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Structural Properties of the Human Acidic Ribosomal P Proteins Forming the P1–P2 Heterocomplex

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The ribosome has a morphologically distinct structural feature called the stalk, recognized as a vital element for its function. The ribosomal P proteins constitute the main part of the eukaryotic ribosomal stalk, forming a pentameric structure P0–(P1–P2)$_2$. The group of P1/P2 proteins in eukaryotes is very diverse, and in spite of functional and structural similarities they do not fully complement one another, probably constituting an adaptive feature of the ribosome from a particular species to diverse environmental conditions. The functional differences among the P1/P2 proteins were analysed *in vivo* several times; however, a thorough molecular characterization was only done for the yeast P1/P2 proteins. Here, we report a biophysical analysis of the human P1 and P2 proteins, applying mass spectrometry, CD and fluorescence spectroscopy, cross-linking and size exclusion chromatography. The human P1/P2 proteins form stable heterodimer, as it is the case for P1/P2 from yeast. However, unlike the yeast complex P1A–P2B, the human P1–P2 dimer showed a three-state transition mechanism, suggesting that an intermediate species may exist in solution.

Key words: ribosome, ribosomal P protein, stalk.

Abbreviations: CD, Circular dichroism; PBS, phosphate-buffered saline buffer; FPLC, fast protein liquid chromatography; bis-ANS - (4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; MS, mass spectrometry.

The ability of proteins to bind to one another in a highly specific manner is an important feature of a variety of biological functional complexes and also is the basis of regulatory mechanisms in numerous biological processes. The ribosome, a macromolecular machine *par excellence*, is composed of tens of proteins interacting with one another and with an intricate mesh of rRNAs represents a very sophisticated biological complex, where interplay among all its components constitutes the crucial element in its performance (*1*). One site, where extensive protein–protein interactions take place is the GTPase-associated-centre, recognized as a landing platform for translation factors (*2*). This center forms, on the 60S ribosomal subunit, a very conserved, morphologically distinct feature with a prominent structure called the stalk. The stalk represents the only ribosomal structure made exclusively of proteins, which occur in multiple copies (*3–6*). In prokaryotic cells, the stalk is composed of L12 protein homodimers anchored to the ribosome through the L10 protein, which together with L11, forms the stalk base. A species-dependent variability has been documented in the stoichiometry of the stalk complexes, showing a lack of uniformity for prokaryotic ribosomes in respect to L12. In thermophiles, there are three, whereas mesophiles have two L12 dimers, forming heptameric L10(L12)$_6$ or pentameric L10(L12)$_4$ complexes, respectively (*7*, *8*). However, for proper functioning of the ribosome only one dimer is enough (*9*), implying that additional L12 dimers may have arisen as regulatory elements. The eukaryotic stalk structure is composed of P proteins that have little similarity in the primary structure to the prokaryotic counterparts, but probably are of alike molecular organization to the bacterial stalk, having the same function on the eukaryotic ribosome. There are two types of P proteins. The first one comprises two small 11 kDa proteins P1 and P2, representing eukaryotic orthologs of the L12 protein, and the second group contains only one P0 protein equivalent to the prokaryotic L10. The P proteins form a pentameric structure P0–(P1–P2)$_2$ on the ribosome (*10*, *11*), where the P1/P2 proteins constitute biologically relevant heterodimers P1–P2 (*12–17*), attached to P0 through the P1 proteins (*15*, *17*). The stalk is a vital element for cell survival (*18*), but the P1/P2 proteins are not absolutely required for the proteosynthetic activity of the ribosome or cell viability (*19*), unlike the P0 protein which is essential for protein synthesis and cell viability (*20*). Beside their primary function on the ribosome in supporting the basic mechanism of protein synthesis, recent reports indicate that P1/P2 proteins may have additional function (*21*), for example, in facilitating translation of certain mRNAs (*22*, *23*).

The tertiary structure of the P proteins has not been fully described, in contrast to their prokaryotic orthologs

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whose structural organization at the atomic level is known (*8*, *24*). Biophysical analyses of lower eukaryotic proteins (yeast P1A and P2B) brought a preliminary structural characterization of the P proteins. The individual P proteins showed characteristics of 'molten globule'-like structures (*25*, *26*), but together the P1A and P2B protein formed a stable entity without additional ribosomal components (*27*), and the overall structure of which could be described as rod-like (*28*). This dimer is thought to be the key element in the formation of the pentameric stalk structure and confers full functionality on the stalk; on the other hand, the second yeast dimer P1B–P2A is rather a regulatory element (*21*), and this pair of proteins probably can shuttle between the ribosomal and the cytoplasmic pool of the P proteins (*29*).

In the course of a study, on the ribosomal stalk, we have focused our attention on the human P1/P2 proteins. Previously, we have shown that human P proteins preferentially form the P1–P2 heterodimer (*12*). Higher eukaryotes have only single species of the P1 and P2 proteins, unlike yeast, in which two variants of each are present. Consequently, there is only one type of the P1–P2 heterodimer present on the human ribosomes, in contrast to the yeast one that contains the P1A–P2B and P1B–P2A dimers of slightly different function (*21*). Here, we present experimental data, obtained with the aid of different biophysical methods, which indicate that the human P1–P2 dimer is able to adopt a stable structure without additional ribosomal components, as its yeast counterpart P1A–P2B, but some features distinguish the human dimer from the yeast heterocomplex.

