*This paper is dedicated to Professor Włodzimierz Ostrowski*

# Molten globule as an intermediate on the human prostatic phosphatase folding pathway*

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Human prostatic acid phosphatase (hPAP, EC.3.1.3.2), a secretory homodimeric protein was denatured in 6 M urea, pH 2.5, and refolded by dilution at pH 7.2 with recovery of the enzymatic activity and dimeric structure. Circular dichroism, intrinsic fluorescence and chromatographic analysis of renaturating protein suggested that the kinetic intermediate of the hPAP folding is a monomer which displays a molten globule state (R. Kuciel, A. Mazurkiewicz & W.S. Ostrowski, 1996, *Int. J. Biol. Macromol.* 18, 167–175). To confirm these data experiments were performed to estimate the interaction of the renaturating protein with dyes and amphipathic lipid structures. Increased binding of the hydrophobic probe 1-anilinonaphthalene-8-sulfonate and Congo Red to the refolding enzyme supported the existence of molten globule state with the relaxed β-structure in the renaturating protein. Presence of liposomes, included in the renaturation mixture as a model of acid phospholipid, resulted in perturbations of the human PAP refolding process. Some folding intermediates were bound to phosphatidylserine liposomes or, alternatively, water soluble, inactive aggregates were formed.

Human prostatic acid phosphatase (hPAP) is synthesized as a secretory protein by epithelial cells of the prostate gland. It is a highly glycosylated protein composed of two identical subunits [1–3]. Studies on denaturation-renaturation and subunits reassociation of hPAP [4, 5] indicate that the active form of enzyme is a dimer and that individual monomers show no detectable catalytic activity. That is why the hPAP might be considered a good model to follow folding and assembling of secretory proteins with subunit structure. Previous experiments have shown [5] that, in the process of renaturation, a kinetic intermediate of flexible structure and of relative molecular mass corresponding to the monomer of hPAP was formed. The main objective of the study reported here is further

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Abbreviations: hPAP, human prostatic acid phosphatase; ANS, 1-anilinonaphthalene-8-sulfonate.

description of this intermediate and determination whether it might be considered to be in molten globule state [6, 7]. The folding pathway of hPAP was investigated by analysing of interactions of the refolding protein with dyes and amphipathic lipids. ANS dye is widely used as a hydrophobic probe of high affinity for the molten globule state [8, 9]. Therefore the fluorescence spectra of ANS in the solution along with those of the protein, under different solvent conditions were measured. The sulfonated diazo dye Congo Red penetrates into relaxed β-conformations in proteins and is able to form complexes with unfolding intermediates of proteins [10–12]. Quantitative measurement of the Congo Red binding to native hPAP and to the renaturating enzyme was conducted to determine whether the dye interacts with the native, active protein molecule, and to find out whether any new dye binding motifs of the phosphatase molecule became exposed during the renaturation process.

The extended intention of this work was to collect observations which might help to understand folding and assembly of oligomeric secretory proteins *in vivo*, so renaturation of the denatured enzyme was performed in the presence of phosphatidylserine liposomes to mimic the effect of vicinity of membranes upon the arrangement of biologically active protein. Our data indicate that liposomes containing acidic phospholipids inhibit reactivation of the enzyme.


## MATERIALS AND METHODS

*Materials.* Urea was purchased from Serva Feinbiochemica (Heidelberg, Germany). p-Nitrophenylphosphate (disodium salt) and ANS (1-anilinonaphthalene-8-sulfonate) were obtained from Sigma (St. Louis MO, U.S.A.). Congo Red was purchased from Aldrich Chemical Company. Liposomes were prepared from brain extract, Folch Fraction III Sigma (St. Loius, U.S.A.). All reagents used for HPLC were purchased from Fluka Chemie AG. All other reagents were of analytical grade obtained from POCH, Poland. Prostatic acid phosphatase was purified from human seminal plasma according of Van Et-

ten & Saini [13]. Phosphatase concentration was determined spectrophotometrically by using the hPAP absorption coefficient of $E_{280}^{0.1\%} = 1.44$ [14].

*Phosphatase activity assay.* The activity of hPAP was measured at 37°C using 20 mM p-nitrophenylphosphate in 0.1 M acetate buffer, pH 5.0, as described earlier [5].

*Denaturation of hPAP.* This was performed in 6 M urea dissolved in 0.1 M glycine-$H_3PO_4$ buffer, pH 2.5, containing 1 mM dithiothreitol and 1 mM EDTA. Protein concentration was about 1 mg/ml. The sample was incubated for 2 h at 20°C·

*Renaturation of denatured hPAP.* The renaturation was initiated by tenfold dilution of the denatured enzyme in 0.1 M Tris/HCl buffer, pH 7.2, containing 1 mM EDTA (renaturation buffer). The final protein concentration was approximately 100 μg/ml (1 μM). The residual urea concentration was 0.6 M. To determine the progress of reactivation of the enzyme at defined time intervals, 10 μl aliquots of the renaturation mixture were withdrawn for the phosphatase activity assay. In controls (native enzyme without denaturation), urea was added to the solutions to 0.6 M concentration.

*Gel electrophoresis.* PAGE/SDS electrophoresis was performed according to Laemmli [15].

*Size exclusion chromatography.* This was performed on a TSK G2000 column (7.5 mm × 300 mm) using a KONTRON HPLC system as previously described [5] with 0.1 M phosphate buffer, pH 7.2, containing 0.3 M sodium chloride as eluent. The flow rate was 0.5 ml/min. The absorbance was measured at 220 nm. To identify monomeric, dimeric and aggregated forms of the renaturating enzyme, retention time of the reaction products was compared with mobility of standard proteins: lysozyme (14300), trypsin (23000), carbonic anhydrase (29000), bovine serum albumin (67000), native human prostatic acid phosphatase (100000) and IgG (160000).

