Biochem. J. (1991) 277, 281–284 (Printed in Great Britain)

Requirements for the inactivation of ribosomes by gelonin

Simonetta SPERTI, Maurizio BRIGOTTI, Mariacristina ZAMBONI, Domenica CARNICELLI and Lucio MONTANARO*

Dipartimento di Patologia sperimentale dell'Università di Bologna, Via S. Giacomo 14, 40126 Bologna, Italy

Inactivation of *Artemia salina* and rabbit ribosomes by gelonin requires ATP and a high-M_r factor present in the rabbit reticulocyte-lysate post-ribosomal supernatant. The kinetic constants of the gelonin-catalysed release of adenine from *A. salina* ribosomes are $K_m = 4.35\ \mu$M and $K_{cat.} = 0.1\ min^{-1}$ in the absence of cofactors, and $K_m = 1.15\ \mu$M and $K_{cat.} = 108\ min^{-1}$ in their presence. The last two values are similar to those measured for ricin A chain in the absence of cofactors ($K_m = 2.02\ \mu$M and $K_{cat.} = 317\ min^{-1}$).

INTRODUCTION

Gelonin (M_r 30500), isolated from the seeds of *Gelonium multiflorum* (Stirpe *et al.*, 1980), belongs to the large family of ribosome-inactivating proteins (RIPs), proteins of both plant and bacterial origin which catalytically and irreversibly inactivate the 60 S subunit of eukaryotic ribosomes. RIPs are traditionally considered inhibitors of the elongation step of protein synthesis (Vázquez, 1979), and gelonin, like other RIPs, has *N*-glycosidase activity, specifically cleaving an adenine bond in 28 S RNA (Endo *et al.*, 1988; Stirpe *et al.*, 1988) at the level of a highly conserved region which appears involved in the interaction of ribosomes with elongation factors (Moazed *et al.*, 1988).

Several reports indicate that gelonin, when assayed on the rabbit reticulocyte-lysate system translating endogenous mRNA, has the same capacity to stop protein synthesis as other RIPs (Stirpe *et al.*, 1980; Endo *et al.*, 1988; Brigotti *et al.*, 1989). The activity of gelonin is instead about 500-fold lower than that of ricin A chain and many other RIPs when tested on a poly(U)-directed system containing isolated ribosomes (Brigotti *et al.*, 1989; Cenini *et al.*, 1990). The present work was undertaken with the aim of explaining the different susceptibility to gelonin of ribosomes in the reticulocyte lysate system and in that, more fractionated, translating poly(U).

MATERIALS AND METHODS

Gelonin and ricin were generously given by Professor F. Stirpe of this Department. All dilutions of gelonin were in 1% BSA. Ricin A chain was prepared as previously described (Sperti *et al.*, 1986). Rabbit globin mRNA and Dowex 1 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were as previously described (Brigotti *et al.*, 1989).

Artemia salina 80 S ribosomes and 'S-105' [proteins precipitated from the post-ribosomal supernatant by 75%-satd.-$(NH_4)_2SO_4$] were prepared from undeveloped cysts by the method of Sierra *et al.* (1974). The unfractionated rabbit reticulocyte lysate was prepared as described by Allen & Schweet (1962). The post-ribosomal supernatant (indicated throughout the paper as 'S-140') was obtained from the lysate supplemented with 25 μM-haemin and centrifuged for 2 h at 140000 *g*. The sedimented polysomes were used for the preparation of a ribosomal salt-wash fraction rich in initiation factors (Morrisey & Hardesty, 1972). Where indicated, 'S-140' was gel-filtered through Sephadex G-25 (Jackson & Hunt, 1983); its protein concentration (22 mg/ml) was 37% of that of the parent 'S-140'. Rabbit reticulocyte ribosomes were isolated from the unfractionated lysate preincubated for 7 min at 28 °C in the mixture for the assay of endogenous mRNA translation described by Brigotti *et al.* (1989), modified to contain non-radioactive leucine. The procedure inactivates initiation factor(s) (Jackson & Hunt, 1983), which are naturally lacking in *A. salina* ribosomes (Sierra *et al.*, 1974). At the end of incubation, the rabbit ribosomes were sedimented through 0.5 M-sucrose containing 30 mM-Hepes, pH 7.6, 70 mM-KCl, 2 mM-magnesium acetate and 1 mM-dithiothreitol. When required, KCl-washed ribosomes were obtained by a further sedimentation through a discontinuous sucrose gradient consisting of 4 ml of 1.0 M-sucrose and 4 ml of 0.7 M-sucrose, both containing 30 mM-Hepes, pH 7.6, 2 mM-magnesium acetate and 1 mM-dithiothreitol; KCl was 0.5 M in the 0.7 M-sucrose layer and 70 mM in the 1 M-sucrose layer. The systems for the translation of poly(U) and of added rabbit globin mRNA are described in the legends to Tables. Assays were performed in duplicate, and values in Tables are representative of at least four independent experiments which gave consistent results. The adenine released from ribosomes by the *N*-glycosidase activity of RIPs was converted into its etheno derivative and measured by h.p.l.c. as previously described (Zamboni *et al.*, 1989). When the activity of gelonin was assayed in the presence of 'S-140' and ATP, minor modifications had to be introduced: the enzymic reaction was stopped with trichloroacetic acid, which was then extracted with diethyl ether (McCann *et al.*, 1985); at this stage, before formation of the etheno derivative, ATP was completely removed from the samples by treating them with 10 mg of Dowex 1 (formate form). Protein was measured by the method of Lowry *et al.* (1951).

