Pergamon

Biochemical Pharmacology, Vol. 48, No. 12, pp. 2231–2235, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00415-3

CORRELATION OF THE RESPONSE TO NITROGLYCERIN IN RABBIT AORTA WITH THE ACTIVITY OF THE MU CLASS GLUTATHIONE *S*-TRANSFERASE

SARASWATI R. KENKARE, CHAO HAN* and LESLIE Z. BENET†

Department of Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143-0446, U.S.A.

(*Received* 16 *May* 1994; *accepted* 4 *August* 1994)

Abstract—The relationship between the activity of glutathione *S*-transferases (GSTs), especially the mu isozyme, and the production of responses to nitroglycerin (GTN) was investigated in rabbit aorta. GST mu isozyme activity was measured using *trans*-stilbene oxide (TSO) as a substrate. Each aorta was divided into four parts, two of which were frozen for enzymatic analyses while the remaining two were used to measure the effects of GTN (0.5 μM), i.e. the increase in cGMP levels and the corresponding relaxation. Thus, all three measures were obtained in each individual rabbit aorta. Eight different rabbits were studied. An excellent correlation was obtained between the rise in cGMP and the mu isozyme activity ($r^2 = 0.948$). A good correlation was also obtained between TSO activity and the relaxation response to GTN. Total GST activity did not correlate well with either cGMP increases or percent relaxation. These observations indicate that the activity of the mu isozyme measured using TSO and not the total GST correlates with the responses to GTN in the *in vitro* rabbit aorta model.

Key words: glutathione *S*-transferases; mu isozyme; cGMP; nitroglycerin; rabbit aorta

GTN‡ is believed to be metabolized in the vascular smooth muscle where it stimulates the production of cGMP via nitric oxide or *S*-nitrosothiols. The nature of the process involved in the production of this second messenger is not clear. Different hypotheses have been proposed to describe the mechanism of GTN metabolism. Both enzymatic and non-enzymatic thiol-mediated, as well as thiol-independent, processes have been proposed [1–6]. It is our working hypothesis that GSTs are involved in the metabolic bioactivation of GTN. On the basis of inhibitor studies in homogenates of rabbit liver [7, 8], rabbit aorta [9], and bovine coronary arteries [10], we have been able to demonstrate successfully that GST catalyzes the metabolic conversion of GTN to its dinitrate products. When the effects of a GST inhibitor were studied in an aortic strip model, the relaxation response to GTN was decreased in parallel with reduced metabolism of GTN [9] and also with decreased cGMP concentrations [11], suggesting that the biotransformation of GTN is required to produce pharmacological action.

It is known that GSTs are expressed in the body as multiple isoforms. In rabbit aorta, the distribution of the different GST isozymes has been studied. Chern *et al.* [12] and Lanzo *et al.* [13] found that the majority of GSTs occur as the pi isozyme (85%) while the mu isozyme accounts for approximately 15% of total GSTs. The alpha isozyme could not be detected in rabbit aorta. Using affinity chromatography, immunoprecipitation and immunoblotting techniques, the GST mu isozyme has been shown to mediate GTN metabolism in rabbit aortic cytosol. In immunoprecipitation studies on rabbit aortic cytosol, antiserum against the pi isozyme did not affect GTN metabolism [13], whereas antiserum against mu isozyme decreased GTN metabolism significantly [12]. Furthermore, Tsuchida *et al.* [14] have successfully purified GST mu isozymes with activities towards GTN from the human heart and aorta. In the human aorta, these authors found the same distribution of GST isozymes as in rabbit aorta with the pi being the most abundant, the mu isozyme a smaller subset and the alpha undetectable. Tsuchida *et al.* [14] also showed that the pi isozyme has no activity to GTN, while the mu isozymes exhibit high activities towards GTN. In our earlier studies [11] in aortic strips pretreated with ethacrynic acid, a GST mu preferential inhibitor, a good correlation was observed between decreases in relaxation and cGMP levels induced by GTN. Thus, the mu isozyme of GST, an efficient metabolizer of GTN, appears to be important in the bioactivation of GTN (i.e. the production of pharmacologic effects).

