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The conformational changes of apocytochrome *c* upon binding to phospholipid vesicles and micelles of phospholipid based detergents: a circular dichroism study

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The influence of lipid aggregates on the secondary structure of the mitochondrial precursor protein apocytochrome *c* was investigated by circular dichroism techniques. A conformational change of the protein from a random coil to partially α-helical structures was observed upon binding to negatively charged DOPS SUVs. Also DOPC SUVs showed to induce such a conformational change, but to a lesser extent. The detergents decyl-, lauryl and myristoyl-phosphoglycol or -phosphocholine, were synthesized as micel forming phospholipid analogs and are shown to mimic the phospholipids well in their ability to induce α-helices in the protein. A full assignment of the regions where the possible α-helices are formed is proposed by making use of derived fragments of apocytochrome *c*, prediction methods and the known X-ray structure of cytochrome *c*. Besides a helix at the N-terminus (residues 1–22) and at the C-terminal part (residues 80–101), two regions in the middle section (residues 49–54 and 59–70) are suggested to be helical. It is inferred that the two cysteines in the positions 14 an 17 at the N-terminal part are facing in the same direction, which could facilitate the covalent attachment of the heme group to the precursor in the translocation process.

Introduction

Apocytochrome *c* is the precursor form of cytochrome *c*, a protein functional in the mitochondrial respiratory chain and located in the intermembrane space. After synthesis in the cytosol it has to be translocated over the outer mitochondrial membrane. In contrast to most mitochondrial precursor proteins apocytochrome *c* possessed no cleavable amino-terminal presequence [1,2]. Also no proteinaceous components at the mitochondrial surface, ATP or a membrane potential are required for translocation [3], indicating the uniqueness of the import pathway of apocytochrome *c* [4].

The import of apocytochrome *c* into mitochondria can be resolved into two different steps: first the binding of the protein to the outer membrane and secondly a subsequent translocation into the inner membrane space coupled to heme insertion by the enzyme heme lyase [5]. The molecular mechanisms of the translocation process across the outer mitochondrial membrane are still unknown. From many biochemical and biophysical studies on apocytochrome *c*–lipid interactions [6–11] the following model for the translocation process emerges: (1) there is a preferential binding of the basic protein to negatively charged lipids on an initially electrostatic basis, (2) a translocation competent state is formed, (3) apocytochrome *c* partially inserts into the hydrophobic domain resulting in the translocation of the N-terminal part to the opposite interface of negatively charged lipid containing bilayers, (4) membrane insertion/translocation is accompanied by changes in lipid organization [12,13].

From circular dichroism (CD) studies it was shown that apocytochrome *c* undergoes a conformational change from random structure in aqueous environment

Abbreviations: DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; PS, phosphatidylserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; 10-Pglycol, decylphosphoglycol; 12-Pglycol, laurylphosphoglycol; 12-PN, laurylphosphocholine; 14-Pglycol, myristoylphosphoglycol; SDS, sodium dodecylsulfate; TEA, triethylamine; TCE, trichloroethane; MeOH, methanol; TLC, thin-layer chromatography; CD, circular dichroism; CMC, critical micelle concentration; RMS, root mean square; NMR, nuclear magnetic resonance; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle.

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to partially α-helical conformation upon binding to PS vesicles [14] and SDS micelles [11], whereas no change could be observed upon binding to respectively zwitterionic PC LUVs or neutral Lubrol and octyl glucoside micelles [11,28]. Also by dissolving apocytochrome *c* (and derived fragments) in chloroethanol a partially α-helical structure could be observed [15]. The induction of α-helices could be an important step in the import process in particular since data obtained with SDS indicate that the α-helices are formed in the same regions as found in the folded holoprotein [11].

However, thus far the data obtained by CD of apocytochrome *c* bound to lipid vesicles were of insufficient quality, most likely due to light scattering contributions, to allow a proper analysis of the adoption of secondary structure of the protein. Moreover, monomers of SDS are able to induce conformational changes within proteins, probably due to the denaturating properties of this strong detergent [16].

It is the aim of our research to get quantitative information about the changes in secondary structure upon interaction of apocytochrome *c* with a lipid-like interface. As a first step we report here CD studies of the protein (and derived fragments) in interaction with micelles of a new series of detergents, which were designed to mimic the putative natural target phospholipids and which enable future high resolution NMR experiments. The results obtained are compared to CD data of the protein on interaction with SUVs prepared of the phospholipids DOPS and DOPC. To achieve proper analysis of the CD spectra a criterion will be defined, which reduces the possibility of turbidity artefacts of the samples.