RESULTS

Table 1 shows the effect of ricin A chain and gelonin added to mRNA- or poly(U)-translating systems containing either *A. salina* or rabbit reticulocyte ribosomes. Both RIPs at the concentration of 0.5 nM strongly inhibited the translation of exogenous globin mRNA catalysed by rabbit 'S-140' and initiation factors. In contrast, ricin A chain, but not gelonin, inhibited poly(U) translation measured, as described by Brigotti *et al.* (1989) and by Cenini *et al.* (1990), with $tRNA^{Phe}$ pre-charged with [^{14}C]phenylalanine and *A. salina* 'S-105' as the source of elongation factors.

In the above assays, translation of poly(U) was carried out at 7 mM-Mg^{2+}, and that of mRNA at 2.5 mM-Mg^{2+}. Since it has been reported that high concentrations of Mg^{2+} protect ribosomes from inactivation by some RIPs (Hedblom *et al.*, 1978; Cawley

Abbreviation used: RIP, ribosome-inactivating protein.
* To whom correspondence should be addressed.

Table 1. Effect of gelonin and ricin A chain on mRNA and poly(U) translation by *A. salina* and rabbit reticulocyte ribosomes

The assays of protein synthesis were as follows. mRNA translation: 62.5 μl of 10 mM-Tris/HCl, pH 7.4, containing 100 mM-ammonium acetate, 2 mM-magnesium acetate, energy mixture (1 mM-ATP, 0.2 mM-GTP, 15 mM-phosphocreatine and 48 μg of creatine kinase/ml), 0.05 mM amino acids (minus leucine), 0.1 μCi of L-[^{14}C]leucine, 0.35 μg of rabbit globin mRNA, 25 μl of rabbit 'S-140', 37 μg (as protein) of the ribosomal salt-wash fraction and 2.5 pmol of ribosomes; after 1 h incubation at 28 °C, 1 ml of 0.1 M-KOH was added, the samples were decolorized with two drops of 35% (w/v) H_2O_2 and the acid-insoluble radioactivity was measured. Poly(U) translation: 100 μl of 80 mM-Tris/HCl, pH 7.4, containing 120 mM-KCl, 7 mM-magnesium acetate, 2 mM-dithiothreitol, 2 mM-GTP, 22 pmol of [^{14}C]phenylalanyl-tRNA, 80 μg of poly(U), 90 μg (as protein) of *A. salina* 'S-105' and 2.5 pmol of ribosomes; after 20 min at 28 °C, the hot-acid-insoluble radioactivity was measured. Values in parentheses are percentage inhibitions.

