Proteins of the kidney microvillar membrane

Purification and properties of carboxypeptidase P from pig kidneys

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Carboxypeptidase P has been purified by immunoaffinity chromatography from pig kidneys. A single-step assay with Z-Pro-Met (where Z represents benzyloxycarbonyl) as substrate was used, methionine being determined by using L-amino acid oxidase and horseradish peroxidase. The enzyme constitutes about 1.5% of the kidney microvillar proteins. Triton X-100-solubilized and papain-released forms of the enzyme were isolated. The former had an apparent subunit M_r of 135 000, and the latter form contained two polypeptide chains of M_r 128 000 and 95 000. The undenatured forms were dimeric proteins. In common with other microvillar hydrolases, carboxypeptidase P was a glycoprotein and each subunit contained one Zn atom. $MnCl_2$ (1 mM) in the assay was necessary for maximum activity; in its absence, 0.5 mM- $ZnSO_4$ produced a limited activation, but was inhibitory at higher concentrations. The K_m for Z-Pro-Met, in the presence of $MnCl_2$, was 4.1 mM, and the k_{cat} for freshly prepared enzyme was 1230 min^{-1} . The enzyme lost activity during storage at -20°C . In a limited survey of peptides, hydrolysis was observed only with substrates containing a proline, alanine or glycine residue in the P_1 position, and these included angiotensins II and III. The best substrate in this series was Val-Ala-Ala-Phe.

In 1970, Dehm & Nordwig (1970*a,b*) described two exopeptidases present in a microsomal fraction from pig kidneys. Both enzymes were capable of releasing terminal amino acids linked to proline residues. The enzyme that released alanine from Z-Pro-Ala they named carboxypeptidase P (Dehm & Nordwig, 1970*b*). When purified 330-fold, it required Mn^{2+} for maximum activity, and, although -Pro-Xaa substrates were hydrolysed best, it also attacked -Ala-Xaa and -Gly-Xaa peptides. The reaction catalysed is similar to that by proline carboxypeptidase, but in other respects this enzyme is clearly different. Proline carboxypeptidase has a lysosomal and not a membrane distribution. Furthermore, it has a pH optimum of 5 and is listed (EC 3.4.16.2) as a serine peptidase (Yang *et al.*, 1968; Kakimoto *et al.*, 1973; Taylor & Tappel, 1974; Walter *et al.*, 1980; Ody & Erdős, 1981). For

these reasons it can be distinguished from the membrane carboxypeptidase P. Dehm & Nordwig (1970*b*) assumed that the enzyme was an intracellular peptidase and argued that it was the first example of a non-lysosomal intracellular carboxypeptidase. However, microsomal pellets prepared from kidney cortex are rich in microvilli. We have shown that both aminoacyl-prolyl aminopeptidase and carboxypeptidase P are enriched in microvillar-membrane preparations and that both are released by papain treatment (Kenny *et al.*, 1977). Auricchio *et al.* (1978) have reported that carboxypeptidase P is present in rabbit intestinal brush-border preparations, but in other respects the enzyme has received very little attention in comparison with other microvillar peptidases. In the present paper we report the purification of the carboxypeptidase by immunoabsorbent chromatography and describe for the first time the detergent-released form. Amongst its properties we have shown it to be a Zn^{2+} -containing metalloenzyme.

Abbreviation used: Z, benzyloxycarbonyl.

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Experimental

Materials

Pig kidneys were kindly donated by Asda Farm Stores, Lofthousegate, W. Yorks, U.K.

Peptides were purchased from Sigma Chemical Co., Poole, Dorset, U.K., or from Bachem Feinchemikalien, Bubendorf, Switzerland.

Emulphogen was obtained from Sigma Chemical Co.

Preparation of microvillar fraction

This was as described by Booth & Kenny (1974).

Assays of enzyme activities

Carboxypeptidase P was assayed with Z-Pro-Met as substrate, methionine being determined by L-amino acid oxidase (Nicholson & Kim, 1975). The incubation mixture (final volume 1.6 ml) contained in addition to enzyme 3.1 mM-Z-Pro-Met, 24 mM-sodium barbitone, 25 mM-sodium acetate, 25 mM-NaCl, 0.18 unit of L-amino acid oxidase (Sigma type I, from *Crotalus adamanteus*), 20 µg of horseradish peroxidase (Sigma type II), 1 mM-MnCl₂ and 0.1 mg of *o*-dianisidine, adjusted with HCl to pH 7.75. After incubation for 15–30 min at 37°C the reaction was stopped by the addition of 750 µl of 50% (v/v) H₂SO₄ and A₅₃₀ was determined. One unit of activity is defined as the release of 1 µmol of methionine/min.

