Isolation of a mouse Theta glutathione S-transferase active with methylene chloride

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A glutathione S-transferase metabolizing methylene chloride has been isolated from mouse liver using a variety of chromatographic methods. N-terminal and internal amino acid sequences show that the enzyme, designated GST T1-1*, is closely related to the rat Theta-class GST 5-5. The mouse enzyme, molecular mass 25000 Da, has been isolated to homogeneity in active form with an approximate yield of 2% of the cytosolic activity towards methylene chloride. GST T1-1* has a specific activity of about 5.5 μmol/min per mg of protein whereas the rat GST 5-5 is reported to have a specific activity of about 11 μmol/min per mg of protein [Meyer, Coles, Pemble, Gilmore, Fraser and Ketterer (1991) Biochem. J. **274**, 409–414], demonstrating that both the rat and mouse enzymes have similar activity with this substrate. Limited evidence was obtained for a second enzyme, with a similar molecular mass (25400 Da), which had an N-terminal sequence identical to that of rat GST 12-12. This protein, which was sequenced from a band on a gel, was extremely labile and could not be isolated to homogeneity. The partially purified enzyme was not active with methylene chloride.

INTRODUCTION

When mice were exposed by inhalation to 2000 or 4000 p.p.m. of methylene chloride for 2 years an increased incidence of liver and lung tumours was seen, which was not seen in rats or hamsters exposed under the same or similar conditions [1,2]. The tumours seen in mice have been attributed to metabolites of methylene chloride produced by glutathione conjugation in a reaction catalysed by a glutathione S-transferase (GST) enzyme [3,4]. The specificity is believed to arise from the markedly higher enzyme activity found in mice than in other species including humans [5,6].

Preliminary studies in this laboratory revealed that the enzyme catalysing the conjugation of methylene chloride with glutathione was not one of the then known major classes of glutathione S-transferases (J. Nash, unpublished work). Subsequently Meyer et al. [7] identified one of the enzymes in rat liver responsible for methylene chloride metabolism as GST 5-5, a member of the Theta class [7]. In mammals, there are four distinct families of soluble GST, Alpha, Mu, Pi and Theta, with distinct catalytic and structural properties [7,8]. The Theta family has been identified relatively recently in rat and man. At present three rat Theta-class enzymes are known, 5-5, 12-12 and 13-13, and two human enzymes, T1-1 and T2-2. Theta-class transferases had not previously been isolated from the mouse. Structural information suggests that the family may be representative of an ancient progenitor GST gene as there are structural similarities to plant GSTs and a bacterial dehalogenase enzyme [9]. The Theta-class GSTs have also been linked with nuclear transferases in rat liver and with polymorphism in the human population [10,11].

Although Theta-class GSTs have been identified in rats and humans, the high activity in mice relative to the other species suggests that the mouse enzyme may be structurally different and may have a higher specific activity towards methylene chloride. Alternatively, an enzyme similar to that in the rat may be expressed to a significantly greater extent, or may be concentrated in certain cells of the mouse liver and lung. Since methylene chloride carcinogenicity is linked to high GST metabolic activity, the nature, activity and distribution of these enzymes in different species is a key part of its human safety assessment. A GST enzyme which metabolizes methylene chloride has now been isolated from mouse liver and sequenced as part of a larger study of the species-dependent tissue and cellular distributions of these enzymes.

EXPERIMENTAL

Tissues

Mouse liver was obtained from male $B_6C_3F_1$ mice (20 to 25 g) purchased from Charles River, Maidstone, Kent, U.K.

Enzyme activity

The activity of GST towards methylene chloride was determined by the formation of formaldehyde (Figure 1), which is an *in vitro* metabolic product of the conjugation of methylene chloride with

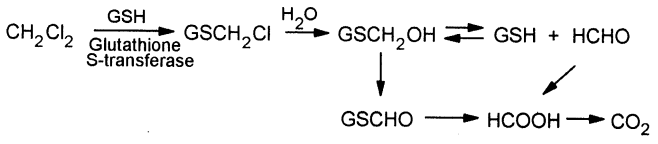

Figure 1 The metabolism of methylene chloride by glutathione S-transferases

Abbreviations used: GST, glutathione S-transferase; DTT, dithiothreitol.

