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The conformation and stability of human Zn-α_2-glycoprotein in aqueous and methanolic solutions

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Abstract

The stability of Zn-α_2-glycoprotein has been studied using UV-spectroscopy and circular dichroism with respect to the influence of temperature, pH, and solvent composition. It has been found that: (1) this protein contains a relatively high proportion of β-sheet (60%) and a very low amount of other periodic structures as estimated from circular dichroic spectra; (2) at pH 7.4, the circular dichroic spectra change reversibly in the temperature range between 25 and 85°C; small disturbances were observed at 265 nm; (3) with the assumption of the two-state process, the temperature of cooperative denaturation was $T_m = 66$°C, van't Hoff's enthalpy of this process was 27 kJ mol^{-1}; (4) up to pH 9.5 the dichroic spectrum appeared the same as at pH 7.4; (5) in the presence of methanol (vol. fraction 50%), no isodichroic points on the circular dichroic spectra were found during temperature denaturation; after cooling from 85°C, the α-helix content was higher than in the native protein; (6) in the molecule of Zn-α_2-glycoprotein, 14 out of 18 tyrosines can dissociate with the instrinsic pK = 11.2; and (7) the temperature perturbation difference spectra yielded nonlinear ΔA vs. T curves with temperature transition corresponding to the values found in the circular dichroic spectra; the numbers of chromophores exposed to the solvent as determined by the temperature difference spectra were: 4 tyrosines, 1 tryptophan, and 1 phenylalanine. In several aspects, a parallel has been found between Zn-α_2-glycoprotein and orosomucoid (acid α_1-glycoprotein), another plasma glycoprotein. © 1997 Elsevier Science B.V.

Keywords: Zn-α_2-glycoprotein; Methanol; Secondary structure; Denaturation; Dissociation; Circular dichroism; UV-spectroscopy

1. Introduction

Using precipitation by zinc ions, Bürgi and Schmid [1] isolated Zn-α_2-glycoprotein (Zn-α_2-gp throughout this paper) from the Cohn's Fraction VI of human blood serum; the name of the newly prepared protein comes from this isolation. Zn-α_2-gp was then characterized in terms of its chemical and physicochemical properties. As found by Bürgi and Schmid [1], this protein was electrophoretically homogeneous in the pH range of 3.9 to 13; its isoelectric point varied between 3.8 and 3.9, the absorption coefficient $E_{1\%,\ 1\ cm}$ was 18 at 278 nm, and the partial specific volume was 0.706 ml g^{-1} (further data were summarized by Schulze and Heremans

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[2]). Zn-α_2-gp is quite stable; it is precipitated neither by boiling water, nor by 8 M urea, or by 1.8 M $HClO_4$. Instability of its molecule was observed at pH values below the isoelectric point, and in extremely alkaline solutions.

A rather high content of tyrosine and tryptophan residues revealed in the Zn-α_2-gp molecule in the first amino acid analysis [3] was later confirmed by Araki et al. [4]. These authors found 18 tyrosines and 18 tryptophans in the molecule of this protein formed of 276 amino acids in a single chain. This chain is arranged into two loops linked by disulfide bridges between Cys 101 and 164, and Cys 203 and 258. Carbohydrate units consisting of three glycans represent 18% of the total molecular weight 38 478 calculated from the amino acid composition [3]. This value lies close to the 41 000 estimated from physicochemical measurements [2]. The three glycans described as sialylated *N*-biantennas are supposed to be located in β-turn regions. Prediction study done by Araki et al. [4] led to the following expected content of secondary structures: 23% α-helix, 27% β-sheet, and 22% β-turn.

From the fact that human seminal Zn-α_2-gp does not contain carbohydrate part, Ohkubo et al. [5] concluded that the presence of this part is not a general feature of Zn-α_2-glycoproteins. These authors supposed that both blood plasma and seminal plasma Zn-α_2-glycoproteins may be expressed from one gene, but the post-translational modifications of these proteins follow different pathways. Comparative study of different proteins allowed to conclude that Zn-α_2-gp is closely related to antigens of the main histocompatibility complex [1]. The biological function of Zn-α_2-gp is not clear yet, but its higher occurrence in patients with breast cancer could have served as a diagnostic tool [6,7]. Recently, two novel isoforms of this protein were isolated from rat liver [8].

