EFFECT OF METHANOL ON THE STRUCTURE OF HUMAN BLOOD SERUM OROSOMUCOID (ACID α_1-GLYCOPROTEIN)

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The effect of methanol (volume fraction 0—70%) on the optical properties of human blood serum orosomucoid (acid α_1-glycoprotein) was studied over the pH range 5—12. The changes of these properties were correlated with the concept of three-dimensional arrangement of the orosomucoid molecule as obtained by a prediction study based on refined conformational preferences. From a complex analysis of the data obtained following conclusions on the orosomucoid structure in solution could be made: *1*) α-helix content increases at constant pH with the methanol volume fraction exceeding 30%. *2*) α-helix content at constant methanol concentration is maximal at pH 9—10. *3*) The changes in conformation brought about by the presence of methanol result in the masking of 1 tyrosine and 1 tryptophan residue in 70% alcohol. It has been observed that the complex action of even the lowest alcohol leads to marked changes at the various levels of the three-dimensional organization of the orosomucoid molecule.

Orosomucoid (acid α_1-glycoprotein, ORS in what follows) belongs to human blood serum glycoproteins which have been intensively studied. The attention which this protein has attracted is due to some of its properties, such as its extraordinarily high solubility in water, very low isoelectric point and considerable heat stability (see reviews by, e.g. Schulze and Heremans[1], Jeanloz[2]). The ORS molecule is built of one chain comprising 181 amino acid residues and amino acid substitutions occur at nineteen sites. Five heteropolysaccharide units are attached by the N-glycosidic bond to asparaginyl residues[3]. Two disulfide bonds crosslink cysteines 5—147 and 72—164 (ref.[4]). A characteristic feature of the ORS molecule is its certain closed-up arrangement. There are 10 masked carboxyl groups[5] and 9 masked tyrosine residues[6] in the ORS molecule. The temperature perturbation difference spectrometry measurements revealed in native ORS four exposed tyrosine residues out of 10—12 and one tryptophan residue of the three residues present in the molecule[7].

Puett and coworkers[8], who tried to cast light on the secondary structure of ORS, studied the circular dichroic spectra of glycopeptides and also the effect of sialic acid on these spectra. Aubert and Loucheux-Lefebvre[9] made an effort to predict

the secondary structure of ORS and compared the data obtained with the values calculated from CD spectra.

This study has been focused on the behavior of human blood serum ORS in a mixed medium water–methanol in which the volume fraction of methanol was 0–80%. A number of optical methods were used and a prediction study based on refined conformational parameters was also undertaken. The aim of the study has been to determine how the secondary structure of this protein and its behavior in alkaline media are affected by dilute methanol.

EXPERIMENTAL

Materials

Human blood serum orosomucoid was isolated from Cohn's fraction VI of human blood serum (IMUNA, Šarišské Michalany) by fractionation of the desalted preparation on CM-cellulose using a method developed on our Laboratory[10]. The purity of the protein isolated was checked by polyacrylamide gel electrophoresis and by immunoelectrophoresis. For all measurements ORS was dissolved in redistilled water and the concentrations of its solutions were determined spectrophotometrically at 280 nm ($E_{1\%,280} = 8{\cdot}9$, ref.[1]) or gravimetrically according to Keil and coworkers[11]. The molecular mass of ORS was taken as 41 000 (ref.[12]). N-Acetyl-L-tryptophan amide (NaTrpA, Lachema, Brno), L-tyrosine (Reanal, Budapest), N-acetyl-L-tyrosine ethyl ester (NATyrE, Serva, Heidelberg), DL-phenylalanine (Lachema, Brno), all of analytical purity, served as standards. Methanol of spectroscopy grade was used in the spectrophotometry measurements. All buffers and volumetric solutions were prepared from analytical purity reagents, 1 mM KOH and NaOH solutions used for the adjustment of higher pH-values were carbonate-free.

Methods

Absorption and difference spectrometry. These measurements were performed on a Specord M 40 (Zeiss, Jena) spectrophotometer over the wavelength range 220–350 nm. The measurements were made in cells of 1 cm optical path, the orosomucoid concentration was $1{\cdot}5 \cdot 10^{-4}$ mol . dm^{-3}, the ionic strength of the solution was adjusted to 0·1 by KCl. A tandem four-cell arrangement was used with the orosomucoid solution in phosphate buffer, pH 7·0, as a reference.

