Esa K. J. Tuominen

Phospholipid–cytochrome c interactions
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ABBREVIATIONS

Aβ    amyloid β-peptide
AFM   atomic force microscopy
CD    circular dichroism
cyt c  cytochrome c
FTIR  Fourier-transform infrared
HF    hydrogen fluoride
Nle91  chemically modified cytochrome c bearing a norleucine residue at position 91
RFI   relative fluorescence intensity
wt    wild type, the naturally occurring form of a protein

[Zn^{2+}-heme] cyt c  cytochrome c with Zn^{2+}-substituted porphyrin
X_{POPG}  mole fraction of a phospholipid species in a liposome of mixed composition

In the text, each phospholipid molecule is given a four letter abbreviation, with the two first letters corresponding to the sn-1 and sn-2 side chain fatty acids, respectively, and the next two letters corresponding to the headgroup. For example, 1-palmitoyl-2-oleyl-sn-glycero-3-phospho-rac-glycerol is abbreviated POPG.

**Phospholipid side chains** are abbreviated as follows:
P     palmitic acid
O     oleic acid
S     stearic acid

**Phospholipid headgroups** are abbreviated as follows:
PC    phosphatidylcholine
PG    phosphatidylglycerol
PE    phosphatidylethanolamine
PS    phosphatidylserine

**Phospholipids with mixed or unspecified side chains** are abbreviated with only two letters that correspond to the headgroup.

**Synthetic and special phospholipids** are abbreviated as follows:
CL    cardiolipin
PPDPG 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-sn-glycero-3-phosphoglycerol
PPDPC 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-sn-glycero-3-phosphocholine
NBD-PG 1-palmitoyl-2-(N-4-nitrobenz-2-oxa-1,3-diazol)aminocaproyl-sn-glycero-3-phosphoglycerol
P(Br_{2})SPG 1-palmitoyl-2-(9,10)-dibromo-sn-glycero-3-phospho-rac-glycerol
brain-PS phosphatidyl serine purified from bovine brain
ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications referred to in the text with the Roman numerals I-IV.


ABSTRACT

The ability of the peripherally associated membrane protein cytochrome c (cyt c) to bind phospholipids in vitro was studied using fluorescence spectroscopy and large unilamellar liposomes.

Previous work has shown that cyt c can bind phospholipids using two distinct mechanisms and sites, the A-site and the C-site. This binding is mediated by electrostatic or hydrophobic interactions, respectively. Here, we focus on the mechanism underlying these interactions.

A chemically modified cyt c mutant Nle\(^{91}\) was used to study the ATP-binding site, which is located near the evolutionarily invariant Arg 91 on the protein surface. This site was also demonstrated to mediate phospholipid binding, possibly by functioning as a phospholipid binding site.

Circular dichroism spectroscopy, time resolved fluorescence spectroscopy of zinc-porphyrin–modified [Zn\(^{2+}\)-heme] cyt c and liposome binding studies of the Nle\(^{91}\) mutant were used to demonstrate that ATP induces a conformational change in membrane-bound cyt c. The ATP-induced conformational changes were mediated by Arg 91 and were most pronounced in cyt c bound to phospholipids via the C-site.

It has been previously reported that the hydrophobic interaction between phospholipids and cyt c (C-site) includes the binding of a phospholipid acyl chain inside the protein. In this mechanism, which is known as “extended phospholipid anchorage,” the \(sn\)-2 acyl chain of a membrane phospholipid protrudes out of the membrane surface and is able to bind in a hydrophobic cavity in cyt c. Direct evidence for this type of binding mechanism was obtained by studying cyt c/lipid interaction using fluorescent [Zn\(^{2+}\)-heme] cyt c and fluorescence quenching of brominated fatty acids and phospholipids.

Under certain conditions, cyt c can form fibrillar protein-lipid aggregates with negatively charged phospholipids. These aggregates resemble amyloid fibrils, which are involved in the pathogenesis of many diseases. Congo red staining of these fibers confirmed the presence of amyloid structures. A set of phospholipid-binding proteins was also found to form similar aggregates, suggesting that phospholipid-induced amyloid formation could be a general mechanism of amyloidogenesis.
Structure of cytochrome c
Cytochrome c (cyt c) is a globular 13-kDa protein. It is water soluble and basic, with a net positive charge of +8 at neutral pH and an isoelectric point of almost 10. The native conformation of cyt c consists of a predominantly α-helical structure folded around a covalently bound heme group (Bushnell et al, 1990). In eukaryotes cyt c is encoded by a nuclear gene that is translated by cytosolic ribosomes into a precursor form known as apocytochrome c (Moore & Pettigrew, 1990). Apocytochrome c is transported into the mitochondria, where it is converted to cytochrome c by the enzyme cytochrome c heme lyase, which covalently adds the heme group. The covalent addition of the heme group induces the spontaneous folding of cyt c into its native conformation from the random coil conformation of apocytochrome c without the aid of chaperones (Stuart & Neupert, 1990). In multicellular organisms, cyt c is an electron transfer protein and an initiator of apoptosis. In mitochondria, it peripherally binds to the inner mitochondrial membrane and shuttles electrons from cytochrome c reductase (complex III) to cytochrome c oxidase (complex IV) (Moore & Pettigrew, 1990). During programmed cell death, cyt c is released from the mitochondria into the cytoplasm, resulting in the induction of apoptosis (Ow et al, 2008).

Mammalian cyt c is 104 amino acids long, and 27 of these residues are invariably conserved across eukaryotes. Furthermore, only conservative substitutions are present in many other residues. The conservation of these amino acids indicates that these locations are likely to be critical to the structure and function of this protein. Clearly, most of these locations have a role in maintaining the secondary and tertiary structure of the protein or are responsible for interactions between cyt c and its redox partners in the respiratory chain. While specific amino acids in natively folded cyt c are known to mediate the apoptotic function of cyt c (Hao et al, 2005), other conserved amino acids, including the highly invariant Arginine 91, do not seem to be involved in any of the known functions of cyt c, and the reason for their evolutionary conservation remains unclear.

The prosthetic heme group is covalently linked to the peptide chain at Cys 14 and Cys 17. Under physiologic conditions, the heme iron is additionally bound by two coordinate axial ligands, His 18 and Met 80 (Figure 1) (Bushnell et al, 1990). Met 80 is particularly
important, as changes in its coordination give rise to specific changes in the absorption and CD spectra, reflecting conformational changes during lipid binding (II, III) (Santucci & Ascoli, 1997; Zucchi et al, 2003).

**Phospholipid binding by cyt c**

Cyt c has been the paradigm for peripherally associated membrane proteins for more than three decades (Nicholls, 1974). It is of central biological importance and is readily available as a reagent; therefore, it has become the most commonly used model for protein-membrane interactions and phospholipid binding. A considerable body of literature focusing on cyt c–lipid interactions has accumulated (Kalanxhi & Wallace, 2007).

Cyt c binds to acidic phospholipid-containing membranes at low and moderate ionic strengths. A critical surface charge density and an overall negative charge on the membrane are required for binding (Mustonen et al, 1987): uncharged and zwitterionic phospholipid membranes are not readily bound by cyt c. After electrostatic connections to the phospholipid are made, hydrophobic interactions also develop, as demonstrated by the inability to dissociate cyt c from the membranes with ATP or by increasing the ionic strength (Rytömaa & Kinnunen, 1994; Rytömaa & Kinnunen, 1995; Rytömaa et al, 1992). The hydrophobic interactions are also evident in isolated mitochondrial membranes, as a portion of cyt c remains attached to the membrane even following exposure to high ionic strength solutions (Cortese et al, 1998). Acidic phospholipid binding has been demonstrated using numerous methods, including resonance energy transfer, ultracentrifugation, tryptophan fluorescence, circular dichroism, NMR, differential scanning calorimetry, resonance Raman spectroscopy and surface plasmon resonance (de Jongh et al, 1992; Heimburg et al, 1991; Muga et al, 1991b; Mustonen et al, 1987; Pinheiro et al, 1997; Salamon & Tollin, 1996a; Salamon & Tollin, 1996b).