MATERIALS AND METHODS

Expression, Purification of Recombinant Human P Proteins, and Complex Preparation—Recombinant human proteins P1 (GenBank accession number NP_000994) and P2 (GenBank accession number NP_000995) were prepared according to the procedure established previously for the yeast P proteins (*30*). Briefly, DNA was amplified from a cDNA library of HeLa cells with the aid of the polymerase chain reaction (PCR) using specific primers; for P1 (forward, 5′-gga gaa ttc gca tgg cct ctg tct ccg agc tc-3′, reverse 5′-gcg gga tcc cat gtt ata aaa gag gtt tag tc-3′), and for P2 (forward 5′-gcg gca tcc cgc tac gtc gcc tcc tac-3′, reverse 5′-gcg aag ctt tta atc aaa aag gcc-3′). The amplified PCR fragments for P1 and P2 were introduced into expression vectors pLM1 and pQE30, respectively. The proteins were expressed in *Escherichia coli* BL21(DE3) cells and P1 protein was purified by procedure as described previously (*30*). P2 was expressed as a fusion protein with 6xHis at the N-terminus, and was purified on by affinity chromatography on the Ni-column (Sigma-Aldrich). The human P1–P2 heterocomplex was prepared by following the denaturation/renaturation procedure established for the yeast complex (*13*, *27*).

Circular Dichroism Spectroscopy—Circular dichroism (CD) spectra were collected on a Jasco J-720 spectropolarimeter equipped with a PTC-343 Peltier-type thermostatic cell holder. Far-UV CD spectra were usually acquired at 20°C, and the protein concentration was 50 μg/ml, using PBS buffer (phosphate-buffered saline) (pH 7.5), supplemented with 10 mM $MgCl_2$. For pH 2, glycine buffer was used (25 mM glycine, pH 2.1, 150 mM NaCl and 10 mM $MgCl_2$). The analysis was carried out in a cuvette with a 1 mm path. The obtained spectra are averages of three scans and are reported as the mean residue ellipticity [Θ] (degrees per square centimetre per decimole), the data were calculated as described before (*27*).

CD temperature melting curves were determined by monitoring changes in the dichroic intensity at 222 nm as a function of temperature. Thermal denaturation experiments were performed in the range of 20–90°C and a heating rate of 0.5°C/min. Thermal scans were collected using 1 mm cells at a protein concentration of 50 μg/ml. The reversibility of the thermal transition was checked after cooling the thermally denatured sample and subsequently recording a new scan under the same conditions. Analysis of urea-induced equilibrium unfolding was carried out as presented earlier (*27*).

Size-exclusion Chromatography (SEC)—An Akta Purifier FPLC system from GE Healthcare Life Sciences, equipped with the Superose 12 HR 10/30 FPLC gel filtration column was used. For native conditions, the column was equilibrated with a buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$; for denaturating conditions the column was equilibrated with the aforementioned buffer supplemented with 8M urea and the protein sample was equilibrate for 12 h in denaturating buffer. The flow rate was 0.2 ml/min. The protein elution profile was monitored at 280 nm and analysed using UNICORN program v. 4.0 supplied with the FPLC system.

Fluorescence Spectroscopy—Fluorescence spectra were recorded on a Perkin-Elmer luminescence spectrometer (LS50B). Samples of proteins were prepared at 4.5 μM in PBS buffer or 20 mM Tris–HCl (pH 7.8) and 150 mM NaCl, and all buffers were supplemented with 10 mM $MgCl_2$. Samples were excited at 280 nm for tryptophan and tyrosine or at 295 nm for tryptophan fluorescence only. The intrinsic fluorescence emission spectra were collected in the 295–450 or 310–450 nm range, respectively, at a scan speed of 100 nm/min. The bis-ANS binding studies were performed using 5 μM bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid) and 0.5 μM of protein. Fluorescence emission spectra of the bis-ANS were collected from 420 to 600 nm, at a scan speed of 100 nm/min and excited at 380 nm for direct excitation. In energy transfer experiments, the sensitized fluorescence was recorded from 295 nm to 540 nm, with excitation at 280 nm.

