

## ATP Induces a Conformational Change in Lipid-bound Cytochrome *c*\*

Received for publication, January 30, 2001  
Published, JBC Papers in Press, March 12, 2001, DOI 10.1074/jbc.M100853200

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**Resonance energy transfer studies using a pyrene-labeled phospholipid derivative 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-sn-glycero-3-phosphoglycerol (donor) and the heme (acceptor) of cytochrome *c* (cyt *c*) have indicated that ATP causes changes in the conformation of the lipid-bound protein (Rytömaa, M., Mustonen, P., and Kinnunen, P. K. J. (1992) *J. Biol. Chem.* 267, 22243–22248). Accordingly, after binding cyt *c* via its so called C-site to neat phosphatidylglycerol liposomes (mole fraction of PG = 1.0) has commenced, further quenching of donor fluorescence is caused by ATP, saturating at 2 mM nucleotide. ATP-induced conformational changes in liposome-associated cyt *c* could be directly demonstrated by CD in the Soret band region (380–460 nm). The latter data were further supported by time-resolved spectroscopy using the fluorescent cyt *c* analog with a Zn<sup>2+</sup>-substituted heme moiety. A high affinity ATP-binding site has been demonstrated in cyt *c* (Craig, D. B., and Wallace, C. J. A. (1993) *Protein Sci.* 2, 966–976) that is compromised by replacing the invariant Arg<sup>91</sup> to norleucine. Although no major effects on conformation and function of cyt *c* were concluded due to the modification, a significantly reduced effect by ATP on the lipid-bound [Nle<sup>91</sup>]cyt *c* was evident, implying that this modulation is mediated via the Arg<sup>91</sup>-containing binding site.**

Cytochrome *c* (cyt *c*)<sup>1</sup> is a mitochondrial peripheral membrane protein functioning in the respiratory chain in the inner mitochondrial membrane, shuttling electrons from cyt *c* reductase to cyt *c* oxidase. An acidic phospholipid, cardiolipin, either alone or complexed with cyt *c* oxidase, provides the membrane-binding site (1, 2). ATP can modulate the electron transfer rate between cyt *c* and its redox partners (3, 4). However, whether

these effects are due to nucleotide binding to cyt *c* or to cyt *c* oxidase, or both remains unclear as both cyt *c* and cyt *c* oxidase have been shown to contain at least one nucleotide-binding site (5, 6). In cyt *c* part of the high affinity ATP-binding site is constituted by the invariant Arg<sup>91</sup> (5, 7–10), and binding of ATP to this decreases the rate of electron flow through the mitochondrial electron transport chain (9). Accordingly, when the ATP-binding site of cyt *c* is occupied by a covalently bound ATP, the electron-transfer activity of cyt *c* with reductase and oxidase are inhibited to 41 and 11–15%, respectively, of the values measured for the native protein (3). However, the redox potential of the above modified cyt *c* remains close to the value of the native form (11). Several studies have indicated ATP-induced changes in the structure of cyt *c*. For example, auto-oxidation of reduced cyt *c* takes place upon its gel filtration in the presence of ATP (7), whereas this is not observed in the absence of the nucleotide (12). ATP has also been shown to reduce the thermal stability of cyt *c* (13).

Interestingly, release of cyt *c* from mitochondria to cytoplasm has been found to be of critical importance in processes connected to programmed cell death (apoptosis), raising a novel and central point of interest in its properties (14, 15, 16). Furthermore, this release represents in most cases the commitment step for the full activation of the cell death program (17). Outlines of the apoptotic function of cyt *c* have been elucidated lately. In cytoplasm cyt *c* forms a complex with a protein called Apaf-1 and caspase-9, and formation of this “apoptosome” complex leads to the activation of the cascade of proteases executing apoptosis in cells. The presence and hydrolysis of ATP or dATP are required for the formation of the apoptosome and thus also make these nucleotides mediators of the programmed cell death (18). The concentration range of ATP present in cytoplasm of living cells is millimolar (19), which is sufficient for its binding to cyt *c* (7). Based on the recognition of different conformations of cyt *c* by monoclonal antibodies, it was recently suggested that the apoptotically active cyt *c* is membrane-bound (20). The combined effects of ATP on cyt *c* and its membrane binding properties could thus provide a possible mechanism to regulate the activity of cyt *c* in triggering apoptosis.

The membrane association of cyt *c* has been extensively studied (21). We have provided evidence for two distinct acidic phospholipid-binding sites in cyt *c* and have nominated these as the A- and C-sites (22). Accordingly, negative surface charge density of the liposomes, pH, and ionic strength together determine whether cyt *c* is bound electrostatically via its A-site or by hydrogen bonding via its C-site (22, 23). Interaction of cyt *c* with lipids has been suggested to involve additionally a hydrophobic interaction between the protein and an acidic phospholipid due to the so-called extended lipid anchorage (24, 25). In

\* This work was supported by the Academy of Finland (to P. K. J. K.), Research and Science Foundation of Farnos, M.D./Ph.D. program of the University of Helsinki (to E. K. J. T.), and the Natural Sciences and Engineering Research Council of Canada (to C. J. A. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: cyt *c*, cytochrome *c*; [Nle<sup>91</sup>]cyt *c*, modified cyt *c* containing norleucine at position 91 instead of arginine; *N*-*t*-Boc, *N*<sup>α</sup>-tertiary butyloxycarbonyl; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PG, phosphatidylglycerol; PPDPG, 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-sn-glycero-3-phosphoglycerol; X<sub>PG</sub>, mole fraction of PG; [Zn<sup>2+</sup>-heme]cyt *c*, zinc-substituted cytochrome *c*; HPLC, high performance liquid chromatography; wt, wild type.

this mechanism one of the acyl chains of acidic phospholipid is accommodated within a hydrophobic channel in cyt *c* (26), whereas the other chain(s) of the glycerophospholipid remain(s) in the lipid bilayer. ATP is able to dissociate the A-site but not the C-site-mediated interaction of cyt *c* with acidic phospholipids (22, 23, 27).

The effects of ATP on cyt *c* associated with liposomes containing acidic phospholipids have indicated that the nucleotide may induce conformational changes in this protein (22). We report here evidence for a change in the conformation of cyt *c* to be induced by ATP, revealed by CD measurements in the Soret region, and time-resolved fluorescence spectroscopy of a cyt *c* analog with a Zn<sup>2+</sup>-substituted heme moiety. Resonance energy transfer measurements demonstrate this effect of ATP to be strongly attenuated for the lipid bound [Nle<sup>91</sup>]cyt *c* in which the high affinity ATP-binding site is abrogated.