The activity of the enzyme/isozyme of interest can be measured using specific substrates. Seidegard *et al.* [15, 16] have found that TSO is a specific substrate for the GST mu isozyme isolated from human liver, with a 2000-fold higher activity to this isozyme compared with others. In contrast, CDNB is a well-known general substrate of GST [17]. The objective of the present work was to investigate the relationship in rabbit aorta, a site of action of GTN, between

* Present address: Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E OW3.

† Corresponding author. Tel. (415) 476-3853; FAX (415) 476-8887.

‡ Abbreviations: GTN, nitroglycerin, glyceryl trinitrate; GST, glutathione *S*-transferase; TSO, *trans*-stilbene oxide; CDNB, 1-chloro-2,4-dinitrobenzene; cGMP, cyclic 3′,5′ guanosine monophosphate; TCA, trichloroacetic acid.

the activity of GSTs, especially the mu isozyme, and the generation of the responses to GTN (especially the production of cGMP).

MATERIALS AND METHODS

Materials. The techniques utilized here follow procedures described previously by Lau and Benet [9] and Kenkare and Benet [11]. Briefly, eight male New Zealand white rabbits (Nitabell Rabbitry, Hayward, CA), weighing 2–3 kg, were used in this study. The rabbits were anesthestized using s.c. ketamine (40 mg/kg as 1.5 mL of a 100 mg/mL solution in Water for Injection USP) and decapitated 15 min later. The thoracic cavity was exposed and the descending aorta was removed surgically and placed immediately in a beaker containing Krebs buffer (NaCl, 119 mM; KCl, 4.8 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.5 mM; glucose, 11.1 mM), which was gassed continuously with carbogen (95% oxygen and 5% carbon dioxide). After isolation of the aorta, excess fat and connective tissues were removed carefully; during this process, the buffer was replaced constantly with fresh buffer. The endothelium was not disturbed. The helically prepared tissue was cut into four strips (3 cm × 4 mm). Two of the four strips were frozen immediately for enzyme assays to be performed later, while the remaining strips were used for cGMP and relaxation measurements.

The aortic strips frozen for enzyme assays were homogenized with 3 vol. of PBS. The homogenate was centrifuged using an Eppendorf centrifuge at maximum speed, and the clear upper layer was frozen and stored at −80°.

Response measurements. Aortic strips were suspended individually in Krebs buffer (composition described above) contained in 25 mL jacketed circulating water tissue baths maintained at 37°. One strip from each tissue was assigned as the buffer control while the others were treated with GTN. The contraction and relaxation of the strips were recorded via transducers, coupled to a Grass model 7 polygraph (Quincy, MA). The precontraction of the strips was performed as described previously [11]. The relaxation produced by GTN (0.5 μM) was recorded at the end of 5 min, which is about the time for maximal relaxation. Immediately after the 5-min measurement, the strips were freeze-clamped using tongs precooled in liquid nitrogen and frozen on dry ice. Analysis of cGMP was then carried out in each of these strips, usually the next day.

Analysis of cGMP levels. The frozen strips were homogenized in 1 mL of 6% cold TCA to precipitate proteins; the homogenate was then centrifuged at 2200 *g* for 10 min at 2–4° to remove the protein precipitate. The supernatant was transferred into a fresh test tube and washed with a 4-fold volume of diethyl ether saturated with water. This procedure was repeated at least four times. Each time the ether layer was removed carefully, after the tubes were vortexed, and allowed to stand for some time. After removing the residual ether by evaporation under a stream of nitrogen, the TCA-free samples were then assayed for cGMP using a ^{3}H-labeled cGMP radioimmunoassay kit (Amersham, Arlington Heights, IL). Each time a new kit was used, standard curves were made with six concentrations (0, 0.5, 1, 2, 4 and 8 pmol cGMP/100 μL) prepared from the standard solutions of cGMP.

Enzyme assays. The strips frozen for enzymatic analysis were homogenized using three times the volume of PBS, pH 7.4, and spun at 2200 *g* for 5 min. The supernatants were then used for enzyme assays.