Up to now, the available information about the conformational properties of Zn-α_2-gp has been rather limited. Therefore, the aim of the present paper was to study optical properties of this protein and analyze them in terms of secondary structures. Simultaneously, the question of Zn-α_2-gp stability under different conditions (temperature, pH, solvent composition) should also be answered.

2. Materials and methods

Zn-α_2-glycoprotein was an electrophoretically homogeneous preparation of Behringwerke (Marburg, Germany). The concentration of protein solutions was determined spectrophometrically using the absorption coefficient $E_{1\%,\ 1\ cm,\ 278\ nm} = 18$ [2].

n-Acetyl L-Tyrosine Ethyl ester (NAcTyr), *n*-Acetyl L-Tryptophan Ethyl ester (NAcTrp), and *n*-Acetyl L-Phenylalanine Ethyl ester (NAcPhe) of reagent grade purity (all Sigma, USA) were used as model compounds; tetranitromethane (TNM) was a product of Aldrich (USA). Methanol of analytical grade purity was a product of Lachema (Czech Rep.); its content in the solution was expressed as a volume fraction, ϕ (%), throughout this paper. Freshly prepared carbonate-free volumetric solutions of KOH for titration experiments were kept under nitrogen atmosphere.

Circular dichroic (CD) spectra were recorded with an Autodichrographe Mark V (Jobin-Yvon, France) equipped with software Dischrosoft Version A, using 0.1 cm quartz cells. CD spectra of 0.1% Zn-α_2-gp solution in 0.05 M tris–buffer pH 7.4 were recorded in two regions: 205–260 nm (sensitivity, 1×10^{-5}), and 240–320 nm (sensitivity, 1×10^{-6}). In order to reach favorable signal to noise ratio, each spectrum was the result of three scans in steps 0.5 nm.

In the temperature-dependent experiments, the temperature of the sample was kept at the desired value with the precision ± 0.1°C using a water bath. The initial spectrum was recorded at 25°C and the temperature was then raised to 85°C with the 10°C increments. To attain thermal and conformational equilibrium, this solution was kept in the sample cell for 15 min at each temperature. After attaining the maximal temperature, the sample was cooled down to 25°C in the same manner as in the heating step. The real temperature in the cell was measured with a thermistor probe; the temperatures registered in the water bath were corrected using this calibration.

The relative content of individual secondary structures in Zn-α_2-gp molecule was determined from the CD spectra using a program developed in the laboratory of one of the authors (M.K.); the curve was calculated as the linear combination of the model spectra [9].

$$K = \frac{[\Theta]_T - [\Theta]_N}{[\Theta]_D - [\Theta]_T} \quad (1)$$

For thermodynamic analysis, we used Eq. (1) where $[\Theta]_T$ is the ellipticity at the temperature T; $[\Theta]_N$ and $[\Theta]_D$ are the limiting ellipticities of the native and denatured form, respectively. The value of H was calculated from the slope of the van't Hoff's plot of ln K vs. $1/T$.

Spectrophotometric titration was performed in a continuous way with a Shimadzu UV-160A double-beam spectrophotometer using 1 cm quartz cells and both absorption and difference spectra of the protein were recorded. The temperature was maintained at 25 ± 0.5°C using a water bath. In the difference technique, the reference sample contained the protein dissolved in 0.05 M tris–buffer pH 7.4.

The temperature perturbation difference spectra (TPDS) were recorded with a Shimadzu UV-160A double-beam spectrophotometer using 1 cm quartz cells. The reference cell was kept at 25°C; after 15 min of equilibration, the temperature of the sample cell was raised to 85°C. The spectra of the model compounds were taken in 10°C intervals, of the protein in 5°C intervals. In each experiment, the sample was kept at the appropriate temperature for 15 min to attain thermal and conformational equilibrium; the cooling followed subsequently in the same way as the heating.

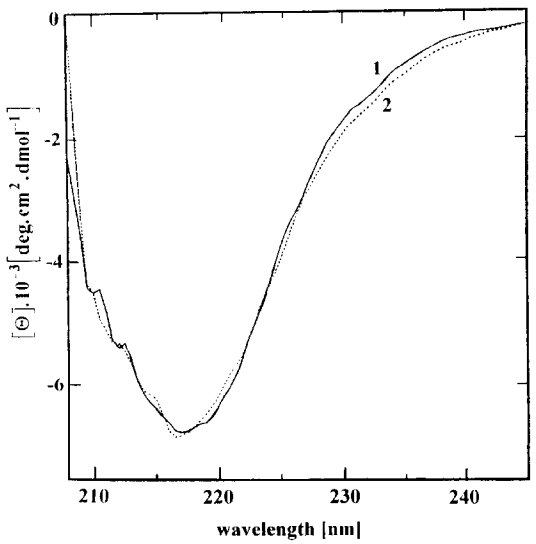

Fig. 1. The CD spectrum of Zn-α_2-gp at pH 7.4; (1) the native protein at 25°C, (2) the sample after cooling from 85 to 25°C.