Phospholipid binding by cyt c is mediated by two different types of interactions that occur on different regions on the surface of the protein. At a neutral pH and with membranes containing a low mole fraction of acidic phospholipids, the dominant interaction is electrostatic. This mode has been termed “A-site” binding. At a low pH and with membranes containing higher levels of acidic phospholipids, increasing amounts of hydrogen bonding and hydrophobic interactions are seen. This mode of interaction which involves hydrophobic interactions has been termed “C-site” binding (Rytömaa & Kinnunen, 1994). Hydrophobic binding to membranes is thought to arise as a consequence of extended lipid anchorage, in which one of the fatty acid chains of a membrane phos-
**Figure 1**

A 3-D model of cyt c. The backbone structure is shown in green cartoon graphics. The heme porphyrin (red), the iron atom (orange), the coordinate axial ligands His 18 (light blue) and Met 80 (yellow) and the proposed lipid binding amino acids Arg 91 (blue) and Asn 52 (cyan) are represented as ball and stick models. The image was created in PyMOL software from the crystal structure datafile 1hrc.pdb (Bushnell et al, 1990).
phospholipid protrudes out of the membrane and incorporates into a hydrophobic channel in cytochrome c, while the other chain remains in the lipid bilayer (Kinnunen et al, 1994; Kinnunen, 1996; Rytömaa & Kinnunen, 1994). This type of phospholipid conformation, which promotes hydrophobic interactions on membrane surfaces has also been described during membrane fusion (Holopainen et al, 1999). The presented model has now gained general acceptance as a mechanism underlying the interactions with also other peripheral membrane proteins (Greenberg et al, 2008; Hagan et al, 2008).

Several studies have reported changes in the tertiary structure of cytochrome c upon lipid binding, including the unfolding of the protein, a reduction in the α-helical content (Muga et al, 1991b; Pinheiro et al, 1997; Spooner & Watts, 1991), changes in the heme iron coordination state (Zucchi et al, 2003) and both increases and decreases in the redox potential (Huang & Kimura, 1984; Salamon & Tollin, 1997). Some have also suggested that cytochrome c partially or completely incorporates into the phospholipid membrane (Heimburg & Marsh, 1995; Salamon & Tollin, 1996a). The conformational changes induced by membrane binding depend on the acyl chain length and the degree of saturation of the membrane phospholipid acyl chains, indicating that the protein interacts with the hydrophobic acyl chains in the lipid bilayer (Nantes et al, 2001; Stewart et al, 2000; Zucchi et al, 2003).

While there are conflicting data in the literature, it can be concluded that the binding of cytochrome c to acidic phospholipids induces a conformational change in the protein that results in a structure with decreased organization and reduced thermal stability that retains a native-like secondary structure (Muga et al, 1991a). Overall, this conformation resembles a molten-globule state (Pinheiro et al, 1997). In some systems, the Met 80–heme coordination bond is disrupted as a result of this conformational change (Oellerich et al, 2004; Stewart et al, 2000). The degree of conformational change depends on the surface charge density of the membrane, in keeping with the requirements for the two different types binding (A- and C-sites) (Muga et al, 1991b).

In phospholipid monolayer experiments, cytochrome c displays two different types of interactions with phosphatidylethanolamine. At low surface pressures, cytochrome c penetrates the monolayer, whereas at higher pressures cytochrome c is not able to penetrate the monolayer. A considerable amount of cytochrome c is bound to the monolayer surface at higher pressure, however, and this binding is sensitive to increasing salt concentrations or perfusion of the subphase with fresh buffer. With monolayers of cardiolipin (instead of phosphatidylethanolamine), this high surface pressure adsorption cannot be abolished with salt. The authors of this study concluded that the cytochrome c–cardiolipin complex undergoes a time-
Figure 3
A model of cyt c binding to CL according to Sinibaldi et al., 2010 displaying the proposed acyl chain binding sites for two acyl chains in extended conformation. Image was kindly provided by Dr. Fabio Polticelli.
The lipid-binding sites on cyt c have been identified using mutant proteins prepared by bacterial co-expression of cyt c and cytochrome c heme lyase (Kalanxhi & Wallace, 2007; Sinibaldi et al, 2010). A hydrophobic channel exists inside cyt c that can accommodate an extended phospholipid acyl chain. One end of this hydrophobic channel opens to the surface near the invariant Asn 52 (Figure 3). Mutation of this residue to isoleucine, which drastically distorts the hydrophobicity of the channel, reduces the CL-induced effects on the conformation of cyt c (Sinibaldi et al, 2010). The location of the other lipid-binding site is less clear. The area around Arg 91 constitutes an ATP-binding site (II) and mediates an interaction with the membrane (I). In the 3D structure, another hydrophobic crevice is apparent in the vicinity of Arg 91; this site has been proposed to be the binding site for free fatty acids (Kalanxhi & Wallace, 2007; Stewart et al, 2000). In conclusion, there is now considerable evidence to support the idea that there are two distinct phospholipid-binding sites on the surface of cyt c located in the proximity of Arg 91 and Asn 52.

**Cyt c in apoptosis**

The canonical function of cyt c is the transfer of electrons in the mitochondrial electron transport chain. It is now recognized that cyt c has a second crucial activity that is fundamentally important for the development of multicellular organisms. During programmed cell death (apoptosis), cyt c is released from the mitochondria into the cytoplasm (Kluck et al, 1997; Liu et al, 1996). After its release, cyt c binds to proteins Apaf-1 and procaspase-9 to form a multimeric complex known as the apoptosome (Ow et al, 2008). Apoptosome formation requires the binding and hydrolysis of ATP or dATP. Even though dATP is a more effective activator of the apoptosome, ATP is more likely to be the physiologic ligand as it is more readily available in the cytoplasm (Kim et al, 2005; Li et al, 1997b). The formation of the multimeric apoptosome activates caspase-9, a proapoptotic protease that initiates the activation of downstream executioner caspases, such as caspase-3 and caspase-7. This apoptotic pathway is generally known as the intrinsic or mitochondrial pathway, and the release of cyt c into the cytoplasm is the rate-limiting step. Indeed, it has been demonstrated that microinjection of exogenous cyt c into the cytoplasm is sufficient to initiate programmed cell death (Li et al, 1997a; Zhivotovsky et al, 1998). Therefore, simply the presence of cyt c in the cytoplasm is sufficient to induce apoptosis, emphasizing the importance of the release of cyt c from the mitochondria for regulating cell death. Proper cyt c function in the apoptosome requires both the entire polypeptide sequence in its natively folded...
conformation and the heme group. However, while the presence of the heme group is required, its electron transfer activity is not, as [Zn²⁺-heme] cyt c is functional in the apoptosome (Liu et al, 1996). Experiments using apopotosomes reconstituted from highly purified recombinant components have demonstrated that binding of cyt c to Apaf-1 induces ATP hydrolysis by Apaf-1, however, the cyt c–binding site on Apaf-1 is distinct from the ATP hydrolysis site. (Kim et al, 2005). The modification of Lys 72 on the surface of cyt c decreases its proapoptotic potential but does not significantly affect its function in the electron transfer chain (Hao et al, 2005). The same region of the cyt c surface is thought to be involved in lipid binding (Kalanxhi & Wallace, 2007; Rytömaa & Kinnunen, 1995; Sinibaldi et al, 2010). To date, however, there is no direct evidence suggesting that interactions with phospholipids are involved in apoptosome formation after the release of cyt c from the mitochondria.

