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Cytochrome *c* forms complexes and is partly reduced at interaction with GPI-anchored alkaline phosphatase

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Abstract

Cytochrome (cyt) *c* forms complexes, undergoes a conformational change and becomes partly reduced at interaction with membrane anchored alkaline phosphatase (AP), a glycoprotein which is released into the body fluid in forms differing in hydrophobicity. The proportion of products formed in the mixtures depends on pH, ionic strength, temperature and the buffer composition. The reaction terminates in an equilibrium between cyt *c*(FeII) and other cyt *c* conformers. Optimal conditions for the rate of the reaction are 100 mM glycine/NaOH, pH 9.7–9.9, at which 68–74% of cyt *c* is found in the reduced state. The interaction affects compactness of the haem cleft as shown by changes induced in CD spectra of the Soret region and changes in optical characteristics of phenylalanine, tyrosine and tryptophan residues. Differential scanning calorimetry of AP+cyt *c* mixtures revealed a creation of at least two types of complexes. A complex formed by non-coulombic binding prevails at substoichiometric AP/cyt *c* ratios, at higher ratios more electrostatic attraction is involved and at 1:1 molar ratio an apparent complexity of binding forces occurs. The rapid phase of the cyt *c*(FeII) formation depends on the presence of the hydrophobic alkylacylphosphoinositol (glycosylphosphatidylinositol) moiety, the protein part of the enzyme participates in an electrostatic and much slower phase of cyt *c*(FeII) creation. The results show that non-coulombic interaction may participate at interaction of cyt *c* with cellular proteins.

Keywords: Cytochrome *c*; Cytochrome *c* complex; Aromatic amino acid; Alkaline phosphatase; Glycosylphosphatidylinositol anchor; Non-coulombic binding; Apoptosis

1. Introduction

The markedly high structural flexibility of the cytochrome (cyt) *c* molecule was, until recently, almost exclusively studied in point of its ability to transfer electrons between natural respiratory complexes. Cyt *c* freely diffusible in the intermembrane space of mitochondria is transiently docked by its affinity binding sites to negatively charged complexes of cyt bc_1 and cyt aa_3 to maintain electron transport. These binding sites are constituted by positively charged amino acid residues whose location on the molecule surface has been well documented (cf. [1,2]). At present, it becomes clear that cyt *c* harbours a function in sensitising cells to apoptosis by inducing stimuli prerequisite for caspase-9 processing. It is released from the mitochondrial intermembrane space and interacts with apoptotic protease activating factor-1 (APAF-1) to form an oligomeric complex thereby initiating the proteolytic cascade [3,4]. The release is induced by proapoptotic proteins of the Bcl-2 family (e.g. Bax, Bid, etc.) which translocate in the mitochondria [5,6]. Most of them carry clusters of positively charged residues, which seem to have a critical role in the releasing process. It was suggested that the releasing effect of diverse polycations might be due to a dual mechanism: (i) binding to the cardiolipin on the inner membrane and induction of permeability transition pores, and (ii) direct binding to the outer membrane leading to its rupture ([7], and references therein). A pronounced specificity for the interaction between several polyamines and phosphatidylinositol, the major negative phospholipid of the outer membrane, was detected [7]. Similar experiments with the anionic phospholipids phosphatidylserine, phosphatidic acid and phosphatidylglycerol showed much lower affinity of polyamines to these phospholipids.

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On the other hand the polyamines which interacted strongly appeared to be more potent in direct destabilisation of the outer membrane [7]. Cyt *c* itself as a highly charged cationic protein might be involved in the process [8].

We found [9,10] that cyt *c* underwent a partial reduction and formed complexes with an isozyme of mammalian alkaline phosphatase (AP), a cell membrane-bound protein containing a glycosylphosphatidylinositol (GPI) anchor. Several GPI anchored proteins have been identified as a part of receptor complexes [11,12]; the GPI anchor in membrane microdomains was identified as a high affinity receptor for the channel-forming protein aerolysin [13]. Three mammalian tissue-specific APs, placental, germ cell, and intestinal forms, are known to be more than 90% homologous in their protein part whose crystal structure has been recently obtained with the placental isozyme and determined at atomic resolution [14,15]. All three isozymes attract major attention for their wide applicability in human diagnosis and therapeutic monitoring [16–18]. We have chosen the intestinal isoform whose secondary structure was found to be well preserved up to pH 10.4 and whose conformation was stable over a wide temperature range [19]. Although previously believed to be expressed only in the intestine, this isoform was recently identified in human pancreas, liver and kidney [18,20]. The variants show a large heterogeneity both in the hydrophobic and hydrophilic constituents of the GPI anchor [21] which further associates with (lipo)protein complexes and is thus protected from degradation by specific phospholipases [22,23]. An intestinal AP variant containing a membrane binding domain was found in serum samples of patients with malignancy [17,24]; three other variants consisting of hydrophobic dimers, more complex hydrophobic structures and hydrophilic dimers were detected in human plasma [22]. The variants did not show a marked difference in the enzymatic phosphomonoesterase activity, thus the reason of the variety released into the body fluid is not known. We found that cyt *c* binds to samples of intestinal AP and in longer time intervals irreversibly coaggregates with the enzyme. After incubation periods of >24 h the free cyt *c* was almost undetectable in the reaction mixture. High molecular aggregates of AP+cyt *c* were detected with an AP sample containing a larger portion of non-catalytic hydrophobic part whereas mainly a modified AP dimer of AP+cyt *c* species was found with a sample containing a higher amount of catalytic protein [10]. The early stage of the interaction was characterised by transition of a part of the cyt *c*(FeIII) into the reduced state. In the present paper we show that this redox transition is not a one-step process. The appearance of cyt *c*(FeII) is preceded by creation of transient complex(es) between the two proteins and accompanied by structural changes in the haem cleft of the cyt *c*(FeIII). Coulombic as well as non-coulombic binding forces are involved in the interaction.

