Isolation of a human hepatic 60 kDa aspartylglucosaminidase consisting of three non-identical polypeptides

Marc BAUMANN,*|| Leena PELTONEN,† Pertti AULA‡ and Nisse KALKKINEN§

*Department of Medical Chemistry, University of Helsinki, 00170 Helsinki 17, †Laboratory of Molecular Genetics, National Public Health Institute, 00290 Helsinki 29, ‡Department of Medical Genetics, University of Turku, 00520 Turku 52, and §Recombinant DNA Laboratory, University of Helsinki, 00380 Helsinki 38, Finland.

We have characterized the properties of human aspartylglucosaminidase (EC 3.5.1.26), the lysosomal enzyme which is deficient in the human inherited disease aspartylglucosaminuria. The purification procedure from human liver included affinity chromatography, gel filtration, strong-anion- and strong-cation-exchange h.p.l.c., chromatofocusing and reverse-phase h.p.l.c. In a denaturing SDS/polyacrylamide-gel electrophoresis, the 6600-fold purified enzyme was shown to be composed of three non-identical inactive polypeptide chains of molecular masses 24, 18 and 17 kDa. In a native polyacrylamide-gel electrophoresis, these polypeptide chains ran as one active enzyme complex. As judged from the elution position of the native enzyme in a Biogel P-100 gel filtration, the approximate molecular mass of this complex was 60 kDa. The enzyme had a pI of 5.7, a pH optimum at 6, a K_m of 0.48 mM and a specific activity of 200 000 nkat for the substrate 2-acetamido-1- β -(L-aspartamido)-1,2-dideoxy-D-glucose. The enzyme showed a 57% loss of activity at 60 °C after 45 h but was practically inactive after incubation at 72 °C for a few minutes. The molecular structure, K_m and specific activity as well as the thermostability of the enzyme described here are different from those reported previously for human aspartylglucosaminidase.

INTRODUCTION

Mammalian *N*-aspartyl- β -*N*-acetylglucosamine amidohydrolase (aspartylglucosaminidase, AGA) (EC 3.5.1.26) hydrolyses β -aspartylglycosylamine linkages (Makino *et al.*, 1966), which are the most common glycosidic bonds in glycoproteins (Neuberger *et al.*, 1966). AGA has been shown to be a lysosomal enzyme (Mahadevan & Tappel, 1967), and enzyme activity has been detected in a number of human tissues and body fluids (Conchie & Strachan, 1969; Palo *et al.*, 1971; Dugal, 1977). Aspartylglucosaminuria is an inherited lysosomal storage disorder in which AGA activity is reduced in several tissues and body fluids of the patients (Pollitt *et al.*, 1968; Palo *et al.*, 1972). Aspartylglucosaminuria is characterized by slowly progressive mental deterioration and some connective tissue changes (Pollitt *et al.*, 1968; Autio, 1972). The faulty degradation of glycoproteins leads to accumulation of aspartylglucosamine and other glycoasparagines in several tissues of patients with aspartylglucosaminuria (Palo *et al.*, 1973; Maury, 1980) and also to urinary excretion of these compounds (Jenner & Pollitt, 1967; Lundblad *et al.*, 1976). Purification of the human hepatic AGA has been reported earlier, and its properties have been studied to some extent (Savolainen, 1976; Dugal & Strömme, 1977; McGovern *et al.*, 1983), but there has been some inconsistency in the physical and chemical data. In order to study the molecular pathology of aspartylglucosaminuria, we have further characterized the structure and properties of AGA. Our results suggest that the native human hepatic AGA is a protein of molecular

mass of approx. 60 kDa, which is denatured to three polypeptide chains. Dissociation of these three polypeptide chains resulted in the loss of the catalytic activity.

MATERIALS AND METHODS

Materials

Four separate identical purification procedures were carried out on human liver tissue: the first using fetal liver (38 g total), obtained immediately after termination of four second-trimester pregnancies, the second and third with liver tissue from newborns (260 g total), obtained at autopsy within 1–2 h of death, and the fourth with adult liver, removed at autopsy within 4 h of death. All liver samples were stored immediately at –70 °C. Since no differences in the physical or chemical characteristics of the purified enzyme could be detected between AGA purified from fetal, newborn or adult liver tissue, only the purification procedure from newborn liver tissue (106 g) obtained at autopsy within 1 h of death is described in detail.