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glutathione [3]. The protein samples in 200 mM Tris/HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 5 mM glutathione, were placed in sealed Reactivials with zero headspace. The vials were preincubated at 37 °C before the addition of methylene chloride to a final concentration of 37 mM. The reaction was stopped after 30 min by addition of 20 % trichloroacetic acid to precipitate the protein, and the supernatant analysed for formaldehyde by the method of Nash [12].

Protein assay

Protein concentrations were measured using the method of Bradford [13], with reagents obtained from Bio-Rad Laboratories. Enzyme purity was assessed by SDS/PAGE using the method of Laemmli [14] with gels containing 12.5 % polyacrylamide. Proteins were stained with Coomassie Blue. The molecular masses of the Theta-class GSTs were determined with reference to purified Alpha-, Mu- and Pi-class GSTs [15].

Enzyme purification

All procedures were carried out at 4 °C unless otherwise stated. Livers were removed from male $B_6C_3F_1$ mice and thoroughly washed in ice-cold 1.15 % KCl. A Potter–Elvehjem homogenizer was used to prepare a 20 % (w/v) homogenate in a 0.25 M sucrose, 5.4 mM EDTA, 20 mM Tris buffer (pH 7.4), containing 25 μM PMSF. A soluble supernatant fraction (cytosol) was prepared by centrifugation of the homogenate at 100000 ***g*** for 1 h and applied to an S-hexylglutathione affinity column (1.5 cm × 20 cm) using a 20 mM Tris/HCl buffer, pH 7.8, containing 5 mM dithiothreitol (DTT), 2 mM EDTA and 5 μM PMSF at a flow rate of 2 ml/min. The flow-through fraction was collected and desalted using a column (2.5 cm × 25 cm) of G25 Sephadex (Pharmacia LKB, Uppsala, Sweden) equilibrated with 10 mM sodium phosphate buffer, pH 6.8, containing 0.2 mM DTT, 2 mM EDTA and 25 μM PMSF at a flow rate of 2 ml/min before being applied to an Orange A (Amicon, Lexington, MS, U.S.A.) column (2 cm × 50 cm) equilibrated with the same buffer at 2 ml/min. The bound protein was eluted with a gradient from 0 to 20 mM NaCl over 30 ml, followed by a gradient from 200 mM to 1 M NaCl over a further 30 ml. Fractions which metabolized methylene chloride were eluted at about 0.8 M NaCl and pooled prior to concentration by ultrafiltration (Amicon ultrafiltration stirred cell, PM10 10 kDa cut-off membrane). The pooled fraction contained approx. 15 % of the original cytosolic protein and 65 % of the methylene chloride activity with a 5-fold increase in the specific activity.

The buffer containing the active enzyme fraction from the Orange A column was exchanged using a G25 Sephadex column (2.5 cm × 25 cm) equilibrated with 30 mM sodium phosphate buffer, pH 6.8, containing 0.2 mM DTT, 2 mM EDTA and 10 % (v/v) glycerol, at 2 ml/min. The protein which was eluted from the G25 column was applied to a hydroxyapatite column (2 cm × 40 cm) equilibrated with 30 mM sodium phosphate buffer, pH 6.8, containing 0.2 mM DTT, 2 mM EDTA and 10 % (v/v) glycerol, at a flow rate of 0.6 ml/min and at ambient temperature. The column was developed with a linear gradient of a volume of 85 ml between 30 and 180 mM sodium phosphate buffer, pH 6.8, containing 0.2 mM DTT, 2 mM EDTA and 10 % (v/v) glycerol. Fractions active with methylene chloride were eluted between 80 and 135 mM sodium phosphate, pooled, concentrated and buffer-exchanged using a PD10 column (Pharmacia LKB, Uppsala, Sweden) into 75 mM Tris/acetic acid buffer, pH 9.5, which contained 0.2 mM DTT, 10 % (v/v) glycerol and 0.5 mM glutathione. The protein fraction was then applied to a chromatofocusing column (Mono P, 5/20; Pharmacia LKB, Uppsala, Sweden), which had been pre-equilibrated in the same buffer at a flow rate of 0.4 ml/min. The pH gradient was developed with 10 % polybuffer 96/acetic acid, pH 6.0, containing 0.2 mM DTT, 10 % (v/v) glycerol and 0.5 mM glutathione. The active fractions were eluted at approx. pH 7.6, pooled, concentrated and transferred into 100 mM sodium phosphate, pH 6.8, containing 0.2 mM DTT, 2 mM EDTA, 10 % (v/v) glycerol, 0.5 mM glutathione and 1.5 M ammonium sulphate using a PD10 column. The sample was applied to a hydrophobic interaction column (phenyl-Sepharose; Pharmacia LKB, Uppsala, Sweden) equilibrated at 0.3 ml/min with the above buffer. The column was developed using a linear gradient with a volume of 25 ml of 20 mM sodium phosphate, pH 6.8, containing 0.2 mM DTT, 2 mM EDTA, 10 % (v/v) glycerol and 0.5 mM glutathione. The active fractions were eluted at about 100 mM ammonium sulphate and contained a single protein on SDS/PAGE with a molecular mass of about 25000 Da. Partial amino acid sequence data were then obtained on this band.

