H^+ Ion Equilibria in Solutions of Copolypeptides of L-Lysine and L-Aspartic Acid and of L-Lysine and L-Glutamic Acid

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pH and conductometric titration curves of copolypeptides containing L-lysine and either L-glutamic acid or L-aspartic acid residues have provided necessary evidence to believe that in the copolypeptides the zwitterion formation is complete at the isoionic point. The amounts of acid/alkali used up at the final inflexion/break in the titration curves agreed with the analytically determined quantity of basic/acid groups in the copolypeptides. Besides, the titration curves showed additional inflexions/breaks which distinguished between the various kinds of acid/basic groups present.

Polyamino acids, in particular, copolypeptides, serve as good model systems for understanding the behaviour of proteins. Investigations of the H^+ ion equilibria of copolypeptides in aqueous solutions had been shown[1-4] to be useful in studying conformational changes that occur on reaction with an acid or base. The role of vicinal charged sites on the titrations of polyacids, polybases, and polyampholytes, in general, had been worked out by Katchalsky *et al.*[5,6] but this aspect has received a rather limited attention in the study of polypeptide titrations. The abnormal behaviour of some proteins, however, had been explained[7-10] as arising out of such effects. Using non-peptidic ampholytes, the authors[11,12] had shown that a clear differentiation could be made, studying the titration behaviour of non-equimolar polyampholytes, between groups that take part and those that do not, in the formation of a zwitterion. Further, it had also been demonstrated that conductometric titrations, carried out for the first time on such systems, proved extremely useful in the identification and estimation of different kinds of prototropic groups. The present study is an application of the same kind of approach to the amphoteric polypeptides made up of L-lysine and L-aspartic acid/L-glutamic acid.

Experimental

N-Carboxy anhydrides (NCA) required for the polymerization were prepared starting from the corresponding amino acids, suitably protected, by using either directly phosgene or Leuch's method. The amino acids were modified as follows: L-glutamic acid to γ-benzyl L-glutamate,[13] L-aspartic acid to β-benzyl L-aspartate,[14] and L-lysine to α,ε,N-dicarbobenzoxy L-lysine.[15] Copolymerization was effected in dioxane (4%) with sodium methoxide (A/I=100) as initiator. After polymerization the protecting groups were removed simultaneously by treatment with HBr in acetic acid. The hydrobromide was converted to the free base by passing through a column of Amberlite IRA-400 in its hydroxy form. The isoionic form was obtained by passing through a mixed bed column of Amberlite IRA-120 and IRA-400.

Number-average molecular weights were determined following Sela and Berger.[16] Their amino acid composition was obtained following the method of Giri *et al.*[17]

The isoionic copolypeptide (10—15 mg) was dissolved in 20 ml of water in a titration vessel in which nitrogen could be swept continuously and was titrated with HCl/KOH (0.07—0.1N). pH/specific conductivity was measured after equilibration (2—5 min) after each addition of acid or alkali. A Leeds-Northrup conductivity (drum-type) bridge was used in conjunction with certified Sullivan resistances and a Muirhead Audio frequency oscillator. pH was measured with a Beckman Zeromatic pH meter. All adequate precautions such as using carbonate free alkali and maintaining an inert atmosphere were taken. Titrations were performed at room temperature (25—27°C).

Results and Discussion

Table 1 gives the amino acid composition of the copolypeptides, obtained as stated above, as also the amounts of acidic COOH groups and basic (NH_2) groups expected to be present. It can be seen that while A and C are acidic, B and D are basic. Considering these polypeptides as zwitterions, it follows that whereas the polypeptides under consideration have the acidic or the basic monomer in excess, at the isoionic point, there would be available in the molecule "free" carboxyl or amino groups which have not taken part in zwitterion. In other words, copolypeptides A and C would have the following prototropic groups available: i) COO^- anions, ii) COOH groups, and iii)

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Table 1. Amino acids analysis

Copolypeptide	Ratio of lys. to asp. acid residues	Ratio of lys. to glu. acid residues	mequiv. basic groups/100 g of copolypeptide	mequiv. acid groups/100 g of copolypeptide	Mol. wt.
A	1.00/1.59		322	516	21500
B	2.65/1.00		573	220	10700
C		1.00/1.27	342	436	15200
D		2.54/1.00	557	219	10700

NH_3^+ as the titratable groups. IR spectra fully support this in that B and D do not show any characteristic absorption for COOH group (1400 cm^{-1}).[18] Consequently, the copolypeptides A and C have only one kind of basic group, *viz.*, COO^-, to react with an acid, while B and D would have two kinds, COO^- and NH_2. In other words, copolypeptides having an excess of basic groups would be expected to have a two-stage reaction with acid and those with excess of acidic groups a two step reaction with alkali.