#### EXPERIMENTAL PROCEDURES

**Materials**—1-Palmitoyl-2-[10-(pyren-1-yl)decanoyl]-sn-glycero-3-phosphoglycerol (PPDPG) was purchased from K&V Bioware (Espoo, Finland). Horse heart cyt *c* (type VI, oxidized form), egg PG, and egg PC were from Sigma. The Na<sub>2</sub> salt of ATP was from Roche Molecular Biochemicals. No impurities were detected in the above lipids upon thin layer chromatography on silicic acid using chloroform/methanol/water/ammonia (65:20:2:2, v/v) as the solvent system and examination of the plates for pyrene fluorescence or after iodine staining. All other reagents were of reagent grade from Sigma.

**Preparation of Liposomes**—Lipids were dissolved in chloroform and mixed in this solvent to obtain the desired compositions. PPDPG (*X* = 0.01) was used as the fluorescent lipid probe. The solvent was removed under a stream of nitrogen, and the lipid residue was subsequently maintained under reduced pressure for at least 2 h. The dry lipids were then hydrated in 20 mM Hepes, 0.1 mM EDTA, pH 7.0 or 4.0, at room temperature to yield a lipid concentration of 1 mM. To obtain unilamellar vesicles, the hydrated lipid dispersions were extruded with a Liposo-Fast small-volume homogenizer (Avestin, Ottawa, Canada). Samples were subjected to 19 passes through two polycarbonate filters (100 nm pore size, Nucleopore, Pleasanton, CA) installed in tandem (28). Minimal exposure of the lipids to light was ensured throughout the above procedure. Subsequently, the liposome solution was divided into proper aliquots and diluted with the buffer to a final lipid concentration of 25 μM.

**Steady-state Fluorescence Measurements**—The lipid binding of cyt *c* was assessed as described previously (22–24, 29, 30) by monitoring resonance energy transfer (31, 32) between the pyrene containing lipid PPDPG and the heme of cyt *c*. The measurements were conducted with a PerkinElmer Life Sciences LS50B spectrofluorometer using 2.5 and 4.0 nm band passes for excitation and emission beams, respectively. The excitation wavelength was 344 nm, and the quenching of PPDPG fluorescence by the heme of cyt *c* was measured at 398 nm. Two ml of liposome solution were placed into a magnetically stirred 4-window quartz cuvette in a holder thermostated with a circulating water bath at 25 °C. Subsequently, ATP was included to yield up to 5 mM final concentration, and then 5- or 10-μl aliquots of a 20–40 μM solution of native or Arg<sup>91</sup>-modified cyt *c* were added, and the quenching of pyrene fluorescence by the heme of cyt *c* was observed. In experiments at pH 4 ATP was added after the addition of cyt *c*. Changes in fluorescence were allowed to stabilize for ~40 s, and then the intensity of pyrene monomer fluorescence was recorded. Because of the low concentrations of both lipids and cyt *c* generally utilized, minimal interference by the inner filter effect was expected. The merits as well as limitations of the use of pyrene-labeled lipids in energy transfer measurements have been discussed elsewhere (22, 33–35).

Formation of a hexagonal H<sub>II</sub> phase has been demonstrated as a consequence of the cyt *c*-cardiolipin interaction (36). Accordingly, although cardiolipin is likely to constitute the membrane-binding site for cyt *c* in the inner mitochondrial membrane (1, 2), the present experiments were conducted using PG as the acidic phospholipid so as to avoid ambiguities in the interpretation of the results arising from the formation of a H<sub>II</sub> phase. Except for interference presumably arising from the formation of the inverted hexagonal phase as well as peculiarities in the extent of protonation of the two vicinal phosphates of cardiolipin, we have found no evidence for significant differences in the binding of cyt *c* to these two lipids, cardiolipin and PG.

**Circular Dichroism Spectroscopy**—CD spectra were collected with an Olis RSF 1000F CD spectrophotometer (On-line Instrument Systems

Inc., Bogart, GA). Soret region CD spectra in the 380–460 nm region were recorded with 10-mm path length cells containing 20 mM Hepes, 0.1 mM EDTA, pH 7.0, 10 μM cyt *c*, LUVs, and different concentrations of ATP where indicated. Measurement cell was thermostated to 25 °C with a circulating water bath. Final spectra, representing the average of at least three tracings, were corrected for the background. CD is expressed as difference of the extinction coefficients for left and right circularly polarized light calculated per heme.

**Time-resolved Fluorescence Spectroscopy of [Zn<sup>2+</sup>-Heme]cyt *c***—The intrinsic Trp fluorescence in cyt *c* is nearly imperceptible due to quenching by the vicinal strongly absorbing heme, preventing the use of this fluorescent amino acid as an intrinsic probe for the conformation of cyt *c*. Substitution of Zn<sup>2+</sup> for Fe<sup>2+</sup> in the porphyrin of cyt *c* yields an intensely fluorescent derivative of cyt *c* (37). This analog has been characterized in considerable detail and has been shown to resemble closely the parent protein in most qualities thus representing a good model to study the conformation of cyt *c* (38). This cyt *c* derivative was prepared from horse cyt *c* according to Vanderkooi and Erecinska (39). Iron-free cyt *c* was first made, which was subsequently treated with ZnCl<sub>2</sub> to yield [Zn<sup>2+</sup>-heme]cyt *c* (37). Also in the [Zn<sup>2+</sup>-heme]cyt *c* the energy transfer was very efficient, and practically no Trp emission could be measured (data not shown).

A commercial laser spectrometer (Photon Technology International, Ontario, Canada) was used to measure fluorescence lifetimes. A train of 500-ps pulses at a repetition rate of 10 Hz was produced by a nitrogen laser, pumping a dye (rhodamine 6G, Merck, 5 mM solution in methanol) laser and followed by frequency doubling to yield the excitation pulses at 298 nm. Excitation maximum for the [Zn<sup>2+</sup>-heme]cyt *c* is at approximately 415 nm. In order to maximize the spectral separation between the excitation and emission bands, we used energy transfer from Trp for the excitation of the Zn-porphyrin, *i.e.* exciting the single Trp residue at 295 nm. Fluorescence from the Zn-porphyrin at 640 nm is thus due to Förster type resonance energy transfer (31, 32) from the Trp residue (donor) to the Zn-porphyrin (acceptor) of cyt *c*. Two ml of 10 μM [Zn<sup>2+</sup>-heme]cyt *c* in 20 mM Hepes, 0.1 mM EDTA, pH 7.0, was placed in a magnetically stirred 4-window quartz cuvette in a holder thermostated with a circulating water bath at 25 °C. For the measurement of fluorescence lifetimes, the average of five emission decay curves at 640 nm was analyzed by the non-linear least squares method and fit to a sum of exponentials using the software provided by the instrument manufacturer. Instrument response functions were measured separately using aqueous glycogen solution. The validity of the fit of a particular model was judged by the value of the reduced χ<sup>2</sup> (40, 41).

