Mutagenesis of residue 157 in the active site of human glyoxalase I

Marianne RIDDERSTRÖM*, Alexander D. CAMERON†, T. Alwyn JONES† and Bengt MANNERVIK*[1]

*Department of Biochemistry, Uppsala University, Biomedical Center, Box 576, S-751 23 Uppsala, Sweden and †Department of Molecular Biology, Uppsala University, Biomedical Center, Box 590, S-751 24 Uppsala, Sweden

Met-157 in the active site of human glyoxalase I was changed by site-directed mutagenesis into alanine, glutamine or histidine in order to evaluate its possible role in catalysis. The glyoxalase I mutants were expressed in *Escherichia coli* and purified on an *S*-hexylglutathione affinity gel. The physicochemical properties of the mutant proteins were similar to those of the wild-type enzyme. The glutamine mutant exhibited the same high specific activity as wild-type glyoxalase I, whereas the alanine and histidine mutants had approx. 20 % of wild-type activity. The k_{cat}/K_m values of the mutant glyoxalase I determined with the hemithioacetal adduct of glutathione and methylglyoxal were reduced to between 10 and 40 % of the wild-type value. This reduction was due to lower k_{cat} values for the alanine and histidine mutants and a twofold increase in the K_m value for the glutamine mutant. With the hemithioacetal of glutathione and phenylglyoxal, the kinetic parameters of the mutants were also of the same magnitude as those of wild-type glyoxalase I. Studies with the competitive inhibitors *S*-hexyl- and *S*-benzyl-glutathione revealed that the affinity was reduced to 7–11 % of the wild-type affinity for the glutamine and alanine mutants and to 30–40 % for the histidine mutant, as measured by a comparison of K_i values. The results show that Met-157 has no direct role in catalysis, but is rather involved in forming the substrate-binding site of human glyoxalase I. The high activity of the glutamine mutant suggests that a structurally equivalent glutamine residue in the N-terminal half of *Saccharomyces cerevisiae* glyoxalase I may be part of a catalytically competent active site.

INTRODUCTION

The glyoxalase system is of importance for detoxication of reactive 2-oxoaldehydes via their transformation into the corresponding non-toxic 2-hydroxy acids using glutathione as a cofactor [1–3]. Since 2-oxoaldehydes react with functional groups in essential biomolecules, such as guanyl nucleotides in nucleic acids to form imidazopurinone adducts [4], and with cysteine, lysine and arginine residues in proteins [5], it is obvious that elimination of these compounds may be essential to proper functioning of a cell. The pathophysiological correlates of certain diseases, *e.g.* diabetes, appear to be related to elevated levels of 2-oxoaldehydes [2]. The physiological substrates of glyoxalase I are assumed to be methylglyoxal, glyoxal and 4,5-dioxovalerate [1–3]. Methylglyoxal is formed as a by-product from the triosephosphate isomerase reaction in glycolysis [6], from acetone in ketone body metabolism [7] and from aminoacetone in threonine catabolism [8]. Glyoxal is formed by lipid peroxidation and degradation of glucose and glycated proteins [9]. 4,5-Dioxovalerate is a metabolite deriving from the degradation of 4-aminolaevulinate [10]. The glyoxalase system is believed to be ubiquitous, and it has been found in most human tissues [11,12] as well as in a wide variety of biological species.

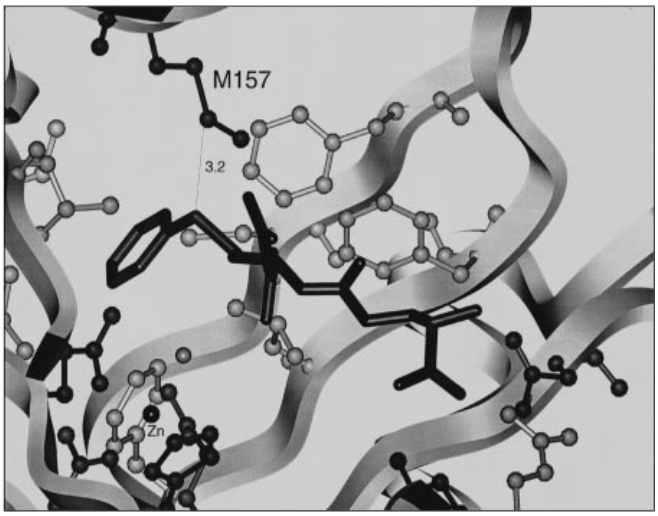

Figure 1 Schematic representation of active-site residues of human glyoxalase I

S-Benzylglutathione represents the ligand bound in the crystal structure [20]. The Zn^{2+} is shown together with its five ligands, Gln-33, Glu-99, His-126, Glu-172 and water. The sulphur of the Met-157 is present 3.2 Å from the expected position of the C-1 of the S-substituent of the glutathione derivative.