Total GST activity (CDNB activity). Total GST activity was measured using the procedure of Habig *et al.* [17]. Briefly the assay was carried out in a 3-mL plastic cuvette that contained 1 mM CDNB, 5 mM glutathione, 0.1 mM potassium phosphate buffer, pH 6.65, and 10 μL of the aortic supernatant preparation. The increase in absorbance was measured at 340 nm at room temperature. The extinction coefficient was 9.6 mM^{-1} cm^{-1}, and one unit of activity was defined as 1 μmol CDNB/min. Activities were expressed in micromoles CDNB per minute per milligram protein. The variability of this activity measure was less than 20%.

GST mu isozyme activity (TSO activity). GST mu isozyme activity was measured using a radiometric assay similar to that described by Seidegard *et al.* [15]. Briefly, the samples were incubated with 4 mM glutathione and 250 μM [^{3}H]TSO (sp. act. 15 Ci/mmol) in PBS, pH 7.4, at 37° for 10 min. The reaction was terminated by extraction with 200 μL of hexanol. The percent radioactivity in the aqueous phase (product of TSO and glutathione) was calculated from the radioactivity measurements in the organic phase and aqueous phase using liquid scintillation counting. The unit of activity was expressed as nanomoles TSO per minute per milligram protein or normalized to total GST activity as nanomoles TSO per minute per CDNB unit. The variability of the GST mu activity measure was less than 15%.

Protein assays. The analysis of proteins was conducted using the classical procedure [18]. Protein concentrations were expressed in milligrams per milliliter.

Data analysis. GTN-induced relaxation was measured as percent decrease in tension below the elevated tension elicited by precontracting the strips. Fold increases in cGMP were calculated as cGMP in GTN-treated strips divided by cGMP in buffer control. Linear regressions for all the correlations were carried out using Cricket Graph software.

RESULTS

GST mu activity compared with the responses to GTN in aorta. Basal cGMP levels in the aortas of the eight rabbits were found to average 14.5 ± 2.1 pmol/g wet wt. The rise in cGMP upon GTN treatment in an individual rabbit aorta was compared with its own basal cGMP level. Upon treatment with 0.5 μM GTN, the cGMP levels in the aortic strips increased from 2- to 4.2-fold relative to basal levels. A good correlation ($r^2 = 0.789$) was observed between the fold increase in cGMP and the percent relaxation in the eight rabbit aortas.

Six of the eight rabbit aortas had TSO activities ranging from 1.42 to 2.27 nmol/min/CDNB unit. These rabbit aortas also showed a 2.2- to 3.5-fold

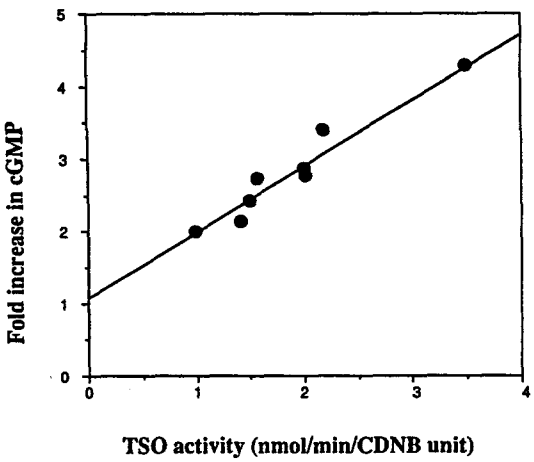

Fig. 1. Correlation between the fold increase in cGMP levels in response to GTN (0.5 μM) and the TSO activity (GST mu activity, normalized to number of CDNB units) in eight rabbit aortas ($r^2 = 0.948$).

increase in cGMP levels and a corresponding 56 to 100% relaxation. One rabbit aorta, with a GST mu activity of 0.99 nmol/min/CDNB unit, exhibited a much lower increase in cGMP and a correspondingly lower percent relaxation in response to GTN. The eighth rabbit aorta, with a very high GST mu activity of 3.58 nmol/min/CDNB unit, yielded the greatest increase in cGMP in response to the same concentration of GTN. When the GST mu activity was compared with the increase in cGMP levels, an excellent correlation was found ($r^2 = 0.948$, Fig. 1). A significant correlation was also observed when cGMP concentrations themselves were compared with TSO activity (normalized to CDNB units, $r^2 = 0.899$).

