Comparison between the monomeric and binary-complex forms of procarboxypeptidase A from whole pig pancreas*

M. Carmen MARTÍNEZ, Francesco X. AVILÉS, Blanca SANSEGUNDO and Claudi M. CUCHILLO
Departament de Bioquímica, Facultat de Ciències and Institut de Biologia Fonamental, Universitat Autònoma de Barcelona, Bellaterra, Spain

(Received 12 December 1980/Accepted 26 February 1981)

Two different forms of procarboxypeptidase A (I and II) were obtained from pig pancreas extracts. The M_r values, the pattern found on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, and the sedimentation coefficients indicate that form I is a binary complex formed by two different subunits, whereas form II is a monomer. The carboxypeptidase A-precursor subunit of form I and the form II monomer are very similar with respect to M_r value, amino acid composition and fragmentation by CNBr and iodosobenzoic acid. The activation process of both forms is unspecific with respect to the activating enzyme, the peptide released during activation is unusually long (M_r approx. 12500) and, in the case of the binary complex, the activation with trypsin follows a rather complex pattern, suggesting that the accompanying subunit of form I might play a modulating role in the activation process. Although the appearance of enzymic activity is rather slow, a protein with an M_r equivalent to that of active carboxypeptidase A is found very early in the activation process. Both zymogens are glycoproteins (so far no carbohydrate has been reported in any procarboxypeptidase A) and both contain two strongly bound Zn^{2+} ions/molecule. Other chemical and physical properties were also determined.

The procarboxypeptidase-A system is, in some species, much more complicated than that of other pancreatic zymogens such as trypsinogen and chymotrypsinogen. The main differences are in the number of protein subunits, the specificity of the activating enzyme and the length of the peptide(s) released during activation. For the bovine proenzyme, the complexity of the system was shown by Brown *et al.* (1963), who demonstrated that there are only two forms of the zymogen, one of them being a binary complex and the other a ternary complex. Kobayashi *et al.* (1978) showed the existence of a binary complex and a monomeric procarboxypeptidase A in pig pancreatic secretion. The binary-complex form consists of the carboxypeptidase A-precursor subunit plus a zymogen (proprotease E) which gives rise, on activation, to an elastase-like activity.

The relationship between the oligomeric structure

Abbreviation used: SDS, sodium dodecyl sulphate.

* This paper is dedicated to the memory of Professor E. Concustell (1935–1980), Head of the Departament de Bioquímica, Facultat de Medicina, Universitat Autònoma de Barcelona, in whose laboratory part of this work was carried out.

and either the regulation of the proenzyme intrinsic activity or the activation process is not yet known. Although the binary- and ternary-complex forms of bovine procarboxypeptidase A have been extensively studied (Yamasaki *et al.*, 1963; Uren & Neurath, 1972), the lack of the corresponding monomeric form in the native state has made it difficult to solve such problems, because it is impossible to compare the properties of the different forms (monomeric and oligomeric) from the same source. However, such a comparative study could be carried out with the monomeric and binary-complex forms of pig pancreatic procarboxypeptidase A, once the large-scale preparation is accomplished and the structural and functional characterization has been carried out.

In the present work, the existence of two forms of procarboxypeptidase A in whole pig pancreas, a more easily available source than pancreatic secretion, is reported, and a method for their isolation in rather large quantities is described. A partial characterization of both forms was achieved. The results indicate that both proenzymes show great similarities to their homologous bovine proteins with respect to relative molecular mass, sedimentation coefficient, content of some amino acids, position of

certain amino acid residues within the polypeptide sequence and activation process. However, some important differences, with regard to the number of strongly bound Zn^{2+} ions, carbohydrate content (not previously detected in any procarboxypeptidase A) and in the process of activation to the active enzyme are also apparent. For these reasons all comparisons between the procarboxypeptidase A of both species are subject to some limitations.

