Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4443–4447, May 1991
Biochemistry

Cloning, expression, and characterization of a class-mu glutathione transferase from human muscle, the product of the *GST4* locus

(cDNA sequence/gene conversion/expression in *Escherichia coli*/reaction mechanism)

WILLIAM R. VORACHEK, WILLIAM R. PEARSON*, AND GORDON S. RULE

Department of Biochemistry, University of Virginia, Charlottesville, VA 22908

Communicated by Oscar L. Miller, Jr., January 7, 1991

ABSTRACT **A class-mu glutathione transferase cDNA clone, GTHMUS, was isolated from human myoblasts and its sequence was determined. The sequence predicts a protein of molecular weight 25,599 whose 24 amino-terminal residues are identical to those of the class-mu isoenzyme expressed from the *GST4* locus. The GTHMUS cDNA shares 93.7% nucleotide sequence identity with a human liver cDNA clone, GTH411, that is encoded at the *GST1* locus. Comparison of the liver and muscle cDNA sequences shows two regions of remarkable sequence conservation: a 140-nucleotide region in the 5′ coding portion of the molecule that has a single silent nucleotide substitution, and a 550-nucleotide region, including the entire 3′ noncoding region, that has only three nucleotide substitutions or deletions. This sequence conservation suggests that gene conversion has occurred between the human *GST1* and *GST4* glutathione transferase gene loci. The human muscle and liver glutathione transferase clones GTHMUS and GTH411 have been expressed in *Escherichia coli*. The kinetic mechanism of the muscle enzyme was examined in product inhibition studies. The inhibition patterns are best modeled by a steady-state ordered bi–bi reaction mechanism. Glutathione is the first substrate bound and chloride ion is the first product released. Chloride ion inhibits the muscle enzyme.**

The glutathione transferases (RX: glutathione R-transferase, EC 2.5.1.18) are a family of proteins that detoxify chemical carcinogens, either by conjugating glutathione to reactive electrophilic sites or by direct binding (1, 2). The soluble glutathione transferase enzymes have been grouped into three classes according to protein sequence similarity and antibody crossreactivity—alpha, mu, and pi (2). Members of the same class share 70–95% protein sequence identity, while interclass alignments show 25–30% sequence identity. These enzymes are abundant in mammalian liver. Class-alpha and -pi isoenzymes have been characterized from human, mouse, and rat liver. Genetic loci for six human glutathione transferase enzymes have been described: *GST1–GST6* (3–5). Three of these loci encode class-mu isoenzymes: *GST1* is a polymorphic locus that encodes an enzyme expressed in liver and peripheral blood (3, 6), while the products of *GST4* and *GST5* are expressed in muscle and brain, respectively (5, 7). We report the sequence of the mRNA encoded at the *GST4* locus below.†

Three alleles have been described at the *GST1* locus: a null allele, *GST1-1*, and *GST1-2* (4, 6). Approximately 50% of the population is homozygous for the null allele (4, 8). These individuals lack an enzyme activity on the substrate *trans*-stilbene oxide (8) and are more likely to contract cancer if they are heavy smokers (9). This loss is caused by a gene deletion (4, 10). The mRNA products of the *GST1-1* and *GST1-2* alleles have been cloned (10, 11). The two cDNA clones differ at a single base pair in the protein coding region. GTH411 [the product of *GST1-1* (10)] encodes an asparagine at residue 172 and is also referred to as GST-ψ (12). GTH4 encodes a lysine at this position and is thus more basic; it is referred to as GST-μ (6). The equivalence of *GST1-1* with GST-ψ and *GST1-2* with GST-μ has been confirmed by expression of the products of both alleles (13).

Southern blots of human DNA probed with human class-mu glutathione transferase cDNA probes suggest that there are at least two additional class-mu glutathione transferase genes (10). Additional class-mu isoenzymes have been characterized from muscle (*GST4*), brain (*GST5*) (5), and testes (14). No polymorphisms at these alleles have been described (3, 5, 7). We refer to the gene product of the *GST4* locus as GST4 (human muscle glutathione transferase).

To characterize further the glutathione transferase isoenzymes expressed in human tissues, we have isolated a cDNA clone from human myoblasts that is encoded by *GST4*. The mRNA that encodes the muscle isoenzyme shares extensive sequence identity with the mRNA encoded at *GST1*, suggesting that gene conversion has taken place between these two genes. We have expressed both GST-ψ and muscle (GST4) enzymes in *Escherichia coli*.

EXPERIMENTAL PROCEDURES

cDNA Cloning. The GTHMUS cDNA clone was isolated from a myoblast cDNA library that was the kind gift of Sonia Pearson-White (University of Virginia). The sequence of the insert was determined using the Erase-a-Base system (Promega); both strands were sequenced and each nucleotide was determined an average of six times.