A lower but obviously significant correlation ($r^2 = 0.715$) was observed upon comparison of TSO activity with percent relaxation. When the enzyme activity was normalized to protein content (nmol TSO/min/mg protein) rather than total GST content, the correlations were lower than those previously obtained. That is, comparison of the TSO activity normalized to protein content with the fold increase in cGMP yielded an r^2 of 0.776 (while absolute cGMP values gave an r^2 of 0.714) and an r^2 of 0.539 with percent relaxation.

Total GST activity compared with the responses of GTN in aorta. The total GST activities of the different rabbit aortas were compared and were found to range from 0.18 to 0.6 μmol CDNB/min/mg protein. When total GST activities were compared with responses to GTN in aortas of individual rabbits, no correlation was found with the increase in cGMP levels ($r^2 = 0.099$) or the percent relaxation ($r^2 = 0.067$) in the eight rabbits studied. Table 1 summarizes the correlations observed between the different isozyme/enzyme activities and the responses to GTN in the individual rabbits.

DISCUSSION

Metabolism of GTN is known to be important for the production of pharmacologic effects of this drug in vascular tissue. Different theories have been proposed to explain the metabolism of GTN in vascular and nonvascular tissues. Feelisch and Noack [1] proposed the non-enzymatic thiol-dependent decomposition of GTN, while more recently several investigators [2, 3, 5, 19, 20] have suggested that cytochromes P450 may be involved in the formation of nitric oxide from GTN. Brien *et al.* [21] have proposed that the biotransformation of GTN in rabbit aorta occurs due to a direct interaction of one of the nitrate groups of GTN with the iron (Fe^{2+}) of guanylate cyclase bound heme. Chung and Fung [22] proposed that the enzyme responsible for the activation of GTN to nitric oxide is located in the plasma membrane and is thiol dependent. Most recently, Fung *et al.* [23] reported that this enzyme is a 160 kDa protein that is distinct from GSTs. We and others [7–10, 24, 25] have suggested that GSTs are important in the metabolic activation of GTN and that the majority of the metabolism is associated with the cytosolic fraction.

Three different classes of GST isozymes, designated alpha, pi and mu, have been identified. These isozymes are expressed to different extents in different organs of the body and in different species [26]. As mentioned earlier, the results of Tsuchida

Table 1. Summary of the correlations between the enzyme activities and GTN responses

X variable	Y variable	r^2
cGMP increase	% Relaxation	0.789
GST mu (nmol TSO/min/CDNB unit)	cGMP increase	0.948
	Absolute cGMP levels	0.899
GST mu (nmol TSO/min/mg protein)	cGMP increase	0.776
	Absolute cGMP levels	0.714
GST mu (nmol TSO/min/CDNB unit)	% Relaxation	0.715
GST mu (nmol TSO/min/mg protein)	% Relaxation	0.539
Total GST activity (μmol CDNB/min/mg protein)	cGMP increase	0.099
	Absolute cGMP levels	0.092
Total GST activity (μmol CDNB/min/mg protein)	% Relaxation	0.067

et al. [14], Chern *et al.* [12] and Lanzo *et al.* [13] indicate that in human and in rabbit vasculature, the GST mu isozyme metabolizes GTN while the pi isozyme, the most abundant isozyme, is not active in metabolizing GTN. The alpha isozyme was not detectable. We have shown recently that GTN responses in rabbit aortic strips (measured as percent relaxation and elevation of cGMP levels) were modulated when the strips were pretreated with a GST mu selective inhibitor [11].

In the present study, the activity of total GST enzymes and the activity of the mu isozyme were measured together with the response to a specific concentration of GTN in the same rabbit aorta. Since the basal cGMP levels were also measured in the same animal, the cGMP increases represent specific rises in the level of the second messenger in the aorta of each rabbit. As the enzyme activity measurements and the response measurements were done on each individual rabbit aorta, it was possible to correlate individual tissue responses with the enzyme activities in those rabbits.