Materials and methods

Assay of enzymic activities

The carboxypeptidase-A (EC 3.4.17.1) activity was measured spectrophotometrically at 254 nm by the method of Folk & Schirmer (1963) with hippuryl-L-phenylalanine (Merck, Darmstadt, Germany) as substrate. The procarboxypeptidase A content was determined as the increase in carboxypeptidase-A activity after incubation with pig trypsin (Cambrian Chemicals, Croydon, Surrey, U.K.). For routine analysis 0.5 ml of sample was incubated with 10 μl of trypsin solution (final concentration in the reaction mixture 0.6 mg/ml) at 30°C for 40 min.

Chymotryptic activity was measured spectrophotometrically with 10 mM-*N*-acetyltyrosine ethyl ester (Chemapol, Prague, Czechoslovakia) as substrate (Schwert & Takenaka, 1955). The chymotrypsinogen content was determined as the increase in chymotrypsin activity after incubation with trypsin under the same conditions as for procarboxypeptidase A.

Protein concentration

This was determined by a modification of the Lowry method (Hartree, 1972), with bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) as standard.

N-Terminal amino acid determination

The protein (0.25 mg) was first oxidized with performic acid as described by Hirs (1967), and the *N*-terminal amino acid was dansylated (5-dimethyl-aminonaphthalene-1-sulphonylated) by the method of Gray (1972). After peptide hydrolysis with 6 M-HCl at 110°C for 22 h, the dansyl derivatives were identified by two-dimensional t.l.c. on polyamide sheets (10 cm $\times$ 10 cm) (Cheng Chin Trading Co., Taiwan) by the methods of Hartley (1970) and Wesenberg & Thibert (1977). The method of Gray (1967) was used to prepare the dansyl-amino acid standards.

Analysis of sugars

Hexoses were determined directly by the orcinol method (Glazer *et al.*, 1975), with a mixture of galactose and mannose (Merck) as standards;

2.5 mg of protein was used. Hexosamines were determined in an amino acid analyser with 0.2 M-sodium citrate buffer, pH 5.28; a mixture of 10 mM each of glucosamine, galactosamine, tryptophan and lysine (Merck) was used as standards. The amino sugars were previously released from the protein (11 mg) by hydrolysis in 4 M-HCl at 100°C for 6 h.

Sialic acid was determined colorimetrically by the method of Aminoff (1961), with *N*-acetylneuraminic acid (Boehringer, Mannheim, Germany) used as standard to obtain the calibration curve; 10 mg of protein was used.

Determination of free thiol groups

This was done with 5,5'-dithiobis-(2-nitrobenzoic acid), by the method of Habeeb (1972); 1.5 mg of protein was used.

Determination of zinc

Zinc was determined by atomic absorption spectrometry at 213.9 nm. Buffers were prepared with double-distilled water and were treated with a solution of 0.03% dithizone (Merck) in CCl_4 (Fuwa *et al.*, 1964). Samples (approx. 1.5 mg of protein) were dialysed overnight against 5 mM-Tris/HCl buffer, pH 7.5, which had been treated as described above, and were digested with concentrated ($d = 1.186$) HNO_3 (Carlo Erba, Milano, Italy). The calibration curve was obtained with zinc metal (Merck), by using HCl ($d = 1.19$) (Merck) to dissolve it. Blanks were obtained in the same way. The glassware had been kept immersed in concentrated HNO_3 during the previous night.

Amino acid analysis

Amino acid analyses were carried out in a Carlo Erba autoanalyser by the method of Spackman *et al.* (1958); 0.5 mg of protein was used. The tryptophan content was determined colorimetrically by the method of Spies & Chambers (1948); 1.2 mg of protein was used.

Other methods

Disc electrophoresis on polyacrylamide gels was carried out by the method of Davis (1964). Slab-gel (1 mm thick) electrophoresis on polyacrylamide in the presence of SDS was carried out by the method of Laemmli (1970).

Relative molecular masses were estimated by gel-filtration chromatography carried out, at 4°C, on a Bio-Gel P-150 (superfine grade; Bio-Rad, Richmond, CA, U.S.A.) column (1.5 cm $\times$ 90 cm) equilibrated with 12.5 mM-Tris/acetate, pH 6.6, containing 0.25 M-KCl. Sephadex G-150 (superfine grade; Pharmacia, Uppsala, Sweden) and Ultrogel (LKB, Bromma, Sweden) were also used under the same experimental conditions.