2. Materials and methods

2.1. Materials

Alkaline phosphatases were commercially lyophilised products from Sigma, type VIII, from rabbit intestine, lot 102F8190, designated in the paper as AP (AP_I); two other samples, type VIII, rabbit intestine, lot 31H8125 (AP_{II}), and the bacterial type III-L, from *Escherichia coli* ($AP_{E.c.}$), were used in experiments given in Section 3.3. The $AP_{E.c.}$ was taken as a structural model of the catalytic AP protein lacking the GPI anchor [25] and as a sample missing the surface domains present in mammalian APs [14,15,26]. The analysis of the used AP samples was given previously [10]. Cytochrome *c* (horse heart), C 7752 (cyt *c*), and the chemicals used in this work were of the same provenance. Solutions of the AP samples were prepared freshly by solubilising 2 mg of enzyme solid in 1 ml of 10 mM phosphate buffer (pH 7.2) and left 30 min at 22°C for equilibration before use. They were tested on the presence of free thiol groups by addition of 5 mM *N*-ethylmaleimide and 1 mM mersalyl sodium and on the presence of residual sialic acid by treatment with neuraminidase. The effect of these reagents on the reaction with cyt *c* was insignificant. Measurements of phosphomonoesterase activity using *p*-nitrophenyl phosphate as a substrate were performed as described [9]. The enzymatic activity of the APs was not altered by the addition of cyt *c*.

2.2. Optical spectroscopic techniques

Absorption spectra in the visible and the UV regions were obtained using Ultrospec 2000 and Shimadzu UV 3000 spectrophotometers. The changes in the AP+cyt *c* mixtures were estimated by measuring in an absolute or in a difference mode as stated in the figure captions. Unless otherwise stated the reaction was started by the addition of 100 μg AP to the 75 μg cyt *c*(FeIII) in 1 ml volume of a buffer in a sample cuvette (standard conditions) and measured at 550 nm. The buffers used in 100 mM concentration controlled the pH within ± 0.1 pH units. The reaction mixtures containing 10 mM buffer concentrations resulted in time-dependent pH changes of ≤ 0.3 pH. The pH of the mixtures was measured by a Sensorex glass electrode. The addition of 0.5 mM magnesium and 20 μM zinc ions, which protect the native state of the enzyme [26], did not affect the reaction. The correction for the slight increase (0.024–0.025) of absorption due to the AP addition was made by subtraction. The standard error of the mean and the standard deviation were estimated for the whole series of experiments as shown in Fig. 1. The evaluation was made by means of the Microsoft Excel 7.0 program.

Fluorescence measurements were performed on a Shimadzu RF 5000 spectrofluorometer equipped with a temperature controlled cuvette holder in the range from 20°C

to 80°C. The heating rate was 1°C/min. The excitation wavelength of the Trp residue was at 290 nm and the fluorescence emission was followed at 350 nm.

Circular dichroism spectra were recorded on a Jasco model spectropolarimeter using 1 cm cells at 22 ± 1°C as described in [9]. The smoothed curves presented in the figures are the average of five scans collected at 20 nm/min.

2.3. FPLC analysis

FPLC was performed on a LCC-500 model, Pharmacia, with a Mono S column equilibrated with 50 mM phosphate buffer, pH 7.0 (solvent A). Solvent B was 1 M NaCl in solvent A. The elution profile of cyt *c*(FeIII), cyt *c*(FeII) and other protein components was followed by absorption measurements at 405 nm and 280 nm.

2.4. DSC measurements

Differential scanning calorimetry was performed with a DASM-4 microcalorimeter. Prior to use and during the experiment 1.3 mg of cyt *c* (110 μM) was kept in a fully oxidised state by addition of potassium ferricyanide. Control samples of alkaline phosphatase (1.5–9 mg) showed no thermal transitions up to more than 90°C. The heating curves were corrected using an instrument baseline by heating the buffer [27].

3. Results

3.1. Reduction of cyt c in the presence of AP is a multi-step process

We have already shown that the addition of cyt *c*(FeIII) to the solution of AP results in a profound change in the spectral characteristics of the haem which could be attributed to the formation of cyt *c*(FeII) and the creation of a

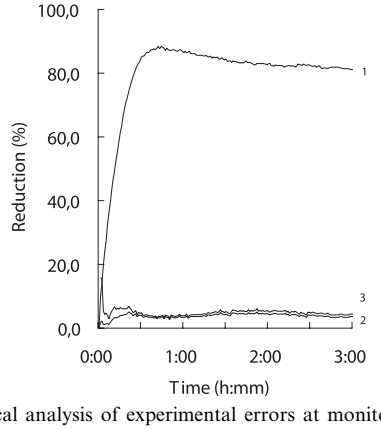

Fig. 1. Statistical analysis of experimental errors at monitoring cyt *c* absorption in a mixture with alkaline phosphatase. Standard conditions in 100 mM glycine/NaOH, pH 9.7. 1, the mean; 2, standard deviation; 3, standard error of the mean.

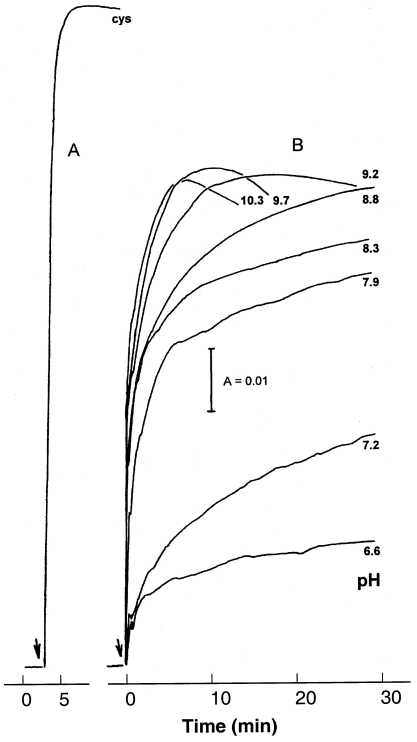

Fig. 2. pH dependence of cytochrome *c* reduction in the presence of alkaline phosphatase. (A) Reduction of 0.075 mg cyt *c*(FeIII) in 10 mM pyrophosphate buffer of pH 8.8 at the addition (arrow) of 1 mM cysteine–HCl. (B) Cyt *c*(FeIII) addition to 0.1 mg alkaline phosphatase preincubated for 10 min at 25°C in 1 ml of 10 mM phosphate (pH 6.6–7.8), pyrophosphate (pH 8.3–9.2), and glycine/NaOH (pH 9.7–10.3). The absorption at 550 nm was measured as the difference versus cyt *c* in the corresponding buffers.