Other microvillar peptidases were assayed as previously described (Fulcher & Kenny, 1983).

Hydrolysis of peptides by carboxypeptidase P

This was determined by h.p.l.c. analysis with a gradient (0–70%, v/v) of acetonitrile (Matsas *et al.*, 1983). The incubation mixture (final volume 60 µl) contained 2–5 mM-peptide, in the same assay buffer, pH 7.75, including 1 mM-MnCl₂. The reaction was stopped, after incubation for 15–60 min, by heating at 100°C for 5 min, and any precipitate was removed by centrifugation before analysis.

Preparation of a specific antiserum and immuno-adsorbents to carboxypeptidase P

A stepwise procedure, similar to that employed to generate an anti-(aminopeptidase A) serum (Danielsen *et al.*, 1980), was followed. The DE-52-DEAE-cellulose-eluted papain-solubilized enzyme was subjected to line electrophoresis in agarose into an IgG fraction of an antiserum raised to kidney microvilli. Portions of the line precipitates were checked by staining for carboxypeptidase P, as described previously (Booth *et al.*, 1979). The precipitate was then excised and stored at –20°C. The electrophoresis was repeated ten times and the

accumulated protein was used to immunize a rabbit (Danielsen *et al.*, 1980), yielding antiserum 1. Line electrophoresis was repeated with a sample of enzyme that had been further chromatographed by a gradient (0–300 mM) of NaCl on DEAE-cellulose. The agarose now contained antiserum 1, and the precipitates identified as those containing carboxypeptidase P from repeated runs were pooled and used to generate antiserum 2. An IgG fraction was prepared and coupled to CNBr-activated Sepharose 4B (5 mg of IgG/ml). This column was used to purify (as described below) a sample of a detergent-solubilized form of the enzyme. After absorption of this sample by antibodies raised to microvillar-membrane proteins, the preparation was used to generate antiserum 3. An IgG fraction of antiserum 3 was coupled to CNBr-activated Sepharose 4B (35 mg of IgG/5 ml of column). This column yielded about 0.8 mg of enzyme from each purification run.

Purification of carboxypeptidase P

A microvillar fraction was prepared from 100 g of frozen kidney cortex. For detergent solubilization, the pellet was resuspended in 200 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 1% (w/v) Triton X-100, corresponding to a detergent/protein ratio of 4:1. It was shaken for 1 h at 4°C. For solubilization by digestion with papain the pellet was resuspended in 10 mM-sodium phosphate buffer, pH 6.9, and, after activation of the papain for 15 min at 37°C with 2.5 mM-2-mercaptoethanol, it was added to give a papain/protein ratio of 1:10. After 90 min incubation at 37°C iodoacetamide was added to 10 mM. In both solubilization procedures a supernatant fraction was obtained by centrifugation at 32000g for 2 h. Papain was removed by chromatography on a column (50 ml) of DEAE-cellulose (Whatman DE-52). After loading, the column was washed with 500 ml of 50 mM-Tris/HCl buffer, pH 7.8, and batch-eluted with 300 mM-NaCl in the same buffer. The subsequent purification of both forms of the enzyme depended on an immunoaffinity column. Triton X-100 (1%, v/v) was present in all buffers used for the detergent form. The samples were loaded in 50 mM-Tris/HCl buffer, pH 7.8, containing 150 mM-NaCl. After a washing (10 column volumes) with the same buffer the activity was eluted with 2 mM-Tris/HCl buffer, pH 8.0. The eluted activity was concentrated on a column (0.5 ml) of DE-52 DEAE-cellulose, by elution with 0.3 M-NaCl in 50 mM-Tris/HCl buffer, pH 7.8. Traces of other microvillar proteins were removed by absorption at 4°C for 18 h with 25 µl of an immunoabsorbent prepared by coupling to Sepharose 4B an IgG fraction of an antiserum raised to microvillar-membrane proteins (Danielsen *et al.*, 1980).

elution. For this reason a final step was necessary involving the absorption of the contaminants by antibodies raised to all microvillar components.

