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Thermal stability of the human blood serum acid α_1-glycoprotein in acidic media

Kateřina Hofbauerová[a,b], Vladimír Kopecký Jr.[b,c], Jan Sýkora[a], Vladimír Karpenko[a,*]

[a]*Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Albertov 6, CZ-12843 Prague 2, Czech Republic*

[b]*Institute of Physiology, Czech Academy of Sciences, Vídeňská 1083, CZ-14220 Prague 4, Czech Republic*

[c]*Institute of Physics, Charles University, Ke Karlovu 5, CZ-12116 Prague 2, Czech Republic*

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Abstract

Thermal stability of human α_1-acid glycoprotein and its desialyzed form were studied in the pH range of 1.5–5.2, i.e. about its p*I*. Circular dichroism, fluorescence and UV-absorption were used to determine the conformational changes and their reversibility in the temperature range 25–80 °C. These changes were tested in a three step process—heating, cooling and a second heating. Principal component analysis was applied for analyzing the spectral sets obtained in these experiments. Fully reversible behavior of Trp residues, as characterized by fluorescence spectroscopy, was observed during the heating process at all pH values. Nevertheless, three different types of the protein motion (reversible, irreversible and rearrangement of the protein core) were determined by UV-absorption spectroscopy. Thus, an environment of Tyr and Phe is modified or reversibly rearranged during the heating process in acid media. These types of α_1-acid glycoprotein behavior were not significantly affected by desialyzation.

Keywords: Orosomucoid; Thermal stability; UV-spectroscopy; Fluorescence; Circular dichroism; Principal component analysis

1. Introduction

α_1-Acid glycoprotein (AGP), also known as orosomucoid, is one of the most thoroughly studied glycoproteins of human blood serum. Its molecule consists of a single polypeptide chain of 183 amino acids with possible substitutions at 21 positions. Two disulfide bridges are formed between cysteines 5–147 and 72–164 [1]. Five branched heteropolysaccharide units, amounting to approximately 42% of the total mass of the AGP molecule [2], are *N*-glycosidically linked to the asparaginyl residues in the N-terminal half of this molecule [3]. Sialic acid residues at the terminal ends of polysaccharide chains represent approximately 10–12% of the total content of carbohydrates and contribute to the extremely low isoelectric point of AGP, which varies between pH 1.8 and 2.7 according to the buffer used [4,5]. The carbohydrate components of AGP are also responsible for its high solubility and the unusual stability of this

*Corresponding author. Tel.: +420-2-2195-2448; fax: +420-2-2491-9752.

E-mail address: karpenko@prfdec.natur.cuni.cz (V. Karpenko).

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protein in neutral solutions, as confirmed by its physicochemical properties [6]. Partial deglycosylation was found to be an important part of the mechanism determining the half-life of AGP in blood [7].

The study of the genetic polymorphism of AGP [8] has revealed three different phenotypes of this protein. Further investigation has eventually led to inclusion of AGP into the lipocaline proteins family, which forms part of an overall structural superfamily of calycins [9]. The sequence identity in this family was found to be only between 15–20%, but in known X-ray structures of lipocalines the root mean square deviation in the positions of C_α-atoms is between 1.5 and 1.7 Å; the content of secondary structures is thus almost the same in these structures. It can be concluded from homology modelling of two forms of AGP [5] and lipocalines that 16 out of 21 amino acids, which are different in two genetic types of AGP, are of similar kind. Therefore, these substitutions should not have significant influence on the secondary structure of AGP. From the remaining 5 amino acids, 2 are located in turns, structures with high flexibility. What concerns the last three amino acids, they form a part of very conservative structure. Independently of the kind of these amino acids, they were always found in the beta sheet in lipocalines. Both forms of AGP have three tryptophans in the same positions. The first tryptophan is conserved in all members of the lipocaline family in the PDB database. AGP is considered to be a major member of the acute phase protein family; yet its physiological function is still not well understood. Nevertheless, a number of this protein's potentially significant physiological roles have been described, such as its ability to bind basic drugs [10,11] and certain steroid hormones [12,13].

