ALKALINE DENATURATION OF OROSOMUCOID STUDIED BY THE TEMPERATURE JUMP METHOD AND STOPPED-FLOW-TEMPERATURE-JUMP METHOD

X.SVOBODOVÁ*, V.KARPENKO and V.KALOUS

Department of Physical Chemistry,
Charles University, 128 40 Prague 2

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Using the temperature-jump relaxation method alone and combined with the stopped-flow method (the stopped-flow-temperature-jump method), the association-dissociation equilibria of tyrosine residues in alkali-denatured forms of orosomucoid have been studied. Analysis of the relaxation data showed that the denaturation exposed the masked groups. The denaturation scheme, derived previously from stopped-flow data, was supplemented by results of the relaxation measurements. The problems of the overall reversibility of denaturation and of the microscopic reversibility of the individual processes have been solved.

The preceding paper[1] describes a study of the state of tyrosine residues in orosomucoid[2] by differential spectrophotometry in the UV region. The kinetics of the alkaline denaturation of orosomucoid, manifesting itself by rapid changes in absorbance and fluorescence intensity, have also been dealt with. The measurements were carried out by the stopped-flow method[3]. The results obtained allowed us to propose an incomplete denaturation scheme of orosomucoid.

The present study, making use of the temperature jump method[4] and the stopped-flow-temperature-jump method[5] (combination of the former method with the stopped-flow method), deals with the microscopic and macroscopic reversibility in the alkaline denaturation of orosomucoid and characterizes the denatured forms.

EXPERIMENTAL

Materials and Methods

The orosomucoid employed was specified in the preceding paper[1]. The chemicals were of A. R. grade (Lachema). The working solution was 0·025% in orosomucoid and 0·1M in KCl. The exact concentration of KCl was chosen in relation to pH, so that the conductance of the solution was the same at all pH's.

* Present address: Výskumný ústav liečiv, 800 01 Bratislava.

pH, brought to desired values by additions of KOH, was measured with a pH-meter Radelkis (Hungary) with a combined electrode. The temperature-jump and the stopped-flow-temperature-jump measurements were carried out using a Stopped-Flow Spectrophotometer D-115 combined with Temperature-Jump Accesory D-115 (Durrum), with optical detection in the UV region. The signal was intercepted on the screen of a memory oscilloscope Tectronic 5103 and photograped with a Polaroid apparatus.

The measurements were carried out at a wave length of 244 nm at 20°C (thermostat). The slit width was 0·5 mm, the optical path was 20 mm. The conductivity of all the solutions was $1{\cdot}43 \,.\, 10^{-2}$ ($S.cm^{-1}$), the voltage applied was 5 kV. The temperature jump delays were zero, 10 ms, 100 ms, 1 s, 10 s and 100 s.

The heating pulse width was 100 μs. Two relaxations were measured; one ("fast") at an oscilloscope delay of 100 μs and time constant 500 μs, the other ("slow") at a delay of 500 μs and time constant 1 ms.

The relaxation curves were evaluated by the least-square method, using the equation for simple relaxation[4],

$$A = B + \Delta A \exp(-k \,.\, t) \qquad (1)$$

where A denotes absorbance at time t, B the absorbance after the relaxation, ΔA the difference between the original and the final absorbance (the so-called extent of relaxation), and $k = 1/\tau$ (reciprocal value of the relaxation time τ).

RESULTS

The relaxation curves were recorded with a series of solutions of different pH's in the alkaline region. Results of mathematical analysis of the relaxation curves (reciprocal values of the relaxation times, $1/\tau$, and total changes of absorbance produced by relaxation) are given in Table I. A "fast" and a "slow" relaxation process were detected. The slow one occurred throughout the pH range investigated (7–12), the fast process was observed in the range pH 9·5 to 11·7. The fast relaxation was

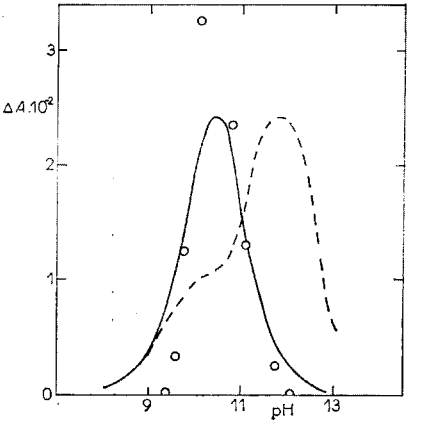

FIG. 1

Total Relative Change of Absorbance in Relation to pH Determined from Temperature Jump Data for the Fast Relaxation

○ Experimental points, —— least-square-fitting curve, ------ theoretical curve involving relaxation of the masked groups.

associated with a decrease in absorbance, in the slow relaxation the absorbance increased. The extent of the fast relaxation was strongly pH-dependent (Fig. 1).