Many factors regulate cyt c release and the initiation of the mitochondrial apoptosis pathway. The activation of proapoptotic signaling molecules, including BID, BAX and BAK, promotes cyt c release, while antiapoptotic molecules, such as BCL-2 and BCL-xL, inhibit release (Basanez et al, 2002; Kagan et al, 2005; Li et al, 1997a; Yang et al, 1997). Importantly, none of the regulatory proteins that control the release of cyt c from the mitochondria have been shown to interact with cyt c itself. Rather, these proteins predominantly contribute to the permeability of the outer mitochondrial membrane and the structure of the cristae formed by the inner mitochondrial membrane during apoptosis (Kagan et al, 2009). What seems most important for cyt c release is the attachment of cyt c to the inner mitochondrial membrane. Translocation through the outer mitochondrial membrane after detachment seems to be a secondary event and is not specific to cyt c, as many proteins of similar size are also released after the initiation of the mitochondrial apoptosis pathway (Kagan et al, 2009; Ow et al, 2008).

The physiological binding site of cyt c in the mitochondrial membrane is CL (Speck et al, 1983; Vik et al, 1981). This interaction is important both for the function of cyt c in the electron transfer chain and for preventing the release of cyt c from the mitochondrial intermembrane space. Recently, it has been demonstrated that mitochondrial cyt c binds to CL using the extended lipid anchorage (Sinibaldi et al, 2010). This mode of membrane binding has been proposed to induce a conformational change in cyt c and promote the emergence of lipid peroxidase activity in cyt c (Kagan et al, 2009; Kagan et al, 2005; Kagan et al, 2004). Based on this model, yet unknown factors would trigger the peroxidase activity of membrane-bound cyt c in the mitochondria. The subsequent peroxidation of mitochon-
drial CL would simultaneously lead to decreased membrane integrity and the detachment of cyt c from CL due to its reduced affinity for peroxidated CL side chains (Belikova et al, 2006; Mattila et al, 2008). This model suggests that a phospholipid-mediated mechanism could control apoptosis and further highlights the importance of understanding the interactions between cyt c and phospholipids. The peroxidase activity also potentially explains the rationale for having heme-containing cyt c also function as an apoptotic protein. Altogether, the discovery of the dual functions of cyt c has challenged the old biological dogma of one gene producing one protein with only one unique function.

**ATP binding**

*In vitro*, cyt c binds ATP with a low millimolar affinity (Craig & Wallace, 1991a). A similar concentration of ATP is present in the mitochondrial intermembrane space and cytosol (Bjursell & Skoog, 1980). The binding of ATP to cyt c is mediated through a site that contains the invariant Arg 91 and nearby amino acids. ATP binding at this site can be abrogated by photoaffinity labeling or amino acid substitutions (McIntosh et al, 1996). Photoaffinity-modified 8-azido-ATP cyt c also has reduced activity in the electron transfer chain (Craig & Wallace, 1995; Lin et al, 1995). Therefore, it has been suggested that the modulation of the reactivity of cyt c via ATP binding may comprise a feedback mechanism that regulates the electron transfer chain.

Physiological concentrations of ATP inhibit the conformational changes in cyt c that are induced by oleic acid. As a result, lipid binding–induced peroxidase activity is also reduced (Sinibaldi et al, 2005). Therefore, ATP binding influences both protein conformation and reactivity, thereby functioning as a regulatory mechanism for respiration and apoptosis.

**Zinc-heme cyt c**

Synthetic cyt c species have been created by replacing the heme iron in the heme group with other metal ions. The Fe$^{2+/3+}$ ion in the heme group can be removed with hydrogen fluoride to generate an iron-free porphyrin cyt c. Porphyrin cyt c readily binds soluble metal ions to create metalloporphyrin cyt c species. The most widely studied of these species is a cyt c protein bearing a Zn$^{2+}$ ion ([Zn$^{2+}$-heme] cyt c) in place of the iron molecule. [Zn$^{2+}$-heme] cyt c lacks the redox capabilities of wt cyt c but retains a very similar 3D structure (Anni et al, 1995; Vanderkooi et al, 1976; Vanderkooi & Erecinska, 1975). Also, both of the coordinating bonds between the Zn$^{2+}$ ion and His 18 and Met 80 and are present, indi-
cating that this protein is likely to retain wt properties (Qian et al, 2003). The [Zn$^{2+}$-heme] is intrinsically fluorescent and can be used for fluorescence spectroscopy without the addition of other chemical moieties (Anni et al, 1995). Wt cyt $c$ is not fluorescent in its folded conformation because the heme group effectively quenches the fluorescence of the tryptophan residues in cyt $c$. For the same reason, even wt cyt $c$ species that are labeled with conventional fluorescent moieties are not useful for fluorescence studies. The fluorescent [Zn$^{2+}$-heme] can be used alone or as a resonance energy transfer pair with tryptophans to monitor changes in cyt $c$ structure (II) (Ensign et al, 2008).

[Zn$^{2+}$-heme] cyt $c$ supports active apoptosome formation in cell-free apoptosis models. Therefore, the redox activity of cyt $c$ is not required for the induction of apoptosis after its release from the mitochondria (Liu et al, 1996), further indicating that [Zn$^{2+}$-heme] cyt $c$ behaves similarly to the wt protein.

**Amyloid fibers**

Some proteins or peptides can convert from their native soluble conformations into ordered fibrillar aggregates. The accumulation of these fibrous aggregates in tissues occurs during many diseases, including neurodegenerative Alzheimer’s disease, prion diseases and systemic amyloidosis. These highly organized fibrillar aggregates are called amyloid deposits because the pathologist who discovered them first believed they were composed of carbohydrates (Virchow, 1854). The deposits found in diseased individuals actually consist predominantly of polymerized polypeptides in a repeating β-sheet conformation (Aguzzi & O'Connor, 2010; Chiti & Dobson, 2006). Electron microscopy and AFM have shown that these aggregates are organized as protofilaments that are twisted together to form rope-like ribbons. Each protofilament is approximately 2–5 nm in diameter, and bundles of protofilaments form up to 30 nm wide ribbons. These elongated structures are termed fibrils, and they can be macroscopic in scale (Saiki et al, 2005; Serpell et al, 2000). Because they are not crystalline enough to be studied using diffraction methods and are too heterogeneous for NMR spectroscopy, it has been a challenge to acquire any detailed structural information regarding these aggregates. Recent advances in spectroscopic methods, however, have revealed more about the molecular structure of these aggregates. While the amyloid structures are basically polymers that are rich in pleated β-sheets, there is now also evidence for structural heterogeneity. Indeed, several different structural forms of the same amyloidogenic polypeptide have been found in individual fibers (Chiti & Dobson, 2006; Chiti & Dobson, 2009; Luhrs et al, 2005; Sawaya et al, 2007).
Overall, more than 40 diseases with fibrous protein aggregate deposits have been described. Each of these diseases has a distinct clinical profile and is associated with the aggregation of a single dominant protein or peptide. Some of these conditions are hereditary, caused by mutations in the dominant polypeptide, but most are considered sporadic, in which the deposited protein or peptide has a wt sequence. In addition, there are transmissible forms of amyloid diseases, such as transmissible spongiform encephalopathy. In these diseases, the transmission of a small amount of the fibrous deposits to a healthy individual triggers amyloid deposition in the recipient. No abnormal or foreign genetic material, such as bacterial or viral DNA or RNA, is needed to transfer these diseases. Therefore, it seems that the abnormal folding of an otherwise normal protein is the underlying mechanism of these diseases. Interestingly, the proteins or peptides that cause these diseases do not seem to share any structural or conformational homology until they are in the aggregated state. They range in size from 23 to over 1000 amino acids. In their native forms, all types of secondary and tertiary structure are represented. Many of these proteins are water-soluble, globular proteins with no propensity to polymerize. In fact, the factors that contribute to the amyloidogenicity of a protein remain largely unknown (Aguzzi & O’Connor, 2010; Chiti & Dobson, 2006).

