THERMAL STABILITY OF HUMAN BLOOD SERUM OROSOMUCOID (ACID α_1-GLYCOPROTEIN) IN WATER AND WATER–ETHANOL SYSTEMS

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Thermal stability of human serum orosomucoid (acid α_1-glycoprotein) was studied in water and in water–ethanol systems by the differential scanning microcalorimetry and temperature perturbation difference spectrometry. This investigation has led to the following results: (i) the change in heat capacity C_p of isoionic orosomucoid with temperature was irreversible; different values of ΔH were obtained on repeated heating; (ii) in buffered solution at pH 7, hysteresis in temperature perturbation difference spectra was observed within the wavelength range of 250 – 266 nm; (iii) a strong destabilizing effect of ethanol on the orosomucoid molecule was observed in the alkaline region at an alcohol volume fraction of 20%. This effect corresponds to the anomalous behaviour of the water–ethanol systems.

Orosomucoid (acid α_1-glycoprotein; henceforth ORS) is among the most thoroughly studied glycoproteins of human blood serum. Its molecule is formed by one chain consisting of 181 amino acids linked by two disulfide bridges between cysteines 5 – 147 and 72 – 164. Primary structure determination revealed a high number of possible substitutions – at 21 positions of the peptide chain[1]. Characteristic is a low isoelectric point of this protein caused by the high number of carboxylic groups; the pI value varies between 1.8 and 2.7 according to the buffer used for its determination (for details see Schulze and Heremans[2], and Jeanloz[3]). The excellent solubility of ORS in water is due to joint effects of many charged amino acid residues in its peptide chain and to the high carbohydrate moiety content, which is 40% of the total molecular mass. A significant contribution comes from sialic acid, which constitutes approximately 11%. ORS presumably possesses a compact spatial arrangement, characterized by numerous masked groups: approximately 10 carboxyls[4], 9 tyrosyls[5], 2 tryptophyls[6], and almost all phenylalanines[7]. The extraordinary thermal stability is another remarkable property of ORS. A calorimetric study of this protein carried out at pH 7.4 provided a ΔH value of the transition equal to 119 kcal mol^{-1}; the process was found to be apparently a multistate one[8]. This kind of behaviour is supposed to result from the existence of polypeptide variants of ORS. Further examination of this problem by fluorescence[9], however, led to a significantly lower ΔH value. In both cases some experiments were done with a small

addition of ethanol as a compound exerting characteristic effects on proteins. Recent study[10] of the unfolding behaviour of ORS induced by heat or guanidine hydrochloride resulted in the conclusion that, from the residue 122, the polypeptide chain may be "loosely" folded. Thermal unfolding of ORS, according to that work, departs from the two-state model; at least one species different from the native or unfolded protein was detected. The present study was aimed at gaining more information about the thermal stability of the ORS molecule in general and about the influence of higher ethanol concentrations in particular.

EXPERIMENTAL

Materials

Human serum orosomucoid was isolated from Cohn's Fraction VI of human blood serum (Imuna, Sarisske Michalany) by fractionation on a CM-cellulose column[11]. Prior to this isolation, salts were removed from Fraction VI by desalination on a Sephadex G-25 column. The content of impurities was negligibly small and the protein preparation was homogeneous, as checked by polyacrylamide gel electrophoresis and by immunoelectrophoresis. Redistilled water was used in all experiments. The protein concentration in solutions was determined from the absorbance at 280 nm ($\varepsilon_{1\%,280}$ = 8.9; ref.[2]), and/or by gravimetry when a small sample of the solution was dried to the constant weight at 105 °C in a vacuum above P_2O_5. Isoionic ORS was prepared by further purification of the desalted sample. In this case, residual inorganic impurities were removed by dialysis or electrodialysis of ORS previously desalted on Sephadex. The pH of ORS so prepared was 3.67 (at a concentration of 2.4 wt.%); electrical conductivity was 74 . 10^{-6} Ω^{-1}cm^{-1} (0.35 wt.% solution in conductivity water). The molecular mass of ORS was taken to be 41 000 (ref.[12]). Ethanol of spectroscopy grade was used in this work; its content in solution is expressed as the volume fraction ϕ (%). *N*-Acetyl-L-tyrosine ethyl ester (Serva, Heidelberg), and *N*-acetyl-L-tryptophanamide (Lachema, Brno) were used as model compounds. All inorganic reagents were of analytical grade purity.

