

Evidence of Complex Formation between Alkaline Phosphatase and a Pro-apoptotic Hemoprotein Cytochrome *c*

V. DADÁK, P. JANDA AND O. JANICZEK

*Department of Biochemistry, Faculty of Science, Masaryk University,
Brno, Czech Republic*

Abstract. Two proteins, alkaline phosphatase (AP) and cytochrome *c* (cyt *c*) which seem to be involved in the apoptotic cell death program were examined on their interaction. Intestinal AP affects ferricytochrome *c* (cyt *c*(FeIII)) by changing its optical properties, redox state and conformation. The effect proceeded over the course of hours with a gradual decrease in free cyt *c*(FeIII) as the AP concentration increased. A heme containing high molecular species was created in the first stage of interaction of the proteins in neutral, acidic (pH 2.6), alkaline (pH 8.3), low ionic strength (10 mmol/l phosphate), and high ionic strength (0.5 mol/l NaCl) media. Further complexation was favored by higher pH values and temperature. Differential scanning calorimetry revealed a decrease in enthalpy of the thermodenaturation temperature (T_m) of cyt *c* at 84.5°C due to the AP addition. Increments of AP in the mixtures resulted in the appearance of T_m peaks at 68°C and 61°C. Electrophoretic analysis of the commercial samples of intestinal APs showed main fractions from 63.2 kDa to 72.9 kDa and from 172.9 up to 179.0 kDa. Changes in positions and intensities of the bands were detected upon longer incubation (24 h) with cyt *c*. The electrophoretic pattern of the bacterial AP was homogeneous with one fraction of 43.7 kDa showing no alteration due to the cyt *c* presence. Gel permeation chromatography of incubated mixtures of intestinal APs and cyt *c* confirmed the creation of new heme containing complexes.

Key words: Alkaline phosphatase intestinal — Alkaline phosphatase bacterial — Aggregation — Cytochrome *c* complexation — Apoptosis

Abbreviations: AP, alkaline phosphatase; cyt *c*(FeIII) and cyt *c*(FeII), ferri and ferro cytochrome *c*.

Introduction

The enzyme termed alkaline phosphatase (AP) [orthophosphoric-monoester-phosphohydrolase, E.C. 3.1.3.1] hydrolyzes a variety of monophosphate esters at high pH optima. Its natural substrate and exact function in metabolism have not yet been elucidated. APs are widely distributed in organisms ranging from bacteria to man (cf. McComb et al. 1979; Coleman and Gettins 1983; Harris 1989 for a review). The bacterial APs are water soluble metalloproteins without glycosylation and fatty acid attachment and are consequently characterized the best (Sowadski et al. 1985; Sone et al. 1997; Sun et al. 1999). Homodimer of *E. coli* AP with a molecular mass of 94 kDa and 449 residues per monomer is mainly considered a model for APs since the amino acid sequences of the catalytic part are highly conserved in evolution and the kinetic properties are similar to all variants of the enzyme (Kim and Wyckoff 1989, 1991). In mammals four structural genes encoding placental, intestinal, germ-cell and tissue non-specific (liver, bone, kidney) isoenzymes have been detected, sequenced and mapped to chromosomes (see Millán 1986; Henthorn et al. 1987; Martin et al. 1989; Moss 1992; Weissig et al. 1993). They are expressed as highly glycosylated homodimers with subunit molecular masses ranging from 60 to 80 kDa. The dimeric APs contain two to four ions of zinc and one to two ions of magnesium (Sowadski et al. 1985). The mammalian isoenzymes retain the core of the three-dimensional structure established for AP of *E. coli* with modification (mainly insertions) on the surface loop regions of the enzyme which considerably expand the molecule. The enzyme may serve as a phosphate/calcium-binding protein (Hui et al. 1997). In addition, it is known that it also possesses a collagen binding domain on its surface (Harris 1989). The variability in the surface region among isoenzymes, homologies between APs and proteins known to mediate binding and the presence of regions promoting cell adhesion indicate an important role of the AP in the physiology of the cell life and aging (Deng et al. 1992; Bublitz et al. 1993; Armesto et al. 1996; Hui et al. 1997; Souvannavong et al. 1997). In fact, it was found that chondrocyte-derived apoptotic bodies contain AP activities that most probably contribute to cartilage calcification (Hashimoto et al. 1998).

Cyt *c* is a soluble redox active hemoprotein localized in the intermembrane space of mitochondria. Release of cyt *c* into the cytosol and its complexation with cellular proteins seems to be an essential step in initiating the programmed cell death (see Mignotte and Vayssière 1998 for a review). We found previously (Dadáková et al. 1996) that AP from rabbit intestine induced profound changes in the circular dichroism spectra of cyt *c*(FeIII). The observed conformational change was dependent on the cyt *c* : AP ratio and on the length of the incubation period. An increase in 550 nm absorption indicated subsequent generation of cyt *c*(FeII). In the present study we focused on the assumed generation of complex(es) between the two enzymes. The results of spectral measurements, differential scanning calorimetry and chromatographic techniques showed evidence of complexation between the proteins. The resulting complexes were characterized by chromatographic and electrophoretic analysis.