Mass Spectrometry—Protein solutions were analysed by using a modified Q-ToF 2 mass spectrometer (Waters, Manchester, UK) described in (*31*). For the analysis, protein solutions were exchanged into 200 mM ammonium acetate (pH 7.5) and aliquots of ~2 μl were introduced into the mass spectrometer via nanoflow capillaries. The following experimental conditions were used: capillary voltage 1.2 kV, extractor voltage 2 V, cone voltage 80 V, accelerating voltage was varied at 4–120 V

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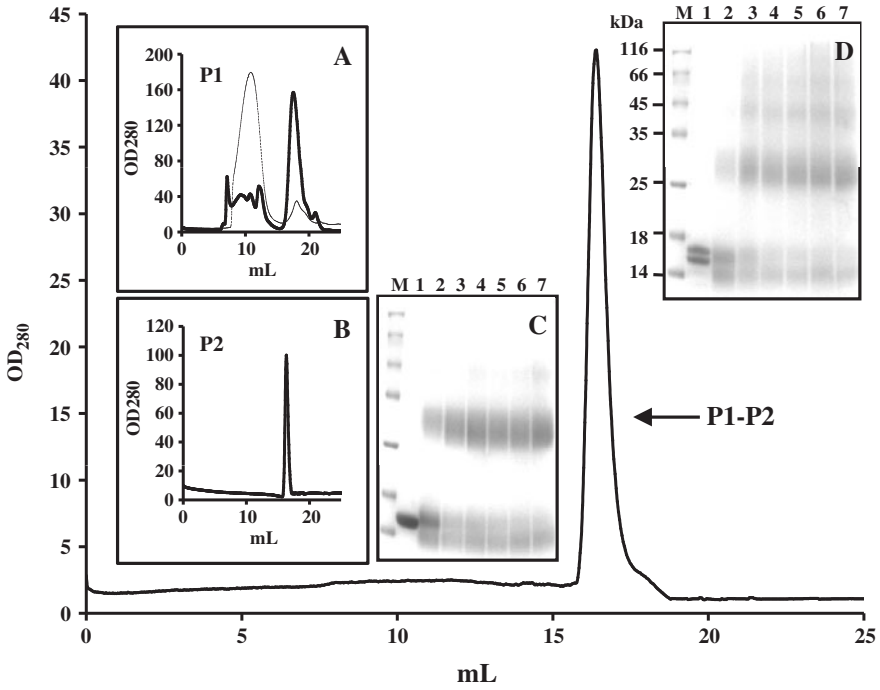

Fig. 1. **Size exclusion chromatography (SEC) of recombinant human P1/P2 proteins.** Positions of the P1–P2 heterodimer is marked with an arrow. (A) SEC analysis of P1 protein. Thin line represents the elution profile in native conditions, the thick one shows the elution of P1 in denaturating conditions. (B) SEC analysis of P2 protein. (C and D) cross-linking experiment of recombinant P2 and P1–P2 complexes, respectively. M, molecular mass markers; lanes 1–7, time titration of the proteins with glutaraldehyde, 0, 5, 10, 15, 20, 30, 60 min.

at an argon pressure of 3.0×10^{-2} mbar in the collision cell to study in-cell CID. Pressure at the ion transfer stage was maintained at 1.81×10^{-2} mbar for yeast and human P1/P2 dimers and at 5.52×10^{-3} mbar for human P1/P2 dimer. The pressure at the front end of the instrument was adjusted for optimal signal.

Protein Analysis—Protein concentration was determined from the absorbance at 280 nm using an extinction coefficient calculated from the amino acid composition of the human P1/P2 complexes, following published method (*32*). Chemical cross-linking of the recombinant ribosomal human P proteins was performed with glutaraldehyde as previously described (*13*). SDS-PAGE in 14% acrylamide slab gels was carried out according to the method of Laemmli (*33*).

RESULTS

Expression and Preliminary Characterization of Human P1/P2 Proteins—The human P1/P2 proteins were cloned, expressed and purified as described in MATERIALS AND METHODS section. SEC analysis showed that in native conditions pure P1 is eluted as a very broad peak at the void volume of the column, indicating that the protein occurs as a set of oligomeric forms with a molecular mass in the rage of hundreds of kDa (Fig. 1, inset A). On changing the protein environment in the SEC analysis to denaturing conditions, some portion of the P1 protein eluted as a sharp peak with a low molecular mass, showing that the protein was converted to the monomeric form (Fig. 1, inset A). On the other hand, the P2 protein showed a single symmetrical peak in the SEC analysis, demonstrating that it occurs in a monodisperse form (Fig. 1, inset B). Additionally, cross-linking gave a clear indication that P2 can form homodimers (Fig. 1, inset C). The human P1–P2 heterodimer was obtained after denaturation/renaturation procedure of a mixture of P1 and P2 proteins, as reported for P1/P2 proteins from other species, which ensures the biological activity of the complex (*27*, *34*). SEC analysis showed that the P1–P2 complex predominantly exists in a monodisperse form (Fig. 1), and cross-linking approach demonstrated that the dimer represents the dominant fraction, with some higher oligomeric forms visible as well (Fig. 1, inset D).