Sedimentation analyses were performed in a Beckman model E or an MSE Centriscan 75 ultracentrifuge. Samples were dissolved in 10 mM-sodium phosphate buffer, pH 6.5, containing 0.25 M-NaCl, and the observed sedimentation coefficients at infinite dilution were corrected to 20°C and water.

The absorption coefficient was determined gravimetrically. Solutions of approx. 5 mg of protein/ml were prepared in 20 mM-potassium phosphate buffer, pH 8.0, and were extensively dialysed against the same buffer. The absorbance at $\lambda_{\max}$ was measured and the samples were dried in an oven at 90°C for 3 days. After that they were desiccated over P_2O_5 under vacuum until constant weight. Blanks of the buffer were treated in exactly the same way and were subtracted from the experimental results. Analyses were carried out in triplicate.

Fragmentations with CNBr and with iodosobenzoic acid were carried out by the methods of Gross & Witkop (1962) and of Mahoney & Hermondsen (1979) respectively. In both cases the cleavage products were dialysed against 10% (v/v) acetic acid in acetylated bags (Visking; Scientific Instrument Centre, London W.C.1, U.K.), freeze-dried and analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970).

Results and discussion

Purification of the zymogens

The procedure used in the purification of pig pancreatic procarboxypeptidase A is based on that of Folk & Schirmer (1963). However, in order to obtain a purer product and at the same time to simplify the procedure, some modifications were introduced: (i) in the early stages of purification only one $(NH_4)_2SO_4$ precipitation, at 35% saturation,

was carried out; (ii) soya-bean trypsin inhibitor was added at a greater concentration (approx. 0.5 mg/ml); (iii) the third chromatography on DEAE-cellulose (pH 8.0) was not carried out; instead, the fractions containing the procarboxypeptidase A activity were pooled and precipitated with solid $(NH_4)_2SO_4$ (50% saturation) and were chromatographed on a Bio-Gel P-150 (superfine grade) column (2.5 cm $\times$ 90 cm) equilibrated with 20 mM-sodium phosphate buffer, pH 8.0, containing 0.25 M-NaCl. In this way two procarboxypeptidase A fractions were obtained (I and II), which were stored separately at 4°C after precipitation with $(NH_4)_2SO_4$. The purification was 37-fold for form I and 40-fold for form II, with a joint yield of 17%. The values for the intrinsic activity were 12.5% for form I and 18% for form II, and the specific activities, with hippuryl-L-phenylalanine as substrate, were 13 (form I) and 14 μ mol of substrate/min per mg of protein (form II). Both forms of procarboxypeptidase A were practically homogeneous, as checked by polyacrylamide-gel electrophoresis (Fig. 1). Chymotrypsinogen C was completely removed in the second DEAE-cellulose chromatography (pH 6.0).

Physical properties

Ultraviolet spectra and absorption coefficient.

Form I shows a u.v. spectrum with $\lambda_{\max} = 281.5$ nm and $A_{281.5}^{1\%} = 20.7$, whereas form II has $\lambda_{\max} = 278$ nm and $A_{278}^{1\%} = 15.55$.

Sedimentation velocity. The sedimentation coefficients of both forms decrease linearly with protein concentration. The concentration-dependence of s follows the equation

$$s = s^0(1 - gc)$$

Fig. 1. Separation of two forms of procarboxypeptidase A (I and II) by gel-filtration chromatography on Bio-Gel P-150 —, Transmittance at 280 nm; O, activity against hippuryl-L-phenylalanine after incubation with trypsin; Δ , free activity against hippuryl-L-phenylalanine (no incubation with trypsin). No chymotryptic activity was detected either before or after trypsin activation. Insert: Polyacrylamide-gel electrophoresis of the two main peaks of the chromatogram, showing a high degree of homogeneity.