Materials and Methods

Alkaline phosphatases were commercial lyophilized products from Sigma: Type VIII from rabbit intestine, No. P2265 designated as Lot 102F8190 (AP_I) and Lot 31H8125 (AP_{II}), and Type III-L No. P5931 from *E. coli* (AP_{E_c}). Cytochrome *c* (horse heart), C 7752 was of the same provenance. The optical analysis of the solutions demonstrating a slow autoredox was described previously (Dadáková et al. 1992). Solutions of AP were prepared as stated in Dadáková et al. (1996) except that *n*-dodecyl β -D-maltoside was omitted. The incubation of samples for chromatographic and electrophoretic analysis was carried out in 10 mmol/l phosphate buffer, pH 7.2, supplemented with 1 mmol/l MgCl₂ and 20 μ mol/l ZnCl₂; 60 μ l of the mixture contained 100 μ g of AP and 75 μ g of cyt *c*, the incubating temperature varied as given in the legends.

Absorption spectra were obtained using Ultrospec 2000 Pharmacia and Shimadzu UV 3000 spectrophotometers. The absorption changes in the mixtures after long incubation due to autoredox of cyt *c*(FeIII) and AP self-aggregation were eliminated by measuring samples in a difference mode.

The differential scanning calorimetric measurements of cyt *c*(FeIII) and its mixtures with AP were made with a highly sensitive DASM-4 microcalorimeter according to Antalík and Bágellová (1995). Control samples of alkaline phosphatase (0.6–7.5 mg) showed no thermal transitions in the conditions used (up to more than 90°C) either free in solution or in a mixture containing 1.5 mg of protamine. Cytochrome *c* in the samples (110 μ mol/l) was converted into the fully oxidized form (Bágellová et al. 1994). The heating curves were corrected using an instrument baseline obtained by heating the buffer.

FPLC was performed on a LCC-500 model (Pharmacia) with an Mono S column (10 $\times$ 150 mm), equilibrated with 50 mmol/l NaK-phosphate buffer pH 7.0 (Solvent A). Solvent B was 1 mol/l NaCl in Solvent A, the flow rate was 1 ml/min using the scale of 0.2 cm/ml. The elution profile of components was followed by measuring absorption wavelengths at 280 nm and 405 nm.

Gel permeation chromatography was carried out using an Ultropac TSK G3000 SW column of the same provenance (600 mm length, 7 mm i.d.), 50 mmol/l Tris/HCl buffer, pH 7.2 was used as the mobile phase. The calibration curve of the column was obtained with globular protein standards in the range of molecular weights from 13.7 to 150 kDa.

Polyacrylamide gel electrophoresis (PAGE) was performed using 0.75 mm slab gels with Mini Protean, (Bio Rad). SDS PAGE was carried out according to Laemmli (1970), stacking gel (T 4.9%, C 0.1%) was in 0.240 mol/l Tris/HCl, pH 6.8, separating gel (T 7.5%, C 0.4%) contained 0.37 mol/l Tris/HCl, pH 8.6, the electrophoresis buffer was 38 mmol/l Tris and 160 mmol/l glycine at pH 8.3. The samples of AP and mixtures of AP with cyt *c* were diluted with sample buffer containing 1% SDS, 20% glycerol, without mercaptoethanol, and treated at 100°C for 5 min before analysis. Non-denaturing PAGE was carried out either with the continuous or with the discontinuous buffer systems, the stacking gel (T 5.4%, C 0.15%) contained

0.12 mol/l Tris/HCl, pH 6.8, the separating gel (T 7.5%, C 0.24 %) and the electrophoresis buffer were 12.5 mmol/l and 50 mmol/l glycine, pH 10.0, respectively. Protein staining was performed with Coomassie Brilliant Blue R-250 and evaluated in a VD 620 videodensitometer. The calibration curve was constructed using the high molecular weight standards (kit I Bio Rad).

Results

Cytochrome c binding to alkaline phosphatase

In Fig. 1A, B the long wavelength absorption spectra of cyt *c*(FeIII) incubated in a slightly buffered solution of neutral pH supplemented with incremental additions of AP are presented. About 10% of cyt *c* in control samples was found in the reduced state after 24 hours incubation (cf. Fig. 1A, b) and its complete (> 95%) reduction was obtained on addition of ascorbate (Fig. 1B, b). It may be seen that the 24 h standing of the 1:1, 1:2 and 1:4 (cyt *c*/AP, w/w) mixtures (curves 1B c, d, e,) decreased the reducibility of cyt *c*(FeIII) (41%, 34%, 26% respectively) and caused an apparent diminution of the cyt *c*(FeIII) absorption (Fig. 1A). The shape of the 1A c, d, e, curves indicates an increase in unspecific absorption accompanied by a decrease in cyt *c*(FeIII) absorption. The change in absorption might be ascribed to an increased turbidity of the mixtures. At pH lower than 8.0 the enzyme might adopt a novel less stable conformation owing to the loss of some metal (Sowadski et al. 1985). On the other hand the rise in turbidity may be due to partial self aggregation of the native enzyme (Bublitz et al. 1993). To prove the alternative of coaggregation we carried out experiments at alkaline pH and examined the effect of temperature. In Fig. 2A, B, C the ultraviolet absorption spectra of mixtures incubated at three different temperatures are compared. The spectra were measured at pH 8.8 using a difference mode i.e. versus a solution of cyt *c* either incubated in parallel (a) or prepared freshly (b). It is seen (Fig. 2B, C) that the curves obtained at the two lower temperatures (8°C and 16°C) exhibit a distinct maximum at 280 nm of the tryptophan absorption. In contrast to this, the shape of the curves obtained at 25°C is clearly different showing a steep slope partly concealing the maximum at 280 nm. The finding supports our assumption of the coaggregation of the proteins. The difference in the trough between 360–320 nm seen on the curves can be explained by various amounts of cyt *c*(FeIII) and cyt *c*(FeII) due to autoreduction (the δ absorption maxima) in the samples.

