REVERSIBLE THERMAL DENATURATION OF IMMOBILIZED CHYMOTRYPSINOGEN

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The reversible thermal denaturation of chymotrypsinogen A bound to the insoluble poly(2-hydroxyethyl methacrylate) Spheron matrix was investigated by the fluorescence method. The applicability of fluorescence data in the calculation of the thermodynamic parameters of denaturation was tested. Equilibrium data were obtained for immobilized chymotrypsinogen and chymotrypsinogen in solution at various pH in the range 2–6. The binding to Spheron shifts the thermodynamic equilibrium in favour of the denatured state. An increase in pH above 3 did not affect the denaturation of immobilized chymotrypsinogen. The denaturation is controlled by the first-order kinetics. The rate constants and magnitudes of changes of free energy, enthalpy and entropy were calculated for the transition from the native state into the activated state, and from denatured state into the activated state. The temperature dependence of the rate constants for the denaturation of immobilized chymotrypsinogen is qualitatively different from for chymotrypsinogen in solution. The results were interpreted as effects of the physical interaction between the denatured protein and polymeric matrix.

The interest aroused by the practical utilization of proteins bound to polymeric matrices and acting as affinity sorbents, heterogeneous catalysts *etc.*, gives rise to attempts to elucidate causes leading to changes in the properties of bound proteins with respect to the properties of these proteins in solution. As a model for the study of the effect of immobilization on the properties of proteins we chose the reversible thermal denaturation of chymotrypsinogen, which in solution is a characteristic prototype of the conformational transition of globular proteins. The denaturation of chymotrypsinogen in solution is sufficiently reversible only at low ionic strength values in the pH range from 2 to 3·7. Beyond this range, the denatured chymotrypsinogen undergoes irreversible aggregation[1,2]. It was shown, that reversible denaturation in solution may be approximated with sufficient accuracy by a two-state model of cooperative transition between the native and denatured states[3–6]. The values of themodynamic transition quantities, directly measured by the calorimetric method[5–7], are in good agreement with those calculated according to the two-state model from transition curves obtained by measuring changes of various physical parameters characterizing the protein conformation[3,6,8]. (Here, the transition curve means the temperature dependence of the value of some physical or chemical para-

meter characterizing the protein conformation[3]). A semiempirical theoretical analysis of the denaturation transition of chymotrypsinogen in solution was suggested by Brandts[4].

EXPERIMENTAL

Materials and methods. Bovine chymotrypsinogen, recrystallized five times (Léčiva, Czechoslovakia) was used without further purification. The crosslinked macroporous poly(2-hydroxyethyl methacrylate) Spheron 300 described by Čoupek and coworkers[9] was employed as the insoluble carrier. Samples used in the measurements were a mixture of beads having the maximum diameter 0·2 mm. Spheron was purified from undesirable fluorescence impurities by extraction with benzene for several days, followed by acetone. The denaturations were carried out in solutions 0·01M-KCl + 0·01M-HCl; for pH higher than 3·6, the buffer 0·1M citric acid + 0·2M-Na_2HPO_4 was used.

Chymotrypsinogen was bound onto a matrix modified by a method suggested by Porath[10]. The initial reaction of hydroxyl groups with cyanogen bromide was performed in a solution with constant pH (10·5). The chymotrypsinogen was bound in 0·1M-$NaHCO_3$. After the reaction lasting 15 h the product was washed with the borax (0·1M-$Na_2B_4O_7$ + 1M-NaCl, pH 9·2) and acetate (0·1M-CH_3COONa + 0·1M-CH_3COOH + 1M-NaCl, pH 4·6, and 0·01M-CH_3COONa + + 0·01M-CH_3COOH, pH 4·6) buffers, and after that with twenty litres of distilled water for 5 h. By using various ratios of the initial protein and carrier concentrations in the binding reaction, samples were obtained having different concentration of immobilized chymotrypsinogen on the polymer surface in the range 100—3 mg of bound chymotrypsinogen per one gram of dry gel. The amount of bound protein was determined by an analysis of amino acids after acid hydrolysis of the samples according to Axén and Ernbach[11]. Fluorescence was measured with a Hitachi Perkin–Elmer MPF-2A Spectro fluorimeter. The experimental arrangement and cell for the investigation of denaturation on insoluble samples by means of fluororescence measurements in reflexion arrangement have been described in our earlier paper[1]. Additional measurements of the absorption of solutions and of the diffusion reflectance of insoluble samples were carried out with an Optica Milano CF4 R spectrophotometer.