macromolecular species composed of the two proteins. The rate of the cyt *c*(FeIII) → cyt *c*(FeII) transition and the proportion of the individual species were dependent on the AP/cyt *c* ratio, pH and the ionic strength of the medium [9]. In Fig. 2A,B we show the kinetics of the cyt *c*(FeII) formation in dependence on the pH value of the AP/cyt *c* mixture, and compared with the addition of cysteine as the reducing agent. An immediate increase in the 550 nm absorption indicating the cyt *c*(FeIII) → cyt *c*(FeII) transition was observed at the neutral and alkaline pH region. The maximum value of the cyt *c*(FeII) formed in 10 mM buffers increased from 37% at pH 6.6 to 68–74% at the apparent optimum between pH 9.7 and 9.9. An apparent steady state was obtained after 3–4 h at neutral pH and after 10–15 min at the pH optimum. The rate of the cyt *c*(FeIII) transition as well as the maximum value of the cyt *c*(FeII) were lowered at higher concentration of phosphate and pyrophosphate buffers. The 50 mM and 100 mM phosphate at pH 7.2 slowed down the reduction to 72% and 51% in comparison to the value found with the buffer of 10 mM concentration (100%); a decrease to 80% and 71% was found for pyrophosphate buffer at pH 8.8 under the same conditions. On the contrary, a higher concentration of glycine/NaOH buffer accelerated the cyt *c*(FeII) formation. The values found in the interval of 10

min after mixing with 10 mM and 50 mM glycine/NaOH of pH 9.7 were 64% and 86% of those found with 100 mM glycine medium. Low concentrations of KCl (up to 100 mM) slightly accelerated the reaction, higher concentrations were inhibitory. The addition of ascorbate to the reaction mixture caused almost complete reduction of cyt *c*, indicating that the alkaline forms of the cyt *c*(FeIII) were present only in insignificant amounts. The slow decrease in the 550 nm absorption observed after reaching the steady state (cf. Fig. 2B) continued also after repeated pulses of ferricyanide and was sensitive to ionic strength and to the presence of a metal complexing agent EDTA (not shown). The results also indicate that the reverse reaction leading to cyt *c*(FeIII) does not take place and hint at the transition of the cyt *c*(FeII) to the high spin form [9]. To minimise these additional changes most of the experiments were performed below the pH optimum in the region of pH 8.3–8.8.

A concomitant reaction proceeding upon mixing cyt *c* and AP was a creation of macromolecular species composed of both proteins. This was proven by measuring the protein and haem *c* absorption in high molecular fraction eluted from the Mono Q column. Protein complexes in the mixtures were detected over the whole pH range used (pH 2.6–8.8) and their content was dependent on temperature [10]. The portion of cyt *c* built into the complexes had a similar pH dependence as was found for the cyt *c*(FeII) formation. The lowest amount ($3.4 \pm 3\%$) of cyt *c* in the protein complex measured after an interval of 30 min was detected at pH 2.6. The shift to higher pH values favoured complex formation: 7.2% and 9.7% of cyt *c* were detected in macromolecular complexes at neutral pH and pH 8.3, respectively [10]. These values further increased on longer intervals of incubation and were paralleled by the increments of cyt *c*(FeII) in the mixtures (see Fig. 3A). The amount of macromolecular species detected at the acidic pH did not change with time. The addition of 0.5 M KCl to the mixture lowered but did not prevent the formation of protein complexes (see Fig. 3B). The main effect of KCl was found in the course of longer incubation periods as it prevented the increase in macromolecular species and in cyt *c*(FeII). The results indicate the existence of a reaction process consisting of several steps which may have a different sensitivity to ionic strength.

3.2. The compactness of the haem c cleft is affected by interaction with alkaline phosphatase

The circular dichroism spectrum of cyt *c*(FeIII) exhibits a characteristic doublet in the Soret region with a band of positive ellipticity at ~400 nm and a negative peak at 417 nm. The doubly peaked spectrum is replaced by a broad single positive peak at about 425 nm after the cyt *c*(FeIII) → cyt *c*(FeII) transition (cf. Fig. 4A). As shown previously the interaction of AP and cyt *c*(FeIII) at neutral pH was characterised by a profound change in ellipticities of the Soret Cotton effect [9]. Intensities in ellipticities were affected by the AP/cyt *c* ratio; the largest change was found at the ratio of ~5:1. In that case the intensity of the positive peak increased several times with a simultaneous shifting to 395 nm, and the intensity of the negative ellipticity at 417 nm was weakened. The weakening of the 417 nm negative ellipticity in the AP/cyt *c* mixture (pH 7.0) after 30 min was linked to a profound change in ellipticities at 245 nm and 270 nm (see Fig. 4B), which is in the region of the tyrosine Cotton effects [28]. The 0.5 M KCl added before the AP addition diminished the spectral changes but had only little effect when added after the incubation period (Fig. 4A). The rise in pH caused a complete elimination of the negative ellipticity and a more distinct appearance of the positive 425 nm band attributable to cyt *c*(FeII). The gradual appearance of the 425 nm band was accompanied by a concurrent decrease in the positive ellipticity at 395 nm. This indicates that the