*Spectroscopic techniques.* Absorption spectra of proteins in the range from 220 nm to 320 nm were recorded with a Hitachi U 2000 spectrophotometer. Spectra of intrinsic fluorescence of the protein were recorded at

310–400 nm with a Hitachi F 4000 spectrofluorimeter using an excitation wavelength of 295 nm. Fluorescence spectra of external ANS probe were registered with excitation at 400 nm. Absorbance of the measured samples was less than 0.1 absorbance unit at the excitation wavelength. The emission spectra of ANS were observed in the range of 420–600 nm. Solutions used in ANS fluorescence measurements were prepared by adding ANS stock solution to the renaturation buffer (1 ml) to obtain final concentration of ANS in the range of 1–100 µM in the samples. Portions of 0.1 ml of the denatured hPAP were introduced into these samples. Final concentration of the protein was about 1 µM. After intensive mixing the emission spectra of ANS were recorded at about 2 min intervals up to 20 min of renaturation. For comparison, the fluorescence spectra of native and denatured hPAP, in the presence of ANS at any given concentration, were recorded.

Quantum yield for ANS bound to hPAP was measured by comparing areas under the fluorescence emission spectra of ANS bound to hPAP with the areas of the emission spectra obtained with equally absorbing samples of ANS in 1-propanol. The number of molecules of ANS bound to the renaturating hPAP was determined from a linear least-squares fit to a double-reciprocal plot of the fluorescence data.

***Measurements of Congo Red binding to native hPAP.*** Native hPAP at 0.1–2 µM concentration prepared in 0.1 M Tris/HCl, pH 7.2, containing in 1 ml 1 mM EDTA, 0.6 M urea and 25 µM Congo Red. As a reference, a control sample of 25 µM solution of Congo Red in the same buffer was prepared. After 5 min incubation of the protein with the dye at room temperature, a dense suspension of Sephadex G-25 gel (0.4 ml), preswollen in the renaturation buffer, was added. This mixture was vortexed for 3 min and then Sephadex G-25 particles were removed by centrifugation. Supernatants were collected and their absorption spectra were recorded between 220–620 nm. The molar absorption coefficient at 490 nm for Congo Red in the renaturation buffer was determined as $2.96 \times 10^4$

$M^{-1} cm^{-1}$ and this value was used to calculate the dye concentration in the solution.

***Effect of ANS and Congo Red on the activity of native hPAP.*** The phosphatase activity assay was performed in the presence of ANS (1–100 mM) or Congo Red dye (0.1–5 mM) added into the substrate (0.1–20 mM) solution. Kinetic data of the catalytic reaction were analysed using the program ENZYME [16]. The types of inhibition and inhibitor constants were also evaluated according to this program.

***Effect of ANS and Congo Red on reactivation of denatured hPAP.*** Renaturation of hPAP was carried out in the presence of ANS (1–100 µM) or Congo Red (1–25 µM) in the renaturation buffer. At defined time intervals, activity of the renaturating enzyme was compared with the activity of hPAP refolding in the renaturation buffer without any additives.

***Measurement of Congo Red binding to renaturating hPAP.*** Renaturation of hPAP was performed by tenfold dilution of the denatured enzyme in 0.1 M Tris/HCl buffer, pH 7.2, containing 1 mM EDTA, to obtain an approximate 1 µM final protein concentration. At different time intervals, 1 ml samples were withdrawn from the renaturating mixture and immediately 100 µl of the Congo Red stock solution in the buffer was added to 25 µM concentration of the dye in each of the renaturating samples. After 5 min incubation at room temperature, the suspension of preswollen Sephadex G-25 particles (0.4 ml) was added to each sample. These mixtures were vortexed for 3 min and the gel was removed by centrifugation. Absorption spectra of supernatants were recorded between 220–620 nm.

***Liposome preparation.*** Large unilamellar vesicles, composed mainly of phosphatidylserine (brain extract, Folch fraction III), were prepared in 0.1 M Tris/HCl buffer by the extrusion method of Hope *et al.* [17]. Polycarbonate membranes with pore size of 0.1 µM were used. Final concentration of the lipid was 10 mg per 1 ml of suspension.

***Effect of liposomes on reactivation of the denatured hPAP.*** In several parallel samples renaturation of the denatured hPAP

was initiated by dilution into the renaturation buffer containing no liposomes. After 1 min renaturation, the suspension of liposomes (50 µl) was added to one of the renaturating samples to reach a lipid to protein molar ratio of about 500:1. In another renaturating sample, liposomes were added after 5 min renaturation. The next sample was renatured for 10 min and then liposomes were added. In one experiment, a sample of denatured hPAP was mixed with the renaturation buffer containing liposomes. The activity of renaturating hPAP was then measured and compared with the activity of the control sample renaturating without liposomes. The activity of native hPAP in solutions with and without liposomes was also determined.

*Binding of denatured, partially renatured and native hPAP to liposomes.* Samples of native, denatured or partially renatured hPAP were diluted with the renaturation buffer containing liposomes (final protein concentration was about 100 µg/ml, lipid to protein molar ratio was 500:1). After incubation for 45 min at room temperature, phosphatase activity was measured in each sample. Then, the liposomes were separated by centrifugation ($120000 \times g$ for 2 h). In the clear supernatants absorption fluorescence spectra and the enzymatic activity were recorded. Analysis of soluble proteins in supernatants was performed using the size-exclusion HPLC chromatography. The pellets containing liposomes were collected and resuspended each in 3.5 ml of renaturating

buffer to measure the phosphatase activity. In another experiment, pellets of liposomes were dissolved in 0.2 ml of SDS sample buffer, then 50 µl of each sample was analysed by PAGE/SDS for protein content.