RESULTS AND DISCUSSION

Fluorescence of Chymotrypsinogen and Thermal Denaturation

The fluorescence spectrum of native chymotrypsinogen in the solution 0·01M-KCl + + 0·01M-HCl, pH 3, has a maximum at 331 nm. The fluorescence intensity decreases with increasing temperature almost linearly, while the shape of the spectrum remains unchanged. In the range of denaturation temperatures, the spectrum is gradually shifted towards longer wavelengths. At temperatures above the denaturation region, where all the molecules are in the denatured state, the shape of the spectrum does not change any more, and the intensity decreases again almost linearly with increasing temperature. The fluorescence spectrum of completely denatured chymotrypsinogen has its maximum at 343 nm (Fig. 1).

The utilization of fluorescence data as a parameter for the construction of transition curves and for the calculation of equilibrium thermodynamic quantities of dena-

turation was tested. Assuming a two-state transition model, the fluorescence intensity at the given wavelength in our experimental arrangement can be expressed by

$$Q^{\lambda} = I_{exc}(a_N^{\lambda} C_N + a_D^{\lambda} C_D), \qquad (1)$$

where Q^{λ} is the fluorescence intensity, I_{exc} is the intensity of excitation light in the sample layer from which emission is detected, C_N and C_D respectively are concentrations of the native and denatured form, a_N^{λ} and a_D^{λ} respectively are coefficients proportional to the quantum yield and to the extinction coefficient of the native and denatured form. The intensity of excitation light in the sample layer from which emission was detected was approximately given by

$$I_{exc} = I_{exc}^0 e^{-Al}, \qquad (2)$$

where I_{exc}^0 is the intensity of excitation light incident on the surface of the measuring cell with solution, A is the absorption coefficient of solution, and l is the distance of the solution layer (0·5 cm) from which emission was detected from the cell surface. (The error in the application of Eq. (2) due to the convergency of the excitation beam did not exceed 0·8% in our case, and is not considered further). The absorption coefficient, $A = E_N C_N + E_D C_D$, introduced nonlinear dependence on the concentrations C_N and C_D. E_N and E_D respectively are the extinction coefficients of the native and denatured form. For this reason, corrected quantities

$$Q_{kor}(T) = Q(T)/e^{-0.5A(T)}, \qquad (3)$$

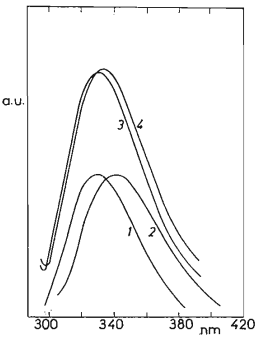

FIG. 1

Fluorescence Spectra of Chymotrypsinogen A in 0·01M-KCl + 0·01M-HCl (pH 3)

1 Native free protein, *2* denatured free protein, *3* native protein bound on Spheron, *4* denatured protein bound on Spheron. Spectra of native protein were recorded at 24°C, the intensity of spectra of the denatured form measured at higher temperatures was extrapolated to 24°C.