The outcome of these studies show that upon addition of negatively charged DOPS SUVs apocytochrome *c* undergoes a conformational change from random coil to partially α-helical structures. The detergents show to mimic phospholipids very well in the ability to induce α-helices in the protein. By using fragments of apocytochrome *c* we are able to identify the regions where these helices are formed.

Material and Methods

Materials

The phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were synthesized and purified according to established methods [17,18]. The synthesis and purification of the detergents decyl-, lauryl-, and myristoylphosphoglycol (10-, 12-, and 14-Pglycol) is described below. The synthesis of laurylphosphocholine (12-PN) was carried out as described by Brown et al. [19,20].

Apocytochrome *c* was prepared by removal of the heme group of horse heart cytochrome *c* (type VI, Sigma) as described by Fisher et al. [21]. The protein migrated as a single band in SDS-polyacrylamide gel electrophoresis with an appearent molecular mass of 11.5 kDa.

The fragments 1–65H, 66–80 and 81–104 were prepared by cyanogen bromide cleavage of cytochrome *c* as described by Corradin and Harburry [22]. The heme containing fragment, 1–65H, was used for the preparation of the heme free fragment, 1–65, by removal of the heme group as described Fisher et al. [21].

Synthesis of alkylphosphoglycol

The synthesis of alkyl-phosphoglycol is mainly based on reactions described by Eibl [23]. The reaction-scheme is outlined in Fig. 1. All steps are done under nitrogen gas using dried solvents (distillation in the presence of calcium hydrid).

0.1 mol fatty acid (Merck, Darmstadt (F.R.G.)) dissolved in 100 ml diethyl ether was added dropwise at room temperature to 10 ml (approx. 0.12 mol) oxalyl chloride (Merck, Darmstadt (F.R.G.)). After 8 h the excess oxalyl chloride was evaporated and 8 ml of MeOH was added, yielding a 100% conversion of the fatty acid into its methyl ester. Small aliquots (upto approx. 6 g) of $LiAlH_3$ were added to the fatty acid methyl ester, dissolved in 100 ml diethyl ether and 50

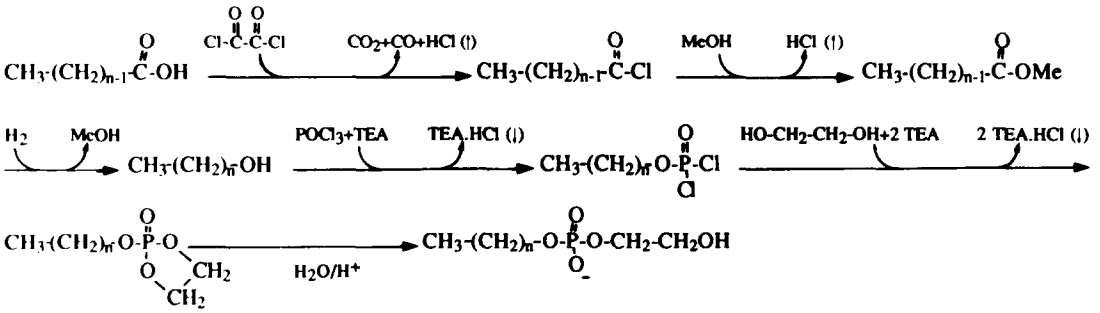

Fig. 1. Reaction scheme of the synthesis of alkyl phosphoglycol.

ml tetrahydrofuran. After removal of the excess hydride by ethyl acetate, the reaction was quenched by 250 ml aqueous 10% H_2SO_4 solution. The product, extracted with diethyl ether, revealed by TLC analysis on silicagel (eluens hexane/diethyl ether (1 : 1, v/v)) one spot of fatty alcohol (yield: 0.093 mol).

A 100% conversion of the fatty alcohol to the alkyl phosphoric acid dichloride was obtained by adding dropwise 0.025 mol fatty alcohol and 0.0275 mol TEA in 100 ml dried hexane to 0.0275 mol $POCl_3$ in 10 ml hexane at 2° C under nitrogen-gas, letting it react for 3 h revealing the white precipitate TEA · HCl. After removal of the precipitate by filtration, the solvent was evaporated.

