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On the Solvent Dependence of the Protonation and Dimerization Equilibria of the Basic Tripsin Inhibitor (Kunitz Base)

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The solvent dependence of coordination chemical equilibria of macromolecular polypeptides is strongly influenced by the solvent dependence of the secondary structure (conformation) of the macromolecule [1]. Thus besides the usual solvation effects, *e.g.* the different solvation of the functional groups in solutions of different composition, the differences in the dielectric properties of the system *etc.*, steric effects caused by the conformation change of the molecule determine the solvent dependence of protonation and metal complexation equilibria of polypeptides and proteins. This latter conformational effect may differ not only in size but also in direction in the different systems. This is why the same solvent exchange may have completely opposite effects in different model systems.

Since the biological activity of polypeptides and proteins depends on their conformation and since because of technological and stability reasons their solutions made with mixed solvents are used in pharmaceutical practice, the study of the solvent dependence of their coordination equilibria is of importance from a practical and also from a theoretical point of view. Specially solvents having no undesirable physiological effects, as *e.g.* propylene glycol, have attracted the attention of biochemists. The coordination chemical role of this solvent is enhanced by its ability to form strong chelate type solvates with ions and even with some functional groups.

Previous investigations [2] have shown that the replacement of 50 v/v % of the water by propylene glycol in the solution of corticotropin or its fragments resulted in an increase in the protonation constants of the carboxylates and in a decrease of those of the amino and phenolate groups of the peptide, but the extent of the solvent effect was found to be different in the case of fragments having different chain lengths. The aim of our present investigation was the determination of the solvent effect in the solution of the Basic Pancreas Tripsin Inhibitor (BPTI) polypeptide made with the same solvent mixture.

BPTI is a polypeptide consisting of 58 amino acid residues. Its amino acid sequence [3, 4] and atomic structure [5, 6] is known. BPTI was shown to dimerize even in diluted aqueous solution. Contradictory mechanisms were published [7, 8], however, concerning this dimerization process. Our previous investigations [8] have indicated that the dimerization of BPTI in solution is due to the formation of intermolecular H-bonds and so the pH-dependence of this process is determined by the protonation–deprotonation equilibria of the peptide. The study of the solvent dependence of these equilibria could also provide new information on the mechanism of the dimerization process.

Experimental

The Basic Tripsin Inhibitor Kunitz base (BPTI) used in the measurement was the product of the G. Richter Chemical Works, Budapest to whom the authors express their thanks.

The commercial BPTI was transformed to its perprotonated product by dry freezing with an equivalent amount of hydrochloric acid. The protonation equilibrium measurements were performed according to our procedure [9]. The total peptide concentration used was between 10^{-4}–10^{-3} M dm^{-3} in each titration; the composition of the solvent 50 v/v % water–propylene glycol. The ionic strength was kept constant at 0.3 with sodium chloride. All solutions were termostated at 25.00 °C. The accuracy of the emf measurement was found to be ±0.1 mV and that of the volume measurement ±0.005 cm^3. A computer evaluation procedure was used for the determination of the composition (bound proton: peptide ratio) of the species formed in the solution and of the corresponding equilibrium constants. The reliability of the evaluation was checked by simulating the experiment mV *vs.* pH curves on the basis of models assuming the stepwise formation of protonated species of various composition. The model resulting in the best fitting curve to the experimental points was accepted as the correct one.

Results and Discussion

The composition of the protonated species and the corresponding equilibrium constants are presented in Table I. For comparison the analogous data measured in aqueous solution are also given.

TABLE I. Protonation Constants of BPTI in Aqueous Solution and in 50 v/v % Water–Propylene Glycol Mixture (logarithmic values).

Functional groups	Proton:Peptide ratio	Successive protonation constants	
		in water	in solvent mixture
Phenolic OH	2:1	22.28	–
	4:1	21.16	–
ϵ-NH_2	6:1	19.88	–
	7:1	9.47	–
	8:1	8.97	10.33
Terminal NH_2	9:1	7.90	8.50
$-COO^-$ +	19:2	8.27*	–
Dimer formation	20:2	–	13.61**
$-COO^-$	20:2	4.36	–
	22:2	8.71	8.28
	24:2	7.41	6.07
	27:2	9.94	6.42

It is to be seen that the substitution of 50 v/v % of the water by propylene glycol in the solution resulted

$$*K = \frac{[H_{19}P_2]}{[H_9P]\,[H_{10}P]}\ ; \qquad **K = \frac{[H_{20}P_2]}{[H_9P]\,[H_{11}P]}$$

in this system in a decrease of the protonation constants of the carboxylates and in an increase of those of two amino groups, showing just the opposite trend as that in the corticotropin systems. One has also to notice that the protonation–deprotonation equilibria of only two amino groups (probably those of the terminal one and one another) of the five appeared in the equilibrium measurements and none of the phenolic hydroxides. This shows that the solvent exchange increased the basicity of these groups to such extent that they did not show up in the pH range investigated.

The data in Table I clearly indicate also that the solvent change favours the dimerization process. This is not only reflected in the increase of the dimer formation constant but also in the fact that the association of the monomeric species containing unprotonated carboxylate and protonated amino groups (resulting in the formation of the dimer) is accompanied in aqueous solution by the uptake of one proton and in the solvent mixture by uptake of two. This shows that the dimerization of BPTI is due to the formation of one intermolecular H-bond in aqueous solution and two such bonds in the solvent mixture, in both cases carboxylate oxygens serving as one of the pillar atoms (in the monomeric species before dimerization namely, only the carboxylate groups are unprotonated).

To get comparable dimerization constants we calculated from the equilibrium constants

$$*K = \frac{[H_{19}P_2]}{[H_9P]\,[H_{10}P]} \text{ and } K_{20,2} = \frac{[H_{20}P_2]}{[H_{19}P]\,[H^+]}$$

measured in water the constant

$$**K = \frac{[H_{20}P_2]}{[H_9P]\,[H_{11}P]}$$

analogous to that measured in the solvent mixture. The comparison of the value calculated for aqueous solution (1g *K 12.63) with that measured in the solvent mixture (1g **K = 13.61) indicated that the introduction of the mixed solvent increased the protonation constant of those carboxylates which take part in the dimer forming H-bonds. It decreases only the protonation constant of the other carboxylates in the dimer. This latter effect may be due to steric reasons in the macromolecular dimer.

The increase of association constants (for protonation and dimerization processes) in the propylene glycol containing solutions is probably due to the decreased relative permittivity (ϵ) of the mixed solvent system (ϵ = 59.0, measured by dielectrometry). The decrease in the association constants most probably indicates however specific interactions, *e.g.* specific solvation of functional groups, steric effects caused by the secondary structure of the macromolecule *etc.*

On the basis of the latter suggestions the solvent dependence of the protonation of BPTI contributes to a better understanding of that of the corticotropin fragments [2]. The decreased protonation constants of the ϵ-amino and phenolate groups in the propylene glycol containing solutions of the corticotropin fragments seem to be due to the favoured chelate type solvation of the unprotonated functional groups by

propylene glycol. The same functional groups in BPTI are however not attainable by the solvent molecules because of the rather rigid secondary structure of this peptide. Thus the dominant part of the solvent effect in the protonation equilibria of ϵ-amino and phenolate groups in the corticotropin systems seems to be their specific solvation and in the BPTI system the change in the dielectric properties of the solvent.

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