were employed in the transition curves; here, the $A(T)$ values as a function of temperature were ascertained in an independent absorption measurement of the sample. Instead of measuring the fluorescence intensity at a single wavelength, the intensity was measured at two wavelengths, and the quantity

$$\Delta Q^{\lambda_1\lambda_2} = Q^{\lambda_1}_{\mathrm{kor}} - Q^{\lambda_2}_{\mathrm{kor}} . \qquad (4)$$

was used in the transition curves (Fig. 2). The wavelengths were usually chosen so that for the native state $\Delta Q^{\lambda_1\lambda_2}_{\mathrm{N}} = 0$. The $\Delta Q^{\lambda_1\lambda_2}_{\mathrm{D}}(T)$ values for the denatured state in the denaturation temperature range were obtained by extrapolation of the temperature dependence $\Delta Q^{\lambda_1\lambda_2}_{\mathrm{D}}$ measured above the denaturation temperature range. The transition curves were used to calculate the equilibrium constant of the denaturation reaction at various temperatures according to

$$\frac{\Delta Q^{\lambda_1\lambda_2}(T) - \Delta Q^{\lambda_1\lambda_2}_{\mathrm{N}}(T)}{\Delta Q^{\lambda_1\lambda_2}_{\mathrm{D}}(T) - \Delta Q^{\lambda_1\lambda_2}(T)} = \frac{C_{\mathrm{D}}}{C_{\mathrm{N}}} = K(T) . \qquad (5)$$

The equilibrium constants $K(T)$ were independent of the wavelengths at which the denaturation change in the parameter $\Delta Q^{\lambda_1\lambda_2}(T)$ was measured. Thus, in the case of chymotrypsinogen the denaturation intermediate states, the fluorescence spectrum of which would differ from that of the native or denatured form, are not operative. The $K(T)$ values calculated from fluorescence measurements did not differ from $K(T)$ values calculated from measurements of the denaturation change in the absorption coefficient. Earlier, the measurement of the change in the absorption coefficient has been used successfully in the calculation of the thermodynamic quantities of denaturation of chymotrypsinogen according to the two-state model by other authors[3,6]. The values of the thermodynamic parameters calculated from fluorescence measurements for the denaturation of chymotrypsinogen in solution (Table I) are

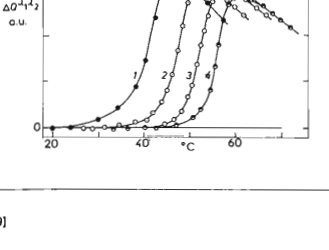

FIG. 2

Transition Curves of Denaturation of 0·01% Chymotrypsinogen A in 0·01M-KCl + 0·01M-HCl Obtained as the Temperature Dependence of the Fluorescence Parameter $\Delta Q^{\lambda_1\lambda_2}$ (*cf.* text)

pH: 1 2, 2 2·4, 3 2·8, 4 3·05.

in good agreement with the published values obtained calorimetrically[5-7]. One may assume, therefore, that the change in fluorescence adequately reflects the co-operative transition of chymotrypsinogen between the native and denatured state and that the fluorescence parameters may be employed in the calculation of the thermodynamic quantities of denaturation according to the two-state model.

With increasing temperature, the fluorescence spectrum of chymotrypsinogen bound to Spheron behaves similarly to that of chymotrypsinogen in solution. In the case of denaturation, the emission spectrum is also shifted towards longer wavelengths (Fig. 1). While the emission fluorescence spectrum of the native form of chymotrypsinogen bound to Spheron has the maximum at 331 nm (similarly to that of chymotrypsinogen in solution), the maximum of the spectrum of denatured immobilized chymotrypsinogen lies at 336 nm, *i.e.* at shorter wavelengths than that of the denatured chymotrypsinogen in solution. The decrease in the shift of the spectrum in the denaturation may be caused by an interaction of the denatured protein with the matrix, or may be only apparent as a result of the scatter of excitation light from the insoluble matrix[1]. In order to obtain transition curves of the denaturation of chymotrypsinogen bound to Spheron, we used the uncorrected parameter $\Delta Q^{\lambda_1\lambda_2}$ analogous to the corrected one from Eq. (*4*) for chymotrypsinogen in solution. As has been discussed in our earlier paper[1], in the reflexion arrangement used by us it is necessary to correct the fluorescence intensity for the change in the diffusion reflectance of the sample during the denaturation. In our case the recorded denaturation change in diffusion reflectance did not exceed 3%, and the correction was neglected. Similarly to chymotrypsinogen in solution, the $K(T)$ values calculated for the denaturation

