ON THE KINETICS OF THE ALKALINE DENATURATION OF OROSOMUCOID

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Spectrophotometric titration of blood orosomucoid revealed three accessible tyrosine residues, of pK 10·0, and nine masked residues, pK 11·8. Rapid changes of absorbance and fluorescence intensity of orosomucoid in mildly alkaline media were investigated by the stopped-flow method. These changes are ascribed to the effect of the altering protein conformation on the aromatic chromophores. A scheme of the denaturation steps of orosomucoid is propounded and the individual steps are characterized by their rate constants.

Orosomucoid (acidic α_1-glycoprotein[1]) is a blood plasma protein whose biology and chemistry has recently been paid increasing attention[2]. Some authors[3-6] deal with the structure and physical chemistry of this protein, as this approach is expected to elucidate its extraordinary resistance to denaturing effects, both physical and chemical[1,7].

In studying the stability of a protein molecule attention is often given to masked functional groups[8,9] and their uncovering in denaturation. The tyrosine residue is a group whose dissociation or unmasking can be followed by spectrophotometric titration[10-13]. This was studied by Yamagami and coworkers[6], who observed that after an addition of an alkali hydroxide to a solution of orosomucoid absorbance of the solution increased with time.

We have dealt with similar experiments and found that absorbance of orosomucoid not only increased, but, under different conditions, also unexpectedly decreased with time. The latter phenomenon, which was probably due to conformation changes, was studied by the "stopped-flow" method[14,15], which detected the primary change in the molecule caused by the denaturant. In addition to following the fast changes in absorbance, intensity of fluorescence was measured as well.

EXPERIMENTAL

Materials

Orosomucoid was isolated from Cohn's[16] fraction VI of human blood serum (from Imuna, Nat. Corp.) by the method used in our laboratory[17]. The preparation was tested by immunoelectro-

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phoresis and electrophoresis in starch gel; the impurities stainable by Amido Black 10B did not exceed 2%. All experiments were carried out with isoionic solutions of orosomucoid, prepared by desalting on a mixed ion exchanger Serdolit MB (Serva, West Germany). Concentration of these solutions was determined by drying aliquot portions in a desiccator over P_2O_5 at 105°C *in vacuo* to constant weight. Solutions of KOH were carbonate-free; they were kept under nitrogen.

Methods

pH was measured with a pH meter pHM 28 equipped with a glass electrode G 202 B and a calomel electrode K 100 (Radiometer products, Denmark), connected to a recorder EZ 2 (Laboratory Instruments, Prague). In kinetic measurements a pH meter Radelkis (Budapest) and a glass electrode combined with a calomel electrode were used. The electrodes were calibrated with the aid of an acetate buffer, pH 4·64. Changes of pH were measured in a 0·25% solution of orosomucoid, which was 0·1M in KCl. In all experiments the temperature was kept thermostatically to 25°C.

Optical spectra were registered on a spectrophotometer Specord UV VIS (Carl Zeiss, Jena), connected to a recorder EZ 2 (Laboratory Instruments, Prague). The optical path in a cell was 10 mm. The absorption spectra were recorded in the region 200—300 nm with orosomucoid solutions of conc. $2 \cdot 10^{-3}$ to $5 \cdot 10^{-2}$%, at pH 7·40 (0·1M phosphate buffer), pH 10 and pH 12·50. The optimum concentration proved to be approx. 0·01%, at which the difference spectra showed maximum separation of the signal from the noise. The difference spectra were measured in the usual tandem arrangement[9]. The working solution was obtained by mixing a 0·02% solution of orosomucoid, 0·1M in KCl, with aqueous KOH of a suitable concentration. The reference pH was 7·40 (0·1M phosphate buffer). The measurements covered a pH range of 6·5—13·8 at four-minute intervals. The limit values of absorbance, corresponding to the reactions that had occurred, were used in the calculations.

The stopped-flow experiments were performed on a Stopped-Flow Spectrophotometer, model D-115 (Durrum, U.S.A.) with optical detection in the UV region, and a right-angle fluorimetric detection, connected to a memory oscilloscope Tectronix 5103 (U.S.A.). The light whose absorbance was measured had a wave length of 244 nm, the optical path was 20 mm, the slit width was 0·5 mm. In the fluorescence measurements the wave length of the excitation light was 244 nm and the slit width was 2 mm. The time constant in the "stopped-flow" measurements was chosen as approximately 1/10 of the expected reaction time. The reference sample was a 0·025% solution of orosomucoid in 0·1M-KCl. The measured solutions had the same composition, except that they contained, in addition, suitable amounts of KOH.