The detection and characterization of amyloid protein aggregates in histological samples and in vitro relies on specific dyes that intercalate into the pleated-β sheet structure of the amyloid fibers. The most commonly used compound is the azo-dye Congo Red, although other dyes, such as Thioflavins, can also be used. Amyloid-bound Congo Red molecules that are intercalated into the pleated-β sheet are oriented such that they emit an apple-green birefringence upon exposure to polarized light (Nilsson, 2004). The detection of this birefringence has remained a standard technique for amyloid detection (Alakoskela et al, 2006; Heikenwalder et al, 2007). The specificity of Congo Red staining has been questioned as Congo Red has also been shown to stain smaller protein aggregates that do not strictly fit the classic definition of amyloid deposits in tissue (Khurana et al, 2001). However, these aggregates may still be significant disease-related protein deposits (Aguzzi & O’Connor, 2010).

Amyloid formation initiates with proteins that are in their native functional conformation and the physicochemical steps leading to the formation of the amyloid structure and the subsequent polymerization into fibrils remain unclear. Proteins associated with pathogenic amyloid deposits, as well as many non-disease associated proteins, form amyloid fibrils in vitro (Rochet & Lansbury, 2000). Usually, the native form of the amyloidoid-
genic protein is fully folded, nontoxic and soluble. In vitro, amyloid formation is promoted by denaturing conditions, such as low pH, high temperature, high pressure or the presence of non-aqueous solvents (Schmittschmitt & Scholtz, 2003). A reduction in the overall net charge and a low dielectric environment also enhance rapid fiber formation (Rochet & Lansbury, 2000). These data support a model in which a conformational change, possibly the partial unfolding of the protein, is required to initiate the aggregation and subsequent folding into the amyloid-type conformation. Once aggregation has started, the aggregate may serve as a template for additional protein molecules, leading to rapid aggregation. Most of the known mutations that are associated with the hereditary forms of amyloid deposition diseases have been shown to decrease the conformational stability of the native fold (Sekijima et al, 2005). Accordingly, any condition that promotes changes in the conformation of the protein may also promote amyloid formation by making alternative folding pathways thermodynamically more accessible. The conditions used to induce amyloid formation in vitro are rather harsh and probably not physiologically relevant. Instead, it may be feasible that binding to acidic phospholipids would induce the conformational changes that initiate amyloid formation under physiological conditions.
AIMS OF THE STUDY

This dissertation focuses on the interaction between phospholipids and the peripheral membrane protein cytochrome c. Cytochrome c is an important biological molecule and a model of peripherally associated membrane proteins.

These aims provided the basis for the studies:
1) To investigate the molecular mechanism underlying the interaction between cytochrome c and phospholipids. I–IV
2) To demonstrate that extended phospholipid anchorage is an effective mechanism for peripheral protein–membrane interactions. III
3) To characterize amyloid-containing protein fibers formed by peripheral membrane proteins following interaction with acidic phospholipids. IV
MATeRIALs AND METHODS

Reagents
Pyrene-labeled phospholipid derivatives (PPDPC, PPDPG) were obtained from K&V Bioware (Espoo, Finland). Other phospholipids and NBD-PG were obtained from Sigma or Avanti Polar Lipids (Alabaster, AL). The modified cyt c species were obtained from our collaborators in the group of Dr. Carmichael Wallace. ATP was purchased from Roche Molecular Biochemicals. The phospholipid purity was routinely assayed using thin layer chromatography on silicic acid using chloroform:methanol:water:ammonia (65:20:2:2, v/v) as a solvent system; plates were examined for fluorescence or following iodine staining. All other reagents were obtained from typical commercial suppliers.

Cyt c solutions
We used oxidized, horse heart–derived, wild type cyt c from Sigma unless otherwise specified. To prepare the stock and working solutions, dry, lyophilized protein was weighed and dissolved either in H$_2$O or buffer, usually 20 mM HEPES/0.1 mM EDTA, pH 7.0. The redox state and concentrations were determined by spectrophotometry (Margoliash & Frohwirt, 1959). The redox state of the dissolved protein from the commercial source was consistently at least 98% oxidized and was used as such in experiments with oxidized cyt c. To obtain the reduced form of cyt c, solid sodium dithionite was added to a concentrated cyt c solution until no further change in solution color could be seen. After reduction, the solution was eluted from a disposable gel column in the desired buffer (Sephadex PD-10, Amersham Biosciences, Uppsala, Sweden) to remove the inorganic reaction products and excess reducing agent. The fractions containing cyt c were pooled and used as a stock solution for experiments after determining the concentration. Cyt c solutions were stored at -20°C and kept on ice after thawing.

The cyt c derivative in which Arg 91 was replaced with norleucine (Nle$^{91}$) was prepared by Dr. Wallace and his group using the advanced protein chemistry methods described in the Methods section of paper II and the references therein.
**Preparation of the [Zn$^{2+}$-heme] cyt c**

The substitution of Zn$^{2+}$ for Fe$^{2+}$ in the porphyrin of cyt c yields an intensely fluorescent derivative (Vanderkooi et al., 1976). This analog has been characterized in considerable detail and has been shown to closely resemble the parent protein in most qualities. Therefore, it is a good model for studying the conformation of cyt c (Anni et al., 1995). The [Zn$^{2+}$-heme] cyt c derivative was prepared from horse cyt c as described by Vanderkooi and Erecinska (Vanderkooi & Erecinska, 1975). To obtain iron-free cyt c, HF was first added to generate porphyrin cyt c. The porphyrin cyt c was then treated with ZnCl$_2$ in an aqueous solution to generate [Zn$^{2+}$-heme] cyt c (Vanderkooi et al., 1976). The final product was purified using ion-exchange chromatography and then transferred into the desired buffer using a disposable gel filtration column, as described for wt cyt c. In the experiments, [Zn$^{2+}$-heme] cyt c was handled similarly to wt cyt c.

**Fluorescence spectroscopy**

Lipid binding to cyt c was monitored by measuring the resonance energy transfer between pyrene-labeled lipid PPDPG and the heme group of cyt c, as described previously. When the absorbing heme group is in close proximity to the fluorescent pyrene group in the liposomal membrane, Förster resonance energy transfer quenches the fluorescence. This effect is used to measure the association of heme-carrying cyt c molecules with liposomes (Förster, 1948; Rytömaa & Kinnunen, 1995; Stryer, 1978). The measurements were conducted using a Perkin-Elmer LS50B spectrofluorometer with bandpass filters of 2.5 nm to 5.0 nm for excitation and emission, as required by the signal intensity. Liposome solutions with phospholipids at final concentration of 25 μM were added to a magnetically stirred 4-window quartz cuvette. The cuvette holder was equilibrated at 25°C. Cyt c was titrated in aliquots of 5–10 μl. The indicated amount of ATP was added either to the buffer before preparing the liposomes or after cyt c binding. When ATP was added after cyt c binding, it was titrated in 5 μl aliquots of a 200 mM stock solution until the desired final concentration was reached. The pH of the ATP stock solution was adjusted to pH 7.0 with NaOH. For steady state measurements, the change in fluorescence was allowed to stabilize for 40 s before the signal was recorded. Because of the low concentrations of both cyt c and lipids that were used, only minimal interference from the inner filter was expected.
90-degree light scattering
The 90-degree angle scattering of 500 nm light was used to assay cyt c binding to large unilamellar vesicles (LUVs), as described by Nelsestuen and Lim (Nelsestuen & Lim, 1977). The intensity of light scattering at 90-degrees was measured using a spectrofluorometer with bandwidths set to a minimum at a 500 nm wavelength. The signal was recorded continuously to produce an uninterrupted signal/time graph. Otherwise, the conditions used were the same as those described in the Fluorescence Spectroscopy section. The scattering from buffer or unbound cyt c in the absence of liposomes was negligible.

