Interaction of Pepstatin with Pig Pepsinogen

By JOHN KAY and COLIN W. DYKES

Department of Biochemistry, University College, Cardiff CF1 1XL, Wales, U.K.

(Received 4 May 1976)

In contrast with pepsin, pepsinogen does not bind pepstatin at pH values between 5.3 and 2.5. Pepsinogen is not retarded by pepstatin immobilized on to aminohexyl-Sepharose at pH 5.3 or 4.1, whereas at pH 3.0 activation takes place during the chromatography, with retardation of the resultant pepsin.

We have shown (Dykes & Kay, 1976) that, at low pH values, pepsinogen converts itself into pepsin by a sequential release of peptides from the *N*-terminus of the zymogen, and not in a one-step transformation. This was achieved by using pepstatin (Aoyagi *et al.*, 1971) to trap the first intermediate formed in the activation process. It was important for the success of this scheme that pepstatin should not react with pepsinogen. However, since it has been shown (Al-Janabi *et al.*, 1972; McPhie, 1972; Sanny *et al.*, 1975) that pepsinogen activates itself below pH 3 by an intramolecular mechanism, the same active site (involving at least two, well-characterized, β -carboxyl groups of aspartic acid residues 32 and 215) must be responsible in the zymogen for its own activation as is operative in the resultant pepsin. It was therefore decided to see whether pepstatin would interact with pepsinogen, especially since there is much evidence to suggest that activation is preceded and accompanied by conformational changes (Funatsu *et al.*, 1971; Wang & Edelman, 1971; MaPhie, 1972).

Materials and Methods

Pig pepsinogen was purified to homogeneity from pig stomachs (Ryle, 1970) with a final step of chromatography on polylysine-Sepharose (Kay, 1972). Pepsin was prepared by activating pepsinogen (Rajagopalan *et al.*, 1966) and pepstatin was a generous gift from Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. 1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide was from Sigma (London) Ltd., Kingston-upon-Thames, Surrey, U.K. Rabbit muscle lactate dehydrogenase (EC 1.1.1.27) and deoxyribonuclease (EC 3.1.4.5) were generous gifts from Miles Research Laboratories, Stoke Poges, Slough, Berks., U.K. Aminohexyl-Sepharose was purchased from Pharmacia Fine Chemicals Ltd., Uppsala, Sweden.

Pepsinogen, deoxyribonuclease and lactate dehydrogenase concentrations were determined by using $E_{280}^{1\%}$ = 1.47, 1.15 and 1.49 respectively (Miles

Laboratories, 1972). Pepstatin was dissolved in methanol and its concentration was determined by (1) amino acid analysis for alanine and valine on a Locarte analyser after hydrolysis for 24 h *in vacuo* with 6M-HCl at 110°C and (2) titration against a known amount of pepsin (Aoyagi *et al.*, 1971; Kunimoto *et al.*, 1974).

Pepstatin was coupled through its carboxyl group to aminohexyl-Sepharose by using a water-soluble carbodi-imide. Pepstatin (12 mg in 0.5 ml of dimethyl-formamide) was added to 2.4 ml (0.6 g) of aminohexyl-Sepharose suspended in 2 ml of water at pH 6.0. Carbodi-imide (200 mg in 0.2 ml of water) was added slowly, the pH being kept at 6.0 by the addition of 1 M-HCl or 1 M-NaOH. Stirring was continued at room temperature (about 20°C) overnight, after which the resin was washed successively with 50% (v/v) methanol, 0.1 M-NaHCO₃/1 M-NaCl, 1 mM-HCl/1 M-NaCl, water, and 0.1 M-sodium acetate, pH 5.3.

It was found by (a) amino acid analysis and (b) titration against standard pepsin that the amount of pepstatin immobilized was 3.2 and 3.0 μ mol/g of dry resin respectively.

Proteolytic-activity measurements were made against haemoglobin at pH 2 (Anson, 1948), and Azocoll at pH 5.3 (Kay, 1975).

Experiments to test for binding of pepstatin to pepsinogen were performed by using the 'competitive-assay' method of Feinstein & Feeney (1966). Pepsinogen (0.25 mg = 6.25 nmol) was preincubated with pepstatin (7 μ g = 10 nmol) for 5 min at 0°C at pH 5.3 or pH 3.0, or for 2 min at pH 2.5 (in 0.0032 M-HCl). The pH was then adjusted to 5.3 by addition of a predetermined volume of 0.2 M-sodium acetate buffer, pH 5.3. At this time, exogenous pepsin (0.45 mg = 12.5 nmol) was added in 0.05 M-acetate buffer, pH 5.3, and an additional 1 min was allowed for it to combine with the pepstatin remaining. The residual pepsin activity was then measured against Azocoll at pH 5.3 (Kay, 1975).