Mass Spectrometry Analysis—Figure 2 shows mass spectra of yeast and human P1–P2 heterodimers and human P2–P2 homodimer at accelerating voltages of 4 V and 40 V. In all cases, the dimers represent the dominant form. The charge states observed for the yeast P1A–P2B dimer range from 9+ to 11+ and for both human homo- and hetero-dimers from 10+ to 12+. For all protein dimers, increasing the accelerating voltage from 4 V up to 120 V resulted in an increase of peak intensities for the dissociated proteins in the spectra (data not shown). For all the samples, no dimers were observable at accelerating voltages above 80 V (data not shown). Representative data are presented in Fig. 2, showing dissociation of the protein dimers at the accelerating voltage of 40 V, giving peaks for highly charged monomers and monomers at lower charge states. For example, human P2 homodimer at 11+ charge state dissociates to

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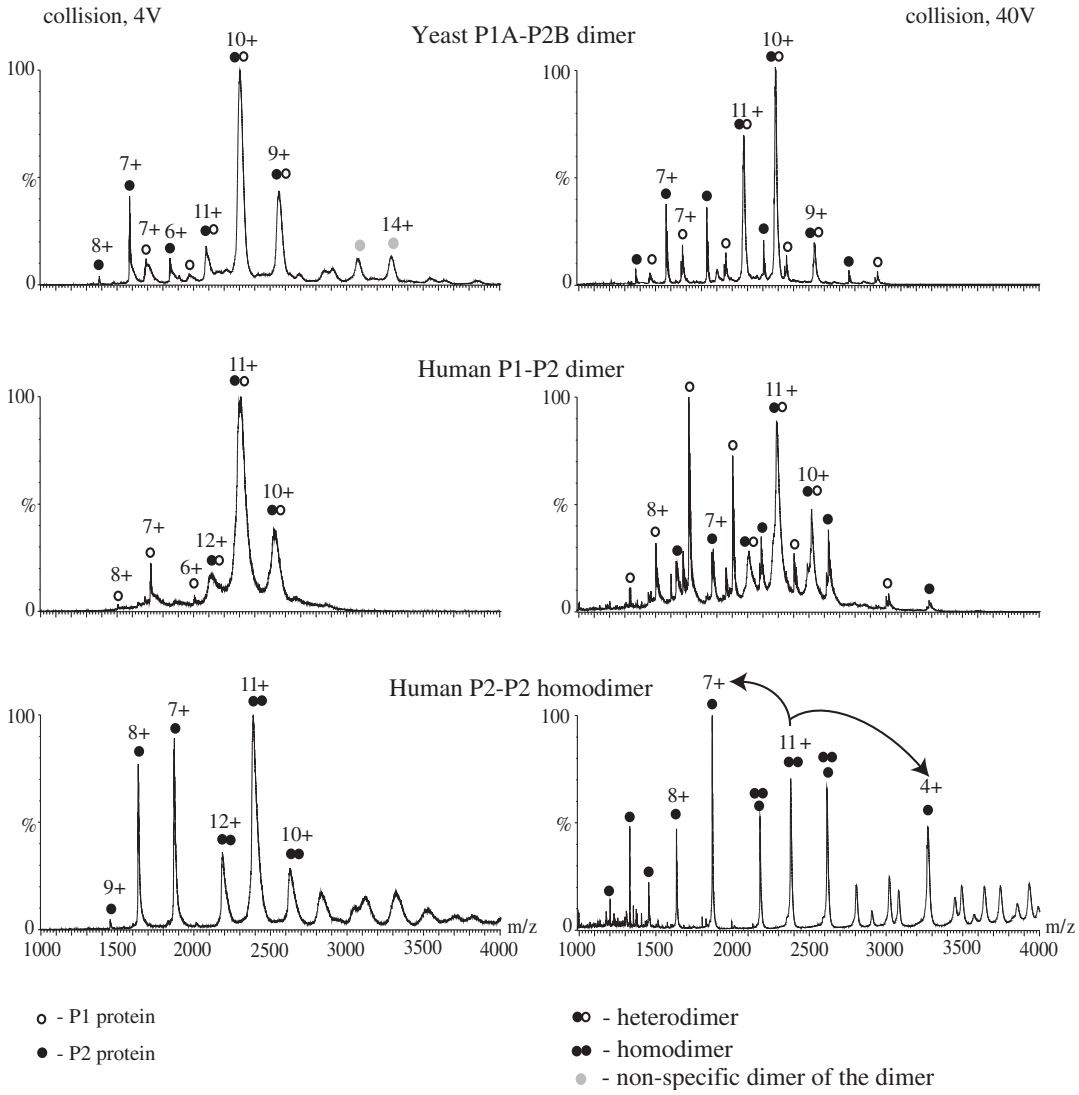

Fig. 2. **Mass spectrometry analysis of human and yeast P1/P2 complexes.** Left panel, accelerating voltage 4 V, right panel, 40 V.

P2 monomers at charge states of 7+ and 4+, as indicated by arrows. On the same spectrum the 6+ and 5+ charge states of the P2 monomer overlap the 12+ and 10+ charge states of the P2–P2 homodimer. The accelerating voltage at which both homo- and hetero-dimers begin to dissociate is approximately the same. The human P2–P2 homodimer appears less stable in the gas phase than the human P1–P2 heterodimer, as the relative intensity of the monomer/dimer signals of the human P2–P2 homodimer was found to be higher at the accelerating voltage 4 V and 40 V. Comparing the yeast and human P1–P2 heterodimers, the spectra for those complexes are similar in terms of their behaviour at low and higher accelerating voltage. The heterodimers begin to dissociate at approximately the same accelerating voltage of 40 V, indicating that the analysed complexes exhibit similar stability in the gas phase.