Temperature perturbation difference spectrometry (TPDS). These spectra were also measured on a Specord M 40 spectrophotometer using solutions of protein concentration equal to that used for absorption spectrometry. The concentrations of the model compounds were following: NATrpA $8{\cdot}1 \cdot 10^{-6}$ mol dm^{-3}, NATyrE $1{\cdot}1 \cdot 10^{-5}$ mol dm^{-3}. The reference sample was thermostated at 22–25°C during the measurement. The measured sample was always heated by 2–5°C to the final value of 40–45°C. The values measured were evaluated according to the equations[7]

$$x\left(\frac{\Delta\varepsilon}{\Delta T}\right)_{\mathrm{Tyr}_{288}} + y\left(\frac{\Delta\varepsilon}{\Delta T}\right)_{\mathrm{Trp}_{292}} = \left(\frac{\Delta\varepsilon}{\Delta T}\right)_{\mathrm{ORS}_{292}}$$

$$x\left(\frac{\Delta\varepsilon}{\Delta T}\right)_{\mathrm{Tyr}_{295}} + y\left(\frac{\Delta\varepsilon}{\Delta T}\right)_{\mathrm{Trp}_{300}} = \left(\frac{\Delta\varepsilon}{\Delta T}\right)_{\mathrm{ORS}_{300}}$$

The values of the slopes ($\Delta\varepsilon/\Delta T$) for model compounds were taken from the work of Kálal and Kalous[7] and were also verified in own measurements.

CD *Spectrometry.* A Jasco ORD/UV-5 spectropolarimeter with the CD module was employed. All experiments were performed at 20°C in cells of 1 cm optical path. For the calculation of the mean residual ellipticity Θ' the mean relative molecular mass of amino acid residue in the ORS molecule was taken to be equal to 119·1. The estimates of the percentual α-helix content of the ORS molecule was made with the aid of the equation of Greenfielder and Fasman[13]:

$$\% \alpha H = 100\,(\Theta' + 3\,900)\,.\,39\,600^{-1}$$

Fluorescence spectrometry. All fluorescence measurements were made on a Perkin Elmer LS 58 fluorimeter. The emission and excitation spectra of ORS and of model compounds were measured over the range optimal for the samples examined. The optimal concentrations were following: ORS $3\,.\,10^{-6}$ mol dm^{-3}, NATrpA $1\,.\,10^{-6}$ mol dm^{-3}, NATyrE $3\,.\,10^{-6}$ mol . . dm^{-3}, mixture of NaTrpA and NATyrE (1 : 4) $3\,.\,10^{-5}$ mol dm^{-3}. The emission spectra were always recorded for several wavelengths of excitation radiation over the range 280–300 nm. All experiments were carried out at 20°C.

pH *Measurement.* The problems in pH measurement in mixed media containing aliphatic alcohols have been studied by many authors[14–18]. Two corrections of the measured values are usually made: the first one concerning the potential of the glass electrode and the second one the activity coefficients of the system components. Based on these considerations an equation was proposed[16] expressing the relation between the pH-value measured and the following parameters:

$$\mathrm{pH} = \mathrm{p}m_{\mathrm{H}} - \log {}_{\mathrm{m}}\gamma_{\mathrm{H}} - \log {}_{\mathrm{s}}\gamma_{\mathrm{H}} + E_{\mathrm{j}}\,,$$

where $\mathrm{p}m_{\mathrm{H}}$ is the actual concentration of H_3O^+ ions, the activity coefficients γ express the influence of mixed medium ($_{\mathrm{m}}\gamma_{\mathrm{H}}$) and the salt effect ($_{\mathrm{s}}\gamma_{\mathrm{H}}$), respectively, E_{j} stands for the potential of the liquid boundary between saturated KCl solution and the mixed medium. Tanford[19] proposed a simplified formula in which both activity coefficients are included in one term and potential E_{j} is disregarded:

$$\mathrm{pH} = -\log c_{\mathrm{H}^+} - \log \gamma_{\mathrm{H}^+}\,,$$

where pH is the pH-meter reading, c_{H^+} the actual concentration of H_3O^+ ions in the solution. The values of the apparent activity coefficient $-\log \gamma_{\mathrm{H}^+}$ are obtained by plotting the measured pH-values versus the negative logarithm of the concentration of volumetric solution of the acid or base, respectively. The deviation of this plot from a straight line corresponding to the values for which $\gamma_{\mathrm{H}^+} = 1$ is the sought correction factor. Tanford's approach has been used in this study and it was confirmed that at methanol volume fractions below 30% the deviation of the measured values from the real ones was not more than 0·05 at pH below 12·5. The correction is necessary, however, at higher alcohol concentrations. The measurements were made on a OP-265 pH-meter (Radelkis) equipped with combined electrode OP-0808 P supplied by the same manufacturer.

RESULTS

Prediction of Orosomucoid Structure

An effort has been made in this study to refine the data of Aubert and Loucheux-

Lefebvré[9] particularly those on α-helix. The conformational parameters calculated by Davidová[20] by the statistical method of Chou and Fasman[21,22] and based on the data on 62 proteins were used. These conformational parameters differed markedly for some amino acids (by up to 25%) from earlier data; moreover, in this set cysteine was distinguished from cystine.

The prediction of α-helix and β-turn in this study was based on sliding averages of six conformational parameter values and in regions where it was necessary these data were complemented by sliding averages obtained for value quartets. The modified method of Chou and Fasman and the values of parameter P according to Levitt[23] were applied simultaneously. The prediction of the β-turns was based on values of products obtained for quartets of amino acid frequencies[24].

Structures for which $\langle P_\alpha \rangle_6 > 1{\cdot}00$ or $\langle P_\alpha \rangle_6 > \langle P_\beta \rangle_6$ were predicted as α-helix; for further refinement of helix they had to comply with the condition that $\langle P_\alpha \rangle_4 > \langle P_\beta \rangle_4$. Other marginal conditions had also to be fulfilled concerning the beginning and end of the helical stretch and the occurrence of amino acids with a strong (H), medium (I) and weak (B) tendency to form helix or β-sheet in the sextets examined[25].

The prediction of the secondary structure of ORS provides evidence that this protein contains relatively few helical structures. Using the procedure described above it becomes discussable whether α-helix involves positions 4–9 (ref.[9]). Position 5 is occupied by cystine for which the preference parameters[20] are $P_\alpha = 0{\cdot}085$, $P_\beta = 1{\cdot}60$ and moreover β-turn can be predicted for Leu 8.

The areas of the probable occurrence of α-helix are the following:

33–36, in position 32 $P_\alpha \simeq P_\beta$, then four residues follow occurence of which in α-helix corresponds to the combination H_2I_2 while β-turn is predictable for position 36.

Adjoining region, 37–46, repeatedly complies with the condition for β-turn in positions 38, 40, 41, 42, 43 and 44, the highest values being those for Val 41 and Glu 43. This decreases the probability of occurrence of α-helix in this segment which according to reference 9 should be located in the region between residues 41 and 46; for Val 41 $\langle P_\alpha \rangle_4 > \langle P_\beta \rangle_4$.

The other regions with the possible occurrence of α-helix are:

95–102, if Glu is residue 95; for Arg 95 the helix involves residues 96–102. In this region $\langle P_\alpha \rangle_6 > \langle P_\beta \rangle_6$. β-turn, however, is predictable for Leu 101, but in the region 99–104 there is the combination H_5I with a strong preference for α-helix;

135–146, following after Pro 131, yet residues 133 and 134 (Thr in both cases) rather prefer the β-structure. This helix is terminated by cystine 147;

167–181, following after Pro 166. For the whole region $\langle P_\alpha \rangle_6 \gg \langle P_\beta \rangle_6$. The values of $\langle P_\beta \rangle_6$ vary between 0·65 and 0·78.

Simultaneously the distribution of hydrophobic residues in the ORS molecule was examined by the procedure of White and Jacobs[26] which takes Phe, Met, Leu, Ile, Val, Cys, Ala, Pro, Gly, Trp and Tyr for hydrophobic residues with the assignment of the value 1 while the remaining amino acids are assigned the value 0. Using a sliding window comprising 11 amino acid residues we were able to assess the distribution of hydrophobic amino acids in ORS. The results of this analysis suggest that the N-terminus is very hydrophobic, in contrast to the C-terminus which is only slightly hydrophobic.