During the development of the methodology used to isolate the enzyme described above, the active fraction from the Orange A column was analysed by anion-exchange chromatography. This procedure involved transfer by gel filtration (PD10) of the active fraction into 30 mM piperazine/HCl buffer, pH 9.5, containing 0.2 mM DTT and 10 % (v/v) glycerol. This was then applied on to a Mono Q 5/5 anion-exchange column (Phamacia LKB, Uppsala, Sweden) pre-equilibrated with the same buffer. The column was developed with a linear gradient of 0–150 mM NaCl over 50 ml and then 150 mM to 1 M NaCl in 15 ml. A number of protein fractions from this column were analysed by SDS/PAGE and a number of bands with molecular masses of ~25000 Da were N-terminally sequenced.

Primary sequence analysis

SDS/PAGE was performed using the method of Laemmli [14], the resolving gel containing 12.5 % polyacrylamide. The gel was blotted on to a Problot membrane (Applied Biosystems) and the stained band excised and cut into two 1 mm × 5 mm strips. These strips were washed in 20 % (v/v) methanol for 5 min before the N-terminal sequence was determined using an Applied Biosystems 477 protein sequencer with on-line 120A phenylthiohydantoin analysis. The internal sequence was obtained from bands (5–10 μg of protein) cut from the gel and digested overnight in 1 ml of 70 % formic acid containing 2 mg of CNBr under a nitrogen atmosphere, prior to electrophoresis in a 16.5 % Tris-Tricine gel and blotting on to Problot membranes. Sequence data were obtained in the same way as for the N-terminal sequence.

RESULTS AND DISCUSSION

Enzyme purification

The bulk of the GSTs were removed from the cytosol by elution through an S-hexylglutathione affinity column. The Theta-class GSTs have a high K_m for glutathione and do not therefore bind to this column. A combination of dye matrix affinity, hydroxyapatite, chromatofocusing and hydrophobic interaction chromatography were used to purify the enzyme metabolizing methylene chloride. The dye matrix column bound less than 15 % of cytosolic protein, including the Theta-class GST enzymes. Subsequent purification steps were carried out in the presence of at least 10 % (v/v) glycerol in order to stabilize the protein's metabolic activity. In addition, glutathione was found to be an

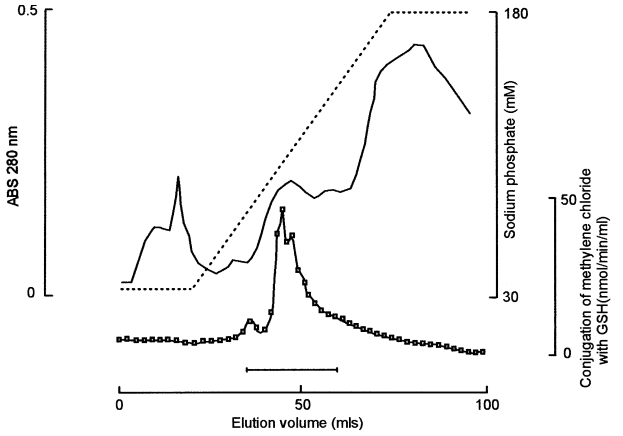

Figure 2 Hydroxyapatite chromatography to purify Theta-class GSTs from active fractions obtained from the Orange A column