Expression in *E. coli*. The vector pMG27N-S (15) was obtained from SmithKline Beecham. This vector contains the left promoter (P_L) of λ phage and sites for *N*-gene utilization (Nut). Gene expression from P_L is repressed by a temperature-sensitive *c*I gene product. The vector pMG27N-S provides a λ cII ribosome binding site and an *Nde* I restriction endonuclease site at the N-terminal methionine codon.

The glutathione transferase cDNA clones were inserted into pMG27N-S by using a synthetic oligonucleotide adapter that extended from the 5′ *Bst*XI site to the initiation codon and contained an *Nde* I cohesive end at the N-terminal methionine codon. Constructs were propagated on a cI$^+$ strain and transformed into a temperature-sensitive cI strain for induction of the enzyme.

Purification of Glutathione Transferases. For production of human glutathione transferase in *E. coli*, 2 liters of cells were grown in 2× YTE (10 g of yeast extract, 16 g of tryptone, and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; GS-DNB, conjugate between GSH and CDNB.

*To whom reprint requests should be addressed at: Department of Biochemistry, Box 440 Jordan Hall, University of Virginia, Charlottesville, VA 22908.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M63509).

5 g of NaCl per liter) in a New Brunswick Scientific Bioflow III fermentor. Transferase synthesis was induced when the cells reached an OD_{550} of 0.6–0.9, by increasing the temperature of the culture from 32°C to 42°C over 25 min, followed by a reduction in temperature to 40°C. Maximum activity of the glutathione transferase was obtained 3–5 hr after induction. Enzymatic activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate; activities are reported as μmol of CDNB consumed per min.

Harvested cells were suspended in ≈70 ml of 10 mM Tris Cl/5 mM 2-mercaptoethanol, pH 8.0 (buffer A). Lysozyme was added (0.1 mg/ml), followed by incubation on ice for 20 min. The cells were sonicated until lysed and were centrifuged in a Beckman 50.2 Ti rotor for 15 min at 30,000 rpm. The pH of the supernatant was adjusted to 8.0 with Tris base and the supernatant was loaded onto a 50-ml DEAE-Sepharose (Pharmacia) column equilibrated with buffer A. Both the liver and muscle transferases bound to the column and were eluted with a 500-ml linear salt gradient (0–3 M NaCl) in buffer A. Both enzymes were eluted in the first 20% of the gradient.

The eluted transferase was precipitated by the addition of ammonium sulfate to 80% saturation. The precipitated proteins were resuspended in 10 mM Tris Cl/5 mM 2-mercaptoethanol, pH 7.0, and were chromatographed on a 500-ml column of Sephacryl S-200 (Pharmacia) in the same buffer. The peak fractions were pooled and adjusted to pH 7.0 and loaded directly onto a 30-ml hexyl-glutathione (GSH) affinity column (Sigma). After the column was washed with 1 column volume of Tris buffer (10 mM, pH 7.0), the transferases were eluted with 50 mM Tris Cl/5 mM GSH/5 mM 2-mercaptoethanol, pH 9.0. The peak fractions were neutralized with 0.5 M Tris·HCl, concentrated by ultrafiltration, and stored at −80°C.

Enzyme Characterization. Standard assay conditions for glutathione transferases were followed (16). The conjugate between CDNB and GSH (GS-DNB) was synthesized according to Schramm *et al.* (17). Kinetic measurements in the presence of chloride ion were performed in the presence of Na_2SO_4 to maintain a constant ionic strength equivalent to 1 M NaCl. Sulfate did not inhibit enzyme activity. Protein content was determined by the method of Bradford (18) using bovine serum albumin as the standard. Isoelectric pH (pI) values of GST4 were determined by chromatofocusing (Pharmacia) and isoelectric focusing in polyacrylamide gels.

RESULTS

Isolation of a Class-mu Glutathione Transferase cDNA from Human Myoblasts. We isolated and sequenced a class-mu glutathione transferase cDNA from a human myoblast cDNA library. The mRNA for the library came from an individual who was homozygous for a null allele at the *GST1* locus. About 200,000 plaques from the library were probed with a ^{32}P-labeled human liver class-mu cDNA (GTH411); four cross-hybridizing plaques were isolated and purified. Each clone contains an 1100-base-pair insert; the four inserts appear to be identical on the basis of limited sequence data. The complete sequence of one clone, GTHMUS, is shown in Fig. 1*A*. The GTHMUS insert shares 93.7% nucleotide sequence identity with the human liver GTH411 insert. It encodes a protein of 217 amino acids (25,599 Da). The 24 N-terminal residues predicted from the nucleotide sequence of the GTHMUS insert are identical to those determined for the isoenzyme encoded at *GST4* (7, 24). The sequence of the isoenzyme predicted from the GTHMUS insert shares 84.3% amino acid identity with the human liver glutathione transferases encoded at *GST1* and 84.8% identity with the rat 6-6 (Yb3) isoenzyme expressed in rat brain (19). The muscle isoenzyme shares 68% amino acid sequence identity with a class-mu isoenzyme expressed in human brain and testis (14); it shares about 75% sequence identity with class-mu isoenzymes found in rat liver (Fig. 1*B*).

