Apocytochrome *c* Interaction with Phospholipid Membranes Studied by Fourier-Transform Infrared Spectroscopy†

Arturo Muga,‡ Henry H. Mantsch, and Witold K. Surewicz*
Steacie Institute for Molecular Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6
Received October 3, 1990; Revised Manuscript Received December 6, 1990

ABSTRACT: Apocytochrome *c*, the heme-free precursor of cytochrome *c*, has been used extensively as a model to study molecular aspects of posttranslational translocation of proteins across membranes. In this report, we have used Fourier-transform infrared spectroscopy to gain further insight into the mechanism of apocytochrome *c* interaction with membrane phospholipids. Association of apocytochrome *c* with model membranes containing the acidic lipid dimyristoylphosphatidylglycerol (DMPG) as a single component results in a drastic perturbation of phospholipid structure, at the level of both the acyl chains and the interfacial carbonyl groups. However, in a binary mixture of DMPG with acyl chain perdeuterated dimyristoylphosphatidylcholine (DMPC-d_{54}), the perturbing effect of the protein on the acidic phospholipid is greatly attenuated. In such a membrane with mixed lipids, the physical properties of the DMPG and DMPC components are affected in a similar fashion, indicating that apocytochrome *c* does not induce any significant segregation or lateral-phase separation of acidic and zwitterionic lipids. Analysis of the apocytochrome *c* spectrum in the amide I region reveals that binding to phospholipids causes considerable changes in the secondary structure of the protein, the final conformation of which depends on the lipid to protein ratio. In the presence of a large excess of DMPG, apocytochrome *c* undergoes a transition from an essentially unordered conformation in solution to an α-helical structure. However, in complexes of lower lipid to protein ratios ($\leq\sim$40:1), infrared spectra are indicative of an extended, intermolecularly hydrogen-bonded β-sheet structure. The latter is suggestive of an extensive aggregation of the membrane-associated protein.

Apocytochrome *c* is the heme-free precursor form of the inner mitochondrial membrane protein cytochrome *c*. Interest in exploring molecular details of the interaction between apocytochrome *c* and membrane phospholipids is at least 2-fold. First, apocytochrome *c* provides a convenient model for studying a specific class of lipid–protein interaction which involves initial electrostatic binding of a water-soluble protein to the membrane surface, followed by a hydrophobic penetration of the protein into the bilayer (Rietveld et al., 1983). This membrane binding and/or penetration is believed to be accompanied by a change in protein conformation (Rietveld et al., 1985). Second, studies on the apocytochrome *c*–lipid interaction provide important clues regarding the mechanisms of membrane translocation and assembly of mitochondrial proteins. Like many other mitochondrial proteins, cytochrome *c* is synthesized in its precursor form on free ribosomes in the cytoplasm and is subsequently transported to its functional site at the outside of the inner mitochondrial membrane (Hay et al., 1984; Nicholson et al., 1987). While the mechanism of this mitochondrial translocation of apocytochrome *c* remains ambiguous, a growing number of experimental data indicate that at least some steps involved in protein import may be mediated by lipid–protein interactions (Nesmeyanova, 1982; Rietveld et al., 1983, 1986; Dumont & Richards, 1984; Rietveld & de Kruijff, 1986; Nicholson et al. 1987; Demel et al., 1989).

De Kruijff and co-workers have demonstrated that apocytochrome *c* has a high affinity for acidic but not for zwitterionic phospholipids (Rietveld et al., 1983, 1986b; Görrisen et al., 1986). Subsequent biophysical studies have revealed a number of molecular details of the interaction between the protein and model membranes of negatively charged lipids (Rietveld & de Kruiff, 1986; Rietveld et al., 1985; Demel et al., 1989; Görrissen et al., 1986; Li-Xin et al., 1988; Pilon et al., 1987). However, acidic phospholipids constitute only a fraction of the total mitochondrial membrane lipids. It is therefore of importance to understand how the apparently strong and specific interaction between acidic phospholipids and the protein is influenced by other lipid components of the membrane and what are the consequences of this interaction on the lateral distribution of different lipid classes. While previous studies indicate that in model membranes containing a mixture of acidic and zwitterionic lipids apocytochrome *c* associates preferentially with the former (Rietveld et al., 1986a; Berkhout et al., 1987), the description of such binary lipid systems in the presence of the protein is far from being complete.

In this paper, we report the results of a Fourier-transform infrared spectroscopic investigation of the interaction between apocytochrome *c* and phosphatidylglycerol or a phosphatidylglycerol–phosphatidylcholine mixture. Our data demonstrate that the interaction of the protein with the acidic phospholipid is strongly modulated by the zwitterionic lipid component of the binary mixture. This interaction does not lead to a lateral-phase separation between different lipid classes. Moreover, we use infrared spectroscopy to probe the effect of membrane binding on the conformation of apocytochrome *c*.

Materials and Methods

Dimyristoylphosphatidylglycerol (DMPG)[1] and acyl chain perdeuterated dimyristoylphosphatidylcholine (DMPC-d_{54}) were obtained from Avanti Polar Lipids, Birmingham, AL.

† The NATO Scientific Affairs Division provided travel support under Grant 0050-87. Issued as NRCC Publication No. 32302.
* Author to whom correspondence should be addressed.
‡ Permanent address: Department of Biochemistry, University of the Basque Country, Bilbao, Spain.

[1] Abbreviations: DMPG, dimyristoylphosphatidylglycerol; DMPC-d_{54}, acyl chain perdeuterated dimyristoylphosphatidylcholine; SDS, sodium lauryl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid.