The glyoxalase system is composed of two distinct enzymes [13], glyoxalase I (EC 4.4.1.5; lactoylglutathione lyase) and glyoxalase II (EC 3.1.2.6; hydroxyacylglutathione hydrolase). Glyoxalase I catalyses the formation of *S*-D-lactoylglutathione from the hemithioacetal adduct formed spontaneously between methylglyoxal and GSH. *S*-D-Lactoylglutathione is a substrate for glyoxalase II, which hydrolyses the *S*-ester into D-lactate and glutathione.

Human glyoxalase I is a dimer with a molecular mass of 43 kDa [14,15]. Two allelic subunit variants differing in charge (residue 110), although with similar properties and function, have been found by purification of the enzyme from erythrocytes [16] and analysis of cDNA encoding glyoxalase I [17,18]. Glyoxalase I requires Zn^{2+} for its catalytic function [19]. The interaction of the protein with the metal has been elucidated in the three-dimensional crystal structure [20], in which the metal is found to be bound to four amino acid side chains, two residues deriving from each of the two subunits. The Zn^{2+} is believed to

[1] To whom correspondence should be addressed.

	157	172
Human[a]	VKKPDDGK**M**KGLAFIQDPDGYWIEILNP	
Tomato[b]	----L---**M**--I---K---------FDT	
S. cerevisiae N[c]	K-RLSE-R**Q**-DI--ALG--------TYS	
S. cerevisiae C	SP-FNQ-R**M**-NI--LK-----S--VVPH	
S. pombe N[d]	K--LS---**M**-HI--AL---N----LVSQ	
S. pombe C	K--LT--R**M**-DI--LL---N--V-VIEQ	
P. putida[e]	Q-RLS--R**M**NH----K------V-VIQ-	

Figure 2 Alignment of amino acid sequences corresponding to the region surrounding the active-site Met-157 of human glyoxalase I

Amino acid 157 (Met) is the mutated active-site residue and amino acid 172 (Glu) is one of the ligands to the active-site Zn^{2+}, as evidenced by the crystal structure of human glyoxalase I [20]. The yeast sequences *S. cerevisiae* and *Schizosaccharomyces pombe* can be divided into an N- and a C-terminal half, each apparently homologous to the human glyoxalase I subunit [25]. *P. putida* is *Pseudomonas putida*. Data came from the following references as indicated: [a] [14,15,17,18]; [b] [22]; [c] Gentles et al. (1995), Swiss Protein Bank accession number P50107, [23]; [d] Devlin et al. (1995), Swiss Protein Bank accession number Q097519; [e] [24].

be of importance for catalysis, which involves formation of an ene-diolate intermediate during the isomerization of the hemithioacetal adduct into the *S*-ester [21]. The suggested catalytic mechanism requires an active-site residue that can effect abstraction of the hydrogen from C-1 and deliver it to C-2. However, the crystal structure of glyoxalase I does not reveal any obvious candidate that could serve this function in the active site, except for the ligands of the metal. On the other hand, Met-157, the sulphur of which is only 3.2 Å away from C-1 in *S*-benzylglutathione, a substrate and product analogue bound at the active site (Figure 1), may play a role in catalysis. This residue is conserved among several established primary structures of the enzyme and the putative glyoxalase I amino acid sequences from eukaryotes, except in the N-terminal half of the yeast *Saccharomyces cerevisiae*, where a glutamine is present (Figure 2) [20,25]. To study the influence of the conserved Met-157 on the activity of the enzyme, this amino acid in human glyoxalase I was mutated into alanine, glutamine and histidine. The glyoxalase I mutants were heterologously expressed in *Escherichia coli*, purified by affinity chromatography, and functionally characterized.

