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H.p.l.c. separation and study of the charge isomers of human placental glutathione transferase

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Glutathione transferase (GST) from human placenta was purified by affinity chromatography and anion-exchange h.p.l.c. The enzyme exhibited different chromatographic and electrophoretic behaviours according to the concentration of GSH, suggesting a possible change in the net charge of the molecule and a concomitant conformational change due to ligand binding. Two interconvertible forms were quantitatively separated into distinct catalytically active states by h.p.l.c. Depending upon the GSH concentration, polyacrylamide-gel electrophoresis revealed the presence of one or two bands. A K_d of 0.42 mM for GSH was determined fluorimetrically. The loss in intrinsic fluorescence also suggested a conformational change in the enzyme. Kinetic studies using ethacrynic acid were conducted to determine whether the presumed conformational change could effect the catalytic capability of placental GST. A biphasic response in initial velocities was observed with increasing concentrations of GSH. Two apparent K_m values of 0.38 and 50.27 mM were obtained for GSH, whereas $V_{max.}$ values showed a 46-fold difference. It was concluded that the enzyme assumes a highly anionic form in the presence of a low GSH concentration, whereas it is converted into relatively weaker anionic form when its immediate environment contains a high GSH concentration. Since the average tissue concentration of total GSH was estimated at 0.11 mM for term placenta, the results suggest that the high-affinity–low-activity conformer would predominate *in vivo*.

INTRODUCTION

The placenta is capable of metabolizing xenobiotics before fetal exposure. Occurrence of significant amounts of placental GST and its proven ability to detoxify xenobiotics (Pacifici & Rane, 1981; Radulovic *et al.*, 1986) suggest that it may be integral in the detoxification of chemical fetotoxicants. Indirect evidence suggesting the microheterogeneity of placental GST, which has previously been reported to be a single protein composed of identical subunits (Guthenberg *et al.*, 1979; Polidoro *et al.*, 1980), is derived from several studies. Polidoro *et al.* (1981) observed two interconvertible forms of human placental GST on polyacrylamide-gel electrophoresis. Two forms of human placental GST were detected on the basis of reduction by GSH of Nitro Blue Tetrazolium salt (Ricci *et al.*, 1984). In the presence of GSH, human placental GST has been reported to adopt a conformational state that prevents inhibition by cephalosporins (Polidoro *et al.*, 1984). Vander Jagt *et al.* (1981) were able to kinetically discern the presence of three conformational states: (1) in the absence of GSH; (2) in the presence of GSH; and (3) in the presence of CDNB without GSH. Despite these reports, the necessary tangible evidence for the existence of multiple conformational states by their physical separation from one another is still lacking. Very little is known about their biochemical properties and the conditions that lead to their formation.The major obstacle is the lack of a suitable method to isolate these conformers in a catalytically active state. In this regard, our previous study (Radulovic & Kulkarni, 1985) indicated that h.p.l.c., which is a procedure quite different from the f.p.l.c. one described for the purification of rat liver isoenzymes (Ålin *et al.*, 1985), possesses the required high-resolution capability and may provide direct evidence of these forms by physical separation.

The results of the present study suggest that human term-placental GST does undergo a conformational change resulting in the formation of two charge isomers according to the concentration of GSH. These conformers can be physically separated from each other by using the h.p.l.c. procedure. The change in conformational state was also found to alter catalytic activity.

MATERIALS AND METHODS

CDNB, ethacrynic acid, GSSG, NADPH, DTNB, and glutathione reductase (EC 1.6.4.2; 178.6 units/ml) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). GSH was bought from United States Biochemical (Cleveland, OH, U.S.A.). All other chemicals were purchased commercially.

Purification of the enzyme

The isolation and purification of term placental GST was performed by affinity chromatography and h.p.l.c. as previously described (Radulovic & Kulkarni, 1985). A

Abbreviations used: GST, glutathione transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); f.p.l.c., fast protein liquid chromatofocusing; DTT, dithiothreitol.