FPLC analysis was introduced to explore changes occurring in cyt *c* during incubation with AP. With the aid of the Mono Q column and by measuring the heme and the protein absorption of eluted fractions, we distinguished the presence of the free AP protein from the oxidized and reduced species of cyt *c* besides their polymeric form and the assumed aggregate with the AP protein. Control samples of cyt *c* were incubated under the conditions of the experiments (pH 2.6, 7.0 and 8.3). Their elution profiles were characterized by a sharp peak of cyt *c*(FeIII) preceded by a tiny shoulder belonging to small amounts of cyt *c*(FeII) present in the sample.

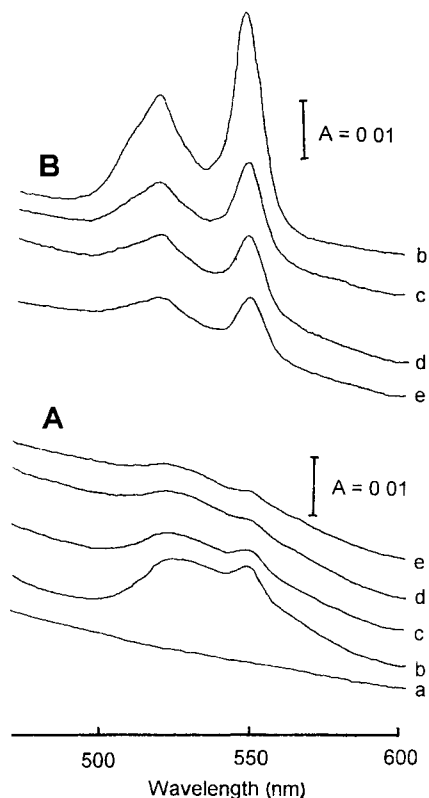


Figure 1. Long wavelength absorption spectra of cytochrome *c* after 24 hours incubation with alkaline phosphatase (A) Solutions of 0.1 mg of AP (a) and 0.1 mg of cyt *c* (b) in 1 ml of 10 mmol/l phosphate buffer, pH 7.2, were incubated at 25°C separately and in mixtures with increasing w/w ratios of AP, 1:1 (c), 1:2 (d), 1:4 (e) (B) Sample of cyt *c* (b) and the mixtures with AP (c, d, e,) were treated as in 1A and measured after addition of ascorbate

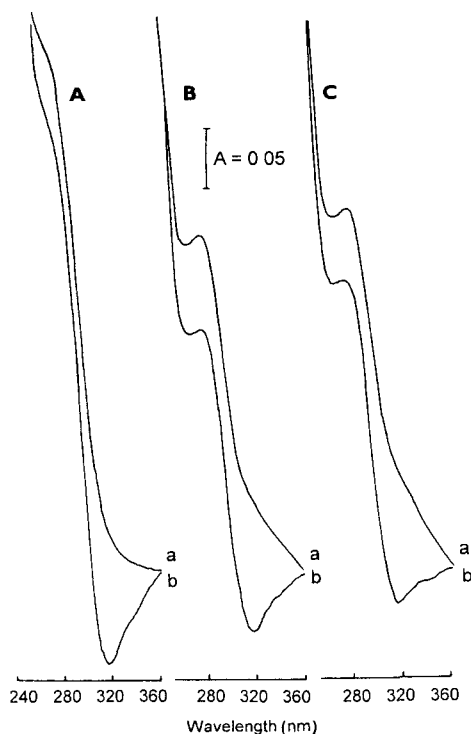


Figure 2. The ultraviolet absorption of cytochrome *c* incubated with alkaline phosphatase at different temperatures. Samples of 0.075 mg cyt *c* were mixed with 0.1 mg AP in 10 mmol/l pyrophosphate buffer, pH 8.8 and incubated for 24 hours at 25°C (A), 16°C (B), and 8°C (C). The spectra were measured as difference versus solutions of cyt *c* incubated at the same conditions (a), or prepared freshly (b).

