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The effect of polycations on the activity of pepsin

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A study has been made of the interaction of selected polycations with pepsin A (E.C. 3.4.23.1). Protamine, polybrene, spermine, spermidine and poly(L-lysine) all acted as inhibitors of the enzyme at low concentrations, but at higher concentrations of the polycations the inhibition was less pronounced. A more detailed study of the anomalous inhibition was made using protamine, polybrene and poly(L-lysine) and it was shown experimentally that, when used by themselves, each of these polycations acted as a weak proteolytic catalyst. The order of catalytic effectiveness was: protamine > polybrene ≫ poly(L-lysine). Thus, it is now possible to explain why the observed inhibition of pepsin decreases when the concentration of the inhibiting polycation is increased.

Using haemoglobin as a substrate, Katchalski et al (1954) were the first to show that pepsin was inhibited at pH 1·7 by the polycation poly(L-lysine). It was established that the inhibition was caused by a rapid reaction between the enzyme and the inhibitor as pre-incubation did not alter the extent of the inhibition. Herriott (1962) commented that poly(L-lysine) behaved similarly to the natural pepsin inhibitor which he had previously isolated from pepsinogen. One difference between the two was that the natural inhibitor was active at pH 5, but not at pH 2 whilst the poly(L-lysine) was active at both pH values. As a result of several studies (Dellert & Stahmann 1955; Anderson 1970; Anderson et al 1980), it has been shown that with a given concentration of pepsin there is an optimum concentration of poly(L-lysine) that causes maximum inhibition. Further increases in the concentration of the polycation cause a decrease in the amount of inhibition. Anderson et al (1980) have shown that the amount of inhibition is also dependent on the molecular weight of the poly(L-lysine) and the pH of the reaction medium.

In the present study we have found that pepsin is inhibited by the polycations protamine, polybrene, spermine and spermidine. We have also shown that protamine, polybrene and poly(L-lysine) all behave as weak catalysts when used alone. The latter observation has been used to give for the first time a simple explanation of the fact that although each polycation inhibits pepsin, in all cases, activity is restored if the concentration of the polycation is increased above a certain level.

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MATERIALS AND METHODS

Materials

Pepsin (E.C. 3.4.23.1), from porcine stomach mucosa had been twice crystallized and the lyophilized powder was obtained from the Sigma Chemical Company and stored at 0–5 °C in a desiccator. Haemoglobin which had been prepared from washed, lysed and dialysed bovine erythrocytes, was obtained from Sigma (Type II) and stored at 0–5 °C in a desiccator. Sigma also supplied protamine sulphate (Grade X) which had been obtained from salmon and gave a negative Millon test. We used the material in extensive gel electrophoresis experiments and found it to be homogenous. Poly(L-lysine hydrobromide), molecular weight 2500 Daltons; and polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) were both of synthetic origin and were obtained from Sigma, as were spermine tetrachloride, spermidine trichloride and Folin and Ciocalteu's phenol reagent. All these materials were stored under appropriate conditions. The other chemicals used were AR.

Concentrations of each polycation have been expressed as 'base moles per litre' (bm litre^{-1}) where one base mole is the mass of a polycation associated with one positive charge. Details are in Table 1.

Methods

Using haemoglobin as the substrate, we determined the activity of pepsin at 25 °C and pH 2·1 with a method based on that of Anson (1938). Folin and Ciocalteu's reagent was used to increase the precision of the determination. With azocasein as the substrate (25 °C, pH 5·3–5·5) there was a more rapid reaction, but the increased sensitivity was offset by a lack of reproducibility in the results. Thus, most of the work was done with haemoglobin as the substrate

(25 °C, pH 2·1). In each case a blank experiment was done in the absence of pepsin to correct for the small amount of hydrolysis of haemoglobin at pH 2·1. Absorbance values were reproducible to within ±0·002 absorbance units.

The effect of protamine sulphate on the reaction of pepsin with haemoglobin was studied at pH 2·1 and 25 °C. All the solutions were pre-equilibrated at 25 °C for 30 min before mixing. The polycation (10^{-3} bm litre^{-1}) in 0·01 M sodium acetate–hydrochloric acid buffer (pH 2·1) was added in carefully chosen amounts to the enzyme (10^{-6} M in the same buffer, pH 2·1) followed by a measured volume of the buffer solution and well mixed by shaking. The mixture was incubated for 5 min at 25 °C and then appropriate amounts of haemoglobin solutions (1·5% in buffer pH 2·1) were added to make a final volume of 5 ml. The mixture was incubated for 25 min at 25 °C before the reaction was stopped by the addition of trichloroacetic acid (5 ml of 10%) and the mixture was cooled in an ice bath before centrifuging at 4 °C. The extent of the hydrolysis of haemoglobin was determined by the method of Anson (1938) using freshly prepared Folin and Ciocalteu's reagent (diluted 1 : 2 with distilled water) and sodium hydroxide (0·5 M). The absorbances of the solutions were measured at 420 nm. It was found that the Folin and Ciocalteu's reagent reacted to a small extent with the polycation and a blank experiment was done in each case to allow the results to be corrected for that effect.