In addition to the measurements described above we repeated relaxation with the same reaction mixture to see whether the relaxation itself produced any irreversible changes in the protein molecule.

In the combined (stopped-flow-temperature-jump) experiments correlation of relaxation to the processes observed in the stopped-flow measurements was investigated. The fast relaxation was found to occur independently of the temperature-jump experiment delay after the stopped-flow mixing. The relaxation had the same parameters for all delays. The slow relaxation followed the stopped-flow mixing with a delay of at least 100 ms.

DISCUSSION

To interpret the facts observed it was first necessary to elucidate the effect of the temperature jump on the equilibrium of the system. As the measurements were carried out in a very great excess of water and since the ionic product of water is strongly temperature-dependent, the temperature jump was supposed, with a given composition of the solution, to decrease pH at a rate much higher than the rate of any of the processes under study. If so, the solution underwent a mediated pH jump[5].

TABLE I

Reciprocal Values of Relaxation Times (s^{-1}) and Values of the Total Change of Absorbance A For Relaxation at Different pH's Obtained from Analysis of Fast Relaxation and Slow Relaxation Curves (temperature jump method)

pH	Fast relaxation			Slow relaxation	
	$1/\tau$ s^{-1}	$10^{-3}\Delta A$ %	$10^{-2}\Delta A$	$1/\tau$ s^{-1}	ΔA %
8·6	[a]	0	0	11·5	2·52
9·3	[a]	0	0	11·1	2·49
9·5	$1{\cdot}9 \cdot 1^3$	0·487	0·345	5·61	3·19
9·7	$1{\cdot}9 \cdot 1^3$	1·88	1·27	11·9	2·25
10·1	$2{\cdot}3 \cdot 1^3$	5·71	3·28	16·3	1·42
10·8	$2{\cdot}3 \cdot 1^3$	5·71	2·37	13·7	3·43
11·1	$2{\cdot}1 \cdot 1^3$	4·70	1·32	9·9	3·38
11·7	$2{\cdot}5 \cdot 1^3$	1·82	0·257	15·3	3·49
12·0	[a]	0	0	19·2	3·49

[a] No relaxation occurred.

Let us now interpret the fast relaxation. All experimental facts suggest association of the protein with hydrogen ions. A decrease in absorbance would be expected since the association would reduce the number of dissociated tyrosine residues, which absorb strongly under the conditions of the measurement. The fact that a relaxation faster still was never observed (although the apparatus could have detected it) also indicates association of the protein with hydrogen ions, since a sequence of denaturation processes is started by interaction with hydrogen or hydroxyl ions (see *e.g.* Joly[6]). The strongest support for our interpretation of the relaxation is analysis of the dependence of the extent of relaxation upon pH (Fig. 1), described below.

The extent of fast relaxation, ΔA, was found to be related to pH by an equation whose form fits an acidobasic process. As can be derived[7,8], the equation is

$$\Delta A = \sum_{i=1}^{N} n_i \varepsilon_i K_i h / (K_i + h)^2 , \qquad (2)$$

where ΔA designates the extent of relaxation; n_i, ε_i and K_i denote respectively the number, absorptivity and dissociation constant of the i-th group of the tyrosine residues, h is concentration of hydrogen ions and N the number of different classes of tyrosine residues.

Using the least-square method it was calculated from the experimental data that these fit *best to equation* (2) (*1*) *for* $N = 1$ and pK 10·4. The theoretical course of the function is confronted with experimental data in Fig. 1. The agreement is very good. This seems to justify the assumption that the fast relaxation is represented by association of the protein with hydrogen ions, resulting in an acidobasic equilibrium. The tyrosine residues participating in this equilibrium have a pK of 10·4, which value corresponds to the upper limit of the temperature jump, 35°C. As follows from the spectrophotometric titration[1], pK 10·4 corresponds to the dissociation constant of the freely accessible tyrosine residues.