AP alone was not retained on the column. It was eluted as a sharp peak at the beginning, as detected by absorption at 280 nm (not shown). The AP presence in the samples of cyt *c*(FeIII) caused a change in the elution profile (Fig. 3A). The changes proceeded gradually for hours (Fig. 3B). From the elution profiles of the

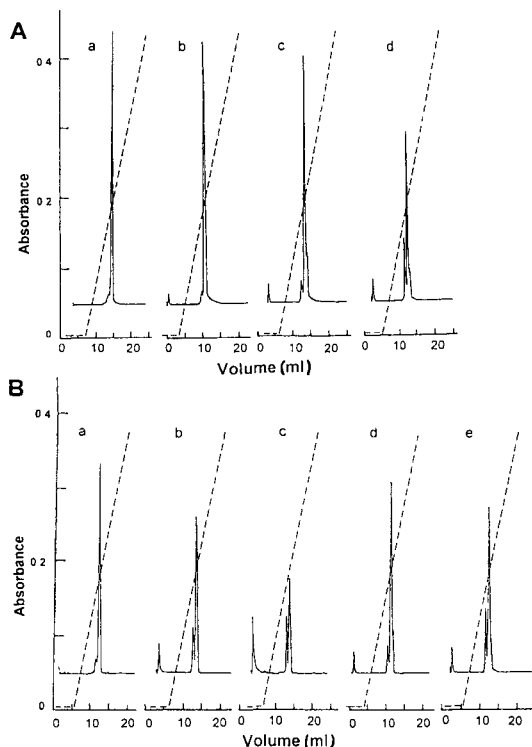


Figure 3A,B. Elution profiles of cytochrome *c* in mixture with alkaline phosphatase. Absorbance at 405 nm was plotted versus the elution volume. The conditions of separation were as follows 0–5 min solvent A, 5–25 min 0–100% solvent B. The linear gradient of solvent B is overlaid. (A) The analyzed samples (0.2 ml) contained 0.15 mg cyt *c* (a), with the addition of 0.1 mg AP (b, c, d), either in diluted hydrochloric acid, pH 2.6 (b), or in 8 mmol/l phosphate buffer, pH 7.0 (c), or in 8 mmol/l pyrophosphate/HCl buffer, pH 8.3. The separation started after 30 min incubation at 22°C. (B) Samples of cyt *c* (a), and the mixtures with alkaline phosphatase (b, c, d, e) were kept 20 h either at 22°C (a, b, c), or at 7°C (d, e) in solutions with pH 7.0 (b, d) or pH 8.3 (a, c, e) composed as given in A.

AP plus cyt *c* mixtures in Fig. 3A,B we conclude i) an increase in the proportion of cyt *c*(FeII) as the pH of the mixture increases, (small at pH 2.6 and mostly pronounced at pH 8.3), ii) the appearance of a new peak indicating the heme presence in the high molecular protein fraction, iii) the increased inhomogeneity of the elution profile of cyt *c* which was apparent mainly at the alkaline pH. The results show that the addition of AP to the solution of cyt affects the structure of the hemoprotein. The presence of potassium ferricyanide in control experiments prevented the cyt *c*(FeII) formation but did not influence its complexation with AP. Finally, the reaction was followed in the presence of 0.5 mol/l sodium chloride. The

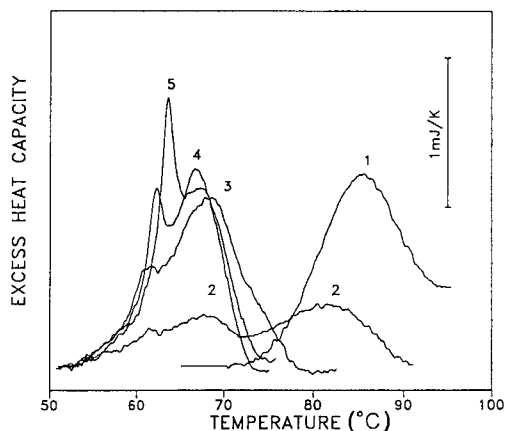


Figure 4. Differential-scanning-calorimetry (DSC) of cyt *c*Fe(III) in the presence of alkaline phosphatase. DSC scans were performed in 5 mmol/l phosphate buffer, pH 7.0, containing 1.3 mg (110 μ mol/l) cyt *c* and 110 μ mol/l potassium ferricyanide (1). The cyt *c*/AP ratios were 2:1 (2), 1:1 (3), 1:2 (4), 1:5 (5).

high ionic strength of the medium slowed down the reaction, nevertheless, it did not prevent the complexation of the proteins (not shown).

Next, differential scanning calorimetry was used to support the assumption of complexation between cyt *c* and AP. It has already been shown that a complex formation of cyt *c*Fe(III) either with its physiological or artificial reaction partners destabilizes the molecule in terms of thermodenaturation (Yu et al. 1985; Bágeľová et al. 1994; Murphy et al. 1995; Sedlák et al. 1997). The thermal denaturation profile of cyt *c*Fe(III) in our reaction media was found to be similar (84–85.4°C) to that reported in the literature (Yu et al. 1985; Bágeľová et al. 1994). The alkaline phosphatase (sample of AP₁) showed no thermal transition up to ~95°C. In Fig. 4 we show thermograms of cyt *c*Fe(III) in the presence of increasing concentrations of AP measured at low ionic strength and neutral pH. The addition of AP gave rise to a new thermodenaturation peak at $T_m = 68^\circ\text{C}$ while the original peak was decreased and its maximum shifted to 82°C. An increase in AP in the mixture up to a ratio of 1:1 enlarged the peak at 68°C and caused a new peak to appear at 61°C whilst the peak of transition of the free cyt *c* disappeared. Thermograms of mixtures containing a surplus of AP (see Legend to Fig. 4) were characterized by increasing and sharpening of the maxima concomitantly with their slight shifting. In Fig. 5 we show thermograms of the cyt *c* and AP (1:1) mixture measured in the presence of increasing amounts of sodium chloride. The increase in NaCl concentration gradually shifted both peaks to higher T_m values. It can be seen that between 0.2 mol/l and 0.5 mol/l NaCl the peak of the lower T_m was lost and only one peak at 76.3°C was present. The results suggest the existence of new substance(s) in the