TABLE I

Transition Temperatures T_0 and Enthalpy of Transition at these Temperatures ΔH_0 (at T_0 the free energy of transition is zero) for the Thermal Denaturation of Chymotrypsinogen in a 0·01% Solution and of Chymotrypsinogen Bound to Spheron at Various pH in 0·01M-KCl + + 0·01M-HCl

pH	Chymotrypsinogen bound to Spheron		Chymotrypsinogen in solution	
	T_0, K	ΔH_0, J/mol	T_0, K	ΔH_0, J/mol
1·9	303	138 000	—	—
2·25	309·2	142 000	—	—
2·4	313·6	167 000	320·2	423 000
2·65	318·9	222 000	—	—
3·0	320·5	243 000	329·1	557 000

of bound chymotrypsinogen were independent of the wavelengths at which the parameter $\Delta Q^{\lambda_1\lambda_2}$ was measured. Thus, also for the bound chymotrypsinogen the denaturation is reflected in fluorescence measurements as a transition between two states. Unfortunately, there is no further evidence which would rule out the existence of denaturation intermediate states with a fluorescence spectrum identical with that of the native or denatured state.

Reversibility of the Denaturation Reaction

In pH ranges smaller than 3·7, after cooling of denatured chymotrypsinogen in solution below the denaturation transition temperature range, the fluorescence and absorption spectra were changed within several tens of seconds into spectra characteristic of the native state. However, even at low chymotrypsinogen concentrations used by us (0·01 – 0·05%), the measured optical quantities ΔQ exhibited some 5% of irreversible changes. These irreversible changes may be attributed to the irreversible association of denatured chymotrypsinogen reflected in a rise in the light scattering from solution. Under the same conditions, after cooling below denaturation transition temperature range the shape of the fluorescence spectra of denatured chymotrypsinogen bound onto Spheron also returned to the original shape of the spectra of the native sample. In this case, however, the reversible change took some 20 h, and the fraction of irreversible changes was rather high. In the case of samples having a concentration about 100 mg of chymotrypsinogen bound on 1 g of dry gel the change in the spectra was almost irreversible. The fraction of reversible denaturation increased at lower concentrations of immobilized chymotrypsinogen, and the reversibility was improved by washing freshly prepared samples with a major volume of distilled water.

The dependence of reversibility on the concentration of bound protein and on washing shows that association of denatured proteins on the surface or irreversible interaction of nonspecifically bound proteins with the matrix may participate in the reversible denaturation. Samples with 3 – 9 mg of chymotrypsinogen bound on 1 g

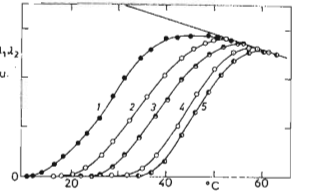

FIG. 3

Transition Curves of Denaturation of Chymotrypsinogen A Bound on Spheron in 0·01M-KCl + 0·01M-HCl Obtained as the Temperature Dependence of the Fluorescence Parameter $\Delta Q^{\lambda_1\lambda_2}$ (*cf.* text)

pH: *1* 1·9, *2* 2·25, *3* 2·4, *4* 2·65, *5* 3.

of dry gel used by us in the study of denaturation exhibited approx. 25% of irreversible change. In repeated denaturations of renatured samples, the same transition curves were obtained as in the first denaturation, and also the fraction of the nonreversible component remained the same.