The results of this study showed that total GST activity, measured as μmol CDNB/mg protein, did not correlate well with the GTN-induced increase in cGMP levels of each rabbit aorta ($r^2 = 0.099$). However, upon comparing the GST mu isozyme activity, measured as nmol TSO/CDNB unit, a very good correlation ($r^2 = 0.948$) was observed between these activities and the increase in cGMP levels. This indicates that GST mu activity (TSO activity) and not the total GST activity is a good marker of the biochemical response to GTN in rabbit aorta. The increase in cGMP levels and the percent relaxation in the different rabbits correlated well ($r^2 = 0.789$) as seen in our previous study [11], which is in agreement with the hypothesis that cGMP is the second messenger of GTN-induced relaxation in vascular smooth muscle. A marked but somewhat lower correlation ($r^2 = 0.715$) was observed between the mu isozyme activity and the percent relaxation in aortas. This decreased correlation is predictable since relaxation of aortic strips is a secondary response to GTN, preceded by the cGMP increase, a more primary biochemical response.

We also observed a decrease in the r^2 values when cGMP increases and percent relaxation were correlated with GST mu isozyme activity/mg of protein rather than when GST mu isozyme activity was normalized to CDNB measurements. Theoretically, we would expect the correlation between the nmol TSO/min/mg protein vs cGMP increase to be as good as that between nmol TSO/min/CDNB unit vs cGMP increase. However, practically, due to our technique of sample preparation, which was limited by sample size, there was an added variability in the protein measurements in the supernatants. Thus, nmol TSO/min/mg of protein did not correlate as well with cGMP increase as nmol TSO/min/CDNB unit.

It could be argued that the observed relationship between the TSO activity and the response to GTN may be coincidental, and the increased GST mu isozyme activity was due to a higher level of expression of all proteins in individual rabbits. However, this explanation is probably unlikely, since rabbit aortas with high GST mu activities did not have high total GST activities (data not shown).

The results of this study indicate that mu isozyme activity (TSO activity) in vascular tissue and the pharmacologic response to GTN correlate well in the rabbit model. This study does not exclude the possibility of other enzymes being involved in the metabolism and/or bioactivation of GTN; however, together with evidence obtained previously, it does indicate that the mu isozyme of GST may play a role in the activation of GTN in the rabbit aorta in the *in vitro* system used in this study.

It would be interesting to study this enzyme activity in extravascular tissues in rabbits to probe the possibility of using a peripheral tissue, such as blood, as a predictor of the enzyme activity and GTN effects. Furthermore, it would be worthwhile to determine if there is a correlation between the mu isozyme activity in different tissues and to study the possible involvement of these isozymes in tolerance development, a prominent clinical problem of organic nitrate dosing. Studying this enzyme in human vascular tissues and determining its role in the action of nitrates in human vascular tissues would be of clinical relevance. These questions are the subject of further ongoing investigations in our laboratory.

REFERENCES

1. Feelisch M and Noack EA, Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur J Pharmacol* **139**: 19–30, 1987.
2. Delaforge M, Servent D, Wirsta P and Ducrocq C, Particular ability of cytochrome P-450 CYP3A to reduce glyceryl trinitrate in rat liver microsomes: Subsequent formation of nitric oxide. *Chem Biol Interact* **86**: 103–117, 1993.
3. McDonald BJ and Bennett BM, Cytochrome P-450 mediated biotransformation of organic nitrates. *Can J Physiol Pharmacol* **68**: 1552–1557, 1990.
4. Schröder H and Schror K, Inhibitors of cytochrome P-450 reduce cyclic GMP stimulation by glyceryl trinitrate in LLC-PK1 kidney epithelial cells. *Naunyn Schmiedebergs Arch Pharmacol* **342**: 616–618, 1990.
5. Servent D, Delaforge M, Ducrocq C, Mansuy D and Lenfant M, Nitric oxide formation during microsomal hepatic denitration of glyceryl trinitrate: Involvement of cytochrome P-450. *Biochem Biophys Res Commun* **163**: 1210–1216, 1989.
6. Schröder H, Cytochrome P-450 mediates bioactivation of organic nitrates. *J Pharmacol Exp Ther* **262**: 298–302, 1992.
7. Lau DT and Benet LZ, Differential formation of dinitrate metabolites from glyceryl trinitrate in subcellular fractions of rabbit liver. *Biochem Pharmacol* **38**: 543–546, 1989.
8. Lau DT and Benet LZ, Nitroglycerin metabolism in subcellular fractions of rabbit liver. Dose dependency of glyceryl dinitrate formation and possible involvement of multiple isozymes of glutathione *S*-transferases. *Drug Metab Dispos* **18**: 292–297, 1990.
9. Lau DT and Benet LZ, Effects of sulfobromophthalein and ethacrynic acid on glyceryl trinitrate relaxation. *Biochem Pharmacol* **43**: 2247–2254, 1992.
10. Lau DT, Chan EK and Benet LZ, Glutathione *S*-transferase-mediated metabolism of glyceryl trinitrate in subcellular fractions of bovine coronary arteries. *Pharm Res* **9**: 1460–1464, 1992.