Preparation of liposomes
The small-pore extrusion method was used to prepare large unilamellar phospholipid vesicles in an aqueous solution (MacDonald et al, 1991). Phospholipids were mixed from stock solutions in CHCl₃ or 9:1 CHCl₃:MeOH to yield the desired lipid compositions. The organic solvent was removed by evaporation under a gentle stream of nitrogen, and the tube was kept under reduced pressure for at least 2 h to remove the remaining trace amounts of solvent. The dried lipids were hydrated in buffer at room temperature with vigorous vortexing to yield concentrations of 1–3 mM. The hydrated lipid dispersions were extruded 15–19 times through double 100 nm polycarbonate membranes using a LiposoFast pneumatic small-volume homogenizer (Avestin, Ottawa, Canada). According to published data, this method produces a uniform population of unilamellar vesicles with an average diameter between 111 and 117 nm (Berger et al, 2001; MacDonald et al, 1991; Wiedmer et al, 2001). The extruded liposome solutions were then divided into aliquots and diluted with buffer to the desired concentrations. Liposomes were stored at +4°C for a maximum of 2 days prior to use.

After publication of the experiments described in this dissertation, the vesicle size was measured directly using a dynamic light scattering instrument (Malvern Instruments, Worcestershire, United Kingdom). After preparing the liposomes in 20 mM HEPES/0.1 mM EDTA, pH 7.0, using this method, the average size was 111.1 ± 14.35 nm and 100.3 ± 17.5 nm for the X₂₀/X₈₀ and X₂₀ liposomes, respectively.

Circular dichroism spectroscopy
Circular dichroism (CD) spectra were collected using an Olis RSF 1000F CD spectrophotometer (On-line Instrument Systems, Bogart, GA, USA). This instrument is a dual beam setup with two photomultiplier tubes used for gathering circular dichroism data.
The light source was a water-cooled 150 W Xenon lamp. The CD spectra were collected in cylindrical CD cells with quartz windows. The measurement cell was thermally controlled with a circulating water bath; a temperature of 25°C was used for all experiments.

The Soret region CD spectra of cyt c in the 380–460 nm range were recorded in 10 mm path-length cells. The final spectra, representing the average of at least three tracings, were background-corrected. The cyt c CD data are expressed as the difference between the extinction coefficients for left- and right-circularly polarized light calculated per heme.

**Brominated lipids**

The brominated lipid derivatives 9,10-dibromostearic acid and 1-palmitoyl-2-(9,10)-dibromo-sn-glycero-3-phospho-rac-glycerol used in the study were produced from oleic acid and POPG, respectively. Br₂ reacts with the double bond of the oleic acid moiety by addition (Figure 5).

Lipids (approximately 1 µmole) were dissolved in 1 ml of octane, and a solution of 5% Br₂ (w/v) in octane was added in 2 µl aliquots until the mixture became the color of molecular bromine. The solutions were immediately stored at ~20°C for at least 24 h. Unreacted bromine was removed by evaporation under reduced pressure and by chromatography on a silicic acid column eluted with CHCl₃:MeOH (7:3, v/v). The purity of the products was verified by thin layer chromatography in chloroform:methanol:water:ammonia (65:20:2:2, v/v); a single band was evident following iodine staining (Dawidowicz & Rothman, 1976).

**Fiber formation and imaging of the fibers**

To form the protein-lipid fibers that were studied in paper IV, protein was slowly added to a magnetically stirred liposome solution (25 µM lipid concentration), and the solution was incubated overnight with continuous stirring. The fibers were collected by pipette, transferred onto glass slides and observed using bright field, phase contrast or fluorescence microscopy with an Olympus IX 70 inverted microscope (Olympus Optical, Tokyo, Japan). Where indicated, the fibers were incubated with 10 µM Congo red, and the resulting birefringence was observed using crossed polarizers in the excitation and emission paths. Images of NBD-PG-labeled fibers were taken with a confocal fluorescence microscope equipped with a spinning disk confocal scanner (Yokogawa, Tokyo, Japan) and a 488 nm krypton ion laser light source (Melles Griot, Carlsbad, CA). A long pass filter
dependent conformational change that is triggered by an interaction with the phospholipid acyl chains (Quinn & Dawson, 1969a; Quinn & Dawson, 1969b).

Usually, extended lipid anchorage is not considered a possible mechanism for protein-phospholipid binding in the studies mentioned above. The ability of a peripheral membrane protein to interact with membrane fatty acids without inserting into the membrane could readily explain many of these observations (Gennis, 1989; Hagan et al, 2008; Jensen & Mouritsen, 2004; Kinnunen, 1996; Kinnunen, 1999; Mouritsen, 2005).

The adoption of an extended phospholipid conformation would only require the rotation of the C₂-C₃ carbon bond of the glycerol backbone. This rotation will lead to the protrusion of the sn-2 acyl chain out of the lipid bilayer, allowing it to interact with peripheral membrane proteins (Hauser et al, 1980; Hauser et al, 1988). In natural phospholipids, the presence of a saturated side chain in sn-1 and an unsaturated side chain in sn-2 is conspicuous (Gennis, 1989). The greater structural diversity of sn-2 side chains suggests that they are indeed more likely than sn-1 side chains to take part in biological reactions (Figure 2). This reactivity would be possible in an extended phospholipid conformation.

More recently, the extended lipid anchorage model has been accepted as a valid mechanism for the interaction between cyt c and lipids (Kagan et al, 2009; Kalanxhi & Wallace, 2009).

**Figure 2**

Panel A: The chemical structure of the phospholipid POPG showing structural components of a phospholipid molecule.

Panel B: A CPK model of a POPG molecule in the extended conformation. The sn-2 acyl chain readily adopts this conformation by rotation of a single carbon bond in the glycerol backbone.
 (>510 nm) was used to filter out the excitation light when monitoring NBD-fluorescence. Images were acquired with a Hamamatsu monochrome camera (C4742-95-12 NRB, Hamamatsu Photonics K.K., Hamamatsu, Japan) operated with AquaCosmos software from the manufacturer. Color bright field, phase contrast and fluorescence images were taken using a CMOS SLR camera (Canon EOS-10D) connected to the microscope. Adobe Photoshop software was used to process the microscope images into a suitable format for publication. The image manipulation techniques used during processing were restricted to adjustments of the contrast, color balance and printing resolution.

Figure 4
A schematic representation of a large unilamellar vesicle with cyt c molecules bound onto its surface. The diameter of the vesicles used in this study was approx. 100 nm.
RESULTS

The invariant Arg⁹¹ is required for the aggregation of liposomes by cyt c (I)

The Nle⁹¹ mutant cyt c was prepared to study the previously reported high affinity ATP-binding site of cyt c (Corthesy & Wallace, 1986; Corthesy & Wallace, 1988; Craig & Wallace, 1991a; Wallace & Rose, 1983). The binding of this mutant to large unilamellar vesicles was measured using resonance energy transfer between the membrane-embedded pyrene and the heme group of cyt c. The resonance energy transfer assay following lipid binding revealed no differences between the mutant and the wt proteins (I, Fig. 1). At the end of the experiment, however, increased turbidity was observed in the wt samples, but not in the samples containing the Nle⁹¹ mutant. The resonance energy transfer assay strictly monitors the average proximity of the heme group to the donor lipid. Especially when there is nearly saturated binding and the majority of the fluorescence is quenched, it does not account for changes in the lipid bilayer structure or for aggregation of the liposomes.

