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The Effect of Solvents on Protonation Equilibria of Corticotropin (ACTH) Fragments

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Potentiometric equilibrium measurements were used to determine the protonation group constants characteristic of the individual donor groups in the polyfunctional molecule of two Corticotropin fragments, the N-terminal $ACTH_{1-4}$ containing 4 amino-acids and the N-terminal $ACTH_{1-32}$ containing 32 amino acids, in aqueous solution and in trifluoroethanol–water (50 v/v %) and propylene glyco–water (50 v/v %) solvent mixtures. The protonation group constants of the N-terminal Corticotropin fragment $ACTH_{1-14}$ containing 14 amino acids were also established in water. The results reflect the effect of the formation of intramolecular H-bonds and solvation on the protonation processes

The biological activity of macromolecular polypeptides is known to depend on the conformation of the molecule. Circular dichroism studies [1] have indicated that the conformation of Corticotropin [2] (ACTH) in aqueous solutions is a function of the pH of the solution. The effect of pH-dependent rearrangements accompanied by changes in conformation of ACTH fragments on their protonation equilibria was reflected also by the potentiometric equilibrium study of the system [3]. CD investigation in various solvents [1, 4] confirmed the dependence of the conformation of ACTH on the nature of the solvent. The polypeptide globules showing some initial order in aqueous solution assume the α-helix structure in trifluoroethanol. In solvent mixture water–trifluoroethanol the degree of order varies with the ratio of solvent components.

In the present work, we study the effect of the solvent and solvent-dependent conformation change of ACTH fragments on their protonation equilibria. This effect is also reflected in the protonation macro constants determined in the usual way in different solvent mixtures [5]. Up to now, unambiguous and exact determination of this effect was hindered by the fact that protonation processes of several donor atoms with similar basicities in the molecule overlap so much that protonation constants suitable for quantitative characterisation of the individual donor groups could not be obtained. The protonation macro constants describe quantitatively the protonation process as a whole and give the changes in the composition of the species (peptide: bound proton ratio) present in the solution as a function of pH, but they are not suitable for the exact characterisation of the individual groups because of the formation of several protonation isomers of the polyfunctional ligand.

The solution of the problem was made possible by the introduction of our new evaluation procedure [6] leading to the determination of "group constants" suitable for quantitative characterisation of stepwise protonation of polyfunctional ligands.

As a next step, a method was to be developed for the quantitative consideration of the effect of solvent mixtures (having lower relative permittivities and solvation properties different from water) on the experimental data of pH-metric equilibrium measurements. This method and the results obtained by its use in investigations on protonation of ACTH fragments will be discussed in this paper.

Determination of the Autoprotolysis Constant of a Solvent Mixture

In order to obtain data suitable for equilibrium calculations (that is, which can be directly correlated to the concentration of free hydrogen ions) from e.m.f. measurements in solvent mixtures, first the autoprotolysis constant of the solvent mixtures was to be determined. This was carried out by means of data obtained from $HClO_4$–NaOH titration curves, which were obtained in solutions of analogous composition with those used in the protonation studies on ACTH fragments but not containing the peptide. Only those regions of the titration curves were taken (16–20 points in each titration) which had slopes between about 0.5 and 2, thus the effect of errors in the measurement of e.m.f. and in volume reading is commensurable. The evaluation was based on the following equations which are the modifications of those of Dyrssen and Ingri *et al.* [7, 8]:

$$E = E'_o - x \lg \left[\frac{\frac{C_H V_H - C_{Na} V_{Na}}{V_H + V_{Na}} + \left[\left[\frac{C_H V_H - C_{Na} V_{Na}}{V_H + V_{Na}}\right]^2 + 4K_v \right]^{1/2}}{2} \right] \quad (1)$$

$$K_v = \frac{\left[2 \cdot 10^{(E'_o - E)/x} - \frac{C_H V_H - C_{Na} V_{Na}}{V_H + V_{Na}}\right]^2 - \left[\frac{C_H V_H - C_{Na} V_{Na}}{V_H + V_{Na}}\right]^2}{4} \quad (2)$$

$$x = \frac{m \Sigma E \lg [H^+] - \Sigma \lg [H^+] \Sigma E}{m \Sigma (\lg [H^+])^2 - (\Sigma \lg [H^+])^2} \quad (3)$$

$$E_o = \frac{\Sigma E - x \Sigma \lg [H^+]}{m} \quad (4)$$

where E is the measured e.m.f., E'_o is the pH-independent constant of the galvanic cell, x is the experimental value of the Nernst expression RT/2.303 F, C_H and V_H and C_{Na} and V_{Na} are the analytical concentrations and volumes, respectively, of perchloric acid and sodium hydroxide, K_v is the autoprotolysis constant of the solvent mixture in a solution of 0.3 mol dm^{-3} ionic strength and 25 °C temperature, m is the number of measured points, $[H^+]$ is the equilibrium concentration of hydrogen ions (the logarithmic part of eqn. (1)).