For the sake of comparison Fig. 1 also gives the curve for an acidobasic equilibrium in which the masked tyrosine residues, of pK 11·8, are included. (The numbers of the two classes of tyrosine residues were taken from the results of spectrophotometric titrations[1]). As is seen, the course of this curve deviates from that determined experimentally. This demonstrates that only the portion of orosomucoid containing accessible tyrosine residues (pK 10·4) participated in the acidobasic equilibrium. The previous stopped-flow experiments[1] show that only alkali-denatured forms of orosomucoid were present in the solution under the given conditions. Analysis of the extent of relaxation in relation to pH shows that these forms contain no masked tyrosine residues. This fact is a certain characteristic of the denatured forms and, thus, of the denaturation process.

Let us now calculate the rate constants of association and dissociation. With the fast relaxation it applies

$$D_i^{(x+y)^-} + yH^+ \underset{k_{2,1}}{\overset{k_{1,2}}{\rightleftharpoons}} D_i^{(x)^-} \qquad (A)$$

where D represents the denatured form of orosomucoid carrying a charge $(x + y)^-$ or $(x)^-$. In such reaction, assuming that the concentration of H^+ ions is not considerably changed, the relaxation time τ is related to the rate constants of association $(k_{1,2})$ and dissociation $(k_{2,1})$ by the equation[5]

$$\tau^{-1} = k_{1,2}[H^+] + k_{2,1} . \qquad (3)$$

It further holds that

$$K_A = k_{2,1}/k_{1,2} \qquad (4)$$

where K_A is the acid dissociation constant.

Consequently,

$$k_{1,2} = 1/\tau(K_A + [H^+]) . \qquad (5)$$

From these formulae the rate constants of association and dissociation were calculated; pK_A was set equal to 10·4, which value was obtained by analysis of the dependence of the extent of relaxation upon pH. The rate constant of association has been found to have a high value (Table II).

We shall now treat the slow relaxation. Interpretation of the fast relaxation and the results of repeated relaxation allow us to judge that the slow relaxation corres-

TABLE II

Rate Constants of Association, $k_{1,2}$, and Dissociation, $k_{2,1}$, for Tyrosine Residues of Orosomucoid at Different pH's (temperature jump method)

pH	Association $k_{1,2}$, $s^{-1}.mol^{-1}.l$	Dissociation $k_{2,1}$, s^{-1}
9·5	$5{\cdot}3 . 10^{12}$	$2{\cdot}12 . 10^2$
9·7	$7{\cdot}9 . 10^{12}$	$3{\cdot}13 . 10^2$
10·1	$19{\cdot}2 . 10^{12}$	$7{\cdot}68 . 10^2$
10·8	$41{\cdot}0 . 10^{12}$	$16{\cdot}4 . 10^2$
11·1	$43{\cdot}0 . 10^{12}$	$17{\cdot}2 . 10^2$
11·7	$64{\cdot}0 . 10^{12}$	$25{\cdot}6 . 10^2$

ponds to a conformation change of the type

$$A \underset{k_{2,1}}{\overset{k_{1,2}}{\rightleftharpoons}} B \qquad (B)$$

where $k_{1,2} \gg k_{2,1}$, so that the inverse values of relaxation times, given in Table I, can be regarded as the rate constants of the process[5]. In view of what has been deduced from the combined experiments it seems that only a denatured form, *i.e.* one that has found a proton according to equation (*A*), can undergo this conformation change, which then can be expressed schematically as

$$D^{x^-} \rightarrow N^{x^-}. \qquad (C)$$

Here D^{x^-} denotes the denatured form, N^{x^-} the form after an x-fold dissociation but before the conformation change, *i.e.* a kind of the native conformation carrying the charge x (ref.[1]). Back-conversion to the native form is here assumed since in repeated relaxations, when a mixture was allowed to cool down and then heated in a temperature jump, the system was always exposed to a certain "denaturation step" in the cooling, which caused its alkalization. As, however, there appeared to be no differences between the results of the individual relaxation experiments the process is evidently reversible, at least by the criteria of these measurements. Therefore, we assume that the reaction product is the form N^{x^-}.