11. Kenkare SR and Benet LZ, Effect of ethacrynic acid, a glutathione-*S*-transferase inhibitor, on nitroglycerin-mediated cGMP elevation and vasorelaxation of rabbit aortic strips. *Biochem Pharmacol* **46**: 279–284, 1993.
12. Chern WH, Serabjit-Singh CJ, Lanzo CA and Han BJ, The metabolism of nitroglycerin (GTN) in rabbit aorta correlates with the activity of the mu class glutathione *S*-transferase (GST), but not the appearance of nitric oxide. *FASEB J* **5**: A1220, 1991.
13. Lanzo CA, Chern WA, Burkhart W and Moseley MA, Isolation and characterization of the cytosolic mu class glutathione *S*-transferase active in the metabolism of nitroglycerin. *FASEB J* **6**: A1588, 1992.
14. Tsuchida S, Maki T and Sato K, Purification and characterization of glutathione transferases with an activity toward nitroglycerin from human aorta and heart. Multiplicity of the human class *Mu* forms. *J Biol Chem* **265**: 7150–7157, 1990.
15. Seidegård J, De Pierre JW, Birberg W and Pilotti A, Characterization of soluble glutathione transferase activity in resting mononuclear leukocytes from human blood. *Biochem Pharmacol* **33**: 3053–3058, 1984.
16. Seidegård J, Guthenberg C, Pero RW and Mannervik B, The *trans*-stilbene oxide-active glutathione transferase in human mononuclear leucocytes is identical with the hepatic glutathione transferase mu. *Biochem J* **246**: 783–785, 1987.
17. Habig WH, Pabst MJ and Jakoby WB, Glutathione *S*-transferases: The first step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139, 1974.
18. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
19. Schröder H, Barann M, Bennett BM and Leitman DC, Cross-tolerance to L-arginine-dependent guanylate cyclase activators in nitrate-tolerant LLC-PK1 kidney epithelial cells. *Pol J Pharmacol Pharm* **42**: 259–263, 1990.
20. Schröder H, Organic nitrates. New knowledge about the mechanism of action of an old class of substances. *Med Monatsschr Pharm* **15**: 134–139, 1992.
21. Brien JF, McLaughlin BE, Breedon TH and Bennett BM, Biotransformation of glyceryl trinitrate occurs concurrently with relaxation of rabbit aorta. *J Pharmacol Exp Ther* **237**: 608–614, 1986.
22. Chung SJ and Fung HL, Identification of the subcellular site for nitroglycerin metabolism to nitric oxide in bovine coronary smooth muscle cells. *J Pharmacol Exp Ther* **253**: 614–619, 1990.
23. Fung HL, Chung SJ, Bauer JA and Chong S, Biochemical mechanism of organic nitrate action. *Am J Cardiol* **70**: 4B–10B, 1992.
24. Hill KE, Hunt RJ, Jones R and Hoover RL, Metabolism of nitroglycerin by smooth muscle cells. Involvement of glutathione and glutathione *S*-transferase. *Biochem Pharmacol* **43**: 561–566, 1992.
25. Needleman P, Blehm DJ and Rotskoff KS, Relationship between glutathione-dependent denitration and the vasodilator effectiveness of organic nitrates. *J Pharmacol Exp Ther* **165**: 286–288, 1969.
26. Mannervik B, Alin P, Guthenberg C and Jensson H, Identification of three classes of cytosolic glutathione transferase common to several mammalian species: Correlation between structural data and enzymatic properties. *Proc Natl Acad Sci USA* **82**: 7202–7206, 1985.