The effects of the polycations polybrene, poly(L-lysine), spermine and spermidine at pH 2·1, were investigated using similar procedures. Appropriate blank experiments were carried out.

The effect of protamine sulphate on pepsin at pH 5·5 was studied using azocasein (25 mg ml^{-1}) as the substrate following the method of Kay & Kassel (1973).

RESULTS

Initial studies (Mekras 1982) showed that the hydrolysis of haemoglobin by pepsin was comparatively slow and that the rate was linear only over the first minute, after which it slowly declined. Due to the experimental difficulties of determining the initial velocity of the hydrolysis, we have used the absorbance values after a fixed time interval (25 min), as a direct measure of the extent of the hydrolysis of haemoglobin by pepsin.

The effect of protamine sulphate on the hydrolysis of haemoglobin by pepsin is shown in Fig. 1. All determinations were duplicated and because of the unusual nature of the results the work was repeated several times. We found that using vigorous shaking, the interaction of the polycation with pepsin was rapid and no advantage was found in allowing the enzyme and polycation to incubate beyond 5 min. Fig. 1 shows clearly that at low concentrations (b and c) protamine sulphate inhibits the action of pepsin, but at higher concentrations (d and e) the apparent activity of the system is actually increased beyond that of the enzyme alone.

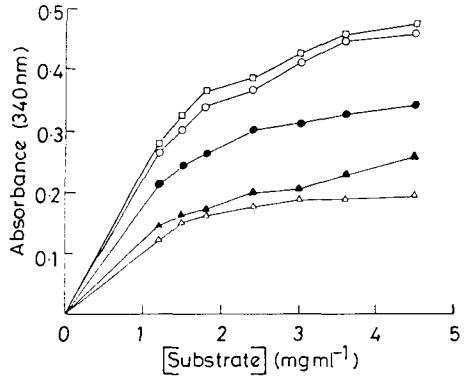

FIG. 1. The effect of protamine sulphate on the hydrolysis of haemoglobin by pepsin (10^{-7} M) in acetate buffer (0·01 M, pH 2·1) at 25 °C. Protamine sulphate concentrations (base mol litre^{-1}): (a) ●, zero; (b) △, 2×10^{-5}; (c) ▲, 4×10^{-5}; (d) ○, 10×10^{-5}; (e) □, 20×10^{-5}.

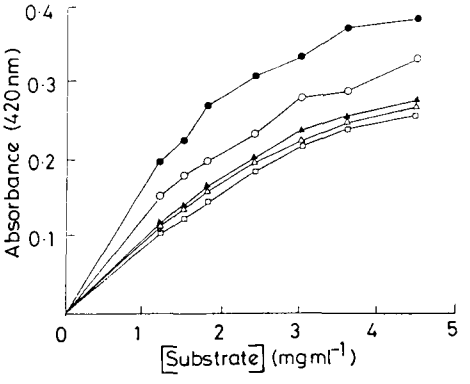

FIG. 2. The effect of polybrene on the hydrolysis of haemoglobin by pepsin (10^{-7} M) in acetate buffer (0·01 M, pH 2·1) at 25 °C. Polybrene concentrations (base mole litre^{-1}: (a) ●, zero; (b) ○, 2×10^{-5}; (c) □, 4×10^{-5}; (d) △, 10×10^{-5}; (e) ▲, 20×10^{-5}.

The effect of polybrene on the activity of pepsin is shown in Fig. 2. The same type of behaviour was observed although in this case the reversal was less pronounced.

Figs 3 and 4 show the behaviour of pepsin in the presence of spermidine and spermine respectively.

Again the same trend was found as the concentration of the polycation was increased.

Although we found the results less reproducible with azocasein, the same general trend was observed when protamine sulphate was added to the pepsin-azocasein system. The effects are shown in Fig. 5 for a single, constant concentration of azocasein. Again, low concentrations of the polycation causes inhibition, but higher concentrations cause enhanced activity. These observations agree with those reported in Fig. 1.