Fractional intensities *I*(*t*) were calculated according to Equation 1,

$$I(t) = \alpha_i \tau_i^j \sum_{i=1}^N \alpha_i \tau_i \quad (\text{Eq. 1})$$

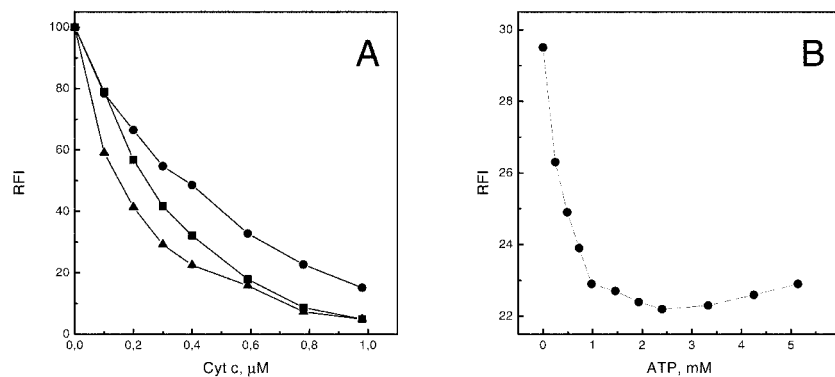
where α<sub>*i*</sub> is amplitude and τ<sub>*i*</sub> is lifetime.

Fluorescence emission decay for [Zn<sup>2+</sup>-heme]cyt *c* in solution had an average lifetime  $\bar{\tau}$  of ~4.2 ns. The emission decays for [Zn<sup>2+</sup>-heme]cyt *c* could be best fitted to a two-exponential process, yielding short (τ<sub>1</sub>) and long (τ<sub>2</sub>) lifetime components (Table I).

At this point it is relevant to note that differences were evident in the lifetimes of different [Zn<sup>2+</sup>-heme]cyt *c* preparations. Yet, the qualitative changes measured under the different conditions used were highly reproducible.

**Semisynthesis and Characterization of [Nle<sup>91</sup>]cyt *c***—Solid-phase peptide synthesis was used to first obtain a synthetic fragment representing residues 66–104 of the horse cyt *c* sequence, with the naturally occurring Arg<sup>91</sup> replaced by norleucine, *i.e.* with a straight aliphatic chain instead of the alkylguanidine. Methods for monitoring reaction progress, resin cleavage, deprotection, and purification of peptides were as described previously (42). Glu (the C-terminal residue) was coupled via a 4-carboxyamidomethyl-benzyl ester to a cross-linked polystyrene resin (0.4 mmol), and the peptide chain was extended with symmetrical anhydrides of side chain-protected *N*-*t*-Boc amino acids (or 1-hydroxy-benzotriazole esters of Boc-Asn or Boc-Glu) in an automated system. Purity was checked by analytical reversed-phase HPLC, amino acid analysis, and mass spectrometry. The 39-residue peptide (163 mg) had the expected amino acid composition and a molecular mass of 4529.9 ± 0.8 (calculated 4530.4). The complementary fragment 1–65 with the covalently linked heme at Cys<sup>14</sup> and Cys<sup>17</sup> was prepared by CNBr cleavage from natural horse cyt *c*. Its purification and coupling at equimolar ratio to synthetic 66–104 to create the semisynthetic holo-cytochrome were as reported previously (42). After ligation the product

FIG. 1. Effect of ATP on the C-site membrane association of native cyt *c* liposomes. Panel A, binding of cyt *c* in the presence of ATP to liposomes containing  $X_{PG} = 1.0$ , pH 7. The concentration of ATP was 0 (●), 1 (■), or 2 (▲) mM. Panel B, effect of subsequently added ATP on C-site bound cyt *c*. The protein was added prior to the addition of ATP to a final concentration of 0.15  $\mu\text{M}$ . The mole fraction of PG in PC liposomes was 0.20 at pH 4. Total lipid concentration in 20 mM Hepes, 0.1 mM EDTA was 25  $\mu\text{M}$ .



was separated from unreacted peptides by Sephadex G50 gel exclusion chromatography in 7% HCOOH, with a crude yield of 50%. The protein was renatured by buffer exchange from 8 M urea into 50 mM potassium phosphate, pH 7, and finally purified by low pressure cation-exchange chromatography. The semisynthetic cyt *c* analog revealed a single component by HPLC and was 97% reducible by ascorbate. The UV-visible absorption spectra for both oxidation states of the analog were fully superimposable with those of the native protein (data not shown). The above indicate no significant disruption of the coordination sphere or the environment of the heme in the interior of the protein.

Upon elution of the  $[\text{Nle}^{91}]$ cyt *c* in both oxidation states on a Waters 600E HPLC system using an SP5PW cation-exchange column and a gradient of phosphate buffer (40–400 mM, pH 7), the elution times were exactly as expected on the basis of deletion of a single positive charge (Table II), mirroring the absence of the basic guanidino group (42). Titrations of the disappearance of the 695 nm charge transfer band that signals heme iron-Met<sup>80</sup> ligation with pH change were performed in 50 mM phosphate buffer (43). A minimal change was noted in the resistance of the protein to the conformational transition precipitating the ligand exchange reaction at alkaline pH (44), but a somewhat larger effect on the more complex denaturation process at acid pH was observed (12). It is possible that in the native structure the proximity of the guanidino group to the carboxylate of Glu<sup>69</sup> could be stabilizing at low pH, forming an ion pair in the absence of ATP.

The association of ATP with  $[\text{Nle}^{91}]$ cyt *c* was assessed by two methods. First, elution times on an ATP-agarose column equilibrated in 35 mM phosphate were measured. The observed retention times in this column vary widely for different cyt *c* analogs and provide a measure of the affinity of the protein for ATP (7). This method revealed  $[\text{Nle}^{91}]$ cyt *c* to bind ATP substantially less effectively than the native protein, and the elution time from a column with immobilized ATP is half that of cyt *c* (12 versus 24 min). Second, equilibrium gel filtration of both native protein and the  $\text{Nle}^{91}$  analog was undertaken in 10 mM Tris cacodylate buffer, pH 6.95, containing 0.1 mM ATP, as described by Corthésy and Wallace (5). This method gave the nucleotide/cyt *c* stoichiometry of 0.37 for the analog and 1.14 for the native protein.  $[\text{Nle}^{91}]$ cyt *c* thus represents a model for the parent protein in which the only function compromised is ATP binding.