Although AGP was crystallized as a lead salt in 1953 [4], its structure has still not been successfully studied by X-ray analysis [14]. Indirect insight into the spatial arrangement of the AGP molecule yielded data about the accessibility of some of its amino acids. Chemical modification and temperature perturbation difference spectroscopy revealed numerous residues that were completely or partially buried in the native state: 5–7 tyrosines [15,16], 2 tryptophans [15,17] and almost all phenylalanines [18]. These findings allowed us to conclude that the molecule of AGP contains a compact hydrophobic core. According to acidobasic titrations, approximately 10 out of the total 44–47 carboxylic groups (including sialic acid) do not dissociate even at the most acidic pH values that can be reached before the titration curve becomes disturbed and irreversible [19]. The strong ability of AGP to bind small inorganic ligands in acid solutions [20] explains the wide range of p*I* values of this protein.

The data obtained from CD spectra [21] indicate a low content of α-helix (below 10%) and a high amount of β-sheet (approx. 60%), in the native structure of AGP at neutral pH. The content of β-turns is close to 10%. The proposed three-dimensional model of the AGP molecule [22], based on the lipocaline family sequence homology, shows that the core of AGP adopts a β-barrel-like conformation. The native molecule was found to be an ellipsoid with an axial ratio 6:1, which decreases to 4:1 after desialyzation [23].

Up to the present, the thermal stability of AGP has been studied in neutral aqueous solutions [24,25] and in the alkaline region in mixed solvents (water with small aliphatic alcohol) [26]. These studies confirmed the stabilizing effect of methanol on helical structures in AGP with simultaneous destabilization of the rest of its molecule. Another interesting feature of AGP is the presence of an intermediate stage in its thermal unfolding [27].

The aim of the present study was to investigate the thermal stability and reversibility of the thermally-induced changes of AGP molecules in the acidic region, including its p*I*. UV absorption spectroscopy, fluorescence and CD spectroscopy were used to follow this behavior. Each of these methods provides different kind of information and the analysis of the results yields a complex description of the nature and extent of these conformational changes. For the precise analysis of the experimental data multivariate statistical methods were used to clarify the types of AGP peptide chain motions.

2. Materials and methods

The AGP used in this study was an electrophoretically homogeneous preparation from Behringwerke (Marburg, Germany). The purity of this preparation was checked by SDS electrophoresis. The concentration of the protein solution was determined spectrophometrically using the absorption coefficient $E_{1\%,\ 1\ cm,\ 278\ nm} = 8.9$ [28] and the value of 41 000 was chosen for the relative molecular weight of AGP [26]. All reagents used in our experiments were of analytical grade purity.

Sialic acids were removed from AGP by *Clostridium perfringens* neuraminidase attached to beaded agarose (Sigma). 40 mg of AGP were dissolved in 4 ml of 50 mM acetate buffer pH 5.0. 1 U of neuraminidase dissolved in 1 ml of 50 mM acetate buffer pH 5.0 was added to this solution and the mixture was incubated for 23 h at 37 °C with a droplet of toluene to prevent the growth of microorganisms. Thereafter this solution was dialyzed for 3 days against distilled water and subsequently freeze-dried. The degree of desialyzation was determined according to Svennerholm [29] and the homogeneity of the obtained sample was checked by SDS polyacrylamide gel electrophoresis (desialyzed preparation is denoted ASI–AGP throughout this paper). To adjust individual pH values, the following 50 mM buffers were used: citrate–phosphate pH 6.0 and 3.5, citrate pH 5.0 and acetate pH 4.0 and 5.0. Lower pH values (3.0, 2.5, 2.0, 1.5) were adjusted by adding the appropriate volume of HCl. Determination of pH was performed with a pH-meter pHM 93 (Radiometer, Denmark), equipped with a combined microelectrode pHC 4400 of the same manufacturer. The temperature dependence of pH in the temperature range of our experiments was determined experimentally for individual pH values.