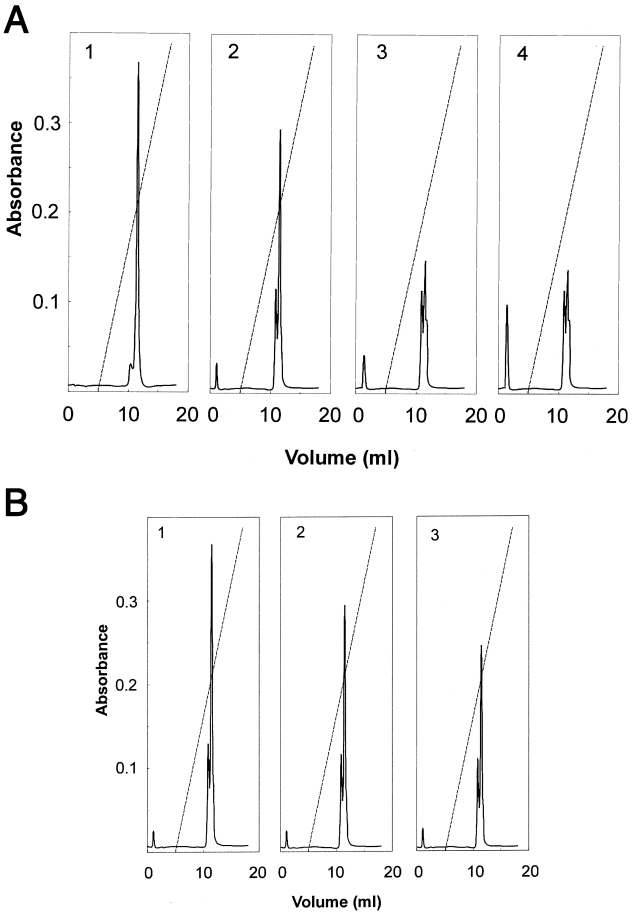

Fig. 3. Elution profiles of cytochrome *c* in a mixture with alkaline phosphatase. Absorbance at 405 nm was plotted versus the elution volume. The conditions of separations were: 0–5 min solvent A, 5–25 min 0–100% solvent B. The linear gradient of solvent B is overlaid. (A) The samples (0.2 ml) contained 0.15 mg cyt *c*, (1) with the addition of 0.1 mg AP, (2–4) in 10 mM pyrophosphate/HCl buffer, pH 8.3. The separation started after an incubation period of 30 min (2), 3 h (3), or 20 h (4), at 22°C. (B) The mixture of cyt *c* with alkaline phosphatase of pH 8.3 (see A) supplemented with 0.5 M potassium chloride was incubated for 30 min (1), 3 h (2), 20 h (3).

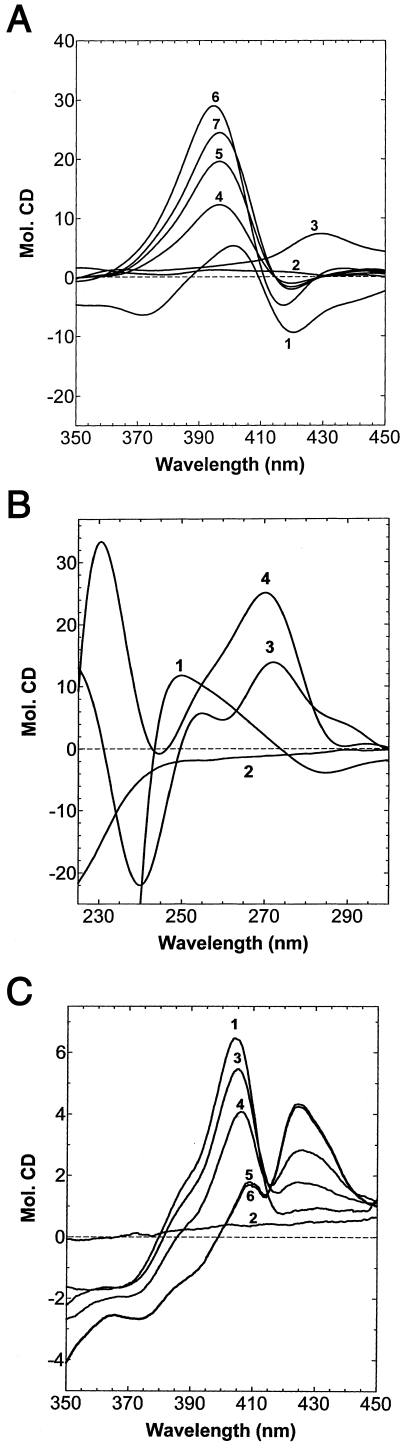

Fig. 4. Circular dichroism spectra of cytochrome *c* and mixtures with alkaline phosphatase in 10 mM phosphate, pH 7.2. (A) Soret region. 0.13 mg cyt *c*(FeIII) (1); and cyt *c*(FeII) (3); 1.15 mg alkaline phosphatase (2); and mixtures of 1 and 2 in 10 mM phosphate incubated at 22°C for 30 min (4), 3 h (5), 20 h (6), and 20 h and then 0.6 M KCl added (7). (B) UV region. Cyt *c*(FeIII) (1); alkaline phosphatase (2); mixtures of 1 and 2 after 30 min (3), and after 3 h (4). (C) CD spectra of cytochrome *c* and alkaline phosphatase in 100 mM glycine/NaOH of pH 9.7. Cyt *c*(FeIII) (1); alkaline phosphatase (2); mixtures of 1 and 2 measured after mixing: ~1 min (3); 5 min (4); 30 min (5); 20 h (6).

form responsible for the high 400 nm → 395 nm Soret peak precedes the cyt *c*(FeII) formation and may be a conformational precursor in the cyt *c*(FeIII) → cyt *c*(FeII) transition. This suggestion was strengthened by an experiment performed in 100 mM glycine buffer, pH 9.7, i.e. under optimal conditions for cyt *c*(FeII) formation (Fig. 4C). In this case the maximum intensity of the 400 nm was slightly red shifted (406 nm) and was rapidly decreasing with the concomitant increase of the cyt *c*(FeII) band. The addition of 0.5 M KCl to the glycine reaction mixture affected only slightly the rate of the reaction. The proportion among various forms of cyt *c* in the presence of KCl did not change. Two conclusions in favour of the suggestion made above could be drawn from the CD experiments: (i) the transition of cyt *c*(FeIII) to cyt *c*(FeII) does not go to completion and exerts a marked steady state between cyt *c*(FeII) and yet unidentified cyt *c* conformers, and (ii) cyt *c*(FeII) formed in the mixture is not subjected to a reverse oxidising reaction.