	[^{14}C]Amino acid incorporated (d.p.m.)	
Ribosomes from...	*A. salina*	Rabbit reticulocytes
mRNA translation		
No inhibitor	670	3598
Gelonin (0.5 nM)	210 (69)	1053 (71)
Ricin A chain (0.5 nM)	134 (80)	683 (81)
Poly(U) translation		
No inhibitor	7787	6715
Gelonin (150 nM)	8013	6884
Ricin A chain (0.5 nM)	2370 (70)	2031 (70)

Table 2. Inhibition of poly(U) translation by gelonin

The assay was as follows: 62.5 μl of 10 mM-Tris/HCl, pH 7.4, containing 100 mM-ammonium acetate, 7 mM-magnesium acetate, energy mixture (1 mM-ATP, 0.2 mM-GTP, 15 mM-phosphocreatine and 48 μg of creatine kinase/ml), 0.05 mM amino acids (minus phenylalanine), 0.15 μCi of L-[^{14}C]phenylalanine, 80 μg of poly(U), 25 μl of rabbit 'S-140' and 2.5 pmol of ribosomes. Incubation was for 1 h at 28 °C. In parallel assays without poly(U) no label was incorporated by *A. salina* ribosomes; the phenylalanine incorporated by rabbit reticulocyte ribosomes (564 and 310 d.p.m. in the absence and in the presence of gelonin, respectively) was subtracted. Values in parentheses are percentage inhibitions.

	[^{14}C]Phenylalanine incorporated (d.p.m.)	
Gelonin Ribosomes from...	*A. salina*	Rabbit reticulocytes
Absent	3662	13712
Present (0.5 nM)	1501 (59)	8227 (40)

Table 3. Requirements for the inhibition of poly(U) translation by gelonin

A. salina ribosomes (20 pmol) were preincubated in the absence and in the presence of 5 nM-gelonin for 10 min at 28 °C in 20 μl of 10 mM-Tris/HCl, pH 7.4, 100 mM-ammonium acetate and 2 mM-magnesium acetate containing phosphocreatine (PC, 15 mM), creatine kinase (PCK, 48 μg/ml), ATP (1 mM), GTP (either 0.2 mM or 1 mM) and 1 μl of gel-filtered rabbit 'S-140' (22 μg as protein) as indicated. At the end of preincubation, samples containing 2.5 pmol of ribosomes were withdrawn, adjusted to 7 mM-magnesium acetate, supplemented with poly(U), L-[^{14}C]phenylalanyl-tRNA and *A. salina* 'S-105', and assayed for poly(U) translation in the 100 μl system described in Table 1. Values in parentheses are percentage inhibitions.

	[^{14}C]Phenylalanine incorporated (d.p.m.)	
Additions to preincubation Gelonin...	Absent	Present
'S-140', ATP, 0.2 mM-GTP, PC/PCK	7913	508 (94)
ATP, 0.2 mM-GTP, PC/PCK	8218	8113 (1)
'S-140'	8281	7395 (11)
'S-140', PC/PCK	8112	4299 (47)
'S-140', ATP	7799	517 (93)
'S-140', ATP, PC/PCK	7518	601 (92)
'S-140', 1 mM-GTP	8479	2842 (66)
'S-140', 0.2 mM-GTP, PC/PCK	7893	1367 (83)

et al., 1979; Rodes & Irvin, 1981), the possibility of a protective effect of Mg^{2+} in the poly(U)-directed system was investigated. A non-enzymic initiation complex between aminoacyl-tRNA, poly(U) and *A. salina* ribosomes was formed during a preliminary incubation at 7 mM-Mg^{2+} (Mosteller *et al.*, 1968; Konecki *et al.*, 1975). *A. salina* 'S-105' and GTP were then added, and the Mg^{2+}

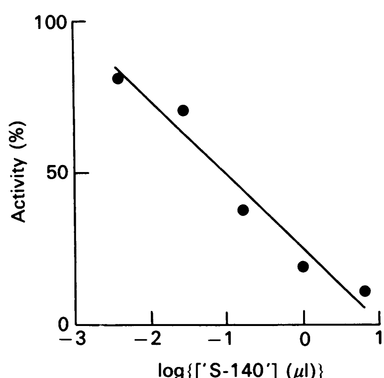

Fig. 1. Inactivation of ribosomes by gelonin as a function of gel-filtered rabbit 'S-140'

A. salina ribosomes (20 pmol in 20 μl) were preincubated with 5 nM-gelonin in the presence of 1 mM-ATP, 0.2 mM-GTP, 15 mM-phosphocreatine, 48 μg of creatine kinase/ml and different amounts of gel-filtered rabbit 'S-140' (0.0036–6.5 μl). Preincubation and the subsequent assay of poly(U) translation were as described in Table 3.