When these data are compared with the structure predicted from this viewpoint a difference between the two long helical regions becomes obvious. One half of the residues located in the region 135–146 are the hydrophobic ones whereas there is only one fifth of hydrophobic residues in the region 167–181. There is one hydrophobic residue only between 170 and 181 (Fig. 1.). From our prediction the presence of β-sheet can be expected in the following regions:

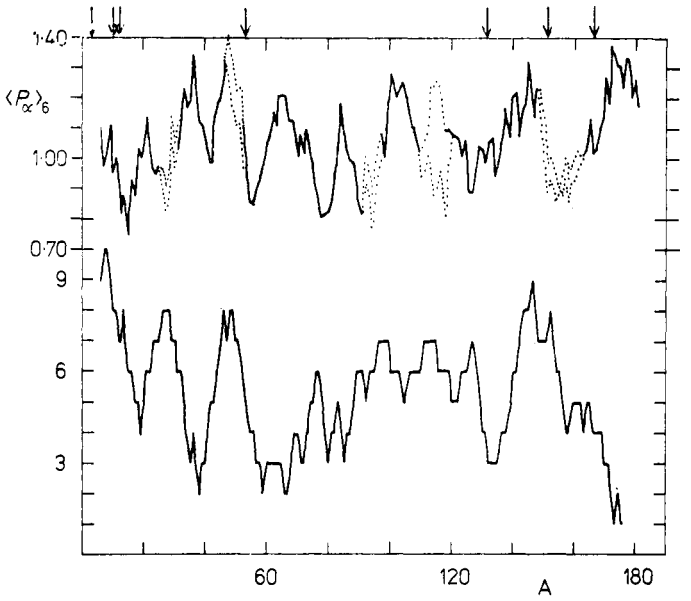

FIG. 1

Values of sliding averages $\langle P_\alpha \rangle_6$ for prediction of helical structure of ORS (upper curve). Regions with possible substitutions of amino acid residues are marked by a dotted line. The occurrence of the helix is probable for values higher than 1·10. The hydrophobic ORS profile (bottom curve) as sliding window of eleven amino acid residues. The individual amino acids A (1–181) of the primary structure of ORS are designated on the abscissa, the positions of proline residues are marked by arrows

11–18, following after Pro 10. Starting with Asp 19 the values of $\langle P_\alpha \rangle_6$ and $\langle P_\beta \rangle_6$ are very similar;

24–31, where $\langle P_\beta \rangle_6 > \langle P_\alpha \rangle_6$;

74–83, with the possible β-turn in position Tyr 78. For the whole region $\langle P_\beta \rangle_6 > \langle P_\alpha \rangle_6$;

90–94, the occurrence of β-sheet is probable provided that Val occupies 92;

125–129, where $\langle P_\beta \rangle_6 > \langle P_\alpha \rangle_6$;

155–161, provided position 156 is occupied by Val.

Absorption and Difference Spectra

These spectra were measured over the range of 230–310 nm. The dependence of the absorbance maximum on methanol content over the methanol volume fraction range of 0–70% (all data on methanol content which follow are given as volume fractions, φ_{Me} (vol. %)) at pH 5, 7, 10 and 12 is shown in Fig. 2. This maximum was found at 280 nm and its position remained unchanged with the exception of a shift to 285 nm observed at pH 12 when the methanol content changed from 40 to 70%. The dependence on alcohol concentration at pH 5 and 7 is almost identical, sigmoidal, increasing with the change in methanol content from 30 to 50%. A greater dispersion of the values measured was observed at higher pH-values, nevertheless a decrease of the absorbance maximum can be observed at pH 10 starting with the methanol volume fraction 40–50%; in contrast no change is observed at pH 12.