Processing of Experimental Data

A spectrophotometric titration curve was expressed as a dependence of absorptivity, referred to 10^5 g of the protein, on pH. On disregarding the electrostatic factor, which is permissible in this pH range[6,12,13], the equation of a spectrophotometric titration curve[9] takes the form

$$\varepsilon_{(\mathrm{pH})} = \sum_{i=1}^{N} \frac{n_i \varepsilon_i \, 10^{-\mathrm{p}K_i}}{10^{-\mathrm{pH}} + 10^{-\mathrm{p}K_i}} , \qquad (1)$$

where $\varepsilon_{(\mathrm{pH})}$ is the absorptivity of the protein (calculated from absorbance) no longer changing with time (the limit absorbance at a given pH), $\mathrm{p}K_i$ is pK of the i-th class of tyrosine residues, n_i the number of tyrosine residues in the i-th class, ε_i the absorptivity of the i-th class and N the number of classes of tyrosine residues.

From this equation, using the least-square non-linear method[16] and assuming no differences within an i-th group, we calculated the values of pK_i and n_i. One set of calculations referred to $N = 2$, another to $N = 3$; the two sets of results were then compared. Using the values of pK_i and n_i best fitting equation (*1*) we calculated the theoretical course of the function, and from this equation the root-mean-square deviations of experimental points from the theoretical curve (see Fig. 2).

The kinetic data were also evaluated by the least-square non-linear method using the equation for a consecutive reaction[18,19]

$$A_{(t)} = B + \sum_{i=1}^{N} C_i \exp\left(-k_i \cdot t\right), \qquad (2)$$

where $A_{(t)}$ denotes absorbance or decrease in fluorescence in time t, B the final value of a measured quantity, C_i the pre-exponential factor, k_i the rate constant of the i-th step of the consecutive process considered, t the time and N the number of steps of the process. The calculations refer to $N = 1$ and $N = 2$; a calculator Hewlett-Packard HP 9830 A and the language BASIC were used.

RESULTS AND DISCUSSION

State of Tyrosine Residues in Orosomucoid Inferred from Difference Spectra

Examples of the difference spectra of orosomucoid for various pH's are shown in Fig. 1. These contain the absorption peak at 295 nm used by Yamagami and coworkers[6] in studying the tyrosine residues, and peaks at 244 and 211 nm. The maximum at 244 nm corresponds to the light absorption by dissociated tyrosine residues[9], that

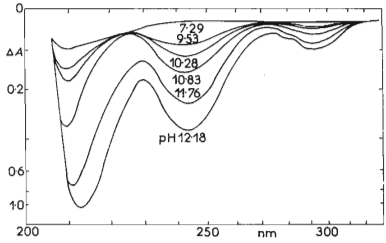

FIG. 1

Difference Absorption Spectra of 0·01% Orosomucoid in 0·1M-KCl at Different pH's in the Alkaline Region

Reference pH 7·40.

at 295 nm to the absorption by dissociated tyrosine and tryptophan residues[9] and the peak at 211 nm to non-specific absorption by the protein[9].

The absorbances at 244 nm are plotted in Fig. 2; the titration curve was drawn using the least-squares curve fitting.

The numbers of tyrosine residues, the corresponding pK values for 2 or 3 classes of tyrosine residues and the root-mean-square deviation of experimental points from the theoretical curve, obtained by mathematical analysis of the titration curve, are given in Table I, together with the results obtained by Yamagami and coworkers[6] from graphical analysis of the titration curve of a 0·14% solution of orosomucoid in 1M-KCl at 295 nm. The two sets of results differ in the numbers of the free and the masked tyrosine residue (for the same total of 12). Since these data depend on con-

TABLE I

Analysis of Spectrophotometric Titration Curve for a Wave Length of 244 nm

N^a	pK_1	pK_2	pK_3	$n_1{}^b$	n_2	n_3	s^c
2	9·97	11·83	—	3	9	—	1·996
3	9·94	11·74	11·90	3	2	7	2·096
3[d]	9·9	11·0	11·8	5	2	4	omitted

[a] Number of classes of tyrosine residues. [b] Number of tyrosine residues. [c] Root-mean-square deviation [d] Yamagami and coworkers[6].