Chromatography of pepsinogen or pepsin on insolubilized pepstatin was carried out by applying

the zymogen (or enzyme) in 0.1M-sodium acetate buffer, pH7.3, containing 0.35M-NaCl, or 0.1M-sodium formate buffer, pH4.1, containing 0.15M-NaCl. It was necessary to include NaCl, to prevent non-specific adsorption. Control experiments were done by using lactate dehydrogenase at pH5.3 and deoxyribonuclease at pH4.1 (where lactate dehydrogenase is precipitated) to measure recoveries and to assess non-specific interaction with pepstatin-aminohexyl-Sepharose.

Chromatography of pepsinogen or lactate dehydrogenase at pH5.3 in the absence of NaCl on aminohexyl-Sepharose (i.e. without pepstatin) showed that both proteins were retarded non-specifically until 0.35M-NaCl was included in the elution buffer.

Non-biospecific interaction of lactate dehydrogenase with the aminohexyl spacer arm has been reported previously (O'Carra *et al.*, 1974). Inclusion of at least 0.3M-NaCl in their buffer was required to prevent this effect.

Results

The effect of pepsinogen on the ability of pepstatin to inhibit pepsin is shown in Table 1. At three pH values, none of the pepstatin was removed by complexing with pepsinogen, at least on exposure for up to 5 min, so that it was all available to inhibit the pepsin added. An excess of pepsin over pepstatin was used deliberately so that there was always a positive value of pepsin activity to be measured. Control incubations showed that there was no activation of pepsinogen under the conditions used, nor was there any activation of pepsinogen by pepsin at pH5.3.

The results obtained from chromatography of pepsinogen on immobilized pepstatin at several pH values are given in Table 2. At pH5.3 and 4.1, all of the applied pepsinogen was eluted immediately from the affinity column. Recoveries were comparable with those of lactate dehydrogenase and deoxyribonuclease

respectively, neither of which should be retarded by the pepstatin-containing column. At pH3.0, however, a significantly lower yield of protein was obtained and the 'missing' material was eluted from the column by 0.1M-NaHCO₃/1M-NaCl, pH9. It seemed likely that, since about 10 min was required for 1 column-volume to pass, some activation of pepsinogen to pepsin might have taken place under the acidic conditions.

To investigate this possibility further, a larger amount of pepsinogen [4mg in 0.4ml of 0.1M-formate (pH3.0)/1M-NaCl] was applied to the pepstatin-containing column (0.5cm×2.2cm). The column was developed with the same buffer, and fractions (1 ml) were collected into tubes containing 1 ml of 0.1M-sodium phosphate buffer, pH6.5, and 50 μ l of 1M-NaOH (sufficient to adjust the final pH to 6.0). The recovery of material (fraction A) which was not retarded by the column was 58%. The pepstatin column was then washed with 0.1M-NaHCO₃ (pH9.0)/1M-NaCl, which eluted a second fraction (fraction B). The recovery of this material was 23% of the pepsinogen applied originally.

Fraction A (total vol. 8ml) was placed in a 10ml Amicon concentration cell fitted with a UM-05 membrane (mol.wt. cut-off 500), and the salt was removed by several cycles of concentration followed by dilution with 0.05M-sodium phosphate buffer, pH6.5. Finally the desalted material (2ml) was applied to a column (0.5cm×6.0cm) of polylysine-Sepharose 4B and elution was continued with 0.05M-sodium phosphate buffer, pH6.5. Peptide material (which is not retarded by this column; Harboe *et al.*, 1974) was collected in tubes 2-4 (fraction A1), and the protein fraction was eluted by a linear 0-1M-NaCl gradient.

Samples of fractions A1 and B were hydrolysed with 6M-HCl and the amino acid compositions were determined (see Table 3). The recovery of peptide material (based on aspartic acid content) in fraction

Table 1. *Interaction of pepsinogen with pepstatin*

The activity of pepsin (0.45 mg in 0.1 ml) was measured against Azocoll in 0.05M-sodium acetate buffer, pH5.3 (final volume 3 ml), in the absence and presence of pepstatin (7 μ g). Pepsinogen (0.25 mg) was preincubated alone or with pepstatin (7 μ g) for 5 min at 0°C in a volume of 0.25 ml of 0.05M-acetate buffer, pH5.3, or 1M-HCl, or for 2 min at 0°C in 0.0032M-HCl before adjustment of the pH to 5.3 with a predetermined volume of 0.2M-acetate buffer, pH5.3. Pepsin (0.45 mg/0.1 ml) was added, and the mixtures were left for 1 min at 20°C. Pepsin activity remaining was measured against Azocoll in 0.05M-acetate buffer, pH5.3 (final volume 3 ml). Activities are expressed relative to that found for pepsin alone.