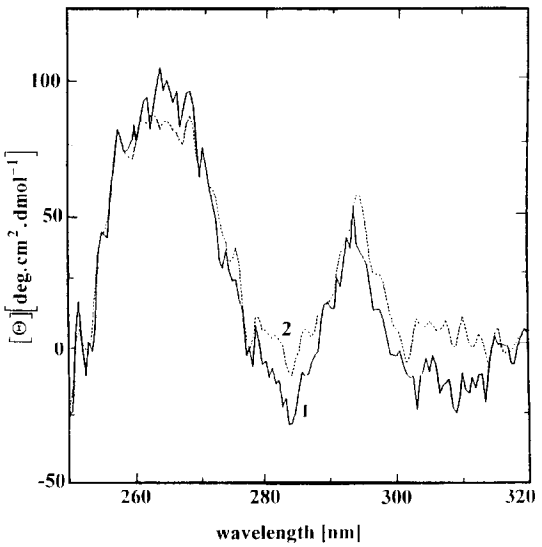

Fig. 2. The CD spectrum of Zn-α_2-gp at pH 7.4; (1) the native protein at 25°C, (2) the sample after cooling from 85 to 25°C.

The appropriate wavelengths suitable for further calculations were selected from absorption spectra of model compounds, of Zn-α_2-gp recorded in our experiments, and from the literature data [10]. The numbers of exposed chromophores were estimated using a system of two Eq. (2) of the general form [11]:

$$x\frac{d(\Delta A_{\lambda_1})}{d(\Delta T)_Y} + y\frac{d(\Delta A_{\lambda_1})}{d(\Delta T)_W} = \frac{d(\Delta A_{\lambda_1})}{d(\Delta T)_{Zn}}$$

$$x\frac{d(\Delta A_{\lambda_2})}{d(\Delta T)_Y} + y\frac{d(\Delta A_{\lambda_2})}{d(\Delta T)_W} = \frac{d(\Delta A_{\lambda_2})}{d(\Delta T)_{Zn}}$$

where x and y denote the numbers of exposed tyrosine (Y) and tryptophan (W) residues per protein (Zn) molecule, respectively; the slopes of ΔA vs. ΔT curves were determined for the wavelengths λ_1 and λ_2. In our experiments, an attempt was also made to estimate the approximate numbers of exposed phenylalanines by the same approach. The numbers of exposed tyrosines and tryptophans were calculated using following pairs of wavelengths: 283 and 300 nm, 280 and 288 nm. For phenylalanines in combination with tyrosines, the pairs 252 and 258, and also 252 and 263 nm led to acceptable results.

In all experiments, pH was measured with a PHM93 pH-meter (Radiometer, Denmark) equipped

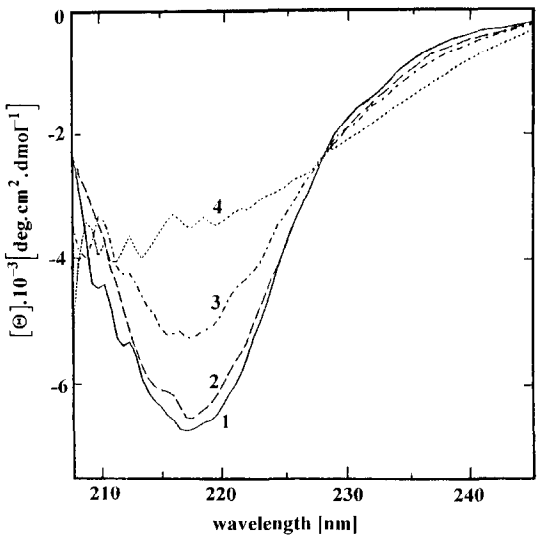

Fig. 3. The CD spectrum of Zn-α_2-gp (pH 7.4) at the different temperatures: (1) 21.5°C, (2) 46°C, (3) 66°C, (4) 86.2°C.

with a combined microelectrode pHC 4400 of the same manufacturer. In mixed solvents, pH-meter readings should be corrected for the effect of the solvent on the activity coefficient of H_3O^+ ions and also for its direct influence on both electrodes; this correction was done as described elsewhere [12].