0006-2960/91/0430-2629$02.50/0

SDS (98% pure) and horse heart cytochrome *c* (type VI) were purchased from Sigma (St. Louis, MO). Deuterium oxide (99.8% pure) was from MSD Isotopes, St. Louis, MO. Apocytochrome *c* was prepared by removal of the heme group from the holoprotein as described by Fisher et al. (1973), and then subjected to a renaturation procedure according to Henning and Neupert (1983). The protein was stored at 4 °C in 100 mM NaCl–50 mM HEPES (pH 7.4), at a concentration of 1–1.5 mg/mL, and was used within 3 days after renaturation. Protein concentration was determined spectrophotometrically as described previously (Stellwagen et al., 1972).

For preparation of samples used to study the effect of apocytochrome *c* on the molecular organization of membrane lipids, a dry lipid film (obtained either from 1 mg of DMPG or from 1 mg of DMPG plus 1 mg of DMPC-d_{54}) was hydrated with 1 mL of buffer (50 mM Hepes–100 mM NaCl, pH 7.4) or with apocytochrome *c* solution (approximately 1 mg/mL) in buffer. The mixtures were gently vortexed and incubated at 28 °C for 30 min. Lipid–protein complexes were then collected by centrifugation (16000*g*, 5 min) and washed twice with a buffer prepared in ^2H_2O (50 mM HEPES–100 mM NaCl, p^{2}H 7.4).

To study infrared amide bands of apocytochrome *c* in the presence of different amounts of lipids, in most cases unilamellar phospholipid vesicles were first prepared by sonication of DMPG or a DMPG–DMPC mixture in a deuterated buffer (composition as above) at a lipid concentration of 50 mg/mL. Vesicles were then mixed at desired proportions with apocytochrome *c* solution in the same (deuterated) buffer, and samples were incubated at 28 °C for 30 min. Samples used to study the rate of hydrogen–deuterium exchange in membrane-bound protein were prepared as described above, except that all steps were performed in fully protonated buffer. The aqueous solvent was subsequently exchanged by dialysis of the lipid–protein recombinants for 2 h against three changes of the deuterated buffer. The same samples were also used for circular dichroism measurements, except that sodium chloride was removed by dialysis against NaCl–free buffer.

Samples for infrared spectroscopy were assembled between two calcium fluoride windows separated by a 50-μm-thick Teflon spacer. Infrared spectra were recorded with a Digilab FTS-60 instrument. Typically, 100–200 interferograms were co-added and Fourier-transformed to give a resolution of 2 cm^{-1}. To eliminate spectral contributions of atmospheric water vapor, the instrument was continuously purged with dry nitrogen. Temperature was controlled by the computer and during data acquisition was stable within 0.1 °C. The average heating rate was 10 °C/h. The frequencies of the lipid C–H or C–^{2}H stretching bands were determined with an accuracy of 0.01 cm^{-1} by use of a center of gravity algorithm (Cameron et al., 1982). Overlapping infrared bands were resolved by using Fourier self-deconvolution procedures (Kauppinen et al., 1981).

Circular dichroism spectra were acquired at 28 °C on a Jasco J-600 spectropolarimeter, using a 0.2-mm path-length quartz cell. Eight spectra were averaged to improve the signal-to-noise ratio.

Results

Structural Properties of Membrane Lipids. The effect of apocytochrome *c* on the conformational properties of DMPG acyl chains was studied by following changes in the infrared band near 2850 cm^{-1} which represents the symmetric stretching vibration of the methylene groups. The wavenumber of the CH_2 symmetric stretching mode (ν_s CH_2) has been widely used as a measure of the trans:gauche ratio in lipid acyl chains (Casal & Mantsch, 1984; Lee & Chapman, 1986; Mendelsohn & Mantsch, 1986), although it is also sensitive to the strength of the interchain interactions. Moreover, the temperature dependence of this parameter has been used to monitor thermotropic phase transitions in membrane lipids.

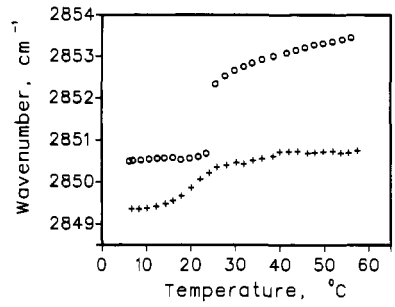

FIGURE 1: Temperature dependence of the position of the CH_2 symmetric stretching band of DMPG alone (O) and that of a DMPG–apocytochrome *c* complex at a lipid to protein molar ratio of (20 ± 2):1 (+).

The wavenumber versus temperature plots of the ν_s CH_2 mode for the aqueous dispersion of DMPG and DMPG complexed with apocytochrome *c* are shown in Figure 1. The sharp increase in the CH_2 stretching frequency of DMPG by ~2 cm^{-1} at around 24 °C represents a cooperative transition of the lipid acyl chains (Surewicz et al., 1987c). Upon complexation with apocytochrome *c*, this cooperative transition is largely abolished, although the small increase in wavenumber between approximately 14 and 28 °C suggests that the protein-bound lipids may undergo a residual, broad transition in this temperature range. The wavenumbers of the ν_s CH_2 mode of apocytochrome *c* complexed DMPG are shifted to considerable lower values compared to those of the protein-free phospholipid, particularly in the temperature range that corresponds to the liquid-crystalline state of the pure lipid (Figure 1).

The effect of apocytochrome *c* on a binary mixture of acidic and zwitterionic phospholipids was studied by using a 1:1 mixture of DMPG and acyl chain perdeuterated DMPC. The advantage of using one lipid component with deuterated chains is that the C–^{2}H and C–H stretching modes appear in different regions of the infrared spectrum. This allows for an independent monitoring, using the same sample preparation, of the conformational order and thermotropic behavior of each individual component of the lipid mixture (Dluhy et al., 1985; Jaworsky & Mendelsohn, 1986). Plots of the temperature-induced variation in the C–H and C–^{2}H symmetric stretching frequency of the DMPG and DMPC-d_{54} components show that upon mixing the two lipids undergo a single cooperative transition with a midpoint at 23 °C (Figure 2).