EXPERIMENTAL

Materials

Enzymes used for PCR and recombinant DNA work were obtained from Boehringer-Mannheim (Mannheim, Germany). Oligonucleotides were custom-synthesized by Operon Technologies (Alameda, CA, U.S.A.). [α-^{32}P]dCTP and Sequenase version 2.0 were purchased from Amersham International (Amersham, Bucks., U.K.). Glutathione, methylglyoxal dimethyl acetal and phenylglyoxal were obtained from Sigma (St. Louis, MO, U.S.A.). Methylglyoxal was prepared by hydrolysis of the methylglyoxal dimethyl acetal, effected by boiling of the starting material in 10 % (v/v) H_2SO_4 for 10 min [26]. *S*-Hexyl- and *S*-benzyl-glutathione were synthesized as described previously [27]. Other chemicals were commercial products of the highest purity.

Site-directed mutagenesis via PCR

Mutagenesis was performed by PCR in a 100 μl reaction mixture containing 10 mM Tris/HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each dNTP, 0.8 μM 5′ and 3′ primers and 20 ng of template DNA. Human glyoxalase I cDNA inserted in the pKK-D vector [28] was used as a template in the reaction. Two consecutive PCRs were performed. In the first, the 5′ part of the cDNA was amplified with a vector-specific primer pKKFor (5′-AAT TGT GAG CGG ATA ACA AT-3′) and the mutagenesis primers HGIM157A (5′-TGC CAG GCC TTT CGC TTT ACC ATC-3′) and HIM157QH (5′-TGC CAG GCC TTT STG TTT ACC ATC-3′, where S = G/C) for 20 cycles. The 3′ part of the cDNA was subsequently amplified using an internal primer GI5A (5′-TTG AAT TCG AGT TGA CGC ATA ACT GGG G-3′) and a stop primer GIEXSTOP (5′-TAA GTC GAC TGC AGT TAT TAC ATT AAG GTT GCC ATT TTG TTA GG-3′). The products of each PCR were combined (20 μl from each reaction mixture) and digested with the restriction endonuclease *Stu*I, followed by ligation at 37 °C for 30 min. A second PCR with the primers pKKFor and HGIEXSTOP was carried out for 25 cycles with the ligated 5′ and 3′ parts of the glyoxalase I cDNA as template. The amplified 560 bp fragment was digested with *Eco*RI and *Sal*I, ligated into the expression vector pKK-D, and transformed into *E. coli* JM109. All of the constructed mutants were sequenced [29] to verify that no unwanted mutations were present.

Expression and purification

The mutant forms of glyoxalase I were heterologously expressed in *E. coli*, cultured in the presence of 1 mM $ZnSO_4$, as described previously [18]. The mutants were purified on *S*-hexylglutathione affinity gel and eluted with 5 mM *S*-hexylglutathione in 10 mM Tris/HCl, pH 7.8, containing 0.2 mM dithiothreitol. The previously estimated absorption coefficient at 280 nm for human glyoxalase I (1.68 $ml \cdot mg^{-1} \cdot cm^{-1}$) was used to determine the protein concentration.

Physicochemical characterization

The relative molecular mass of the mutant proteins was estimated on precast SDS/8–20 % (w/v) polyacrylamide gels from Pharmacia Biotech using the PhastSystem. The protein bands were visualized by staining with silver.

Kinetic measurements

Enzyme activities were determined at 30 °C in a 1 ml reaction system consisting of 0.1 M sodium phosphate, pH 7.0, and 2 mM each of methylglyoxal and glutathione. The absorption coefficient at 240 nm used for monitoring *S*-ester formation was 3370 $M^{-1} \cdot cm^{-1}$ [13]. Kinetic constants were determined with the hemithioacetals of methylglyoxal and phenylglyoxal with GSH as substrate. The equilibrium constants for methylglyoxal and phenylglyoxal used for calculation of substrate and free GSH concentrations were 3.0 and 0.60 mM respectively [30]. The free GSH concentration was kept constant at 0.3 mM. Formation of the *S*-ester product *S*-D-lactoylglutathione was monitored at 240 nm, and formation of *S*-mandeloylglutathione at 263 nm (absorption coefficient 1100 $M^{-1} \cdot cm^{-1}$). The experimental data were analysed using the SIMFIT computer package [31] and Graphpad Prism 2.0 (GraphPad Software Inc., San Diego, CA, U.S.A.).

Inhibition studies

K_i values were determined with the competitive inhibitors *S*-hexyl- and *S*-benzyl-glutathione used in the micromolar concentration range. The adduct concentration of methylglyoxal

and GSH was varied between 20 and 200 μM and the free GSH concentration was kept constant at 0.1 mM. The data were analysed using Graphpad Prism 2.0.