The examination of the dissociation of tyrosine residues of ORS in the presence of methanol was considerably complicated. These measurements, carried out at φ_{Me} equal to 30, 55 and 70% could be evaluated approximately only in the first two cases where the absorbance at 244 nm follows a typical dissociation curve. Because

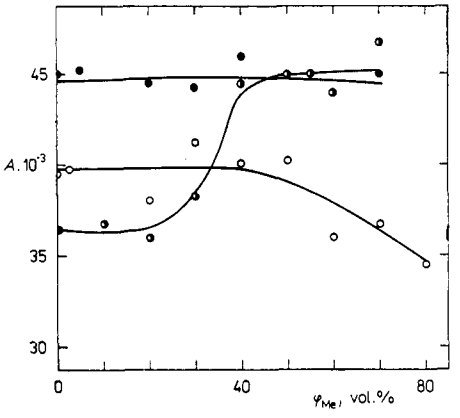

FIG. 2

Molar absorbance A of ORS at 280 nm as a function of methanol content φ_{Me} at ◑ pH 5, ● pH 10, ○ pH 12

of the limited quantity of material available the curve was determined in orienting experiments only. The latter showed that the inflection point of the curve, which lies at pH 11 in 30% methanol, is shifted to pH 9 in 55% methanol. The pK value of the tyrosine residues in 30% methanol is therefore in accordance with our previous analyses which were carried out in aqueous solutions[6].

During all experiments carried out in the presence of methanol at higher pH, another maximum in the difference spectrum of ORS can be observed lying between 260 and 270 nm. For $\varphi_{Me} = 30\%$ this maximum can be observed at pH 11·5, for 55% at pH 11 and for 70% at pH 10. At both lower methanol concentrations this maximum is shifted toward shorter wavelengths with increasing pH and lies at 260 nm at pH 13 whereas in 20% methanol is at 260 nm from the very beginning. This maximum obviously corresponds to the phenylalanine residues.

Temperature Perturbation Difference Spectra

These measurements were based on the study by Nicola and Leach[27], some data on model compounds $(\Delta\varepsilon/\Delta T)_{X_\lambda}$ were partly taken from the work of Kálal and Kalous[7]. The values of $(\Delta\varepsilon/\Delta T)_{ORS_\lambda}$ are listed in Table I. From the latter the numbers of exposed chromophores (Tyr and Trp) in the orosomucoid molecule could be calculated (Table II). As mentioned elsewhere[27] the values of the slope of the temperature dependence curve at 266 nm can yield information on the state of the phenylalanine residues. Such measurements, however, were unsuccessful in the case of ORS.

CD *Spectra*

The helix content as a function of methanol concentration was determined at pH 5, 7, 10 and 12 (Fig. 3). The values for pH 5 and 7 are almost identical. Characteristic of all these plots is an increase in α-helix content at methanol volume fractions higher than 30%. In the absence of alcohol the initial values decrease with the decreasing pH. α-Helix content as a function of pH was determined for the following methanol volume fractions: 0, 10, 55 and 70%. These plots are characterized by a flat maximum which is shifted in the presence of alcohol approximately by two pH units toward alkaline values (Fig. 4).

Fluorescence Spectra

When excited at a wavelength of 285–290 nm the dependence of fluorescence intensity on methanol content passes through a minimum lying for the individual compounds in the range corresponding to 30–40% of alcohol; the minimum for model mixture NaTyrE : NATrpA 4 : 1 corresponds to 30–50% of methanol (Fig. 5).

TABLE I

TPDS Slopes for ORS at different pH and methanol content (φ_{Me})

φ_{Me} vol. %	pH	$\left(\frac{\Delta\varepsilon}{\Delta T}\right)_{ORS_{292}}$	$\left(\frac{\Delta\varepsilon}{\Delta T}\right)_{ORS_{300}}$
30	5	0·6128	0·5317
55	5	0·5774	0·5085
70	5	0·4183	0·3057
0	7	0·0699	0·0541
0	10	0·0875	0·0612
30	12	0·3699	0·4122
55	12	0·5294	0·5773
70	12	0·3443	0·2792

TABLE II

Numbers of exposed tyrosine and tryptophan residues of ORS at different values of pH and methanol content (φ_{Me})

Amino acid	pH	φ_{Me}, vol. %		
		30	55	70
Tyr	5	2·5	2·4	1·0
	12	2·5	3·5	1·1
Trp	5	2·0	1·8	1·0
	12	1·6	2·2	1·0

TABLE III

Wavelength of fluorescence intensity maximum (nm) for model mixture NATyrE : NATrpA 4 : 1 as a function of pH and methanol content (φ_{Me})

pH	φ_{Me}, vol. %			
	0	30	55	70
5	—	359·5	358·5	357·5
7	363·0	360·5	358·0	358·0
10	363·0	361·0	358·5	357·5
12	363·0	363·0	359·0	358·0

As observed in measurements with the model mixture the position of the fluorescence maximum depends both on pH and on the methanol content (Table III).