CD Spectroscopy—A preliminary CD analysis showed that individual human P proteins exhibit a curve pattern characteristic for α-helical proteins, especially P2 had two well-recognized minima, a distinctive feature for proteins where α-helices constitute the major component (Fig. 3). The α-helical content, calculated on the basis of the far-UV CD signal recorded at 222 nm, was 74 and 52% for P1 and P2 proteins, respectively. In the case of the P1–P2 heterodimer, the spectra exhibit features typical for α-helical proteins as well, where α-helices constitute about 49%. The obtained values are in good agreement with the previously published data, for the yeast heterocomplex (*27*).

To evaluate the structural stability of the human homo- and hetero-complexes, equilibrium denaturation by urea was applied. The changes in CD far-UV spectra at 222 nm were collected as a function of urea concentration. The analysis showed that the heterodimer is characterized by fully cooperative denaturation, with a two-state transition mechanism. The unfolding of the complex started at 2.5 M urea and was completed at

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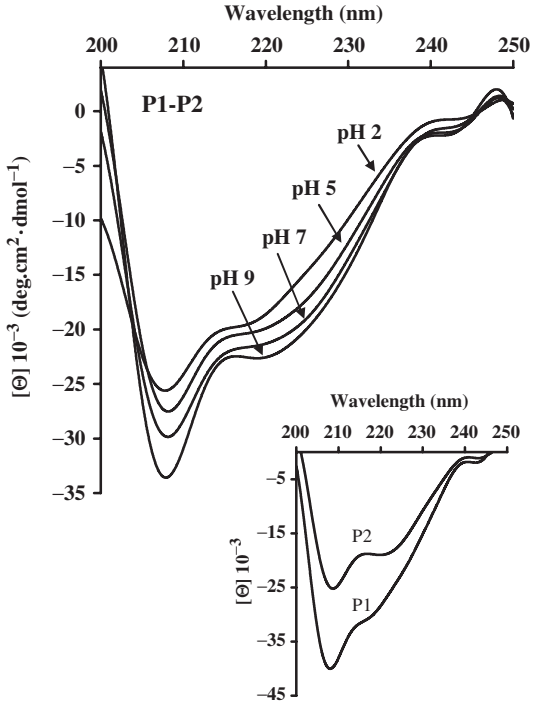

Fig. 3. **Far-UV CD spectroscopy of P1–P2 heterocomplex at various pH values.** Inset, CD spectra of individual P1 and P2 proteins.

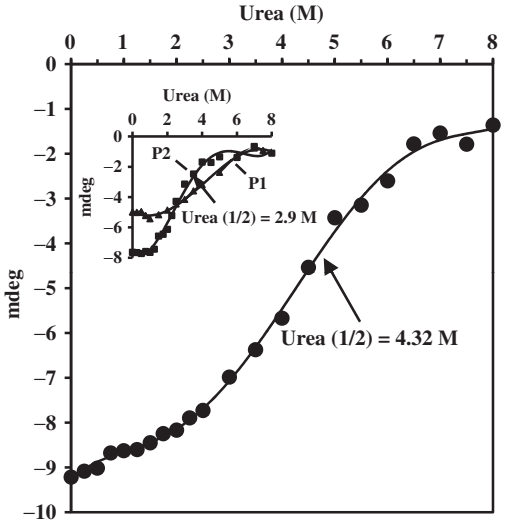

Fig. 4. **Changes in CD at 222 nm of P1–P2 heterodimer as a function of urea concentration.** Inset, CD data recorded at 222 nm of individual P1 and P2 proteins versus urea concentration. The transition midpoints for P2 dimer and P1–P2 heterodimer are indicated by arrows.

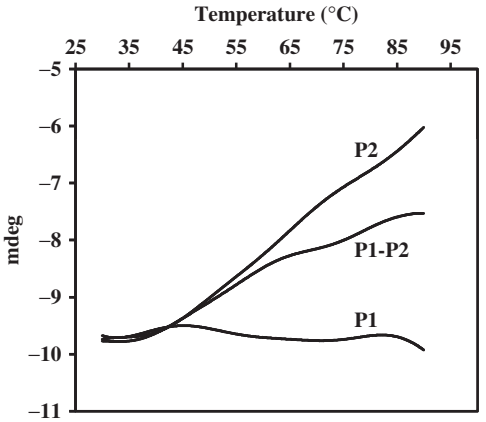

Fig. 5. **Thermal unfolding of human P1/P2 proteins.** Ellipticity at 222 nm was monitored as a function of temperature at neutral pH.