Nitration of Zn-α_2-gp was performed in 0.05 M tris–buffer of pH 8 by the method of Riordan and Vallee [13]. TNM was diluted by ethanol (96 wt.%) to the final concentration 0.84 mol dm^{-3} and this solution was added to Zn-α_2-gp in tenfold excess with respect to its tyrosine content. The solution was stirred 60 min and then dialyzed 24 h against the same buffer; the extent of nitration was checked spectrophotometrically. This procedure was performed with the native protein and with the sample after TPDS experiment, both in 2×10^{-5} mol dm^{-3} concentration.

3. Results

3.1. CD spectroscopy

The principal feature of the CD spectra of the native Zn-α_2-gp at pH 7.4 and 25°C was an intense negative peak at 218 nm (Fig. 1). The best fit for the spectrum between 210 and 250 nm was found with a linear combination of 3% α-helix, 60% β-sheet, and 1% β-turns; the remaining 36% represented aperiodic structure. Such data indicate, taking in consideration the principal limitations in chiroptical methods, that Zn-α_2-gp contains a high amount of β-sheets and a very low amount (if any) of the other periodic structures. In the 250–320 nm range (Fig. 2), a positive peak was observed at 265 nm most probably corresponding to disulfide chromophores. Further positive peak, at 295 nm, is typical of aromatic chromophores.

With increasing temperature, the peak at 218 nm disappeared gradually (Fig. 3), but after cooling back from 85°C to 25°C, the final CD spectrum was identical with the original one of the native protein shown in Fig. 1. This result indicated that secondary structures regenerated fully; in other words, heat-induced changes were reversible at pH 7.4 in the studied temperature range (Fig. 4). Zn-α_2-gp is a protein with relatively high content of aromatic residues (compare the value $E_{1\%,\ 1\ cm,\ 278\ nm}$). The heat induced changes in the region 230–240 nm indicate that short-wavelength aromatic transitions

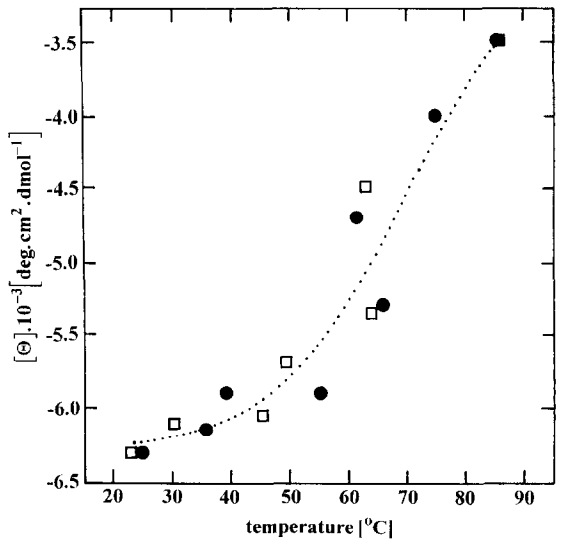

Fig. 4. Temperature dependence of the ellipticity Θ_{218} for Zn-α_2-gp in 0.05 M tris–buffer (pH 7.4): (□) heating, (●) cooling.

can influence the shape of CD spectra and, consequently, interfere in the determination of the periodic structures based on this spectral method. However, the aromatic bands do not seem, in the case of this protein, to modify the peptide bands in the way that would make the standard procedure of analysis impossible. Sigmoidal shape of this curve as well as the isodichroic point ($\lambda = 228$ nm) (Fig. 3) allowed the conclusion that the heat induced transition of Zn-α_2-gp between native and denatured form could be considered, in the first approximation, as a simple two-state process. As the curve in Fig. 4 does not reach its limiting value, the value of Θ_D ($-3000°$ cm^2 dmol^{-1}) was estimated by optimalization from the inflection point determined from the first derivative of this curve. Then, using van't Hoff's procedure, the heat change of this process was calculated as $\Delta H = 27$ kJ mol^{-1}. The temperature of cooperative transition (T_m) estimated from this curve was 66°C. Due to the limited amount of the protein, no further experiments could be performed to obtain a sufficiently reliable value of Δc_p.