Analysis of similar plots in the presence of apocytochrome *c* clearly shows that the perturbing effect of the protein on DMPG in its mixture with DMPC-d_{54} is much less dramatic than that described above for DMPG alone, although the ratio of protein to DMPG in both samples remains the same (cf. Figure 1 and Figure 2A). Thus, in the lipid mixture, the protein induces only a slight broadening of the DMPG transition, accompanied by a moderate conformational perturbation of the acyl chains at temperatures below and above the transition region. Furthermore, though apocytochrome *c* does not interact with pure phosphatidylcholine membranes (Rietveld et al., 1983; unpublished data from this laboratory), in a mixture of DMPC-d_{54} with DMPG the protein perturbs both lipid components. The response of the DMPC-d_{54} component is similar to that of DMPG, with respect to both the ther-

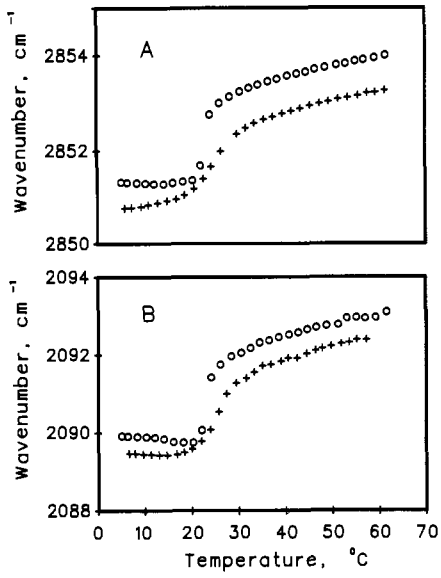

FIGURE 2: Temperature dependence of the position of the CH_2 symmetric stretching mode of DMPG (A) and of the $C-^2H_2$ mode of DMPC-d_{54} (B) in a 1:1 mixture of DMPG and DMPC-d_{54} in the absence (○) and the presence (+) of apocytochrome c at a DMPG to protein molar ratio of (20 ± 2):1.

motropic behavior and the conformational ordering and/or packing (Figure 2).

Information about the structure of the interfacial region of the lipid bilayer is provided by vibrational modes of phospholipid carbonyl groups (Casal & Mantsch, 1984). The ester C=O stretching mode of DMPG consists of at least two overlapping component bands; in the gel phase, the bands are at 1742 and 1721 cm^{-1} and shift to 1744 and 1727 cm^{-1} in the liquid-crystalline phase (Figure 3). Initially, these two bands were assigned to the carbonyl groups of the *sn*-1 and *sn*-2 chains, respectively (Bush et al., 1980; Mushayakarara & Levin, 1982), though recent experiments (Blume et al., 1988) point to a more complex interpretation of the lipid C=O mode. The splitting of this mode may reflect not only the conformational nonequivalence of the interfacial region of two acyl chains but also the coexistence of hydrogen-bonded and non-hydrogen-bonded populations of C=O groups in both the *sn*-1 and *sn*-2 chains.

Association of DMPG with apocytochrome c brings about considerable changes in the lipid carbonyl stretching mode, the most prominent of which is the emergence of a strong band at 1735 cm^{-1} (Figure 3). At present, it would be too speculative to propose a detailed molecular interpretation of the C=O stretching mode of protein-bound DMPG. Nevertheless, the spectral changes depicted in Figure 3 clearly show that the perturbation of the lipid structure induced by apocytochrome c is not limited to the acyl chains, but also extends into the interfacial (and possibly the head group) regions of DMPG. In the presence of DMPC as a second lipid component, the effect of apocytochrome c on the C=O stretching mode is largely diminished; the C=O band contour of the DMPG–DMPC mixture in the presence of the protein (spectrum not shown) is very similar to that of the lipid mixture without the protein.

Protein Structure. The secondary structure of apocytochrome c may be probed by examining infrared spectra in the region characteristic of protein amide bands (Byler & Susi, 1986; Surewicz & Mantsch, 1988).

Trace 1 in Figure 4A shows the infrared spectrum, between 1500 and 1800 cm^{-1}, of apocytochrome c solution in ^2H_2O buffer. The conformation-sensitive amide I mode consists of

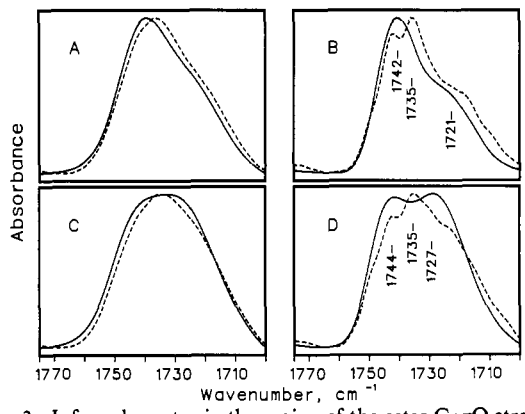

FIGURE 3: Infrared spectra in the region of the ester C=O stretching band of DMPG alone (—) and of DMPG complexed with apocytochrome c at a lipid to protein molar ratio of (20 ± 2):1 (---). Top panels represent spectra at 7 °C before (A) and after band narrowing by Fourier self-deconvolution (B). Bottom panels represent spectra at 50 °C before (C) and after band narrowing by Fourier self-deconvolution (D).

