# THE TYROSYL AND TRYPTOPHYL RESIDUES OF OROSOMUCOID STUDIED BY DIFFERENCE AND DERIVATIVE SPECTROPHOTOMETRY

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The accessibility of the tyrosyl and tryptophyl residues in orosomucoid (an acid $\alpha_1$-glycoprotein) was studied by the methods of temperature-perturbation difference spectrophotometry and derivative spectrophotometry. It has been found that four residues of tyrosine and one residue of tryptophan are on the surface of the molecule, exposed to the solution. The method of derivative spectrophotometry has been modified to be applicable to proteins containing both tyrosine and tryptophan.

Orosomucoid (an $\alpha_1$-glycoprotein)[1] is the most acid plasma protein, whose content increases in a number of disorders[2]. A considerable part of the molecule is constituted by saccharides, bound to the residues of asparagine in the polypeptide chain[3,4]. The protein skeleton of orosomucoid is represented by one polypeptide chain, composed of 198 amino acid residues[5]. On the basis of knowledge of location of the oligosaccharide chains and disulphide bonds, Schmid and coworkers[4] have propounded a model of the orosomucoid molecule.

As for the tyrosyl and tryptophyl residues exposed to the solution, the literature gives rather discrepant values. Thus Yamagami and coworkers[6] state that 5 to 8 tyrosyl residues out of the total of 12 are exposed to the solution, Schmid and coworkers[7] found 6, Svobodová and Kalous[8] only 3.

The present paper is an attempt to make these results more accurate by the use of two more spectrophotometric methods.

## EXPERIMENTAL

### Materials

Orosomucoid was isolated from the Cohn Fraction VI of blood serum (Imuna). N-Acetyl-L-tyrosine ethyl ester was from the firm Serva, L-tryptophan from the firm Calbiochem.

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The solutions of the protein and the model compounds were prepared fresh prior to each measurement by dissolving them in a phosphate buffer, 0·05 mol $dm^{-3}$, pH 6·8. To determine the exact concentrations of orosomucoid and the model compounds the following molar absorptivities were employed: $\varepsilon_{278} = 3{\cdot}57 \cdot 10^6$ $m^2$ $mol^{-1}$ for orosomucoid[9], $\varepsilon_{280} = 5{\cdot}55 \cdot 10^5$ $m^2 \cdot mol^{-1}$ for L-tryptophan[10] and $\varepsilon_{275} = 1{\cdot}5 \cdot 10^5$ $m^2$ $mol^{-1}$ for N-acetyl-L-tyrosine ethyl ester[11].

Before the spectrophotometric measurements each solution was filtered through a Millipore microfilter, pore size $0{\cdot}45 \cdot 10^{-6}$ m.

## Methods

Two spectrophotometric techniques were used to determine the accessibility of the tyrosyl and tryptophyl residues: the temperature-perturbation difference spectrophotometry[11-13] (TPDS) and the derivative spectrophotometry[14].

For TPDS we employed an apparatus Pye Unicam SP 8-400 with quartz cells Silica 0, optical path 1·000 cm, the absorbance range being set to 0—0·1 across the width of the paper. The concentrations of the substances under study were determined accurately by means of their molar absorptivities. The cells with the measured and the reference samples of the protein or a model compound were brought to a required temperature with a precision of ± 0·1°C.

Prior to TPDS the two cells were filled with the same solution of the substance to be measured and after equilibration of temperature to 25°C we recorded the zero line, corresponding to zero difference of absorbance. Then the temperature of the solution to be measured was lowered to 15°C. After a precise determination of temperatures in the two solutions the difference spectrum was immediately recorded. Further difference spectra were obtained in the same way, the temperature of the measured sample being stepwise elevated by 3°C up to a temperature of 38°C. Finally we tested reproducibility of the zero line (to verify reversibility of the process) at 25°C in both cells.