To learn more about this process, we used the 90° light scattering assay described by Nelsestuen and Lim. For large unilamellar vesicles and peripherally bound proteins, the scattering intensity mostly reflects the mass of the scattering particle, i.e., the amount of protein bound on the liposome surface (Nelsestuen & Lim, 1977). Scattering was recorded continuously during the addition of wt or Nle⁹¹ cyt c to assess the kinetics of binding. For the wt protein, the light scattering intensity was found to drop drastically after the cyt c concentration exceeded 0.4 µM. The scattering intensity was actually lower than the level of scattering caused by liposomes alone (I, Fig. 2), probably due to the aggregation and subsequent rupture and fusion of the liposomes. The particle size was also dramatically different, and the contributors to signal intensity did not match those of the original experimental setup. While a detailed analysis of the kinetics of this process could not be achieved with our experimental setup, we did note that the addition of excess cyt c after the initial aggregation further accelerated the process. With the Nle⁹¹ mutant, the scattering saturated at a well-defined level correlating with the amount of membrane bound protein bound to the membrane and there was no indication of liposome aggregation.
Furthermore, the signal appeared to reach a steady state after each addition and rapid binding of Nle\textsuperscript{91} to liposomes (I, Fig. 2).

The cyt c concentration required for liposome aggregation roughly corresponded to the concentration required to neutralize the surface charge on the liposomes. Yet, the modification of a single amino acid on the protein surface was found to prevent aggregation. This result suggests that lipid aggregation is mediated by a specific site-mediated lipid interaction rather than being nonspecific aggregation due to decreased repulsion between the liposomes after protein binding.

**ATP binding induces a conformational change in C-site lipid–bound cyt c (II)**

Previous work has demonstrated that while ATP can dissociate cyt c bound to phospholipid liposomes through its A-site, it does not reverse the hydrophobic interactions mediated by the C-site (Rytömaa & Kinnunen, 1995). In publication II, the effects of ATP to lipid-bound cyt c in the conditions that promote C-site interactions are described.

Experiments using fluorescence resonance energy transfer to study the binding of cyt c to large unilamellar liposomes containing pyrene-labeled phospholipid analog PPDPC demonstrated that enhanced transfer occurs in the presence of ATP (II, Fig. 1). This effect has also been reported elsewhere and can be explained either by increased membrane association of cyt c molecules or by changes in the orientation of the heme groups related to the fluorescently labeled membrane. The latter can occur as a result of conformational changes caused by binding of ATP to membrane bound cyt c (Rytömaa & Kinnunen, 1994). This possibility was further studied using circular dichroism spectroscopy (CD) of the heme group absorbance in the Soret region (380–440 nm). The underlying mechanisms of dichroic optical activity in the Soret region are not exactly defined, and therefore the peaks in the CD spectrum are not specifically assigned to any chemical moieties. Nevertheless, changes in the CD spectra in this region are sensitive indicators of the conformational environment of the heme group (Blauer et al, 1993). The trough in the spectrum at 420 nm has been previously assigned to the coordinating bond between the heme iron and the Met 80 residue of cyt c by denaturation studies (Santucci & Ascoli, 1997).

The CD spectra for cyt c in solution and for A-site lipid–bound cyt c are similar in shape and match previously published results (Santucci & Ascoli, 1997). ATP does not induce any detectable change in the CD spectrum of cyt c under these conditions (II, Fig. 2).
The recorded CD spectrum of C-site membrane-bound cyt c (at \( X_{\text{POPG}}=1.0 \) in pH 7.0) is very different from the spectrum of soluble and A-site-bound cyt c. The peak at 410 nm and the trough at 420 nm are reversed and a wide positive band emerges at 390 nm. In the presence of 3 mM ATP, the shape of the spectrum changes even more, with a new peak forming at 405 nm (II, Fig. 2 panel C).

The spectrum recorded for the C-site-bound cyt c suggests that lipid binding disrupts the Fe-Met 80 coordinating bond, indicating that the overall conformation of the protein is different. ATP induces additional changes in the spectrum of the C-site lipid-bound cyt c. Both of these spectra have distinct shapes, including sharp peaks and a rotational strength equal to unbound protein. Therefore, they probably represent organized conformations rather than a simple molten globule or denatured random coil states.

Time-resolved fluorescence spectroscopy of \([\text{Zn}^{2+}\text{-heme}]\) cyt c was used to further investigate the lipid- and ATP-induced conformational changes. The \([\text{Zn}^{2+}\text{-heme}]\) group is intrinsically fluorescent, and changes in its emission lifetime reflect differences in the environment surrounding this prosthetic group. The emission lifetime data for the \([\text{Zn}^{2+}\text{-heme}]\) cyt c were consistent with the results of the CD spectroscopy. Only modest differences were detected in the emission lifetimes of soluble and A-site lipid-bound \([\text{Zn}^{2+}\text{-heme}]\) cyt c. For the C-site-bound \([\text{Zn}^{2+}\text{-heme}]\) cyt c, the presence of 1 mM ATP induced a dramatic increase in the average emission lifetime, indicating that a conformational change occurred (II, table I).

The high-affinity ATP-binding site of cyt c is compromised in the Nle\(^{91}\) analog. Changing the arginine side chain to a straight aliphatic chain deletes the positive charge that contributes to ATP binding without disturbing the overall protein conformation. The C-site lipid-binding properties of this modified cyt c were found to be identical to wt cyt c. In the presence of ATP, the enhanced quenching effects were found to be somewhat diminished yet noticeable (II, Fig. 4). These data were interpreted as a partial loss of the ATP-binding site in the Nle\(^{91}\) analog. Affinity column data confirm this incomplete loss of ATP binding in the Nle\(^{91}\) analog (II, table II). Together, these results suggest that this Arg 91-containing site is important in the ATP-induced conformational changes.

**Extended lipid anchorage (III)**

The interaction between reduced cyt c and oleic acid induces changes in the absorption spectrum that resemble those caused by the oxidation of cyt c. Previous studies have shown that this effect is caused by a spin state change in the heme iron after disruption
of the Met 80–heme coordinating bond due to a conformational change rather than the oxidation of the protein (Nantes et al, 2001; Stewart et al, 2000).

Consistent with previously published data, the spectrum of reduced cyt c lost its double-peak nature, and a shift to a lower wavelength in the Soret band region was detected (III, Fig. 1). These effects are detectable even with concentrations of oleic acid that are below the critical micellar concentration, suggesting that this effect is due to an interaction between the fatty acyl chain itself and cyt c, rather than being a nonspecific detergent effect.

We investigated whether fatty acyl chains could induce similar spectral changes when interacting with cyt c in phospholipid bilayers. Cyt c was incubated with LUVs containing various XPG and excess lipid concentrations. An identical change in the spectrum of cyt c was observed for liposomes containing XPG=1.0, suggesting that the liposomal phospholipid acyl chains are able to interact with membrane-bound proteins in a manner similar to free oleic acid in solution (III, Fig 2). These fatty acyl chain–induced changes did not occur with liposomes at a low XPG, but evolved rapidly when the acidic phospholipid content was raised over XPG=0.6 (III, Fig. 3). The dependence of this effect on the phospholipid composition is identical to what has been observed for hydrophobic C-site binding of cyt c (Rytömaa & Kinnunen, 1994), suggesting that these two interactions share a common mechanism.

The fluorescence of [Zn$^{2+}$-heme] cyt c depends on the immediate environment of the zinc-porphyrin group. We took advantage of this property to study the mechanism of fatty acid binding. Bromine is a well-known collision quencher that can quench the emission of fluorophores following direct contact (Bolen & Holloway, 1990). We used brominated fatty acids and phospholipids with brominated acyl chains to detect fatty acid binding in the interior of cyt c by measuring the quenching of the [Zn$^{2+}$-heme] cyt c fluorescence.