6.5 M urea, with the transition midpoint at 4.32 M urea (Fig. 4). The free energy difference in the absence of denaturant ΔG_{H2O} was calculated to be 2.26 kcal/mol. The equilibrium denaturation experiment indicates that the P1–P2 dimer is a stable protein complex, which may adopt its tertiary structure without additional ribosomal components. Interestingly, the unfolding of P2 also resembles cooperative behaviour, exhibiting a typical two-state transition mechanism, but in this case the unfolding started at 1.25 M urea and was completed at 4 M, with the transition midpoint at 2.9 M urea (Fig. 4, inset), indicating that the P2 protein might exist as a stable dimer but with the structural stability lower than that of the heterodimer supporting the observation made with the MS analysis. Rather intriguing data were obtained in a thermal denaturation experiment. Thermal unfolding of P proteins was followed by monitoring changes in dichroic intensities at 222 nm upon heating from 25°C to 95°C (Fig. 5). P1 did not show any significant changes in the entire range of the temperature, indicating that it was already in an aggregated form, and was resistant to heat denaturation. In contrast, the P2 protein showed thermal denaturation in the whole range of the applied temperature, but the unfolding was non-cooperative, resembling the profile characteristic for molten globule-like proteins, as previously reported for individual yeast P1/P2 proteins (*25*, *27*). The P1–P2 heterocomplex showed cooperative thermal unfolding, but the curve had a distinct inflection point separating two unfolded phases (Fig. 5). This indicates that unfolding of the heterocomplex is not a two-state process and the transition curve could be fitted rather to a three-state folding-unfolding transition. Such behaviour suggests that the P1–P2 complex may have two independently folding domains, probably belonging to the two monomers that constitute the dimer. This situation is different from that previously observed for the yeast P1A–P2B heterodimer, which showed a two-state transition (*27*).

Intrinsic Fluorescence Properties—Fluorescence analysis is a very sensitive tool to follow minute changes in the tertiary structure organization of proteins. It could very conveniently be applied to the acidic ribosomal P protein complexes, with only one tryptophan residue present in the human P1 protein at position 43, making the intrinsic fluorescence of the dimer an excellent probe for investigating conformational changes in the environment around that fluorophore. Initially, fluorescence

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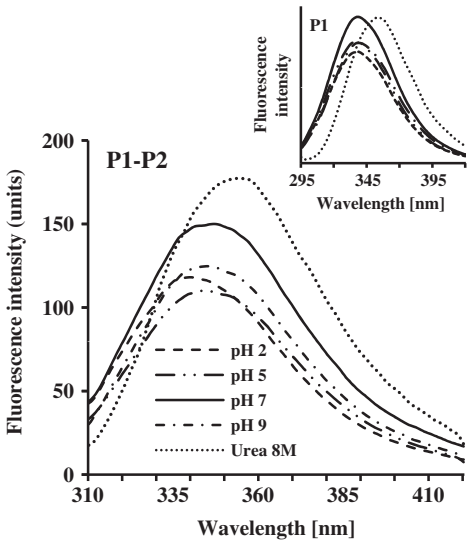

Fig. 6. **Intrinsic fluorescence of P1–P2 heterocomplex at various pH values.** Excitation at 295 nm. Inset, intrinsic fluorescence of P1 protein.

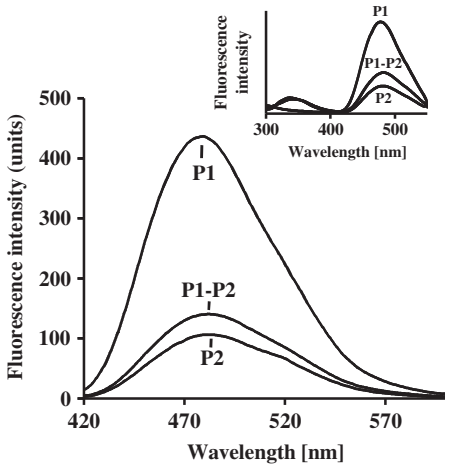

Fig. 7. **Fluorescence spectra of bis-ANS-labelled P1 and P2 proteins.** Inset, energy transfer from the intrinsic fluorophores to bis-ANS using excitation at 280 nm.

emission spectra of the P1–P2 dimer were collected at different pH values, using an excitation wavelength of 280 nm and 295 nm for tyrosine and tryptophan, and for tryptophan, respectively. At neutral pH, the emission spectrum characteristic for tryptophan (excitation at 295 nm) was centred at 347 nm, at the alkaline pH there were no significant perturbations in the emission spectra, showing a maximum at 345 nm, but the fluorescence intensity was slightly decreased. Exposition of the heterodimer to pH 2 shifted the fluorescence maximum toward shorter wavelengths centred at 339 nm. In 8 M urea, the dimer was completely unfolded, exhibiting a fluorescence maximum at 355 nm, diagnostic of an indolic group completely exposed to the solvent (Fig. 6). A similar fluorescence pattern was observed for excitation at 280 nm (data not shown). In contrast to the dimer, the P1 protein alone showed no changes of the fluorescence maximum (excitation at 295 nm) at various pH. The maximum was centred at 338 nm (Fig. 6, inset), which indicates that the protein was in an aggregated form, supporting the analyses presented earlier. The P2 protein was not studied because it does not contain tryptophan residue, which is suitable for fluorescence analysis.