Figures 1 and 2 give the representative titration curves of the copolypeptides A and B with HCl and KOH, respectively. It can be seen that the conductometric titration curves marked (2) and (4), show fairly sharp breaks indicating the culmination of one type of reaction. The pH curves have corresponding inflexions, *i.e.*, changes in curvature, which are however less prominent. (In arriving at the position of the inflexions care was taken to scan the regions as carefully as possible with very small additions of acid or alkali and also checking with repetitive experiments. Also refer to modified plots.) Titration curves for the

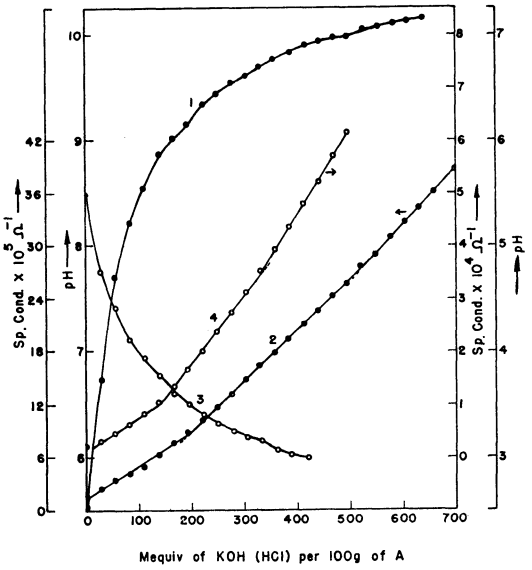

Fig. 1. Variation in pH (1,3) and sp. cond. (2,4) on addition of KOH/HCl to aq. solutions of isoionic copolypeptide A.

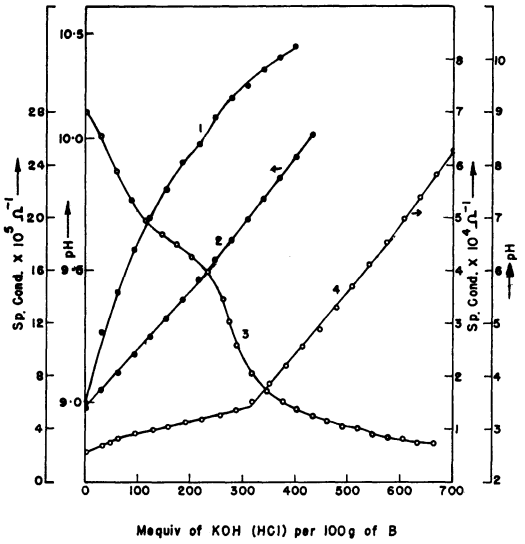

Fig. 2. Variation in pH (1,3) and sp. cond. (2,4) on addition of KOH/HCl to aq. solutions of isoionic copolypeptide B.

Table 2.

Copoly-peptide	Measurement	HCl added in mequiv./100 g at inflexion/break			KOH added in mequiv./100 g at inflexion/break		Total no. of prototropic groups	
		1st	2nd	3rd	1st	2nd	By titration	By analysis
A	pH	155	330		200	515		
	Cond.	150	330		190	520		
	Average	152	330		195	517	847	838
B	pH	55	275	550	230			
	Cond.	65	315	560	225			
	Average	60	290	555	227		782	793
C	pH	190	340		105	440		
	Cond.	195	335		110	435		
	Average	192	337		107	437	774	774
D	pH	65	315	535	235			
	Cond.	95	335	540	225			
	Average	80	325	537	230		767	776

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copolypeptides C and D are not given as they are very similar in nature to those of A and B. However, Table 2 summarizes the amounts of acid/base which had to be added to reach the various inflexions/breaks for all the copolypeptides.

In the first place it can be seen that the total amounts of acidic/basic groups which titrate with alkali/acid between the final inflexion/break in the titration curves are in fair agreement with the amount obtained from amino acid analysis. As to the fine features, it is interesting to note in what follows that as anticipated from the zwitterion structure the titration with alkali of A and C did show a two-stage reaction as against the one step reaction in copolypeptides B and D.