In all three optical methods described below, experiments with changing temperature were performed in three cycles: (1) heating from 25 to 80 °C and to 70 °C only in circular dichroic spectra, (2) cooling from the peak temperature back to 25 °C and (3) a second heating to 80 or 70 °C, respectively. Upon reaching any of the temperature extremes, the sample was kept at this temperature for 10 min to attain conformational equilibrium.

The circular dichroic (CD) spectra were measured with a Jobin–Yvon Autoradiograph MARK V using quartz cells with a 1-mm optical path. The concentration of all samples was 0.5 mg/ml. The data were recorded at 0.5 nm intervals, with a sensitivity set at 2×10^{-6}. Each measurement was the result of 3 repeated records. The temperature of the sample was changed in 10 °C steps in all cycles.

The fluorescence spectra were recorded with a Perkin–Elmer LS 50B luminescent spectrometer using quartz cells with a 1-cm optical path. The spectra were recorded at a speed of 240 nm/min in the range of 285 to 485 nm, using the excitation wavelengths of 286 and 295 nm. The spectral width of the slit was 2.5 nm and the concentrations of protein solutions varied between 0.2–1.2 mg/ml. The temperature of the sample was changed in 5 °C steps in all cycles. In CD and fluorescence experiments, the temperature of the sample was maintained by an external water bath.

The UV-absorption spectra were measured with a Cecil 800 spectrophotometer (BUCK Scientific) equipped with a temperature regulation block CE 245 that maintained the temperature with a precision of ± 0.3 °C. Quartz cells with 1-cm optical path were used. The spectra were recorded in the wavelength range of 240 to 320 nm, with a recording speed of 60 nm/min and the concentration of the samples varied between 0.6–0.8 mg/ml. The temperature of the sample was changed in 5 °C steps in all cycles.

A principal component analysis (PCA), using a singular value decomposition algorithm, was applied to reduce spectral series $\{Y_i(\nu),\ i=1,...,\ N\}$ to their lowest dimension without loss of spectroscopic information. Then each spectrum $Y_i(\nu)$ can be unambiguously expressed as:

$$Y_i(\nu) = \sum_{j=1}^{M} W_j V_{ij} S_j(\nu) \qquad (1)$$

where W_j is the diagonal matrix of singular values, V_{ij} is the unitary matrix of coefficients and $S_j(\nu)$ corresponds to the matrix of the orthonormal subspectra (eigenvectors). M represents the number of independent spectral species found in the set of analyzed data. The simplest determination

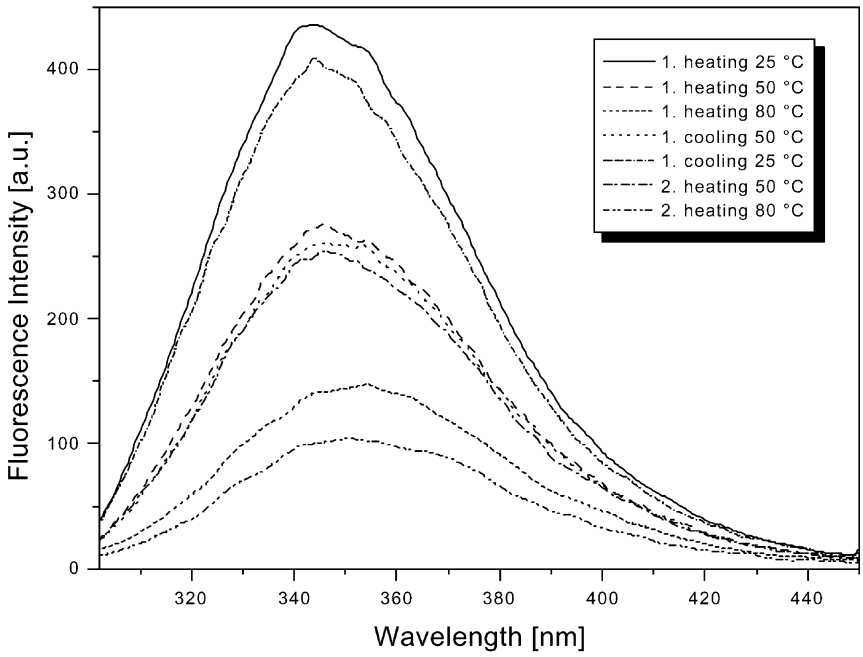

Fig. 1. Fluorescence spectrum of AGP at pH 1.5 during three heating cycles (heating, cooling and the second heating) in the temperature range 25–80 °C (only curves for the temperature extremes and for 50 °C are shown). Fluorescence intensity is expressed in arbitrary units.