We first assessed the effect of non-brominated lipid species on the fluorescence. We observed that the addition of non-brominated oleic acid caused a biphasic change in the fluorescence of [Zn$^{2+}$-heme] cyt c. After an initial decrease in fluorescence intensity at protein:lipid ratios up to 1:20, the intensity recovered and reached saturation at approximately 90% of the initial level at approximately a 1:40 protein lipid ratio (III, Fig. 4). Similar, yet more pronounced behavior was detected for POPG vesicles (III, Fig. 5). The biphasic response suggests that the interaction could occur through a two-step binding process, or that there are two different binding sites on cyt c. Other authors have also pro-
Brominated oleic acid [(9,10)-dibromostearate] and the corresponding brominated phospholipid P(Br₂)SPG were found to induce a significantly more pronounced decrease in fluorescence intensity than unbrominated fatty acids (III, Figs 4 and 5), presumably because the proximity of the bromine groups in the fatty acid chains to the [Zn²⁺-heme] group inside the protein caused quenching. This effect is even easier to appreciate when the relative [Zn²⁺-heme] fluorescence was normalized to the intensity measured for the identical concentration of nonbrominated fatty acids. We observed pronounced quenching by (9,10)-dibromostearate and P(Br₂)SPG, which saturates at 20–30 µM lipid, corresponding to a cyt c:lipid molar ratio of 1:30–1:40 (III, Fig. 6). The efficient quenching by the brominated fatty acid moiety provides direct evidence for an interaction between this lipid and the [Zn²⁺-heme] moiety in the interior of cyt c.
Fibrous cyt c–phospholipid aggregates (IV)

During the course of the experiments described in paper I, we observed the formation of red fibrous aggregates of cyt c and phospholipids. During the 90-degree light scattering experiments, thin red fibers were visible in the cuvettes after titrating in cyt c. This particular experiment takes up to an hour to complete, as the cyt c solution is slowly titrated into a continuously stirred solution of liposomes. The conditions that seemed to support fiber formation included the presence of acidic phospholipids, an incubation time of 1 hour or more, continuous stirring and rather dilute lipid concentrations, i.e., 10–100 µM lipid.

When the fibers were viewed under a bright field microscope, several different populations of fibers could be observed, even within a single incubation sample. The fibers were typically between 0.2-5.0 mm long and 5-50 µm thick; no correlation between length and thickness was observed. The fibers appeared to have relatively straight, ribbon-like segments that were occasionally twisted into helical structures. Some fibers or parts of fibers appeared frayed or had smaller fibers sticking out of the surface of the main core fiber. The fiber structure was composed of smaller thin fibrils; the size of these fibrils was smaller than what could be readily observed using optical microscopy. The helicity also suggested that the fiber was a periodic superstructure composed of repeating structural motifs (IV, Fig. 1).

A spectrum of colors could be seen in the fibers. Some of the fibers were predominantly red, as can be expected from the absorption spectrum of cyt c, but nearly colorless fibers could also be observed. A small fraction of fibers actually had a blue or magenta tint. Furthermore, a single fiber could exhibit structural transitions along its length. For example, a single fiber could have both blue and red segments, segments with visibly separated ribbons or segments that transition between a rippled and smooth surface. The thickness of a fiber was fairly constant, even with the heterogeneity in the superstructure. This heterogeneity makes the study of its structure in more detail challenging.

The fibers formed under a large range of conditions, although they seemed to require an acidic phospholipid (e.g., phosphatidylglycerol, phosphatidylserine or cardiolipin). A threshold amount of negatively charged phospholipid was required to initiate fiber formation. When cyt c was incubated with liposomes composed of pure zwitterionic POPC, no fibers formed. Experiments with varying lipid compositions indicated that a threshold concentration of approximately $X_{PS}= 0.1$ was required for fiber formation with cyt c. No differences could be detected between liposomes composed of POPG/POPC,
brain-PS/POPC and brain-PS/SOPC. These data resemble the known requirement for a critical surface charge density for electrostatic cyt c membrane binding, suggesting that A-site (or C-site) membrane binding is required to initiate fiber formation (Kinnunen et al, 1994; Rytömaa & Kinnunen, 1994).

To further characterize the fiber structure, we incorporated trace amounts of a fluorescent phospholipid analogue NBD-PG into liposomes prior to fiber formation. Under the fluorescence microscope, these fibers had evenly distributed fluorescence, demonstrating the presence of phospholipids throughout the fiber structure. Strictly speaking, this fluorescence pattern only indicates the presence of the fluorescent phospholipid analogue but does not provide information regarding the localization of the protein in this structure (IV, Fig. 3). Given the known robust interaction between cyt c and phospholipids and the fact that the conditions required for fiber formation were identical to those required for binding of cyt c to liposomes, we think it is highly probable that the fiber is an organized protein-phospholipid microstructure.

The appearance of the cyt c–phospholipid fibers resembled amyloids. To test this hypothesis, we used Congo red to stain the fibers and found that following visualization with cross-polarized light, the fibers almost uniformly emitted the green birefringence characteristic of amyloid structures (IV, Fig. 2). This suggests the presence of an amyloid-like structure (Nilsson, 2004).

Many soluble proteins are known to bind acidic phospholipid membranes in a manner analogous to cyt c. Therefore, we wondered if the formation of fibers with an amyloid-like structure is a universal behavior for acidic phospholipid–binding proteins. To test this hypothesis, we examined the behavior of lysozyme, insulin, glyceraldehyde-3-phosphate dehydrogenase, myoglobin, transthyretin, histone H1 and α-lactalbumin; all of these proteins resemble cyt c in that they are generally water-soluble proteins with clusters of cationic amino acids on their surface. All except transthyretin have been previously reported to bind anionic phospholipid membranes in vitro through a mechanism similar to cyt c (see Table I in the original publication IV for references).

Indeed, we found that all of the tested proteins were capable of inducing the formation of protein-lipid aggregates upon incubation with liposomes made of brain-PS/POPC. The resulting fibers were almost indistinguishable from one another, although the visible red color of the cyt c fibers was absent from fibers produced by other proteins. The variation in individual fibers of a single sample was larger than that for fibers formed from different proteins. As a consequence, we were unable to use our methodology to
study such heterogeneous samples. Later, the structure of the cyt c–phospholipid fibers was studied with FITR- and AFM, and the presence of amyloid-like beta-sheet protein structures was confirmed (Alakoskela et al, 2006). These studies also demonstrated significant variation in the protein structure of these phospholipid–cyt c aggregates. Segments of individual continuous fibers displayed both amyloid (beta-sheet) and native cyt c conformations. The blue color seen in some of the fibers was found to be linked to the amyloid-containing segments, probably as a result of the periodic structure of the fiber causing light interference (Alakoskela et al, 2006).
DISCUSSION

Lack of liposome aggregation by Nle\textsuperscript{91} (I)
Previous studies have provided evidence for multiple phospholipid-binding sites in cyt c (Rytömaa & Kinnunen, 1994). The aggregation of liposomes by cyt c is consistent with this model. At low cyt c: negatively charged phospholipid ratios, the electrostatic repulsion of the liposomes seems to prevent aggregation. After surface charge neutralization, the wt cyt c molecule is able to bind two separate liposomes and cause aggregation; ultimately, this aggregation results in rupture and/or fusion of the liposomes and the formation of macroscopic aggregates. Because the aggregation behavior can be disrupted with the perturbation of a single amino acid, Arg\textsuperscript{91}, this activity is likely to be mediated by a defined site. Importantly, the Arg\textsuperscript{91} residue is evolutionarily invariant, suggesting that this amino acid may constitute an evolutionarily conserved phospholipid-binding site. Other than phospholipid and ATP binding, no other hypothesis has been put forth to account for the conservation of this residue (Ow et al, 2008)(II).