In this fashion we obtained a series of temperature-perturbation difference spectra for various differences in temperature. The differences in absorbance at the indiivdual wavelengths were recalculated to differences of molar absorptivities, $\Delta\varepsilon$, according to the Lambert–Beer law. The plot of $\Delta\varepsilon$ *vs* the temperature differences in the two cells gave a line. Its slope, $(\Delta\varepsilon/\Delta T)_\lambda$, for the model compounds (N-acetyl-L-tyrosine ethyl ester, L-tryptophan) and for orosomucoid was used to calculate the numbers of the exposed tyrosyl and tryptophy residues. These numbers were calculated from the following general equations[7]:

$$x(\Delta\varepsilon/\Delta T)_{288}^{\mathrm{TYR}} + y(\Delta\varepsilon/\Delta T)_{292}^{\mathrm{TRP}} = (\Delta\varepsilon/\Delta T)_{292}^{\mathrm{PROTEIN}}$$

$$x(\Delta\varepsilon/\Delta T)_{300}^{\mathrm{TYR}} + y(\Delta\varepsilon/\Delta T)_{300}^{\mathrm{TRP}} = (\Delta\varepsilon/\Delta T)_{300}^{\mathrm{PROTEIN}}$$

where $x$ and $y$ denote the numbers of the exposed tyrosyl and tryptophyl residues respectively, and $(\Delta\varepsilon/\Delta T)_\lambda$ the slopes of the substances at the given wavelength.

To determine the heterogeneity of the tyrosyl residues, a new method has been proposed, based on that of Brandts and Kaplan[14], who used the derivative spectra. According to these authors the heterogeneity, H, of the tyrosyl residues can be expressed by the product of the heterogeneity parameters R (ratio of heights of the peaks *I* and *II* in a derivative spectrum) and $\lambda_{1/2}$ (half-width of the derivative band I); see Fig. 1 With an increase in H, the heterogeneity of the tyrosyl residues in the protein increases too.

The derivative spectra of orosomucoid and the model compound (L-tryptophan) were obtained by numerical differentiation of the absorption spectra with respect to the wave length. The spectra

were digitalized by a step of 0·5 nm. In the calculation we used the formula for a symmetrical derivative[15] $dA/d\lambda = A(\lambda + \Delta\lambda) - A(\lambda - \Delta\lambda)/2\Delta\lambda$, where $dA/\Delta\lambda$ is an element of the derivative spectrum, $A(\lambda + \Delta\lambda)$ and $A(\lambda - \Delta\lambda)$ the elements of digitalized spectrum, and $\Delta\varepsilon$ the step of digitalization. The absorption spectra employed were recorded, at a scale range of two absorbance units, with an apparatus Pye Unicam SP8-400 at a temperature 22°C.

Since orosomucoid also contains three tryptophyl residues, we could not use the method of Brandts and Kaplan, applicable only to proteins without them. For the presence of tryptophyl residues deforms the tyrosyl bands, thus distorting the results. Therefore, we subtracted from the derivative spectrum of the protein the derivative spectrum of L-tryptophan, multiplied by the number of tryptophyl residues in the protein. For the derivative spectra thus obtained we determined the parameters $R$ and $\lambda_{1/2}$, according to the above-given definition. The assumption was that both the exposed (accessible) and the masked (buried) tryptophyl residues contributed equally to the spectrum of the protein.

## RESULTS

### *Temperature-Perturbation Difference Spectrophotometry*

The model compound for the temperature perturbation of the tyrosyl residues in a molecule of orosomucoid was N-acetyl-L-tyrosine ethyl ester. At wavelengths of 288 nm and 300 nm the following slopes $(\Delta\varepsilon/\Delta T)_\lambda$ were calculated for it from five independent measurements: $(\Delta\varepsilon/\Delta T)_{288}^{TYR} = 0{\cdot}268\ m^2\ mol^{-1}\ K^{-1}$, $(\Delta\varepsilon/\Delta T)_{300}^{TYR} = 0{\cdot}059\ m^2\ mol^{-1}\ K^{-1}$.

The model compound for the perturbation of tryptophyl residues was L-tryptophan, for this we obtained $(\Delta\varepsilon/\Delta T)_{292}^{TRP} = 0{\cdot}656\ m^2\ mol^{-1}\ K^{-1}$ and $(\Delta\varepsilon/\Delta T)_{300}^{TRP} = 0{\cdot}352\ m^2\ mol^{-1}\ K^{-1}$.

With orosomucoid we analogously obtained $(\Delta\varepsilon/\Delta T)_{292}^{PROTEIN} = 1{\cdot}891\ m^2\ mol^{-1}\ K^{-1}$ and $(\Delta\varepsilon/\Delta T)_{300}^{PROTEIN} = 0{\cdot}665\ m^2\ mol^{-1}\ K^{-1}$.