## RESULTS

### Binding of ANS to native, denatured and renaturating hPAP

The fluorescence emission of ANS in a solution of the native hPAP is low (spectrum 1 in Fig. 1A) indicating poor binding between the ANS dye and the native protein. There was no increase of the fluorescence intensity of ANS in the presence of denatured hPAP, either (spectrum 2 in Fig. 1A). Moreover, ANS at concentrations of 1–100 µM did not influence the catalytic activity of hPAP and did not affect the rate of reactivation of the renaturating hPAP. Therefore, the fluorescence spectra of ANS that was initially present at different concentrations in the renaturation buffer were recorded during the course of renaturation of the denatured hPAP. The fluorescence of ANS increased considerably after dilution of the denatured hPAP, then, gradually decreased with the progress of renaturation (spectra 3–9 in Fig. 1A). Figure 1B shows that reduction of the ANS fluorescence observed during the renaturation of hPAP was accompanied by recovery of catalytic activity of the phosphatase. It was calculated that, at the initial
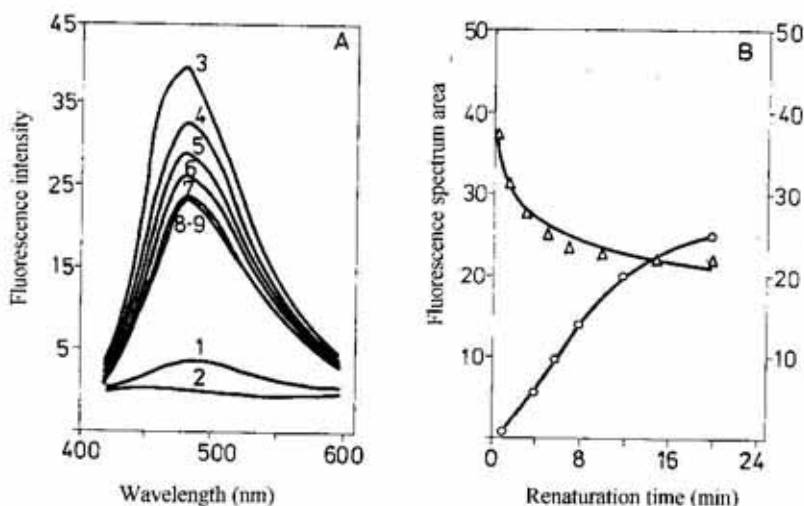


**Figure 1. Fluorescence spectra of ANS in the presence of native, denatured and renaturating hPAP.**

A. Kinetics of refolding of hPAP (1 µM) monitored by ANS (100 µM) fluorescence ($\lambda_{exc}$ 400 nm). (1) Native hPAP; (2) denatured hPAP; (3–9) spectra of ANS monitored at 20 s, 90 s, 3 min, 5 min, 7 min, 10 min and 15 min, respectively, after dilution of the denatured hPAP. B. Renaturation of hPAP followed by monitoring changes in ANS fluorescence (Δ) and reactivation of enzyme (O).

stage of hPAP renaturation, 4 to 5 molecules of ANS were bound per subunit of the renaturating hPAP.

### Binding of Congo Red to native hPAP and effect of the dye on the catalytic activity of enzyme. Binding of Congo Red to renaturating hPAP

Free Congo Red dye (25 µM) dissolved in 0.1 M Tris/HCl buffer, pH 7.2, containing 1 mM EDTA and 0.6 M urea (spectrum 1 in Fig. 2A) could be easily removed from the solution by adsorption on Sephadex G-25 particles (spectrum 2 in Fig. 2A). Native hPAP, when present in solution, interacted with the dye and soluble Congo Red-protein complexes were not adsorbed on the surface of the G-25 gel. Absorption spectra of supernatants, after removal of free Congo Red with gel particles, were recorded at different concentrations of hPAP (Fig. 2B). These data allowed to calculate that one molecule of the native hPAP



**Figure 2. Congo Red dye binding to native hPAP.**

A. Spectrum of: (1) 25 µM solution of Congo Red in renaturation buffer containing 0.6 M urea; (2) supernatant after Congo Red adsorption on Sephadex G-25 gel. B. Spectra of the Congo Red-native hPAP complexes: (1) 0.3 µM hPAP (without dye); (2) 0.3 µM hPAP-Congo Red; (3) 0.7 µM hPAP-Congo Red; (4) 1.4 µM hPAP-Congo Red.

dimer is able to bind about 3.2 molecules of Congo Red. This means that on average 1.6 molecules of the dye were bound per monomer of the native protein molecule.

Congo Red inhibits the catalytic activity of hPAP. In the computer analysis [16] of experimental data of the phosphatase reaction performed at different concentrations of $p$-nitrophenylphosphate as a substrate (0.1–20 mM) and at different concentrations of Congo Red as inhibitor (0–5 µM), a mixed non-competitive model provided the best fit. The final values of $V_{max}$, and $K_m$ were 6.44 $\times 10^{-4}$ M/min$^{-1}$ µg$^{-1}$ and 1.36 $\times 10^{-4}$ M, respectively. The value of $K_{i1}$ was 4.72 $\times 10^{-7}$ M and the value of $K_{i2}$ was 5.27 $\times 10^{-6}$ M. This indicates that two inhibitory complexes of Congo Red and the native hPAP were formed, of the binding constants which differ by one order of magnitude.

To examine the effect of Congo Red on reactivation of denatured hPAP, the renaturation was performed in the presence of the dye (1–25 µM). The recovery of catalytic activity of the denatured enzyme was inhibited by Congo Red, and the extend of inhibition depended on the dye concentration (Fig. 3). It appeared that in 25 µM Congo Red solution, reactivation of the denatured hPAP was completely suppressed.