Poly(L-lysine hydrobromide) has been extensively studied at pH 5 (Anderson 1980). In Fig. 6 it is clearly shown that poly(L-lysine) is also an effective inhibitor of pepsin at pH 2·1 at low concentration (line b), but as the concentration is raised, the inhibition becomes less pronounced. The behaviour is in agreement with that of the other polycations.

Protamine, polybrene and poly(L-lysine) were each examined to see if they catalysed the hydrolysis of haemoglobin at pH 2·1. The results, after the subtraction of appropriate blanks, are shown in Fig. 7. The best catalyst is protamine followed closely by polybrene, while poly(L-lysine) is much less effective. In comparison with pepsin (line d), of course, the polycations are less efficient. Fig. 8 shows a possible explanation for the unusual behaviour of protamine that was reported in Fig. 1. In Fig. 8 we have compared the catalylic effect of pepsin plus protamine (2×10^{-4} bm litre^{-1}, line a) with that of pepsin (line b) and also with a theoretical line (d) compiled from the experiments using pepsin (b) and experiments using protamine alone (2×10^{-4} bm litre^{-1}, line c). This simple addition over estimates the final catalysis because some protamine will be bound to the pepsin in the pepsin protamine system (a), thus making the actual concentration less than 2×10^{-4} bm litre^{-1}. (Based on other experiments (Mekras 1982) it was estimated that one mole of pepsin binds ten base moles of protamine.)

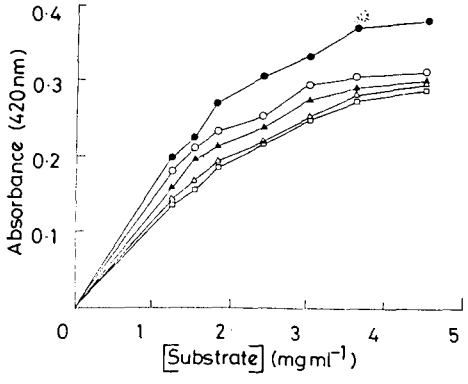

FIG. 3. The effect of spermidine on the hydrolysis of haemoglobin in pepsin (10^{-7} M) in acetate buffer (0·01 M, pH 2·1) at 25 °C. Spermidine concentration (base mol litre^{-1}): (a) ●, zero; (b) ○, 2×10^{-5}; (c) ▲, 4×10^{-5}; (d) □, 10×10^{-5}; (e) △, 20×10^{-5}.

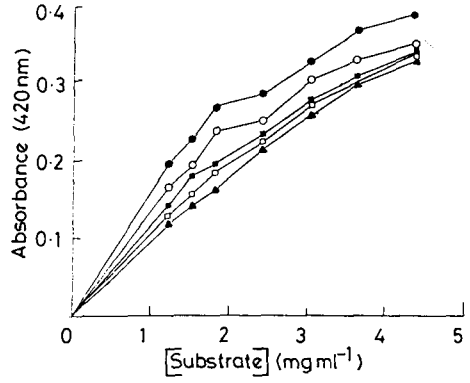

FIG. 4. the effect of spermine on the hydrolysis of haemoglobin by pepsin (10^{-7} M) in acetate buffer (0·01 M, pH 2·1) at 25 °C. Spermine concentrations (base mol litre^{-1}): (a) ●, zero; (b) ○, 2×10^{-5}; (c) ■, 4×10^{-5}; (d) ▲, 10×10^{-5}; (e) □, 20×10^{-5}.

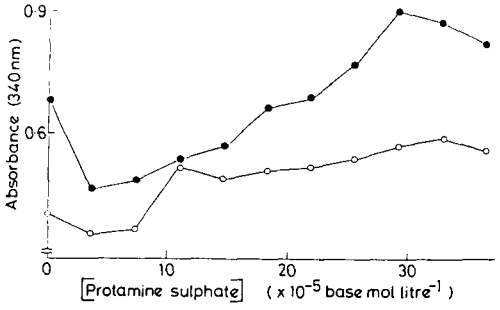

FIG. 5. The effect of protamine sulphate on the hydrolysis of azocasein by pepsin (10^{-7} M) in acetate buffer (0·01 M) at pH ranges: (a) ○, 5·0–5·4; (b) ●, 5·4–5·8.

