the native cyt c caused a very rapid and extensive formation of highly aggregated structures. In a typical experiment such as illustrated in Fig. 2 the intensity of scattered light started to decrease after the concentration of cyt c exceeded 0.4 μM, subsequently

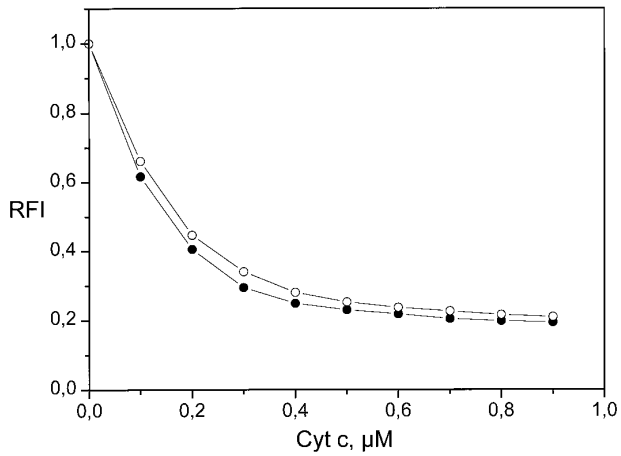

FIG. 1. Binding of cytochrome c to liposomes as revealed by resonance energy transfer. Relative fluorescence intensity (RFI) at different concentrations of wild type cyt c (●) and the Nle^{91} cyt c mutant (○). Total lipid concentration was 25 μM. The mole fractions of the lipids in liposomes were $X_{brain\ PS}$= 0.20, X_{POPC}= 0.79, and X_{PPDPC}= 0.01. Fluorescence intensities were normalized to one assigned to the emission measured prior to the addition of cyt c.

falling off to a level lower than the original scattering due to liposomes only. The resulting aggregate is readily amenable also to visual inspection as increased sample turbidity. Aggregation by cyt c of vesicles containing acidic phospholipids has been reported before, as well as fusion and formation of multilamellar liposomes (18,19,20). In contrast to the native protein for the Nle^{91} mutant the scattering saturates at a well defined level, correlating to the amount of protein bound to the membrane (17) and without any indication of liposome aggregation (Fig. 2).

DISCUSSION

As translocation of cyt c through the outer mitochondrial membrane and possibly also its downstream effects in apoptosis could involve interactions of this protein with phospholipids, we have undertaken to characterize the lipid binding sites of cyt c. We have previously provided evidence for multiple acidic phospholipid binding sites in cyt c (21). The functional significance of these lipid binding sites remains to be elucidated. The aggregation of liposomes by cyt c is, however, in keeping with such multiple phospholipid binding sites in cyt c as this readily allows for cyt c mediated linkage of two separate vesicles, so as to ultimately result in rupture of liposomal membranes and the formation of macroscopic aggregates in the presence of cyt c. Our present data demonstrate this property of cyt c to require a well defined site in the protein, the invariant Arg^{91} being essential. It is usually assumed that residues which have been conserved throughout evolution perform

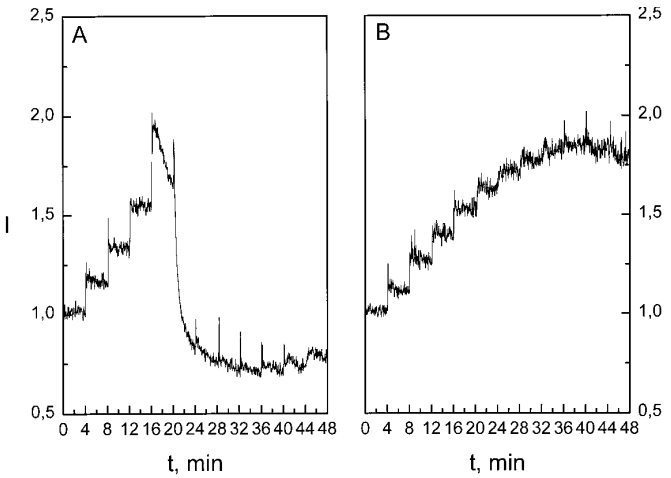

FIG. 2. Intensity of scattered light at 90° due to wild type cyt c (A) or Nle^{91} mutant (B) binding to liposomes. The protein concentration was increased at 4 min intervals, with each addition resulting in a 0.1 μM increment. Total lipid concentration was 25 μM. The mole fractions of the lipids in liposomes were $X_{brain\ PS}$= 0.20 and X_{POPC}= 0.80. Scattering intensities were normalized to one assigned to the scattering measured prior to addition of cyt c.

an essential role in a protein's biological function, thus providing the evolutionary pressure for their conservation. As Arg^{91} appears to be dispensable in terms of the properties of cyt c as a substrate for the mitochondrial redox partners, cyt c reductase and cyt c oxidase, it seems plausible to suggest that the invariance of Arg^{91} relates to other functions of cyt c. In this regard lipid-protein interaction could be important in both release of cyt c from mitochondria as well as perhaps also in the hitherto unknown downstream effects of this protein in apoptosis. The import of the cyt c precursor protein apocytochrome c (lacking the heme) into the mitochondria appears to be lipid mediated and involves acidic phospholipids. Importantly, translocation of apocytochrome c involves neither signal sequence nor protein machinery (22). Drastic effects of cyt c on lipid membrane structures have been shown in vitro and there is experimental evidence indicating that translocation of cyt c through liposomal model membranes may take place (18,20). The possibility that the translocation of cyt c in apoptosis could involve a lipid mediated mechanism thus cannot be excluded. Likewise, the ability of cytochrome c to rupture membranes could be relevant to the apoptotic function on cyt c subsequent to its release from mitochondria. To this end, loss of integrity of internal cell organelle structures is one of the hallmarks of apoptosis. Investigations on the effects of cyt c along these lines are currently in progress in our laboratory.

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