where $s^0 = 5.0$ (± 0.07 S.E.M.; $n = 5$) and $g = 0.0113$ for form I, and $s^0 = 3.8$ (± 0.05 S.E.M.; $n = 5$) and $g = 0.0115$ for form II. These values are normalized to 20°C and water. These values are in agreement with those found by other authors for the binary-complex procarboxypeptidase A-S5 from bovine pancreas, either native (Brown *et al.*, 1963) or reconstituted (Puigserver & Desnuelle, 1977), and with the isolated subunit I (Puigserver & Desnuelle, 1977) respectively. These results suggest that, by analogy with the bovine proenzymes, form I is a binary complex and form II monomeric.

Relative molecular mass. Two methods were used to estimate the M_r of both zymogens: gel electrophoresis in the presence of SDS and gel filtration. Fig. 2 shows the polyacrylamide-gel electrophoresis in SDS of both forms I and II, together with the products of their tryptic hydrolysis and the corresponding M_r standards. The M_r that corresponds to

Fig. 2. SDS/polyacrylamide-gel electrophoresis of forms I, II and their tryptic-activation products

(a) Tryptic digest of form II at 25°C for 2 min (trypsin:zymogen ratio 1:4.2); (b) form II; (c) M_r markers (in decreasing order of M_r): immunoglobulin G (L + H chains), bovine serum albumin, egg albumin, carbonic anhydrase, soya-bean trypsin inhibitor and egg-white lysozyme; (d) form I; (e) tryptic digest of form I (same conditions as for a). Abbreviations: PCPA, carboxypeptidase A-precursor; CPA, carboxypeptidase A. For other details see the text.

each component and the values obtained by gel filtration are also listed in Table 1. The results found with SDS are in rather good agreement with those found by Kobayashi *et al.* (1978). There is a binary-complex form (I) and a monomeric form (II), although the results of Kobayashi *et al.* (1978) give a somewhat higher value for the subunit corresponding to the carboxypeptidase-A precursor from both forms. There are, however, large discrepancies between the above values and the M_r values found by gel filtration: form I (binary complex) shows an M_r which is only approx. 68% of the sum of those of the precursor subunit and the proprotease E (as found by SDS/polyacrylamide-gel electrophoresis), and the monomer form II has an M_r by gel filtration which is approx. 73% of that found by SDS/polyacrylamide-gel electrophoresis. The very high value deduced for the activation segment ($M_r = 12\,500$ approx.) lost by the zymogen after trypsin treatment should also be noticed, in contrast with those reported for other procarboxypeptidases, which are in the range 7000–10000 (Fresheim *et al.*, 1967a; Lacko & Neurath, 1970; Reeck & Neurath, 1972).

The determination of M_r values by electrophoresis in the presence of SDS is subject to important errors, as has been shown with bovine carboxypeptidase A (Jacobson *et al.*, 1973). Careful analytical ultracentrifugation of pig carboxypeptidase A (Folk & Schirmer, 1963) gives a M_r of 34 800, and thus the electrophoretic method underestimates the actual value by about 8–9%. Whether this difference is also applicable to the other molecular species in the present study is not known.

The M_r values obtained by gel filtration show very marked deviations from the above results. The values obtained are much lower, suggesting a strong interaction between the proteins and the gel matrix. These interactions should be of a non-specific nature, as the results are very similar whether a dextran-type gel such as Sephadex, an acrylamide-type gel such as Bio-Gel or a mixed gel such as Ultrogel is used. It is unlikely that they are of an electrostatic nature, as the same behaviour is obtained in the range 40–300 mM-NaCl.