To determine the number of Congo Red molecules interacting with the refolding enzyme, the renaturation of hPAP was terminated at different times after initiation by adding the dye to the reaction mixture to a concentration of about 25 µM. Then, the unbound Congo Red was removed from the protein solution by adsorption on Sephadex G-25 particles and absorption spectra of protein-dye complexes in supernatants were recorded (Fig. 4A). From these spectra it was calculated that after the first 30 s of renaturation one subunit of hPAP was able to bind 7–8 molecules of Congo Red (as compared to 1.6 molecules per monomer of the native enzyme). With the progress of renaturation, the amount of the dye bound to the renaturating sample decreased and this change was accompanied by a gradual increase of the catalytic activity of protein (Fig. 4B).
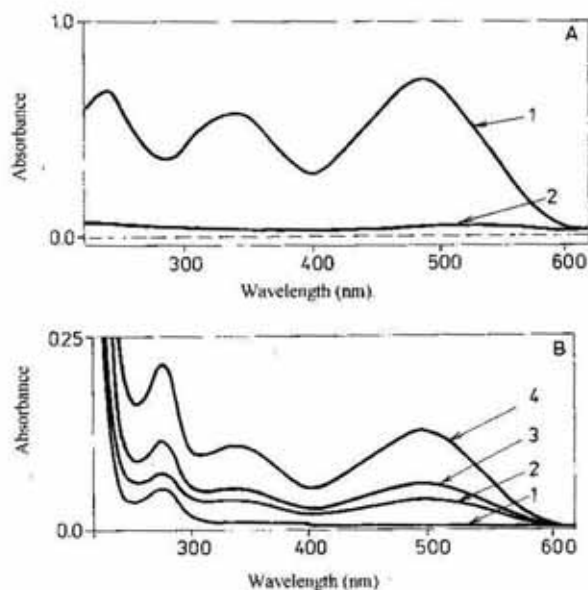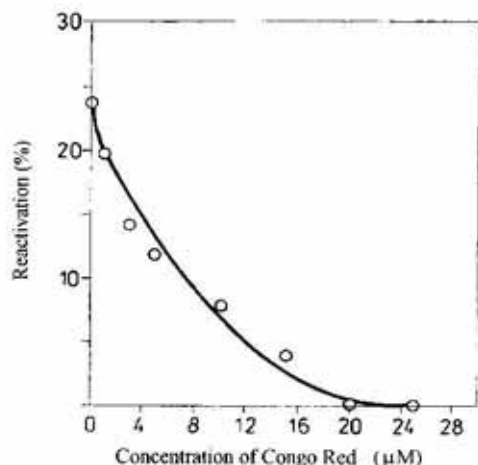
**Figure 3. Reactivation of denatured hPAP in the presence of Congo Red.**

The activity of phosphatase was measured 15 min after the initiation of renaturation.

## Effect of phosphatidylserine liposomes on renaturation of denatured hPAP

Negatively charged phosphatidylserine liposomes, when present in the renaturation buffer, completely arrested the process of hPAP reactivation (Fig. 5). Moreover, liposomes added at different times to the renaturating sample of hPAP decreased the final

yield of recovery of catalytic activity. At the same time they did not influence the activity of native hPAP (Fig. 5). These observations suggested that it would be of considerable interest to study closer the effect of liposomes on the renaturating hPAP. The samples containing native, denatured and renaturating hPAP with and without liposomes were prepared (Table 1). The enzyme refolded for 45 min without liposomes regained about 40% of the catalytic activity of the native hPAP (sample (f) in Table 1). There was no reactivation of the denatured enzyme if dilution was carried out in the presence of liposomes (sample (e) in Table 1). When liposomes were added 1 min after the dilution, some activity was regained, but the extent of recovery was low (sample (d) in Table 1). Absorption spectra recorded after removal of liposomes by centrifugation show that concentrations of protein in supernatants of the native hPAP incubated with or without liposomes were similar (samples (b) and (c)). In samples d–f containing hPAP renaturating with or without liposomes about 70–80% of protein was found in supernatants. Intrinsic fluorescence