Methods

The enzymic activity was measured by detecting the release of *N*-acetylglucosamine, as originally described by Makino *et al.* (1966). The incubation was done either for 4 h at 37 °C or for 2 h at 50 °C. All purification procedures were performed at room temperature if not otherwise indicated.

Abbreviations used: AGA, aspartylglucosaminidase; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide; ConA, concanavalin A.

|| To whom correspondence should be addressed.

Liver was thawed in a 10 mM-sodium phosphate buffer, pH 7.0, containing 0.1 mM-phenylmethanesulphonyl fluoride (PMSF), 1 mM-*N*-ethylmaleimide (NEM) and 0.2% (w/v) NaN_3 , and then homogenized at 0 °C with a Polytron Type pT 10/30 cutter for 3 × 3 min. The homogenate was further treated with a Teflon Potter–Elvehjem homogenizer at 300 rev./min (internal diameter 2 cm) for 3 × 2 min at 0 °C and then centrifuged at 25000 *g* for 30 min at 4 °C. The supernatant was stored at –20 °C. The remaining pellet was resuspended in the same buffer as used above, centrifuged and the supernatant stored at –20 °C. This washing procedure was continued until no AGA activity could be detected in the remaining resuspended pellet.

Affinity chromatography was performed at 4 °C on a concanavalin A (ConA)–Sephacrose (Pharmacia, Uppsala, Sweden) column (1.6 cm × 18 cm), equilibrated with 10 mM-sodium phosphate (pH 7.0)/0.1 mM-PMSF/1 mM-NEM/0.2% (w/v) NaN_3 (flow rate of 20 ml/min). The enzyme was released from the column with 0.6 M- α -methylglucose.

Gel filtration was performed at 4 °C on a BioGel P-100 column (2.5 cm × 70 cm) equilibrated with 20 mM-triethanolamine acetate (pH 7.2)/0.1 mM-PMSF/1 mM-NEM/0.2% (w/v) NaN_3 (flow rate of 15 ml/h). The strong-anion-exchange h.p.l.c. was performed on a Mono Q column (HR 5/5, Pharmacia) equilibrated with 20 mM-triethanolamine acetate, pH 7.2, with a linear gradient of sodium acetate (0–0.7 M in 10 min) in the equilibration buffer (flow rate of 1 ml/min). The strong-cation-exchange h.p.l.c. was performed on a Mono S column (HR 5/5; Pharmacia) equilibrated with 50 mM-sodium acetate, pH 5.5, with a linear gradient of sodium acetate (0–0.25 M in 15 min) in the equilibration buffer (flow rate of 1 ml/min). Chromatofocusing h.p.l.c. was performed on a Mono P column (HR 5/20; Pharmacia), equilibrated with 25 mM-Bis-Tris/HCl, pH 7.2 (flow rate of 1 ml/min). The elution was carried out with 40 ml of the polybuffer 96, pH range 7–4 (Pharmacia).

Reverse-phase h.p.l.c. was performed with a VYDAC 218 TP 54 column (0.46 cm × 25 cm) equilibrated with 0.1% trifluoroacetic acid, with a linear gradient of acetonitrile (0–70% in 30 min) (flow rate of 1 ml/min).

SDS/polyacrylamide-gel electrophoresis (PAGE) was carried out as described by Kalkkinen *et al.* (1980). The silver staining was carried out as described by Tunon & Johansson (1984), with the following modifications. Prefixation steps 1 and 2 were carried out for only 20 min followed by rinsing with water for 15 min and fixation for only 5 min at 50 °C, washing then with water (2 × 5 min) and with aqueous ethanol (2 × 5 min). The ammoniacal silver solution treatment was performed for only 10 min. The AGA activity from the native PAGE was measured by cutting out the band according to its position in a stained lane run in parallel. The enzyme was electrophoretically eluted from the gel piece as described by Allington *et al.* (1978).

The amount of protein was measured by the Bio-Rad protein assay as described by Bradford (1976) and/or by measuring the coloured SDS/PAGE band intensities stained with Coomassie Brilliant Blue.

The K_m was graphically measured by means of a Lineweaver–Burk plot, and the specific activity was calculated from obtained values. The pH optimum was detected by following the amount of released *N*-acetylglucosamine at various pH values.