Chemical properties

Table 1 lists some relevant chemical features of the two procarboxypeptidases A isolated from pig pancreas. The *N*-terminal amino acid is histidine in both proteins, in contrast with the results of Kobayashi *et al.* (1978), who found only lysine as *N*-terminal amino acid for both enzyme forms. They only found histidine at position 7 and half-cystine as the *N*-terminal residue of zymogen E. A possible explanation for this discrepancy could be that the *N*-terminal region of both procarboxypeptidases A had undergone partial hydrolysis during isolation

Table 1. Some chemical and physical properties of the dimeric (I) and monomeric (II) forms of pig pancreatic procarboxypeptidases A

Form	Hexoses (%, w/w)	Galactosamine (%, w/w)	Sialic acids	N-Terminal amino acid	Free thiol groups	Zn ²⁺ (mol/mol)	M_r	
							Gel filtration	SDS electrophoresis
I	0.5	0.86	No	His	No	1.68*	49 000	45 000 + 26 000‡
II	0.2	0.55	Traces	His	No	1.71†	34 500	45 000

* Based on M_r 71 000.† Based on M_r 45 000.‡ M_r values of the two components of form I.

from the glands, by contrast with the zymogens obtained by Kobayashi *et al.* (1978) from pancreatic secretion.

The amino acid compositions of both forms agree reasonably well with those found by Kobayashi *et al.* (1978), except for the tryptophan content, especially in the binary complex (5.7 mol % in the present work, 2.3 mol % by Kobayashi *et al.*, 1978). No free thiol groups were found, and thus all half-cystine residues must be involved in the formation of disulphide bonds.

The presence of small amounts of hexoses and galactosamine had not been previously reported for this type of proteins, although sugars are found in some pancreatic hydrolases (Reinhold *et al.*, 1968). It is not possible at present to make any hypothesis about their probable biological role.

Another remarkable feature is the high Zn²⁺ content: 1.6–1.8 atoms per molecule of each procarboxypeptidase A, which cannot be removed by extensive dialysis. This suggests that both zymogens have two sites for the strong binding of the metal. A similar amount of zinc has been reported for bovine pancreatic carboxypeptidase A (Vallee *et al.*, 1960) after dialysis against saturating concentrations of the metal ion. However, in this latter instance only one zinc atom remains bound after extensive dialysis against buffer that has been freed from the metal ion (Vallee *et al.*, 1960).

Identity between form II and the carboxypeptidase A-precursor subunit of form I

On the basis of the identity of M_r between the larger subunit of the procarboxypeptidase A binary complex, obtained from pancreatic secretion, and the monomeric protein, and the very similar amino acid composition and coincidence of the first seven N-terminal amino acid residues, Kobayashi *et al.* (1978) concluded that both proteins were identical.

In the present work, the same M_r for the precursor subunit and for the monomeric form was found. The amino acid composition could not be compared, owing to the high affinity of zymogen E for the precursor subunit, which made it very difficult to

Fig. 3. Polyacrylamide-gel electrophoresis of CNBr fragments

(a) Form II; (b) form I; (c) zymogen E. Protein samples were treated with a 50-fold excess of CNBr in 70% formic acid at 25°C for 18 h. Samples were then dialysed against 10% acetic acid in acetylated bags and freeze-dried. For electrophoresis conditions see the text.

separate both polypeptide chains. The procedure for their separation, in preparative amounts, described by Kobayashi *et al.* (1978) was difficult to reproduce, and for this reason no amino acid analysis of the precursor subunit from form I was possible. However, peptide 'mapping' of the CNBr fragments of form I, form II and of the precursor subunit and zymogen E (previously separated in very small amounts by SDS/polyacrylamide-gel electrophoresis) was carried out (Fig. 3). The results show that form I gives a CNBr-peptide 'map' very similar to that for form II, except for two bands which belong to zymogen E and its larger fragment. Similar experiments carried out with active carboxy-

peptidase obtained from form II (results not shown) also indicate a close homology with the positions of the three methionine residues in bovine pancreatic carboxypeptidase A. The fourth methionine residue of the proenzyme would be located in the activation segment, as deduced from the M_r values of the different fragments.