An iteration method was developed for the calculation of constants E'_o, x and K_v. Equation (1) gives the dependence of the electromotive force on analytical concentration and volume data and on the former constants characteristic of the given system. Using the principle of least squares in linear minimization of error, the slope and intercept with the best fit to all data can be calculated by eqns. (3) and (4). Since simultaneous calculation of K_v in a similar manner cannot be achieved, in the first approximation a hypothetic autoprotolysis constant had to be inserted in the expression, its error caused distortion of the E'_o and x values. In order to avoid this, eqn. (2) and E'_o and x obtained were used to calculate the autoprotolysis constant of the solvent mixture in all points, they were averaged and the arithmetic mean of the values obtained in the ith and $(i - 1)$th steps was taken. In the $(i + 1)$th step, these values are used for calculating E'_o and x. The whole procedure was repeated several times to achieve slow but definite convergence of constants E'_o, x and K_v to the real values, which could be checked by calculating the corrected experimental scattering obtained from calculated and measured E values. The autoprotolysis constants determined in this way are $10^{-11.04}$ in a mixture of CF_3CH_2OH and H_2O (50 v/v %) and $10^{-13.53}$ in a mixture of $HOCH_2CHOHCH_3$ and H_2O (50 v/v%). Relative permittivities measured by dielectrometry of the solvent mixtures were 55 and 59, respectively.

Determination of Protonation Group Constants of ACTH Fragments

For the potentiometric determination of the protonation constants the Corticotropin fragments produced by the Richter Gedeon Works, Budapest, were subjected twice to freeze-drying with equivalent amounts of hydrochloric acid. The resulting perprotonated products were titrated with 0.01 *M* an 0.1 *M* NaOH standard solutions, respectively, as given in previous papers [3, 5, 6]. The measurements were carried out in aqueous solutions and 1:1 mixtures of trifluoroethanol and water and propylene glycol and water (v/v ratios). Protonation of $ACTH_{1-14}$ was investigated only in aqueous solutions. The equilibrium data were obtained from the alkalimetric titration curves and the $\bar{H}$–pH functions constructed from them ($\bar{H}$ is the average number of protons dissociated by one peptide molecule at each given pH of the solution).

In computerized processing, the $\bar{H}$–pH data pairs yielded directly the corresponding group constants [6] K_A, K_B and K_C (K_i) of the donor groups with similar basicities which are in the following correlation with the complex products β calculated in the usual way, given for example for a three-step process:

$$\beta_1 = K_A + K_B + K_C$$

$$\beta_2 = K_A K_B + K_A K_C + K_B K_C$$

$$\beta_3 = K_A K_B K_C$$

The above correlation can be analogously given also for multi-step processes.

On the basis of the alkalimetric titration curves of perprotonated compounds, as well as from the $\bar{H}$–pH functions derived from them it could be stated that the proton dissociation processes are stepwise for $ACTH_{1-4}$ and enitrely overlapping and covering the whole pH range examined for $ACTH_{1-32}$.

Evaluation of the titration curves and determination of the group constants was made difficult by several factors. In the pH range of the deprotonation of the phenolic hydroxyls of tyrosines and the ϵ-ammonium groups of lysines the deprotonation processes of the guanidinium groups of arginine are not negligible. In the 5×10^{-4}–10^{-3} mol dm^{-3} concentration of peptides employed by us, the respective macro constants of the protonation of quanidino groups (lg K = 12–13) could be determined only with

TABLE I. Protonation Group Constants of ACTH Fragments in Aqueous Solutions (Logarithmic Values).