In order to study the assembly of the hetero- and homo-dimers, changes of the solvent accessibility surface area of hydrophobic amino acids were analysed by binding of a fluorescent dye, bis-ANS. Upon non-covalent binding to hydrophobic parts of a protein the two naphtyl rings of the bis-ANS molecule become oriented in parallel, which brings about a significant increase in fluorescence quantum yield; therefore, fluctuating globular structures, in which the hydrophobic core is not yet complete (molten globule-like structures), are very sensitive to the bis-ANS probe. Fig. 7 shows that the human P1 and P2 proteins bind the bis-ANS probe in a completely different way. The increase in the quantum yield of the bis-ANS fluorescence upon binding to P1 is about four times greater than upon binding to P2. The P1–P2 heterodimer binds the chemical probe less efficiently comparing to the P1 protein, but slightly better than the P2 homodimer. bis-ANS can be excited either directly or indirectly via energy transfer from intrinsic fluorophores. Using the energy transfer approach with excitation at 280 nm, the chemical probe was sensitized to similar extend as in the direct excitation analysis, underscoring the fact that much more energy is transferred to bis-ANS from the P1 protein (Fig. 7, inset), indicating a lack of a well-formed hydrophobic core in this protein.

The stability of the human P protein complexes was studied as a function of urea concentration by steady-state fluorescence, following either fluorescence emission intensity at the constant wavelength of 315 nm to avoid the effect of urea influence on the wavelength, or fluorescence maximum (Fig. 8). The analysis of the P1–P2 heterodimer showed that the transition resembled three-state folding–unfolding process, with a prominent inflection point separating two unfolded phases in the unfolding curve (Fig. 8A). Interestingly, in the range of urea concentrations of 0–1 M, there was an immediate loss of fluorescence intensity accompanied by a partial shift in the emission maximum (Fig. 8A, inset), indicating simultaneous changes in the quaternary and tertiary structures of the complex. The further loss of fluorescence intensity in the range of 1–2 M urea was not accompanied by a shift in the emission maximum, demonstrating that the structural changes were mainly restricted to the quaternary structure. In the range of 2–3 M urea, a plateau in fluorescence intensity was observed, but it was accompanied by changes in the emission maximum that reached plateau at 354 nm at 3 M urea (Fig. 8A, inset), showing that the complex has lost its tertiary fold, evidenced by the full exposition of the tryptophan to the solvent. The results of the urea unfolding analysis followed by steady-state fluorescence did not correspond to those worked out by CD spectroscopy, which showed a typical two-state mechanism.

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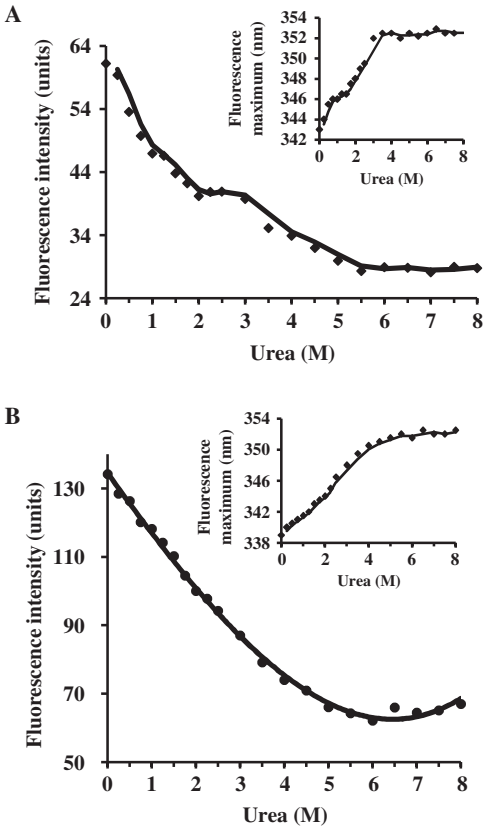

Fig. 8. **Equilibrium unfolding of P1–P2 and P1 protein complexes by urea.** The unfolding was monitored by measuring intrinsic fluorescence intensity at 315 nm (main graph) and emission maximum (inset). (A) P1-P2 heterocomplex. (B) P1 complex.

Please note that in the range of urea concentration of 0–2.5 M, there were no significant changes in CD spectra indicating a lack of alterations in the secondary structure, but the fluorescence spectra underwent the most significant changes, indicating that alterations in quaternary/tertiary structures in the absence of secondary structure modifications. This suggests that denaturation of the heterodimer might proceed through an intermediate species. In contrast, the P1 protein showed non-cooperative unfolding when fluorescence emission intensity or maximum were followed (Fig. 8B).

DISCUSSION

The human P1/P2 proteins represent a group of conserved eukaryotic proteins, components of the ribosomal GTPase-associated-centre (*2*, *6*). However, in spite of the functional and as it is believed also structural similarity, there are differences among eukaryotic P1/P2 proteins. First of all, the number of proteins in the P1 and P2 groups is variable: in mammals there are two polypeptides only, P1 and P2, but lower eukaryotes such as *Saccharomyces cerevisiae* or *Candida albicans* have four (P1A, P1B, P2A and P2B) (*16*) and *Schizosaccharomyces pombe* seems to have even six P1/P2 proteins, three in either group (*35*). Plants have four, with an additional P3 group (*36*). However, what is more important, the P proteins from distinct species are not fully complementary in terms of function, and it seems that they have diverged in ribosome evolution, and apart from their primary function might also represent regulatory element enabling distinct organisms to accommodate their metabolism to changing environmental conditions (*12*, *37–39*).