The equilibrium thermodynamics of denaturation of chymotrypsinogen. Figs 2 and 3 show the transition curves of denaturation at various pH for chymotrypsinogen in solution and for chymotrypsinogen bound to Spheron. While for chymotrypsinogen in solution the denaturation transition at the individual pH lies in the temperature interval 10°C, this interval is extended for immobilized chymotrypsinogen to 20 to 30°C. For the same pH, the denaturation transitions of immobilized chymotrypsinogen lie at lower temperatures than those of chymotrypsinogen in solution. Using measurements of the transition curves, the equilibrium constants were calculated and van't Hoff curves were constructed (Fig. 4). In Table I, the transition temperatures T_0 determined through the intersection of van't Hoff curves with zero axis and the enthalpies of transition at these temperatures, H_0, are summarized for various pH. (At T_0, the free energy of transition is zero, *i.e.* the concentrations of the native and denatured forms are equal).

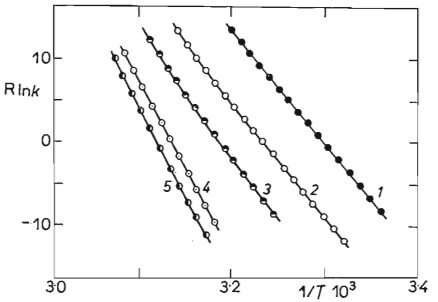

FIG. 4

Van't Hoff Curves for the Denaturation of Chymotrypsinogen Bound on Spheron Calculated from Three Independent Measurements of Transition Curves

pH: *1* 1·9, *2* 2·25, *3* 2·4, *4* 2·65, *5* 3.

Effect of pH *on Denaturation*

It can be seen in Figs 2 and 3 that both for chymotrypsinogen in solution and for immobilized chymotrypsinogen the denaturation range is shifted with decreasing pH to lower temperatures. Hence, in both cases the presence of hydrogen ions reduces the thermodynamic stability of the native state. The pH dependence of denaturation of chymotrypsinogen in solution has been studied by various authors[2-7]. Brandts[4] assigned the dependence of the free energy of transition on pH to the titration of three anomalous acid groups, which in the native state are protected from protonation inside the protein and exhibit an anomalous pK 1·3, while in the uncoiled denatured state they have their natural pK 4·5. In the case of immobilized chymotrypsinogen the dependence on pH is shifted towards the acid region, and already near pH 3 the effect of pH became saturated. A further increase in pH above this value in the range pH 3 – 6 examined by us no more affected perceptibly the transition curves of denaturation of immobilized chymotrypsinogen bound to Spheron. For chymotrypsinogen in solution, the range of saturation of the effect of pH is experimentally inaccessible with respect to its irreversible association at higher pH. With the pH dependence extrapolated according to Brandts's[4] theoretical relation, saturation should occur only near pH 5·5. The shift of the dependence towards the acid range for bound chymotrypsinogen may be due either to a decrease in the local concentration of hydrogen ions in the vicinity of the protein caused by the matrix surface, or to a changed structure of the immobilized protein reflected in a shift of pK of ionizable groups. If we admit that the acid shift is caused by a change in the properties of immobilized protein and apply the theoretical procedure used for chymotrypsinogen in solution, we may estimate approximately that three ionizable groups in immobilized chymotrypsinogen have pH 1·1 in the native state, and pK 2·3 in the denatured state. In this case these groups would not have their natural pK even in the denatured state and would be always partly protected from protonation by the hydrophobic surroundings, or by the participation in the formation of ionic bonds.