Eventually, this rapid liposome aggregation seems to favor the formation of amyloid-containing fibers. However, they seem to be separate processes as turbidity-increasing aggregation is not required for fiber formation. Also, the kinetics of the liposome aggregation detected using scattering is an order of a magnitude faster than the appearance of the fibers (I, IV and unpublished observation). Unfortunately, the availability of Nle\textsuperscript{91} cyt c and technical difficulties prevented us from studying the kinetics of fiber formation in more detail.

ATP binding affects the conformation of lipid-bound cyt c (II)
Over the years, evidence has accumulated in support of a direct role for ATP in the regulation of the biological function of cyt c. The Nle\textsuperscript{91} mutant was designed to perturb the invariant Arg 91 and to assess its effect on ATP binding. The modification of arginine to norleucine removes a charge without altering protein stability or the secondary/tertiary structure. This modification is much more conservative and specific than previous modifications of this binding site, in particular N7,N8-(1,2-dihydroxycyclohex-1,2-ene)diyl-L-arginine 91-cyt c and 8-azido-ATP cyt c, which introduce bulky chemical moieties into
this binding site (Craig & Wallace, 1991b). The affinity filtration data demonstrate that ATP binding of this mutant is markedly lower but not completely lost (II). Accordingly, the ATP-mediated conformational changes were found to be diminished in magnitude but still detectable. The inability of this mutant to aggregate liposomes was dramatically different from the wt protein (I).

All of the methods that were used to study ATP binding to phospholipid-bound cyt c demonstrated a conformational change in C-site-bound cyt c. Using Soret region CD spectroscopy to monitor the heme environment, it became clear that the conformation of cyt c in the presence of ATP is distinct from any of the conformations seen for free or lipid-bound cyt c in the absence of ATP. Therefore, the presence of ATP favors a previously unknown conformational state of membrane-bound cyt c. Subsequent work has demonstrated that ATP affects the lipid-induced peroxidase activity of cyt c. Thus, ATP binding is likely to regulate mitochondrial respiration and apoptosis (Kagan et al, 2005; Sinibaldi et al, 2005).

Extended lipid anchorage (III)

A number of biological reactions are linked to membranes in vivo, and membrane binding provides an additional regulatory step for these reactions. When the diffusion of the reactants is restricted to a two-dimensional membrane surface, the reaction rates can increase 1000 fold. The blood coagulation cascade is a well-known example of an enzymatic cascade being controlled by peripheral membrane association (Kinnunen et al, 1994; Nelsestuen, 1999). The physical state of the membrane can also directly affect the activity of the proteins bound to it (Kinnunen, 1996). Because peripheral membrane association is involved in numerous cellular reactions, it is important to understand the molecular mechanism underlying these interactions.

In addition to electrostatic interactions, hydrogen bonding and partial penetration, extended phospholipid anchorage has been proposed as a mechanism underlying peripheral membrane interactions. This mechanism involves hydrophobic interactions, but the peripherally bound proteins do not penetrate into the membrane. Extended phospholipid anchorage can facilitate membrane binding with a low dissociation constant and allows for the lateral pressure profile of the membrane to regulate membrane binding (Kinnunen, 1996). The lateral pressure profile and H_H11 propensity are two physical properties of membranes and membrane lipids that are defined by the molecular geometry of the membrane components. Phospholipids with bulky hydrophobic acyl chains
and relatively small headgroups have an increased propensity to form non-lamellar lipid phases (e.g., hexagonal H$_{II}$-phase) instead of bilayers to relieve the pressure profile in the membrane. This property would also promote extended phospholipid anchorage of membrane proteins, as extending side chains from the membrane relieves lateral pressure. Therefore, the lipid composition could directly affect the ability of a protein to bind the membrane via an extended anchorage. It should be noted that cardiolipin, which is the physiological membrane ligand for C-site cyt c binding, has four acyl chains and a uniquely high H$_{II}$-propensity. Additionally, the molecular structure of CL readily tolerates the extended conformation of one or possibly two acyl chains, which may be accommodated in the hydrophobic cavities of cyt c (Sinibaldi et al, 2010).

Many previously published results are consistent with extended phospholipid anchorage being the mechanism for the membrane association of cyt c, but the work reported in publication III provides the first direct evidence that an extended membrane phospholipid acyl chain can bind inside a peripheral membrane protein. Other groups have since verified these results (Kagan et al, 2005; Kalanxhi & Wallace, 2007; Sinibaldi et al, 2010).

Figure 6
A schematic representation of a cyt c bound onto a lipid bilayer.
Fibrous protein-lipid aggregates (IV)

Amyloid formation is associated with a number of severe human diseases, including Alzheimer's disease, systemic amyloidosis, Parkinson's disease and prion diseases. While some of these diseases are associated with genetic mutations that result in amino acid changes, most cases occur in individuals with a normal genotype and amino acid sequence. Therefore, the propensity to form amyloid fibrils is probably intrinsic to all proteins at some level. Different environments may favor amyloid formation even for proteins that naturally do not have a high propensity to form amyloids. Therefore, the differences in the amyloidogenicity of proteins are qualitative, not quantitative in nature.

The physicochemical basis of amyloid formation remains poorly understood. In particular, the events leading to the formation of amyloids from normal proteins in vivo are still largely unknown. In general, the conditions that have been found to trigger amyloid formation in vitro, such as low pH, reduced dielectricity and hydrophobic solvents, are not relevant in vivo. Therefore, there are probably unknown additional factors contributing to amyloidogenesis in vivo.

The aggregates that we observed forming in the presence of PS-containing liposomes appear similar to disease-related amyloids. The fact that acidic phospholipid–containing membranes can facilitate amyloid fibril formation at a neutral pH suggests that the interaction between cationic proteins and negatively charged membranes could also be involved in disease-related amyloid formation in vivo. Indeed, this mechanism has already been suggested for several proteins, including the Aβ peptide, α-synuclein and prions (Chi et al, 2008; Morillas et al, 1999; Terzi et al, 1994; Volles et al, 2001). Phospholipids bearing a net negative charge are abundant in various intracellular membranes and are readily available for surface aggregation by proteins. Importantly, the externalization of acidic phospholipids to the outer leaflet of the plasma membrane is increased during inflammation and apoptosis, possibly also favoring lipid-induced amyloidogenesis of extracellular proteins. Externalization of PS also increases in aging blood erythrocyte membranes. The presence of PS on the outer leaflet of erythrocyte plasma membranes has been demonstrated to act as a signal for cell senescence that controls the removal of aged cells from circulation (Boas et al, 1998).

Because we observed that a variety of cationic proteins are capable of forming similar amyloid-containing structures with phospholipids, we suggest that this behavior could be a general phenomenon (IV).
CONCLUSIONS

At high cyt c:acidic phospholipid ratios, we observed rapid aggregation of large unilamellar liposomes. This activity is abolished by the modification of the invariant Arg91, indicating that cyt c possesses multiple phospholipid-binding sites.

ATP binds to both free and membrane-bound cyt c, causing conformational changes in the C-site membrane-bound protein. The ATP-mediated effects are reduced by the modification of Arg91. The evolutionally conserved Arg91 is part of a phospholipid- and ATP-binding site, which perhaps explains its conservation throughout evolution.

Liposomes composed of brominated phospholipids quench the intrinsic fluorescence of [Zn2+-heme] cyt c, indicating that an acyl chain of a membrane phospholipid interacts with the heme group inside the protein. This result provides direct evidence in support of the extended lipid anchorage of cyt c to phospholipid membranes.

We observed the formation of fibrous lipid-protein aggregates under conditions favoring cyt c binding to acidic phospholipids. These fibers had an amyloid-type protein structure. A similar behavior was also observed for a number of other soluble phospholipid-binding proteins. These results suggest that peripheral membrane binding may be a general mechanism by which amyloids form under physiological conditions.
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