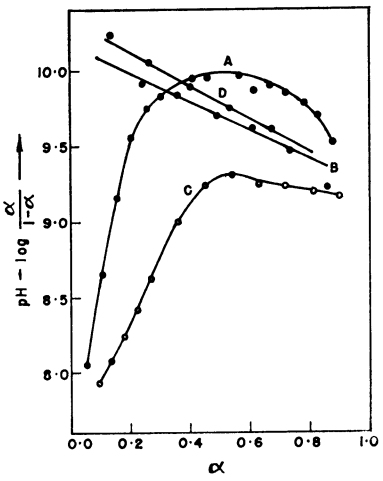

Fig. 3. Dependence of the function, pH−log{α/(1−α)} on degree of ionisation (α) of the copolypeptides in the alkaline region.

The first inflexion/break in the titration of A with KOH (Fig. 1, Curves 1, 2) corresponds to an addition of 195 milliequivalents (mequiv.)/100 g of A. The modified plot, α *versus* pH−log {α/(1−α)}, Fig. 3, shows a sharp change in curvature about the same region (α~0.35). Being an acidic polypeptide this should have two kinds of groups reacting with alkali and the first to react would be the COOH groups, not involved in zwitterion formation, followed by NH_3^+ cations. Amino acid analysis indicates (Table 1) that there would be 194 mequiv. (516—322) of COOH groups and 322 mequiv. of NH_3^+. The titration data leads to values of 195 and 322 mequiv./100 g which is in very good agreement with the expected values. In Fig. 3 can also be seen the modified plot of the acidic copolypeptide C, which is very similar to that of A and showing the expected behaviour of a two-stage neutralization phenomenon (Table 2).

Figure 1 (Curves 3, 4) gives the titration curve of copolypeptide A with HCl and the corresponding modified plots, β *versus* pH+log {β/(1−β)}, are presented in Fig. 4, which bring out the similarity between A and C more clearly. There is a two-stage neutralization against the one stage anticipated. It can be seen from Fig. 1 that it is the second inflexion/break at 322 mequiv./100 g of A that corresponds with the total anticipated COO^- anions of 330 mequiv./100 g (Table 2). The modified plots in Fig. 4 make it clear that in

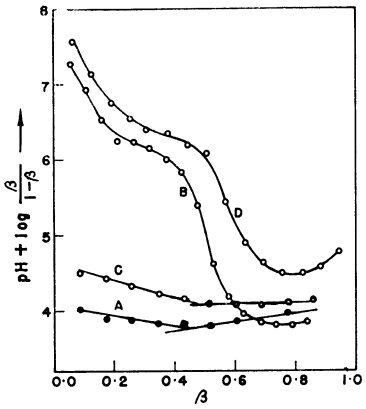

Fig. 4. Dependence of the function, pH+log {β/(1−β)} on the degree of ionisation (β) of the copolypeptides in the acidic region.

both A and C about half of the COO^- groups are stronger (the two segments in the modified plots cannot extrapolate to same pK) indicating the different energies of protonation for the carboxylate anions. This difference perhaps arises out of specific interactions such as hydrogen bond formation[19] and/or interactions between vicinally situated similar charges.[5]

Copolypeptides B and D, as suggested earlier, would be expected to have the ammonium cations as the only kind of acidic group to react with KOH. The modified plots, α *versus* pH−log {α/(1−α)} (Fig.3), show a linear behaviour and the representative titration curves of B (Fig. 2, Curves 1 and 2) show a single inflexion/break at the expected value (Table 2).

Titrations with the acid (HCl), as in the case of those with A and C, seem to deviate from the expected behaviour in having three instead of the two-stage reaction (see Table 2). Figure 3, showing the modified plots, also suggests the same and here again it appears that the protonation of NH_2 groups takes place in two stages followed by a single stage reaction of COO^- anions. This behaviour is again similar to that found in the case of polyvinylamine by Katchalsky *et al.*[5] where the vicinally situated prototropic groups were having a marked influence on their reactivity with acid.

To summarize, the amounts of COO^-, NH_2, and NH_3^+ groups found in the copolypeptides on titration are what one would expect on the basis of the assumption that zwitterion formation is complete at the isoionic point, and consistent with their chemical composition. The sequence in which the groups titrate follows the order $COO^- < NH_2 < NH_3^+$ of their pKs. The difference in the lengths of the side chains of L-aspartic acid and L-glutamic acid residues does not appear to influence the titration pattern of the copolypeptides, though this difference has been found to influence their conformation.[20]

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