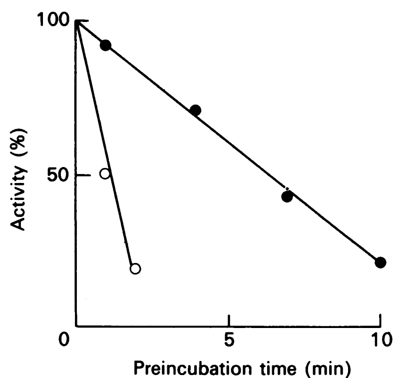

Fig. 2. Effect of preincubation of ribosomes with rabbit 'S-140' on the rate of inactivation by gelonin

A. salina ribosomes (30 pmol in 30 μl) were preincubated in the presence of ATP, GTP, phosphocreatine, creatine kinase and gel-filtered rabbit 'S-140' (0.3 μl) as described in the legend to Fig. 1: ●, 5 nM-gelonin added at the beginning of preincubation; ○, 5 nM-gelonin added after 10 min of preincubation. At different times from the addition of gelonin, samples containing 2.5 pmol of ribosomes were withdrawn and assayed for poly(U) translation as described in Table 3. Inactivation of ribosomes is expressed as percentage of poly(U) translation by controls parallely run in the absence of gelonin.

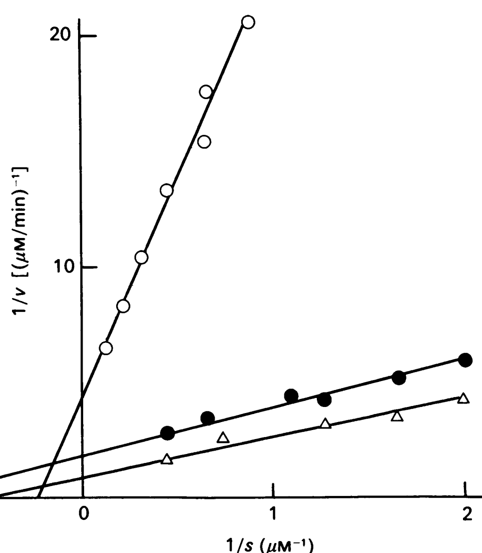

Fig. 3. Lineweaver–Burk plot of the initial velocity of adenine release from *A. salina* ribosomes catalysed by gelonin and by ricin A chain

○, *A. salina* ribosomes (1.1–6 μM) in 200 μl of 80 mM-Tris/HCl (pH 7.4)/120 mM-KCl/7 mM-magnesium acetate/2 mM-dithiothreitol were incubated for 10 min at 24 °C with 2.9 μM-gelonin. △, *A. salina* ribosomes (0.5–2.2 μM) were incubated for 1 min at 24 °C with 3.7 nM-ricin A chain in 200 μl of the same buffer. ●, *A. salina* ribosomes (0.5–2.2 μM) were preincubated for 10 min at 24 °C in 200 μl of 10 mM-Tris/HCl (pH 7.4)/100 mM-ammonium acetate/2 mM-magnesium acetate containing ATP, GTP, phosphocreatine, creatine kinase and gel-filtered rabbit 'S-140' (2 μl) as described in the legend to Fig. 2; gelonin (5 nM) was then added and incubation was allowed to proceed for 2 min at 24 °C.

concentration was either maintained at 7 mM or lowered to 2.5 mM by dilution. The subsequent phenylalanine polymerization was not inhibited by gelonin (150 nM) at either concentration of Mg^{2+} (results not shown). Replacement of K^+ with NH_4^+ slightly increased the sensitivity of the poly(U)-translating system to gelonin [IC_{50} (concn. giving 50% inhibition) = 145 nM]. However, the inhibitor remained completely ineffective at a concentration of 0.5 nM.

In contrast, as shown in Table 2, 0.5 nM-gelonin did inhibit poly(U) translation, by ribosomes from both *A. salina* and rabbit reticulocytes, when the assay was performed in a mixture that mimicked that used for the translation of rabbit globin mRNA in Table 1. The main differences with respect to the previous poly(U)-translation assay were the replacement of *A. salina* 'S-105' with rabbit 'S-140' and that of [^{14}C]phenylalanyl-tRNA with [^{14}C]phenylalanine, ATP and energy-regenerating components. Both substitutions were necessary. When, in the same system, *A. salina* 'S-105' was the source of enzymes, no inhibition was observed with *A. salina* ribosomes; with rabbit ribosomes a slight inhibition occurred, which was no longer observed when KCl-washed ribosomes were used (results not shown).