3.3. *Aromatic amino acids participate in the structural changes of cyt c*

It is commonly accepted that the change of the induced Soret CD of cyt *c*(FeIII) arises from variations in the symmetry of the haem environment and reflects loosening of the haem crevice [28,29]. The change in the negative ellipticity at 417 nm was assigned to the distance and orientation of Phe-82, which is positioned on the methionine side of the haem plane [30,31]. The disturbance of coupling between the haem group and aromatic residues in its proximity was proven by ultraviolet absorption spectra and fluorescence measurements. Fig. 5A shows the difference spectra of AP+cyt *c* versus AP which can be ascribed to alteration of the cyt *c* structure. It is evident that at lower temperatures at which the coaggregation of the two proteins was suppressed [10] a marked change in absorption of aromatic amino residues took place. The 282 nm maximum of the Trp-59 was shifted to shorter wavelengths, indicating involvement of tyrosine residues and exhibiting the vibrational fine structure of phenylalanine residues (see Fig. 5A, insert). The shift in the Trp maximum was accompanied by an increase in the 320–330 nm absorption that is in the region of the transient tryptophan species [32]. These radicals are strong reducing agents and could transfer electrons readily along the cyt *c* molecule [33]; however, they should be immediately quenched by oxygen [34]. We generated the Trp excited species in the anaerobic AP+cyt *c* mixtures, but the spectral profiles obtained were not in favour of this idea. The experiments given in Fig. 5B performed with cyt *c* in the absence of AP showed that the changes in the two maxima did not proceed concomitantly. Longer incubation periods of cyt *c* in alkaline media brought about only a minor change in the 282 nm maximum. The absorption at 320 nm increased depending on the incubation conditions and

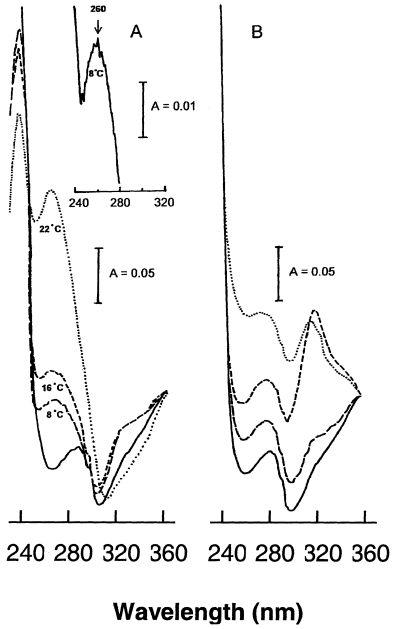

Fig. 5. Changes in the UV absorption of cytochrome *c* with alkaline phosphatase. (A) Samples of cyt *c* (———) and mixtures with AP (see legend to Fig. 1) were kept in 10 mM pyrophosphate buffer of pH 8.8 for 20 h at 8°C (— — —) and insert, 16°C (- - -), or 22°C (···), and measured as the difference versus alkaline phosphatase. (B) Spectra of cyt *c* in buffer: freshly dissolved (———), with few grains of potassium borohydride (- - -), after incubation for 24 h at 22°C aerobically (— — —), or anaerobically (···). For obtaining anaerobic conditions, see [33].

was most pronounced under exclusion of oxygen. A similar absorption profile was obtained after the addition of a few grains of potassium borohydride to a fresh solution of cyt *c*(FeIII). It was assumed that the appearance of the 320 nm absorption might belong to the δ peak of the cyt *c*(FeII). Experiments comparing the intensities of the δ cyt *c*(FeII) and the α cyt *c*(FeII) (550 nm) maxima (not shown) confirmed that the absorption changes at 320 nm were mainly due to transition of cyt *c*(FeIII) to cyt *c*(FeII) forms.

The measurement of the Trp-59 fluorescence is a good probe for investigating deformations in its environment. In a native state of cyt *c* the Trp-59 does not show any luminescent signals because of the adjacent haem, which is exceedingly efficient at depopulating excited electronic states. An increase in the Trp distance from the haem brings about an increase in the intensity of fluorescence emission and this can be used to detect structural changes occurring around the haem. Fig. 6 shows the change in the intensity of fluorescence emission of AP (trace 1) and mixtures with cyt *c* (traces 2 and 3) dependent on temperature. It may be seen that in the range of 20°C up to 80°C the fluorescence emission of AP was continuously decreasing. Cyt *c* alone did not show an increase in fluorescence emission although it had at least one conformational transition in the temperature range used [29]. The additions of cyt *c* to the AP solutions produced a marked decrease in fluorescence intensities of AP (see onsets of traces 2 and 3) but at 55°C up to 65°C the fluorescence intensity increased. This is the temperature range at which the first step in the denaturation process of cyt *c*, i.e. the opening up of the haem crevice, occurs [28,29]. Traces 4 and 6 show control experiments performed with free Trp which exclude the possible filtering effect of cyt *c*. Trace 5 shows that the increase in the fluorescence intensity was sensitive to ionic strength. The results given in Fig. 6 bring evidence that the deepest part of the haem crevice is also affected at the interaction and indicate an apparent increase in the distance of the Trp-59 from the haem.

3.4. Non-coulombic binding forces are involved in AP+cyt c interaction

It is known that the interaction of cyt *c* with natural as well as artificial reaction partners destabilises the molecule in terms of denaturation [35,36]. The temperature of cyt *c*(FeIII) denaturation in our experiments measured by differential scanning calorimetry was at ∼84°C in all the used media. The sample of AP had no thermal transition up to 95°C; the sample of AP_{II} showed aggregation at ∼80°C and was used only in control experiments. In Fig. 7A we show thermograms of cyt *c* in the presence of increasing concentrations of AP. It may be seen that the addition of AP at a low concentration, i.e. at a 1:2 ratio of AP:cyt *c* (w/w), resulted in an almost complete disappearance of the T_m peak of cyt *c*. Further additions of AP resulted in the creation of new peaks of thermode-

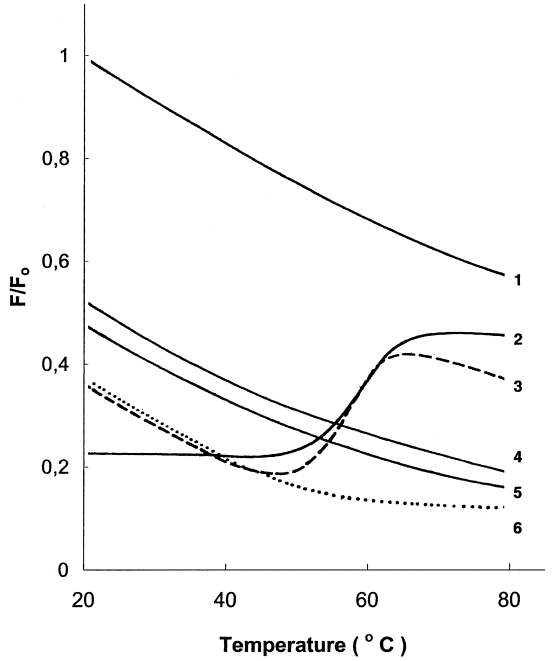

Fig. 6. Temperature dependence of fluorescence of cyt *c* in mixtures with alkaline phosphatase and free tryptophan. Samples of 0.34 mg AP in 2.5 ml of 10 mM phosphate, pH 7.2 and 5 mM potassium ferricyanide (1,2,3,5) were mixed with 0.53 mg cyt *c* (2) or 0.26 mg cyt *c* (3,5) and 0.5 M KCl (5). In samples containing 1.6 μM tryptophan (4,6) the cyt *c* was present in the two concentrations.