0.025 mol ethylene glycol (Merck, Darmstadt (FRG)) and 0.095 mol TEA in 60 ml hexane were added with 1 h to the alkylphosphoric acid dichloride dissolved in 60 ml hexane at room temperature, after which the temperature was raised to 40° C for another 3 h. After filtering off the precipitated TEA · HCl, the filtrate was evaporated and the remaining product, alkylphosphoric acid glycolester, was dissolved in 250 ml 2-propanol. TLC analysis on silicagel (eluens TCE/MeOH (70 : 20, v/v)) showed a product at $R_f = 0.95–1$ which was assigned to a closed five-ring (as described by Eibl [23]). After addition of 120 ml aqueous 20% acetic acid and stirring for 3–4 h complete ring opening occurred as shown by TLC where the spot showed a R_f value of 0.1–0.2 (eluens TCE/MeOH (70 : 20, v/v)), revealing alkylphosphoglycol in a yield of 60–75%.

The products were purified on open silica 60 columns eluted with TCE/MeOH (70 : 20, v/v). TLC analysis showed in all cases single spots (UV detection, I_2 reagens), reacting positive on phosphorus reagens. The alkyl-phosphoglycols and -phosphocholines dissolved in $CDCl_3/CD_3OD$ (1 : 1, v/v) could be positively identified by both ^{31}P- and ^{13}C-NMR on a MSL-300 at resonance frequencies of, respectively, 121.497 and 75.468 MHz and no contamination could be detected.

Methods

Sample preparation

For the CD experiments stock solutions of 0.3 mM apocytochrome *c* or fragments in 10 mM phosphate (pH 7.0) buffer were used. 0.1 ml of protein solution was added to 0.2 ml of lipid- or detergent-containing 10 mM phosphate (pH 7.0) buffer, reaching final concentrations of 0.1 mM of protein and the desired lipid- or detergent concentration ranging from 0 to 50 mM. Stock solutions of SUVs of DOPS or DOPC (35 mM lipid) were prepared by sonication using a Branson B-12 tip sonicator for 30 times 0.5 min at 50 Watt, while cooling on ice. A stock solution of 50 mM detergent was used.

Critical micelle concentrations

The different CMC values for the various detergents were estimated by turbidity measurements performed on a Hitachi U-3200 spectrophotometer. The measurements were carried out at various wavelengths (280, 300 and 336 nm) to exclude specific absorption effects, using a 10 mM phosphate buffer and a 1 ml quarts cuvet. The values were estimated to be 1.1 mM, 0.6 mM, and < 0.05 mM for, respectively, 10-, 12- and 14-Pglycol. The CMC of 12-PN was found to be 1.2 mM, in good agreement with van Dam-Mieras et al. [24].

Circular dichroism experiments

The measurements were performed at room temperature in 0.1 and 0.2 mm light pathway cells on a Jasco-600 spectropolarimeter, interfaced to a Laser 386 computer, while flushing the cuvet-chamber with nitrogen gas. For each sample 6–10 CD spectra were accumulated from 260 to 185 nm. After subtraction of the control protein-free sample and smoothing of the spectrum the data were stored with a resolution of 0.1 nm. The scan speed was 20 nm/min, the time constant 0.5 sec, the band width 1 nm and the sensitivity range of the photomultiplier was 20 to -20 mdeg.

CD spectral analysis

The observed CD spectra are expressed as a linear combination of four reference spectra for pure α-helical, β-strand, β-turn and random coil structures. The coefficients are calculated by a least-squares method. Each spectrum is analyzed twice: (1) using the constraint that the sum of the coefficients must be equal to 1 (giving 100%), and (2) without this restriction. By comparing these two analysis and knowing the length of the light pathway of the cell used, the protein concentration and the number of peptide bonds within the observed protein, one is able to check a possible loss of intensity due to turbidity of the samples. All data presented in this paper arise from the constrained procedure. As reference spectra for the α-helical, β-sheet and random conformation we used the data of the CD spectra of $(Lys)_n$ as described by Greenfield and Fasman [25] and for the β-turn we took the average of fifteen proteins as described previously by Chang et al. [26]. This set of reference spectra gave in all cases the lowest RMS values (defined by Brahms and Brahms [27]) which are ranging, if not mentioned otherwise, from 1 upto a maximum of 10. Analysis of the CD spectrum of horse heart cytochrome *c* in 10 mM phosphate buffer (pH 7.0) revealed with this reference set 39% α-helix, which is in good agreement with the literature value of 40% obtained by X-ray diffraction [28].