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minor modification was made in the h.p.l.c. protocol to demonstrate the effects of different concentrations of GSH in the mobile phase on the chromatographic behaviour of GST. Typically, GST partially purified by affinity chromatography was concentrated, divided into suitable portions, and dialysed against 10 mM-potassium phosphate buffer, pH 6.0, containing the indicated concentration of GSH before anion-exchange h.p.l.c. A Synchrom (Linden, IN, U.S.A.) AX-300 column was utilized. The mobile phase consisted of a citrate/potassium phosphate buffer (19.7 ml of 25 mM-citric acid + 30.3 ml of 50 mM-dibasic potassium phosphate/100 ml; Buffer A) and Buffer A containing 400 mM-KCl (Buffer B). Both Buffers A and B used to elute GST from the AX-300 column also contained 1 mM-DTT and the indicated GSH concentration, pH 5.85. In the purification experiments, CDNB was used as the second substrate to monitor GST activity in the various fractions. Protein content was determined by the method of Bradford (1976).

Enzyme assay

Ethacrynic acid was chosen as the second substrate on the basis of previous reports that it represents one of the better spectrophotometric substrates for acidic isoenzymes other than CDNB (Polidoro *et al.*, 1980; Guthenberg & Mannervik, 1981; Koskelo, 1983) and the lack of substrate inhibition with high GSH concentration. The purified GST was dialysed overnight twice against 1 litre of 10 mM-potassium phosphate buffer, pH 7.4, to remove GSH before kinetic studies. Enzymic activity was determined spectrophotometrically on an Aminco DW-2 instrument. Assays were conducted, with 3 ml of incubation medium maintained at 37 °C, by the method of Habig *et al.* (1974), with minor modification. The incubation mixture consisted of 0.20 mM-ethacrynic acid, various concentrations of GSH (0.1–4.0 mM), 1.0 μg of GST and 0.1 M-potassium phosphate buffer, pH 6.5. Incubation mixture without enzyme served as a control. GST activity towards CDNB was determined as previously described (Radulovic & Kulkarni, 1985).

Electrophoresis

After h.p.l.c. purification of placental GST in the presence of 10 mM-GSH, the fractions that comprised the major peak were pooled and concentrated to approx. 1.5 ml by using an Amicon PM-10 membrane. The concentrated sample was dialysed overnight against 10 mM-potassium phosphate buffer containing 10 mM-GSH, pH 6.0, before electrophoresis. Polyacrylamide-gel electrophoresis was conducted as previously described (Radulovic & Kulkarni, 1985), with no, 5.0 mM- and 10.0 mM-GSH in the running buffers.

Fluorimetry

The changes in intrinsic fluorescence of the tryptophan residues of placental GST due to GSH were assessed as the decrease in fluorescence, using 100 μg of GST in the presence of a prescribed concentration of ligand at 25 °C. Measurements were made in 10 mM-potassium phosphate buffer, pH 7.4, with a Perkin–Elmer 650–40 spectrofluorimeter. Excitation and emission wavelengths were 283 and 334 nm respectively, with slits set at 10 nm. Stock solutions of GSH were made up in the same buffer and the pH adjusted to 7.4 with 1 M-KOH.

Determination of glutathione

The tissue concentration of GSH + GSSG was determined by Method-I of Akerboom & Sies (1981), with minor modification. A 2 ml portion of placental homogenate was heated in a boiling-water bath for 5 min to precipitate protein. The heat-treated homogenates were centrifuged at 4000 *g* for 15 min to pellet the precipitated protein, and an appropriate portion of the resulting supernatant was used in the assay. The assay mixture (1.1 ml) consisted of 63.6 μM-DTNB, 0.2 mM-NADPH, 20 μl of glutathione reductase (6 units/ml), 50 μl of term-placental supernatant and 1 ml of 0.1 M-potassium phosphate containing 1 mM-EDTA, pH 7.0. The rate of formation of 5-thio-2-nitrobenzoate at 25 °C was monitored at 412 nm in an Aminco DW-2 spectrophotometer. Reaction mixture without glutathione reductase was used as a control. The assay was standardized by using 12.5, 25, 50, 75 and 100 μl of 10 μM-GSSG.