RESULTS

Purification of AGA

The purification of AGA involved affinity chromatography, gel filtration, and strong-anion- and strong-cation-exchange chromatography on h.p.l.c. The enzyme activity was measured in each purification step. Only fractions with the highest enzyme activity from each purification step were processed further.

Embryonic, newborn and adult livers were investigated separately but no differences in the physical or chemical behaviour of AGA purified from each liver type were observed. Thus, results from only the purification procedure used with newborn liver tissue are described.

The glycoprotein fraction from the supernatant of the liver homogenate was retained in ConA–Sephacrose 4B (Fig. 1a). ConA showed a strong affinity to AGA (> 90%) but only 31% of the AGA activity present in the original tissue homogenate could be eluted in a few main fractions, the rest of the activity tailing in later fractions.

The ConA fractions with highest AGA activity were pooled and applied to a BioGel P-100 gel-filtration column. As shown in Fig. 1(b), this molecular sieve step clearly separated the enzymically active fraction in a single peak from the main protein fraction. The enzyme activity was recovered in a peak corresponding to a molecular mass of approx. 60 kDa, as judged from the elution positions of the standard proteins used (indicated by arrows, Fig. 1b). Gel-filtration chromatography was performed in a buffer allowing a direct injection of the active fractions to the strong-anion-exchange column (Mono Q) (Fig. 1c). Of the total enzyme activity applied, 80% was eluted as one sharp peak (Fig. 1c, peak 6.95, indicated with an arrow) with only a slight tailing into another fraction (peak 7.32).

The Mono Q peak 6.95, containing most of the AGA activity, was further purified on a strong-cation-exchange column (Mono S) on h.p.l.c. Total enzyme activity of AGE was also eluted as one sharp peak in this purification step, resulting in 6600 × final purification of the enzymically active AGA protein (Fig. 1d, peak 2.18, indicated by an arrow).

Purity of AGA preparation

Analysis of the enzymically active fraction of the final purification step, Mono S, by SDS/PAGE, resulted in three non-identical protein bands with approx. molecular masses of 24, 18 and 17 kDa (Fig. 2, lane b).

In a native PAGE performed at pH 8.6, only one protein band could be detected (Fig. 3, lane b). Electroelution of this single protein band from the gel resulted in 50% recovery of the enzyme activity. The three protein bands with molecular masses of 24, 18 and 17 kDa were again detected when the elution product was analysed on denaturing SDS/PAGE. Also in a chromatofocusing h.p.l.c., the purified AGA protein was eluted as single enzymically active peak with a pI of 5.7 (Fig. 1f). In an SDS/PAGE analysis, we again verified that this fraction contained all three polypeptide chains with molecular masses 24, 18 and 17 kDa.

Further analysis of the purified AGA protein in reverse-phase h.p.l.c. resulted in the inactivation of the enzyme and separation of two polypeptide fractions (Fig. 1e, peak 20.88 and peak 25.14, indicated by arrows). When these two fractions were analysed by SDS/PAGE,

Fig. 1. Purification of AGA

(a) ConA-Sepharose affinity chromatography of the human liver extract. Elution of adsorbed material was performed with 0.6 M α -methylglucoside. The elution was monitored at 280 nm. Fractions (3.6 ml) were collected and analysed for enzyme activity (bars). (b) Chromatography of the enzymically active fractions from the ConA-Sepharose chromatograph on a BioGel P-100 gel filtration column. Elution was monitored at 280 nm and the enzyme activity of the collected fractions (3.5 ml) was measured (bars). The positions of the molecular mass markers bovine serum albumin (BSA), ovalbumin (Ova) and lysozyme (Lys) are indicated by arrows. (c) Strong-anion-exchange h.p.l.c. of the enzymically active fractions from the BioGel P-100 column. The chromatography was performed with a gradient of sodium acetate in 20 mM-triethanolamine acetate, pH 7.2. The enzymically active peak (6.95) is indicated by an arrow. (d) Strong-cation-exchange h.p.l.c. of the enzymically active peak (6.95) from the Mono Q run. The sample was adjusted to pH 5.5 with acetic acid and applied to the column equilibrated with 50 mM-sodium acetate, pH 5.5. The chromatography was performed with a linear gradient of sodium acetate in the equilibration buffer. The enzymically active fraction is indicated by an arrow. (e) Reverse-phase h.p.l.c. on a Vydac 218 TP 54 column of the enzymically active peak (2.18) from the Mono S run. The chromatography was performed with an increasing amount of acetonitrile in 0.1% trifluoroacetic acid in water. The separated polypeptides are indicated by arrows and the contaminating impurities from the water by asterisks. (f) Chromatofocusing of the enzymically active peak (2.18) from the Mono S run. The column was equilibrated with 25 mM-Bistris/HCl, pH 7.2, and the elution performed with 40 ml of Polybuffer 96 (pH range 7–4). The fractions were measured for the pH (●) and the enzyme activity (bars).