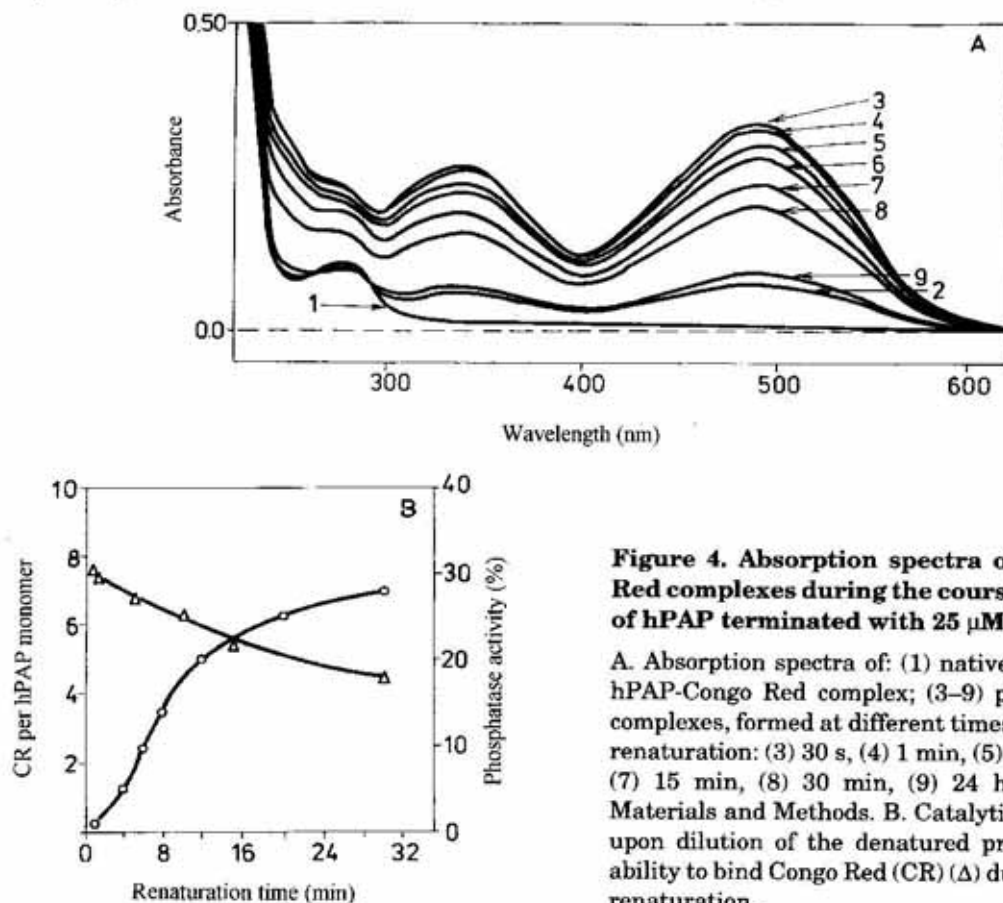Wavelength (nm)



Renaturation time (min)

**Figure 4. Absorption spectra of protein-Congo Red complexes during the course of reactivation of hPAP terminated with 25 μM Congo Red dye.**

A. Absorption spectra of: (1) native hPAP; (2) native hPAP-Congo Red complex; (3–9) protein-Congo Red complexes, formed at different times after initiation of renaturation: (3) 30 s, (4) 1 min, (5) 5 min, (6) 10 min, (7) 15 min, (8) 30 min, (9) 24 h. For details see Materials and Methods. B. Catalytic activity of hPAP upon dilution of the denatured protein (O) and its ability to bind Congo Red (CR) (Δ) during the course of renaturation.
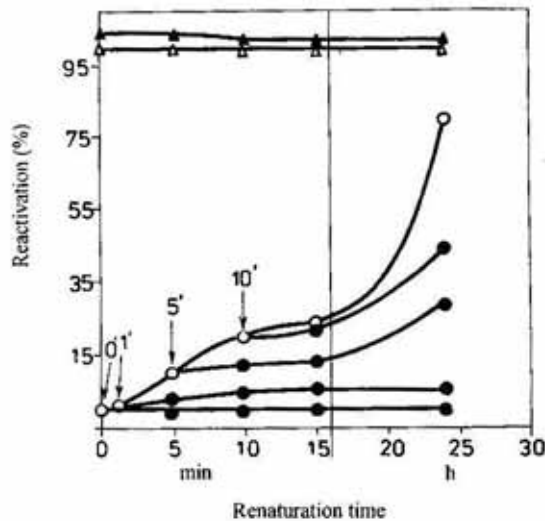
**Figure 5. Reactivation of denatured hPAP in the presence of phosphatidylserine liposomes.**

At the time indicated by arrows, samples of renaturating mixture were withdrawn and transferred to a suspension of liposomes. Phosphatase activity in the samples renaturating without liposomes (O), with liposomes (●). The activity of native enzyme without liposomes (Δ), with liposomes (▲).

spectra of all supernatants, excited at 295 nm show a fluorescence maximum at 337 nm, similar to that of the native enzyme. Phosphatase activity, if present, was located mainly in supernatants. The liposomes, supernatants and pellets were separately incubated overnight at room temperature. The only sample in which an increase of activity was observed was the supernatant of the sample (f) which contained hPAP renaturating from the beginning in the absence of liposomes. The activity of the samples at 24 h after removal of liposomes remained unchanged (Table 1).

**Table 1. Renaturation of human prostatic phosphatase (hPAP) in the presence of liposomes**

| Samples | Phosphatase activity in the whole sample incubated for 45 min at 20°C (%) | Phosphatase activity after centrifugation of samples for 2 h at $120000 \times g$ | | Spectra of supernatants recorded after centrifugation of samples | | Phosphatase activity in the supernatants and the pellets incubated separately for 24 h at 20°C | |
|---|---|---|---|---|---|---|---|
| | | Supernatants (%) | Pellets (%) | Absorbance at 280 nm | Max. of fluoresc. ($\lambda_{exc}$ 295 nm) (nm) | Supernatants (%) | Pellets (%) |
| **a.** Liposomes in the renaturation buffer | 0 | 0 | 0 | 0.0 | – | 0 | 0 |
| **b.** Native hPAP and liposomes in the renaturation buffer | $100 \pm 4$ | $88 \pm 6$ | $6 \pm 1$ | 0.15 | 337 | $90 \pm 5$ | $8 \pm 1$ |
| **c.** Native hPAP in the renaturation buffer | $100 \pm 3$ | $86 \pm 5$ | $3 \pm 0.5$ | 0.15 | 337 | $86 \pm 3$ | $5 \pm 1$ |
| **d.** hPAP renaturating for 1 min and then liposomes added | $7 \pm 1$ | $9 \pm 1$ | 0 | 0.10 | 337 | $9 \pm 1$ | 0 |
| **e.** Denatured hPAP diluted into renaturating buffer containing liposomes | 0 | 0 | 0 | 0.12 | 337 | 0 | 0 |
| **f.** hPAP renaturating without liposomes | $40 \pm 2$ | $60 \pm 2$ | $2.5 \pm 0.5$ | 0.12 | 337 | $70 \pm 2$ | $5 \pm 0.5$ |