## RESULTS

**Effects of ATP on the Binding of cyt *c* to Liposomes**—At neutral pH ATP dissociates cyt *c* from membranes with a low content ( $X = 0.20$ ) of acidic phospholipids (22, 23). This A-site interaction of the protein with lipids is mainly electrostatic in nature, and the dissociation has been concluded to result from a competition between ATP and the deprotonated phosphate group of acidic lipids for the binding to cyt *c* (22). In the presence of ATP the ionic strength required to dissociate cyt *c* from the inner mitochondrial membrane decreases (3) in keeping with our results on the binding of cyt *c* to liposomes *in vitro* (23). If the content of acidic phospholipids in membranes is increased or, alternatively, the pH of the medium is lowered at a low acidic phospholipid content in the liposomes, the membrane association of cyt *c* changes from A- to C-site interaction (22, 23). The C-site interaction of cyt *c* is not dissociated by nucleotides, and elevated ionic strength is able to dissociate the protein only if it causes the deprotonation of acidic phospholipids, thus changing the interaction from C- to A-site.

We have previously reported the C-site interaction of cyt *c* with liposomes at acidic pH to be modulated by ATP, and we have suggested a conformational change in the membrane-bound protein to be induced by the nucleotide (22). This is seen in the data measured at neutral pH and at  $X_{PG} = 1.0$  (Fig. 1, panel A). Accordingly, efficiency of quenching of pyrene fluorescence due to cyt *c* bound to membranes was augmented in the presence of increasing nucleotide concentrations; this effect was saturated at 2 mM ATP. We have suggested the enhanced fluorescence quenching to be due to an ATP-induced conformational change in cyt *c*, which then results in a more efficient resonance energy transfer (*i.e.* dipole-dipole coupling) between pyrene and the heme of cyt *c* (23). The latter could be caused by an altered orientation of the heme of the membrane-bound cyt *c* with respect to the relaxation dipole of pyrene. Instead of increasing the negative surface charge density of liposomes at neutral bulk pH by increasing  $X_{PG}$ , the C-site interaction of cyt *c* can also be achieved at  $X_{PG} = 0.2$  by decreasing pH to 4.0, thus promoting the protonation of the acidic phospholipids (22). A conformational change in cyt *c* due to ATP was also indicated under these conditions (pH 4.0 and at  $X_{PG} = 0.2$ ) by the increase in fluorescence quenching due to the addition of ATP after the membrane association of cyt *c* had commenced (Fig. 1, panel B).

**CD Spectroscopy of Membrane-bound cyt *c***—The circular dichroism of heme group in cyt *c* was measured in order to monitor structural changes in cyt *c*. The exact underlying mechanism resulting in optical activity in the Soret region near 400 nm is uncertain, and the peaks in the cyt *c* spectrum cannot be assigned with absolute certainty to specific chemical moieties. Nevertheless, CD spectra of this region are strongly dependent on the immediate conformational environment of the heme group and provide a sensitive indicator of small scale conformational changes in the protein (45). Denaturation studies have indicated that the trough at 420 nm relates to the heme iron–Met (80) bond in cyt *c* (46). The spectrum of cyt *c* in solution (Fig. 2, panel A) was similar to data published previously (46, 47) and was composed of a distinct shoulder at 390 nm and a trough at  $\sim 420$  nm. The spectrum measured in the presence of 3 mM ATP was identical to the spectrum of free cyt *c*, indicating no changes in the heme environment upon interaction of ATP with cyt *c* in solution.

The spectrum for A-site membrane-bound cyt *c* ( $X_{PG} = 0.2$ , Fig. 2, panel B) displayed similar features as free cyt *c* but with decreased peak intensities. In the presence of 1 mM ATP cyt *c* remains membrane-bound (Fig. 3, panel B), yet should also bind ATP based on the equilibrium gel filtration measurements (5). However, 1 mM ATP caused no changes in the spectrum. In contrast, the CD spectrum of cyt *c* bound to liposomes via the C-site at  $X_{PG} = 1.0$  (Fig. 2, panel C) was very different from the free cyt *c* in solution as well as from the A-site bound protein. More specifically, a wide positive band was centered at 390 nm,

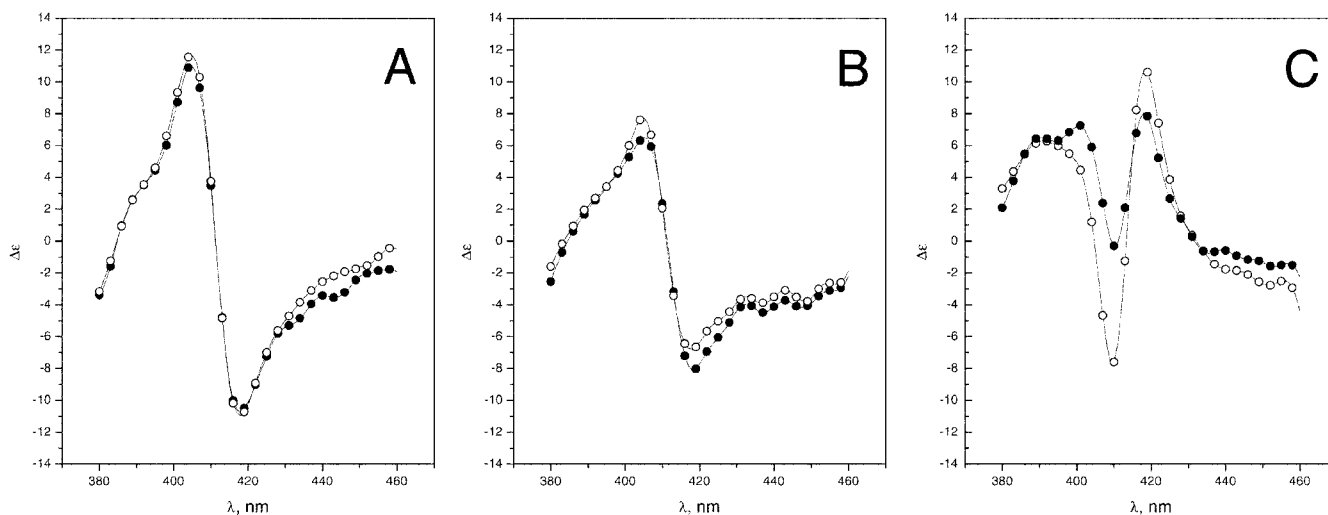
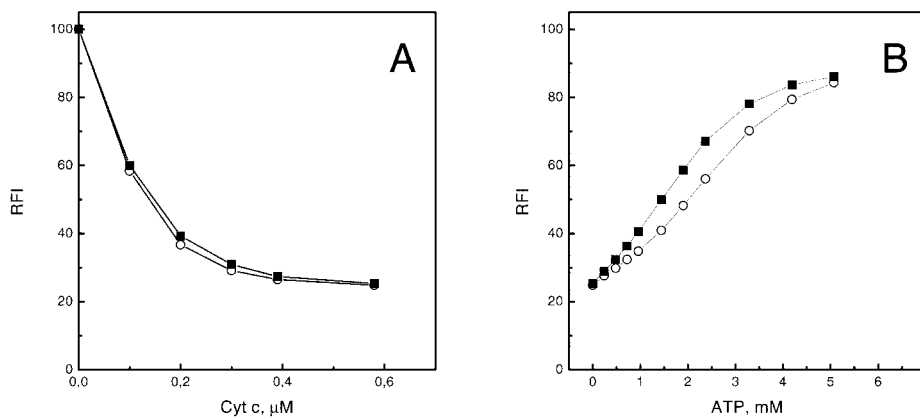