RESULTS

As reported previously (Radulovic & Kulkarni, 1985) and noted in the present study, the presence of 1.0 mM-DTT and $\geqslant$ 1 mM-GSH is required to obtain essentially complete recovery of placental GST from the h.p.l.c. column. The results given in Fig. 1 indicate that placental GST can exist as two distinct forms differing in charge, depending upon the concentration of GSH in the immediate environment. When GST is dialysed and chromatographed with buffer containing 0.6 mM-GSH, the enzyme was eluted as a highly anionic conformer (AA) with a retention time (R_T) of 13.6 min (Fig. 1*a*), comprising 99% of the recovered activity. However, when both dialysis and h.p.l.c. were performed in the presence of 10.0 mM-GSH, GST appeared as conformer A, which was eluted at 5.8 min (Fig. 1*b*) and comprised 96% of the recovered activity. In addition, the h.p.l.c. profile of GST dialysed and chromatographed in the presence of 1.0 mM-GSH yielded both A and AA conformers. However, the percentage distribution based on the recovered activity was approx. 91% A and 9% AA.

Re-chromatography of A, isolated by using 10 mM-GSH, and AA, isolated by using 0.6 mM-GSH, was conducted under identical conditions to determine whether the conversion could be an artefact of the h.p.l.c. procedure. On re-chromatography both conformers retained approx. 98% (A) and 99% (AA) of the activity in the original form. The facts that the R_T values for both forms are highly reproducible and that no other peaks were observed suggest that the observed charge isomers are not artefacts of h.p.l.c.

Since various proportions of the two conformers were resolved when different concentrations of GSH were used in the mobile phase, the question arose as to whether the conversion could be bi-directional. Hence GST, which was previously dialysed against 0.6 mM-GSH, was chromatographed in the presence of 10 mM-GSH. The sole conformer isolated was A, exhibiting an elution profile similar to that obtained with GST dialysed and chromatographed with 10 mM-GSH (Fig. 1*b*). Since both A and AA were isolated by using 1 mM-GSH, the re-chromatography of presumably distinct forms was conducted to determine whether

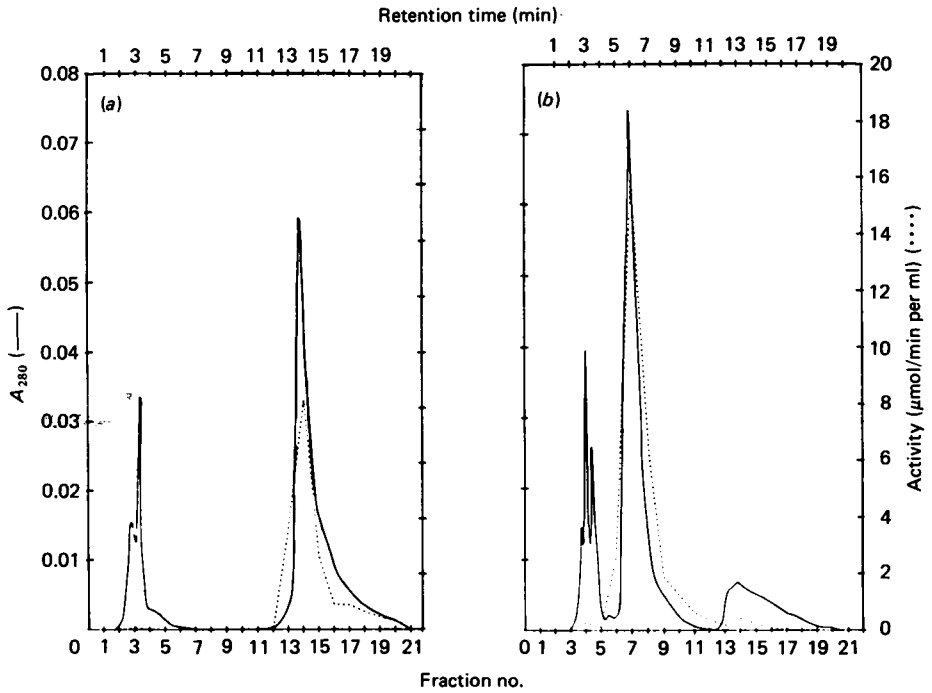

Fig. 1. Effect of glutathione concentration on the h.p.l.c. profiles of human placental GST

Partially purified GST isolated from affinity chromatography was divided into equal portions and dialysed against the indicated concentration of GSH before anion-exchange h.p.l.c. (*a*) The sample applied (90 units, 145 μg of protein) was dialysed against buffer containing 0.6 mM-GSH and chromatographed in the presence of 0.6 mM-GSH. (*b*) The sample applied (90 units, 152 μg of protein) was dialysed and chromatographed in the presence of 10 mM-GSH. A similar elution profile was obained when GST dialysed against 0.6 mM-GSH was chromatographed with 10 mM-GSH in the mobile phase. For further details, see the Materials and methods section.