Fractions active with methylene chloride were eluted from the Orange A column, pooled and concentrated before being transferred into 20 mM phosphate buffer, pH 6.8, containing 10% (v/v) glycerol, 0.2 mM DTT and 2 mM EDTA prior to loading on to a hydroxyapatite column. Fractions were eluted with a gradient of the same phosphate buffer from 30 to 180 mM (broken line). The absorbance of the eluate was monitored at 280 nm (solid line) and activity with methylene chloride (metabolism to formaldehyde) determined (□). The active fractions combined for further purification are indicated by the horizontal bar.

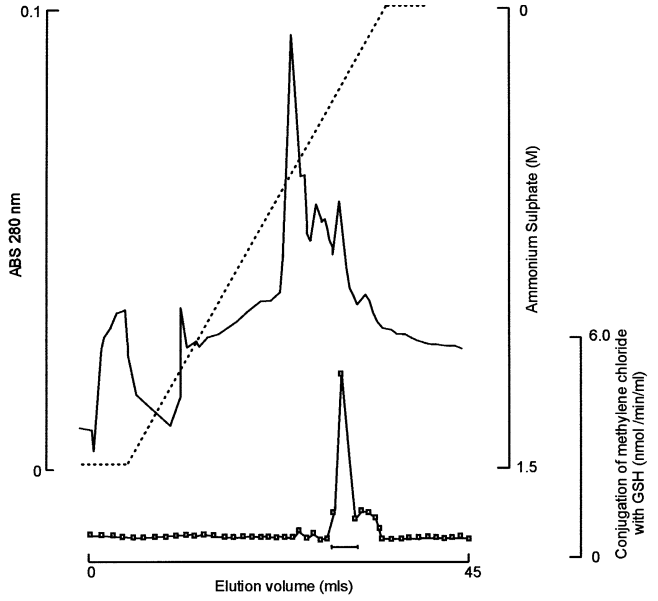

Figure 4 Purification of mouse Theta-class GSTs by hydrophobic interaction chromatography

Fractions eluted from the chromatofocusing column which were active with methylene chloride were transferred to a 100 mM phosphate buffer, pH 6.7, containing 1.5 M ammonium sulphate, glycerol, DTT, EDTA and GSH as described in the text. Absorbance at 280 nm (solid line) and GST activity with methylene chloride (□) were assayed from 1 ml fractions. The gradient into 20 mM phosphate buffer containing glycerol, DTT, EDTA and GSH is shown (broken line). The horizontal bar indicates the active fractions taken for sequence analysis.

essential buffer component of the chromatofocusing and the hydrophobic interaction steps.

Following hydroxyapatite chromatography (Figure 2) and chromatofocusing (Figure 3), the active fractions eluting from the chromatofocusing column at pH 7.6 were stabilized by the addition of glutathione, and further purified by hydrophobic interaction chromatography (Figure 4), to give a single protein band by SDS/PAGE with an N-terminal sequence identical to that obtained for the rat GST 5-5 (Table 1). The mouse enzyme, designated GST T1-1*, was recovered with a yield of about 2% of the initial methylene chloride-metabolizing activity and 0.01% cytosolic protein (Table 2), whereas the yield from rat liver was about 0.002% GST 5-5 protein [7], indicating that the mouse has more of this enzyme than the rat.