Expression of Glutathione Transferases. We inserted the GTH411 and GTHMUS cDNAs into the expression vector pMG27N-S. Constructions were verified by sequencing 200 nucleotides of the 5′ end of the cDNA insert. For expression from the λP_L promoter in the presence of the *N* gene product, the yield in the crude lysate was ≈10,000 units (or ≈50 mg) of either GST-ψ (the product of the GTH411 insert) or GST4 (the product of the GTHMUS insert) per liter of bacterial culture; 1–2 mg/liter was obtained in the absence of the *N* gene product.

Purification of Glutathione Transferases. The high level of expression of these enzymes permitted a simple three-column purification procedure. The specific activities of GST4 in the cell lysate, in the peak fraction of the DEAE column, in the peak fraction of the S-200 column, and in the peak fraction of the affinity column were 9, 36, 51, and 196 μmol of CDNB per min per mg of protein, respectively. GST4 was quite stable unless it was stored at a pH less than 7.0, in which case the activity dropped by 50% in 1 day. GST-ψ rapidly lost activity in the absence of GSH.

The pI of GST4 was determined by chromatofocusing and isoelectric focusing in polyacrylamide gels. Isoelectric focusing produced a single band at a pH of 5.2. The elution profile of the chromatofocusing column showed a peak of activity at pH 6.0 (containing 70% of the activity) and a broad peak at pH 5.2 (containing 30% of the activity). The additional species found with chromatofocusing was not an impurity; purified GST4 appears homogeneous in an SDS/polyacrylamide gel and readily forms crystals in crystallization trials. We have observed that GST4 is unstable at a pH less than 7.0. Our interpretation of the chromatofocusing profile is that the native form of GST4 has a pI of 6.0, whereas the protein eluted at pH 5.2 is denatured.

Substrate Specificity. The specific activities of GST4 and GST-ψ on various substrates are shown in Table 1. The specific activity of GST4 on CDNB was quite reproducible in different enzyme preparations. In contrast, the specific activity of GST-ψ on CDNB varied from batch to batch. The specific activities given in Table 1 are representative for GST-ψ, but specific activities measured with CDNB as the substrate were as high as 160 μmol per min per mg in some preparations.

Kinetic Mechanism of GST4. Given the nature of the chemical reaction catalyzed by glutathione transferases, it is reasonable to assume a bi–bi reaction mechanism. For this type of reaction, GSH and the substrate must both be present in the active site before a reaction can occur, and the conjugated substrate and a leaving group (chloride in the case of CDNB) are produced. There are at least 14 different bi–bi mechanisms that have been characterized by their product inhibition patterns (see ref. 25).

We measured initial enzymatic rate data in the presence of various concentrations of GSH and CDNB. Representative data are shown in Fig. 2. A nonlinear least-squares program (NONLIN; ref. 26) was used to fit the data to the following kinetic models: rapid-equilibrium ordered bi–bi with GSH binding first; rapid-equilibrium ordered bi–bi with CDNB binding first; steady-state ordered bi–bi with GSH binding first; steady-state random sequential bi–bi; and an equilibrium random sequential bi–bi mechanism. The best fit was obtained with an ordered steady-state mechanism; the fitted curves are shown in Fig. 2.

To confirm that the mechanism is an ordered steady-state mechanism, we measured product inhibition using either chloride ion or GS-DNB as inhibitors. All of the Lineweaver–Burk plots obtained in the presence of these inhibitors were linear. The inhibition patterns were classified as either competitive, uncompetitive, or mixed type (noncompetitive).