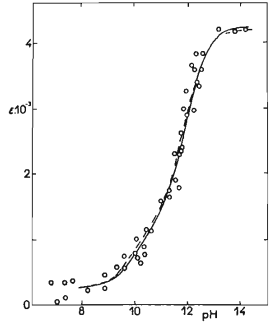

FIG. 2

Spectrophotometric Titration Curve of Orosomucoid at 244 nm

Circles designate the experimental values. The curves were fitted with the aid of a computer under the assumption that orosomucoid contains two (dashed curve) or three (solid curve) classes of tyrosine residues. ε in $cm^2\ g^{-1}$.

formation of the protein the differences between our results and Yamagami's may be due to unequal experimental conditions, *i.e.* to differences in conformation. The effects of ionic strength and protein concentration were probably the strongest ones. It is also possible that the two, not quite identical, preparation procedures had left different marks on the protein conformation. It should also be considered that the titration curves refer to different wave lengths and were evaluated differently (graphically by Yamagami and by the least-square method by us).

Mathematical analysis has shown that the root-mean-square deviations for two or three classes of tyrosine residues are almost identical. This, however, only suggests the same probability of the two sets of results so long as the numerical values are concerned. Comparison of the very close values of pK_2 and pK_3 favours the assumption that only two classes of tyrosine residues are present in orosomucoid. Since sub-classification[6] of masked tyrosine residues by analysis of a spectrophotometrical titration curve has a more or less phenomenological character and since our kinetic data (see below) do not justify any sub-classification we can only deduce from the analysis that orosomucoid contains 2 – 3 free (accessible) tyrosine residues of $pK \approx 10{\cdot}0$ and 9 – 10 masked tyrosine residues of pK 11·8.

The deviation of experimental points from the calculated titration curve of the polyelectrolyte in the pH region 11·5 – 12·5 (Fig. 2) seems to be caused, judging from our kinetic data, by the greater changes of absorbance with time as a result of denaturation, commencing in this region.

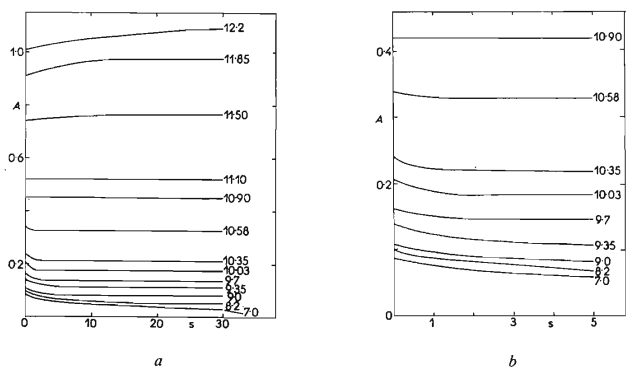

FIG. 3

Absorbance in Relation to Time at Different pH's in the Alkaline Region

a) The whole range of the dependence, *b*) part of the range exhibiting rapid decrease in absorbance.

Fast Changes of Absorbance at 244 nm *Followed by the "Stopped-Flow" Method*

The time dependences of absorbance for different pH's were investigated in periods from a few seconds to one minute. The family of curves thus obtained is shown in Fig. 3*a*,*b*. Calculation suggests that at certain pH's the process is a one-step reaction, at others a two-step one (Table II). The rate constants for the one-step process are pH-dependent.

Since at "zero time" of the apparatus the absorbance of the reaction mixture (measured against orosomucoid in 0·1M-KCl without an addition of KOH) is already different from zero, the reaction occurring on mixing the solutions of orosomucoid and KOH is so fast that it cannot be detected by the "stopped-flow" method. At higher pH's the reaction produces a rapid increase in absorbance, which is probably due to dissociation of the tyrosine residues; for the non-dissociated tyrosine residues practically do not absorb wave lengths 295 and 244 nm.

The increase in absorbance with time (Fig. 3) can be interpreted[3,6,10–13,18] in two ways. Firstly it can be assumed that on the very fast dissociation of the tyrosine residues further increase in absorbance is due to a conformation change, *i.e.* to

TABLE II

Rate Constants for the First (k_1) and the Second (k_2) Steps from Analysis of Stopped-Flow Absorption Curves

pH	12·20	11·85	11·50	11·10
k_1	0·08	0·47	—[a]	—[a]
k_2	0·031	0·032	0·015	—[a]
pH	10·90	10·58	10·35	10·03
k_1	—[a]	1·06	0·91	1·60
k_2	—[a]	—[b]	—[b]	—[b]
pH	9·70	9·40	9·35	9·00
k_1	0·49	0·84	0·75	0·36
k_2	—[b]	—[b]	—[b]	—[b]
pH	8·20	7·20	6·70	6·50
k_1	0·28	0·36	—[a]	—[a]
k_2	0·039	0·046	—[a]	—[a]

[a] No reaction (absorbance does not change with time). [b] Absorbance changes in the second step were not measurable.

a change in the neighbourhood of an aromatic chromophore. In the other interpretation, preferred by Yamagami and coworkers[6], the increase in absorbance is produced by a slow dissociation of masked tyrosine residues.