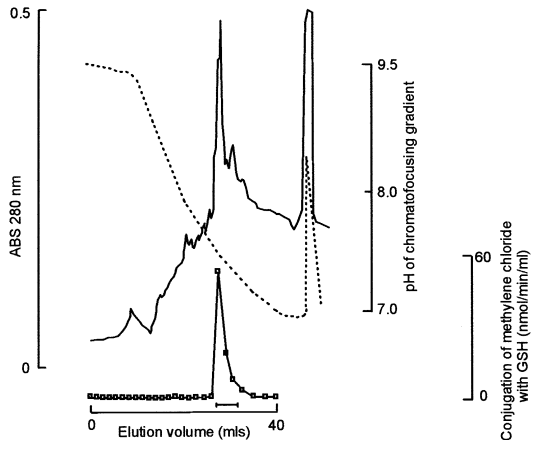

Figure 3 Purification of mouse Theta-class GSTs by chromatofocusing

Active fractions from the hydroxyapatite column were buffer exchanged into 75 mM Tris/acetic acid, pH 9.5, containing EDTA, DTT, GSH and glycerol as described in the text and subjected to chromatofocusing. Absorbance at 280 nm (solid line) and pH (broken line) were monitored and 0.5 ml fractions assayed for GST activity with methylene chloride (□). The horizontal bar indicates the active fractions that were combined for further purification.

Method development studies analysing the Orange A fractions by anion-exchange chromatography provided preliminary evidence for a second Theta-class GST enzyme in mouse liver. One of the bands from the SDS/PAGE gels had an N-terminal sequence which was identical to that of rat GST 12-12. However, at the present time this enzyme has not been isolated to homogeneity and its identity has not been confirmed by internal sequencing. Similar difficulties have been experienced by others with rat GST 12-12 which is extremely labile and has not yet been isolated as a single protein [7]. Methylene chloride is clearly not an appropriate substrate with which to study this enzyme and attempts to identify both alternative substrates and methods of purifying the mouse enzyme are continuing and will be reported at a later date. A human Theta-class GST, designated GST T2-2 [16], has been isolated which is closely related to the rat GST 12-12. This enzyme was also found to be labile, although activity towards menaphthyl sulphate was maintained throughout the purification scheme.

Activities

The specific activity of the mouse enzyme GST T1-1* was found to be similar to that reported by Meyer et al. [7] for rat GST 5-5 (5.5 and 11 μmol/min per mg respectively), although the assay conditions were not identical and exact comparisons cannot be made. The difference seen in total methylene chloride-metabolizing activity between mouse and rat *in vivo*, or in cytosol fractions, is more than 10-fold, which does not appear to be attributable to a higher specific activity of mouse GST T1-1*

Table 1 Amino acid sequences of Theta-class GST subunits from rat and mouse

(A) The N-terminal primary structures of subunits of rat GSTs 5 and 12 [7] are compared with those of the subunits of mouse GST T1-1* and the mouse enzyme (GST 2-2*) tentatively identified as equivalent to GST 12-12. (B) Internal sequence structures of two isolated CNBr-cleaved fragments of Theta-class GST T1-1* compared with the rat GST 5-5 sequence from [7]. Residues given in parentheses are tentative assignments; (?) indicates unknown residue. Abbreviation: a.a., amino acids.

Subunit	Sequence																			
(A) N-terminal primary structures																				
Rat subunit 5	V	L	E	L	Y	L	D	L	L	S	Q	P	C	R	A	I	Y	I	F	A
Mouse subunit GST T1-1*	V	L	E	L	Y	L	D	L	L	S	Q	P	(S)	R	A	I	Y	I		
Mouse subunit GST T2-2*	G	L	E	L	Y	L	D	L	(L)	S	Q	(P)	(S)	R	A	V	Y	(S)	(?)	A
Rat subunit 12	G	L	E	L	Y	L	D	L	L	S	Q	P	C	R	A	V	Y	I	F	A
(B) Internal sequence structures																				
Rat GST 5-5 (a.a. 176–192)	P	V	E	G	G	G	C	P	V	F	E	G	R	P	R	L	A	A		
GST T1-1* 6000 band	P	V	E	G	G	G	(?)	P	V	F	E	G	(?)	P	R	L	A	A		
GST T1-1* 8000 band	D	(?)	(?)	F	T	L	(?)	E	(?)	(?)	A	I	L	(?)	Y	L				
Rat GST 5-5 (a.a. 58–73)	D	G	G	F	T	L	C	E	S	V	A	I	L	L	Y	L				

Table 2 Purification of Theta-class GST T1-1* from mouse liver

Activity was measured with methylene chloride as the substrate as described in the text. Abbreviation: HIC, hydrophobic interaction column.