Results

It is known that apocytochrome *c* shows in buffer a featureless CD spectrum, typical for a random confor-

mation of the protein [10,15,21]. However, as is shown in Fig. 2 (curve a–e), the addition of increasing amounts of DOPS SUVs induces three extremes of ellipticity at 190, 209 and 222 nm typical for α-helical structures [8,21]. These spectra are well suited for spectral analysis, which showed by using a constrained and non-constrained fit-procedure that the loss of intensity is less than 5% upto a DOPS-concentration of 15 mM. Therefore, flattening of the spectrum due to turbidity of the sample could be excluded. Under these conditions all spectra intercept around 204 nm, an iso-dichroic point, which is typical for a conversion of random coil to α-helical structures [29]. Above a DOPS-concentration of 15 mM (Fig. 2 curve f) spectral changes could be observed, such as a large red shift, a merging of the two minima and a loss of intensity, indicative for turbidity effects of the phospholipid vesicles [14], prohibiting a proper spectral analysis.

The two CD spectra in Fig. 3A show that SUVs of the negatively charged DOPS (spectrum a) are more effective in inducing secondary structure in apocytochrome *c* then those prepared from DOPC (spectrum b). Fig. 3B furthermore compares quantitatively the percentages of α-helix in apocytochrome *c* as found by analytical curve fitting as a function of the lipid concentration of DOPS and DOPC. Spectral analysis of the DOPC spectra could not reveal the same quality fits (RMS-values of 10–20) compared to those obtained for DOPS. With increasing lipid concentration an increasing percentage of α-helices is induced in the protein by both lipids. Above a lipid concentration of 10 mM both curves reach a maximum percentage of α-helix induction, which is 22% for DOPS and 14% for DOPC SUVs.

Spectral analysis reveals also that the relative high amount of β-strand (38.5%) does hardly change upon addition of the phospholipids and the amount of β-turn is in all cases neglible; the increase of α-helix content with increasing lipid concentration results completely from a loss in random coil.

The use of lipid SUVs implicates turbidity of the samples which becomes unacceptable at higher vesicle concentrations. To overcome this problem and to enable future high resolution NMR experiments, we synthesized the detergents alkyl-Pglycol and alkyl-PN, which resemble more closely membrane phospholipids, than the often used strong detergent SDS. The alkyl-Pglycol was designed to mimic the anionic and the alkyl-PN the zwitterionic phospholipids in the mitochondrial outer membrane. Fig. 4A shows the CD spectra of apocytochrome *c* with 10 mM 12-Pglycol and 12-PN. Just as for the phospholipid SUVs, the induction of secondary structure is more effective for the 12-Pglycol than its zwitterionic counterpart. The percentages of α-helices induced in apocytochrome *c* as obtained by spectral analysis are shown in Fig. 4B as a function of the concentration 12-Pglycol and 12-PN. Comparing the α-helix induction in apocytochrome *c* by phospholipids and by the detergents one obtains qualitatively similar results. The amount of α-helix increases with increasing detergent concentration and reaches, just as for DOPS and DOPC a plateau value at a concentration of 10 mM, corresponding to a stoichiometry of 1 protein per 100 lipids. For the negatively charged 12-Pglycol we obtain a much higher maximum value than for the zwitterionic 12-PN, respectively, 61 and 28% α-helix, which are both higher than found upon the addition of lipid vesicles. The dashed part of the curve of 12-Pglycol, from 1 to 5 mM, (shown more clearly in Fig. 4C) reflects a region in which extensive visible precipitation of protein-detergent complexes occurs. The CD spectra in this region show a large reduc-

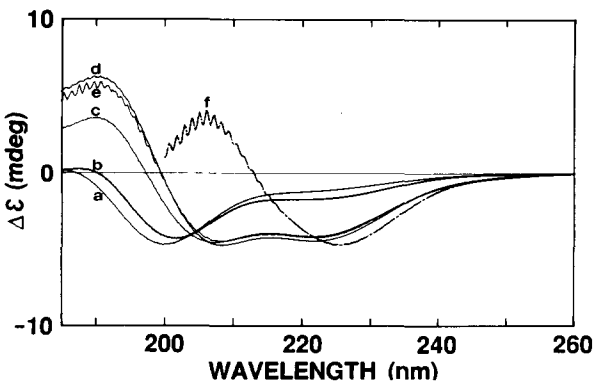

Fig. 2. CD spectra of 0.1 mM apocytochrome *c* in 10 mM phosphate buffer (pH 7.0) containing 0 (curve a), 2 (b), 5 (c), 10 (d), 15 (e) and 20 (f) mM DOPS SUVs. Path length is 0.1 mm.