interconvertibility was possible. Re-chromatography of A and AA in the presence of 1 mM-GSH yielded both conformers in each case. However, the recovered activity exhibited an A/AA ratio of 3:1 and 1:9 for A and AA respectively. These results suggested that the GSH-dependent conversion of GST is a relatively rapid bi-directional process that is governed by the concentration of GSH in the immediate environment and independent of conditions before h.p.l.c. The GSH-dependent conversion cannot simply be attributed to the binding of GSH to its site, since the ratio of mol of GSH in the mobile phase to mol of GST applied to the AX-300 column varies from 186:1 in the case of 0.6 mM-GSH to 2959:1 for 10 mM-GSH, assuming an M_r of 45000 for placental GST. This suggests that one or more non-specific sites other than the GSH-binding site are involved in the conformational change.

Fig. 2 is a composite photograph of three different gels. Electrophoresis was conducted with 100 μg of purified GST. GSH was included in the running buffer at 0 (lane A), 5 (lane B), and 10 (lane C) mM. The two protein bands observed in both lane A and B appeared as a single band when 10 mM-GSH was used (lane C).

The loss of intrinsic fluorescence of tryptophan was also monitored to study the effect of GSH binding on placental GST. Analysis of the data obtained from different purified preparations by Scatchard plot yielded a single binding site with a K_d of 0.42 mM. The maximum loss of intrinsic fluorescence averaged 18.7±2.6% (mean±S.E.M., $n = 3$) with 10 mM-GSH, suggesting that the immediate environment of the tryptophan moieties of placental GST had altered.

Fig. 3 is a representative Lineweaver–Burk plot showing the effect of GSH concentrations on initial velocities of purified GST with ethacrynic acid as the second substrate. A discontinuity in linearity of the activity–response curve was evident at about 1.25 mM-GSH, suggesting differences in affinity of the two conformers towards GSH. In the 0.1–1.0 mM-GSH range, the double-reciprocal plot yielded an apparent K_m (mean±S.E.M., $n = 3$) value of 0.38±0.07 mM for the high-affinity form, whereas an apparent K_m value of 50.27±11.01 mM was determined for the 1.25–4.00 mM-GSH range for the low-affinity form. Differences in average $V_{max.}$ estimations were approx. 46-fold (6.28 versus 289.98 μmol of ethacrynic acid conjugated/min per mg respectively).

An average glutathione concentration (mean±S.E.M., $n = 6$) of 0.11±0.01 mM was estimated for term placenta.

DISCUSSION

Various studies on GST from different tissues (Simons & Vander Jagt, 1980; Vander Jagt *et al.*, 1982, 1983; Mannervik, 1985) have attributed incongruous kinetic results to enzyme 'memory' mechanism, involving slow conformational changes in the protein molecule. However, a procedure that can readily isolate quantitatively different conformational forms of GSH in a catalytically active state has not been available until now.

The evidence presented here (Fig. 1) suggests that the h.p.l.c. procedure, which we developed for the purification of GST (Radulovic & Kulkarni, 1985), is capable of