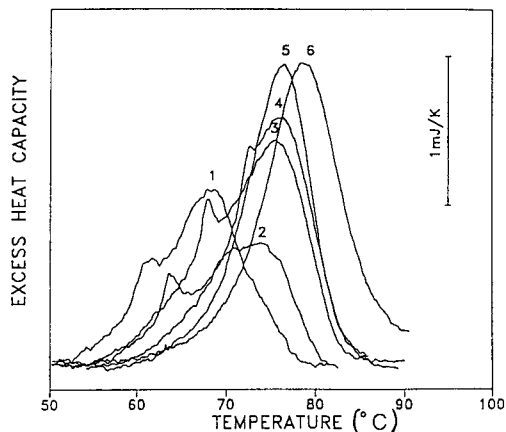


Figure 5. Denaturation of cyt *c* – alkaline phosphatase complex as a function of NaCl concentration. DSC scans were performed with a 1 : 1 mixture of cyt *c*/AP ratio (1). The additions of NaCl to samples were 50 mmol/l (2), 100 mmol/l (3), 200 mmol/l (4), 500 mmol/l (5), 500 mmol/l in the absence AP (6). For other conditions, see legend to Fig. 2.

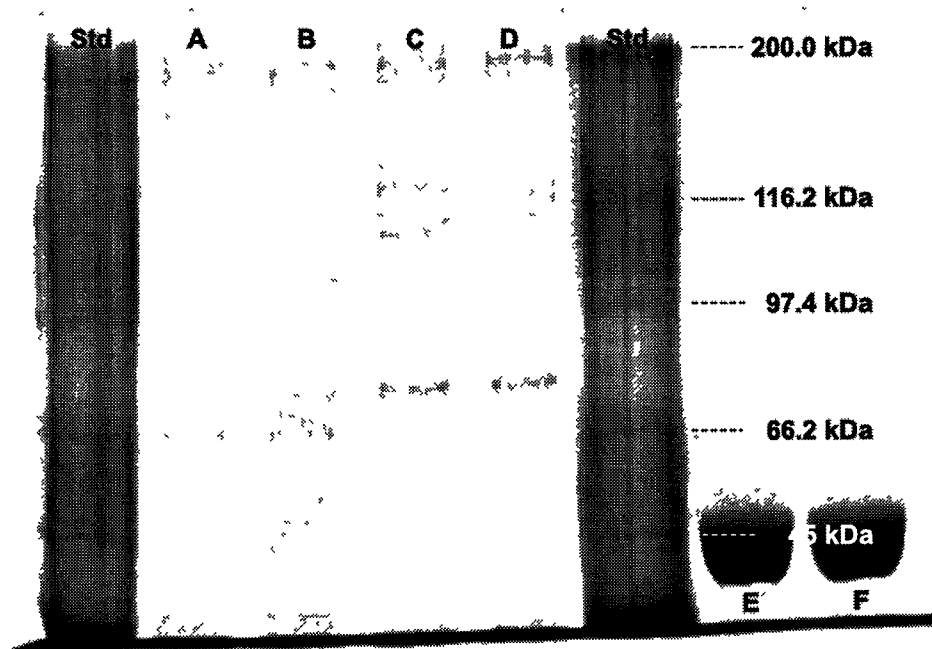


Figure 6. SDS PAGE of intestinal and bacterial alkaline phosphatases. Lanes A and B, 10 µg AP_I, lanes C and D, 10 µg AP_{II}, lanes E and F, 10 µg AP_{Ec}, lanes st, 10 µg marker proteins.



Figure 7. Non-denaturing PAGE of alkaline phosphatases and mixtures with cytochrome *c* after incubation. Discontinuous buffer system. Lane A, 10 μg AP_I , freshly dissolved, lane B, 10 μg AP_I , incubated, lanes C and D, 10 μg AP_I , incubated with 10 μg *cyt c*, lane E, 10 μg AP_II , freshly dissolved, lane F, 10 μg AP_II , incubated, lanes G and H, 10 μg AP_II , incubated with 10 μg *cyt c*, lane I, 10 μg of AP_{Ec} , incubated, lane J, 10 μg AP_{Ec} , incubated with 10 μg *cyt c*. The incubation was carried out in 10 mmol/l phosphate buffer, pH 7.2, for 24 hours at 16°C.

mixtures. These are most probably complexes of the two proteins which differ in their sensitivity to ionic strength.

Characteristic of the AP – cyt c complexes

Electrophoretic analysis was used to characterize the protein pattern of the two samples of the intestinal enzyme (AP_I and AP_II) and the soluble bacterial AP_{Ec} . SDS PAGE of the AP samples is shown in Fig. 6. It can be seen that the two samples of intestinal AP (see Materials and Methods section) differed somewhat in their composition. Besides similar fractions (63.2 kDa to 72.9 kDa and 172.9 kDa to 179.0 kDa), proteins of lower as well as higher molecular weights were detected in both samples. Fractions of higher molecular weight were more specified in AP_II showing the presence of proteins with molecular weights from 112.6 kDa to 127.0 kDa (see lanes C,D). The sample of the bacterial AP was homogeneous with a single band of 43.7 kDa proving the presence of the AP monomer. The incubation of the AP samples in a mixture with *cyt c* did not markedly change the electrophoretic distribution of the SDS PAGE fractions. Somewhat increased intensities of some bands were observed after 24 hours and longer incubation periods. The change was within the range of reproducibility on densitometric evaluation.