At pH 9.5, the CD spectrum of Zn-α_2-gp did not differ from the spectrum at pH 7.4. Yet, deep changes were observed at pH 12: a significant decrease in ellipticity between 205 and 240 nm (Fig. 5) as a sign of the decreasing content of periodic structures. In the range 240 to 320 nm, only a pronounced shift of both maxima to shorter wavelengths was apparent, but, compared with the peptide part of the CD spectrum, their intensity was only slightly pH dependent. This observation allows us to attribute the maximum at 295 nm (Fig. 2) to the electronic transitions of the tryptophan side chain because in alkaline pH region, the tyrosine phenolic groups would dissociate (see below), and their near UV transition would be red-shifted.

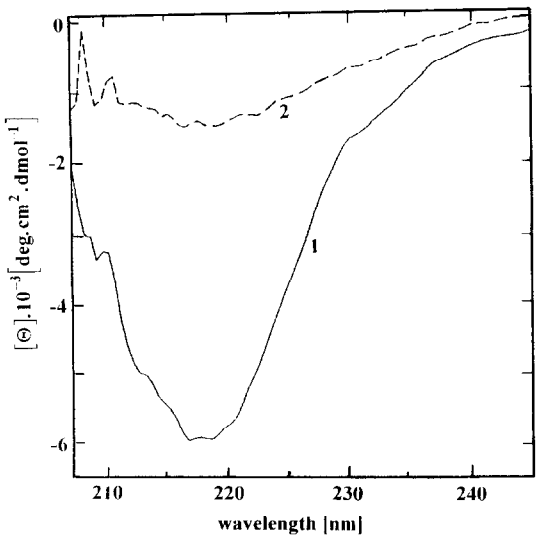

Fig. 5. The effect of pH on the CD spectrum of Zn-α_2-gp at 25°C; (1) pH 9.5, (2) pH 12.

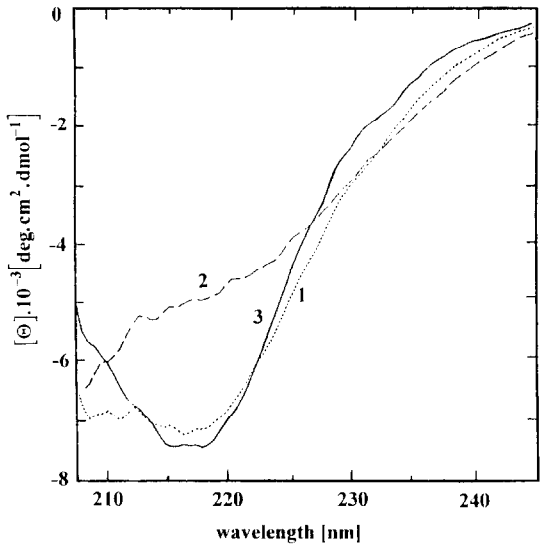

Fig. 6. The effect of temperature on the CD spectra of Zn-α_2-gp (pH 7.4) in the presence of methanol ($\phi = 50$ vol.%); (1) 25°C, (2) 75°C, (3) the sample after cooling to 25°C.

In the presence of methanol ($\phi = 50$ vol.%) at pH 7.4 and 25°C (Fig. 6), a little difference in the far UV CD spectra was observed compared with the aqueous solution. The α-helix content was slightly higher in methanol as given in Table 1. With increasing temperature, however, this effect was more pronounced: at 75°C the α-helix content reached 12%. As no isodichroic points appeared, these conforma-

Table 1
The content of secondary structures (%) of Zn-α_2-glycoprotein in aqueous and methanolic ($\phi = 50$ vol.%) solution at pH 7.4 as estimated from the circular dichroic spectra

	H_2O; 25°C	MEOH; 25°C	MEOH; 75°C
α-helix	3	6	12
β-sheet	60	59	1
β-turn	1	1	1

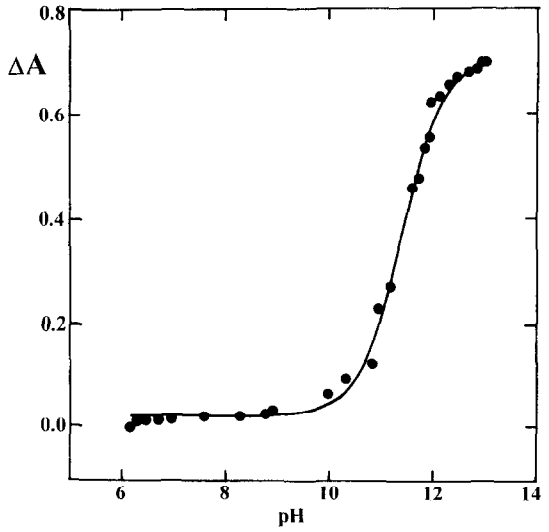

Fig. 7. Spectrophotometric titration curve of Zn-α_2-gp at 293 nm (25°C) plotted as the molar difference absorbance (ΔA) vs. pH.