The two proteins, procarboxypeptidase A I and II, also show a close similarity in the pattern of fragmentation with iodosobenzoic acid, a reagent that hydrolyses peptide bonds in which there is a tryptophan residue. As with the CNBr experiments, in this comparative analysis, no interference owing to the presence of zymogen E was found. This is because its fragments are very small and do not appear in the electrophoresis. Probably this is a consequence of the high tryptophan content of zymogen E (Kobayashi *et al.*, 1978), a feature shared by other proelastases (Gertler *et al.*, 1977). The results thus very probably reflect the similarity between form II and the precursor subunit of form I. Although these results do not prove the identity of both proteins, they show the existence of a close similarity and relatedness.

Activation of the zymogens

Figs. 4(a) and 4(b) show the activation of procarboxypeptidase A I and II by trypsin, chymotrypsin and a mixture of both at 25°C, pH 7.5. The ratio of activating enzymes to zymogen was 1:4.2 (w/w).

Some features can be observed in the activation process of both procarboxypeptidases A. (i) In the first stages chymotrypsin is more effective than trypsin, although it is less effective than a mixture (equivalent in weight) of both. (ii) However, the final activity is the same in all cases. (iii) The first stages in the activation process of form II (monomeric) are faster than those of form I (binary complex), although in both cases the activation with trypsin reaches a stable value only after approx. 90 min incubation. (iv) A complicated pattern is also noticeable in some of the activation progress curves, suggesting that the activation is a rather complex process. This complicated pattern has been reproduced several times, and thus it is not an artefact. (v) The electrophoretic study shows that both proenzymes are converted into carboxypeptidase A very early in the process (2 min or less).

It is difficult to compare these results with those found for other procarboxypeptidases A, because of the variety of experimental conditions used by the different authors. The existence of a general pattern relating the quaternary structure to the rate of the activation process is not straightforward. Thus, although the precursor subunit of bovine pancreatic carboxypeptidase A in its monomeric form, isolated by means of succinylation (Fresheim *et al.*, 1967b)

Fig. 4. Activation of procarboxypeptidase A I (a) and II (b) in 50 mM-Tris/HCl (pH 7.5)/0.5 M-NaCl, 25°C. ●, Activation by pig trypsin; ▲, activation by bovine chymotrypsin; ■, activation by a trypsin/chymotrypsin mixture (1:1, w/w). In all cases the procarboxypeptidase A concentration was 1 mg/ml and the activating-enzyme:zymogen ratio was 1:4.2.

or dimethylmaleylation (Puigserver & Desnuelle, 1977), is activated very rapidly, the naturally occurring monomeric procarboxypeptidase A from the pancreas of the dogfish is very resistant to activation (Lacko & Neurath, 1970). No multiphasic activation process as observed for form I has previously been described, except for bovine pancreatic subunit I isolated after succinylation (Fresheim *et al.*, 1967b).

In the tryptic activation of the pig proenzymes, the formation of a protein of the same M_r as the active enzyme is an early event. However, there is a slow development of measurable activity which may be due, as in the activation of pepsinogen (Keilova *et al.*, 1977; Dunn *et al.*, 1978), to the fact that the activation peptide(s) remains bound to the protein, blocking its active site. The release of the activation segment probably takes place only when extensive degradation has occurred. Hence the long period required for the development of full activity.

The experiments on the activation of pig procarboxypeptidases A also show that the process is unspecific with respect to the activation enzyme. Chymotrypsin, trypsin and thrombin are capable of activating both proenzymes (the activation with thrombin yields results which are very similar to

those found with trypsin). Uren & Neurath (1972) also noticed the unspecific character of the activation of the bovine system. It must be pointed out that the presence of the activated form of pro-peptidase E has an influence on the rate, but not on the extent, of the procarboxypeptidase A activation, as deduced from the graphs shown in Fig. 4. It is not unreasonable to think that in the intestine the combination of several proteolytic enzymes can bring about the activation of procarboxypeptidase A much more rapidly than in the experiments *in vitro*. This is supported by the fact that bovine pancreatic procarboxypeptidase A is activated much more rapidly in the pancreatic juice than *in vitro* (Keller *et al.*, 1958). The activation of the pig procarboxypeptidase A system is also much faster when it is measured in the acetone-dried-powder extract than in the purified proteins, reaching full activity within 15 min (results not shown).