one fraction (20.88) contained the 18 and 17 kDa polypeptide fractions (Fig. 4, lane b), and the other (25.14) the 24 kDa polypeptide (Fig. 4, lane c). The activity of the AGA protein could not be recovered by combining the fractions separated by reverse-phase h.p.l.c. under neutral conditions.

A similar separation of two inactive polypeptide fractions could be achieved when the purified AGA protein was applied to the strong-cation-exchange h.p.l.c. (Mono S) at a pH lower than 5.5. The 18 and 17 kDa polypeptides eluted again as a separate fraction from the 24 kDa polypeptide, and the enzyme was inactivated. The

Fig. 2. SDS/PAGE of purified AGA

Lane a, molecular mass standards; lane b, Mono S strong-cation-exchange h.p.l.c. peak 2.18 from Fig. 1(d). Bands were revealed by silver staining.

Fig. 3. PAGE of purified AGA under native conditions

Lane a, molecular mass standard; lane b, Mono S strong-cation-exchange h.p.l.c. peak 2.18 from Fig. 1(d). Bands were revealed by Coomassie Brilliant Blue staining.

Fig. 4. SDS/PAGE of polypeptide fractions from inactivated AGA

Lane a, molecular mass standards; lane b, reverse-phase h.p.l.c. peak 20.88 from Fig. 1(e); lane c, reverse-phase h.p.l.c. peak 25.14 from Fig. 1(e). Bands were revealed by Coomassie Brilliant Blue staining.

amount of inactivation, when the Mono S run was performed at various pH values from 6.0 to 5.0, correlated directly with the amount of separated polypeptide fractions.

Thermostability, pI and pH optimum of the purified AGA

The thermostability of AGA was remarkable. After having established the approximate thermostability of the purified enzyme protein by incubation at various temperatures (30–90 °C) for 1, 2, 3 and 4 h, we examined the crude liver homogenate, the Mono-Q-partially-purified enzyme fraction and the Mono-S-purified AGA protein in more detailed thermostability studies. The fractions were incubated for 10 min at various temperatures (60–80 °C) followed by incubation for 2 h at 50 °C. All fractions examined were active up to 68 °C (± 1 °C) but were practically inactive at 72 °C (± 1 °C) (Fig. 5a). Of the initial activity (4 h at 37 °C), 43% could be measured after prolonged incubation at 60 °C (45 h). Incubating the enzyme protein with the substrate did not stabilize the enzyme. We could not show the existence of a stabilizing agent as proposed by McGovern *et al.* (1983).

A pI of 5.7 was established by analysing small amounts (e.g. 100–500 pmol) of the purified enzyme protein in chromatofocusing h.p.l.c. The effect of pH on AGA

activity was measured by incubating the enzyme at various pH values, ranging from 4.0 to 10.5. A pH optimum close to 6 was demonstrated, and the enzyme was practically inactive at pH values below 4 (Fig. 5b).

Fig. 5. Effects of temperature (a) and pH (b) on AGA activity

(a) Purified AGA enzyme was incubated without the substrate 2-acetamido-1- β -(L-aspartamido)-1,2-dideoxy-D-glucose for 10 min at various temperatures (60–80 °C). The substrate was then added and the incubation was continued for 2 h at 50 °C; the enzyme activity was then measured. 100 % of the enzyme activity (control) is defined as the activity measured after incubation for 2 h at 50 °C. (b) Purified AGA enzyme was incubated for 4 h at 37 °C at various pH values using 100 mM concentrations of citrate/phosphate buffer at pH 3–7, phosphate buffer at pH 8–10 and glycine/NaOH buffer at pH 10.5 in the incubation mixture. The enzyme activity was then measured. The pH was determined in the final assay mixture. 100 % of the enzyme activity (control) is defined as the activity measured after incubation for 4 h at 37 °C using 50 mM-potassium phosphate buffer, pH 6.1, as the incubation buffer.