Methods

Differential scanning calorimetry (DSC). Calorimetric studies were carried out with a DASM-1 differential scanning microcalorimeter (Mashpriborintorg, Moscow) using isoionic ORS samples. Protein concentration ranged from 5 to 7 mg ml^{-1}; cell volume was 1 ml, scan rate 0.5 K min^{-1}. Degassing during heating was prevented by using an extra constant pressure of 101 kPa over the liquids in the cells. To get information on the reversibility of the observed changes, the protein samples were heated at 90 °C and immediately cooled down to 5 °C, and the heating was repeated. With the highest ORS concentration (7 mg ml^{-1}), this cycle was repeated three times.

Temperature perturbation difference spectroscopy (TPDS). The spectra were recorded on a Specord M 40 spectrophotometer (Zeiss, Jena) equipped with a TSA 1 temperature regulating block of the same manufacturer. Temperature was changed over the range 20 – 75 °C with a precision of ±0.1 °C. Hermetically closed cells 1 cm optical path length were used.

TPDS experiments were performed in three modes:

a) Absorbance changes at a fixed wavelength as a function of temperature. Slit width was 100 cm^{-1}, recording speed 0.5 mm sec^{-1}, temperature change 2.0 K min^{-1}. Absorbance values were recorded at 5 °C steps. The following wavelengths were used: 262, 266, 269, 280, 286, 288, 292, 295, and 300 nm.

b) Difference spectra at a constant temperature over the wavelength region of 240 to 300 nm. In the temperature range 25 to 75 °C, the full spectrum was recorded at 10 °C steps. Instrument parameters were the same as sub *a*).

c) Cyclic experiments were performed in the same way as sub *b*), but the spectra were recorded in three steps: the sample was heated from 25 to 75 °C (step 1), cooled down to the starting temperature (step 2), and then heated again to 75 °C (step 3). The spectra were recorded at 10 °C steps. The three steps followed each other immediately, with no pauses between them.

Experiments in modes *a*) and *b*) were carried out at pH 7; cyclic experiments were performed at pH 7, 9, and 11. For each pH value the spectra were recorded at ethanol contents ϕ_{EtOH} = 0, 20, and 40 vol.%.

The numbers of exposed residues were estimated by using a system of two equations of the general form[13]

$$x\left(\frac{\Delta\varepsilon_\lambda}{\Delta T}\right)_{TYR} + y\left(\frac{\Delta\varepsilon_\lambda}{\Delta T}\right)_{TRP} = \left(\frac{\Delta\varepsilon_\lambda}{\Delta T}\right)_{ORS}, \qquad (1)$$

where x and y denote the numbers of exposed tyrosines and tryptophans, respectively. For the actual calculation of the number of exposed residues, the values of difference absorbance $\Delta\varepsilon_\lambda$ at the wavelength λ_1 were substituted into the first equation, and at λ_2 into the second one.

All TPDS experiments were performed with ORS solutions whose ionic strength was held at 0.15 by addition of solid KCl. Phosphate buffer pH 7 (ionic strength 0.13) was used to attain this pH; higher pH values were reached by addition of appropriate amounts of volumetric (carbonate-free) KOH solutions.

Determination of pH. The pH values were measured with an OP-265 pH-meter (Radelkis, Budapest) using an OP-0808 P combined electrode of the same manufacturer. In mixed solvents, the pH-meter readings were corrected for the solvent effect on the activity coefficients of H_3O^+ ions, and for its direct influence on the two electrodes as described previously[14]. For all experiments, the pH values refer to the initial temperature of 25 °C.