In this report, we present a biophysical analysis of the human P1/P2 proteins, providing new data on the molecular diversity among acidic ribosomal proteins. As has previously been reported, the human P proteins preferentially form the P1–P2 heterodimer (*12*), as it is also the case for other P proteins, but the human ones were not further characterized. The presented analyses show that the P1–P2 complex represents the most stable form indeed, similarly to the yeast P1A–P2B dimer, but new features were observed unrecognized in the yeast heterodimer (*27*).

The analyses of individual human P1 and P2 proteins have shown that they behave in a completely different manner. The P1 protein has a high propensity to aggregate, as showed by SEC analysis, similarly to its yeast counterpart. Additionally, the CD and fluorescence analyses showed that P1 protein exhibits non-cooperative low level of structure complexity, which can be described as molten globule-like structure, similarly to the yeast P1A protein (*26*, *27*). Thus, human P1 has similar properties to P1 proteins from other species, probably because it constitutes the link between the P1–P2 dimer and the P0 protein (*15*, *17*), and therefore is subjected to evolutionary pressure to maintain its primary structural function. On the other hand, P2 protein showed a biophysical profile unlike that of P1. The protein was able to form a fully soluble dimer, as shown by the cross-linking and MS analyses, and in the SEC analysis the homodimer exhibited a single symmetrical peak, indicating that the complex had monodisperse characteristics in solution. Additionally, the urea unfolding CD approach demonstrated that the P2 homodimer behaved in a cooperative manner, thus suggesting that the P2 protein may exist in solution as a stable homodimeric complex. However, unfolding experiment showed that P2 possesses much lower stability than human P1–P2 heterodimer. This feature significantly distinguishes the human P2 protein from yeast P2B, which is unable to exist in a stable dimeric form, but is similar to the behaviour of the yeast P2A or silkworm P2 proteins, which are able to form homodimers as a monodisperse species in solution (*13*, *14*). However, the human P1–P2 heterodimer represents a dominant entity, as shown by the SEC and MS analyses, but a tendency for aggregation was noticed, by the cross-linking experiment, where several minor oligomeric forms were seen, indicating that the heterodimer might not be a fully stable form. Nevertheless, the complex has a well-formed tertiary structure as could be inferred from the CD and fluorescence analyses, showing fully cooperative folding/unfolding properties. Therefore, the P1–P2 complex, having a much higher stability than the P2 homodimer, represents the energetically preferred structural form for the human P1/P2 proteins, and its structural stability resembles that found for the yeast complex, with ΔG_{H2O}

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of 2.26 kcal/mol versus 2.37 kcal/mol (*27*), respectively. However, the most interesting data were provided by equilibrium denaturation by urea followed by measuring steady-state fluorescence (Fig. 8). The loss of fluorescence intensity upon urea denaturation of the heterocomplex may suggest that its unfolding proceeds via a three-state transition mechanism, where the quaternary and tertiary structures of the complex undergo significant parallel/unparallel changes, suggesting that an intermediate species with two independently folding domains may exist in solution. At this stage of analysis, it is difficult to evaluate whether this transient form is monomeric or dimeric. The fluorescence analysis was also supported by the CD/thermal unfolding experiment, which showed a three-state transition, once again underscoring the fact of existence of the intermediate species in solution (Fig. 5).

This is the first evidence showing that the P1–P2 heterodimer of the human ribosomal P proteins may possess intermediate species in solution, which are probably represented by two independently folding domains, which might belong to the P1 and P2 proteins. The P2 protein requires special attention. Our analyses indicate that human P2 may exist in solution as a properly folded monodisperse homodimer. Since the P1–P2 heterodimer may have intermediate species in solution, as suggested by the observed three-state transition, one is tempted to conclude that the P2 protein may be able to leave the heterodimeric complex at some stage of its activity and exist independently. This idea is supported by our latest analysis of the whole yeast stalk structure, where we found that it is possible to detach the P2A protein from the stalk in a specific manner (Grela *et al*., manuscript in preparation). The behaviour of the yeast P2A protein, which has similar properties to human P2, suggests that protein of this class may periodically leave the ribosomal stalk and in this manner function as a potential regulatory element on the ribosome. The data presented earlier represent the first indication supporting the much earlier observations about the exchangeability of P proteins (*29*, *40*).

In conclusion, the data presented in this report have brought a new insight into the understanding of the P proteins, showing that the human P1–P2 dimer showed a three-state transition mechanism, with the quaternary and tertiary structure of the complex undergoing significant parallel/unparallel changes suggesting that an intermediate species may exist in solution. This feature distinguishes the human complex from the yeast P1A–P2B, which exhibited two-state folding/unfolding reaction.

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