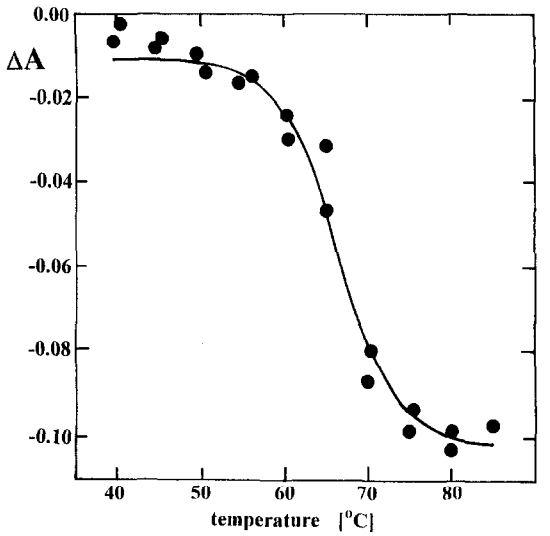

Fig. 9. The temperature dependence of the molar difference absorbance (ΔA) of Zn-α_2-gp at 281 nm (pH 7.4).

tional changes apparently follow more complicated pathway. The negative peak at 218 nm disappeared in methanolic solution at higher temperatures, but after cooling of the sample, this peak was deeper than for the native protein and corresponded to the α-helix content of 9%.

3.2. Spectrophotometric titration

The titration curve of Zn-α_2-gp did not exhibit any significant deviation from the sigmoidal shape and reached the limiting value above pH 12 (Fig. 7). It can be thus expected that titratable tyrosines form one group; their intrinsic pK was 11.1 when calculated for the wavelength 244 nm, and 11.4 for 293 nm. The number of dissociated tyrosine residues calculated with the assumption of the additivity of absorbance led to results 13.5 (244 nm) and 14.3 (293 nm), respectively. In can thus be summarized that between pH 9.5 and 12.5 approximately 14 out of total 18 tyrosine residues dissociate in Zn-α_2-gp with pK 11.2.

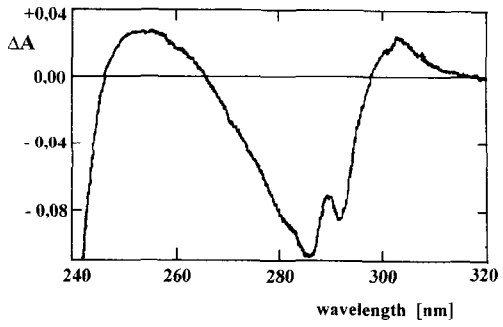

Fig. 8. Temperature perturbation difference spectrum of Zn-α_2-gp at 75°C (pH 7.4)

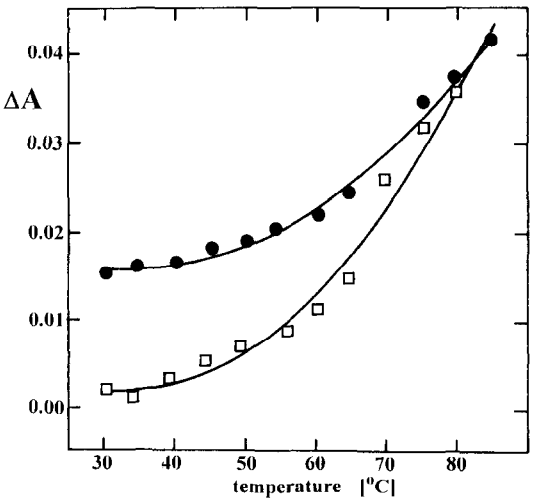

Fig. 10. The temperature dependence of the molar difference absorbance (A) of Zn-α_2-gp at 252 nm pH 7.4); (□) heating, (●) cooling.