Determination of free sugars. A chromatographic method for saccharides[15] was used to determine whether any saccharide residues were split off from the ORS molecule in the extreme conditions of alkalinity and high temperatures. A 20 µl sample of 0.25 wt.% ORS solution was chromatographed on Whatman 3 paper in the butanol–acetic acid–water system (10 : 1 : 3) along with sugar standards. Dried chromatograms were soaked in a solution of $AgNO_3$ in acetone (2 g of $AgNO_3$ in 1 ml of H_2O were dissolved in 199 ml of acetone), dried again, and soaked in 0. 35 M NaOH solution. When spots became visible, the chromatogram was stabilized in ammonia solution.

RESULTS

Calorimetry

Calorimetric experiments produced rather flat curves (Fig. 1) with a broad maximum. The first gradual increase in C_p was observed at low temperatures, above 20 °C, the highest value was attained at 58 °C. Then a sharper decrease in C_p followed, ending at 72 °C. After cooling, however, the C_p value was always higher than for the fresh sample. A lowering and broadening of the maximum was observed during subsequent heating. Because of the limited number of experiments, only an approximate value of

the van't Hoff enthalpy (ΔH_{vH}) varying between 3.9 and 490 kJ mol^{-1} could be calculated for the first heating cycle, e.g. for the native protein.

This result with isoionic ORS (pH 3.67) is in a good agreement with the value obtained by Hallsal[8] for pH 7.4, while fluorescence measurements[9] have led to about one-half of this value. Neither of the works cited mentions irreversibility observed in our experiments. After cooling, the difference in C_p between the original and the new state was approximately 17 kJ K^{-1} mol^{-1}. In the second heating the curve was shifted by this value upwards, the maximum appeared in the temperature region of 42 – 72 °C and was significantly lower than in the first heating. For the second and subsequent heatings the ΔH_{vH} value lay in the range of 120 – 150 kJ K^{-1} mol^{-1}.

Temperature Perturbation Difference Spectra

This technique has been applied to aqueous solutions of ORS by Kálal and Kalous[16]. As evidenced by chromatography of ORS samples in our experiments, no degradation of the carbohydrate moiety occurred after cyclic TPDS in the aqueous or water–ethanol systems at any of the pH values examined.

The wavelengths checked in these experiments were selected on the basis of properties of the UV-chromophores[17] (peaks of dissociated Tyr at 244 nm, the main peak of Phe at 266 nm) as well as from the previous data[7] obtained from the analysis of the fourth-derivatives of the absorption spectra of ORS. Then the main peaks are found at the wavelengths of 250, 282, and 293 nm. The TPD spectra changed substantially with temperature (Fig. 2); their shape in aqueous solution was in a good agreement with the part of spectrum between 270 and 300 nm as reported by Rojo-Dominguez and coworkers[10]. A broad minimum between 270 and 300 nm appearing at higher tempera-

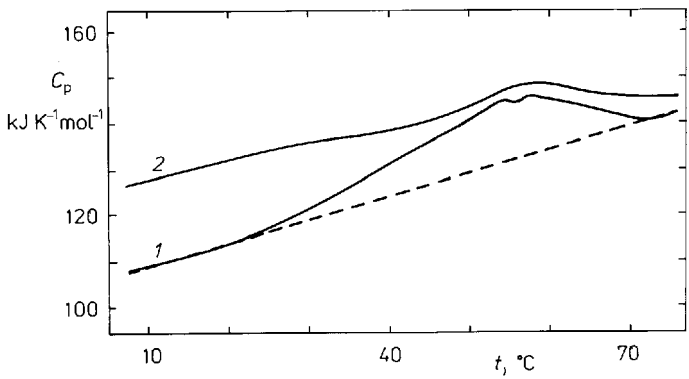

FIG. 1
Temperature dependence of the partial specific heat capacity C_p of isoionic ORS: *1* first heating, *2* second heating; protein concentration 7 mg ml^{-1}