The denaturation kinetics of chymotrypsinogen bound to Spheron. After the jumpwise rise in temperature, the denaturation change of the parameter $\Delta Q^{\lambda_1\lambda_2}$ with time was measured. Similarly to the equilibrium data, the time dependence was independent of the wavelengths chosen. As has been discussed in our earlier paper[1], assuming the first-order kinetics it holds that

$$\frac{\Delta Q_\infty^{\lambda_1\lambda_2} - \Delta Q^{\lambda_1\lambda_2}(t)}{\Delta Q_\infty^{\lambda_1\lambda_2} - \Delta Q_0^{\lambda_1\lambda_2}} = e^{-(k_1+k_2)t}, \qquad (6)$$

where $\Delta Q^{\lambda_1\lambda_2}(t)$ is the fluorescence parameter at a time t, $\Delta Q_0^{\lambda_1\lambda_2}$ is the value for $t = 0$, $\Delta Q_\infty^{\lambda_1\lambda_2}$ is the extrapolated value for time $t \to \infty$, *i.e.* the equilibrium value at a given

temperature, and k_1, k_2 respectively are the rate constants of transition from the native into the activated state, and from the denatured into the activated state. Fig. 5 demonstrates that the reaction satisfies indeed the first-order kinetics. The equilibrium constants $K = (k_1/k_2)$ corresponding to the equilibrium values of the parameter $\Delta Q^{\lambda_1\lambda_2}$ calculated from the transition curves, the rate constants of the establishment of equilibrium, $k = k_1 + k_2$ determined from time dependences in Fig. 5 and rate constants k_1, k_2 calculated from the respective K and k values are summarized in Table II for the denaturation of immobilized chymotrypsinogen at pH 2·6.

From the dependence of $\ln k_1$ on $1/T$ and from equilibrium data, the following quantities were calculated for the transition temperature $T_0 = 318$ K: the equilibrium change in enthalpy $\Delta H = 210000$ J/mol, for the transition from the native into the activated state the change in free energy $\Delta F_1^* = 10000$ J/mol, enthalpy $\Delta H_1^* = 250000$ J/mol and entropy $\Delta S_1^* = 760$ *e.u.*, and for the transition from the denatured into the activated state $\Delta F_2^* = \Delta F_1^*$, $\Delta H_2^* = 40000$ J/mol and $\Delta S_2^* = 94$*e.u.*.

Characterization of the Denaturation Transition of Chymotrypsinogen Bound to Spheron

According to the equilibrium thermodynamic quantities, the denaturation of immobilized chymotrypsinogen possesses typical properties of the denaturation transition of globular proteins[12], consisting in a large positive change in entropy compensated by a positive change in enthalpy. Hence, the denaturation transition of immobilized chymotrypsinogen may be characterized as uncoiling of the native state of a protein, accompanied by solvation of nonpolar side chains, as also indicated by a shift

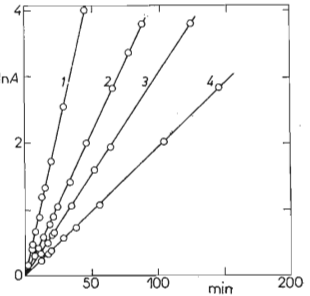

FIG. 5

Kinetics of the Establishment of Equilibrium after a Jumpwise Rise in Temperature in the Denaturation of Chymotrypsinogen Bound on Spheron in 0·01M-KCl + 0·01M-HCl (pH 2·6)

$$A = \frac{\Delta Q_\infty^{\lambda_1\lambda_2} - \Delta Q^{\lambda_1\lambda_2}(t)}{\Delta Q_\infty^{\lambda_1\lambda_2} - \Delta Q_0^{\lambda_1\lambda_2}} = e^{-(k_1+k_2)t} .$$

°C: *1* 47·8, *2* 46·3, *3* 43·9, *4* 39·3.