Non-denaturing PAGE (see Fig. 7) confirmed the presence of at least three distinct bands. The bands near the origin found in all samples of the intestinal

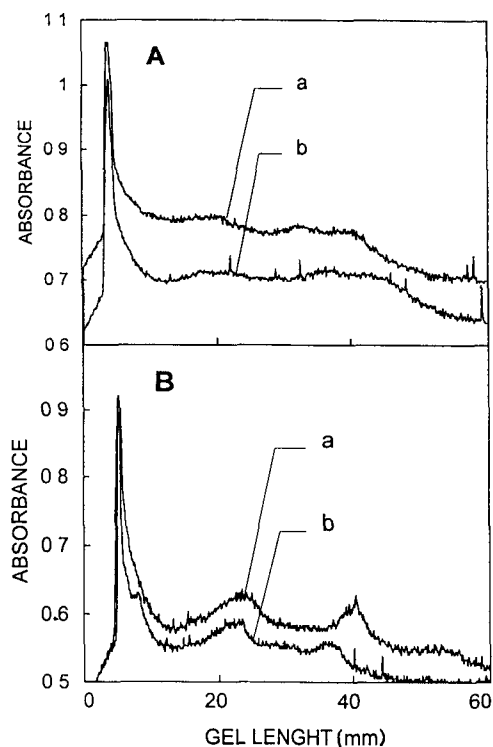


Figure 8. Densitograms of non-denaturing PAGE of alkaline phosphatases and their mixtures incubated with cytochrome *c*. Continuous buffer system AP_I (A), AP_{II} (B), (a) alkaline phosphatase, (b) mixture with cyt *c*. For conditions of incubation and the composition of the samples, see legend to Fig 7. For experimental details see Materials and Methods section.

APs were obviously aggregates of the enzyme. Incubation of the APs with cyt *c* for 1 hour, 3 hours, and 6–12 hours had no detectable influence on the electrophoretic pattern. However, marked differences in the intensities of the bands as well as in their positions could be detected after 24 hours and longer periods of incubation. The bands of intestinal APs incubated alone showed, in most cases, slightly faster anodal mobilities and a difference in the overall intensity. Typical patterns selected from many reproductions are shown in Fig 8A,B. No similar alterations were detected after the incubation of cyt *c* with AP_{E_c} (not shown).

The distribution of the molecular weights of the native enzyme and products originating from its incubation with cyt *c* were examined by means of gel permeation chromatography (see Fig 9). The retention profiles of the two enzyme samples are given in Fig 9A,C. The species with low elution volumes seen in both preparations can be assigned to the presence of enzyme aggregates. The proteins eluted at higher retention volumes may be attributed to enzyme dimer and fractions around 120 kDa. In general, the AP_{II} sample showed a better differentiation of proteins of high as well as low molecular weights. The analysis of the mixtures after incubation indicated the formation of some new species. These could be detected at the high as well as at the low molecular weight fractions. Further examination of the

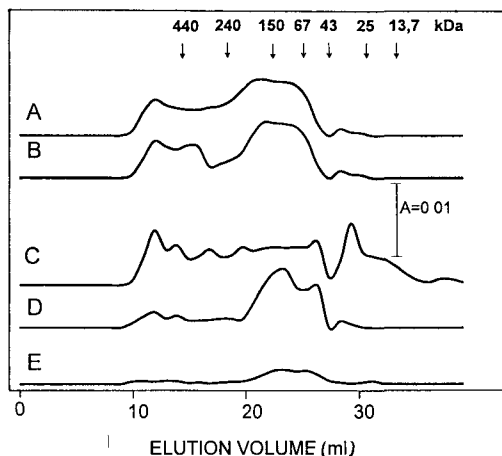


Figure 9. Gel chromatography of intestinal alkaline phosphatases and mixtures with cyt *c*. On Ultropac TSK G3000 SW column were applied. 20 μ g AP_I (A), 20 μ g AP_I incubated with 20 μ g cyt *c* (B), 20 μ g AP_{II} (C), 20 μ g AP_{II} incubated with 20 μ g cyt *c* (D, E). The detection of A, B, C, D was at 280 nm, that of E at 405 nm. For the procedure of the incubation of the mixtures, see legend to Fig. 7.

AP_{II} and cyt *c* mixtures (Fig. 9D, E) revealed new heme containing complexes. The heme absorption at 405 nm of free cyt *c* expected at high elution volumes was almost invisible (Fig. 9E).