a broad band contour with a maximum at 1644 cm^{-1}. As with many other proteins, this amide I contour is a composite of overlapping component bands that represent different elements of protein secondary structure. Some of these components are usually resolvable by the computational procedure of band narrowing by Fourier self-deconvolution. The deconvolved spectrum of apocytochrome c in solution (trace 1 in Figure 4B) shows a strong band at 1643 cm^{-1}, together with a weaker and rather poorely resolved component around 1670 cm^{-1}. While the first feature is characteristic of polypeptide chains lacking any ordered secondary structure, the second component is in the spectral region associated in many proteins with turns (Byler & Susi, 1986; Surewicz & Mantsch, 1988). Notably, no component bands could be identified (at least at the resolution level attainable in this study[2]) that are characteristic of ordered secondary structures such as α-helices or β-sheets. The overall features of the amide I mode of apocytochrome c are similar to those found in the spectrum of an aqueous solution of the basic protein from central nervous system myelin; the latter protein is believed to adopt a largely unordered conformation in solution (Surewicz et al., 1987a).

Incubation of a solution of apocytochrome c with DMPG vesicles results in a considerable alteration of the protein amide I mode. The character and extent of this spectral change depend strongly on the lipid to protein ratio in the DMPG–apocytochrome c complexes. Trace 2 in Figure 4A shows the spectrum of the DMPG–apocytochrome c mixture at a lipid to protein molar ratio of 100:1. The amide I band contour has a maximum at 1648 cm^{-1}. Fourier self-deconvolution (trace 2 in Figure 4B) reveals a major band at 1649 cm^{-1}, together with bands around 1637 and 1672 cm^{-1}. While the wavenumber of the 1649 cm^{-1} band is somewhat below the range typical for most α-helical proteins, we believe that in the spectrum of lipid-bound apocytochrome c this band rep-

[2] The limiting factor in achieving a high degree of resolution enhancement, without introduction of artifacts, is the requirement of a high signal-to-noise ratio in the spectra and the elimination of contributions from atmospheric water vapor (Mantsch et al., 1988; Surewicz & Mantsch, 1988). In the present study, the tendency of apocytochrome c in aqueous solutions to aggregate at higher concentration constraints us to perform measurements at protein concentration considerably lower than used in most other infrared spectroscopic experiments. Since this had an adverse effect on the quality of the spectra, the deconvolution parameters used here are more conservative than those of many other studies. Our present analysis of the amide I band contour remains qualitative, with no attempt to quantitate the percentage of different types of secondary structure.

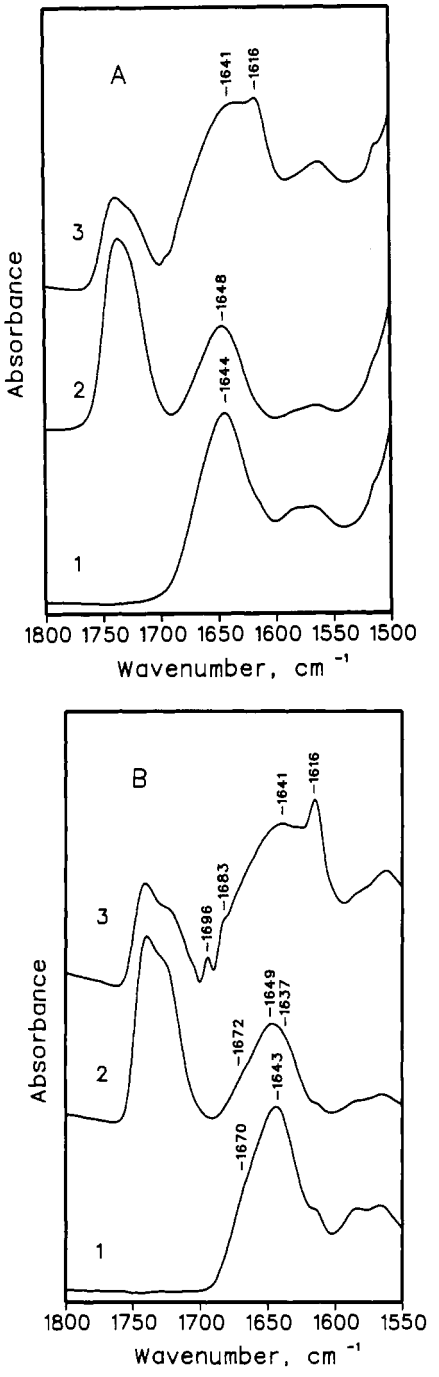

FIGURE 4: Infrared spectra in the amide I region of apocytochrome *c* in solution (1) and apocytochrome *c* in the presence of DMPG vesicles at a lipid to protein ratio of 100:1 (2) and 20:1 (3). (A) Original spectra; (B) spectra after band narrowing by Fourier self-deconvolution using a 15 cm^{-1} half-width Lorentzian line and a resolution enhancement factor (*k* value) of 1.8. The protein concentration in all samples was approximately 3 mg/mL.

resents largely α-helical structures. The low-frequency shift of an "α-helical band" could be explained by the extensive hydrogen–deuterium exchange of the amide groups involved in α-helices. An essentially complete deuteration of the apocytochrome *c* backbone amide groups, regardless of the final protein conformation in its lipid-bound form, should indeed be expected under the conditions of our experiments. Prior to the binding to lipid vesicles, the protein was dissolved in ^2H_2O buffer; the largely unordered conformation in solution (see above) should facilitate a rapid hydrogen–deuterium exchange of the backbone amide groups. The assignment of the 1649 cm^{-1} band in the spectrum of lipid-bound apocytochrome c to α-helices is further supported by the observation that a similar band dominates the spectrum of the protein in the presence of SDS (not shown). Binding to SDS micelles is known to induce a coil–helix transition in apocytochrome *c* (Rietveld et al., 1985). The amide I component around 1637 cm^{-1} is in the region characteristic of β-sheets, although in the spectrum of the mature cytochrome *c* similar bands have been associated with short, extended fragments connecting α-helices (Byler & Susi, 1986).