of the number of independent spectral species is shown by the percentage of the total variance of each eigenvector. For these calculations we used our Matlab [30] based scripts. (Detailed explanation of factor analysis techniques, e.g. PCA, can be found in [31].)

3. Results

The removal of sialic acids was almost complete; after desialyzation of AGP their content decreased below detection limits of the method used for their determination.

3.1. CD spectroscopy

The CD spectra recorded for all samples in the range of 200–260 nm had a negative peak typical for AGP. At pH 7 AGP contains 8% α-helix, 60% β-structure and 10% of β-turn [21]; in ASI–AGP at pH 7.4 there is approximately 4% α-helix, 20% β-structure and 21% of β-turn [6]. In our experiments, in all measured pH ranges (1.5–5.0), the absolute value of the molar ellipticity was higher for AGP than for ASI–AGP. Increasing temperature led to a decrease of β-structure content in both AGP and ASI–AGP. On cooling and subsequent heating, the spectrum changes were reversible. Yet, in contrast to behavior in neutral solutions, no cooperativity was observed. With increasing acidity, the α-helix content decreased slightly in both samples, while changes in the β-structure content were not as apparent. We conclude that, in the acidic region, the CD spectra of both AGP and ASI–AGP did not differ significantly from the previous results at the physiological pH value [21], the only difference being the loss of cooperativity.

3.2. Fluorescence measurements

The tryptophan fluorescence was measured for AGP and ASI–AGP in the pH range 1.5–5.0. The temperature dependence of the fluorescence inten-