A

```
                         ProMetThrLeuGlyTyrTrpAsnIleArgGlyLeuAlaHisSerIleArgLeuLeuLeuGluTyrThrAspSerSerTyrGluGluLysLysTyrThrMe
               10        20        30        40        50        60        70        80        90       100       110       120
gthmus CGCAGCAACCAGCACCATGCCCATGACACTGGGGTACTGGAACATCCGCGGGCTGGCCCATTCCATCCGCCTGCTCCTGGAATACACAGACTCAAGCTACGAGGAAAAGAAGTACACGAT
GST1-1     c                    T             G                  cG                                    t

       tGlyAspAlaProAspTyrAspArgSerGlnTrpLeuAsnGluLysPheLysLeuGlyLeuAspPheProAsnLeuProTyrLeuIleAspGlyThrHisLysIleThrGlnSerAsnAl
              130       140       150       160       170       180       190       200       210       220       230       240
gthmus GGGGGACGCTCCTGATTATGACAGAAGCCAGTGGCTGAATGAAAAATTCAAGCTGGGCCTGGACTTTCCCAATCTGCCCTACTTGATTGATGGGACTCACAAGATCACCCAGAGCAATGC
GST1-1                                                                                            G                     c

       aIleLeuArgTyrIleAlaArgLysHisAsnLeuCysGlyGluSerGluLysGluGlnIleArgGluAspIleLeuGluAsnGlnPheMetAspSerArgMetGlnLeuAlaLysLeuCy
              250       260       270       280       290       300       310       320       330       340       350       360
gthmus CATCCTGCGGTACATTGCCCGCAAGCACAACCTGTGCGGGGAATCAGAAAAGGAGCAGATTCGCGAAGACATTTTGGAGAACCAGTTTATGGACAGCCGTATGCAGCTGGCCAAACTCTG
GST1-1     t  T C                          t     gA     G     A       t TG                  ACC       A  A           G  TGA

       sTyrAspProAspPheGluLysLeuLysProGluTyrLeuGlnAlaLeuProGluMetLeuLysLeuTyrSerGlnPheLeuGlyLysGlnProTrpPheLeuGlyAspLysIleThrPh
              370       380       390       400       410       420       430       440       450       460       470       480
gthmus CTATGACCCAGATTTTGAGAAACTGAAACCAGAATACCTGCAGGCACTCCCTGAAATGCTGAAGCTCTACTCACAGTTTCTGGGGAAGCAGCCATGGTTTCTTGGGGACAAGATCACCTT
GST1-1    cA T     A              g   A G   t  G   A           A   a            G               G          GCA  AA          t

       eValAspPheIleAlaTyrAspValLeuGluArgAsnGlnValPheGluProSerCysLeuAspAlaPheProAsnLeuLysAspPheIleSerArgPheGluGlyLeuGluLysIleSe
              490       500       510       520       530       540       550       560       570       580       590       600
gthmus TGTGGATTTCATCGCTTATGATGTCCTTGAGAGAAACCAAGTATTTGAGCCCAGCTGCCTGGATGCCTTCCCAAACCTGAAGGACTTCATCTCCCGATTTGAGGGCTTGGAGAAGATCTC
GST1-1    a      tC  TC              CCTCC   GTA            A    t    c           t                    c

       rAlaTyrMetLysSerSerArgPheLeuProArgProValPheThrLysMetAlaValTrpGlyAsnLys
              610       620       630       640       650       660       670       680       690       700       710       720
gthmus TGCCTACATGAAGTCCAGCCGCTTCCTCCCAAGACCTGTGTTCACAAAGATGGCTGTCTGGGGCAACAAGTAGGGCCTTGAAGGCAGGAGGTGGGAGTGAGGAGCCCATACTCAGCCTGC
GST1-1                                            T

              730       740       750       760       770       780       790       800       810       820       830       840
gthmus TGCCCAGGCTGTGCAGCGCAGCTGGACTCTGCATCCCAGCACCTGCCTCCTCGTTCCTTTCTCCTGTTTATTCCCATCTTTACTCCCAAGACTTCATTGTCCCTCTTCACTCCCCCTAAA
GST1-1

              850       860       870       880       890       900       910       920       930       940       950       960
gthmus CCCCTGTCCCATGCAGGCCCTTTGAAGCCTCAGCTACCCACTATCCTTCGTGAACATCCCCTCCCATCATTACCCTTCCCTGCACTAAAGCCAGCCTGACCTTCCTTCCTGTTAGTGGTT
GST1-1

              970       980       990      1000      1010      1020      1030      1040      1050      1060      1070
gthmus GTGTCTGCTTTAAAGGGCCTGCCTGGCCCCTCGCCTGTGGAGCTCAGCCCCGAGCTGTCCCCGTGTTGCATGAAGGAGCAGCATTGACTGGTTTACAGGCCCTGCTCCTGCAGCATGGTC
GST1-1               --

   1080      1090      1100      1110      1120      1130
gthmus CCTGCCTTAGGCCTACCTGATGGAAGTAAAGCCTCAACCACAAAAAAAAAAAAA
GST1-1       -
```