tures is a typical feature of TPD spectra of ORS in water. A distinct main peak at 290 nm and two smaller peaks at 284 and 299 nm were observed in this minimum (Fig. 2, curve *4*). A marked increase in Δε with temperature was apparent at shorter wavelengths; at higher temperature, this segment of the curve, below 258 nm, bends downward. This transition appeared between 65 and 75 °C in aqueous solutions.

In ethanol solution at pH 7, the peaks at longer wavelengths did not change substantially (Fig. 3); the downward bending of the curve at shorter wavelengths, however, started at lower temperatures, as shown in Table I. This effect was the same at pH 9 when a distinct broad maximum appeared in the region of 240 – 255 nm.

TABLE I

Temperature of anomaly (t_{an}, °C) in the TPD spectrum within the 240 – 250 nm range at various ethanol contents (ϕ_{EtOH}, vol.%) in ethanol–water mixed solvents at pH 7 at steps 1, 2 and 3

ϕ_{EtOH}, vol.%	t_{an}, °C		
	1	2	3
0	75	75	75
20	65	55	65
40	55	35	45

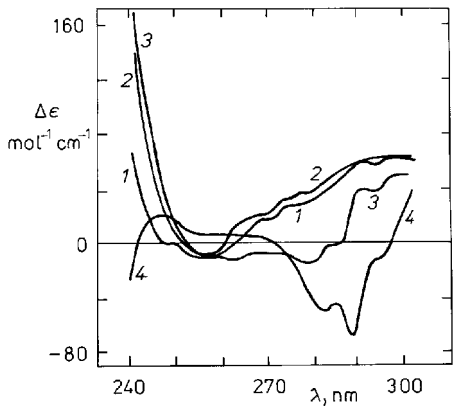

FIG. 2

Temperature perturbation difference spectra of ORS in water at pH 7, ionic strength 0.05; temperature: *1* 35 °C; *2* 55 °C; *3* 65 °C; *4* 75 °C

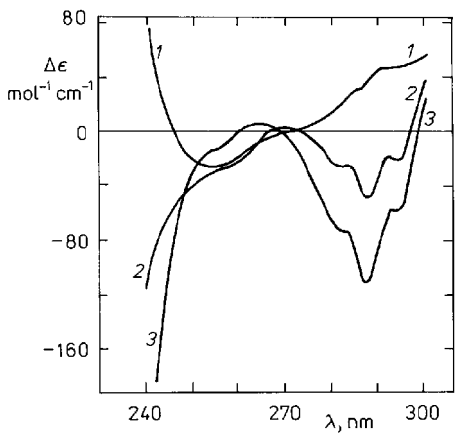

FIG. 3

Temperature perturbation difference spectra of ORS at pH 7, ϕ_{EtOH} = 40 vol.%; temperature: *1* 45 °C; *2* 55 °C; *3* 75 °C

At pH 11 in water, a broad maximum corresponding to dissociated tyrosine residues dominated, while the maxima between 270 and 300 nm were markedly suppressed. In 40% ethanol, Δε increased below 247 nm, and a broad maximum was observed at 260 nm (Fig. 4). The minima at longer wavelengths were very small.