RESULTS

Expression of human glyoxalase I mutants

The glyoxalase I mutants containing alanine (M157A), histidine (M157H) and glutamine (M157Q) in position 157 were heterologously expressed in *E. coli*. All mutants had retained the ability to bind to the *S*-hexylglutathione gel [32] and were purified to homogeneity by affinity chromatography; only a single band corresponding to a subunit of glyoxalase I was visible by SDS/PAGE analysis with silver staining. The yields of the mutant forms per litre of culture medium were 17 mg (M157A), 23 mg (M157H) and 16 mg (M157Q) after purification. All of the mutant proteins were obtained from *E. coli* cultured in the presence of 1 mM $ZnSO_4$ and were assumed to have incorporated Zn^{2+} into the metal-binding site, since the mutations were not targeted towards any of the residues likely to affect the binding of the metal.

The purified mutant forms of glyoxalase I did not contain any unwanted spurious mutations, as verified by sequencing of the expressed cDNA sequences. Within experimental error the relative subunit molecular mass of the mutants was the same as that of the wild-type enzyme, determined to be 22 kDa by SDS/PAGE (results not shown). The isoelectric points of all mutant proteins except M157H were identical with the wild-type value of 4.8. The value for mutant M157H was 4.9 (results not shown).

All of the glyoxalase I mutants were catalytically active, and their specific activities are shown in Table 1. The alanine and histidine mutants had specific activities that were approx. 5 times lower than the wild-type enzyme, as determined with the adduct of methylglyoxal and GSH. Interestingly, glutamine in position 157 of glyoxalase I resulted in a specific activity that even slightly exceeded that of the wild-type enzyme.

Determination of kinetic parameters and inhibition studies

Kinetic constants were determined by steady-state kinetic analysis with the GSH adducts of methylglyoxal and phenylglyoxal. The data were consistent with Michaelis–Menten kinetics. Table 1 shows the kinetic parameters obtained with the adduct of methylglyoxal and GSH as substrate and the concentration of free GSH at 0.3 mM. The k_{cat} values, and the corresponding relative values as compared with the wild-type data, reflect the specific activity. The glutamine mutant retained the high k_{cat} value of glyoxalase I. The K_m value for the wild-type enzyme was estimated at 66 μM. The values for the alanine and the histidine mutants were similar, whereas the K_m value for the glutamine mutant was 2.5 times higher. The k_{cat}/K_m value of the wild-type enzyme (2.3×10^{-7} $s^{-1} \cdot M^{-1}$) was twice that of the glutamine mutant, whereas the histidine and alanine mutants had k_{cat}/K_m values that were 10 and 20 % of the wild-type value. The pH-dependence of the k_{cat}/K_m values was also examined for the wild-type enzyme and the histidine mutant by taking measurements at pH 6.0, 7.0 and 8.0 (results not shown). Maximal activity was obtained at pH 7.0. No obvious difference in the pH-dependence of the two enzyme forms was observed.

Table 1 Kinetic constants of human glyoxalase I mutant forms determined with the hemithioacetal of GSH and methylglyoxal

Measurements were carried out in 1 ml of 0.1 M sodium phosphate, pH 7.0, at 30 °C. The concentration of free GSH was fixed at 0.3 mM. The k_{cat} values are calculated per dimer (43 kD). Values are given as means ± S.D. estimated by non-linear regression analysis. In parentheses are the relative values compared with the wild-type value.

Glyoxalase I	Specific activity (μmol · min^{-1} · mg^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
Wild-type	2340 ± 260 (1)	1500 ± 30 (1)	66 ± 5 (1)	23 (1)
M157Q	2680 ± 170 (1.1)	1650 ± 67 (1.1)	162 ± 17 (2.5)	10 (0.4)
M157A	420 ± 7 (0.2)	260 ± 13 (0.2)	74 ± 10 (1.1)	4 (0.2)
M157H	470 ± 60 (0.2)	300 ± 8 (0.2)	89 ± 8 (1.3)	3 (0.1)

Table 2 Kinetic constants of human glyoxalase I mutant forms determined with the hemithioacetal of GSH and phenylglyoxal

Measurements were carried out in 1 ml of 0.1 M sodium phosphate, pH 7.0, at 30 °C. The concentration of free GSH was fixed at 0.1 mM. The mean k_{cat}/K_m values (calculated per dimer) ± S.D. were determined by non-linear regression analysis. In parentheses are the relative values compared with the wild-type value. Data for the wild-type enzyme are taken from ref. [18].