Discussion

It was reported (Dadáč et al. 1996) that the addition of AP to the solution of cyt *c*(FeIII) brought about a profound change in its optical characteristics which could be partly explained by the generation of cyt *c*(FeII). At AP:cyt *c* ratios higher than 1:1 and at longer incubation intervals the process seemed to have at least two phases, with a slower one leading to a gradual decrease in the total cyt *c*. In the present study we showed long wavelength spectra of mixtures with higher amounts of AP after longer periods (20–24 hours) of incubation (Fig. 1A, B). From the curves in 1A it can be seen that the presence of AP virtually resulted in an apparent decrease in cyt *c*(FeIII). The finding was confirmed by the samples measured after ascorbate addition (Fig. 1B). The shape of the curves agreed with the suggestion that a portion of cyt *c* coaggregated or electrostatically reacted with negatively charged AP molecules. From the experiments an apparent loss between 53% and 74% of the cyt *c* content was calculated. The rationale of experiments conducted at alkaline pH was to protect the native conformation of the AP molecule. Alkaline pH, however, favors a change in cyt *c* conformation especially at longer incubation periods. Spectroscopic measurements performed in the UV region at dif-

ferent temperatures (Fig 2A, B, C) strengthened the view that the two proteins coaggregated. The idea was validated with the aid of FPLC analysis by measuring the elution profiles of the incubated mixtures of AP plus cyt *c* (see Fig 3A, B). The presence of heme was proven in the high molecular fraction thus witnessing the generation of a heme containing high molecular species. The new substance could be detected either at lower or higher (2.6–8.3) pH and at high ionic strength (0.5 mol/l NaCl) of the media.

Differential scanning calorimetry has recently been introduced for proving the existence of complexes between cyt *c* and its physiological (Yu et al 1985, Murphy et al 1995) as well as artificial reactants (Bagelova et al 1994, Sedlak et al 1997). The binding of cyt *c* to reaction partners causes its destabilization as indicated by a large decrease in the denaturation temperature (T_m). The intestinal AP alone did not show thermal transition at the temperature used. From the thermograms of mixtures of increasing AP concentrations (Fig 4) it seemed obvious that a complexation between the two proteins took place. The shape of the thermograms indicated the creation of new complexes. The irreversibility of the thermodenaturation process in our case did not allow us to make a quantitative description of the interaction. Nevertheless, it was of interest that the T_m values of the new peaks were positioned in the region between 61–68°C, which resembled those found upon electrostatic interaction with cytochrome *c* peroxidase and cytochrome *c* oxidase (Yu et al 1985). The close value of thermodenaturation (59.8°C) estimated for cyt *c* in the presence of the natural polyanion heparin (Bagelova et al 1994) supported the view of electrostatic binding. Profiles of thermograms obtained in the presence of increasing concentrations of sodium chloride gave further evidence for this (Fig 5). In these media the screening of electric charges on proteins abolished the splitting of T_m values (68°C and 61°C) and caused their values to be shifted to almost 80°C. However, even with the highest NaCl concentration used (0.6 mol/l) the T_m value of free cyt *c* was not obtained. The results confirmed the view that besides electric attraction other type(s) of binding forces participate in the interaction between AP and cyt *c*.

In an attempt to characterize the change in protein composition after long period of incubation the usual methods such as SDS PAGE, non denaturing PAGE and gel permeation chromatography were used. On SDS PAGE analysis the two samples of intestinal AP (Type VIII designated as AP_I and AP_{II}) somewhat differed in their composition. Besides bands of similar mobilities which could most probably be attributed to the AP monomer and the AP dimer, the two AP samples showed differences in the intensities of additional diffuse zones (Fig 6). It is known that intestinal AP exists as a mixture of at least three isoforms (Deng et al 1992) and may form higher molecular aggregates (Bublitz et al 1993). It is likely that the heterogeneity of intestinal APs explains the variance between the two samples. With the aid of non denaturing PAGE (see Fig 7) modifications of electrophoretic profiles after long lasting incubation were detected. They were evident either in the distribution and/or in the intensities of the bands. From the electropherograms, it may be anticipated that cyt *c* virtually binds to the main components of the sam-

ple (AP dimer, monomer) and, to some extent, also coaggregates with the enzyme. This was confirmed by means of gel permeation chromatography (Fig. 9). Incubation of both intestinal AP samples with cyt *c* resulted in the appearance of new species found within high and low molecular fractions. The marked change in the elution pattern showed the generation of modified AP monomer and dimer (Fig. 9C). In Fig. 9D it is shown that these modified proteins contain the heme proving the complexation of AP with cyt *c*. The cyt *c*, however, was almost undetectable as free protein. The results lead to the conclusion that cyt *c* i) binds to non-aggregated molecules of intestinal AP; ii) partly coaggregates with AP in forming larger aggregates; and iii) may also bind to low molecular fractions present in both samples of the mammalian enzyme. On the contrary, no significant differences were observed on analyzing mixtures of cyt *c* with the soluble enzyme of AP_{E_c}. Our results show that the molecule of cyt *c* has the ability to bind to cell surface glycoproteins such as intestinal AP. According to our knowledge, this is the first report providing evidence of complex formation between pro-apoptotic cyt *c* and the membrane-bound hydrolytic enzyme.