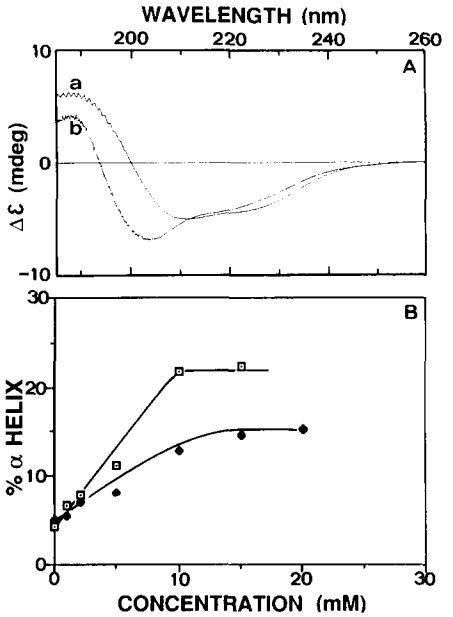

Fig. 3. (A) CD spectra of 0.1 mM apocytochrome *c* in the presence of 10 mM DOPS (a) and DOPC (b). (B) The percentage of α-helix obtained by spectral analysis induced in 0.1 mM apocytochrome *c* as a function of the concentration DOPS (⊡) and DOPC (◆). Path length is 0.1 mm.

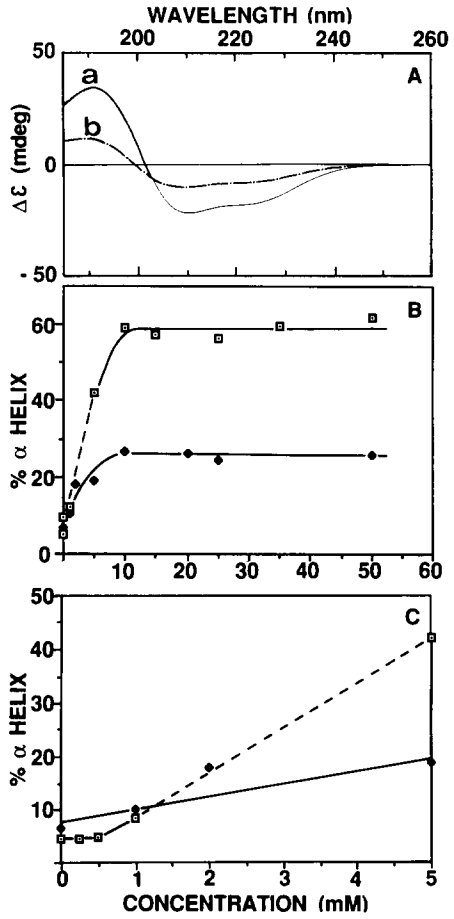

Fig. 4. (A) CD spectra of apocytochrome *c* in the presence of 10 mM 12-Pglycol (a) and 12-PN (b). (B) The percentage of α-helix obtained by spectral analysis induced in 0.1 mM apocytochrome *c* as a function of the concentration 12-Pglycol (⊡) and 12-PN (◆). Path length in 0.2 mm. Fig. 4C is an expansion of the 0–5 mM detergent range shown in Fig. 4B.

tion of the intensity and a small red-shift. Therefore no reliable data on the secondary structure could be obtained in this region. Upto the CMC of these detergents (0.6 and 1.2 mM for, respectively, 12-Pglycol and 12-PN) no significant spectral changes could be observed (Fig. 4C).

Comparable to the phospholipids, spectral analysis showed that the amount of α-helical structures in apocytochrome *c* induced by these detergents is equal to a loss in random coil, whereas the amount of β-structure was not affected.

The dependence of the length of the acyl chain of Pglycols on the conformation of apocytochrome *c* is shown in Fig. 5. The data on 12-Pglycol are included for comparison. The regions where visible precipitation of the samples occurs are 3–20 mM and 0.5–1.5 mM for 10- and 14-Pglycol, respectively. These aggregation regions shift with increasing chain length of Pglycol to lower concentrations, implicating that not only the electrostatic effects play a role in these aggregation phenomena, but perhaps also the packing constrains of the

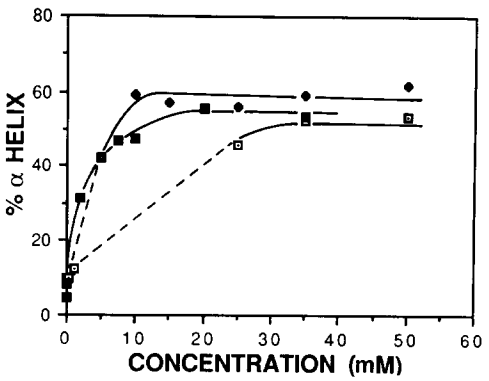

Fig. 5. The percentage α-helix in apocytochrome *c* as a function of the concentration alkyl-Pglycol for the chain lengths 10 (⊡), 12 (◆), and 14 (■) carbon atoms. The dashed parts of the curves indicate the region where visible precipitation of protein-detergent complexes occurs.