B

```
                   10        20        30        40        50        60        70        80        90       100       110       120
gthmus     PMTLGYWNIRGLAHSIRLLLEYTDSSYEEKKYTMGDAPDYDRSQWLNEKFKLGLDFPNLPYLIDGTHKITQSNAILRYIARKHNLCGESEKEQIREDILENQFMDSRMQLAKLCYDPDFE
GST4       ........................
GST1-1     ..I....D......A......................................................A..........C...........T.E.K..V......T..NH...GMI..N.E..
GST1-2     ..I....D......A......................................................A..........C...........T.E.K..V......T..NH...GMI..N.E..
gstmua SCESS.V....D......A......F..T.....R..C.E..........DV....D.........L..KN.................M...T.E.K..V..I...V..F.T..IR...SS.H.

rat3-3     ..I.....V...T.P..............R.A...............................SR........M..L....H....T.E.R..A..V...V..N....IM...N....
rat4-4     .......D......A...F.....T...D...S.............S.................S............LG........T.E.R..V.V....A..T.L...MV..S....
rat6-6     .......D......A..............R.......F..........................S............LG........T.E.R..V......L..N..V..R...N....

              130       140       150       160       170       180       190       200       210            % identity
gthmus KLKPEYLQALPEMLKLYSQFLGKQPWFLGDKITFVDFIAYDVLERNQVFEPSCLDAFPNLKDFISRFEGLEKISAYMKSSRFLPRPVFTKMAVWGNK                  muscle
GST1-1 ....K..EE...K.....E....R...A.N.......LV....DLHRI...N...................................S........            84.3   liver ψ
GST1-2 ....K..EE...K.....E....R...A.N.......LV....DLHRI...K...................................S........            84.3   liver μ
gstmua ....Q..EE..GQ..QF.M..W.FS..A.E.L.....LT..I.DQ.RI.D.K...E.....A.MC...A....A..LQ.DQ.CKM.INN...Q....PVC        68.2   brain/testis

rat3-3 .Q...F.KTI..KM....E....R...A...V.Y...L...I.DQYHI...K...........LA.....K..........Y.ST.I.S.L.Q.S..            76.0
rat4-4 RK.....EG...KM....E......S.A.N...Y...LV....DQHRI...K...........VA.....K...D....G...SK.I.A...F.NP.            76.0
rat6-6 ....G..EQ..G.MR...E....R...A......................AT............A.....K...D...........L.....I..S.            84.8
```

FIG. 1. (*A*) The nucleotide and amino acid sequences of the human class-mu glutathione transferase cDNA clone GTHMUS. A potential poly(A)-addition site (AAGTAAA) is underlined. Also shown are the nucleotide differences between the muscle GTHMUS sequence and the liver GTH411 (GST1-1) sequence encoded at the *GST1* locus. Only the differences are shown; all other residues are the same in both cDNA inserts. Lowercase letters indicate silent changes. (*B*) Comparison of GTHMUS with human and rat class-mu sequences. Amino acid replacements and percent identities with respect to GTHMUS are noted. References for the sequences are as follows: GST4 (7); GST1-1 (10); gstmua (human testis) (14); rat 6-6 (Yb3) (19); rat 4-4 (Yb1) (20, 21); and rat 3-3 (Yb2) (22, 23).

When chloride was used as the inhibitor, we found mixed-type inhibition under three conditions: varying GSH concentration at unsaturating concentrations of CDNB (<0.45 mM), varying concentrations of CDNB at unsaturating concentrations of GSH, and varying concentrations of CDNB at saturating levels of GSH (Fig. 3). We could not perform chloride inhibition experiments under conditions of saturating CDNB because of the low solubility of CDNB in water. The chloride inhibition data exclude all rapid-equilibrium mechanisms.

Table 1. Substrate specificity of GST-ψ (liver) and GST4 (muscle)

Substrate	Specific activity, μmol per min per mg	
	GST-ψ	GST4
CDNB	78	196
1,2-Dichloro-4-nitrobenzene	1.10	1.60
trans-4-Phenyl-3-buten-2-one	0.28	0.01
Ethacrynic acid	0.20	0.22
p-Nitrophenyl acetate	1.10	0.02

Product inhibition studies with GS-DNB were also performed. Reciprocal plots were obtained at various concentrations of GS-DNB by varying the concentration of GSH in the presence of a fixed concentration of CDNB (0.425 mM). These plots intersect above the *x* axis on the $1/v = 0$ line; thus, inhibition is competitive (data not shown). In contrast, no inhibition was observed in the presence of saturating (5 mM) GSH, 0.1 mM conjugate, and CDNB concentrations varying from 0.25 to 1 mM (data not shown). The product