Glyoxalase I	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
Wild-type	27 (1.0)
M157Q	44 ± 0.05 (1.6)
M157A	10 ± 0.1 (0.4)
M157H	10 ± 0.1 (0.4)

Table 3 Inhibition constants of human glyoxalase I mutant forms

Determination of the K_i values (competitive inhibition) was carried out in 1 ml of 0.1 M sodium phosphate, pH 7.0, with the hemithioacetal of methylglyoxal and glutathione at 30 °C; the concentration of free GSH was maintained at 0.1 mM. The formation of *S*-D-lactoylglutathione was monitored at 240 nm. Mean values ± S.D. were determined by non-linear regression analysis.

	K_i (μM)			
Inhibitor	Wild-type	M157Q	M157A	M157H
S-Hexylglutathione	0.37 ± 0.04	5.6 ± 2	3.4 ± 0.9	0.88 ± 0.08
S-Benzylglutathione	0.28 ± 0.03	4.2 ± 1	2.9 ± 0.6	0.85 ± 0.07

k_{cat}/K_m values were also determined with the adduct of GSH and phenylglyoxal (Table 2). The individual parameters k_{cat} and K_m could not be accurately estimated because of the high absorbance at high concentrations of the added phenylglyoxal. With this substrate, the glutamine mutant was 1.6-fold more efficient than wild-type glyoxalase I, whereas the alanine and histidine mutants were 2.5-fold less active.

The results of the inhibition studies with *S*-hexyl- and *S*-benzyl-glutathione are shown in Table 3. The glutamine mutant had K_i values for the two inhibitors that were approximately 15-fold higher than those of the wild-type enzyme. In addition, the alanine mutant had a 9–10 times lower affinity for the glutathione derivatives. The histidine mutant had an affinity that was rather similar to that of the wild-type.

DISCUSSION

Although the three-dimensional structure of human glyoxalase I has been determined [20], uncertainty about the residues directly involved in catalysis remains. In the present study the function of the active-site residue Met-157 was investigated by site-directed mutagenesis. The rationale behind the choice of Met-157 was the proximity of its sulphur atom to C-1 of the *S*-substituent of the

ligand (Figure 1) and hence its possible involvement in catalysis. The proton bound to the corresponding C-1 of the hemithioacetal substrate is transferred to C-2 in the catalytic process. Further, this residue is conserved among several glyoxalase I sequences from eukaryotes. Met-157 was mutated into three alternative amino acids: alanine, histidine and glutamine. Alanine was chosen in order to remove the major part of the sidechain to see whether this extensive change would influence the functional properties of the enzyme. The histidine mutant was studied to find out whether the introduction of an imidazole group that could serve as a base would affect catalysis. Finally, the glutamine mutant was of interest, since glutamine is present in a structurally equivalent position in the N-terminal half of *S. cerevesiae* glyoxalase I, as deduced by alignment of the amino acid sequences (Figure 2). Glutamine also has a sidechain of similar length to that of methionine and introduces an amide group close to the reacting groups of the substrate.

The mutant proteins had physicochemical properties, including relative molecular mass, isoelectric points and binding of glutathione derivatives, similar to those of the wild-type glyoxalase I. The proteins appeared to be stable after purification and storage (+4 °C, 1 week), as indicated by their constant specific activities (not shown).

Glyoxalase I from yeast appears to have arisen by a tandem gene duplication such that the N- and C-terminal halves of the primary structure each correspond to a subunit of the human enzyme [20,25]. The introduction of a glutamine in position 157 was of interest because of the presence of glutamine in the corresponding position in the aligned amino acid sequence of the N-terminal half of the yeast *S. cerevesiae*. In all other eukaryotic glyoxalase I and presumed glyoxalase I sequences, a methionine is present in this position (Figure 2). In prokaryotes, the Met-157 appears to be replaced by serine or threonine. In the prokaryotic sequences, the serine or threonine is preceded by two extra residues, often including glycine, which are not present in the known eukaryotic sequences.