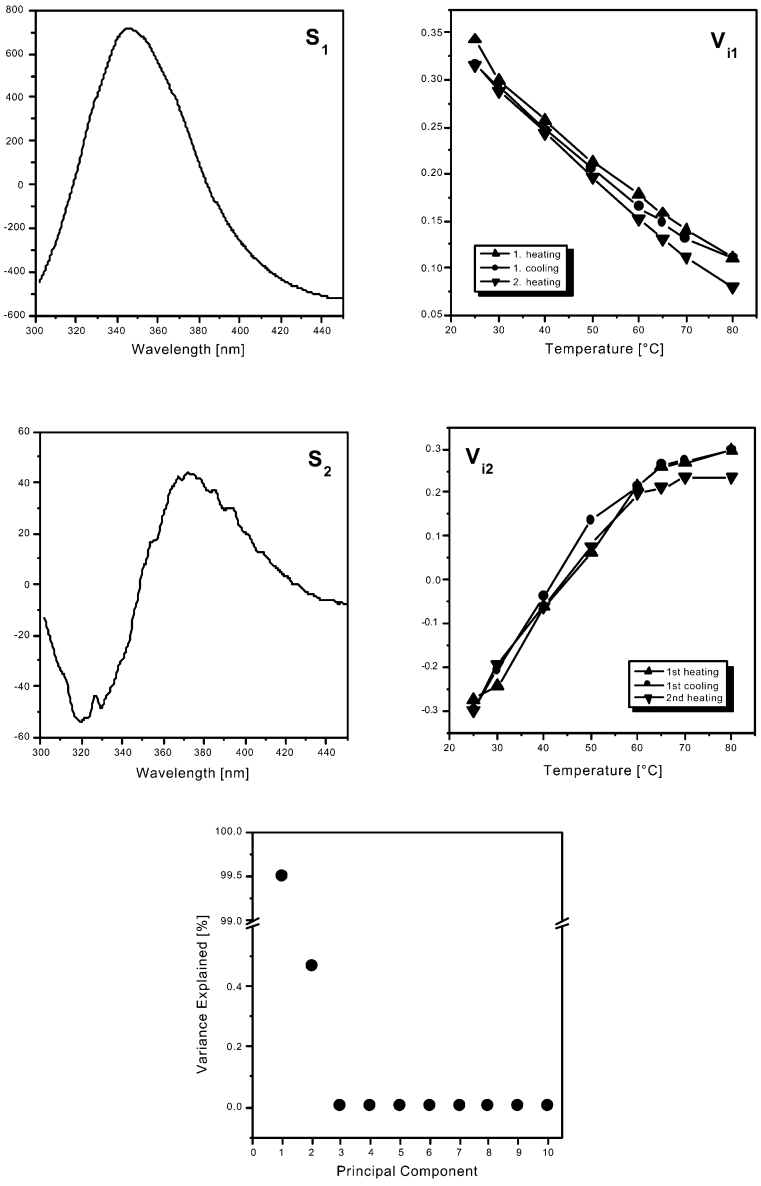

Fig. 2. Results of the principal component analysis applied to the full set of the tryptophan fluorescence spectra of AGP at pH 1.5 (shown in Fig. 1) during repeated thermal cycles. S_j represents the two most significant subspectra. The number of significant subspectra can be determined from the subplot of the explained variance of the spectral set. V_{ij} are the appropriate coefficients indicating the relative contribution of the corresponding subspectrum.

sity followed the same curves for all three cycles of temperature changes and no difference could be observed between the results for AGP and ASI–AGP. As a typical example, the temperature dependence of the emission spectrum of AGP at the most extreme pH value is shown in Fig. 1. As is apparent, the changes of this spectrum were reversible with temperature and were accompanied by a red shift of its maximum from 345 nm to 350 nm with increasing temperature (Fig. 1). This

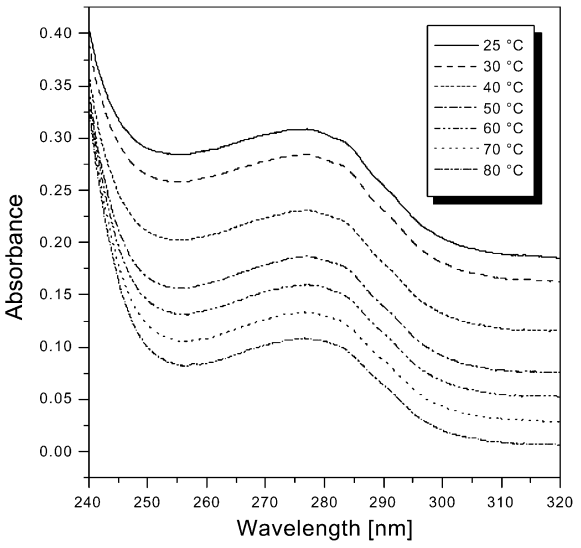

Fig. 3. UV absorption spectra of AGP (in acetate buffer, pH 5.2) during the first heating from 25 to 80 °C.

effect indicates that tryptophan residues became more exposed to the solvent upon heating.

This type of the spectral behaviour, together with the decreasing fluorescence intensity with increasing temperature, can be clearly identified from the PCA results (Fig. 2), which reveal the two components with the highest informational content (see explained variance in Fig. 2). The first principal component (S_1 at Fig. 2) shows the reversible behaviour of AGP during the heating process, which is demonstrated by the appropriate coefficient V_{i1} in Fig. 2. However, the second principal component (S_2), which represents red spectral shift marked by negative band at ca. 320 nm and positive band at ca. 350 nm, indicates a saturation effect beginning approximately at 60 °C.

3.3. UV absorption spectroscopy

The UV absorption spectra follow the behavior of the chromophores (Trp, Tyr and Phe) upon their exposure to the solvent. The examination of the thermal stability of AGP and ASI–AGP yielded the same results for both forms of this protein in the pH range 1.5–5.2. As a typical example, the UV spectra of AGP are shown in Fig. 3 at the highest pH studied, while the fluorescence measurements are shown at the lowest pH. Concerning UV spectra, only a decrease of absorbance with increasing temperature was observed for both AGP and ASI–AGP. The attempt to use the fourth-derivative of UV spectra of AGP failed. Thus, additional information could be obtained from these spectra using the techniques of multivariate statistics.