The fluorescence intensity of ORS versus methanol content is characterized by a similar curve (Fig. 5) also passing through a minimum whose position changes with pH. For pH 5 its position corresponds to methanol volume fraction 30%, at higher pH values to 10%. The dependence of the fluorescence intensity of tryptophan at pH 7 is also shown in identical relative units in Fig. 5. The position of the fluo-

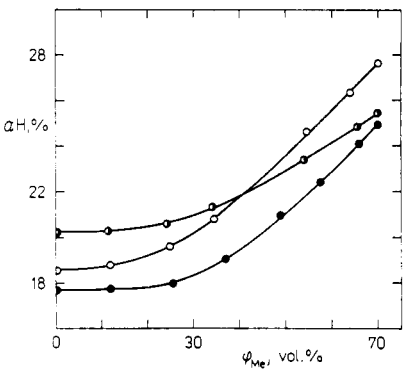

FIG. 3

α-Helix content of ORS molecule as a function of methanol content φ_{Me} at ◑ pH 7, ○ pH 10, ● pH 12

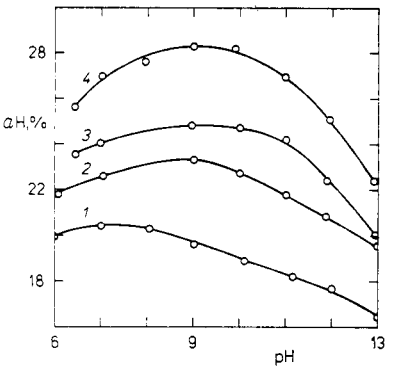

FIG. 4

α-Helix content of ORS molecule as a function of pH; methanol content φ_{Me}: *1* 0; *2* 10; *3* 55; *4* 70

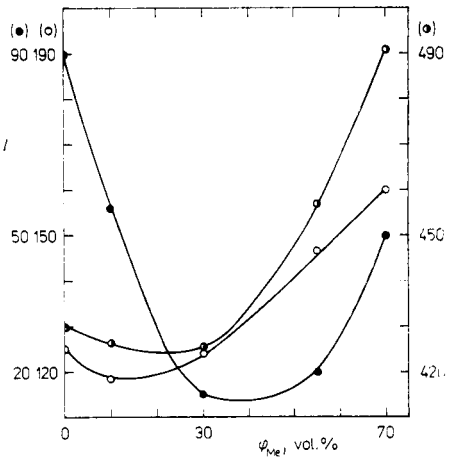

FIG. 5

Intensity of ORS fluorescence *I* (relative units) as a function of methanol content φ_{Me}: ◑ NATrpA; ● mixture of NATyrE : NATrpA 4 : 1; ○ ORS

rescence maximum of orosomucoid (excitation at 280 nm) also changes with pH, except for pH 5, and with the methanol content (Table IV). The fluorescence of ORS as a function of pH at constant methanol content always decreases (Fig. 6). Whereas this decrease is terminated between pH 7 and 8 in the absence of alcohol, the presence of the latter manifests itself by a continuous decrease in fluorescence intensity with a hint of inflection between pH 7 and 10 in 30% alcohol.

DISCUSSION

The behavior of proteins in solutions containing aliphatic alcohols has been intensively studied during last years. The effect of alcohols can be considered representing two contributions. The first one is the effect of alcohols on the structure of water, described by Franks[28] as a competition between water molecules and the alcohol for the cavities in ordered water structures. These processes manifest themselves by outside changes in certain physical or physicochemical parameters. Thus, e.g. the

TABLE IV
Wavelength of ORS emission maximum as a function of pH and methanol content (φ_{Me})

pH	φ_{Me}, vol. %			
	0	30	55	70
7	335·5	335·0	330·5	333·5
10	336·5	334·5	333·0	331·5
12	338·5	335·5	333·5	334·5