Functional group	$ACTH_{1-4}$	$ACTH_{1-14}$	$ACTH_{1-32}$
Terminal COO^-	3.20	3.47	3.54
Glutamic acid + Aspartic acid COO^-		4.19	3.59(!) 4.10 4.25 5.03(!)
Histidine N		6.31	6.43
Terminal NH_2	7.27(!)	7.29(!)	7.47
Lysine NH_2		9.94	9.70(!) 10.13 10.45 10.82
Tyrosine OH	10.70	10.86	10.94 10.98

great uncertainty. At the same time, these processes also affect the constants with lg K > 8 belonging to phenolates and ϵ-amino groups. This problem could be eliminated by increasing the concentration of the peptide and the titrant NaOH solutions, which is however limited. The peptide concentration has to be kept on a low level to affect only to a limited extent the low (0.3) ionic strength of the solution. The increase of the salt concentration for assuring a higher ionic strength could result in the alteration of the conformation of peptides. Another source of error can be the fact that the analytical concentration of these macromolecular peptides can be determined only with an accuracy of ±1–2 rel.%.

In solvent mixtures containing trifluoroethanol, the pH scale is contracted (lg K_v = −11.04, instead of −14 for water), and this further reduces the accuracy of the determination of protonation constants of the strongly basic groups.

Owing to these facts, the accuracy of equilibrium constants determined in the macromolecular systems is necessarily lower than those related to systems containing ligands of smaller size. Thus, in spite of the fact that the experimental mV and derived $\bar{H}$ data could be re-calculated with an accuracy within the limits of the experimental error (±0.1 mV and ±0.02 $\bar{H}$, respectively) from these data, their uncertainty is significant, about 0.05–0.15 lg units.

Therefore, in order to confirm the potentiometric data, UV spectroscopic investigations on protonation of $ACTH_{1-4}$ and $ACTH_{1-32}$ were also carried out.

In Fig. 1, the UV spectra of $ACTH_{1-4}$ recorded in trifluoroethanol–water solvent mixtures of different hydrogen ion concentration are shown. Similar series of curves were obtained in the study of

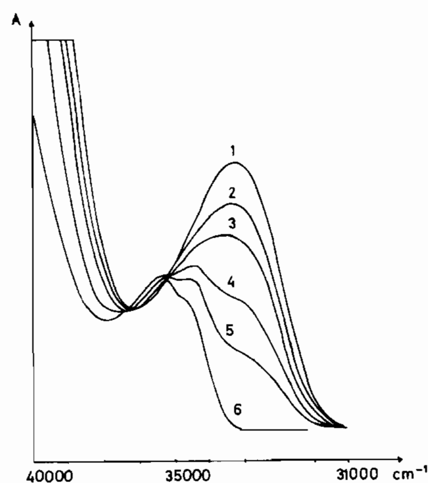


Fig. 1. The UV spectra of $ACTH_{1-4}$ measured in 50 v/v % trifluoroethanol–water solutions of different pH: 1, pH = 11.95; 2, pH = 10.69; 3, pH = 10.38; 4, pH = 9.94; 5, pH = 9.58; 6, pH = 7.95.

the peptide in aqueous solution and in propylene glycol–water solvent mixture. The group constant of the protonation of the phenolic oxygen of tyrosine in $ACTH_{1-4}$ was determined from the pH-dependence of the absorption values of these curves at 34200 cm^{-1}. The values obtained were in agreement with the potentiometrically determined data given in Table I within the limits of the experimental error.

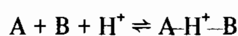
Analogous examination of the peptide $ACTH_{1-32}$, was made difficult by the fact, that in the spectral region about 34200 cm^{-1} sensitive to deprotonation of the tyrosine molecule fragments, the indol ring of

tryptophan present in the molecule produces a very strong absorption, stronger than that of tyrosine phenolate. Furthermore, the aromatic side-chains of phenylalanine and histidine also show some pH-independent absorption. Consequently, the spectrophotometric technique could not provide constants more accurate than those determined potentiometrically.

The protonation group constants of polypeptides $ACTH_{1-4}$, $ACTH_{1-14}$ and $ACTH_{1-32}$ measured in aqueous solutions are summarized in Table I. When comparing the constants of the three molecules with different chain length to each other and to analogous data of the corresponding amino acids, the following conclusions can be drawn regarding the systems examined.

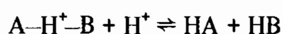
1) The constants assigned to carboxyls of C-terminal amino acids ($ACTH_{1-4}$-methionine: lg K = 3.20; $ACTH_{1-14}$-glycine: lg K = 3.47 and $ACTH_{1-32}$-alanine: lg K = 3.54) were found to be higher by about 1 lg unit than the values determined in the corresponding amino acids. Their order is the same as in the amino acids. Thus the value determined can be explained by the combined action of the peptide bond and the side-chain.