The PCA analysis of the complete sets of AGP's UV spectra recorded during the heating process of the thermal cycles shows three main components (see explained variance in Fig. 4). The first subspectrum (S_1 in Fig. 4) corresponds to reversible conformational changes of the protein, as shown by the appropriate V_{i1} coefficients in Fig. 4. However, the second component, S_2, clearly demonstrates irreversible conformational changes during the heating process. Following the entire heating process (heating, cooling and the second heating), a disturbance at a temperature ca. 50 °C is observed. The most intense disturbance occurred during the second heating, where the trace at V_{i2} in Fig. 4 is far from an ideal zero curvature line. This phenomenon can be explained as a disturbance from the last type of AGP motion during heating and which was characterized by the third component. The V_{i3} coefficients of the third subspectra S_3 (Fig. 4) correspond to a particular type of rearrangement of the AGP molecule during the temperature changes. This type of the rearrangement is fully reversible and thus could be a conformational change of a cooperative character, occurring within the protein core.

4. Discussion

The behaviour of AGP and its desialyzed form in acidic media is a very complex process. The use of the different spectroscopic techniques revealed various aspects of protein chain rearrangement in the molecule of this protein and made it possible to collect crucial pieces of the puzzle surrounding the conformational stability of this protein. The characteristic difference between the behaviour of this protein in the acidic region and at pH 7 [21] is the loss of cooperativity at the lower pH values.