FIG. 2. Effect of ATP on the Soretregion (380–440 nm) CD spectra of cytochrome *c*. cyt *c* concentration was 10  $\mu$ M, and where indicated, LUVs (250  $\mu$ M total lipid) and/or ATP (1 or 3 mM) were added. The mole fraction of POPG in the LUVs was either 0.2 (panel B) or 1.0 (panel C) so as to bind cyt *c* to membrane via A- and C-site, respectively. Medium consisted of 20 mM Hepes, 0.1 mM EDTA, pH 7.0. Panel A, cyt *c* in buffer in the presence of either 0 ( $\circ$ ) or 3 mM ( $\bullet$ ) ATP. Panel B, A-site membrane-associated cyt *c* with liposomes containing  $X_{PG} = 0.2$  in the absence of ATP ( $\circ$ ) or with 1 mM nucleotide ( $\bullet$ ). Panel C, c-site membrane-associated cyt *c* with liposomes containing  $X_{PG} = 1.0$  without ( $\circ$ ) and with 3 mM ( $\bullet$ ) ATP.

FIG. 3. Effect of ATP on the A-site membrane association of native and [Nle<sup>91</sup>]cyt *c*. Panel A, quenching of pyrene fluorescence as a function of native ( $\circ$ ) and [Nle<sup>91</sup>]cyt *c* ( $\blacksquare$ ). Panel B, dissociation of membrane-bound cyt *c* by ATP. The mole fraction of PG in PC liposomes was 0.20. Total lipid concentration in 20 mM Hepes, 0.1 mM EDTA, pH 7, was 25  $\mu$ M.



where a shoulder was seen in the spectrum of free cyt *c*, and a sharp negative deflection was measured at 410 nm, with an opposite peak centered at 420 nm. C-site lipid binding thus seems to change the heme environment dramatically, suggesting the conformation of C-site membrane-bound cyt *c* to be different from both free or A-site-bound cyt *c*. Importantly, CD spectra revealed 3 mM ATP to cause a further conformational change in the C-site membrane-interacting protein (Fig. 2, panel C), evident as diminished intensities of the peaks at 390 and 410 nm and an emerging shoulder at  $\sim$ 405 nm.

**ATP-induced Changes in the Fluorescence of [Zn<sup>2+</sup>-Heme]cyt *c***—The above CD data reveal ATP to induce conformational changes in lipid-bound cyt *c* (22). A fluorescent cyt *c* analog with the heme iron substituted by Zn<sup>2+</sup> described has been previously (37). We utilized time-resolved fluorescence spectroscopy to monitor structural changes in this cyt *c* derivative induced by ATP and upon lipid binding, reflected in the lifetimes of Zn-porphyrin fluorescence at 640 nm. One mM ATP induces a small yet significant increase in the average lifetime  $\bar{\tau}$ , from 4.2 to  $\approx$ 4.7 ns. Also the component lifetimes  $\tau_1$  and  $\tau_2$  were sensitive to the presence of ATP and were prolonged from 1.8 to 2.0 and from 9.1 to 11.2 ns, respectively, with only minor changes in the fractional intensities of the decay components. Compared with the above modest effects of ATP on [Zn<sup>2+</sup>-heme]cyt *c* in solution, much more pronounced effects were evident upon binding to liposomes. These changes also depend on the type of lipid binding involved, determined by the content

of the acidic phospholipid POPG in the liposomes (Table I). Accordingly, the values for the average lifetime  $\bar{\tau}$  of the Zn-porphyrin fluorescence were reduced in the presence of liposomes from 4.2 ns for free cyt *c* to  $\approx$ 2.0 and  $\approx$ 3.5 ns at  $X_{PG} = 0.20$  (A-site) and  $X_{PG} = 1.0$  (C-site), respectively. Significant changes were observed also in the component lifetimes of the Zn-porphyrin emission in the presence of liposomes (Table I). In brief, at  $X_{PG} = 0.20$  the values for  $\tau_1$  and  $\tau_2$  were 0.6 and 2.6 ns, respectively, whereas the corresponding values for [Zn<sup>2+</sup>-heme]cyt *c* bound to liposomes via the C-site at  $X_{PG} = 1.0$  were considerably longer, 1.5 and 8.0 ns. Pronounced differences were seen also in the fractional intensities of the component lifetimes, and at  $X_{PG} = 0.20$   $\sim$ 70% of the excited fluorophore relaxes back to the ground state via the process with the longer lifetime. At  $X_{PG} = 1.0$  the opposite was evident, a conformation with the shorter lifetime representing the dominant species (Table I).