3.3. Temperature perturbation difference spectra

A characteristic spectrum obtained by this method is shown in Fig. 8. Except for 288 nm the difference absorbance vs. temperature curves of Zn-α_2-gp were nonlinear at all wavelengths examined as illustrated on a typical curve (Fig. 9). The transition temperature was in all cases close to 66°C. These effects were reversible during cooling for all wavelengths except for 252 and 258 nm, where a small hysteresis was observed (Fig. 10). The linear part of TPDS curves was so short that precise calculation of exposed chromophores was not possible. Therefore, these numbers could be estimated only roughly as 4 tyrosyls, 1 tryptophyl, and 1 phenylalanine residue at pH 7.

Chemical modification of the native preparation and of the heat treated sample led in both cases to the same result of 11 nitrated tyrosine residues.

4. Discussion

The stability of proteins is one of the most difficult problems to solve, since each method used in this research yields information from its specific point of view. Furthermore, in the explanation of the observed effects several factors usually have to be considered, but only a few of them can be reliably quantified.

In the case of Zn-α_2-gp, the first discrepancy appeared between the content of periodical structures as found by CD spectroscopy and the values estimated by prediction studies [4]. These data differed significantly for all types of structures; as mentioned above, the interpretation of chiroptical methods is limited, but the predicted 23% of helical conformation would undoubtedly form a completely different shape of the CD spectrum than that observed for Zn-α_2-gp (Fig. 1). This protein does not appear to be an exception in this respect because similar discrepancy was found in another glycoprotein, human orosomucoid [9,14]. It should be emphasized here that the prediction methods used did not take into account the influence of saccharide chains. Just the bulky hydrophilic antennae-like structures of these chains prefer contact with the surrounding water. They thus exert a force directed into the bulk of the solution that could have overcome in some cases interactions stabilizing predicted secondary structures. It would be interesting to do a similar study with seminal plasma Zn-α_2-gp that is synthesized without a carbohydrate part.

It is generally expected that the carbohydrate part of a glycoprotein molecule is to a large extent responsible for the solubility of the given protein in an aqueous medium. Some glycoproteins, originally soluble in water, lose this property when freed of carbohydrate moiety. In Zn-α_2-gp, the peptide part of its molecule is rather hydrophobic with 65% of its amino acids belonging to this class. Then, the effect of the carbohydrate part can become very important. Simultaneously, the existence of a core formed of hydrophobic parts of the polypeptide chain should be expected. The presence of such a core in Zn-α_2-gp is confirmed by the high pK value of tyrosine residues as well as by the existence of buried tyrosines that do not dissociate under the nondenaturing conditions. On the other hand, 11 tyrosines acceptable for nitration show that their hydrophobic aromatic rings are not completely shielded from the contact with the solvent.

This observation was further supported by the titration that allows us to conclude that 14 out of 18 tyrosines of Zn-α_2-gp dissociate as a uniform group with pK = 11.2. This value is above the upper limit of pK's found in other simple proteins as well as glycoproteins (8.5–10.9; Ref. [15]). Tyrosines in Zn-

Table 2
Comparison of physicochemical parameters and pK values of tyrosines of some proteins

Protein	Mol. wt.	Total number of Tyr /the number of Tyr with pK given in the next column	pK of tyrosines	The net charge at pH 11
Zn-α_2-gp	38,500	18/14	11.2	−26
Orosomucoid [16]	41,000	12/4	11.8	−34
Hemocyanin [17]	50,800	18/16.5	11.6	−40

α_2-gp are thus hindered in some way in their dissociation. Two mechanisms can be considered: either strong hydrogen bonding with other groups, COO^- belonging among the most probable candidates, or partial masking of tyrosines in the hydrophobic structures of Zn-α_2-gp. If three proteins of similar size are compared (Table 2), it is apparent that pK values of their tyrosines are higher than is generally expected in proteins. This comparison supports the hypothesis that higher pK's of Zn-α_2-gp tyrosines can be attributed to electrostatic effects.

It is the high net charge of this protein that is obviously responsible for the decrease in the content of periodic structures appearing at high pH values. Nothing is known about the interactions of inorganic ions with this protein, but the binding of univalent cations to proteins in alkaline media is not a common feature. If no such binding is supposed for Zn-α_2-gp then the net charge of this protein should exceed 50 at pH 12. The electrostatic repulsion is thus strong enough to break weak interactions that stabilize its spatial arrangement.