However, conversion of the denatured forms into the native one is not governed by the mechanism producing the denatured forms from the native form. This means that the individual reactions (excepting those concerned with acidobasic equilibria) are microscopically irreversible. Analysis of the fast relaxation in relation to pH further shows that the denaturation is associated with unmasking of the buried groups, since no masked groups were present in the denatured forms. The microscopic irreversibility of the denaturation (conformation) changes is then easy to understand, since a prerequisite for masking an accessible dissociated group is its association with a proton. Macroscopically, however, the denaturation appears to be a reversible step, since the denatured forms can be converted back into the native one. The denatured and the native forms are in an equilibrium.

All these facts and the previous findings[1] are included in a scheme of the mechanism of alkaline denaturation of orosomucoid (Fig. 2), which represents a complex equilibrium of the native and the denatured forms.

The scheme illustrates the processes on the dissociation of y protons from a molecule of orosomucoid with a net charge of x^-. After a certain number of protons have dissociated from the tyrosine residues the molecule of orosomucoid undergoes the primary reaction: if the accessible tyrosine residues have dissociated the conformation change will be the result of electrostatic repulsion only, whereas dissocia-

tion of the masked tyrosine residues will produce a more profound conformation change, as a result of severing the bonds by which the non-dissociated masked groups contributed to fixing the molecular structure. The primary reaction is followed by a secondary conformation change, giving rise to a final and rather stable denaturation product. On acidification of the solution all denatured forms bind some protons (in classical experiments only the D_3 form combines with protons since the unstable forms D_1 and D_2 are absent). The form D^{x-} thus produced undergoes a conformational "back-conversion" to the form N^{x-}.

The propounded scheme of alkaline denaturation, derived from our experiments and calculations, reveals the following facts:

1. From the kinetic point of view there is a difference between the masked and the free tyrosine residues. This is apparent from the role of the masked tyrosine residues in fixing a molecule of the protein.

2. Acidification of the protein does not retrace its alkalization. This fact account for the irreversibilities of the acidobasic and the spectrophotometric curves, described by Karpenko[9] and Yamagami[10]. These irreversibilities have been at variance with the deep-rooted idea of orosomucoid molecule as an extraordinarily stable one.

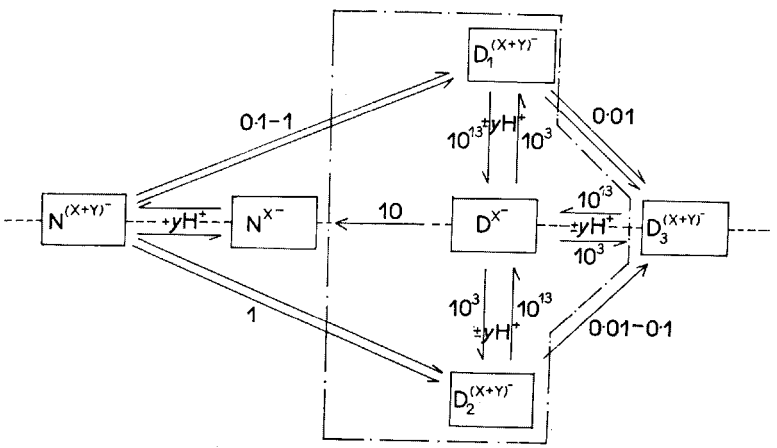

FIG. 2

Propounded Scheme of Alkaline Denaturation of Orosomucoid

N^{x-} and $N^{(x+y)-}$ denote "native" forms in the corresponding degrees of dissociation, D_i^{x-} and $D_i^{(x+y)-}$ denote the denatured forms. The superscript x designates the net charge of a molecule, the numbers on the arrows are the determined rate constants (s^{-1}). The dashed line refers to processes in which masked tyrosine residues dissociated (these processes are illustrated above it); the dot – and – dash line refers to the temperature jump method. The double arrows indicate reactions that were identified by both the absorption and the fluorescence measurements.

From the facts described above it is evident that no irreversibility of denaturation is in question, only one way does not retrace the other.

3. From the viewpoint of denaturation the process is reversible. This means that alkaline denaturation does not affect drastically the constellation of the molecule. Hence, in turn, it follows that electrostatic interaction does not contribute substantially to stabilization of the molecular "core". Our results and measurements of orosomucoid–benzene interactions suggest that the core of the protein is held mainly by strong hydrophobic bonds. The electrostatic interactions are evidently more operative in shaping the envelope of the core and may have a decisive role in interactions of orosomucoid with other constituents of the solution.

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