To detect proteins sedimenting during ultracentrifugation, the pellets containing liposomes were applied onto PAGE/SDS electrophoresis gel (Fig. 6). The results of analysis indicate that there was a negligible, if

renaturating during the run of chromatography (B). The following fractions are visible: a fraction of mobility corresponding to relative molecular mass of the subunit of hPAP (Mo), a fraction of the dimeric native form of the
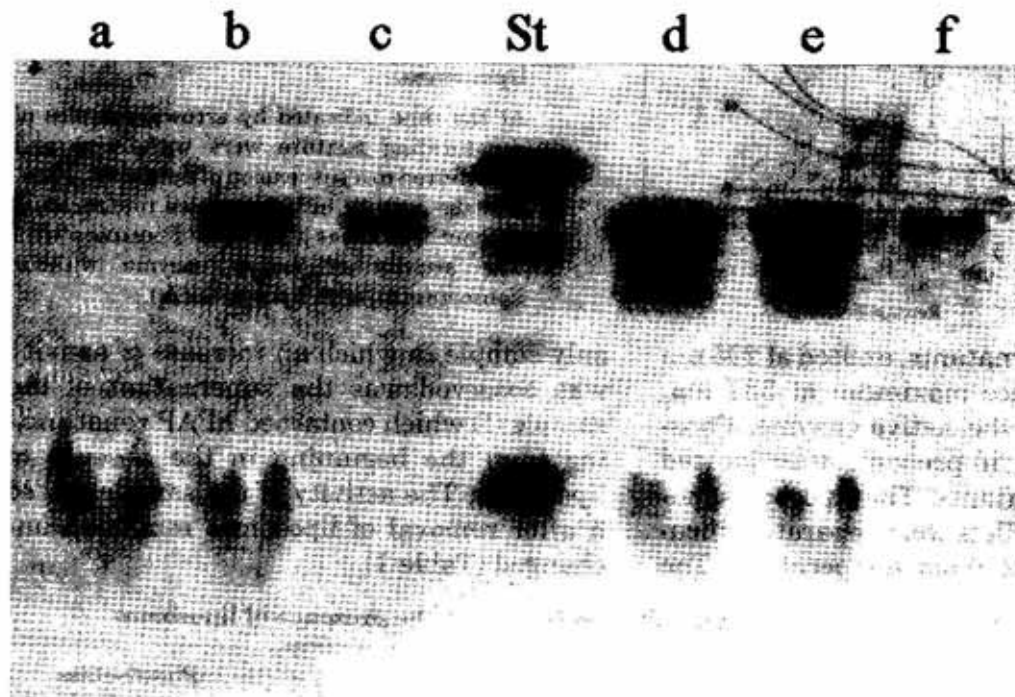


Figure 6. PAGE/SDS electrophoresis of pellets of samples (a–f) (see Table 1) after centrifugation.

Standard proteins (St): bovine serum albumin (67000), IgG (55000 and 25000), ovalbumin (40000) and lysozyme (14300).

any, amount of the native hPAP that was bound to the phosphatidylserine liposomes. On the contrary, the strong bands in the PAGE/SDS gel in samples (d) and (e) suggest that the phosphatase renaturating in the presence of liposomes was trapped into the lipid structure. However, at the same time 70–80% of protein remained in the supernatants after removal of liposomes (Table 1). The question arises why in the soluble fraction of liposome-treated renaturating protein no reactivation of the phosphatase was observed? We tried to find an answer to this question by applying size-exclusion chromatography of the protein remaining in supernatants.

### Size exclusion chromatography of different forms of phosphatase after removal of liposomes

Figure 7 presents a calibration curve of the size-exclusion column (A) and a chromatographic profile of urea-denatured hPAP loaded directly into the column and partially

enzyme (Di), and a fast moving fraction of aggregates (Ag). The supernatants of the samples (a–f) described in Table 1 were analysed in the same system (Fig. 8). Experiments were performed 24 h after the removal of liposomes. In the supernatant of sample (a) no protein fraction has been found. The supernatants of the samples with native hPAP, preincubated with or without liposomes (samples (b) and (c)), show peaks corresponding to the dimeric form of hPAP (about 100000). A soluble part of sample (d) includes a small peak corresponding to the dimeric form of hPAP and a large faster moving fraction of aggregated forms of the protein. In the supernatant of sample (e) that did not regain any catalytic activity, only a fast moving fraction of aggregates was found. The sample (f) which recovered about 70% of the catalytic activity demonstrates a high peak with the retention time corresponding to that of native hPAP and a small peak of the aggregated form. The aggregates are soluble but their relative molecular mass is higher than that of native hPAP. Unfortu-
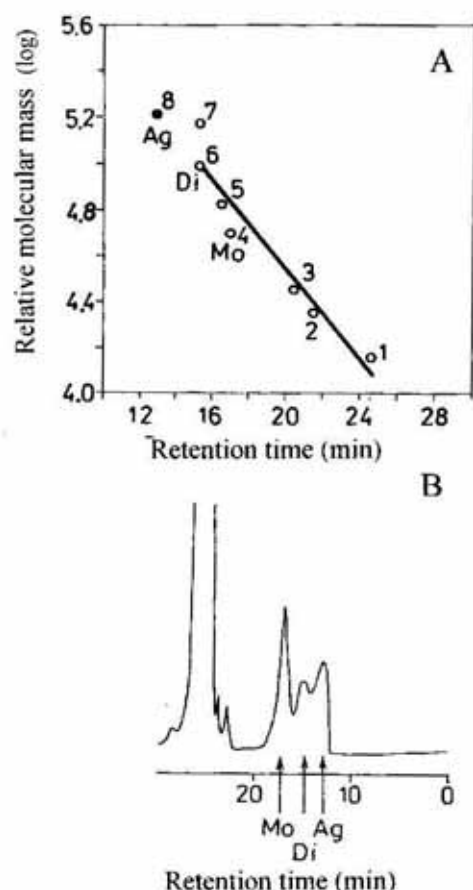
**Figure 7. Calibration of the chromatographic column.**