In an attempt to decide experimentally between the two views two series of experiments were performed. In one of them the dependence of pH upon time was investigated in the time range in which the increase in absorbance had been measured. For should the increase in absorbance be controlled by the time course of dissociation in the investigated time range, pH of the solution would be brought by the released hydrogen ions to lower values. We had calculated that such a decrease in pH would be detected by our apparatus, but none was observed. In the other series of experiments we recorded spectra of a solution of pH 12 in the range 200 to 300 nm at 4-minute intervals; for the conformation changes should manifest themselves[20–22] as a system of hyperchromic and hypochromic phenomena in the protein spectrum. A typical example of the spectra is shown in Fig. 4. A hyperchromic phenomenon occurred at wave lengths 244 and 295 nm, a hypochromic one at 230 nm.

Since the increases in absorbance are not accompanied by pH changes, but are associated with hyper- and hypochromic phenomena in the spectrum it is possible to ascribe them to a more profound change in conformation of the orosomucoid molecule. This change is equal to the very rapid dissociation of tyrosine residues. In view of the pH region where the phenomenon was observed (pH 11) and considering the analysis of the spectrophotometric titration curve, obviously the masked tyrosine residues were involved. Since their dissociation disrupted bonds stabilizing the molecule, a change in conformation followed as a result.

Now we shall deal with the decrease in absorbance observed on exposure of the protein to a mildly alkaline medium (pH 7–11). Such a decrease (Fig. 3), not yet

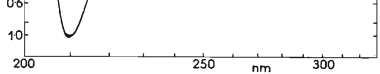

FIG. 4

Difference Spectra of Orosomucoid at pH 12

Measured at intervals 4·4 min. The arrows indicate changes of absorbance.

described in the literature, seems due to changes in the weak interactions of the aromatic chromophore with the surrounding atoms, as a consequence of a small conformation change. Such change (*e.g.* expansion of the molecule) may occur on dissociation of a histidine, tyrosine and lysine residue, since the consequential increase in negative charge enhances repulsion of the negatively charged groups. The repulsion of negative charges, changing the distance of negatively charged groups from the aromatic chromophores, may reduce absorption of electromagnetic radiation by the tyrosine and tryptophan chromophores.

The effect of negative charges on absorption of light has been corroborated by a quantum-chemical calculation, which will be subject of a separate communication.

If we reconsider the behaviour of orosomucoid in the whole pH range investigated it is also possible to explain why at pH 11 the absorbance does not change with time. It has been stated that on dissociation of the accessible groups the molecule expands, and on dissociation of the masked groups a more profound conformation change occurs. In view of the pK values of the accessible and the masked groups the two phenomena are likely to overlap. This circumstance is also evident from Fig. 3. Since the expansion is associated with a decrease in absorbance, whereas a deeper conformation change with its increase, the two processes compete. Around pH 11, where the numbers of the dissociated accessible groups and the dissociated masked groups are roughly equal (see analysis of the spectrophotometric titration curve,

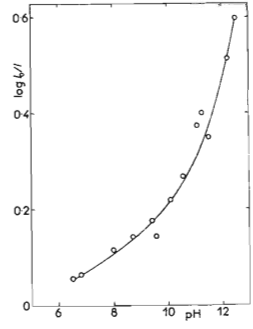

FIG. 5
Fluorimetric Titration Curve

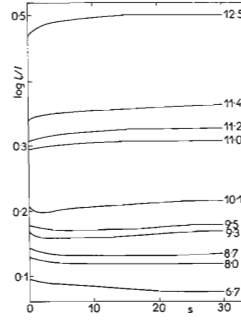

FIG. 6
Decrease in Fluorescence with Time at Different pH's in the Alkaline Region

Table I) and where even the rate constants of the two concurrent processes are comparable (Table III), the effects of the two processes on absorbance compensate for each other (Fig. 3).