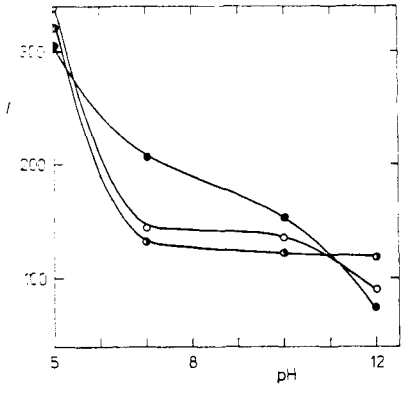

FIG. 6
Intensity of ORS fluorescence I (relative units) as a function of pH; methanol content (φ_{Me} in vol. %): ◑ 0; ○ 10; ● 70

dependence of excess enthalpy of mixing on methanol concentration shows a flat minimum in the region corresponding to 30–60 vol. % of this alcohol. This dependence was decisive in our experiments for the choice of methanol concentrations.

The values measured show as a rule a marked change in the alcohol concentration range described above, a phenomenon confirming the effect of the water structure on the behavior of proteins. An analogous dependence on methanol concentration has been reported by Herskovits and coworkers[29] who observed the highest denaturing effect on various proteins in solutions with 30–50% concentration of this alcohol; similar values were also yielded by the examination of thermodynamic parameters of lysozyme denaturation[30] and of the stability of collagen and tropocollagen[31].

The other effect is the binding of methanol itself to the protein, a phenomenon which has not been investigated in detail as yet. Its qualitative aspects have been studied by Lubas and coworkers[32] who also defined the three destabilizing effects of aliphatic alcohols on proteins. Quantitative data on the binding of small aliphatic alcohols to proteins are also limited and are based mainly on studies of ultraviolet spectra[33]. The existence of a similar possibility was considered in our experiments as well (Fig. 2) since the dependence of ORS absorbance on methanol concentration has at pH 5 a shape resembling the binding isotherms. An evaluation by the Scatchard plot, however, did not lead to realistic values. A plot of the type of v/c_{Me} versus v, where the number of ligands bound v is substituted by the difference between the limiting absorbance value and the absorbance value for the given methanol concentration (c_{Me} being the molar concentration of methanol) is not linear and cannot be used for a reliable determination of even the number of types of binding centers. This plot becomes linear if $v/c_{Me}^{0.25}$ is plotted; this confirms the fact that the mechanism of alcohol interaction with proteins is more complicated as regards the character of the bonds formed.

The dependence shown in Fig. 2 differs for the individual pH-values, the greatest difference showing the curve obtained at pH 5. All three individual absorbance curves are the result of at least three processes which take place simultaneously: of the binding of methanol to ORS, of the conformational changes of this protein and of the dissociation of tyrosine residues. The latter does not occur at pH 5 and therefore the curve for this value is markedly affected mainly by methanol binding; this explains its shape, yet the helicity of ORS increases simultaneously. The curve cannot therefore be processed by usual methods employed for the evaluation of interactions.

When the pH is raised toward alkaline values the dissociation of tyrosine residues takes place; this manifests itself by an absorbance increase at the wavelength of the maximum in the 280–300 nm range. A drop of the curve for pH 10 (Fig. 2), occurring when the methanol fraction volume is 40–50%, is obviously caused by partial masking of tyrosine residues resulting from the increase of the helical content of

ORS (Fig. 3) at this pH. The structure of the ORS molecule at pH 12 is so loose[34] that the increase in the helical content cannot manifest itself in this manner.

As regards helix content of the ORS molecule the refined values of prediction parameters used in this study yielded results differing from the data obtained earlier[9]. It is very little probable that the helix exists between residues 4 and 9, as well as between residues 41 and 46 where on the contrary the presence of β-turns is highly probable. Neither did Aubert and Loucheux-Lefebvre[9] postulate the existence of the helix in either the region 95–102 or in the region of the last four residues of the ORS molecule.

The prediction made in this study permits us to conclude that the helical structures of the ORS molecule comprise 39 amino acid residues, that is 21·5% of the chain; this value is in excellent accordance with the value of 20% determined from the CD spectra.