micelles. The 10-, 12-, and 14-Pglycol induce a maximum of respectively 46, 61 and 55% of α-helix content in apocytochrome *c*. For all these detergents no significant effect on the conformation of apocytochrome *c* could be observed at or below their CMC values, indicating that a possible interaction of apocytochrome *c* with monomer detergents reveals no alteration in its secondary structure; this in contrast to observations of apocytochrome *c* with SDS (data not shown).

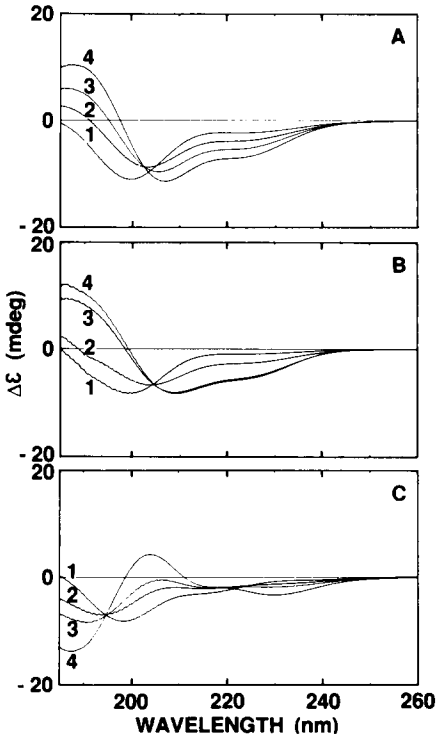

Fig. 6. CD spectra of several fragments of apocytochrome *c*. (A) 1–65H, (B) 1–65 and (C) 66–80 with 0, 1, 5 and 10 mM 12-Pglycol (respectively, spectrum 1, 2, 3 and 4), except Fig. C, curve 2: 2 mM detergent. The concentration of the fragments is 0.1 mM in 10 mM phosphate (pH 7.0) buffer.

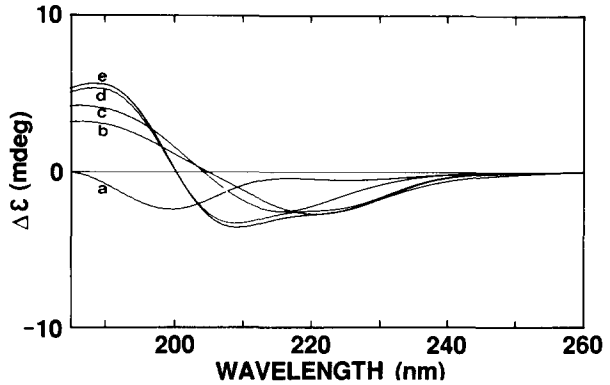

Fig. 7. CD spectra of 0.1 mM C-terminal fragment 81–104 of apocytochrome *c* with (a) 0, (b) 0.5, (c) 2, (d) 10 and (e) 20 mM of 12-Pglycol in 10 mM phosphate (pH 7.0) buffer.

To study the secondary structure of apocytochrome *c* in more detail fragments of the protein were analyzed. The CD spectra of the fragments 1–65, with and without the heme group, and 66–80 are shown in Fig. 6. In the absence of detergents in all cases CD spectra typical for a random coil structure are observed in agreement with previous observations [11,15]. The N-terminal fragments (Fig. 6A and B) show in both cases upon addition of 12-Pglycol an iso-dichroic point around 204 nm for the conversion of random coil to α-helix. For the fragment, 66–80, one can observe an iso-dichroic point at 194 nm and with increasing detergent concentration an increasing maximum of ellipticity at 202 nm, indicative for β-turns [29] (Fig. 6C). The C-terminal fragment, 81–104, shows a more complicated behavior upon titration of 12-Pglycol (Fig. 7). Upto 0.5 mM detergent, thus below the CMC of 0.6 mM, this fragment shows a crosspoint with the detergent-free sample around 208 nm (compare spectra a and b), which is close to the iso-dichroic point for random coil and β-strand. This indicates an interaction of this fragment with 12-Pglycol monomers. Spectral analysis of spectrum b reveals 85% β-strand. With increasing detergent concentration upto 10 mM this crosspoint shifts gradually to lower wavelengths, reaching 204 nm for concentrations 12-Pglycol above 10 mM (spectra d and e), indicative for a conversion into α-helical structures.