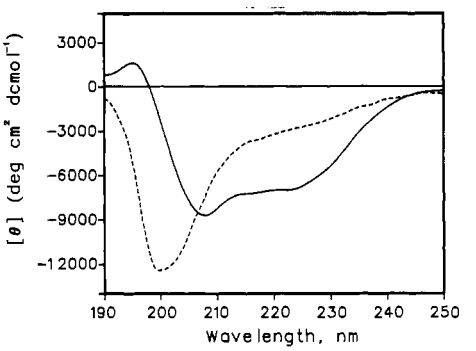

FIGURE 5: Far-UV circular dichroism spectra of apocytochrome *c* in the absence (—) and in the presence (---) of DMPG vesicles at a lipid to protein molar ratio of 100:1. The protein concentration was 0.5 mg/mL.

The amide I mode of the apocytochrome *c*–DMPG complex at a lipid to protein molar ratio of 20:1 (traces 3 in Figure 4A,B) is distinctly different from that of the protein at high lipid concentration. The most characteristic feature of this mode is the strong low-wavenumber band around 1616 cm^{-1}. Bands at a similar position, accompanied by high-wavenumber counterparts above 1680 cm^{-1}, have been previously reported for self-associated peptides which form an intermolecular network of hydrogen-bonded β-strands (Carrier et al., 1990; Surewicz et al., 1987b; Muga et al., 1990). In protein spectra, bands below 1620 cm^{-1} have been seen upon thermal denaturation; they are believed to represent hydrogen-bonded extended structures formed upon aggregation of largely unfolded protein monomers (Surewicz et al., 1990). The features of the amide I mode of the 20:1 DMPG–apocytochrome *c* complex are thus indicative of an extensive aggregation of the lipid-associated protein, with the hydrogen-bonding pattern being similar to that found in intermolecularly associated β-strands.

The spectral pattern characteristic of apocytochrome *c* in an extended β-sheet conformation (represented by trace 3 in Figure 4) has been observed for different DMPG–protein complexes with lipid to apocytochrome *c* molar ratios between approximately 10:1 and 40:1. The spectrum of samples containing a higher proportion of lipid converted gradually (in the range of DMPG:apocytochrome *c* molar ratios from 40:1 to 60:1) to an α-helical pattern represented by trace 2 in Figure 4, and remained essentially unchanged upon further increase in the lipid to protein molar ratio. Conformational preferences very similar to those described above were found also for apocytochrome *c* associated with the membranes composed of an equimolar mixture of DMPG and DMPC.

The conformational assignments of apocytochrome *c* obtained from infrared spectroscopic analysis are corroborated by the results of circular dichroism experiments. In accord with previous studies, the CD spectrum of apocytochrome *c* in solution (Figure 5) shows a pronounced minimum at approximately 200 nm and is fully characteristic of polypeptide chains in a random conformation (Chang et al., 1978). Samples of DMPG–apocytochrome *c* complexes at a lipid to protein molar ratio below approximately 50:1 are highly ag-

gregated, which precludes reliable measurements of CD spectra. However, at higher DMPG:apocytochrome c molar ratios, the aggregation is much less extensive, particularly upon removal of NaCl from the buffer. The circular dichroism spectrum of such samples exhibits a double minimum at 208 and 222 nm (Figure 5), a feature typical for polypeptides and proteins containing a high proportion of α-helices (Chang et al., 1978).

Discussion

It has been demonstrated earlier (Rietveld et al., 1983, 1986b; Görrissen et al., 1986) that apocytochrome c, due to its basic character, binds strongly and specifically to acidic phospholipids. The interaction of the protein with model membranes composed of negatively charged lipids results in a considerable perturbation of lipid acyl chain packing and mobility, as evidenced by differential scanning calorimetry, electron spin resonance, and monolayer experiments (Rietveld et al., 1983, 1985; Demel et al., 1989; Li-Xin et al., 1988; Pilon et al., 1987; Görrissen et al., 1986). The picture emerging from these, as well as other biophysical studies, is that the initial electrostatic binding of apocytochrome c to the membrane surface is followed by its hydrophobic penetration into the bilayer interior (Rietveld & de Kruijff, 1986; Rietveld et al., 1985), or even the translocation of the protein molecule (or at least part of it) across the membrane (Dumont et at., 1984; Rietveld et al., 1986b).

Results of biophysical and biochemical studies on the apocytochrome c interaction with model membranes led to suggestions (Rietveld et al., 1983; Rietveld & de Kruijff, 1986; Demel et al., 1989) that acidic phospholipids may be directly involved in the mitochondrial import of the protein. The specific role of acidic lipids could be to provide binding and (possible) translocation sites, as well as to facilitate the actual translocation event. The latter could be achieved by a local modulation of membrane structure caused by the protein–lipid interaction and/or by lipid-induced changes in the conformation of apocytochrome c. The existing information on structural aspects of the apocytochrome c–lipid interaction was derived largely from studies with model membranes composed exclusively of acidic phospholipids. However, in order to assess the relevance of these observations to interactions taking place in mitochondria, and thus to further advance the models of mitochondrial transport of apocytochrome c, it is important to understand the molecular details of protein interaction with model membranes of increasingly higher complexity. Studies with a binary mixture of acidic and zwitterionic phospholipids provide a first step in this direction.