Proteins present	Activity found (%)		
	Preincubation at pH5.3	Preincubation at pH3.0	Preincubation at pH2.5
Pepsin alone	100	100	100
Pepsin + pepstatin	31	32	34
Pepsinogen + pepstatin + pepsin	30	33	34
Pepsinogen + pepsin	100	100	100

Table 2. *Chromatography of pepsinogen on pepstatin-aminohexyl-Sepharose*

Samples (1–2 mg in 0.1 ml) were applied to a column of pepstatin-aminohexyl-Sepharose (0.5 cm × 2.2 cm) at 4°C in the appropriate buffer. Elution was continued with the same buffer for 3 bed-volumes, when the eluent was changed to 0.1 M-NaHCO₃/1 M-NaCl, pH 9. Recoveries are calculated from protein absorbance at 280 nm.

Protein	pH at which applied	Protein recovered (%) on elution at:		No. of runs
		initial pH	pH 9	
Pepsinogen	pH 5.3 (0.1 M-acetate/0.35 M-NaCl)	83	0	4
Pepsin	pH 5.3 (0.1 M-acetate/0.35 M-NaCl)	0	82	4
Lactate dehydrogenase	pH 5.3 (0.1 M-acetate/0.35 M-NaCl)	83	0	3
Pepsinogen	pH 4.1 (0.1 M-formate/0.15 M-NaCl)	87	0	4
Pepsin	pH 4.1 (0.1 M-formate/0.15 M-NaCl)	0	84	4
Deoxyribonuclease	pH 4.1 (0.1 M-formate/0.15 M-NaCl)	85	0	3
Pepsinogen	pH 3.0 (0.1 M-formate/1 M-NaCl)	56	28	4

Table 3. *Amino acid compositions of fractions obtained by chromatography of pepsinogen on pepstatin-aminohexyl-Sepharose at pH 3.0*

Samples were hydrolysed for 20 h *in vacuo* in 6 M-HCl at 105°C. Values are not corrected for losses on hydrolysis. N.D., Not determined.

Amino acid	Amino acid composition (mol of residue/mol)			
	Fraction A1		Fraction B	
	Found	Expected from activation segment (residues 1–44)	Found	Expected from pepsin
Lys	4.60	9	1.21	1
His	1.34	2	1.20	1
Arg	1.14	2	1.94	2
Asp	4.00	4	42.52	42
Thr	1.28	1	24.75	26
Ser	2.16	2	40.36	43
Glu	2.52	3	26.45	26
Pro	2.26	3	N.D.	16
Gly	2.20	1	37.87	35
Ala	2.88	4	16.35	16
Val	2.20	3	17.82	22
Met	0.00	0	1.97	4
Ile	1.32	1	20.11	26
Leu	4.85	7	27.29	26
Tyr	0.95	1	16.36	16
Phe	1.36	2	14.00	14

A1 was 47% of what would have been recovered if all of the pepsinogen applied to the pepstatin column had activated itself. *N*-Terminal analysis of fraction A1 by using dansyl chloride showed several free *N*-termini.

Discussion

It would seem from the results of the two approaches used in this study that pepsinogen has very little affinity for pepstatin, at least when a very low molar

excess of pepstatin to pepsinogen is used, as in the 'competitive-assay' method. None of the pepstatin appeared to have bound to pepsinogen, even at low pH values, since it was all readily available to inhibit the excess of exogenous pepsin added subsequently. Incubation for 1 min was allowed for formation of the pepsin-pepstatin complex, since it has been reported (Kunimoto *et al.*, 1974) that this process is time-dependent. However, in our experiments, conducted in the absence of any substrate, we did not observe

this effect; the inhibition of pepsin was essentially instantaneous, as has been found for the interaction of pepstatin with cathepsin D (Knight & Barrett, 1976).