The specific activities of the detergent-solubilized and papain-released preparations were 8.4 and 8.5 units/mg of protein respectively. In six preparations the values were in the range 6.0–11.0 units/mg of protein, and for three samples subjected to amino acid analysis, from which precise protein concentrations could be calculated, were 7.8, 9.6 and 9.4 units/mg of protein.

Purity of the enzyme preparation

The detergent form was single-banded on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, but (see below) the papain-released form migrated as two bands of comparable staining intensity (Fig. 1). In 'rocket' and crossed immunoelectrophoresis only one precipitate arc was seen when the purified preparation was run into antiserum raised to pig microvillar-membrane components. By enzymic assays (substrates in parentheses) none of the following was detected: endopeptidase-24.11 (^{125}I -insulin B chain), aspartate aminopeptidase (α -glutamic acid aminomethylcoumarylamide), aminopeptidase N (ala-

nine aminomethylcoumarylamide), γ -glutamyl-transferase (γ -glutamic acid aminomethylcoumarylamide) and dipeptidyl peptidase IV (glycylproline aminomethylcoumarylamide).

Chemical analysis

Amino acid analyses of three preparations of the detergent form are shown in Table 2. They did not reveal any noteworthy features. Carbohydrate was analysed on one of these samples: it contained 332 μg of carbohydrate/mg of protein including 23% mannose, 29% galactose, 30% *N*-acetylglucosamine, 7% glucose and 11% uncharacterized sugars.

Zinc was analysed by atomic emission spectrometry on one sample of carboxypeptidase P. After correction for traces of zinc in the buffer against which the sample had been dialysed, the enzyme contained 1.03 g-atom of Zn/135000 g of protein (determined by amino acid analysis).

Molecular properties of carboxypeptidase P

Subunit M_r . The apparent values by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1) were: detergent-solubilized form, 135000; papain-released form 128000 and 95000. The papain-released form was a hydrophilic protein (see below), and the finding of two polypeptides indicates more than one site of proteolysis. No complementary smaller fragment, e.g. of M_r 30000, was seen on gel electrophoresis. Some of the earlier preparations of the enzyme, isolated over relatively longer periods, showed

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of carboxypeptidase P

A 7–17% polyacrylamide electrophoretic gel was stained with Coomassie Blue. The tracks contained: lane 1, microvillar membranes; lane 2, detergent-solubilized form; lane 3, M_r markers; lanes 4 and 5, detergent-solubilized and papain-released forms before immunoabsorption with IgG fraction raised against kidney microvillar proteins.

Table 2. Amino acid analysis of the detergent form of carboxypeptidase P

The values are means for six analyses on three samples of enzyme hydrolysed (except for the methionine value, which was detected on only one sample). Proline and cysteine were not determined.

Amino acid	Amino acid content (mol/100 mol)
Aspartic acid	10.6 $\pm$ 0.6
Threonine	6.2 $\pm$ 0.4
Serine	7.0 $\pm$ 0.5
Glutamic acid	9.2 $\pm$ 1.0
Glycine	9.6 $\pm$ 1.4
Alanine	7.8 $\pm$ 0.3
Valine	6.8 $\pm$ 0.7
Methionine	(1.0)
Isoleucine	5.3 $\pm$ 0.5
Leucine	9.0 $\pm$ 0.6
Tyrosine	4.8 $\pm$ 0.2
Phenylalanine	4.6 $\pm$ 0.4
Histidine	7.9 $\pm$ 3.3
Lysine	6.0 $\pm$ 1.3
Arginine	5.2 $\pm$ 0.9

bands of M_r 50000 and 65000, suggesting that the enzyme was susceptible to proteolysis at several sites.

Oligomeric structure. Samples (10 μ g) of both the papain-released and detergent-solubilized forms of the enzyme were chromatographed on a column of Superose-6. Markers (Pharmacia Fine Chemicals) were ferritin (M_r 480000), catalase (M_r 232000), lactate dehydrogenase (M_r 140000) and ovalbumin (M_r 43000), and these gave a linear plot, of $\log M_r$ against retention time, which was unaffected by the addition of 0.1% Emulphogen to the buffer when the detergent-solubilized form was run. Duplicate runs of each form yielded M_r values of 245000 (papain-released form) and 291000 (detergent-solubilized form). Both samples of the enzyme had been stored for several weeks and both contained some fragments of $M_r < 100000$ for the papain-released form and < 200000 for the detergent-solubilized form.