Fraction	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Recovery (%)
Cytosol	396	15840	40	100
S-HexylGSH	290	14500	50	92
Orange A	52	10400	200	65
Hydroxyapatite	12	4800	400	30
Chromatofocusing	0.3	260	1300	2.5
HIC	0.05	330	5500	1.7

compared with rat GST 5-5. Based on the relative amounts of Theta-class GST protein recovered from the livers of the two species it seems probable that the higher activity in the mouse is attributable to greater expression of the enzyme in that species.

Sequence analysis

Partial amino acid sequences for the mouse Theta enzymes are compared with the equivalent rat enzymes in Table 1. N-terminal sequences of the two enzymes are very similar. The rat GST 5 and GST Yrs or 12 have been cloned [9,17], as has a human Theta-class GST (GST T1) which shares 82% sequence similarity with the rat GST 5 [18], demonstrating the high degree of conservation between N-terminal sequences of the Theta GSTs so far identified. cDNA clones have been isolated that encode the mouse and rat enzymes and these are being used to express protein from both bacterial and mammalian cells in order to generate protein for antibody production, functional studies and mutation assays. cDNA and oligonucleotide probes to both enzymes are also being used to determine the distribution of these enzymes within the cells and species of interest. Tissue levels of the mRNAs of the Theta-class enzymes are being quantified by the use of Northern blots.

In conclusion, a Theta GST enzyme, designated GST T1-1*, with activity towards methylene chloride, has been isolated and sequenced from mouse liver. The specific activity and amino acid sequence of this enzyme is similar to that of the rat enzyme GST 5-5 and it is believed that the more than 10-fold higher conjugating activity seen in mouse cytosol fractions with methylene chloride is probably due to increased expression of the enzyme in that species. Limited evidence has been obtained for the presence of a second Theta enzyme in mouse liver whose N-terminal sequence is identical to that of rat GST 12-12.

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REFERENCES

1 National Toxicology Programme (1986) NTP Technical Report no. 306
2 Burek, J. D., Nitschke, K. D., Bell, T. J., Wackerle, D. L., Childs, R. C., Beyer, J. E., Dittenber, D. A., Rampy, L. W. and McKenna, M. J. (1984) Fund. Appl. Toxicol. **4**, 30–47
3 Ahmed, A. E. and Anders, M. W. (1978) Biochem. Pharmacol. **27**, 2021–2025
4 Graves, R. J., Coutts, C., Eyton-Jones, H. and Green, T. (1994) Carcinogenesis **15**, 991–996
5 Green, T. (1989) in Biologically-Based Methods for Cancer Risk Assessment (Travis, C. C., ed.), NATO ASI Series, vol. 159, pp. 289–300, Plenum Press, New York
6 Reitz, R. H., Mendrala, A. L. and Guengerich, F. P. (1989) Toxicol. Appl. Pharmacol. **97**, 230–246
7 Meyer, D. J., Coles, B., Pemble, S., Gilmore, K. S., Fraser, G. M. and Ketterer, B. (1991) Biochem. J. **274**, 409–414
8 Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M. and Jornvall, H. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 7202–7206
9 Pemble, S. E. and Taylor, J. B. (1992) Biochem. J. **287**, 957–963
10 Tan, K. H., Meyer, D. J., Gillies, N. and Ketterer, B. (1988) Biochem. J. **254**, 841–845
11 Hallier, E., Langhof, T., Dannapel, D., Leutbecher, M., Schroder, K., Goergens, H. W., Muller, A. and Bolt, H. M. (1993) Arch. Toxicol. **67**, 173–178
12 Nash, T. (1953) Biochem. J. **55**, 416–421
13 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
14 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
15 McLellan, L. I. and Hayes, J. D. (1989) Biochem. J. **263**, 393–402
16 Hussey, A. J. and Hayes, J. (1992) Biochem. J. **286**, 929–935
17 Ogura, K., Nishiyama, T., Okada, T., Kajital, J., Narihata, H., Watabe, T., Hiratsuka, A. and Watabe, T. (1991) Biochem. Biophys. Res. Commun. **181**, 1294–1300
18 Pemble, S., Schroeder, K. R., Spencer, S. R., Meyer, D. J., Hallier, E., Bolt, H. M., Ketterer, B. and Taylor, J. B. (1994) Biochem. J. **300**, 271–276