The observation that apocytochrome c abolishes to a large extent the phase transition of DMPG is in accord with previous differential scanning calorimetry and electron spin resonance data (Rietveld et al., 1983, 1985; Görrissen et al., 1986). The present FT-IR experiments also provide a direct and, importantly, unperturbing insight into the conformational properties of DMPG in the presence of the protein. The observed decrease in the wavenumber of the ν_s CH_2 mode (Figure 1) suggests a decrease in the proportion of the gauche conformers, which is generally consistent with the previously reported ordering and immobilization of spin-labeled phospholipids (Rietveld et al., 1985; Görrissen et al., 1986). However, the low-wavenumber shift of the ν_s CH_2 mode may also be partly due to protein-induced perturbation of interchain interactions. The latter effect is particularly likely to account for the wavenumber shift at low temperatures (below the transition temperature of lipid alone), where the pure lipid should be essentially in an all-trans conformation.

Two noteworthy conclusions can be drawn from experiments with the binary mixture of DMPG and DMPC-d_{54}. First, the perturbing effect of apocytochrome c on conformational properties of the acidic phospholipid is strongly attenuated by the zwitterionic lipid component. While apocytochrome c does not bind to pure phosphatidylcholine membranes, its binding to mixed membrane systems results in the formation of electrostatically stoichiometric complexes in which the ratio of protein to negatively charged phospholipid is preserved, regardless of the concentration of the neutral lipid components (Rietveld et al., 1986b). The above-mentioned attenuation is thus not due to decreased DMPG–apocytochrome c binding in the presence of DMPC-d_{54}. It rather reflects the "modulatory" effect of the latter lipid component, which appears to remain well mixed with DMPG even in the presence of the protein (see also below). The presence of DMPC decreases the separation between the negatively charged DMPG head groups, leading to an overall weakening of the membrane–protein interaction. Second, the protein-induced structural perturbation is not limited to DMPG but extends almost equally to the DMPC-d_{54} component of the mixture.[3] The present demonstration of a weak and essentially equal perturbation of the acidic and zwitterionic lipid components in the mixed model membrane extends earlier observations with spin-labeled phospholipids. Altogether, these data provide solid proof that apocytochrome c does not induce any significant segregation or lateral-phase separation of different lipid classes. From previous fluorescence quenching and energy-transfer experiments (Rietveld et al., 1986a; Berkhout et al., 1987), it was concluded that in mixed model membranes apocytochrome c shows a preferential interaction with negatively charged phospholipids. While this apparent, yet rather poorly defined preference requires further study, it clearly does not lead to the formation of structurally distinct domains in the membrane.

Another aspect of the apocytochrome c–phospholipid interaction that we addressed in this study is the effect of lipid binding on the backbone conformation of the protein. Infrared spectroscopy has proven to be of unique value in studying conformational properties of membrane-associated proteins (Chapman et al., 1989; Surewicz & Mantsch, 1990). Unlike most other optical measurements, vibrational spectra are essentially unaffected by light scattering on large membrane fragments or lipid–protein aggregates.

The infrared spectroscopic data for apocytochrome c in solution are in accord with earlier circular dichroism (CD) studies (Fisher et al., 1973; Rietveld et al., 1985); the solution structure of the protein is largely unordered, devoid of α-helices or β-sheets. Analysis of the infrared spectra of apocytochrome c associated with phospholipid vesicles reveals two distinct types of protein backbone conformation (secondary structure), depending on the lipid to protein ratio. In the presence of a large excess of DMPG, the amide I band of apocytochrome c is indicative of an α-helical conformation. The present observation of a lipid-induced unordered structure–helix transition in apocytochrome c provides a direct experimental confirmation of earlier predictions, based on studies with the protein in the presence of negatively charged detergents (Rietveld et at., 1985). Consistent with an α-helical structure are also

[3] To place this in the same context, at 50 °C the protein causes a decrease in the frequency of the methylene (CH_2 or C^2H_2) symmetric stretching vibration of the DMPG and DMPC-d_{54} components by approximately 0.7 and 0.6 cm^{-1}, respectively. This is to be compared with the protein-induced decrease of 2.6 cm^{-1} found for a pure DMPG membrane.

circular dichroism spectra of apocytochrome *c*–phosphatidylserine recombinants reported by Walter et al. (1986), although the high turbidity of these recombinants precluded detailed interpretation of the CD data. While the increase in helicity suggests that a lipid environment induces structural changes in the apoprotein toward a conformation characteristic of the mature cytochrome *c* in solution (Rietveld et al., 1985), it should be noted that neither CD nor infrared spectra of DMPG-associated apocytochrome *c* are identical with those of the holoprotein in solution. In particular, the infrared band representing an α-helical structure in the lipid-bound apocytochrome *c* is at 1649 cm^{-1}, whereas the corresponding infrared band in the solution spectrum of cytochrome *c* is at 1653 cm^{-1} (unpublished data from this laboratory). This low-wavenumber shift of the "α-helical band" likely reflects a higher degree of hydrogen–deuterium exchange and is fully understandable for those experiments in which, prior to lipid binding, the protein was dissolved (in an unordered conformation) in ^2H_2O (see Results). However, it should be noted here that IR spectra essentially identical with those represented by trace 2 in Figure 4 were obtained also for samples prepared by an alternate method, in which lipid–protein recombinants were formed in fully protonated buffer, and only afterwards was the solvent exchanged by dialysis against a deuterated buffer (see Materials and Methods). The essentially complete hydrogen–deuterium exchange of the backbone amide groups in the latter samples within a 2-h period (as indicated by the position of the amide I band and the lack of the amide II mode at $\sim$1550 cm^{-1}) is rather intriguing. It suggests that, despite the formation of α-helices, the overall tertiary structure of the lipid-associated apocytochrome *c* remains relatively loose (much looser than the structure of the holoprotein in solution), with α-helical backbone amide groups accessible to the aqueous solvent. This water accessiblity is particularly puzzling in view of numerous indications that apocytochrome *c* penetrates deeply into the hydrocarbon region of the lipid membrane (Rietveld et al., 1985, 1986; Görrissen et al., 1986). While we can offer no immediate and unequivocal explanation of this puzzle, two possibilities stand out. First, the previously deduced membrane location of apocytochrome *c* may represent an average picture of a rather dynamic situation in which there is rapid exchange of two populations of the protein, one in the bilayer interior and one in a water-accessible environment on the membrane surface. An alternative explanation could be that apocytochrome *c* induces nonlamellar structures in the membrane (de Kruijff et al., 1985), which provides a more polar and water-accessible environment for the protein, although the presence of such structures in a DMPG–apocytochrome c system has not yet been demonstrated.