We thank the Fundació M. F. de Roviralta (Barcelona) for a grant for the purchase of equipment. M. C. M. was recipient of a fellowship from the Ministerio de Educación y Ciencia. We are grateful to Dr. J. A. Subirana for permission to use the analytical ultracentrifuge and to Dr. J. Ausió for carrying out the experimental determination of the sedimentation coefficients.

References

- Aminoff, D. (1961) *Biochem. J.* **81**, 384–392
- Brown, J. R., Yamasaki, M. & Neurath, H. (1963) *Biochemistry* **2**, 877–885
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dunn, B. M., Deyrup, C., Moeschling, W. G., Gilbert, W. A., Nolan, R. J. & Trach, M. L. (1978) *J. Biol. Chem.* **253**, 7269–7275
- Folk, J. E. & Schirmer, E. W. (1963) *J. Biol. Chem.* **238**, 3884–3894
- Fresheim, J. H., Walsh, K. A. & Neurath, H. (1967a) *Biochemistry* **6**, 3010–3019
- Fresheim, J. H., Walsh, K. A. & Neurath, H. (1967b) *Biochemistry* **6**, 3020–3028
- Fuwa, K., Pulido, P., McKay, R. & Vallee, B. L. (1964) *Anal. Chem.* **36**, 2407–2411
- Gertler, A., Weiss, Y. & Burstein, Y. (1977) *Biochemistry* **16**, 2709–2716
- Glazer, A. N., Delange, R. J. & Sigman, D. S. (1975) in *Chemical Modification of Proteins* (Work, T. S. & Work, E., eds.), pp. 54–55, North-Holland Publishing Co., Amsterdam and Oxford
- Gray, W. R. (1967) *Methods Enzymol.* **11**, 139–151
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
- Gross, E. & Witkop, B. (1962) *J. Biol. Chem.* **237**, 1856–1860
- Habeeb, A. F. S. A. (1972) *Methods Enzymol.* **25**, 457–464
- Hartley, B. S. (1970) *Biochem. J.* **119**, 805–822
- Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427
- Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197–199
- Jacobson, G. R., Schaffer, M. H., Stark, G. R. & Vanaman, T. C. (1973) *J. Biol. Chem.* **248**, 6583–6591
- Keilova, H., Kostka, V. & Kay, J. (1977) *Biochem. J.* **167**, 855–858
- Keller, P. J., Cohen, E. & Neurath, H. (1958) *J. Biol. Chem.* **233**, 344–349
- Kobayashi, R., Kobayashi, Y. & Hirs, C. H. W. (1978) *J. Biol. Chem.* **253**, 5526–5530
- Lacko, A. G. & Neurath, H. (1970) *Biochemistry* **9**, 4680–4690
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Mahoney, W. C. & Hermondtson, M. A. (1979) *Biochemistry* **18**, 3810–3814
- Puigserver, A. & Desnuelle, P. (1977) *Biochemistry* **16**, 2497–2501
- Reeck, G. R. & Neurath, H. (1972) *Biochemistry* **11**, 3947–3955
- Reinhold, V. N., Dunne, F. T., Wriston, J. C., Schwarz, M., Sarda, L. & Hirs, C. H. W. (1968) *J. Biol. Chem.* **243**, 6482–6494
- Schwert, G. W. & Takenaka, Y. (1955) *Biochim. Biophys. Acta* **16**, 570–575
- Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
- Spies, J. R. & Chambers, D. C. (1948) *Anal. Chem.* **20**, 30–39
- Uren, J. R. & Neurath, H. (1972) *Biochemistry* **11**, 4483–4492
- Vallee, B. L., Rupley, J. A., Coombs, T. L. & Neurath, H. (1960) *J. Biol. Chem.* **235**, 64–69
- Wesenberg, J. C. & Thibert, R. J. (1977) *Mikrochim. Acta* **1**, 469–473
- Yamasaki, M., Brown, J. R., Cox, D. J., Greenshields, R. N., Wade, R. D. & Neurath, H. (1963) *Biochemistry* **2**, 859–866