DISCUSSION

It has been shown that the polycations protamine, polybrene, poly(L-lysine) spermine and spermidine are all inhibitors of pepsin at low pH values. In each case the inhibition decreases when the concentration of the polycation is increased above a certain value. Previous workers (Dellert & Stahmann 1955) have observed that type of behaviour for poly(L-lysine) with pepsin. They suggested that it might be due to the insoluble complexes of pepsin and poly(L-lysine) being solubilized by excess of the latter. In our work we have shown (Fig. 7) that the polycations protam-

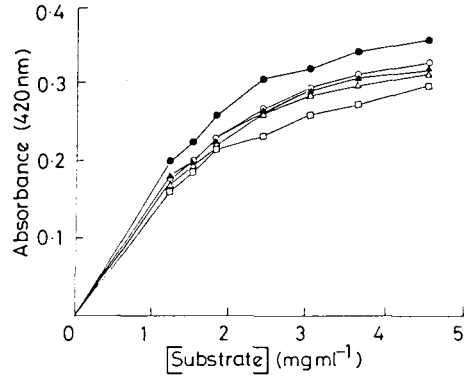

FIG. 6. The effect of poly(L-lysine) on the hydrolysis of haemoglobin by pepsin (10^{-7} M) in acetate buffer (0·1 M, pH 2·1) at 25 °C. Poly(L-lysine) concentrations (base mol litre^{-1}): (a) ●, zero; (b) □, 2×10^{-5}; (c) △, 4×10^{-5}; (d) ○, 10×10^{-5}; (e) ▲, 20×10^{-5}.

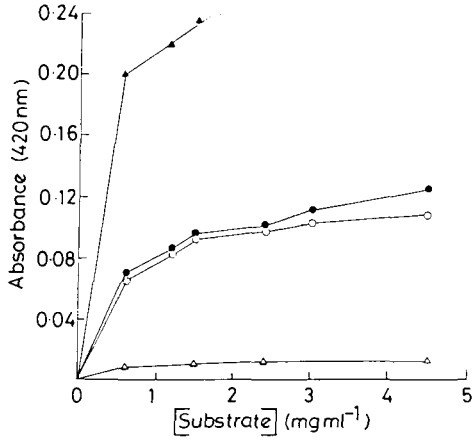

FIG. 7. A study of the catalytic properties of protamine, polybrene, and poly(L-lysine) relative to pepsin using haemoglobin as the substrate in acetate buffer (0·01 M, pH 2·1) at 25 °C. Concentrations: (a) ●, protamine; 20×10^{-5} base mole litre^{-1}; (b) ○, polybrene; 20×10^{-5}; (c) △, poly(L-lysine): 20×10^{-5} base mol litre^{-1}; (d) ▲, pepsin 10^{-7} mol litre^{-1}.

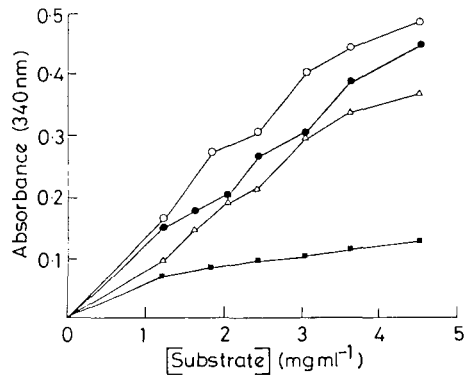

FIG. 8. The behaviour of protamine sulphate as a catalyst for the hydrolysis of haemoglobin compared with that of pepsin at pH 2·1 and 25 °C. The effect of protamine sulphate in the presence of pepsin is also shown, and compared with the separate experiments. (a) ●, pepsin (10^{-7} mol litre^{-1}) plus protamine (2×10^{-4} base mol litre^{-1}); (b) △, pepsin (10^{-7} mol litre^{-1}); (c) ■, protamine (2×10^{-4} base mol litre^{-1}); (d) ○, data calculated from separate experiments with pepsin plus protamine, and protamine alone to show the effect of excess protamine in the presence of pepsin.

Table 1. Percentage inhibition of polycations with pepsin using haemoglobin (1·5 mg ml^{-1}) as the substrate at 25 °C and pH 2·1.