In keeping with the ATP-induced conformational changes observed by CD also the fluorescence decays of lipid-associated [Zn<sup>2+</sup>-heme]cyt *c* were also strongly influenced by ATP. At  $X_{PG} = 0.20$  the lipid-induced changes in the fluorescence lifetimes appear to be somewhat reduced by ATP, and in the presence of 1 mM ATP the value for  $\bar{\tau}$  of A-site lipid-bound cyt *c* was prolonged from 2.0 to 2.7 ns (Table I). For the C-site interaction of [Zn<sup>2+</sup>-heme]cyt *c* with liposomes ( $X_{PG} = 1.0$ ), the nature of the interaction seems to change drastically in the presence of ATP. Accordingly, the value for  $\bar{\tau}$  of [Zn<sup>2+</sup>-heme]cyt

TABLE I

Fluorescence lifetimes of [Zn<sup>2+</sup>-heme]cyt *c*, at excitation and emission wavelengths of 295 and 640 nm, respectively

$\bar{\tau}$  is the average fluorescence lifetime.  $\tau_1$  and  $\tau_2$  represent the short and long lifetime components derived from fitting the curves to two-exponential decays, and  $C_1$  and  $C_2$  are the fractional intensities (in percentage) of  $\tau_1$  and  $\tau_2$ , respectively. [Zn<sup>2+</sup>-heme]cyt *c* concentration was 10  $\mu$ M, and where indicated, 1 mM ATP and/or LUVs (250  $\mu$ M total lipid) were added. Mole fraction  $X_{\text{PG}}$  of the acidic phospholipid POPG in the LUVs was either 0.20 or 1.0 as indicated. Medium consisted of 20 mM Hepes, 0.1 mM EDTA, pH 7.0.

Sample	ATP	$X_{\text{PG}}$	$\bar{\tau}$	$\tau_1 \pm \text{S.D.}$	$C_1$	$\tau_2 \pm \text{S.D.}$	$C_2$
			<i>ns</i>	<i>ns</i>		<i>ns</i>	
[Zn <sup>2+</sup> -heme] cyt <i>c</i>			4.2	1.8 $\pm$ 0.09	67	9.1 $\pm$ 1.5	33
[Zn <sup>2+</sup> -heme] cyt <i>c</i>	+1 mM		4.7	2.0 $\pm$ 0.06	70	11.2 $\pm$ 1.7	30
[Zn <sup>2+</sup> -heme] cyt <i>c</i> + LUV		0.2	2.1	0.6 $\pm$ 0.07	29	2.6 $\pm$ 0.07	71
[Zn <sup>2+</sup> -heme] cyt <i>c</i> + LUV	+1 mM	0.2	2.7	1.2 $\pm$ 0.07	61	5.1 $\pm$ 0.5	39
[Zn <sup>2+</sup> -heme] cyt <i>c</i> + LUV		1.0	3.5	1.5 $\pm$ 0.06	70	8.0 $\pm$ 1.1	30
[Zn <sup>2+</sup> -heme] cyt <i>c</i> + LUV	+1 mM	1.0	8.3	1.8 $\pm$ 0.1	25	10.5 $\pm$ 0.5	75

*c* associated to liposomes via its C-site is 3.6 ns in the absence of ATP and 8.3 ns with 1 mM nucleotide, in keeping with ATP-induced conformational changes. Also the component lifetimes as well as the fractional intensities were affected by the nucleotide (Table I). More specifically, in the presence of 1 mM ATP, the values for  $\tau_1$  and  $\tau_2$  were 1.2 and 5.1 ns at  $X_{\text{PG}} = 0.20$  (A-site interaction) and 1.8 and 10.5 ns for  $X_{\text{PG}} = 1.0$  (C-site interaction). ATP thus appears to partially counteract the effect of A-site-mediated lipid binding on the conformation of cyt *c*.

**Association of Nle<sup>91</sup>-cyt *c* to Liposomes**—The high affinity ATP-binding site in cyt *c* has been demonstrated previously to involve the invariant Arg<sup>91</sup> (5, 7–10). In order to study the possibility that the observed changes induced by ATP in lipid association of cyt *c* would be due to this site, we studied the liposome association of the mutant [Nle<sup>91</sup>]cyt *c* in which the high affinity binding site for ATP is abrogated. Accordingly, we compared the effects of ATP on the membrane association of native cyt *c* and the [Nle<sup>91</sup>] analog. The A-site interaction of cyt *c* with liposomes was not affected by the lack of the Arg<sup>91</sup> guanidino group, and at  $X_{\text{PG}} = 0.20$  and pH 7.0 membrane association of native cyt *c* and its Nle<sup>91</sup> derivative were indistinguishable (Fig. 3). However, compared with the native protein somewhat less ATP was required to dissociate the modified protein. This difference is likely to reflect a small fraction of C-site-associated cyt *c* present at  $X_{\text{PG}} = 0.20$  at neutral pH for which a slight apparent increase in membrane association due to ATP occurred (Fig. 3, *panel B*). These data also show the high affinity ATP-binding site at Arg<sup>91</sup> to be distinct from the lipid-binding A-site, in keeping with our previous results (24, 27).

In the absence of ATP the C-site-mediated membrane association of the modified protein was similar to the native protein indicating that the Arg<sup>91</sup>  $\rightarrow$  Nle modification caused no detectable change in this interaction of cyt *c* with lipids (Fig. 4, *panel A*). However, comparison of these data with those of Fig. 1, *panel A*, shows that 2 mM ATP had a significantly reduced effect on the apparent affinity of [Nle<sup>91</sup>]cyt *c* for neat PG liposomes. Likewise, the effect of ATP on the fluorescence quenching by the modified protein was significantly smaller. Yet, the fact that even for the Arg<sup>91</sup>  $\rightarrow$  Nle mutant a slightly enhanced quenching was seen suggests that the deletion of the guanidino group leaves intact the contribution of the other components of the binding site (48) and that the loss of ATP binding to the mutated site is not complete (Fig. 4, *panel A*), confirming the affinity column data (Table II). Accordingly, compared with the wild type cyt *c*., subsequent addition of ATP to C-site-bound Nle<sup>91</sup> at  $X_{\text{PG}} = 0.2$ , pH 4.0, showed diminished yet still noticeable increase in the quenching by ATP (Fig. 4, *panel B*). These data provide evidence for the high affinity nucleotide-binding site involving Arg<sup>91</sup> to be required for the ATP-induced conformational changes in cyt *c* and the enhancement of energy transfer from the membrane-contained pyrene to the cyt *c* heme in the presence of ATP.