Amphipathic properties. The detergent-solubilized form exhibited charge shift when subjected to crossed immunoelectrophoresis in buffers containing cationic and anionic detergents. Transferrin was run in the same gels as hydrophilic marker protein, and the mobilities of carboxypeptidase P were expressed relative to the migration of transferrin. In Triton X-100 the mobility of the peptidase was 1.3. The addition to Triton X-100 of 0.0125% cetyltrimethylammonium bromide changed the mobility to 1.1, and the addition of 0.2% deoxycholate gave a value of 2.3. Thus cationic detergent slightly decreased mobility, whereas anionic detergent increased it almost 2-fold. When this experiment was repeated with papain-released enzyme, no difference in mobility was observed between non-ionic detergent alone and with the addition of deoxycholate.

Metal ions and chelators. Mn^{2+} was necessary for full activity, and this effect was maximal at 1 mM- $MnCl_2$, as reported by Dehm & Nordwig (1970b). In the absence of Mn^{2+} only 10% of the maximum activity was observed. This activity could be further decreased by the addition of chelators. Both EDTA and 1,10-phenanthroline could inhibit all activity, with IC_{50} values of 0.5 mM and 1 mM respectively. After total inactivation with 10 mM-EDTA, dialysis against 50 mM-Tris/HCl buffer, pH 7.8, containing 0.1% Triton X-100 restored the activity to the level observed when Mn^{2+} was omitted. The addition of $ZnSO_4$ caused a small increase, to about 40% above this level, and this effect exhibited a sharp maximum at about 0.5 mM- $ZnSO_4$, above which concentration the metal ion was strongly inhibitory.

Enzymic properties

Kinetics. K_m was determined on a single prepara-

tion of the detergent-solubilized form of the enzyme by using a Lineweaver-Burk plot. The substrate was Z-Pro-Met, and the assay mixtures contained 1 mM- $MnCl_2$. The mean value for K_m in four experiments was 4.05 ± 0.77 mM. V_{max} decreased during the period in which the enzyme was stored at $-20^\circ C$. The first two experiments yielded values (within a period of 3 days) of 8.40 and 9.78 μ mol/min per mg of protein, equivalent to a k_{cat} value of 1230 min^{-1} for a subunit M_r of 135000; $k_{cat}/K_m = 303 \text{ min}^{-1} \cdot \text{mM}^{-1}$. The third experiment 3 weeks later gave a V_{max} of 4.07 μ mol/min per mg, which fell to 1 μ mol/min per mg of protein a week later. The enzyme lost activity in alkaline conditions. Elution of the immunoaffinity column with a buffer at pH 10.6, conditions that have given good yields of other metallopeptidases, e.g. aminopeptidase A (Danielsen *et al.*, 1980) and endopeptidase-24.11 (Gee *et al.*, 1983), inactivated the carboxypeptidase. For this reason, elution at low ionic strength (2 mM-Tris/HCl buffer, pH 8.0) had to be employed in the immunoaffinity step.

Hydrolysis of peptides. A small number of peptides were investigated as possible substrates for the enzyme (Table 3). A group of peptides containing a C-terminal phenylalanine residue was studied quantitatively. This group showed that a proline residue in the P_1 position was not essential for hydrolysis; alanine and, to a lesser extent, glycine could substitute. Indeed, a tetrapeptide, Val-Ala-Ala-Phe, was hydrolysed 4 times faster than Z-Pro-Phe. Angiotensins were also hydrolysed. The other peptides in this series that were hydrolysed contained an alanine or a proline residue in the P_1 position, and in this group Ala-Ala-Ala-Ala was hydrolysed more rapidly than was the normal assay substrate, Z-Pro-Met. Pro-Hyp, Pro-Pro, His-Leu, Phe-Leu, Phe-Arg, Glu-Tyr and Pro-Leu bonds were not split in those peptides tested that contained these C-terminal sequences. Surprisingly, Ala-Ala-Ala was resistant to hydrolysis, although the tetrapeptide Ala-Ala-Ala-Ala was a relatively good substrate.