In order to identify the factors responsible for the inhibition of poly(U) translation by gelonin, 'S-140' was depleted of low-M_r components by gel filtration, and a two-step experiment was performed in which *A. salina* ribosomes (1 pmol/μl) were pretreated with gelonin (5 nM) in the absence and in the presence of gel-filtered 'S-140' and of the various components of the energy mixture in different combinations. After preincubation, samples containing 2.5 pmol of ribosomes were withdrawn and assayed for poly(U) translation in the 100 μl system described in Table 1. As shown in Table 3, in the absence of 'S-140' ribosomes were not inactivated by gelonin during preincubation. The presence of 'S-140' alone had little effect, whereas the simultaneous addition to the preincubation mixture of 'S-140' and ATP or GTP made ribosomes extremely susceptible to the inactivation by gelonin. The moderate effect of phosphocreatine and creatine kinase in the absence of nucleoside triphosphates may be ascribed to trace amounts of ATP and ADP strongly bound to proteins of the lysate and not completely removed by the gel-filtration procedure.

Thus, for inactivation of ribosomes by gelonin, two factors are required, a high-M_r component present in the rabbit 'S-140' and ATP (or GTP, which was 60% as effective as ATP; see Table 3). Fig. 1 shows the relationship between the amount of gel-filtered 'S-140' in the preincubation mixture and the inactivation of ribosomes by gelonin. A 50% inactivation of ribosomes by gelonin was obtained with 0.08 μl of 'S-140', equivalent to 1.8 μg of protein. This very low value, obtained with a crude protein preparation, suggests that an enzymic activity is involved. Since,

Table 4. K_m and $K_{cat.}$ of gelonin and ricin A chain

The kinetic constants were calculated from the double-reciprocal plots of Fig. 3.

	Substrate	K_m (μM)	$K_{cat.}$ (min^{-1})
Gelonin	*A. salina* ribosomes	4.35	0.1
Gelonin	*A. salina* ribosomes preincubated with 'S-140'	1.15	108
Ricin A chain	*A. salina* ribosomes	2.02	317

as shown in Fig. 2, pretreatment of ribosomes with 'S-140' and ATP greatly accelerates the rate of their subsequent inactivation by gelonin, this enzymic activity appears directed towards ribosomes and not gelonin itself.

In order to quantify the effect of 'S-140' and ATP on the *N*-glycosidase activity of gelonin, ribosomes, at concentrations from 0.5 to 6 μM, were incubated with gelonin in the absence and in the presence of added cofactors, and the amount of adenine released was measured by h.p.l.c. The time of incubation was such that the initial velocity of the reaction was assessed. From the double-reciprocal plots of the data (Fig. 3), the apparent Michaelis constants (K_m) and the turnover numbers (no. of molecules of substrate transformed/min per molecule of enzyme; $K_{cat.}$) reported in Table 4 were calculated. Although in the absence of 'S-140' and ATP the values are only an approximation, since gelonin had to react with ribosomes in non-catalytic conditions in order to obtain a measurable release of adenine (Zamboni *et al.*, 1989), the great divergence in $K_{cat.}$ and the similarity of K_m in the absence and in the presence of added cofactors suggest that 'S-140' and ATP modify the speed of ribosome inactivation rather than the affinity of the ribosome–gelonin interaction. In the presence of cofactors, gelonin becomes almost as effective as ricin A chain in depurinating ribosomes (Fig. 3 and Table 4).