However, about 30 % of the initial activity remained when the purified enzyme protein was incubated at basic pH values, at least up to pH 10.5 (Fig. 5b).

DISCUSSION

AGA is an essential enzyme in the catabolism of glycoproteins in mammalian cells. Deficiency of the enzyme in man causes lysosomal accumulation of non-degraded glycoasparagines, which then leads to aspartylglucosaminuria.

So far we have succeeded in purifying only extremely small amounts of AGA from human liver (Table 1). Our preliminary data on the isolation of the enzyme from human white blood cells suggests that the amount of AGA in these cells is also extremely low (M. Baumann & N. Kalkkinen, unpublished work).

AGA has previously been purified to apparent homogeneity from human liver by several investigators (Conchie & Strachan, 1969; Savolainen, 1976; Dugal & Strömme, 1977; McGovern *et al.*, 1983). The extremely small amount of the active enzyme present in human liver appears to have prevented detailed analysis of the AGA protein in earlier studies. According to the report of Savolainen (1976), only one band separated by PAGE, with a molecular mass of 150 kDa, showed any 2-acetamido-1- β -(L-aspartamido)-1,2-dideoxy-D-glucose cleaving activity after its extraction from the gel. Dugal & Strömme (1977) reported the separation of eight protein bands on PAGE of a 17 500-fold purified enzyme fraction. They did not report how many of these eight protein bands had cleavage activity. Dugal (1978) reported an active enzyme fraction with a molecular mass of 63 kDa as judged from gel filtration. McGovern *et al.* (1983) purified a protein fraction from human liver with AGA activity and a molecular mass of 80 kDa as determined by gel filtration and SDS/PAGE analysis. This enzyme preparation was considered to be a monomer under denaturing conditions.

With the techniques described here, we have now obtained evidence that human AGA is a protein complex of approx. 60 kDa consisting of three non-identical polypeptides of 24, 18 and 17 kDa. Enzyme activity was demonstrated only in protein preparations with all three polypeptide chains present.

The enzyme protein migrated as one protein band in a native PAGE run at pH 8.6 when purified with ConA-Sepharose affinity chromatography and molecular sieve chromatography followed by strong-anion- and strong-

Table 1. Purification of AGA from newborn human liver tissue

Procedure	Protein concn. (mg/ml)	Enzyme concn. (nkat*/ml)	Specific activity (nkat/mg)	Yield (%)	Purity (-fold)
Cell extract	72.0	2170	30	100	1
ConA-Sepharose	2.6	20040	7680	31	250
BioGel P-100	0.2	5840	29830	29	970
Mono Q	0.04	3000	75150	14	2500
Mono S	0.004	800	198340	2	6600

* 1 kat is the enzyme activity which is needed to transform 1 mol of substrate per second at 37 °C to its product.

cation-exchange columns, or by chromatofocusing h.p.l.c.

In a denaturing SDS/PAGE, the purified AGA protein either recovered from the native PAGE or eluted from the chromatofocusing h.p.l.c. behaved identically, resulting in the same three polypeptide chains. In addition, separation of the same three polypeptide chains occurred if the strong-cation-exchange h.p.l.c. was performed at a pH below 5.5, as well as in the reverse-phase h.p.l.c. Both procedures resulted in the inactivation of AGA. The decrease of activity at acidic pH correlates perfectly with the amount of degraded protein complex.

At present, the detailed structure and organization of the human hepatic AGA protein in tissues is partially unclear. We were not able to dissociate the three polypeptide chains found in this study without losing the enzyme activity, but whether all three polypeptide chains represent true subunits of the enzyme molecule or merely products of protein degradation inside the lysosomes remains to be clarified. No differences in the polypeptide-band pattern of the purified AGA protein could be demonstrated by SDS/PAGE analysis under reduced and non-reduced conditions. This and several other observations described in this paper all demonstrate that no disulphide bonds are involved in the quaternary structure of the complex.

The purified enzyme complex could also bind to the mannose-6-phosphate 215 kDa cation-independent receptor, resulting in a decrease of approx. 50% of the enzymic activity (M. Baumann & V. Marjomäki, unpublished work).

All lysosomal enzymes that have been studied so far are synthesized as precursors that are processed to mature forms of lower molecular mass (Tager, 1985; von Figura & Hasilik, 1986). The possibility of lysosomal enzymes requiring 'protective' proteins for their catalytic activity has also been suggested (Verheijen *et al.*, 1985).