The shape of the Δε vs Δ*T* dependence at selected wavelengths was compared for the three steps. Several basic types of changes can be distinguished and are denoted in the following way:

1 2 3 – the change in Δε with temperature was the same in all three steps; this case can be considered a fully reversible process (Fig. 5*a*);

1 3 – steps 1 and 3 were identical, whereas step 2 (cooling) followed a different route; ORS returned to the original conformation via different intermediate stage or stages; this case can be regarded as reversible for the heating of ORS (Fig. 5*b*);

1 2 – the changes in Δε during initial heating and subsequent cooling were identical, whereas the second heating followed a different route; this can be considered a sign of irreversible changes in the ORS molecule (Fig. 5*c*);

TABLE II
Reversibility of TPDS changes of orosomucoid in pure water

pH	λ, nm					
	244	250	266	280	288	292
7	123	13	13	123	123	123
9	12	12	13	123	123	123
11	23	ir	12	ir	ir	12

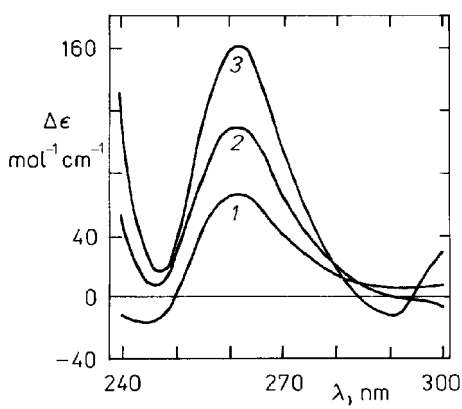

FIG. 4
Temperature perturbation difference spectra of ORS at pH 11, ϕ_{EtOH} = 40 vol.%; temperature: *1* 45 °C; *2* 55 °C; *3* 75 °C

2 3 – cooling and subsequent heating followed the same path, different from the first heating; in this case irreversible changes occurred during step 1 (Fig. 5*d*);

ir – fully irreversible process where steps 1, 2, and 3 followed different paths.

On this basis the TPD spectra were further compared as given in Tables II, III, and IV.

As evident from examples shown in Figs 5*a* – 5*d*, the dependence of $\Delta\varepsilon$ on ΔT was not always linear, and so the possibility of calculating the number of exposed chromo-