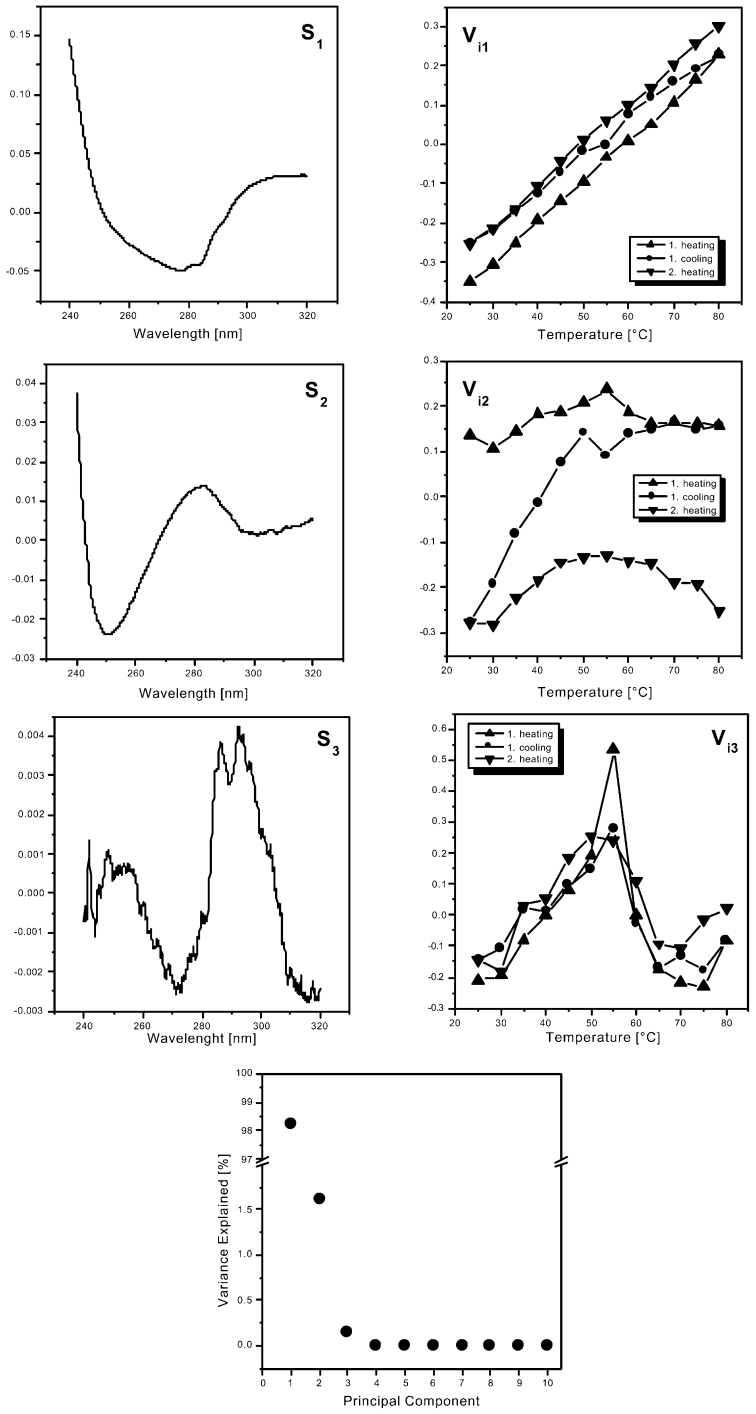

Fig. 4. Results of the principal component analysis applied to the full set of the UV absorption spectra of AGP at pH 5.2 (shown in Fig. 3) during repeated thermal cycles. The meaning of the subplots is the same as in Fig. 2.

Although the conformation of the AGP molecule changes after desialyzation [23], which also was confirmed by our CD measurements, this modification had no observable affect on the thermostability of AGP in acid media. In UV absorption and fluorescence measurements there was also no significant difference between thermostability of the native and desialyzed AGP in the pH region studied. We did not observe any polymeric forms or aggregates of protein. These results allow us to conclude that the thermal stability of the AGP molecule was not significantly affected by desialyzation in this pH region. As this observation is in agreement with the results obtained at physiological conditions [6,32], we can conclude that sialic acid residues do not seem to have a significant role in the molecule-wide dynamics of AGP in the pH range from 1.5 to 8.0.

The PCA results of the fluorescence spectra (Fig. 2) lead to the unambiguous conclusion that thermally-induced changes in the environment of tryptophyl residues are fully reversible in acidic media. The main PCA component of the UV spectra (Fig. 4) probably corresponds to the tryptophyl residues; therefore, reversibility is observed. The observation of the saturation effect can be simply explained by the known positions of the three tryptophyl residues in the AGP molecule. One residue is located near or at the surface of the molecule, while the remaining two are embedded in the matrix [17,33]. The red spectral shift is likely connected with the Trp^{160}, located close to the protein surface, which became yet more exposed to the solvent on heating and became fully exposed at a temperature of approximately 60 °C. At this point, no further conformational changes could be observed and saturation effect appeared. The conformation of the protein core with two other tryptophyl residues could alter its reversibly; it was probably just this effect that contributed to the first PCA component, where no saturation effect is detected (see V_{i1} in Fig. 2).

The most interesting result was found by UV absorption measurements, which revealed conformational changes that had not been previously observed in AGP. We suppose that the irreversibility, clearly demonstrated by the second PCA component (see V_{i2} in Fig. 4), concerns parts of the protein molecule located close to the surface. When we compare the explained variances (Fig. 4), the third PCA component (V_{i3} in Fig. 4) accounted for only approximately 0.14% of total variance of the spectra in contrast with 1.61% of the second PCA component. Thus, the third PCA component should correspond to some very subtle type of movement that seems to occur deeper in the protein molecule. The protein core has a β-barrel-like conformation and thus this effect could be a type of breathing or torsion-like movement of the barrel caused by the motions of other AGP parts in response to the temperature changes. Considering the results of the fluorescence measurements and the above-mentioned irreversibility of the conformational changes, the rearrangement of the AGP molecule in acidic media could not be connected with tryptophyl residues. Thus, the locus of the previously mentioned conformational changes is expected to be found in the environment of Tyr and Phe residues.

We may conclude that AGP and its asialic form are relatively thermostable at low pH values, but show less and different cooperativity in conformation changes than at physiological pH. The hydrophobic core of this protein has a decisive influence on its thermostability in acidic media and the observed conformational changes (reversible and irreversible motions) can be supposed to occur in peripheral free chains of the AGP molecule.

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