By substitution of these values into the above-given equation we calculated $x = 4{\cdot}1$, $y = 1{\cdot}2$. Consequently, the number of the exposed tyrosyl residues in orosomucoid

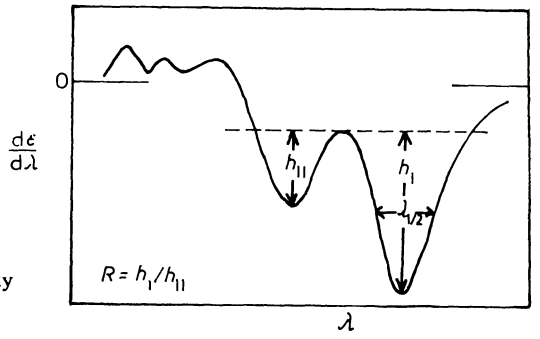


FIG. 1
Definition of parameters of heterogeneity according to Brandts and Kaplan[14]

determined by TPDS is four, the number of the exposed tryptophyl residues is close to one. A typical TPDS of orosomucoid is shown in Fig. 2.

*Derivative Spectrophotometry*

The derivative spectrum of orosomucoid, obtained by differentiating the absorption spectrum, is shown in Fig. 3 (the dotted curve). This spectrum was modified by subtracting the contributions of the three masked tryptophyl residues, a corrected spectrum being thus obtained (Fig. 3, solid curve). The parameters of heterogeneity, obtained as averages of three independent measurements, were $R = 4{\cdot}1$ and $\lambda_{1/2} =$

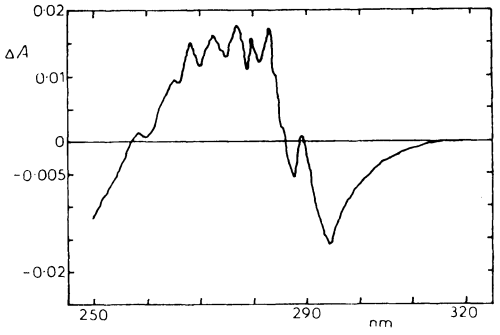


FIG. 2

Temperature-perturbation difference spectrum of orosomucoid, conc. $5{\cdot}5 \cdot 10^{-5}$ mol dm$^{-3}$, $\Delta T = -15$ K, pH 6·8

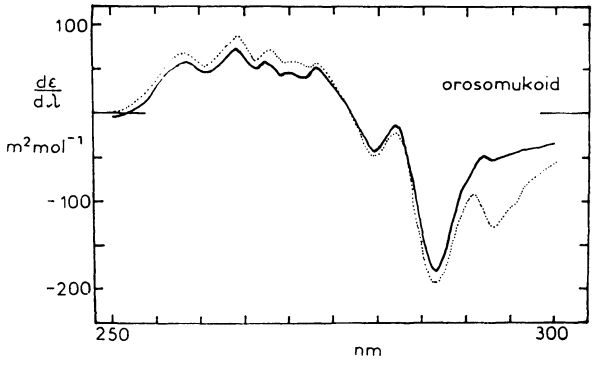


FIG. 3

Derivative spectra of orosomucoid. The original spectrum (·····) and the corrected spectrum (———)

= 4·4, hence H = 18·3. Consequently, the spectral heterogeneity of the tyrosyl residues, as assessed by the method of Brandts and Kaplan[14], is low to medium.

## DISCUSSION

The method of TPDS has shown that a molecule of orosomucoid contains four accessible tyrosyl residues, which are exposed to the solution, whereas the remaining eight are masked in the hydrophobic core of the molecule. This result accords with the constant of heterogeneity, determined as H = 18·3. According to the literature[14], this value suggests that one group of tyrosyl residues predominates, in this case the masked (buried) tyrosyl residues. Our result is very close to that we reported previously[8] (3 exposed tyrosyl residues) and to the result obtained by Schmid and co-workers[7] (5 exposed residues). Either value was determined by spectrophotometric titration. The value obtained by a chemical method[6] (5–8 exposed residues) seems rather unlikely. The chemical modification of the accessible tyrosyl residues probably led to conformational changes of the orosomucoid molecule, by which more tyrosyl residues got exposed.

The number of the exposed tryptophyl residues, as measured by TPDS, is close to 1.

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