A. Retention time *versus* relative molecular mass of protein; (1) lysozyme — 24.6 min; (2) trypsin — 21.6 min; (3) carbonic anhydrase — 20.5 min; (4) a peak corresponding to mobility of the monomeric hPAP subunit (Mo) — 7 min; (5) bovine serum albumin —16.5 min; (6) native dimer of hPAP (Di) — 15.3 min; (8) aggregates (Ag) — 12.6 min. B. Chromatographic profile of denatured hPAP renaturating during the chromatographic run; (Mo) subunit of hPAP; (Di) dimeric native hPAP; (Ag) aggregates.

nately, in this chromatographic system, the retention time of the inactive aggregates was beyond the linearity of the calibration curve of the column, and we could only roughly estimate their relative molecular mass as 180000. It might indicate that these inactive complexes are composed of 3 or 4 subunits of hPAP.

## DISCUSSION

Human prostatic acid phosphatase can be reversibly denatured by urea under acidic

conditions [4, 5, 18]. In the previous study [5], we have reported that within several seconds of renaturation of hPAP an inactive intermediate is formed which exhibits circular dichroism and intrinsic tryptophan fluorescence spectra similar to those of the native protein. The chromatographic analysis of the renaturing enzyme that was carried out at this stage of the reaction, revealed the existence of a compact molecule of a size corresponding roughly to the relative molecular mass of the subunit of hPAP. This structure could be unlike native hPAP, easily degraded by trypsin. With the progress of renaturation, this compound was converted into a dimeric, catalytically active, protease resistant form of hPAP [5].

The results described in the previous paper [5] suggested that the observed kinetic intermediate of the refolding hPAP resembles the molten globule state [6, 7]. Experiments carried out in this study were performed to find additional features of this transient structure in the process of hPAP refolding. In the presence of renaturating hPAP the emission intensity of the external hydrophobic probe ANS was enhanced (Fig. 1A). With the process of renaturation, the emission of ANS was gradually decreased (Fig. 1A). Parallelly with the decreasing ANS fluorescence, phosphatase was regaining its catalytic activity (Fig. 1B). As it was shown before [5], the recovery of catalytic activity of hPAP during renaturation is accompanied by an increase in the content of dimeric molecules of hPAP and by disappearance of a monomeric form of the refolding protein which is sensitive to trypsinolysis. Comparing the time scale of reduction of ANS fluorescence and of the decline in the monomer concentration [5], it might be suggested that ANS interacts with the refolding monomer of hPAP and is eliminated from the protein molecule by the process of dimerization of subunits.

Congo Red, a polyaromatic bis azo dye, has been known for years as a dye, which binds to a β-amyloid peptide of neuritic plaques and cerebrovascular amyloid deposits in patients with Alzheimer's disease [19]. Recently, it was found that heat-aggregated immunoglobulins and antibodies involved in the formation of immune complexes may also bind

Congo Red [10–12]. A new mechanism of the dye-protein interaction has been presented [12]. According to this model, micellar forms of Congo Red can generate polymolecular ligand-protein contacts with periodic motifs in the structure, especially with peptide chains of destabilized β-plates in proteins. Estimations of binding of Congo Red to native hPAP suggest that not more than two



Retention time (min)

**Figure 8. Fast size-exclusion chromatography of supernatants of samples (a–f) (see Table 1).**

(Di) dimeric native hPAP; (Ag) aggregates.

individual molecules of the dye can interact with a subunit of native hPAP. On the other hand, the renaturating hPAP at the initial phase of the reaction binds 7–8 molecules of the dye per monomer of the refolding enzyme (Fig. 4). The progress of renaturation, reactivation (Fig. 4B) and dimerization [5] of the enzyme results in a decreased number of the protein-bound Congo Red molecules (Fig. 4B). A dimer of rat prostatic acid phosphatase — a protein highly homologous to the human enzyme and of similar three-dimensional structure [20, 21] as is formed through the interaction of subunits by edge strands of β-sheets extending the seven-stranded sheet in the monomer into a 14-stranded β-sheet in the dimer [20]. It is likely that binding of Congo Red to the partially refolded subunit of hPAP illustrates an interaction between a supramolecular, micellar form composed of 7–8 molecules of Congo Red and the relaxed fragment of β-sheet in a peptide monomer. The formation of dimer results in recovery of the enzymatic activity and possibly it fixes mutual positions of β-strands due to subunit-subunit interactions, and results also in the inability of this species to bind micellar structures of Congo Red. This suggestion is confirmed by the observation that the Congo Red dye arrests the reactivation of the denatured enzyme (Fig. 3). Probably binding of the dye with the loosen β-sheet structure prevents formation of a native-like pattern of β-sheets, and, as a consequence, formation of the active dimer.