The finding of only one chromosomal location for AGA (Aula *et al.*, 1984) suggests that the active enzyme molecule is coded at one locus in the human genome. This speaks more for the degradation-sensitive sites at the 60 kDa AGA protein than for the subunit structure. In our experiments, using embryonic, newborn and adult livers, obtained at various times after death, we could not find any other fraction with AGA activity, indicating that the precursor of this enzyme is not active as has also been shown for other lysosomal enzymes (Hasilik *et al.*, 1982). The 40-fold higher specific activity when compared with the previous purifications described (Conchie & Strachan, 1969; Savolainen, 1976; Dugal & Strömme, 1977; McGovern *et al.*, 1983) clearly demonstrates the higher purity of our enzyme protein. This homogeneous

AGA protein will facilitate studies on the molecular and cellular pathology in aspartylglucosaminuria.

We thank V. Marjomäki (University of Jyväskylä, Jyväskylä, Finland) for giving us the purified 215 kDa cation-independent receptor.

REFERENCES

- Allington, W. B., Cordry, A. L., McCullough, G. A., Mitchell, D. E. & Nelson, J. W. (1978) *Anal. Biochem.* **85**, 188–196
- Aula, P., Astrin, K. H., Francke, V. & Desnick, R. J. (1984) *Am. J. Hum. Genet.* **36**, 1215–1224
- Autio, S. (1972) *J. Ment. Defic. Res. Monogr. Ser I*, 1–39
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Conchie, J. & Strachan, I. (1969) *Biochem. J.* **115**, 709–715
- Dugal, B. (1977) *Biochem. J.* **163**, 9–14
- Dugal, B. (1978) *Biochem. J.* **171**, 799–802
- Dugal, B. & Strömme, J. (1977) *Biochem. J.* **165**, 497–502
- Hasilik, A., von Figura, K., Conzelmann, E., Nehrkorn, H. & Sandhoff, K. (1982) *Eur. J. Biochem.* **125**, 317–321
- Jenner, F. A. & Pollitt, R. J. (1967) *Biochem. J.* **103**, 48P–49P
- Kalkkinen, N., Jörnval, H., Söderlund, H. & Kääriäinen, L. (1980) *Eur. J. Biochem.* **108**, 31–37
- Lundblad, A., Mason, P. K., Nordén, N. E., Svensson, S., Ockerman, P.-A. & Palo, J. (1976) *Eur. J. Biochem.* **67**, 209–214
- Mahadevan, S. & Tappel, L. (1967) *J. Biol. Chem.* **242**, 4568–4576
- Makino, M., Kojima, T. & Yamashina, J. (1966) *Biochem. Biophys. Res. Commun.* **24**, 961–966
- Maury, P. (1980) *J. Lab. Clin. Med.* **96**, 838–840
- McGovern, M. M., Aula, P. & Desnick, R. J. (1983) *J. Biol. Chem.* **258**, 10743–10747
- Neuberger, A., Gottschalk, A. & Marshall, R. D. (1966) in *Glycoproteins* (Gottschalk, A., ed.), pp. 273–295, Elsevier, Amsterdam
- Palo, J., Riekkinen, P., Arstila, A. & Autio, S. (1971) *Neurology* **21**, 1198–1204
- Palo, J., Riekkinen, P., Arstila, A. U., Autio, S. & Kivimäki, T. (1972) *Acta Neuropathol.* **20**, 217–224
- Palo, J., Pollitt, R. J., Pretty, K. M. & Savolainen, H. (1973) *Clin. Chim. Acta* **47**, 69–74
- Pollitt, R. J., Jenner, F. A. & Merskey, H. (1968) *Lancet* **ii**, 253–255
- Savolainen, H. (1976) *Biochem. J.* **153**, 749–750
- Tager, J. M. (1985) *Trends Biochem. Sci.* **10**, 324–326
- Tunon, P. & Johansson, K.-E. (1984) *J. Biochem. Biophys. Methods* **9**, 171–179
- Verheijen, F. W., Palmeri, S., Hoogeveen, A. T. & Galjaard, H. (1985) *Eur. J. Biochem.* **149**, 315–321
- von Figura, K. & Hasilik, A. (1986) *Annu. Rev. Biochem.* **55**, 167–193