As outlined above, the structure of Zn-α_2-gp exhibits a high thermal stability and quite obvious is the reversible behavior of this protein in thermal denaturation. CD spectrum is reversible between 210 and 240 nm in the temperature range of 25–85°C. Full reversibility of the peptide dichroism and the isodichroic points on CD spectra allow us to consider a simple two-state model of the temperature transition of Zn-α_2-gp. This thermal transition occurs over a very broad range of temperatures (Fig. 4); consequently, the cooperativity of molecular structure is relatively low and segments of β-structures may change their conformation continuously. Nevertheless, this idea does not exclude the usage of the two-state model. Small anomalies are observed in the wavelength range 280–320 nm. In this region, the reflected changes of the tertiary structure are sensitive to the local conformation of disulfide bridges and tryptophyl side chains. At present this observation can be stated, but any further discussion of this effect would be only speculative. Attention should be paid to the sequence Cys 164–Pro 165 and to a possibility of the prolyl peptide bond effect in refolding [18]. Anyway, just because of these small anomalies, two state transition of Zn-α_2-gp was accepted only as a hypothesis that should be further verified. The limited quantity of Zn-α_2-gp we had for this study prevented us to go on this problem. Therefore, the value of van't Hoff's enthalpy should be taken with this limitation.

The interpretation of TPD spectra is difficult due to their high sensitivity to the interaction of chromophores with the surrounding solvent. Without detailed knowledge of the solvent structure any discussion should be limited to the qualitative approach only. In TPD spectra of Zn-α_2-gp two effects should be noticed. The first one is nonlinear dependence of difference absorbance on temperature. This kind of behavior was observed with orosomucoid as well, and cannot be explained reliably at present [19]. These spectra obviously reflect the conformational changes observed in the CD spectra what is further supported by the coincidence of the transition temperature in both cases. Simultaneously, this shape of the TPDS curves makes it difficult, if not impossible, to estimate the numbers of exposed chromophores with sufficient precision. That is why the number of exposed tyrosines in Zn-α_2-gp could be derived more precisely from the data of spectrophotometric titration. The other two chromophores, tryptophan and phenylalanine, should be considered with the same care; one exposed phenylalanine is, however, not surprising. As shown in Fig. 9, the TPD spectrum was reversible at 281 nm, in the region of overlap of tyrosine and tryptophane absorption. On the contrary, a small hysteresis was observed at 252 nm and after cooling the difference absorbance remained higher than before heating. This second effect has also been observed in orosomucoid at the neutral pH in the same wavelength range [19]. A possible explanation is that in the course of cooling Zn-α_2-gp achieves a new conformation not too different from the native one so that it is not registered by CD spectra (compare the CD spectra in Fig. 1). In the new conformation, however, parts of peptide chain or amino acids' side chains assume only a slightly different orientation, in which some groups are more exposed to the solvent than in the native state.

The effect of methanol is rather complex process in which at least three mechanisms are acting simultaneously. The first one is the binding of methanol to the protein via hydrogen bonds or hydrophobic interactions. The second one is the interaction of methanol with water that results in changes of the solvent

structure as well as in hydration layer of the protein. Eventually, the third effect is the enhancement of electrostatic interactions due to decrease in solvent permittivity. As only this last effect can be quantified at present, the influence of methanol on proteins can be discussed only qualitatively.

The volume fraction of methanol chosen in our work coincides with the minimum on the dependence of the excess enthalpy of mixing (ΔH^E) vs. the content of alcohol [20], [21]. At lower concentrations, small aliphatic alcohols are supposed to destroy cluster domains of water, above this concentration, the clustering of alcohols prevails [22]. The minimum coincides with the threshold concentration of alcohol and just this transitional state of water–alcohol system acting on the hydration layer of a protein could destabilize its structure. In the system water–ethanol analogous observation was made with orosomucoid [19]. Similar, although not as pronounced effect, could be expected with methanol.

The effect of methanol at rising temperatures is a picture of 'reconstructive denaturation' during which helical structures are stabilized and their content increases, while other types of secondary structures are gradually destroyed [23]. There is a similar trend with Zn-α_2-gp: the increase of the α-helix content with temperature in the presence of methanol. Yet, doubling of the α-helix content in the methanolic solution between 25 and 75°C cannot be explained by some simple mechanism. In similar experiments with orosomucoid, α-helix content increased to approx. 45°C, but then a decrease followed [9]. As mentioned above, the observed effects could have been related to the alcohol content and thus to the structure of the solvent. The experiments with orosomucoid were performed in 70% methanol, above the minimum of ΔH^E vs. methanol concentration dependence. In the present work, the limited quantity of Zn-α_2-gp did not allow us to investigate this problem in more detail.

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