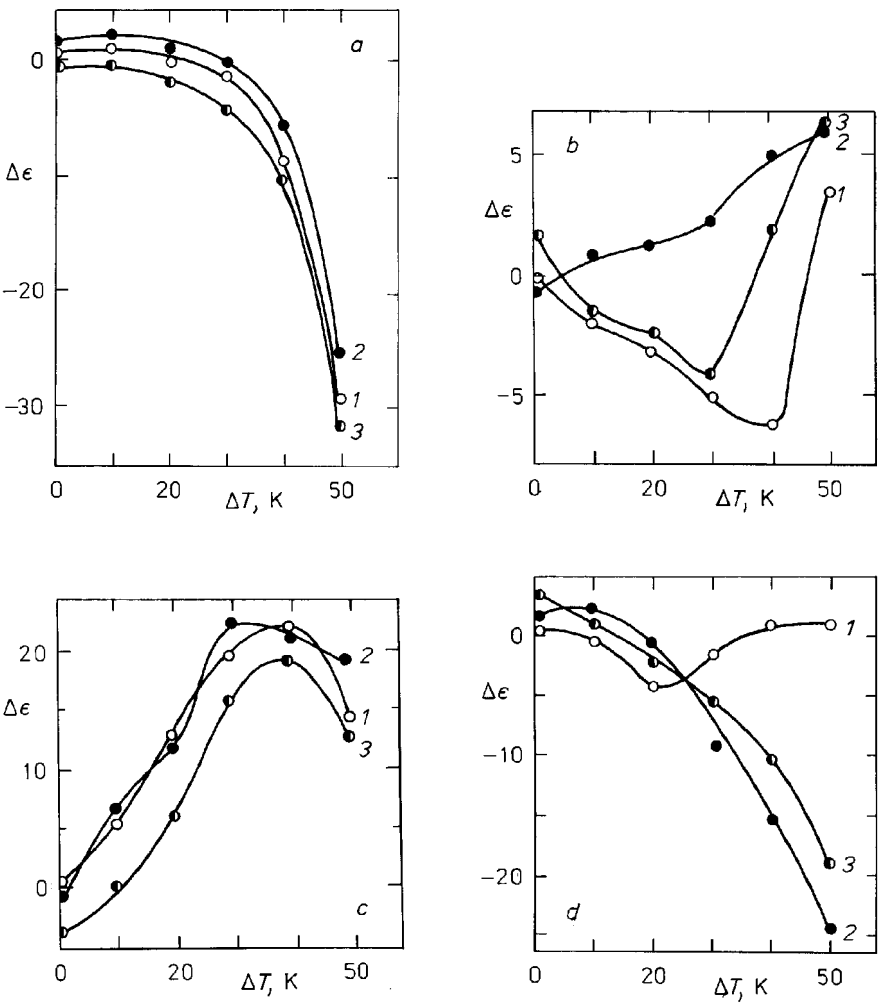

FIG. 5

Temperature perturbation difference spectra of ORS: change of absorbance ($\Delta\varepsilon$) as a function of ΔT; *1* first heating, *2* cooling, *3* second heating; *a* pH 7, ϕ_{EtOH} = 0%, 292 nm, *b* pH 9, ϕ_{EtOH} = 0%, 266 nm, *c* pH 9, ϕ_{EtOH} = 0%, 244 nm, *d* pH 7, ϕ_{EtOH} = 40%, 266 nm

phores using Eq. (*1*) was limited. In spite of this limitation, the approximate numbers of exposed tyrosyl and tryptophyl residues were estimated at pH 7 in 20 and 40% ethanol (Table V). The numbers of exposed tryptophyls could not be calculated with a reasonable precision in the alcohol solution.

TABLE III
Reversibility of TPDS changes of orosomucoid at ethanol content ϕ_{EtOH} = 20 vol.%

pH	λ, nm					
	244	250	266	280	288	292
7	ir	ir	23	23	123	123
9	23	23	23	23	23	ir
11	23	23	23	23	23	23

TABLE IV
Reversibility of TPDS changes of orosomucoid at ethanol content ϕ_{EtOH} = 40 vol.%

pH	λ, nm					
	244	250	266	280	288	292
7	23	ir	23	123	123	123
9	23	13	123	123	123	123
11	ir	ir	12	ir	ir	ir

TABLE V
The numbers of exposed tyrosyls and tryptophyls of ORS at pH 7 in the water–ethanol system ($\phi_{EtOH,}$ vol.%)

ϕ_{EtOH}, vol.%	TYR	TRP
0	4	0.5
20	4	≤ 1
40	6	≤ 1

DISCUSSION

Although ORS is among the thermally most stable proteins, certain doubts in this respect arise from the calorimetric experiments (Fig. 1) as well as from the cyclic TPD spectra (Tables II – IV) both indicating a partially irreversible behaviour of this protein. There is, however, a significant difference between both methods: if the calorimetric data can be rather regarded as a sign of irreversibility, the TPD spectra in comparable conditions (pH 7) are reversible, with a hysteresis in the region of shorter wavelengths that appeared on cooling only. This observation should be discussed in a broader context of the ORS structure, but due to the lack of precise data which are necessary for this, only general qualitative conclusions can be drawn.

In distilled water or in phosphate buffer at pH 7, neither calorimetry nor TPDS indicated a full reversibility, while circular dichroism spectra carried out in the same cyclic manner at pH 7.4 were found to be reversible[18]. The question is, what kind of changes is responsible for this difference. Obviously the changes do not touch the ordered secondary structures to a significant extent such as to influence the CD spectra.

The irreversibility in calorimetry is difficult to explain. The experiments were carried out in slightly different conditions, in full absence of inorganic ions. The only charges then were those of the dissociable groups of ORS. In an isoionic solution, the net charge (z) of a protein is close to zero, in the particular case of ORS, approximately 24 out of the 37 titratable carboxylic groups are dissociated[4] at this pH. Therefore, the number of charges at the ORS molecule is approximately 14 charges lower in the isoionic state than in a solution at pH 7. This difference, and thus a weaker electrostatic stabilization, may be one of the causes why the stability of ORS is decreased. On cooling, the ORS molecule loosened at higher temperature probably acquires a new conformation which is better stabilized by the lower number of charges.