DISCUSSION

The present observations are akin to older evidence obtained with tritin, the RIP from *Triticum aestivum* (wheat germ) and with pokeweed antiviral protein (PAP), the RIP from *Phytolacca americana*. The sensitivity of poly(U) translation to added tritin was greatly increased when ribosomes from Ehrlich ascites cells were preincubated with ATP and tRNA, and the effect was mediated by a ribosome-bound factor (that was neither EF1 nor EF2), which could be removed from ribosomes by high-salt washing (Coleman & Roberts, 1981). Similarly, the presence of ATP and of a post-ribosomal supernatant was required during the preincubation of wheat germ and *A. salina* ribosomes with PAP in order to obtain their inactivation in subsequent poly(U) translation (Ready *et al.*, 1983). This requirement for ATP and for extra-ribosomal protein(s) is highly suggestive of the involvement of a protein kinase activity. Preliminary experiments in our laboratory indicate that gelonin itself is not phosphorylated in the presence of 'S-140' and [γ-^{32}P]ATP. The possibility that phosphorylation of a ribosomal protein might be responsible for the increased susceptibility of ribosomes to gelonin is attractive.

Whatever the mechanism, the present data and the above-reported evidence obtained with tritin and PAP clearly indicate that inhibition of protein synthesis by some RIPs requires conditions more stringent than those present in an assay containing only the factors strictly necessary for poly(U) translation. This assay has been used in a large work of screening of ribosomes isolated from different species for their sensitivity to RIPs (Stirpe & Hughes, 1989; Cenini *et al.*, 1990). It is noteworthy that, of seven metazoan species whose ribosomes were tested (among these, rabbit reticulocyte and *A. salina* ribosomes), seven proved to be highly sensitive to ricin A chain and not one to gelonin. If assayed in a protein-synthesizing system containing the appropriate cofactors, the results would probably have been quite different.

This work was supported by a grant from Consiglio Nazionale delle Richerche, Rome, Italy (89.02698.04).

REFERENCES

Allen, E. H. & Schweet, R. S. (1962) J. Biol. Chem. **237**, 760–767
Brigotti, M., Rambelli, F., Zamboni, M., Montanaro, L. & Sperti, S. (1989) Biochem. J. **257**, 723–727
Cawley, D. B., Hedblom, M. L. & Houston, L. L. (1979) Biochemistry **18**, 2648–2654
Cenini, P., Carnicelli, D. & Stirpe, F. (1990) Comp. Biochem. Physiol. **96B**, 581–584
Coleman, W. H. & Roberts, W. K. (1981) Biochim. Biophys. Acta **654**, 57–66
Endo, Y., Tsurugi, K. & Lambert, J. M. (1988) Biochem. Biophys. Res. Commun. **150**, 1032–1036
Hedblom, M. L., Cawley, D. B., Boguslawski, S. & Houston, L. L. (1978) J. Supramol. Struct. **9**, 253–268
Jackson, R. J. & Hunt, T. (1983) Methods Enzymol. **96**, 50–74
Konecki, D., Kramer, G., Pinphanichakarn, P. & Hardesty, B. (1975) Arch. Biochem. Biophys. **169**, 192–198
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
McCann, W. P., Hall, L. M., Siler, W., Barton, N. & Whitley, R. (1985) Antimicrob. Agents Chemother. **28**, 265–273
Moazed, D., Robertson, M. J. & Noller, H. F. (1988) Nature (London) **334**, 362–364
Morrisey, J. & Hardesty, B. (1972) Arch. Biochem. Biophys. **152**, 385–397
Mosteller, R. D., Culp, W. J. & Hardesty, B. (1968) J. Biol. Chem. **243**, 6343–6352
Ready, M., Bird, S., Rothe, G. & Robertus, J. D. (1983) Biochim. Biophys. Acta **740**, 19–28
Rodes, T. L., III & Irvin, J. D. (1981) Biochim. Biophys. Acta **652**, 160–167
Sierra, J. M., Meier, D. & Ochoa, S. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 2693–2697
Sperti, S., Montanaro, L. & Rambelli, F. (1986) Biosci. Rep. **6**, 1035–1040
Stirpe, F. & Hughes, R. C. (1989) Biochem. J. **262**, 1001–1002
Stirpe, F., Olsnes, S. & Pihl, A. (1980) J. Biol. Chem. **255**, 6947–6953
Stirpe, F., Bailey, S., Miller, S. P. & Bodley, J. W. (1988) Nucleic Acids Res. **16**, 1349–1357
Vázquez, D. (1979) Mol. Biol. Biochem. Biophys. **30**, 67–83
Zamboni, M., Brigotti, M., Rambelli, F., Montanaro, L. & Sperti, S. (1989) Biochem. J. **259**, 639–643

Received 14 January 1991/26 April 1991; accepted 7 May 1991