Infrared spectra of DMPG–apocytochrome *c* complexes at low lipid to protein ratios (below approximately 40:1) are suggestive of an extensive aggregation of the membrane-associated protein. Indeed, acidic phospholipid-promoted oligomerization of apocytochrome *c* has been previously deduced from electron microscopic and biochemical experiments (Rietveld et al., 1983, 1986b). Furthermore, such oligomers have been implicated in the mechanism of protein translocation across the membrane (Rietveld et al., 1986b). An important observation made in the present study is that the backbone conformation of the membrane-bound, aggregated protein is distinctly different from that of the (presumably) monomeric apocytochrome *c* in complexes at high lipid to protein ratios. In the former case, there is little evidence for α-helix, and infrared spectra are compatible with the presence of extended, intermolecularly hydrogen-bonded β-sheets. While the relevance of the above-described conformational preferences of apocytochrome *c* to the mechanism of mitochondrial import of the protein remains to be elucidated, the present observations provide new insight into the apocytochrome *c*–lipid interaction in model systems. In this context, it should be pointed out that most previous spectroscopic and biophysical studies on the effect of apocytochrome *c* on lipid packing and mobility have been performed with complexes of relatively low lipid to protein ratio. Such conditions favor an extended, aggregated structure of the protein and not, as often assumed, an α-helical conformation.

ACKNOWLEDGMENTS

We thank Adele Martin for technical assistance with the preparation of apocytochrome *c*.

Registry No. DMPG, 61361-72-6; DMPC, 18194-24-6; cytochrome *c*, 9007-43-6.

REFERENCES

Berkhout, T. A., Rietveld, A., & de Kruijff, B. (1987) *Biochim. Biophys. Acta 897*, 1–4.

Blume, A., Hübner, W., & Messner, G. (1988) *Biochemistry 27*, 8239–8249.

Bush, S. F., Levin, H., & Levin. I. (1980) *Chem. Phys. Lipids 27*, 101–111.

Byler, M., & Susi, H. (1986) *Biopolymers 25*, 469–487.

Cameron, D. G., Kauppinen, J. K., Moffatt, D. J., & Mantsch, H. H. (1982) *Appl. Spectrosc. 36*, 245–250.

Carrier, D., Mantsch, H. H., & Wong, P. T. T. (1990) *Biopolymers 29*, 837–844.

Casal, H., & Mantsch, H. H. (1984) *Biochim. Biophys. Acta 779*, 381–401.

Chang, C. T., Wu, C. S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.

Chapman, D., Jackson, M., & Haris, P. I. (1989) *Biochem. Soc. Trans. 17*, 617–619.

De Kruijff, B., Cullis, P. R., Verkleij, A. J., Hope, M. J., van Echteld, C. J. A., & Taraschi, T. F. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) pp 131–204, Plenum Press, New York.

Demel, R. A., Jordi, W., Lambrechts, H., van Damme, H., Hovius, R., & de Kruijff, B. (1989) *J. Biol. Chem. 264*, 3988–3997.

Dluhy, R. A., Moffatt, D. J., Cameron, D. G., Mendelsohn, R., & Mantsch, H. H. (1985) *Can. J. Chem. 63*, 1925–1932.

Dumont, M. E., & Richards, F. M. (1984) *J. Biol. Chem. 259*, 4147–4156.

Fisher, W. R., Taniuchi, H., & Anfinsen, C. B. (1973) *J. Biol. Chem. 248*, 3188–3195.

Görrissen, H., Marsh, D., Rietveld, A., & de Kruijff, B. (1986) *Biochemistry 25*, 2904–2910.

Hay, R., Bohni, P., & Gasser, S. (1984) *Biochim. Biophys. Acta 779*, 65–87.

Henning, B., & Neupert, W. (1983) *Methods Enzymol. 97*, 261–274.

Jaworsky, M., & Mendelsohn, R. (1986) *Biochim. Biophys. Acta 860*, 491–502.

Kauppinen, J. K., Moffatt, D. J., Mantsch, H. H., & Cameron, D. G (1981) *Appl. Spectrosc. 35*, 271–276.

Lee, D. C., & Chapman, D. (1986) *Biosci Rep. 6*, 235–256.

Li-Xin, Z., Jordi, W., & de Kruijff, B. (1988) *Biochim. Biophys. Acta 942*, 115–124.

Mantsch, H. H., Moffatt, D. J., & Casal, H. L. (1988) *J. Mol. Struct. 173*, 285–298.

Mendelsohn, R., & Mantsch, H. H. (1986) in *Progress in Protein–Lipid Interactions* (Watts, A., & DePont, J. J. H.