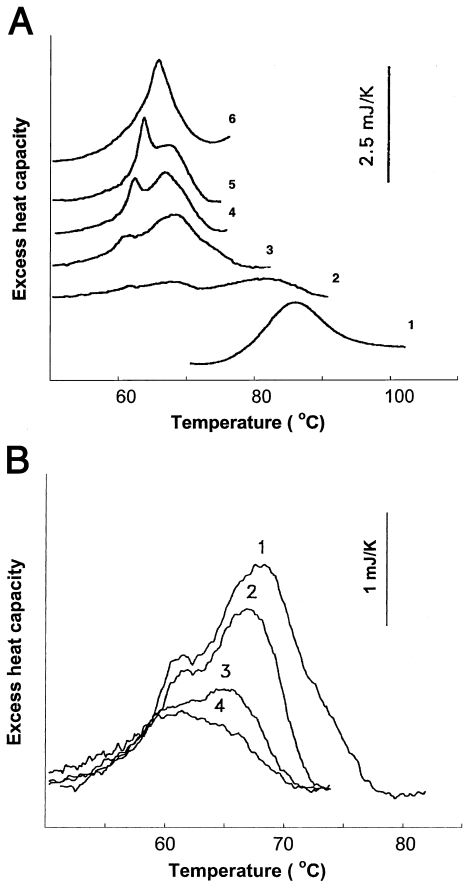

Fig. 7. Differential scanning calorimetry of cytochrome *c* on incremental additions of alkaline phosphatase. (A) DSC scans (1 K/min) in 10 mM phosphate, pH 7.2, of 1.5 mg cyt *c* and 110 μM potassium ferricyanide (1), supplemented with: 0.6 mg AP (2); 1.5 mg AP (3); 3 mg AP (4); 7.5 mg AP (5); 9 mg AP (6). (B) DSC scans of a 1:1 mixture AP/cyt *c* in 10 mM glycine of pH 7.0 (1); 7.4 (2); 7.9 (3); 8.3 (4).

naturation of $T_m = 68°C$ and $T_m = 61°C$. The area of enthalpy at 68°C prevailed at the 1:1 AP/cyt *c* ratio, and the rise in AP concentrations to 2:1 and 4:1 caused a gradual increase in the enthalpy of the transition at 61°C. Subsequent increment in AP led to integration of the two peaks and only one transition at 66°C was observed. The results indicate the creation of new species of cyt *c* in the AP+cyt *c* mixtures. Fig. 7B shows the effect of pH on the two T_m peaks. The increase in pH preferentially affected the structure responsible for the transition at 68°C. The enthalpy belonging to the peak was significantly lowered while the enthalpy at 61°C was much less affected. One broad area with a maximum between 60°C and 65°C was found at pH 8.3. In a previous paper [10] we have shown that the increase in ionic strength of the media affected the two peaks in an opposite way: the area of 68°C was enlarged and sharpened with a concomitant shift to the temperature of 76.3°C, the peak of 61°C was lost. We are led to conclude that the new T_m values found in the AP+cyt *c* mixtures might represent structures (complexes) of cyt *c* which were created by different types, i.e. coulombic and non-coulombic, of binding forces. The non-coulombic binding seems to prevail at a substoichiometric AP/cyt *c* ratio, upon its elevation the coulombic attraction increases, and at the ~6:1 (an equimolar one subunit AP/cyt *c*) ratio an apparent complexity of the binding forces may be observed (cf. Fig. 7A). The basis of the complexity might be sought in the composition of the alkylacylphosphoinositol moiety, which besides its hydrogen bonding ability has a capability of electrostatic bonding due to the presence of phosphate groups.

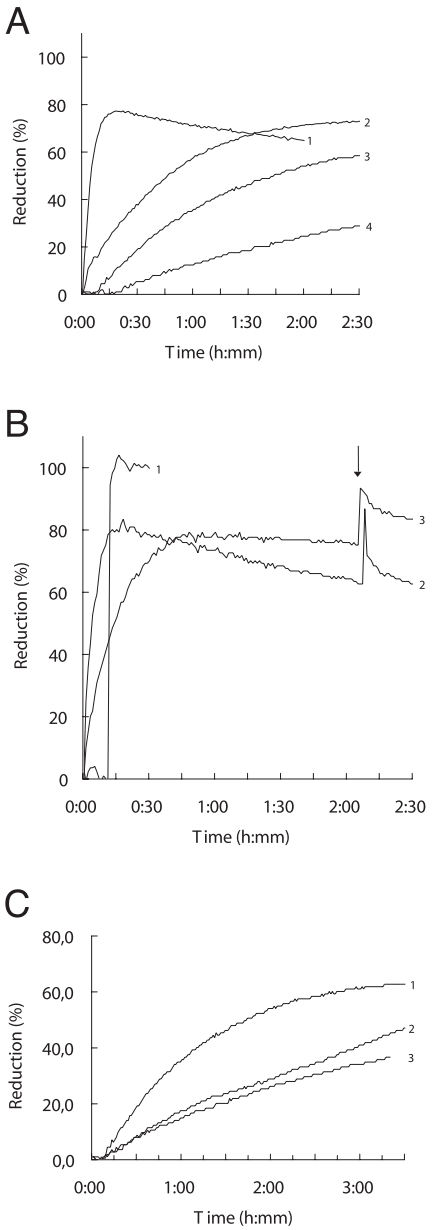

Fig. 8. Reduction of cyt *c* in the presence of different samples of alkaline phosphatase. Standard conditions in 100 mM glycine/NaOH medium, pH 9.7. AP equals AP_I. (A) AP_I (1); AP_{II} (2); $AP_{E.c.}$ (3). For the definition of standard conditions and AP samples see Section 2. (B,C) Effect of ionic strength on the reduction of cyt *c* in a mixture with alkaline phosphatase. (B) AP_I. Ascorbate addition to cyt *c* and 0.5 M KCl (1); cyt *c* and AP_I (2); cyt *c* and AP_I supplemented with 0.5 M KCl (3). Arrow indicates ascorbate addition in 2 and 3. (C) Cyt *c* and $AP_{E.c.}$ (1); cyt *c* and $AP_{E.c.}$ supplemented with 0.5 M KCl (2); cyt *c* (3).