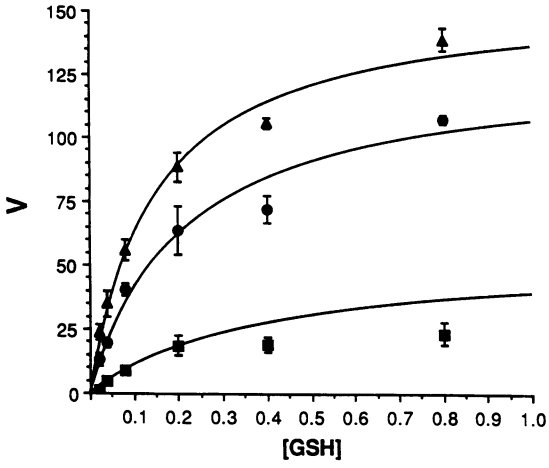

FIG. 2. Plots of initial rate data in the absence of products for GST4. The concentrations of GSH are plotted on the ordinate in units of mM. CDNB concentrations were 0.1 mM (bottom curve), 0.5 mM (middle curve), and 1 mM (top curve). Error bars show the standard error of each triplicate measurement. Curves were drawn for an ordered steady-state model.

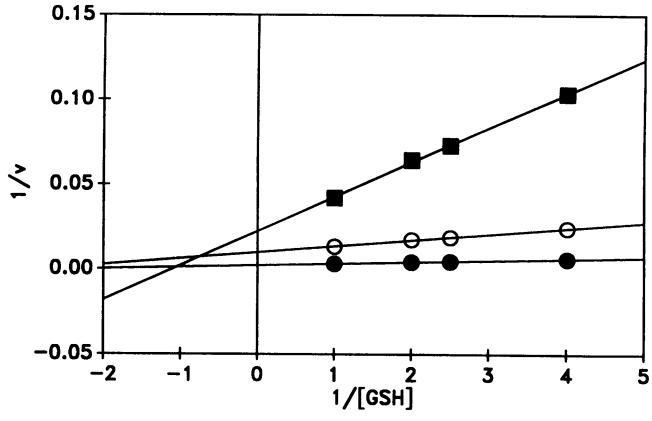

FIG. 3. Reciprocal plots of initial rate data in the presence of chloride for GST4 with varying GSH (mM) and constant CDNB (0.425 mM). Chloride concentrations (as NaCl) were 0.0 M (●), 0.15 M (○), and 0.5 M (■).

inhibition patterns obtained with GS-DNB as the inhibitor are consistent with either a steady-state ordered mechanism or an iso-Theorell–Chance mechanism.

If the reaction mechanism were steady-state ordered bi–bi, then the first substrate bound would be GSH. If the reaction mechanism were iso-Theorell–Chance, then the first substrate bound would be CDNB. We used fluorescence quenching to measure the binding of GSH to GST4 and found a binding affinity $\approx 10^5$ M/liter. These data suggest that the kinetic mechanism of GST4 is an ordered steady-state bi–bi.

The kinetic data therefore suggest an ordered steady-state mechanism in which GSH binds first, followed by CDNB. Chloride is released first, followed by the conjugate. The kinetic constants, as determined from nonlinear regression, are $V_{max} = 264 \pm 30$ units/mg, $K_{ia} = 0.17 \pm 0.07$ mM, $K_{mA} = 0.13 \pm 0.07$ mM, and $K_{mB} = 0.69 \pm 0.15$ mM (see ref. 21 for nomenclature).

DISCUSSION

We have isolated a class-mu glutathione transferase cDNA clone, GTHMUS, from a human muscle library on the basis of homology to the liver glutathione transferase cDNA clone GTH411. GTHMUS encodes a protein that is identical in sequence to the first 24 amino acids of a glutathione transferase found in human muscle (5, 24) that is encoded by the *GST4* locus (5). The isoelectric point of GST4 has been reported to be 5.1 (24) or 5.2 (5), consistent with the 5.2 value for the product of the GTHMUS cDNA clone determined here. Chromatofocusing of the GTHMUS product indicates the presence of an additional species with an apparent pI of 6.0; this has also been observed in another report of human muscle transferases (27). Based on both amino acid sequence identity and isoelectric pH, we conclude that the clone GTHMUS represents mRNA transcribed from *GST4*. The GTHMUS cDNA clone is not a product of *GST1*; the myoblast cell line was obtained from muscle tissue from an individual who is homozygous for the null allele at *GST1*. The protein encoded by the GTHMUS cDNA insert is 68–85% identical to other class-mu glutathione transferases.