## DISCUSSION

Interactions of cyt *c* with lipids have been thoroughly investigated, and cyt *c* is generally taken as a paradigm for an electrostatically membrane-bound peripheral protein bearing a net positive charge and associating with acidic phospholipids (21). Changes in the conformation of cyt *c* upon binding to phospholipid membranes have been described using a variety of techniques including tryptophan fluorescence, CD (49), NMR (50–53), Fourier transform infrared spectroscopy and differential scanning calorimetry (54), resonance Raman spectroscopy (55), and surface plasmon resonance spectroscopy on supported lipid bilayers (56). In brief, these studies have revealed that binding of cyt *c* to acidic phospholipids induces a conformation with less organized tertiary structure (49–53) and reduced thermal stability (54) but with native-like helical secondary structure (49). The lipid-induced conformational changes were demonstrated to depend on the negative surface charge density in the membrane (54). Likewise, cardiolipin was more effective than phospholipids with a single negative charge (51). The above is in keeping with the C-site of cyt *c* to be involved in mediating phospholipid-induced conformational alterations (23). Three different membrane-bound conformations for cyt *c* have been described (57), and these authors demonstrated the presence of an electrostatically bound cyt *c* and two conformationally different membrane-bound types of cyt *c* that were not dissociable from the membrane by increasing ionic strength. Interestingly, the efficiency in electron transport was also different for the latter two conformations (57).

The present data confirm changes in the conformation of cyt *c* upon its binding to liposomes. Most important, we also demonstrate that further conformational alterations are induced by ATP in cyt *c* bound to phospholipids. These ATP-induced changes in the conformation of liposome-associated cyt *c* were further dependent on the lipid composition. Accordingly, they were observed in CD spectra only when using liposomes composed of the acidic PG. Under these conditions cyt *c* binds to membranes via the so-called C-site and attaches to the protonated head groups of PG. The C-site has been suggested to involve residue Asn<sup>52</sup> and a fatty acid-binding hydrophobic cavity in cyt *c* (24). C-site lipid bound cyt *c* is not detached from liposomes by ATP, in contrast to the A-site-bound protein (23).

Although the heme moiety as such is not optically active in solution, its interactions with the neighboring amino acid residues and distortions in its planarity render heme-containing proteins optically active, with the characteristic CD spectra in the Soret region near 400 nm. These CD spectra thus provide information on the variation of environment of the heme group and thus reflect the conformation of cyt *c*. These spectra demonstrate pronounced differences in the conformations of cyt *c* bound to liposomes via the C- and A-sites as well as the conformation of cyt *c* in solution. Moreover, the structure of cyt *c*

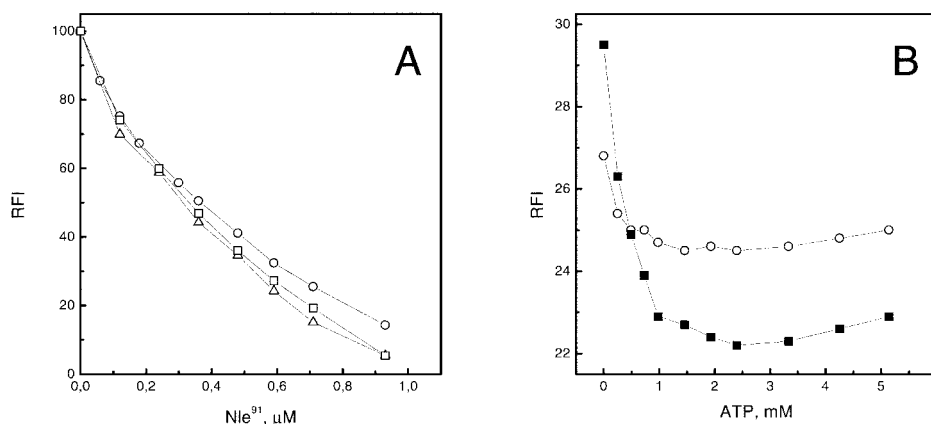


FIG. 4. **Effect of ATP on the C-site membrane association of [Nle<sup>91</sup>]cyt *c* liposomes.** *Panel A*, binding of [Nle<sup>91</sup>]cyt *c* in the presence of ATP to liposomes containing  $X_{PG} = 1.0$ . The concentration of ATP was 0 (○), 1 (□), or 2 (Δ) mM. Comparison of Nle<sup>91</sup> and wt cyt *c*s (displayed in Fig. 1, *panel A*) is aided by taking into account that the graphs for cyt *c*, and the mutants recorded in the absence of ATP are practically indistinguishable. *Panel B*, effect of subsequently added ATP on the C-site-bound [Nle<sup>91</sup>]cyt *c* (○) compared with native cyt *c* (■), data taken from Fig. 1, *panel B*. The proteins were added prior to the addition of ATP to a final concentration of 0.15 μM. The mole fraction of PG in PC liposomes was 0.20, pH 4. Total lipid concentration was 25 μM in 20 mM Hepes, 0.1 mM EDTA.

TABLE II  
Comparison of the properties of [Nle<sup>91</sup>]cyt *c* with the native protein

	HPLC elution time		pK of $\Delta A_{695nm}$	
	Fe <sup>2+</sup>	Fe <sup>3+</sup>	Acid leg	Base leg
[Nle <sup>91</sup> ]cyt <i>c</i>	12.17	13.12	3.5	9.1
Native cyt <i>c</i>	13.18	14.08	2.7	9.2

bound to liposomes via the C-site can be further altered by ATP, whereas no such effect was seen on the protein in solution or when interacting with lipids by its A-site. Although these CD spectra cannot be assigned to specific conformational features, qualitative properties may be discussed. The distinct shape of the spectrum for the C-site-bound cyt *c* with sharp peaks and rotational strength equal to the unbound protein suggests an organized conformation instead of a molten globule-like state. This is in contrast to lipid-induced unfolding of cyt *c* in the presence of phospholipid vesicles (49) revealing a Soret region CD spectrum similar to guanidine hydrochloride denatured species (46). However, in the latter study, conditions different from those used here were employed. More specifically, these authors used phosphatidylserine as the acidic phospholipid and at high concentration (several mM) which could explain the denaturing effect. Most important, we could demonstrate the presence of ATP to have further effects on the CD spectra (Fig. 2), thus revealing an ATP-induced conformational change in lipid-bound cyt *c*. The ATP-induced effect observed in the resonance energy transfer between the heme moiety of cyt *c* and the membrane-embedded lipid (Fig. 4.) could thus be explained by these conformational species having different properties as acceptors in the resonance energy transfer process. Except for decreased amplitudes of the peaks, the CD spectra for the A-site membrane-bound cyt *c* is identical to that of unbound cyt *c* in solution. The difference in amplitudes could be explained by more intense fluctuations in the heme environment of the A-site membrane-interacting protein, where the time-averaged structure would be identical to that of the free cyt *c*. Unfortunately, the amounts of protein required for measuring CD spectra for Nle<sup>91</sup> cyt *c* were prohibitively high.

