ON THE KINETICS OF STRUCTURAL TRANSITION I OF SOME PANCREATIC PROTEINS

F. M. POHL
Max-Planck-Institut für physikalische Chemie, Göttingen, Germany

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1. Introduction

The reversible denaturation of some pancreatic proteins, like trypsin, chymotrypsin and ribonuclease A around pH 2 (transition I) can be described by an equilibrium between two main conformations of the protein, which can be followed by the change of the UV-absorption, optical rotation, fluorescence etc. [1–7]. The kinetic measurements in the second to minute range support such an intramolecular and cooperative "all-or-none" change of the proteins' structure [8–10]. The overall rate constants of the denaturation k_f and the renaturation k_b depend on temperature, pH, ionic strength and solvent composition. From the magnitude of these changes with a variation of the experimental conditions one may estimate the influence of protonation, of electrostatic interactions between charged groups and the magnitude of interactions between nonpolar groups and water in the two states, respectively.

The qualitative kinetic behaviour is very similar for all the proteins studied, while quantitative differences due to structural variation are observed. The pH-dependence indicates that at least 2–3 groups – stabilizing the "native" conformation A – have an unusually small binding constant for protons (pK_a) which increases by 2–3 orders of magnitude in the denaturated state B (pK_b). This low pK_a can be caused, at least partially, by ionic bonds between charged amino acids. The ionic strength dependence of the kinetics seem to support this conclusion. Common to all these proteins is a decrease of the activation energy for the renaturation with increasing temperature. Experiments in solvent mixtures suggest that this is partially due to the interaction of nonpolar side chains with water, similar to the solubility behaviour of small nonpolar molecules [11,12]. Some of the kinetic results obtained with a number of proteins will be summarized and discussed with respect to some hypotheses which might lead to a quantitative description of the observed effects. A more detailed and quantitative account will be given elsewhere.

2. Materials and methods

Trypsin, chymotrypsinogen A, α-chymotrypsin and DIP-chymotrypsin (Worthington Biochem. Corp.), trypsinogen and phosphate free ribonuclease A (Nutritional Biochem. Corp.) from bovine pancreas were used without further purification. The generous gifts of chymotrypsinogen B [13] by L.B.Smillie, anthraniloyl-chymotrypsin [14] by T.Jovin and porcine elastase [15] by D.M.Shotton are gratefully acknowledged.

The time-dependent change of the absorption, optical rotation or fluorescence after heating or cooling the solution in a thermostated microcell within a few seconds (by switching between the circulating fluids of two water baths) were recorded with the aid of a Zeiss PMQ II spectrophotometer, Cary 60 spectropolarimeter or Aminco-Bowmann fluorimeter. Experimental details and the evaluation of the overall rate constants have been reported [9,10].

3. Results and discussion

3.1. *"All-or-none"*

Changing the equilibrium between the native and

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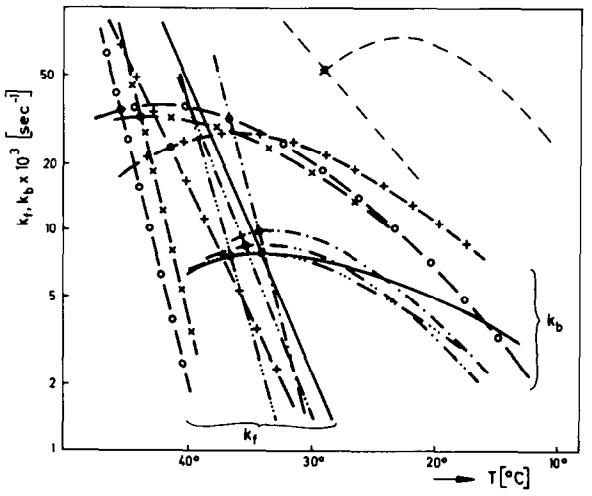

Fig. 1. Temperature dependence of the overall rate constants k_f (denaturation) and k_b (renaturation) of trypsin, trypsinogen (—+—), chymotrypsinogen A (—x—) and B (—○—), α-chymotrypsin (—●—), DIP-chymotrypsin (— · · —), anthraniloyl-chymotrypsin (— · · · —), elastase (— — —) and ribonuclease A (———). Filled circles indicate $k_f = k_b$ (10^{-2} N HCl, 0.2–0.3 mg/ml protein).

denaturated protein by a "sudden" change of the temperature leads to a single relaxation time, which is independent of the protein concentration and the magnitude and direction of the equilibrium change. The amplitude of the relaxation curve under most conditions corresponds to the total change of the equilibrium values. The same time constant for the change of absorption at different wavelengths, of optical rotation and fluorescence is observed. That different parts of the protein alter their environment with the same rate is indicated for example by anthraniloyl-chymotrypsin, where the relaxation time for the change of absorption or fluorescence of the bound chromophore is the same as for the tryptophans and even the same for the energy transfer between both.