Helix content of the ORS molecule is at constant methanol concentration clearly dependent on pH (Fig. 4). The amount of helix increases in the presence of alcohol always between pH 9 and 10, i.e. in the region where the first tyrosine residues dissociate. It is this very region in which the destabilization of the ORS structure is observed[6]. One of the main reasons for this phenomenon is electrostatic repulsion since ORS bears a large negative charge at these pH-values. These phenomena are more marked in Fig. 7 showing the values from Fig. 4 plotted as a function of ORS net charge. For this plot a simplified assumption was made that the dissociation of the protein under these conditions is similar to its dissociation in water. As observed in experiments with albumin[35] the titration curves of proteins are not significantly altered in the presence of small aliphatic alcohols; hence, the titration curve used for ORS was that determined in water[5]. The changes in α-helix content occur when the net charge of the molecule is −20 to −26, the subsequent decrease in

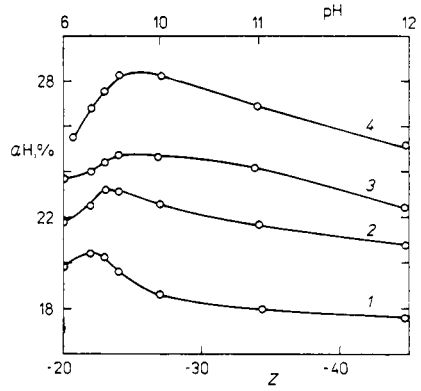

FIG. 7

α-Helix content of ORS as a function of net charge (Z) of protein molecule; methanol content (φ_{Me} in vol. %): *1* 0; *2* 10; *3* 55; *4* 70

α-helix content being almost linear and following the dissociation of the functional groups of ORS.

In spite of the changes which occur in the ORS molecule the majority of tyrosine residues remain masked, as follows from TPDS measurements. We have shown in our earlier experiments[6] that a characteristic feature of the ORS molecule is its content of a higher number of various types of masked groups, a fact which indicates a certain compactness of the molecule with a more pronounced hydrophobic core[5]. If methanol is present it may be expected that it will be bound to ORS both through hydrogen bonds and also through hydrophobic interactions; this may lead to additional masking of the functional groups regardless of the loosening of the tertiary structure.

If the distribution of the tyrosine residues in various structures is compared it becomes obvious that the probability of their additional masking will increase. The tyrosine and tryptophan residues in Table V are classified with respect to structures in which they occur according to our prediction; a region of the chain for which the numerical value of the sliding window (Fig. 1) is 6 or higher is regarded as hydrophobic.

The fluorescence of ORS is determined by its tryptophan residues, as obvious from Fig. 5, and its intensity shows a profile similar both for the protein and for the free amino acid. This curve is markedly different for the model mixture NATyrE : : NATrpA 4 : 1. Likewise, the curves in Fig. 7 point to the decisive role of tryptophan since the fluorescence intensity did not essentially change between pH 7 and 8 at lower alcohol concentrations even though the dissociation of tyrosine residues takes place in this range. General and specific effects of the solvent[36] can be observed at high alcohol concentrations.

As evidenced by the results of fluorescence measurement its quenching occurs when the pH is increased (Fig. 6); this indicates the masking of tryptophan residues. This phenomenon can obviously be ascribed to the binding of methanol via both

TABLE V

Localization of tyrosine and tryptophan residues of ORS in various structures

Amino acid	Structure type				
	β-sheet only	hydrophobic region only	hydrophobic β-sheet	hydrophobic α-helix	undetermined regions
Tyr	74, 157	50, 100, 115	27, 78, 91, 127	142	37, 65
Trp	160		25		122

mechanisms, i.e. via hydrogen bonds and via hydrophobic interactions in view of the character of the amino acid residues in the immediate neighborhood of all three tryptophan residues (Trp 25: Lys, Phe; Trp 121: Asn, Gly; Trp 160: Asp, Lys). The masking of the tryptophan residues is also evidenced by the red shift of the emission spectrum of ORS as a function of pH (Table IV) at constant methanol concentration.

On the other hand the curve of the dependence on methanol concentration at constant pH shows a blue shift indicating the contact of exposed tryptophans with the less polar solvent.

As can be seen in Table I the masking as a result of increasing methanol concentration takes place in native state already. The results of our measurements confirm the data of Kálal and Kalous[7] whereas two tryptophan residues only (25, 160) are masked in native state of ORS according to Schmid[37] even though Trp 122 should also be masked, at least partly. According to our prediction analysis Trp 25, localized in the hydrophobic β-sheet, could be masked. The residue Trp 122 in whose neighborhood a markedly hydrophobic stretch begins, could be masked in the presence of methanol.

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