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References

- Antalík M, Bágellová J (1995) Effect of nucleotides on thermal stability of ferricytochrome *c* *Gen Physiol Biophys* **14**, 19–37
- Armesto J, Hannapel E, Leopold K, Fischer W, Bubltz R, Langer L, Cumme G, A, Horn A (1996) Microheterogeneity of the hydrophobic and hydrophilic part of the glycosylphosphatidylinositol anchor of alkaline phosphatase of calf intestine *Eur J Biochem* **238**, 259–269
- Bágellová J, Antalík M, Bona M (1994) Studies on cytochrome *c*-heparin interactions by differential scanning calorimetry *Biochem J* **297**, 99–101
- Bubltz R, Armesto J, Hoffmann-Blume E, Schulze M, Rhode H, Horn H, Aulwurm S, Hannapel E, Fischer W (1993) Heterogeneity of glycosylphosphatidylinositol-anchored alkaline phosphatase of calf intestine *Eur J Biochem* **217**, 199–207
- Coleman J E, Gettins P (1983) Alkaline phosphatase, solution structure and mechanism *Adv Enzymol* **55**, 381–452
- Dadáč V, Vanderkooi J M, Wright W W (1992) Electron transfer from excited tryptophan to cytochrome *c*: mechanism of phosphorescence quenching? *Biochim Biophys Acta* **1100**, 33–39
- Dadáč V, Vrána O, Nováková O, Antalík M (1996) Interaction of alkaline phosphatase with cytochrome *c* *Biochim Biophys Acta* **1297**, 69–76
- Deng J T, Hoylaerts M F, Van Hoof V O, De Broe M E (1992) Differential release of human intestinal alkaline phosphatase in duodenal fluid and serum *Clin Chem* **38**, 2532–2538
- Harris H (1989) The human alkaline phosphatase: What we know and what we don't know *Clin Chim Acta* **186**, 133–150
- Hashimoto S, Ochs R L, Rosen F, Quach J, McCabe G, Solan J, Seegmiller E, Terkeltaub R, Lotz M (1998) Chondrocyte-derived apoptotic bodies and calcification of articular cartilage *Proc Natl Acad Sci USA* **95**, 3094–3099

- Henthorn P S, Raducha M, Edwards Y H, Weiss M J, Slaughter C, Lafferty M A, Harris H (1987) Nucleotide and amino acid sequences of human intestinal alkaline phosphatase. Close homology to placental alkaline phosphatase. *Proc Natl Acad Sci USA* **84**, 1234—1238
- Hui M, Tenenbaum H C, McCulloch C A G' (1997) Collagen phagocytosis and apoptosis are induced by high level alkaline phosphatase expression in rat fibroblasts. *J Cell Physiol* **172**, 323—333
- Kim E E, Wyckoff H W (1989) Structure of alkaline phosphatases. *Clin Chim Acta* **186**, 175—188
- Kim E E, Wyckoff H W (1991) Reaction mechanism of alkaline phosphatase based on crystal structure. *J Mol Biol* **218**, 449—464
- Laemmli U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* **227**, 680—685
- Martin D, Tucker D, Campbell I, Trowsdale J (1989) Comparison of the three PLAP-related genes on human chromosome 2. *Clin Chim Acta* **186**, 165—170
- McComb R B, Bowers G N, Posen S (1979) *Alkaline Phosphatases*. Plenum, New York
- Mignotte B, Vayssiere J-L (1998) Mitochondria and apoptosis. *Eur J Biochem* **252**, 1—15
- Millán J L (1986) Molecular cloning and sequence analysis of human placental alkaline phosphatase. *J Biol Chem* **261**, 3112—3115 and correction (1991) *J Biol Chem* **266**, 4023
- Moss D W (1992) Perspectives in alkaline phosphatase research. *Clin Chem* **38**, 2486—2492
- Murphy K P, Freire E, Paterson Y (1995) Configurational effects in antibody-antigen interactions studied by microcalorimetry. *Proteins* **21**, 83—90
- Sedlák E, Antalík M, Bágeľová J, Fedurco M (1997) Interaction of ferricytochrome *c* with polyanion Nafion. *Biochim Biophys Acta* **1319**, 258—266
- Sone M, Kishigami S, Yoshihisa T, Ito K (1997) Roles of disulfide bonds in bacterial alkaline phosphatase. *J Biol Chem* **272**, 6174—6178
- Souvannavong V, Lemaire C, Brown S, Adam A (1997) UV irradiation of a B-cell hybridoma increases expression of alkaline phosphatase involvement in apoptosis. *Biochem Cell Biol (Biochim Biol Cellul)* **75**, 783—788
- Sowadski J M, Handschumacher M D, Murthy H M K, Foster B A, Wyckoff H W (1985) Refined structure of alkaline phosphatase from *Escherichia coli* at 2.8 Å resolution. *J Mol Biol* **186**, 417—433
- Sun L, Martin D C, Kantrowitz E R (1999) Rate determining step of *Escherichia coli* alkaline phosphatase altered by the removal of a positive charge at the active center. *Biochemistry USA* **38**, 2842—2848
- Weissig H, Schildge A, Hoylaerts M F, Iqbal M, Millán J L (1993) Cloning and expression of the bovine intestinal alkaline phosphatase gene: biochemical characterization of the recombinant enzyme. *Biochem J* **290**, 503—508
- Yu C A, Gwak S H, Yu L (1985) Studies on protein-lipid interactions in cytochrome *c* oxidase by differential scanning calorimetry. *Biochim Biophys Acta* **812**, 656—664