Discussion

The apparent subunit M_r of carboxypeptidase P (135000) locates this polypeptide in the fuzzy 130 kDa band seen in the sodium dodecyl sulphate/polyacrylamide-gel electrophoretic pattern of microvillar proteins. Dipeptidyl peptidase IV is also present in this band (Macnair & Kenny, 1979), this being an enzyme that amounts to about 4% of microvillar protein (Kenny *et al.*, 1976). Carboxypeptidase P must also be considered to be a major protein in the membrane. The specific activity (mean of 11 preparations) in microvillar preparations was 0.125 unit/mg (Kenny *et al.*, 1977),

Table 3. *Hydrolysis of peptides by carboxypeptidase P*

See the Experimental section for details. The products were analysed by h.p.l.c., and hydrolysis was quantified by the liberation of phenylalanine, monitored at 214 nm. Peptides lacking a C-terminal phenylalanine residue were also studied, but hydrolysis was assessed by decrease in area of the substrate peak, and this has not been fully quantified. Peptides were listed as unhydrolysed only after a 2 h incubation and only when markers corresponding to the putative products were available and shown not to be co-eluted with the substrate.

Peptide	Concentration (mM)	Hydrolysis relative to Z-Pro-Phe	Hydrolysis relative to Z-Pro-Met
Z-Pro-Phe	5	100	
Z-Ala-Phe	5	60	
Z-Gly-Phe	5	13	
Val-Ala-Ala-Phe	5	418	
Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	1.9	20	
Angiotensin III (des-Asp ¹ -angiotensin II)	1.9	26	
Z-Pro-Met			100
Ala-Ala-Ala-Ala	5		~ 300
Arg-Gly-Pro-Phe-Pro-Ile	2		≤ 100
Arg-Tyr-Leu-Pro-Thr	5		< 100
Z-Pro-Ala	5		50
Z-Ala-Met	5		Little hydrolysis
Hip-His-Leu	2.5	}	No hydrolysis observed
Hip-Phe-Arg	2.5		
Ala-Ala-Ala	5		
Gly-Pro-Hyp	5		
Gly-Pro-Pro	5		
Z-Glu-Tyr	5		
Z-Pro-Leu	5		

which, related to the specific activities observed with the purified enzyme, indicates that it is about 1.5% of the microvillar protein. In common with all the microvillar-membrane hydrolases (see, e.g., Kenny & Maroux, 1982), it is a glycoprotein containing, in this case, about 30% carbohydrate. Like aminopeptidase N (Lehky *et al.*, 1973), endopeptidase-24.11 (Kerr & Kenny, 1974; Fulcher & Kenny, 1983) and peptidyl dipeptidase A (Das & Soffer, 1975), it is a Zn²⁺-containing metallopeptidase. Zn²⁺, therefore, provides the commonest catalytic mechanism for microvillar membrane peptidases. Within this group, carboxypeptidase P was unusual in its requirement for Mn²⁺, which is also a feature of the kidney cytosolic enzyme leucine aminopeptidase (EC 3.4.11.1). When Mn²⁺ was omitted from the carboxypeptidase assay mixture, the activity fell to 10% of the maximum. This could be decreased to zero with high concentrations of chelating agents (0.5 mM-EDTA, 10 mM-1,10-phenanthroline). The weak re-activation by Zn²⁺ of chelator-treated enzyme amounted to an increase from 10% to 14% of the Mn²⁺-stimulated activity, and the effect exhibited a sharp maximum at 0.6 mM. This type of response to Zn²⁺ is similar to that observed with endopeptidase-24.11 (Kerr & Kenny, 1974).

The topological features of this enzyme have not been studied in detail, but what information we

have suggests that it resembles other hydrolases in the membrane (Kenny & Maroux, 1982). Thus its release by papain indicates that it is an ectoenzyme with a sufficiently long stalk to permit access by this proteinase. Additionally, the relatively small difference in subunit *M_r* between the detergent-solubilized form and the larger of the two papain-released polypeptides suggests that the hydrophobic and junctional domains comprise only a small proportion (5%) of the total mass of the glycoprotein. In common with other microvillar hydrolases isolated from pig sources, it is a dimeric enzyme. Our *M_r* value for the papain-released form (245 000) is in agreement with the value of 240 000 reported by Dehm & Nordwig (1970*b*) for the trypsin-released form. The detergent-solubilized form, with Triton X-100 exchanged for Emulphogen, gave a higher value (291 000), though less than would be predicted for a dimeric polypeptide binding a micelle of detergent.