A dramatic feature of the GTHMUS/GST4 sequence is its similarity to the liver GTH411 sequence in the 5′ coding and 3′ noncoding regions. Both mRNAs are polyadenylylated at exactly the same position. Furthermore, there are only three differences between the two sequences (two insertions and one deletion) in 450 nucleotides of the 3′ noncoding sequence (nucleotides 671–1120 of GTHMUS). This conservation continues for 90 nucleotides into the C-terminal coding sequence with only one nucleotide substitution. A second highly conserved region is found in the mRNA encoding the N-terminal 40% of the protein, where a 152-nucleotide region (nucleotides 63–214) contains only one silent nucleotide substitution. In contrast, there are 54 nucleotide substitutions, which cause 28 amino acid replacements, in the 280 nucleotides between 277 and 556. The striking conservation of the 3′ halves of the GTH411 and GTHMUS mRNAs suggests that gene conversion has taken place between the *GST1* and *GST4* loci.

Gene conversion has been reported in a number of detoxification genes, including glutathione transferases and cytochrome P450s. Tu and coworkers (28) have reported remarkable conservation of the introns in two rat class-mu glutathione transferase genes, Yb2 and Yb4. Despite the fact that the mRNA encoded by the Yb4 gene differs from the Yb2 mRNA at 53 of 217 possible sites, three of the seven introns in the two genes share >88% nucleotide sequence identity. Gonzalez and coworkers (29) have described a cluster of three human cytochrome P450 genes on chromosome 22, one of which (*CYP2D6*) encodes the enzyme responsible for debrisoquine metabolism. Mutant alleles of the *CYP2D6* gene are responsible for the poor-metabolizer phenotype that is found in 5–10% of Caucasian individuals. *CYP2D6* is closely linked to *CYP2D7* on the chromosome and these two genes share 94–100% identity in six of their eight introns. It has been suggested that recombination and conversion between genes in this cluster may be responsible for the high frequency of mutations in the *CYP2D6* gene (29). Similar interactions between *GST1* and *GST4* may be responsible for the high frequency of deletion of the *GST1* locus.

Both GST-ψ and GST4 show high enzymatic activities on CDNB and 1,2-dichloro-4-nitrobenzene substrates. The specific activities reported here are within the ranges reported for other class-mu glutathione transferases. Despite 84.3% sequence identity with GST4, GST-ψ shows a much higher activity than does GST4 toward *trans*-4-phenyl-3-buten-2-one (tPBO) and *p*-nitrophenyl acetate. The specific activity of GST-ψ toward tPBO is similar to that found for the rat 4-4 class-mu transferase (1.18 μmol per min per mg; ref. 2), which also shares high sequence identity with GST-ψ (81%; ref. 10). The activity of GST4 on tPBO is similar to that of the rat 6-6 isoenzyme (0.02 μmol per min per mg; ref. 2), which shares 84.8% identity with GST4 (Fig. 1*B*). The amino acid sequence of GST-ψ is identical to that of the rat 4-4 class-mu enzyme at 10 out of 11 residues from Leu-158 to Ile-168, while

GST4 differs at 7 of these 11 residues. In contrast, GST4 is identical to the rat 6-6 isoenzyme in this region. Thus, this region may be involved in the catalysis of tPBO.

Of the possible inhibition patterns for bi–bi reaction mechanisms, only the steady-state ordered bi–bi mechanism is consistent with the data. The order of substrate addition is GSH followed by CDNB. The first product released is chloride ion, followed by the GS-DNB conjugate.

This reaction mechanism for GST4 is different from the reaction mechanism reported for the human placental class-pi isoenzyme (30). Product inhibition studies with the GS-DNB conjugate indicate that the mechanism of the placental glutathione transferase is a rapid-equilibrium random mechanism (30).

The kinetic mechanism of a rat class-alpha enzyme was investigated by Pabst *et al.* (31). The kinetic pathway for this enzyme depends on the concentration of GSH. At low GSH concentrations (<0.1 mM), a ping–pong mechanism (CDNB binding followed by chloride release) is found, while at high GSH concentrations (>1 mM) a steady-state ordered mechanism is suggested. In the latter case, the order of substrate and product addition/removal is the same as that reported here for GST4. Our data over a wide range of GSH concentrations do not suggest an alternative pathway for GST4.

It has been reported that a human liver class-mu transferase exhibits a random bi–bi mechanism (32). However, this report was based on fitting initial rate data for various substrate concentrations. Without product inhibition data, it is not possible to distinguish a steady-state ordered reaction from a rapid-equilibrium random mechanism (25).

An ordered reaction scheme may be a general mechanism for glutathione transferases under physiological GSH concentrations. This appears to be true for a number of transferases. However, the reported mechanism for the placental enzyme is an exception to this generalization. An apparent difference in reaction mechanisms for different transferases need not imply a different catalytic mechanism (except in the case of the ping–pong model). The differences between GST4 and the placental enzyme may be due to differences in the affinities of these two enzymes for their substrates.