A significant conclusion of the investigations described previously [5] and presented above is that the kinetic folding intermediate, observed during hPAP renaturation exhibits properties expected for a molten globule [6, 7]: (i) it shows a native-like near UV-CD spectrum with a broad band in the region of 210–230 nm [5]; (ii) it shows a native-like tryptophan fluorescence emission spectrum with a fluorescence intensity maximum at 337 nm [5]; (iii) its high sensitivity towards the protease reflects its structural flexibility [4, 5]; (iv) it binds ANS to hydrophobic regions; (v) it binds Congo Red probably to the relaxed structure of the β-sheet. Size exclusion chromatography indicates that the size of this intermediate is almost as
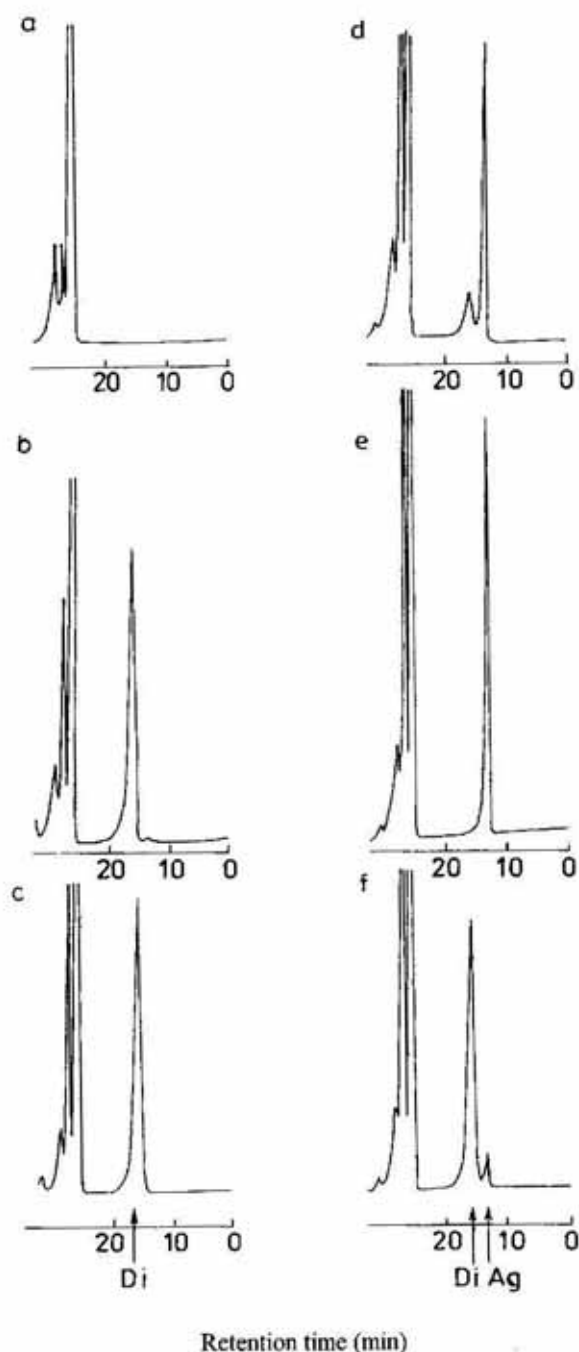
large as should be the size of the partially folded subunit of hPAP [5]. During the refolding performed in the renaturation buffer containing salts and a residual amount of urea, the dimerization of this intermediate was observed [5] as well as reconstitution, with a high yield of the active enzyme (Table 1).

The prostatic acid phosphatase is an example of a secretory homodimeric protein. It is synthesized on ribosomes as a precursor protein with a 32 amino acids long signal sequence [22]. This protein is highly glycosylated [2, 3], then probably folded, assembled and secreted from the gland [23, 24]. The question emerges what is the relation between the spontaneous and almost perfect refolding of this type of oligomeric protein in water solution, in a test tube, and a similar process of folding of the mature protein *in vivo*, in the lipid membranes environment. That is why experiments of renaturation in the presence of artificial membrane structures were designed. The low pH on the surface of anionic phospholipids might induce changes in the native protein structure that facilitate protein insertion into membrane, as it was observed in the case of colicin A [25] or α-lactalbumin [26]. Negatively charged phosphatidylserine liposomes that were used in this study probably do not induce substantial conformational rearrangements within a native hPAP molecule, at least no influence on catalytic activity of enzyme or trapping of the native hPAP into liposomes was observed (Fig. 5). On the other hand, the liposomes inhibit the process of reactivation of denatured hPAP (Fig. 5). A small amount of the refolding hPAP appears to bind to lipid bilayer, but the majority of the renaturating protein forms, in the presence of liposomes, water soluble, inactive aggregates (Fig. 8). Binding and insertion of molten globule-like conformations of proteins into lipid bilayers is well documented [27–29]. Studies on proteins refolding in the presence of lipid structures also suggest that the formed lipid/protein or detergent/lipid micelle complexes might sometimes serve as a template for protein refolding [30, 31]. Additionally, they imply that interaction of amphipathic molecules should be taken into account in considering in the process of three-dimensional formation protein structures.

Proteins entering the secretory pathway are translocated, as at least partially unfolded entities, across the membrane of endoplasmic reticulum through hetero-oligomeric transmembrane protein channels [27]. The translocated proteins fold and assemble within the lumen of the endoplasmic reticulum [28], probably with the help of chaperons which facilitate and control the efficient folding of peptides emerging on the *trans* side of the membrane [29]. The function of chaperons in folding of the monomeric enzymes: dihydrofolate reductase [32], rhodanase [32, 33] and in folding and assembling of oligomeric proteins: citrate synthase [34], bacterial luciferase [35], ribulose 1,5-bisphosphate carboxylase-oxygenase [36] has been demonstrated in experiments *in vitro* and *in vivo*. The results presented in this paper might support the necessity of the chaperone-mediated guidelines of proteins, necessity of preventing premature, inappropriate folding and false assembling of proteins in proximity of the membraneous structures. But, so far, there is no answer why the partially unfolded hPAP molecules which escaped trapping into the lipid bilayer ratchet are unable to reconstruct the active enzyme in water solution.

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