Kinetics of Denaturation Followed by Measuring Decrease in Fluorescence at Excitation Wave Length 244 nm

Before starting the actual kinetic measurements by this method we followed the decrease in the intensity of fluorescence in relation to pH. The results (a fluorimetric titration curve) are given in Fig. 5. As is seen, the curve traces the spectrophotometric titration curve (Fig. 2), in agreement with the observations on model peptides[23] and, *e.g.*, ribonuclease[24]. This phenomenon corresponds to the quenching effect of tyrosine residues and hydrogen ions on the still non-dissociated tyrosine residues and on tryptophan residues.

Further we measured the rapid changes in fluorescence intensity with a series of solutions of different pH's in the alkaline region (Fig. 6). It is evident that the family of curves showing the time-dependence of fluorescence intensity accords with the corresponding absorbance curves. It also reveals that dissociation of the accessible tyrosine residues and dissociation of the masked tyrosine residues are followed by different reactions, overlapping most around pH 11. Further it can be deduced from Fig. 6 that the process proceeded in two steps throughout the pH range investigated, the first depending on whether the accessible or masked tyrosine residues had dissociated and the other on the subsequent change of conformation.

The rate constants of the two steps, obtained by mathematical analysis for each pH, are given in Table III. Mutual comparison of the first-step rate constants in the region below pH 11, obtained from spectrophotometric and fluorimetric curves (data

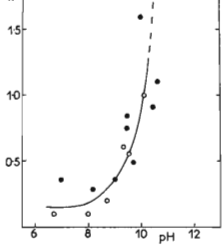

FIG. 7

Spectrophotometric (●) and Fluorimetric (○) Rate Constants (s^{-1}) in Relation to pH

in Tables II and III) and comparison of the pH dependences of the two series of values (Fig. 7) demonstrate that the spectrophotometric and the fluorimetric measurements followed the same process. Changes of fluorescence[25] and absorbance accompanying the process may be due both to relative motion of the charged groups and aromatic chromophores (tryptophan and tyrosine residues), ensuing on dissociation of tyrosine residues as a result of electrostatic repulsion or attraction of charged groups, and to a more essential change of conformation.

On the basis of all the experimental data, their processing and interpretation, we propound a scheme of alkali-induced denaturation of orosomucoid (Fig. 8). The scheme describes processes after pH adjustment of an orosomucoid solution

TABLE III

Rate Constants for the First (k_1) and the Second (k_2) Steps (s^{-1}) at Different pH's obtained by Analysis of Stopped-Flow Fluorescence Data

pH	12·5	11·4	11·2	11·0	10·1
k_1	5·0	5·0	3·0	2·0	1·0
k_2	0·097	0·024	0·30	0·10	0·058
pH	9·5	9·3	8·7	8·0	6·7
k_1	0·56	0·60	0·20	0·10	0·10
k_2	0·048	0·030	0·050	0·010	0·010

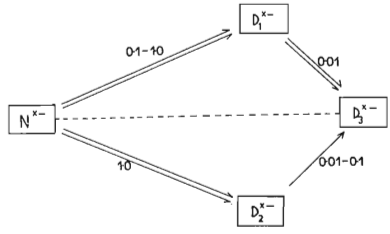

FIG. 8

A Scheme of Alkali-Induced Denaturation of Orosomucoid

N and D denote the native and the denatured forms, respectively; the exponents the net charge of a molecule. The double arrows indicate processes followed both spectrophotometrically and fluorimetrically. The numbers over the arrows are rate constants (s^{-1}). The dashed line separates processes involving masked groups (see text).

to a value corresponding to a charge x, which variable is controlled by dissociation of hydrogen ions. This dissociation gives rise to a form which will here be regarded as the native form, N^{x-}. On dissociation of the accessible groups the consequential increase in negative charge expands the molecule to a form D_1^{x-}. The resulting change of conformation leads to a final denaturation product, D_3^{x-} depending on a given pH. The whole process in accompanied by two-step changes in fluorescence intensity and molecular volume (expansion), along with a decrease in absorbance. If the masked groups dissociate too (at higher pH's), thus disrupting the molecule-fixing bonds, the more profound conformational alterations produce forms D_2^{x-} and D_3^{x-}. These alterations manifest themselves in the intensity of fluorescence and by a system of hyper- and hypochromic phenomena in the spectrum of the protein.

From the kinetic point of view it is possible, as follows from the scheme, to distinguish between the masked and the accessible residues, since their dissociation brings about different changes in conformation.

The problems of microscopic reversibility of the individual steps, reversibility of the whole denaturation change (back-conversion of D into N) and characterisation of the denatured forms remain unsolved. These problems will be treated in the subsequent communication by the "temperature-jump" relaxation method and by its combination with the stopped-flow method (the stopped-flow temperature-jump method).

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