Measurement of fluorescence lifetimes provides an additional and extremely sensitive tool to study changes in the immediate microenvironment of a fluorophore (58). For this purpose we used the intensely fluorescent [Zn<sup>2+</sup>-heme]cyt *c* (38). In addition to confirming that lipid binding induces structural changes in [Zn<sup>2+</sup>-heme]cyt *c* and demonstrating that ATP

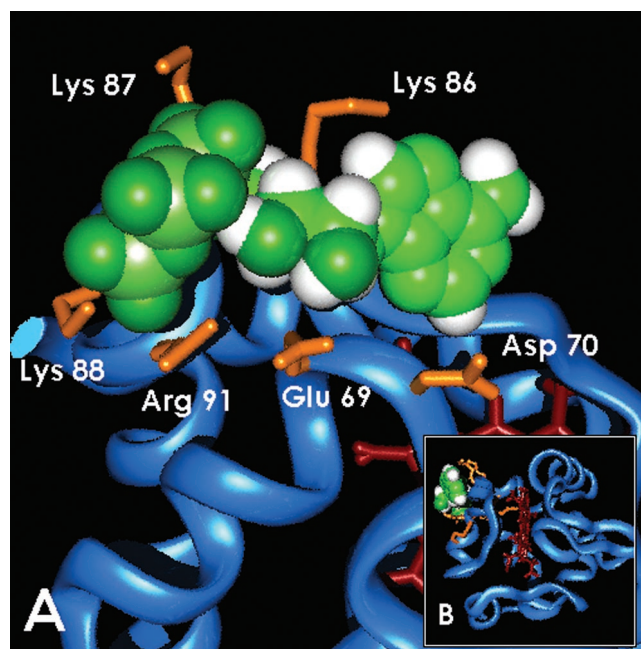


FIG. 5. **Molecular model for the binding of the ATP (green) to a high-affinity binding site in yeast ferrocyanochrome *c* (48).** *Panel A*, interacting side chains are colored in orange, and the heme (brown) is shown for spatial reference. Oxygens in ATP molecule are colored dark green and phosphorus atoms are medium green. *Panel B*, cytochrome *c* presented in conventional orientation.

causes conformational changes in this protein, the present data reveal that ATP has pronounced additional effects on the structure of lipid-bound cyt *c*. Accordingly, our results reveal four different conformations for membrane-bound cyt *c* as follows, depending on the content of the acidic phospholipid in the membrane and the presence of ATP. At low content of acidic phospholipid and at neutral pH the cyt *c* is bound to lipids electrostatically via the A-site (23). Whereas higher concentrations of ATP (>2 mM) reverse this interaction, low [ATP] <2 mM appears to bind to the lipid-associated cyt *c* and to induce a conformational change, as demonstrated by the fluorescence lifetimes and fractional intensities for [Zn<sup>2+</sup>-heme]cyt *c* (Table I). At either  $X_{PG} = 1.0$  or at  $X_{PG} = 0.20$  and at acidic pH the interaction of cyt *c* involves protonated acidic phospholipids and has been nominated as C-site binding (23). Judged from time-resolved fluorescence, the conformations of cyt *c* bound to

lipids via the C-site and A-site are different. Also, in cyt *c* bound to liposomes via the C-site interaction, further structural changes are caused by ATP, evident both in the fluorescence lifetime data for [Zn<sup>2+</sup>-heme]cyt *c* (Table I) as well as in the resonance energy transfer between the fluorescent membrane probe and wt-cyt *c* (Fig. 1). More specifically, for the C-site-bound cyt *c* the changes induced by ATP result in an augmented resonance energy transfer between the fluorescent pyrene-containing lipid analog PPDPG and the heme moiety of wt-cyt *c*. This enhanced resonance energy transfer could result from an altered orientation of the dipoles involved due to changing orientation of the heme with respect to the membrane plane or reduced distance between the dipoles (22).

A high affinity binding site for ATP has been described and shown to involve the invariant Arg<sup>91</sup> (5, 7–10). The involvement of Arg<sup>91</sup> in binding of ATP and consequent modulation of electron transfer by the protein has been investigated previously by semisynthetic analogs of cyt *c* in which this single arginine residue of the 66–104 peptide was chemically modified by cyclohexane-1,2-dione prior to ligation with the 1–65 peptide (10). The [Nle<sup>91</sup>] analog used here binds ATP better than the previously described N<sup>7</sup>,N<sup>8</sup>-(1,2-dihydroxycyclohex-1,2-ene)diyl-L-arginine 91-cyt *c* (7). This difference probably reflects the fact that the latter modification introduces a bulky group, which prevents the approach of the nucleotide to the other moieties constituting this site. Because of this, the choice of norleucine as the substitution for arginine was made specifically with steric considerations in mind, as the side chain of residue 91 (Fig. 5) resides in the hydrophobic face of an amphipathic helix and is fully buried, with the exception of the guanido head group. A straight aliphatic chain of norleucine should thus fulfill the space-filling role of this residue. This analog had strongly reduced affinity for ATP, whereas its other characteristics remained unaffected. In order to study whether the above site was responsible also for the ATP-induced changes in cyt *c* bound to lipids, we examined the effect of ATP on the liposome association of the [Nle<sup>91</sup>]cyt *c*. We have previously demonstrated that the interactions of [Nle<sup>91</sup>]cyt *c* with lipids differ from those of the wt-cyt *c* with lack of aggregation by [Nle<sup>91</sup>] of LUV composed of acidic phospholipid (27). Most important, compared with the wild type the strongly reduced effect of ATP on the resonance energy transfer between the heme moiety of [Nle<sup>91</sup>]cyt *c* and the membrane-embedded fluorescent lipid (Fig. 4, panel B) strongly suggests that the invariant Arg<sup>91</sup> is indeed important in mediating the conformational changes induced by ATP.

The findings that the functional apoptotic cyt *c* is probably membrane-bound (20), and the requirement for ATP (16, 18) in cyt *c*-mediated apoptosis further emphasizes the possible importance of the conformational effects of ATP on cyt *c*. It seems plausible to suggest that the ATP binding and the consequent conformational changes in both free and membrane-bound cyt *c* may represent an evolutionarily conserved way to mediate the reactivity of cyt *c* and may have been adapted for use in the regulation of its biological functions.

**Acknowledgments**—We thank Dr. Christian Blouin for creating Fig. 5. We thank Birgitta Rantala and Angela Brigley for technical assistance.

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