The influence of ethanol on proteins is a complex one; along with deep changes in the structure of the solvent[19,20], direct binding of alcohol to the protein should be taken in account[21]. There are, however, no quantitative data about this process that obviously follows two mechanisms: the binding of ethanol via hydrogen bonds, and by hydrophobic interactions. Simultaneously, electrostatic interactions are enhanced due to the decrease in permittivity of the solvent[22].

As obvious from Tables III and IV, ethanol exerts a strong destabilizing influence on the ORS molecule at a concentration of $\phi_{EtOH} = 20$ vol.%, when a partially irreversible behaviour is already observed at pH 7 and full irreversibility at all wavelengths appears starting from pH 9. In contrast to this, reversible changes occur at $\phi_{EtOH} = 40$ vol.% in the region of longer wavelengths at both pH 7 and 9. The destabilizing effect of ethanol at $\phi_{EtOH} = 20$ vol.% was indicated by the CD spectra as well[7]. This concentration corresponds to a molar fraction of ethanol of 0.07, and is thus only slightly lower than the threshold value of 0.08 below which this alcohol exerts a destructive influence on the low-molecular domains of water[20]. Together with ethanol binding to ORS, this effect

can disturb the hydration layer of this protein destabilizing thus its conformation. The limited volume of information about the molecular domains structure of water–ethanol solutions does not allow a more precise explanation of the observed changes to be suggested.

Conformational changes of ORS observed by TPDS always start in the region of shorter wavelengths, below 260 nm. If phenylalanine residues without possible substitution are considered (Phe 26, 48, 49, 51, 61, 141), only two of them could have been located in structured regions: Phe 26 in the β-sheet and Phe 141 in the α-helix, as predicted previously[14]. Except for Phe 141, the remaining phenylalanines are located in the first half of the ORS molecule which is more hydrophobic. It seems therefore reasonable to assume that on heating, the hydrophobic groups are exposed to water, as can be interred from the increase in C_p. During cooling, the ordered secondary structures are restored in the original shape, whereas some of the unordered structures containing phenylalanines acquire a new conformation via another intermediate state. Thus, cooling does not seem to be a two-stage transition, for certain parts of the ORS molecule at least. In this new conformation some of the hydrophobic groups (phenylalanines from the unordered region) remain more exposed to water than they were in the native state. This higher exposure is reflected by an increased value[22] of C_p (ref.[23]).

In neutral and medium alkaline solutions, phenylalanines appear to be the primary foci of decrease in the thermal stability of ORS. The temperature anomaly (Table I) may be a consequence of this fact, but the downward bending of TPD spectra at lower wavelengths due to a blue shift turns the attention to tyrosines as well. For these chromophores, negative values of $\Delta\varepsilon$ have been observed with model compounds in ethanol[24]. If tyrosines were also responsible for this change, their higher exposure to the ethanol-containing solvent should be expected. This was indeed confirmed by TPDS, as demonstrated by Table V. On heating, the anomaly is thus obviously due both to conformational changes connected with phenylalanines and to exposure of tyrosines to water–ethanol solutions. These changes are irreversible in almost all circumstances. As for the exposure of tyrosines in the presence of ethanol, this effect has been described by Solli and Herskovits[24]. For ORS, a similar picture was obtained with the ethanol–water system in this work, while in methanol–water systems[14] tyrosines are more buried with increasing alcohol concentration.

The dependence of the stability of ORS on pH in the presence of ethanol confirms the previous conclusions that this protein undergoes deep conformational changes at pH above 9. The dissociation of tyrosines in this region contributes further to the increase in the net charge[14] of ORS, but some additional destabilizing mechanism is in action simultaneously.

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