2) The group constant of $ACTH_{1-14}$, being lg K = 4.19 presumably reflects the basicity of a side-chain carboxylate free of hydrogen bonds, is affected only by the peptide bonds. Two group constants with similar values were obtained in the $ACTH_{1-32}$ molecule, too, here however the constants assigned to the other two carboxylates differed strongly. In view of the fact that the group constant belonging to the formation of a hydrogen bond



$$A + B + H^+ \rightleftharpoons A\text{–}H^+\text{–}B$$

should be, in principle, higher by the same value as the group constant of the break-up of the hydrogen bond



$$A\text{–}H^+\text{–}B + H^+ \rightleftharpoons HA + HB$$

is lower than the protonation constants of groups identical with the pillar groups but not involved in hydrogen bonds, the lg K 4.25 and lg K 4.10 constants of $ACTH_{1-32}$ belong most probably to glutamic acid at site 5 and aspartic acid at site 29 being far from each other and thus forming no hydrogen bond, while the constants with values lg K 5.03 and 3.59 should be assigned to γ-carboxyls of glutamic acids at sites 30 and 28. The latter two can form an H-bond and this is reflected in the high difference between the corresponding constants.

3) In $ACTH_{1-4}$ and $ACTH_{1-14}$, the protonation group constans of terminal amino groups are significantly lower than that in $ACTH_{1-32}$. This indicates the possibility of hydrogen bonds between the former ones and a group of higher protonation constant.

4) The highest deviation from the constants assigned to an ϵ-amino group in lysine and in free primary amino groups of the peptide side-chains is produced by the protonation constant lg K = 9.70 in $ACTH_{1-32}$. A similar value was also obtained in the $ACTH_{1-14}$ molecule (lg K = 9.94), this it can be attributed to a fraction also present in the smaller peptide. Such is the ϵ-amino group in lysine at site 11. The low protonation constant indicates its participation in an H-bond with a donor group of higher basicity. Since the letter group is not reflected in the equilibrium data by a protonation constant of higher value, the other pillar of this H-bond with the ϵ-amino group must be a group which becomes deprotonated only at a higher pH not accessible in the present work. Such groups are *e.g.* the alcoholic hydroxyl in serine, guanidinium in arginine or –NH– in a peptide bond.

5) As for the five groups suffering deprotonation in the most alkaline region examined by us (which can be assigned to phenolic hydroxyl and ϵ-ammonium groups being acidic to nearly the same extent in amino acids), their interaction with each other and with other parts of the molecule, as well as their important role in the development of conformation are indicated by the observation that their constants are far from being identical, they differ from the mean value (lg K = 10.66) by ±0.3 lg K units, on the average.

The protonation group constants of polypeptides $ACTH_{1-4}$ and $ACTH_{1-32}$ determined in solvent mixtures are summarized in Table II. By comparing them with each other and the data measured in aqueous solutions, conclusions can be drawn on the effect of solvents on the protonation process.

Variation in the basicity of the most important types of functional groups (carboxylate, amino and phenolic hydroxyl groups) on changing the solvent can clearly be observed in $ACTH_{1-4}$.

Terminal carboxyl groups show higher protonation constant in solvent mixtures (lg K = 3.88 and 3.89, while lg K = 3.20 was obtained in water), which can be attributed to the lower relative permittivity of solvent mixtures favouring the formation of neutral particles ($RCOO^- + H^+ \rightleftharpoons RCOOH$.

The constants lg K = 6.77 and 6.87 belonging to the terminal amino group are lower than those obtained in water (lg K = 7.27), indicating changes in solvation conditions of unprotonated and protonated amino groups favouring the unprotonated state. In $ACTH_{1-32}$, similar statements can be made also with respect to the imidazol group. In fact, the solvent mixture favours the electrically neutral state of these groups, too.

The development of reduced constants of phenolic hydroxyls (as compared to aqueous media) is

TABLE II. Protonation Group Constants of ACTH Fragments in Solvent Mixtures (Logarithmic Values).