Pepsin was retarded by insolubilized pepstatin at pH 5.3 and 4.1, whereas pepsinogen was not retained. Pepstatin immobilized through its carboxyl group should retain most of its inhibitory capacity towards pepsin, as it has been shown that various esters of pepstatin have similar K_i values to that of native pepstatin (Aoyagi *et al.*, 1971; Knight & Barrett, 1976). The necessity to include NaCl to prevent non-biospecific effects has been observed by other workers (O'Carra *et al.*, 1974), and it seems that a complex relationship between ionic strength and pH might exist. At pH 5.3 it was necessary to include salt to a concentration of 0.35M to prevent lactate dehydrogenase and pepsinogen being retarded, whereas at pH 4.1, 0.15M-NaCl was sufficient.

The results at pH 3.0 were somewhat different. Since the pepsinogen was exposed to pH 3 for 10–15 min during the chromatography, some of the zymogen may have converted itself into pepsin. If such an activation had occurred, and since 58% of the protein applied was not retained by the column, the implication is that 40% of the zymogen activated itself to give pepsin plus the peptides from the activation segment. Pepsin should have been retarded by the pepstatin resin, and indeed fraction B has a composition very similar to that of pepsin. The activation-segment peptides, on the other hand, would not have been expected to be retained by the pepstatin column and should have been eluted with the un-activated pepsinogen. By concentration and subsequent chromatography on polylysine-Sepharose, which binds pepsinogen and pepsin but not activation-segment peptides, the fraction A1, which was not retarded, had a composition (and recovery) fairly similar to that expected from the activation-segment sequence. The lower yields of some amino acids may be due to their having been released as small peptides which would have passed through the ultrafiltration membrane and so were lost from the sample applied to the polylysine column. Leucine and lysine are the most predominant amino acids in the activation-segment sequence (Harboe *et al.*, 1974) and several Leu-Lys and Lys-Leu sequences occur, so that this loss would be most apparent in these residues.

Thus it appears that, even at pH 3, pepsinogen has little affinity for pepstatin. Only when it has activated to pepsin can the interaction take place. The implication from this is that, at pH values between 5.3 and 3.0, the active site inherent in pepsinogen is not 'exposed'. Possibly, therefore, the removal of activation-segment peptides is necessary before this active-site region becomes accessible to external reagents, which suggests that the proposed conformational change (Funatsu *et al.*, 1971; McPhie, 1972) may not precede peptide-bond cleavage but might occur after the activation step.

This work was supported by an M.R.C. grant (no. G974/126/B).

References

- Al-Janabi, J., Hartsuck, J. A. & Tang, J. (1972) *J. Biol. Chem.* **247**, 4628–4632
- Anson, M. L. (1948) in *Crystalline Enzymes* (Northrop, J. H., Kunitz, M. & Herriott, R. M., eds.), p. 305, Columbia University Press, New York
- Aoyagi, T., Kunimoto, S., Morishima, H., Takeuchi, T., & Umezawa, H. (1971) *J. Antibiot.* **24**, 687–694
- Dykes, C. W. & Kay, J. (1976) *Biochem. J.* **153**, 141–144
- Feinstein, G. & Feeney, R. E. (1966) *J. Biol. Chem.* **241**, 5183–5189
- Funatsu, M., Harada, Y., Hayashi, K. & Jirgensons, B. (1971) *Agric. Biol. Chem.* **35**, 566–572
- Harboe, M., Andersen, P. M., Foltmann, B., Kay, J. & Kassell, B. (1974) *J. Biol. Chem.* **249**, 4487–4494
- Kay, J. (1972) *FEBS Meet. 8th Abstr.* no. 458
- Kay, J. (1975) *Anal. Biochem.* **67**, 585–589
- Knight, C. G. & Barrett, A. J. (1976) *Biochem. J.* **155**, 117–125
- Kunimoto, S., Aoyagi, T., Nishizawa, R., Komai, T., Takeuchi, T. & Umezawa, H. (1974) *J. Antibiot.* **27**, 413–418
- McPhie, P. (1972) *J. Biol. Chem.* **247**, 4277–4281
- Miles Laboratories (1972) *Enzyme Catalogue*, no. 8
- O'Carra, P., Barry, S. & Corcoran, E. (1974) *FEBS Lett.* **43**, 163–168
- Rajagopalan, T. G., Moore, S. & Stein, W. H. (1966) *J. Biol. Chem.* **241**, 4940–4950
- Ryle, A. P. (1970) *Methods Enzymol.* **19**, 316–336
- Sanny, C. G., Hartsuck, J. A. & Tang, J. (1975) *J. Biol. Chem.* **250**, 2635–2639
- Wang, J. L. & Edelman, G. M. (1971) *J. Biol. Chem.* **246**, 1185–1191