The values of the different secondary structure types of the fragments in buffer (in parenthesis) and at a saturating level of 10 mM of detergent as obtained by spectra analysis are shown in Table I.

TABLE I

Percentages of the different secondary structures induced by saturating amounts of 12-Pglycol in the different fragments of apocytochrome c

The values in brackets are for the peptides in the absence of detergent.

	α-helix	β-strand	β-turn	Random coil
1– 65H	38.4 (6.2)	8.8 (24.5)	0.0 (0.0)	53.0 (69.3)
1– 65	37.6 (4.8)	18.1 (19.7)	0.7 (0.0)	43.6 (75.7)
66– 80	0.0 (0.0)	40.1 (40.7)	20.3 (0.0)	39.5 (59.3)
81–104	73.9 (2.7)	0.0 (28.9)	0.0 (0.0)	26.3 (68.6)

Discussion

Turbidity contributions can be a potential source for artefacts in the estimation by CD of the secondary structure of apocytochrome *c* in membrane environments, as examplified by Walter et al. [14] for vesicular systems at higher lipid concentrations. Also at intermediate concentration ranges of the negatively charged detergents used here a visible precipitation of protein-detergent complexes could be observed, most likely due to a 'salting out' effect, giving rise to turbidity effects on the CD spectrum. In both cases a loss of intensity and a red-shift of the spectrum is observed, which in case of vesicles changes more gradually with the lipid concentration. The visible turbidity in the case of detergent-apocytochrome *c* complexes is accompanied by dramatic spectral changes, suggesting the presence of high amounts of β-stranded structures. We propose that the appearance of an iso-dichroic point in CD spectra upon titration of lipid is a good indication for the conversion of one secondary structure type into another without interfering turbidity contributions and can act as a criterion for quantitative spectral analysis.

Such an iso-dichroic point could be observed around 204 nm, for conversion of random coil to α-helix [10,21] in apocytochrome *c* upon titration of DOPS. The amount of α-helix at 10 mM DOPS (22%) is equal to what Jordi et al. [11] found for apocytochrome *c* with SDS-micelles. The spectrum of apocytochrome *c* in the presence of 20 mM DOPS SUVs is an example where the turbidity of the sample distorts the lineshape; it does not cross the iso-dichroic point as the spectra at lower DOPS concentrations, making a spectral analysis virtually impossible.

Previous reports of CD studies on apocytochrome *c* [11] showed that neutral detergents had no effect on the protein secondary structure [10,11], consistent with the lack of binding of the protein to DOPC LUVs [30]. However, the zwitterionic DOPC SUVs are able to induce significant spectral changes in apocytochrome *c*, but less pronounced as by DOPS. This in contrast to DOPC LUVs which showed under comparable conditions no influence on the CD spectrum of apocytochrome *c* (data not shown). Most likely this is due to the reduced packing of the lipids in the SUVs allowing penetration of the protein. This explanation is fully consistent with the increased ability of the protein to penetrate in PC monolayers at reduced surface pressure [31] and the higher affinity of apocytochrome *c* for small versus large unilamellar vesicles [32].

Although within particular concentration ranges reliable information can be obtained on the secondary

structure of apocytochrome *c* in interaction with vesicles, this system has its limitations as evidenced by the noise in the spectra below 200 nm as a result of turbidity effects. With the used detergents smooth CD spectra are obtained even below 200 nm, showing at least upto 50 mM of detergent an iso-dichroic point at 204 nm, indicating the absence of significant turbidity effects. Qualitatively the detergents show the same headgroup specificity in inducing α-helices in the protein as the phospholipids they mimic, but they are much more effective. The reason for this could lie in the fact that binding of the protein to a phospholipid surface can result in an adsorbed layer of the protein with different conformational properties, as has been shown to occur for DOPS monolayers [31]. Hence, the detergents are more likely to encapsulate the protein as part of the micelle. The 61% α-helix in apocytochrome *c* for 12-Pglycol comes close to the percentage which Toniolo et al. [15] reported for 2-chloroethanol.