Functional group	Trifluoroethanol–water		Propylene glycol–water	
	$ACTH_{1-4}$	$ACTH_{1-32}$	$ACTH_{1-4}$	$ACTH_{1-32}$
Terminal COO^-	3.88	4.08	3.84	4.00
Glutamic acid + Aspartic acid COO^-		4.17 4.73 4.80 5.47		4.21 4.46 5.02 5.29
Histidine N		6.13		6.06
Terminal NH_2	6.77	6.77	6.87	6.82
Lysine ϵ-NH_2		8.41(!) 9.49 9.75 9.82		8.56(!) 9.73 9.84 9.84
Tyrosine OH	9.95	9.84 9.87	10.04	9.84 9.84

probably due to different effects in the two solvents mixtures. In solutions containing propylene glycol, the interaction of phenolate oxygen with propylene glycol being suitable for forming a H-bond of chelate nature with the deprotonated phenolate is responsible for this phenomenon, while in solutions containing trifluoroethanol the specific solvation effected by it on the proton (see, pK_v = 11.04) causes the lower protonation group constants.

In $ACTH_{1-32}$, the solvent-dependence of the protonation group constants differing from those mentioned above can be explained by modifications in the secondary structure.

In both solvent mixtures, the lowest value of the constants assigned to the ϵ-amino groups of $ACTH_{1-32}$ shows a stronger decrease as compared with the corresponding value obtained in aqueous medium, while the constants belonging to the five most basic groups show a much smaller deviation from each other and the mean value (0.11 and 0.03 in trifluoroethanolic and propylene glycol containing solutions, respectively), than in water. According to this, the helical arrangement occurring on decreasing concentration of water favours the development of one hydrogen bond producing an extremely low stability constant, however, it hinders the formation of further hydrogen bonds with the participation of ϵ-amino or phenolic OH groups yielding the globule structure characteristic of the aqueous solutions.

Experimental

The pH-metric equilibrium measurements were effected in solutions adjusted to 0.3 mol dm^{-3} constant ionic strength in the aqueous solutions with potassium nitrate and in the solvent mixtures with sodium perchlorate. The solutions were thermostatted to 25.00 ± 0.1 °C. A precision pH-meter of type Radiometer pH M 64 was used for the measurements and a Radiometer ABU 12 automatic burette for the addition of the 0.01 and 0.1 mol dm^{-3} carbonate-free NaOH titrant. The measuring electrode was a Radiometer G 202 B glass electrode, the reference electrode was a Radiometer P 501 silver electrode being in equilibrium with 0.01 mol dm^{-3} silver nitrate in a Wilhelm bridge [9].

Since amino acids and their derivatives can carry different charges as a function of hydrogen ion activity and this can affect the ionic strength, their concentration was to be low enough to make this effect negligible as compared with ionic strength being 0.3 mol dm^{-3}, but it had to reach or exceed 5×10^{-4} mol dm^{-3}. Thus, all potentiometric measurements were carried out in solutions with 5×10^{-4} and 10^{-3} mol dm^{-3} peptide concentrations.

The perprotonated peptide was dissolved in solution of 0.1 (or 0.01) *M* acid concentration and adjusted to constant ionic strength, then the change in e.m.f. was measured during the titration with 0.01 or 0.1 mol dm^{-3} NaOH standard solution.

The glass electrode was kept in a dilute $HClO_4$ solution made with the solvent in question at least for 16 hrs before the measurements. In aqueous solutions it was calibrated immediately before and after the measurements with a Britton–Robinson buffer [10] adjusted to ionic strength 0.3 mol dm^{-3}. In the solvent mixtures the galvanic cell was calibrated with $HClO_4$–NaOH titrations in 0.01 and 0.1 *M* concentrations. In the concentrations employed, the protein error of glass electrodes (slow setting, poor reproducibility) could not be observed. The equilibria were instantaneously attained.

For the computation of group constants [6] K_i reflecting the protonation of the donor groups not participating in H-bond and of those K_{jf} and K_{jd} reflecting protonation resulting in H-bond formation ($A + B + H^+ \rightleftharpoons A{-}H{-}B$) and in the break-up of a H-bond ($A{-}H{-}B + H^+ \rightleftharpoons AH + BH$), respectively, from the $\bar{H}$ – hydrogen ion concentration data derived from experimental e.m.f.–total concentration values the following equations [11] were used:

$$\bar{H} = \sum_{i=1}^{n-2h} A + \sum_{j=1}^{h} B$$

where

$$A = \frac{1}{1 + K_i[H^+]}$$

$$B = \frac{K_{jf}[H^+] + 2}{1 + K_{jf}[H^+] + K_{jf}K_{jd}[H^+]^2}$$

$\bar{H}$ represents the number of protons dissociated by one peptide molecule at a given pH, n is the number of functional groups taking part in the protonation processes and h is the number of H-bonds.

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