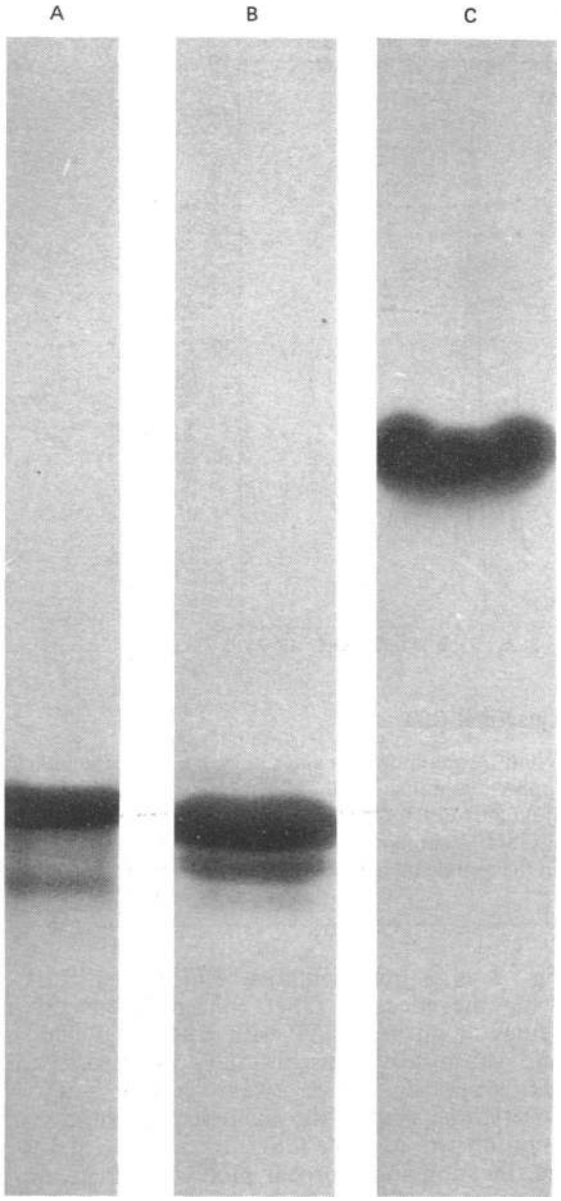

Fig. 2. Gel electrophoresis of human placental GST

Polyacrylamide-gel electrophoresis was performed with 100 μg of GST purified by h.p.l.c., as in Fig. 1(*b*). GSH was included in the running buffers at 0 mM (lane A), 5.0 mM (lane B) and 10.0 mM (lane C), as outlined in the Materials and methods section.

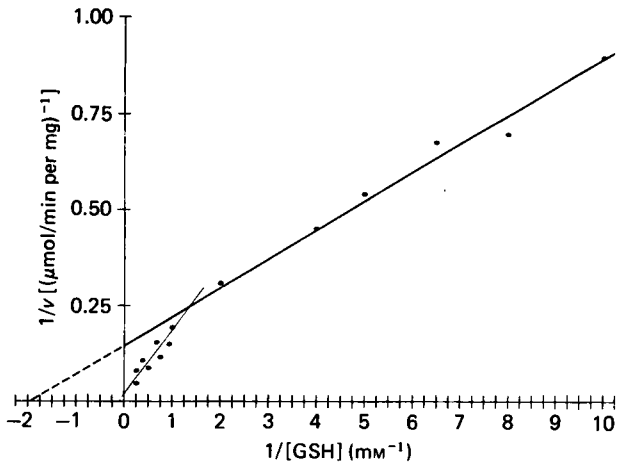

Fig. 3. Reciprocal plot of initial velocities

The assay mixture contained 0.20 mM-ethacrynic acid, various concentrations of GSH and 1.0 μg of GST purified by h.p.l.c. (as in Fig. 1*b*) in 3 ml of 0.1 M-potassium phosphate buffer, pH 6.5. Assays were conducted at 37 °C. Points represent averages of duplicate determinations. See the text for further details.

isolating different conformers differing in surface charge. H.p.l.c. does not result in the generation of multiple 'artefactual' forms of GST, since a series of protein peaks with unpredictable R_T values would be expected, but are not observed.

A possible explanation for the chromatographic behaviour is that GSSG forms a mixed disulphide with GST (Rammage & Nimmo, 1983). However, the possibility of mixed-disulphide formation at pH 5.85 during h.p.l.c. is highly unlikely, since their rate of formation is slow below pH 8.0 (Ziegler, 1985). Furthermore, before h.p.l.c. the samples were dialysed overnight against buffer containing 0.6, 1.0 or 10 mM-GSH at pH 6.0, which should fully reduce any mixed-disulphide linkages formed in the enzyme molecules. In addition, GSH and DTT (1 mM) used in the mobile phase would serve as effective disulphide reductants during h.p.l.c.

It can be argued that the negatively charged GSH may function as a counter-ion and, at higher concentration in the mobile phase, GSH may be sufficient to alter the R_T of GST. This possibility was dismissed for the following reasons: (1) it is highly unlikely that $\leqslant 10$ mM-GSH would be sufficient to elute GST, which requires 400 mM-KCl for elution; (2) it could not account for the presence of both forms, which vastly differ in R_T at 1 mM-GSH; and (3) re-chromatography of A ($R_T = 5.8$ min), which was isolated by using 1 mM-GSH under identical conditions, yielded both A ($R_T = 5.8$ min) and AA ($R_T = 13.6$ min).