in fluorescence towards longer wavelengths. The denaturation transition of immobilized chymotrypsinogen is shifted towards lower temperatures compared to the transition of chymotrypsinogen in solution. As can be seen in Table I, also the change in enthalpy and entropy during the transition is smaller for immobilized chymotrypsinogen. Thus, the binding with Spheron leads to a decrease in the thermodynamic stability of chymotrypsinogen. On the other hand, however, the rate of the denaturation transition of immobilized chymotrypsinogen (Table II) is lower than the rate of transition of chymotrypsinogen in solution at similar temperatures[1,2]. We may therefore speak about kinetic stabilization as a consequence of immobilization. The temperature dependence of the rate constant from the denatured into the activated state k_2 for immobilized chymotrypsinogen (Table II) differs from that for chymotrypsinogen and many other globular proteins in solution[12,13]. The denaturation of proteins in solution is accompanied by an increase in enthalpy and entropy during the transition from the native into the activated state, and by a further rise in enthalpy and entropy during the transition from the activated into the denatured state. At the same time, the thermal capacity remains almost unchanged in the transition from the native into the activated state, but increases considerably in the transition from the activated into the denatured state[12]. Similarly, for immobilized chymotrypsinogen, enthalpy and entropy first increase in the transition from the native into the activated state, but decrease again in the transition from the activated into the denatured state. This decrease may be due to the stabilization of uncoiled protein by an interaction with the polymeric matrix, which at the same time is reflected in a shift in equilibrium in favour of the denatured state and in the just mentioned thermodynamic destabilization of immobilized chymotrypsinogen.

TABLE II

Rate Constants of the Thermal Denaturation of Chymotrypsinogen Bound to Spheron in 0·01M -KCl + 0·01M-HCl (pH 2·6)

K are equilibrium constants calculated from transition curves, $k = k_1 + k_2$ are rates of the establishment of equilibrium, k_1 and k_2 are rate constants of transition from the native into the activated state and from the denatured into the activated state.

Temperature °C	K	$10^4 k$ s^{-1}	$10^4 k_1$ s^{-1}	$10^4 k_2$ s^{-1}
47·8	2·08	13	8·78	4·22
46·3	1·42	7·57	4·44	3·13
43·9	0·78	4·8	2·1	2·7
39·3	0·26	3·06	0·63	2·43

The curved shape of the dependences of the rate constants $\ln k_1$ and $\ln k_2$ on $1/T$ for immobilized chymotrypsinogen may be explained by an increase in the thermal capacity of the protein in the transition from the native into the denatured state and by its new decrease in the transition from the activated into the denatured state. Since the rise in thermal capacity in the denaturation of proteins is attributed to the hydration of nonpolar chains after the uncoiling of the hydrophobic core of the protein[3,4,12], it may be inferred that after the uncoiling of immobilized chymotrypsinogen during the transition into the activated state, hydrophobic domains in which nonpolar amino acid residues of proteins and nonpolar parts of the polymer are present are again formed in the transition from the activated into the denatured state. The formation of hydrophobic bonds between Spheron and protein is possible owing to the structure of the polymer, which in addition to the hydrophilic side groups contains the hydrophobic main chain and hydrophobic crosslinking agent. The hydrophobic and hydrophilic nature of Spheron is adequately reflected in its swelling in both nonpolar solvents and water. The participation of some amino acid residues of denatured immobilized chymotrypsinogen in hydrophobic bonds might also be the cause of the acid shift of pK of ionizable groups in denatured immobilized chymotrypsinogen mentioned above, and of the reduced red shift of the tryptophan fluorescence in the denaturation. The kinetics of renaturation after the jumpwise drop in temperature could no more be expressed through a single exponential curve, and its rate at the same temperatures was approximately three times lower than the rate of denaturation after the jumpwise increase in temperature in Table II. The difference in the rate of establishment of equilibrium after heating and cooling may be attributed to the slow relaxation of the polymeric matrix after the decrease in temperature which influences the stability of the complex denatured protein – polymeric matrix. The existence of a complex between the denatured chymotrypsinogen and matrix in solution is also corroborated by the slowed-down renaturation of denatured chymotrypsinogen in solution in the presence of Spheron and the strong sorption of denatured chymotrypsinogen from solution to Spheron, mentioned in our earlier paper[1]. The assumed complex formation between matrix and denatured immobilized chymotrypsinogen could be an analogy to the irreversible denaturation of proteins, in the course of which physical bonds are formed between uncoiled proteins and the latter undergo irreversible association.

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