These kinetic observations agree with a structural change between two conformational states in an "all-or-none" process with no significant intermediate states and might be characteristic for the three-dimensional network of interactions in these globular proteins.

Fig. 1 summarizes the temperature dependence of k_f and k_b for the different proteins and shows chiefly the qualitative similarities between them.

3.2. *Protons as effectors*

The pH-dependence of k_f, which increases with the second to third power of the proton (= effector) concentration around pH = 2, and of k_b, which decreases slightly, are very similar for these proteins as was discussed for trypsin [10]. A good model for this dependence is a binding constant which can be the same for two or three sites of the protein, but different in the two states. Thus the mechanism corresponds to one which was proposed for subunit enzymes with n identical binding sites for the effector, existing only in two states [16,17]. Under the conditions apparently fulfilled, that the binding process is fast compared to the structure change and a "buffered" effector concentration, the relaxation time for

$$\begin{array}{ccc} \cdot & \overset{k_i}{\rightleftharpoons} & \cdot \\ (n-i)\mathrm{H} + \mathrm{A}_i & \underset{k_i'}{} & \mathrm{B}_i + (n-i)\mathrm{H} \\ \Big\uparrow K_a & & \Big\uparrow K_b \\ (n-i+1)\mathrm{H} + \mathrm{A}_{i+1} & \rightleftharpoons & \mathrm{B}_{i+1} + (n-i+1)\mathrm{H} \\ \cdot & & \cdot \end{array}$$

$(i = 0, 1, ..., n)$

is given by [18]:

$$\frac{1}{\tau} = k_f + k_b = k_o \frac{(1+H/\gamma)^n}{(1+H/K_a)^n} + k_o' \frac{(1+H/\gamma)^n}{(1+H/K_b)^n}$$

($\gamma = k_i K_a / k_{i+1} = k_i' K_b / k_{i+1}'$, H = hydrogen activity, k_n and k_n' the rate constants of the structure change of the completely protonated states). From these expressions follows that the activation energy of k_f and k_b will be independent of the effector concentration only if its binding has an enthalpy of zero. This condition is well fulfilled for the serine-proteinases, especially trypsin, but not for ribonuclease A ($\Delta H^\circ_{\text{protonation}} \approx 3$ kcal/mole). Such an influence on the apparent activation energy seems sometimes to be neglected in denaturation experiments, where, e.g., urea acts as an effector.

3.3. *Possible binding sites*

A $pK_a \approx 1.3$ in "native" trypsin [10] or chymo-

trypsinogen [4] is unusual low for a carboxyl group and is shifted to a pK_b of 3–4.5 in the denaturated state. This indicates that 3 (2 in trypsin) COO^--groups are somehow shielded in the native structure. The number of 3 such groups is consistent with the difference of COOH-groups in the primary structure of chymotrypsin [19,20] and the ones which are titrated at pH = 2 (20°) [21]. A possible explanation for this low pK_a is the formation of ionic bonds to NH_3^+-groups of lysine or arginine. (An aminogroup, e.g., in α-position of a carboxylic acid shifts the pK_{COOH} from 4.8 to 2.3 [22].)

Inspection of the model of α-chymotrypsin, obtained by X-ray diffraction [24], shows that three such bonds are possible at the surface, where one oxygen of COO^- can form an ionic bond to an amino group and the other one a hydrogen bond to a NH-group: glu 21 ... arg 154, asp 128 ... lys 203 and α-carboxyl 245 ... lys 107. The bond involving the terminal carboxyl group seem to be possible in all these serine-proteinases and might lead to a protection against carboxy-peptidase digestion.

3.4. *Electrostatic interactions*

An increase of the ionic strength will destabilize these postulated ionic bonds and k_f will increase. Since the protein carries a high positive net-charge the increase of the counter-ion concentration should also stabilize the native structure (decrease of k_f) and thus a competition between these two effects should be observable. The experimental results, e.g., with trypsin (fig. 2) can be interpreted in the way that at low ionic strength the ionic bonds are influenced to a larger extend, leading therefore to a maximum in k_f. But more conclusive will be the influence of a defined change of the protein's charge pattern on the kinetics.

Another possibility for obtaining information about the electrostatic interactions is to change the dielectric constant D of the solvent and apparently this is the main influence of solvent mixtures on the rate of denaturation. Fig. 3 shows that the destabilization of the native molecule increases linearly with $1/D$, as would be the case if the "effective" distance between the positive charges or their number is larger in the transition state than in the native one.