The interaction of placental GST with the anion-exchange matrix (secondary and tertiary amines) and the resolution of different conformers cannot be explained simply on the basis of a net charge of -1 on the GSH molecule at pH 5.85. If the association of GSH does impart a -1 charge to GST per molecule of GSH bound, then, in the presence of 10 mM-GSH, the native GST must exhibit a strong anionic nature. Such a highly anionic protein is expected to interact strongly with the column matrix and would be eluted much later (R_T much greater than 5.8 min). In contrast with this, the opposite effect in chromatographic behaviour was observed. Without a concomitant change in conformation, GST cannot dissipate the additional negative charge(s), owing to the association of GSH, which would affect its chromatographic and electrophoretic properties. Electrophoresis of GST in the presence of increasing GSH concentration resulted in the elimination of the second, more anionic, protein band (Fig. 2). This observation, which was also reported by Polidoro *et al.* (1981), further refutes the possibility. Further support for the proposed conformational states is provided by fluorescence studies. The observed gradual loss of intrinsic fluorescence

with increasing GSH concentration indicates that a change in the conformation of GST indeed occurs.

GSH-dependent changes in R_T could be attributed to a perturbation of surface charges concomitant with the conformational change. We believe that exposure of acidic residues and/or the masking of basic amino acid residues may be responsible for the changes in surface charge. Hence AA is converted into A in the presence of high GSH concentrations, whereas an intermediate concentration of GSH (1 mM) results in a mixture of conformers, approx. 9:1 (A/AA). Determination of pI values of the two conformers of placental GST was not attempted, since it has been reported that the two electrophoretically distinct forms cannot be resolved by isoelectric focusing (Polidoro *et al.*, 1981).

In most cells, glutathione exists principally in the reduced form (Ziegler, 1985). Hence, the estimations of total glutathione for term placenta are assumed to correspond to GSH. The average tissue concentration of 0.11 mM-GSH for term is somewhat lower than that of 0.56 mM-GSH reported for a single observation (Wellner *et al.*, 1974). However, this concentration of GSH would be sufficient to support the enzyme-mediated reaction via the high-affinity–low-activity conformer. The non-Michaelian behaviour observed in the experiments with various GSH concentrations is not limited to human placental GST (Mannervik, 1985).

Since several investigators have used ethacrynic acid to measure placental GST activity in the presence of 0.25 mM-GSH by the method of Habig *et al.* (1974), the reported rates, 0.006–0.86 μmol/min per mg (Polidoro *et al.*, 1980; Guthenberg & Mannervik, 1981; Koskelo, 1983), may be reflective of the activity *in vivo*, but not of the catalytic potential of GST. Our data (Fig. 3) indicate that precise estimation of the initial velocity would require the GSH concentration in assay to be $\geqslant$ 1.25 mM. It is interesting to note that the minimum GSH concentration (1.25 mM) necessary to convert GST into the low-affinity–high-activity conformer (Fig. 3) is similar to the concentration (1 mM) that resulted principally in the isolation of A (91% on the basis of recovered activity) by h.p.l.c.

Since our introduction of an h.p.l.c. procedure for the purification of GST (Radulovic & Kulkarni, 1985), an independent group (Singh *et al.*, 1986) has confirmed the versatility of this novel technique by resolving multiple acidic forms of GST from human erythrocytes, lung and heart. The advantage of both h.p.l.c. and f.p.l.c. (Ålin *et al.*, 1985) is rapid separation, which is necessary to minimize and/or to eliminate possible formation of oxidation or degradation products of GSTs that can be erroneously interpreted as new isoenzymes (Mannervik, 1985). We have shown, by various biochemical techniques, that these two GSH-dependent conformers do exist. This is in contrast with the single conformational state that Vander Jagt *et al.* (1981) were able to kinetically discern in the presence of GSH. Although no attempts were made to derive a rate equation for the observed kinetics, the current explanation for the non-Michaelian behaviour appears to be conformational changes of GST (Danielson & Mannervik, 1985).

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