The L-amino acid oxidase-linked assay was convenient for the purification and kinetic studies of the enzyme, but had disadvantages in studying effectors, since any influence exerted on the oxidase-peroxidase system needed to be determined at every concentration studied. It also restricted the range of experimental substrates to those in which the C-terminal amino acid residue was a substrate for L-amino acid oxidase, a

consideration that dictated the selection of Z-Pro-Met as substrate. More information on the specificity of the purified enzyme was sought by using h.p.l.c to analyse the products, and here the limitation for precise measurements was the need to detect the product by its absorption at 214 nm. Hence substrates with C-terminal phenylalanine were used. In the series Z-Pro-Phe, Z-Ala-Phe and Z-Gly-Phe we confirm the findings reported by Dehm & Nordwig (1970b) on the effect of residues in the P₁ position. Our data with longer peptides (Val-Ala-Ala-Phe and angiotensins II and III) suggest that residues somewhat distant from the scissile bond may have marked effects on the rates of hydrolysis of the C-terminal phenylalanine residue. The ability of the enzyme to hydrolyse Ala-Ala-Ala-Ala but not Ala-Ala-Ala also suggests a preference for longer peptide chains. These effects deserve further investigation.

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References

- Auricchio, S., Greco, L., de Vizia, B. & Buonocore, V. (1978) *Gastroenterology* **75**, 1073–1079
- Booth, A. G. & Kenny, A. J. (1974) *Biochem. J.* **142**, 575–581
- Booth, A. G., Hubbard, L. M. L. & Kenny, A. J. (1979) *Biochem. J.* **179**, 397–405
- Danielsen, E. M., Norén, O., Sjöström, M., Ingram, J. & Kenny, A. J. (1980) *Biochem. J.* **189**, 591–603
- Das, M. & Soffer, R. L. (1975) *J. Biol. Chem.* **250**, 6762–6768
- Dehm, P. & Nordwig, A. (1970a) *Eur. J. Biochem.* **17**, 364–371
- Dehm, P. & Nordwig, A. (1970b) *Eur. J. Biochem.* **17**, 372–377
- Fulcher, I. S. & Kenny, A. J. (1983) *Biochem. J.* **211**, 743–753
- Gee, N. S., Matsas, R. & Kenny, A. J. (1983) *Biochem. J.* **214**, 377–386
- Kakimoto, T., Oshima, H. S. J. & Erdos, E. G. (1973) *Biochim. Biophys. Acta* **302**, 178–182
- Kenny, A. J. & Maroux, S. (1982) *Physiol. Rev.* **62**, 91–128
- Kenny, A. J., Booth, A. G., George, S. G., Ingram, J., Kershaw, D., Wood, E. J. & Young, A. R. (1976) *Biochem. J.* **157**, 169–182
- Kenny, A. J., Booth, A. G. & Macnair, R. D. C. (1977) *Acta Biol. Med. Ger.* **36**, 1575–1585
- Kerr, M. A. & Kenny, A. J. (1974) *Biochem. J.* **137**, 489–495
- Lehky, P., Lisowski, J., Wolf, D. P. & Wacker, H. (1973) *Biochim. Biophys. Acta* **321**, 274–281
- Macnair, R. D. C. & Kenny, A. J. (1979) *Biochem. J.* **179**, 379–395
- Matsas, R., Fulcher, I. S., Kenny, A. J. & Turner, A. J. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3111–3115
- Nicholson, J. A. & Kim, Y. S. (1975) *Anal. Biochem.* **63**, 110–117
- Ody, C. E. & Erdös, E. G. (1981) *Methods Enzymol.* **80**, 460–466
- Taylor, S. L. & Tappel, A. L. (1974) *Biochim. Biophys. Acta* **341**, 99–111
- Walter, R., Simmons, W. H. & Yoshimoto, T. (1980) *Mol. Cell. Biochem.* **30**, 111–127
- Yang, H. Y. T., Erdös, E. G. & Chiang, T. S. (1968) *Nature (London)* **218**, 1224–1226