In order to address this issue directly we made use of the preparations of commercial APs differing in the proportion of non-catalytic (glycolipid) to catalytic (protein) part. As stated in Section 2, the intestinal AP sample designated in this chapter as AP_I had a preserved GPI anchor. AP_{II} was a preparation of the same origin at which the proportion of the catalytic protein to dry weight was approximately doubled [10]. The bacterial $AP_{E.c.}$ represented a sample lacking the GPI anchor and missing the surface loops characteristic of the mammalian APs [14,15,26]. A marked difference was observed in the pH effect on the cyt *c*(FeII) formation with the three preparations. $AP_{E.c.}$ showed no stimulating effect on the reduction in neutral and slightly alkaline media up to a pH about 8.0. In these media the AP_{II} sample was significantly less active than the AP_I (not shown). The kinetics of cyt *c*(FeII) formation at the optimal pH with the three AP samples are given in Fig. 8. It is evident (see Fig. 8A) that the rapid phase of cyt *c*(FeIII) → cyt *c*(FeII) transition was clearly expressed only with the AP_I whereas it was completely absent with the $AP_{E.c.}$. The AP_{II} showed a trace which could be designated as a kinetics of an intermediate type. The preparations showed a marked difference in response to ionic strength (see Fig. 8B,C). As follows from Fig. 8B the effect of 0.5 M KCl on the reaction with AP_I was relatively small, the rapid phase of the reaction was still obvious and the amount of cyt *c*(FeII) found in the steady state decreased only slightly (5–8%). The addition of KCl was more effective at AP_{II}; in this case the cyt *c*(FeII) formation was lowered by 13–20% (not shown). The most pronounced effect was found with $AP_{E.c.}$ at which the suppression of cyt *c*(FeII) formation owing to the KCl presence was almost complete (see Fig. 8C). In order to confirm the existence of non-coulombic bonding at AP_I the experiments were repeated in the presence of polyanion heparin which is one of the strongest polyanions that occur naturally. It was already shown that heparin forms complexes with cyt *c* by electrostatic bonding but the structure of cyt *c* remains native-like [27]. It could be presumed that it would effectively compete with electrostatic attraction of the negative charges on the surface of AP_I. The results obtained in the presence of 1:2 up to 2:1 heparin/AP w/w ratio showed a similar profile to that found with KCl. The maximal effect of the heparin additions was a slight lowering of the cyt *c*(FeII) formation by 12% and 19%, respectively. The conclusion which can be drawn from the results is that the rapid phase of the cyt *c*(FeII) formation largely depends on non-coulombic (hydrophobic, hydrogen bonding) interaction. The protein portion evidently contributes through its negative charges on the molecule surface which, however, in the case of $AP_{E.c.}$ need some critical density for the interaction. The difference in the reaction kinetics between the bacterial and the AP_{II} preparations can be tentatively ascribed to the binding ability of additional surface domains found in mammalian APs [14,15,26].

4. Discussion

It has been thought that the structural changes in cyt *c* bound to charged surfaces of its natural as well as artificial reaction partners are induced by electrostatic attraction and that other types of interaction, e.g. hydrophobic, van der Waals, etc., which may arise from the specific architecture of the binding site are of minor importance [37]. We have shown previously that cyt *c*(FeIII) interacts distinctly with samples of a mammalian type of alkaline phosphatase differing in the proportion of the negatively charged catalytic protein to the non-catalytic membrane-bound GPI portion [10]. Among products that appeared in the early stage of the interaction was cyt *c*(FeII). We found that in the neutral pH region more than one third of the cyt *c* present in the mixture was converted to the reduced state. The rate of conversion and the portion of cyt *c*(FeII) increased with increasing the AP/cyt *c* ratio and with increasing the pH of the reaction mixture (cf. Fig. 2 and [9]). In this work an attempt has been made to analyse the events that bring about the cyt *c*(FeIII) → cyt *c*(FeII) transition. We show that this transition proceeds with a complex reaction kinetics which involves formation of macromolecular AP+cyt *c* species and is linked to marked changes in the haem *c* crevice. The optimum of the reduction at which 68–74% of the cyt *c* has been reduced is at pH 9.7–9.9. This is, however, the pH region where cyt *c*(FeIII) should adopt the alkaline IVa and IVb conformations not reducible by ascorbate (pK_a values of 8.7–8.9 and 9.1 were reported for the state IVa and IVb transitions of the yeast iso-1-ferricytochrome *c* and horse heart protein, respectively [38,39]). In contrast, ascorbate addition in our experiments altered the obtained cyt *c*(FeII)/cyt *c*(FeIII) ratio to the almost complete ($>95\%$) appearance of cyt *c*(FeII). The spectrum of the cyt *c*(FeII) in the AP mixture could be converted back to that of oxidised cyt *c* by repeated ferricyanide pulses (not shown) and in both cases the spectra returned spontaneously to the original steady state by the same kinetics (cf. Fig. 8B). The conclusion which can be drawn from the results is that the AP+cyt *c* interaction stimulates generation of reducing equivalents in the cyt *c*(FeIII) molecule that rapidly reduce either its own haem iron or an external electron acceptor like ferricyanide.

It has been suggested that the cyt *c* conformers formed upon its binding to natural (e.g. cytochrome oxidase) and artificial polyanion agents are structurally similar to the alkaline conformational states [40,41]. H^1 NMR spectra of cyt *c* variants have shown that in the IVa and IVb states Lys-72/73 and Lys-79 replace Met-80 as axial ligands to the haem iron [39,41]. On the other hand these lysine residues have been implicated as participants in electrostatic interaction with negatively charged binding domains of the reaction partners. A distinct conformational state of membrane-bound cyt *c* was detected by means of the resonance Raman spectroscopy and denoted as B2 [39]. In

the B2 state these lysines are not available for coordination to the haem iron and the formation of the IVa and IVb states is inhibited. We are inclined to suggest that in the AP-bound form the cyt *c* exhibits the B2 or a closely related conformation and remains reducible.