Type of polycation	Concn polycation at max. inhib. (bm litre^{-1})*	Base mol. wt polycation	Inhib. of pepsin by polycation (%)
Protamine sulphate	4×10^{-5}	220	48
Polybrene	4×10^{-5}	187	38
Poly(L-lysine)	4×10^{-5}	197	27
Spermidine	10×10^{-5}	85	32
Spermine	10×10^{-5}	87	28

* base mol litre^{-1}.

ine, polybrene and poly(L-lysine) are weak proteolytic catalysts at pH 2·1 in their own right. We did not observe any formation of an insoluble complex in any of our experiments, possibly because we used a final concentration of pepsin of 10^{-7} mol litre^{-1} as opposed to $7{\cdot}14 \times 10^{-7}$ mol litre^{-1} used by Dellert & Stahmann. Anderson et al (1980) did not report any decrease of the inhibition of pepsin by poly(L-lysine) at pH 5, as the concentration of the polycation was increased, but they used a comparatively high concentration of pepsin ($4{\cdot}8 \times 10^{-7}$ mol litre^{-1}). Our poly(L-lysine) had a degree of polymerisation of about 20 and if one compares the pepsin to polycation ratio, for the nearest poly(L-lysine) used by Anderson et al (1980), lys_{23}, with the one used here, the maximum concentration used by the earlier workers corresponds to approximately 2×10^{-5} bm litre^{-1} in our experiments (Fig. 6). We observed inhibition with concentrations of 2×10^{-5} and 4×10^{-4} bm litre^{-1} which agrees with their results (although they worked at pH 5 and we at pH 2·1).

The relative effectiveness of the polycations as inhibitors of pepsin is shown in Table 1 for one substrate concentration. The order is: protamine > polybrene > poly(L-lysine); and each shows maximum inhibition at a concentration of about 4×10^{-5} bm litre^{-1}. Spermidine and spermine require a higher concentration to produce a maximum inhibition and are less effective as inhibitors.

The data in Fig. 7 shows that protamine, polybrene and poly(L-lysine) are all capable of catalysing the hydrolysis of haemoglobin at pH 2·1. The order

of effectiveness is: protamine > polybrene ≫ poly(L-lysine). Thus, the best catalyst, protamine is the polycation which is also the most effective inhibitor of pepsin when used at low concentrations (Table 1). We therefore consider that it is now possible to give a clear explanation of the phenomena reported in Figs. 1–6. Namely that the pepsin is inhibited by low concentrations of the polycation, but as the concentration of the polycation is increased the observed inhibition becomes less pronounced. In one case (Fig. 1) the pepsin-polycation mixture actually becomes a more effective catalyst than the enzyme. A possible explanation is that initially pepsin forms a complex with each of the polycations which is less active than the enzyme, but when excess polycation is added the catalytic effectiveness is increased because the excess polycation is a catalyst in its own right.

It was noted by Katchalski et al (1954) that pepsin did not hydrolyse poly(L-lysine). We have examined the effect of prolonged incubation on complexes of protamine and pepsin and have established that protamine is not hydrolysed by pepsin.

It is of interest to ask why pepsin should be inhibited by polycations at pH 2·1, since one would expect the interaction to be electrostatic, necessitating the presence of a large number of negative charges on the enzyme. Anderson et al (1980) made the interesting suggestion that the polycation interacted with the carboxyl groups in the active site of pepsin and that maybe the only 'positive-negative' interaction of importance at pH 2·1 where most carboxyl groups would be un-ionized. Pepsin has a total of 36 carboxyl groups and one phosphate group (Herriott 1962). Calculations show that 1·1% of the glutamic acid residues and 1·6% of the aspartic acid residues would be ionized at pH 2·1 assuming pK_a values of 4·07 and 3·90 respectively (Dawson et al 1969). The phosphate group would be expected to be about 47% ionized assuming a pK_1 value of 2·15. However, since pepsin has an isoelectric point below pH 1 (Herriot 1962), even at pH 2·1 a substantial number of negative charges may nevertheless remain on the enzyme which would imply that the actual pK_a values are lower than those quoted. This may account for the interactions that have been observed, but it may be that hydrophobic forces are also important. In that context, it has been reported previously by Schlamowitz et al (1968), that some low molecular weight inhibitors of pepsin are bound to the enzyme principally by hydrophobic forces.

If polycations were to be used to form complexes with pepsin as a method of treating acid peptic disease (Anderson et al 1980), some control of the polycation concentration would appear to be needed. If excess polycation is present at pH values around 2, proteolysis will take place due to the catalytic properties of the polycation.

To summarize, therefore, we have shown that the polycations: protamine, polybrene, poly(L-lysine), spermine and spermidine all inhibit pepsin at low polycation : pepsin ratios. As the concentration of each polycation is increased, the inhibition is reversed and the apparent activity may increase to a level similar to that of the original enzyme. For protamine, polybrene and poly(L-lysine) we have shown that this reversal of behaviour can be explained by observing that each of these polycations is a proteolytic catalyst in its own right. Thus, once a complex has been formed with pepsin, causing inhibition of the enzyme, any excess polycation catalyses the reaction and that is the reason why the initial inhibition is reversed if the concentration of the polycation is increased.

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