This research was supported by research grants from the American Cancer Society to W.R.P. (CN-61660) and G.S.R. (BC-703) and a University of Virginia Biomedical Research Grant (RR-05431) to G.S.R.

1. Jakoby, W. B. (1978) *Adv. Enzymol.* **46,** 383–414.
2. Mannervik, B. & Danielson, U. H. (1988) *CRC Crit. Rev. Biochem.* **23,** 281–334.
3. Board, P. G. (1981) *Am. J. Hum. Genet.* **33,** 36–43.
4. Board, P. G. (1981) *FEBS Lett.* **135,** 12–14.
5. Suzuki, T., Coggan, M., Shaw, D. C. & Board, P. G. (1987) *Ann. Hum. Genet.* **51,** 95–106.
6. Seidegard, J., Guthenberg, C., Pero, R. W. & Mannervik, B. (1987) *Biochem. J.* **246,** 783–785.
7. Board, P. G., Suzuki, T. & Shaw, D. C. (1988) *Biochim. Biophys. Acta* **953,** 214–217.
8. Seidegard, J. & Pero, R. W. (1985) *Hum. Genet.* **69,** 66–68.
9. Seidegard, J., Pero, R. W., Miller, D. G. & Beattie, E. J. (1986) *Carcinogenesis* **7,** 751–753.
10. Seidegard, J., Vorachek, W. R., Pero, R. W. & Pearson, W. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7293–7297.
11. DeJong, J. L., Chang, C. M., Whang-Peng, J., Knutsen, T. & Tu, C. D. (1988) *Nucleic Acids Res.* **16,** 8541–8555.
12. Singh, S. V., Kurosky, A. & Awasthi, Y. C. (1987) *Biochem. J.* **243,** 61–67.
13. Widersten, M., Pearson, W. R., Engstrom, A. & Mannervik, B. (1991) *Biochem. J.*, in press.
14. Campbell, E., Takahashi, Y., Abramovitz, M., Peretz, M. & Listowsky, I. (1990) *J. Biol. Chem.* **265,** 9188–9193.
15. Shatzman, A. R. & Rosenberg, M. (1986) *Ann. N.Y. Acad. Sci.* **478,** 233–247.
16. Habig, W. H. & Jakoby, W. B. (1981) *Methods Enzymol.* **77,** 398–405.
17. Schramm, V. L., McCluskey, R., Emig, F. A. & Litwack, G. (1984) *J. Biol. Chem.* **259,** 714–722.
18. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
19. Abramovitz, M. & Listowsky, I. (1987) *J. Biol. Chem.* **262,** 7770–7773.
20. Ding, G. J. F., Lu, A. Y. H. & Pickett, C. B. (1985) *J. Biol. Chem.* **260,** 13268–13271.
21. Lai, H. C. & Tu, C. D. (1986) *J. Biol. Chem.* **261,** 13793–13799.
22. Ding, G. J., Ding, V. D., Rodkey, J. A., Bennett, C. D., Lu, A. Y. H. & Pickett, C. B. (1986) *J. Biol. Chem.* **261,** 7952–7957.
23. Alin, P., Mannervik, B. & Jornvall, H. (1985) *Eur. J. Biochem.* **156,** 343–350.
24. Singh, S. V., Ahmad, H., Kurosky, A. & Awasthi, Y. C. (1988) *Arch. Biochem. Biophys.* **264,** 13–22.
25. Segel, I. H. (1975) *Enzyme Kinetics* (Wiley, New York).
26. Johnson, M. L. & Frasier, S. G. (1985) *Methods Enzymol.* **117,** 301–342.
27. Hirrell, P. A., Hume, R., Fryer, A. A., Collins, M. F., Dres, R., Bradwell, A. R. & Strange, R. C. (1987) *Biochem. Biophys. Acta* **915,** 371–377.
28. Lai, H. J., Qian, B., Grove, G. & Tu, C. D. (1988) *J. Biol. Chem.* **263,** 11389–11395.
29. Kimura, S., Umeno, M., Skoda, R. C., Meyer, U. A. & Gonzalez, F. J. (1989) *Am. J. Hum. Genet.* **45,** 889–904.
30. Ivanetich, K. M. & Goold, R. D. (1989) *Biochem. Biophys. Acta* **998,** 7–13.
31. Pabst, M. J., Habig, W. H. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249,** 7140–7150.
32. Warholm, M., Guthenberg, C. & Mannervik, B. (1983) *Biochemistry* **22,** 3610–3617.