To ascribe the potential regions within apocytochrome *c* which are able to adopt α-helical structure we investigated fragments of apocytochrome *c*. For the N-terminal fragment 1–65, with and without the heme, percentages of approx. 38% α-helix (about 25 residues) are obtained (Table I). We propose on the following arguments that these helical segments contain the regions 1–18 and 49–54 of the protein. (1) X-ray analysis of horse heart cytochrome *c* revealed a stretch of α-helical residues from 1 through 11 [28,33]. Several prediction methods attributed, besides the region 1–11 also to the segment 12–118 a potentially helical character [34–37]. (2) Also the region 49–54 has been predicted to be α-helical [37] or distorted α-helical [28]. The only influence of the heme group is reflected by the reduction of the amount of β-strands upon addition of detergents (Table I), which hardly affects the iso-dichroic point due to the relative small intensity at 204 nm of a β-stranded ordered polypeptide.

Thus far no characteristic secondary structure type could be identified for the fragment 66–80 upon binding to SDS-micelles [11] or dissolving in 2-chloroethanol [15]. However, our results show a clear conversion of random coil to β-turn upon titration of 12-Pglycol. Spectral analysis reveals no helical structures in this fragment, a small increase of β-strands and an induction of β-turns at cost of random coil upon addition of 12-Pglycol (Table I). The lack of α-helical segments has been confirmed by all prediction methods [34–38] and is probably related to end-group effects [39] and the large number of helix-breaking residues present in this small fragment. The presence of β-turns is predicted by a Chou-Fasman method [10] and is observable in the three-dimensional structure of the holoprotein as determined by X-ray diffraction at Pro-71, participating in a reverse turn and at Pro-76, which is situated in a sharp bend of the polypeptide chain [28].

The C-terminal fragment, 81–104, known to be the most hydrophobic part of apocytochrome *c* [10], shows strong β-strand formation at low detergent concentration, probably as a result of aggregation of the peptide. However, above 10 mM of 12-Pglycol the sample attains a high degree of right-handed α-helical conformation, to be estimated by spectral analysis to be approx. 74% (about 18 residues) (Table I). All the theoretical methods [34,35,37,38] and X-ray analysis on cytochrome *c* [28] identify the region 81–101 to be α-helical.

To evaluate the effect of fragmentation, the secondary structure of intact apocytochrome *c* is compared in Table II to that calculated for the summation of the data obtained on the fragments as presented in Table I. Comparison of the reconstructed protein without the heme group with intact apocytochrome *c* reveals that the intact protein contains 22% (about 22 residues) more α-helical structures and an equal reduction of random coil, than identified for the fragments. This is possibly caused by a loss of helix stability upon reducing the length of helical polypeptide structures. The additional residues involved in helix formation of the intact apocytochrome *c* in the presence of 12-Pglycol are proposed to be located in the 59–70 region. X-ray analysis of horse heart cytochrome *c* demonstrated an α-helix for the residues 62–70 [28], which could be extended, as suggested by many prediction methods [34–38], to Trp-59. This helix is probably disrupted in the fragments due to end-group effects [39]. This makes up to 57 residues which potentially form α-helical structures in apocytochrome *c* bound to a micellar surface. This number is somewhat smaller than obtained by CD analysis for the intact protein (about 63 residues). Besides for experimental errors and uncertainties in the assumptions involved, this difference can be partially reconciled by an extension in the intact protein of the N-terminal α-helix to Lys-22, as suggested by several prediction methods [34,37].

Since the proposed α-helix at the N-terminus includes the two cysteines present in apocytochrome *c* (position 14 and 17), the side chains of these amino acids must be facing the same direction. This could be favourable in coupling the heme group to these two residues. In contrast to the intact holoprotein in aqueous environment, where due to folding of the protein

TABLE II

Comparison of the percentages secondary structure of intact apocytochrome c with the percentages obtained by a summation of the data of the different fragments presented in Table I

	α-helix	β-strand	β-turn	Random coil
Fragments without heme	39.4 (3.8)	18.5 (24.1)	2.1 (0.0)	40.0 (72.1)
Apocytochrome *c*	61.0 (4.8)	12.6 (26.5)	4.6 (0.0)	21.8 (68.7)

around the heme group the helices 12–22 and 49–54 become distorted [28], the stability of the helices present in the N-terminal fragment upon binding to micelles is not affected by the presence of the heme (Table I). This could possibly be caused by the absence of Met-80 in this fragment, which forms in the holoprotein the sixth ligand to the heme.

In the near future we hope to be able to determine the exact structural and dynamical properties of apocytochrome *c* and its derived fragments at a water/lipid interface by 2D-NMR making use of fully deuterated analogs of the detergents, presented in this paper.

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