3.5. *Interaction of nonpolar side-chains with solvent*

While the activation energy for the denaturation is

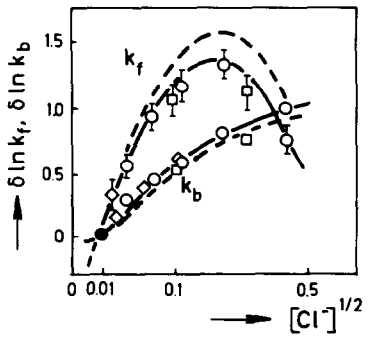

Fig. 2. Ionic strength dependence of k_f and k_b of trypsin. (8×10^{-3} N HCl, 0.25 mg/ml protein, (○) LiCl, (□) KCl, (◇) NaCl). (– –) corrected for the secondary salt effect due to the change of the hydrogen activity with increasing electrolyte concentration [23].

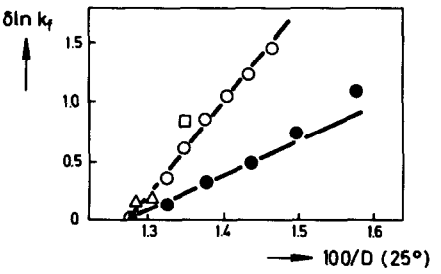

Fig. 3. Change of the rate of denaturation with the dielectric constant D of the solvent for trypsin (open symbols) and ribonuclease A (filled). (○): ethanol–water; (□): dioxane–water; (△): sucrose–water (10^{-2} N HCl, 0.25 mg/ml protein).

temperature independent within the experimental error for all proteins, the one for the renaturation E_b^* decreases with increasing temperature. Such a decrease of the enthalpy is also observed for the transfer of nonpolar amino-acids from water to nonpolar solvents [4,12,24]. (An interaction of nonpolar side chains with water in the denaturated state is also indicated, e.g., by the difference spectrum between states A and B.)

A way to test this hypothesis is to modify the protein, e.g., by introducing an additional nonpolar group, like in DIP- and anthraniloyl-chymotrypsin, or to compare the renaturation kinetics in solvent mixtures with the solubility of nonpolar molecules in the same mixtures. For such a comparison the solubility of argon seems to be a suitable model, since accurate

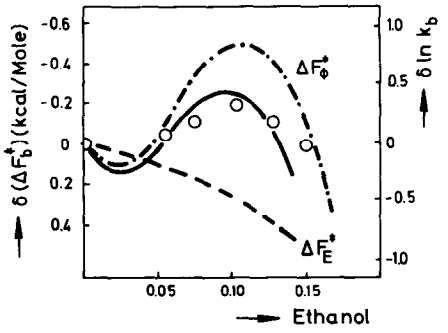

Fig. 4. Rough approximation for the solvent dependence of $\Delta F_b^{\ddagger}$ (———) for the renaturation of trypsin at 18° in ethanol–water mixtures by an electrostatic term $\Delta F_E^{\ddagger}$ (– – –) and by one for the interaction of nonpolar side chains with the solvent $\Delta F_\Phi^{\ddagger}$ (– · – · –). ($\Delta F_\Phi^{\ddagger}$ + 27.2) corresponds to the transfer of 15 moles argon [26] and the number of 15 is estimated from the solvent dependent change of the activation energy at 30° [10].

data in solvent mixtures are available [26]. The thermodynamic transfer parameters of argon to water are the same, within a factor of two, as for an "average" nonpolar side chain [4,12]. Fig. 4 shows how the observed free energy of activation $\Delta F_b^{\ddagger}$ can in a first approximation be divided into an electrostatic and an "hydrophobic" contribution, caused by the change of the solvent properties and qualitatively explains the observed dependence of k_b on the ethanol concentration [10].

Such a comparison, together with the temperature dependence of E_b^* in water, suggests that relatively few additional nonpolar amino acids are exposed to the solvent in the denaturated state and that their number increases from ribonuclease A (3–8) to chymotrypsinogen, DIP-, α- and anthraniloyl-chymotrypsin to trypsin (15–40). Together with hydrodynamic and optical rotation data [27,28] this indicates that the state B is not a random coil with all segments freely movable and in contact with the solvent but might rather be a rearrangement of the protein structure with a certain gain in flexibility.

3.6. *Transition I as a model reaction*

The knowledge of the "native" structure should allow one to test quantitative models for the classes of interactions which can be differentiated by a variation of the experimental conditions and might allow a correlation of the influence of amino acid exchanges on the kinetics. The effect of the proton concentration on the structure of these, relatively simple and well known, proteins may be compared with the influence of "allosteric" effectors, e.g., in subunit enzymes under physiologically more important conditions.

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