The creation of macromolecular complex(es) between the two proteins and the involvement of coulombic as well as non-coulombic binding forces was confirmed by means of FPLC analysis and DSC measurements. The presence of 0.5–0.6 M potassium chloride did not prevent the formation of the complex(es) and did not abolish the appearance of cyt *c*(FeII) in the AP+cyt *c* mixtures. The DSC experiments performed in the high ionic strength media did not show the reversal to the original T_m value of the free cyt *c*(FeIII) [10], as was shown in the case of electrostatically formed cyt *c*–polyanion complexes [27]. This indicates that in the AP-bound forms of cyt *c*(FeIII) non-coulombic binding forces coexist.

Multiple ionic strength dependent conformations of membrane-bound cyt *c* have been recently suggested in spectroscopic CD measurements [42]. In our experiments the CD analysis of the AP+cyt *c* mixtures was limited by the fact that the proteins have in principal similar basic patterns of CD spectra for each type of secondary structure. In order to separate the contribution of transient forms of cyt *c* as they convert over time, measurements of the electronic absorption spectra and of fluorescence emission were undertaken. The results revealed an altered structure of the haem crevice, indicating changes involving residues of aromatic amino acids. The integrity of Phe-82 was found responsible for the 417 nm negative component of the Soret band in the CD spectra [30,31]. In our experiments an immediate drop in the negative ellipticity upon the AP addition was observed. The profound increase in ellipticities observed in the UV region most probably originated from the aromatic Tyr-74 and Tyr-67 which have been implicated in holding the native state of haem crevice [43,44]. From the two tyrosine residues the Tyr-67 seems to be essential in the structural change which preceded the transition of cyt *c*(FeIII) to cyt *c*(FeII), since its hydrogen bonding network affects the electron withdrawing power of the Met-80 ligand and is available to bind to internal water (Wat 166) adjacent to the haem [44,45]. The Tyr-67 or hydroxide ion, provided by this water molecule, has been recently suggested to form obligatory transient species at cyt *c* deprotonation by binding to the haem iron [46]. The Trp-59 constitutes a buried part of the hydrophobic patch of aromatic amino acids forming a channel from the haem to the surface [47]. The increase of the Trp-59 fluorescence observed in the presence of AP and the inhibiting effect of ionic strength on it seem to be in accordance with the model of two different binding sites participating in opening the channel [48,49]. The model will also encompass the inhibiting effect of phosphate and pyrophosphate buffers observed in our experiments through interaction with the positively charged lysine-rich domain around the haem crevice. The opening of the channel might be the basis for a more dynamic behaviour of the haem crevice in the presence of AP, leading to a significant higher flexibility of the haem group. However, the question what precisely triggers these conformational changes and how they serve to stabilise the steady state among the cyt *c*(FeII) and the bound cyt *c* conformers is beyond the scope of our results.

The experiments with the three samples of AP clearly showed that the initial rapid phase in cyt *c*(FeII) formation was dependent on the presence of the non-catalytic membrane anchored portion of the APs which is missing in the bacterial $AP_{E.c.}$. Compared with $AP_{E.c.}$ the mammalian APs preserve a relatively well conserved catalytic site [25] but possess some extra domains on the molecule surface which confer additional binding abilities upon the protein [14,26]. These novel folds may be involved in the slower phase of cyt *c*(FeII) formation as observed with the AP_{II} sample. Besides an interfacial crown domain which contains a collagen binding loop [26], a new calcium binding domain was recently identified on the surface of placental AP isozyme [14]. Several severe mutations occur around this calcium site among AP isozymes [15], suggesting its role in a tissue specific function. Evidence already exists that calcium is involved in the mitogenic effect of placental AP and stimulates DNA synthesis, but neither intestinal nor tissue non-specific APs exert a similar effect [12]. In our experiments neither the metal complexing agents (5.5 mM EDTA, 1 mM 1,10-phenanthroline) nor the steric inhibitors (5 mM L-phenylalanine) had visible influence on the followed interaction. EDTA, however, slowed down the additional reaction leading to disappearance of the 550 nm absorption [9]. Preliminary results obtained with $CaCl_2$ titration of the AP+cyt *c* mixtures could be attributed to the ionic strength effect.

Finally, it is interesting to note that upon AP addition the amounts of cyt *c*(FeII), the pH dependent equilibrium between the two redox states, and the optimal pH value for the cyt *c*(FeII) formation are almost identical with values found at the autoreduction of horse heart cyt *c*(FeIII). However, that reaction measured in glycine/NaOH buffer proceeded at a very slow rate and many hours were needed to obtain steady state values [50]. This apparently inherent ability of cyt *c* to adopt a steady state of a higher cyt *c*(FeII)/cyt *c*(FeIII) ratio might have its basis in the increased stability of the cyt *c*(FeII) to changes in temperature, pH, chaotropes, etc. The well-known resistance of cyt *c*(FeII) to the action of trypsin, subtilisin and other proteases may be relevant to its regulatory function in apoptosis at caspase activation. Moreover, a more effective association of cyt *c*(FeII) with membranes [51], long chain fatty acids [52], and phospholipid liposomes [53] indicates a modulation of the cyt *c* effect by the haem iron oxidation state. The interconversion between the two redox states of cyt *c* might be regarded as an effective binary switch that has a profound effect on the

stability and the binding ability of the cyt *c* protein. In this respect our findings point to the importance of the non-catalytic part of the AP enzyme, which can be found in many membrane anchored proteins. It seems worth noticing that the GPI anchor of the intestinal AP isozyme was found extremely heterogenous with respect to its hydrophobic as well as hydrophilic components [21]. The variance of the non-catalytic portion should obviously result in a variety of similar but not identical binding reactions. In contrast to the free cyt *c* only the lipid-bound protein was affected by ATP [54]. This finding supports the suggestion that the functional apoptotic cyt *c* may act in a membrane-bound form [55]. The interaction between the two proteins described in the present paper provides a further insight into the surface binding-induced perturbation between cyt *c* and cellular proteins comprising covalently connected polar and apolar regions.

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