H. M., Eds.) Vol. 2, pp 103-146, Elsevier Science Publishers, Amsterdam.
Muga, A., Surewicz, W. K., Wong, P. T. T., Mantsch, H. H., Singh, V. K., & Shinohara, T. (1990) *Biochemistry 29*, 2925-2930.
Mushayakarara, E., & Levin, I. (1982) *J. Phys. Chem. 86*, 2324-2327.
Nesmeyanova, M. A. (1982) *FEBS Lett. 142*, 189-193.
Nicholson, D. W., Kohler, H., & Neupert, W. (1987) *Eur. J. Biochem. 164*, 147-157.
Pilon, M., Jordi, W., de Kruijff, B., & Demel, R. A. (1987) *Biochim. Biophys. Acta 902*, 207-216.
Rietveld, A., & de Kruijff, B. (1986) *Biosci. Rep. 6*, 775-782.
Rietveld, A., Sijens, P., Verkleij, A. J., & de Kruijff, B. (1983) *EMBO J. 2*, 907-913.
Rietveld, A., Ponjee, G. A. E., Schiffers, P., Jordi, W., van de Coolwijk, P. J. F. M., Demel, R. A., Marsh, D. & de Kruijff, B. (1985) *Biochim. Biophys. Acta 818*, 398-409.
Rietveld, A., Berkhout, T. A., Roenhorst, A., Marsh, D., & de Kruijff, B. (1986a) *Biochim. Biophys. Acta 858*, 38-46.
Rietveld, A., Jordi, W., & de Kruijff, B. (1986b) *J. Biol. Chem. 261*, 3846-3856.
Stellwagen, E., Rysavy, R., & Babul, G. (1972) *J. Biol. Chem. 247*, 8074-8077.
Surewicz, W. K., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 952*, 115-130.
Surewicz, W. K., & Mantsch, H. H. (1990) in *Protein Engineering—Approaches to the Manipulations of Protein Folding* (Narang, S. A., Ed.) pp 131-157, Butterworth, Stoneham, MA.
Surewicz, W. K., Moscarello, M. A., & Mantsch, H. H. (1987a) *Biochemistry 26*, 3881-3886.
Surewicz, W. K., Mantsch, H. H., Stahl, G. L., & Epand, R. M. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7028-7031.
Surewicz, W. K., Epand, R. M., Orlowski, R. C., & Mantsch, H. H. (1987c) *Biochim. Biophys. Acta 899*, 307-310.
Surewicz, W. K., Leddy, J. J., & Mantsch, H. H. (1990) *Biochemistry 29*, 8106-8111.
Walter, A., Margolis, D., Mohan, R., & Blumenthal, R. (1986) *Membr. Biochem. 6*, 217-237.

Mutant Aminoacyl-tRNA Synthetase That Compensates for a Mutation in the Major Identity Determinant of Its tRNA†

W. Todd Miller, Ya-Ming Hou, and Paul Schimmel*
Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
Received October 1, 1990; Revised Manuscript Received December 6, 1990

ABSTRACT: A single G3·U70 base pair in the acceptor helix is the major determinant for the identity of alanine transfer RNAs (Hou & Schimmel, 1988). Introduction of this base pair into foreign tRNA sequences confers alanine acceptance on them. Moreover, small RNA helices with as few as seven base pairs can be aminoacylated with alanine, provided that they encode the critical base pair (Francklyn & Schimmel, 1989). Alteration of G3·U70 to G3·C70 abolishes aminoacylation with alanine in vivo and in vitro. We describe here the mutagenesis and selection of a single point mutation in *Escherichia coli* Ala-tRNA synthetase that compensates for a G3·C70 mutation in $tRNA^{Ala}$. The mutation maps to a region previously implicated as proximal to the acceptor end of the bound tRNA. In contrast to the wild-type enzyme, the mutant charges small RNA helices that encode a G3·C70 base pair. However, the mutant enzyme retains specificity for alanine tRNA and can serve as the sole source of Ala-tRNA synthetase in vivo. The results demonstrate the capacity of an aminoacyl-tRNA synthetase to compensate through a single amino acid substitution for mutations in the major determinant of its cognate tRNA.

The attachment of amino acids to their cognate tRNAs during protein biosynthesis is catalyzed by aminoacyl-tRNA synthetases. Although the specific details of tRNA recognition by the enzymes are not completely understood, evidence from in vivo and in vitro studies has begun to define determinants for tRNA identity in many systems (Schimmel, 1989; Normanly & Abelson, 1989). In several cases, the aminoacyl-tRNA synthetases have been shown to rely on a small number of nucleotides in the tRNA to determine substrate acceptance. Amber suppression assays in *Escherichia coli* have been used to establish a set of nucleotides sufficient for aminoacylation by glutamine (Yaniv et al., 1974; Ghysen & Celis, 1974; Rogers & Söll, 1988) and serine (Normanly et al., 1986). In these experiments, mutant amber suppressor tRNAs were either isolated or synthesized with nucleotide changes that directed attachment of a noncognate amino acid. The CAU anticodon of *E. coli* $tRNA^{Met}$ has been shown to be an important determinant for aminoacylation by Met-tRNA synthetase in vivo (Varshney & RajBhandary, 1990; Chattapadhyay et al., 1990), and in vitro aminoacylation studies of $tRNA^{Met}$, $tRNA^{Val}$, and $tRNA^{Arg}$ with purified aminoacyl-tRNA synthetases confirm that the anticodon plays an important role in these cases (Schulman & Pelka, 1988, 1989). The anticodon and nucleotides outside of the anticodon appear important for recognition of yeast $tRNA^{Phe}$ (Sampson et al., 1989), and a role for base modifications in tRNA discrimination is indicated by the work of Muramatsu et al. (1988) and Perret et al. (1990).

A variety of mutant $tRNA^{Ala/CUA}$ amber suppressors were created and tested for suppression of the *trpA*(UAG234)

†Supported by Grant GM 23562 from the National Institutes of Health and by a postdoctoral fellowship from the American Cancer Society (W.T.M.).

0006-2960/91/0430-2635$02.50/0