Interestingly, replacement of methionine with glutamine in position 157 of human glyoxalase I resulted in a fully functional enzyme. The K_{cat} and specific activity values show that an equally or, with the phenylglyoxal adduct, even more efficient enzyme than the wild-type glyoxalase I was obtained. The demonstration of a catalytically fully competent human enzyme with glutamine in position 157 suggests that the monomeric glyoxalase I from *S. cerevesiae* may have two different active sites, with methionine and glutamine present as alternative residues in the two positions structurally equivalent to human residue 157. In the other yeast species, *Schizosaccharomyces pombe*, methionine is present in both of the presumed active sites.

In this context it is noteworthy that the kinetic properties of glyoxalase I from *S. cerevisiae* are similar to those of the human enzyme. For example, the yeast enzyme has a 1.4-fold higher catalytic efficiency (k_{cat}/K_m) with phenylglyoxal than with methylglyoxal as substrate [3]. This substrate selectivity ratio is similar to the corresponding value (1.2) for the human wild-type enzyme [18], which is augmented (to a 4.4-fold preference for phenylglyoxal) in the glutamine mutant (see Tables 1 and 2), as could be expected when both active sites contain this amino acid residue.

The present work shows that Met-157 in the active site of human glyoxalase I is not essential for catalytic activity, in spite of its almost complete conservation among known eukaryotic glyoxalase I sequences. The thioether group of the sidechain is probably without functional significance, since the carboxamido group of glutamine in mutant M157Q gives a k_{cat} value similar to that of the wild-type enzyme. The histidine and alanine mutants display comparable kinetic parameters, somewhat more different from the wild-type values. The fact that truncating the amino acid sidechain to an alanine residue results in only a modest decrease in catalytic efficiency indicates that the sidechain of residue 157 has primarily a structural role in the active site. The sidechains of methionine and glutamine are of similar size, and it is possible that they may fulfil essentially the same role in shaping the active site for an optimal fit of the hemithioacetal moiety of the substrate to the active-site residues in the proximity of the Zn^{2+} ion. The K_i values of the competitive inhibitors measure binding properties of the active site, and show somewhat larger differences between the wild-type and mutant values than noted for k_{cat} and K_m values. Nevertheless, the k_{cat}/K_m values, which reflect transition-state stabilization, also suggest that a methionine residue in position 157 is optimal for catalysing the reaction with the natural substrate. The somewhat higher k_{cat}/K_m value noted with the glutamine mutant when the glutathione adduct of phenylglyoxal was used as substrate is probably not important from an evolutionary perspective, since phenylglyoxal is not known as a normal metabolite. In conclusion, it would appear that methionine has been conserved in position 157 in the active site of glyoxalase I, because it optimizes the interactions between the natural substrate and the functional groups of the active site.

We thank Dr. Mikael Widersten for constructive comments on the manuscript. This work was supported by the Swedish Natural Science Research Council and the Carl Trygger Foundation.

REFERENCES

1 Mannervik, B. (1980) in Enzymatic Basis of Detoxication (Jakoby, W. B., ed.), vol. 2, pp. 263–273, Academic Press, New York
2 Thornalley, P. J. (1993) Mol. Aspects Med. **14**, 287–371
3 Vander Jagt, D. L. (1989) in Coenzymes and Cofactors (Dolphin, D., Poulson, R. and Avramovic, O., eds.), vol. 3A, pp. 597–641, John Wiley and Sons, New York
4 Vaca, C. E., Fang, J.-L., Conradi, M. and Hou, S.-M. (1994) Carcinogenesis **15**, 1887–1894
5 Lo, T. W. C., Westwood, M. E., McLellan, A. C., Selwood, T. and Thornalley, P. J. (1994) J. Biol. Chem. **269**, 32299–32305
6 Richard, J. P. (1991) Biochemistry **30**, 4581–4585
7 Casazza, J. P., Felver, M. E. and Veech, R. L. (1984) J. Biol. Chem. **259**, 231–236
8 Elliott, W. H. (1960) Nature (London) **185**, 467–468
9 Thornalley, P. J., McLellan, A. C., Lo, T. W. C., Benn, J. and Sönksen, P. H. (1996) Clin. Sci. Colch. **91**, 575–582
10 Nemeth, A. M., Russell, C. S. and Shemin, D. (1957) J. Biol. Chem. **229**, 415–422
11 Jerzykowski, T., Winter, R., Matuszewski, W. and Piskorska, D. (1978) Int. J. Biochem. **9**, 853–860
12 Larsen, K., Aronsson, A.-C., Marmstål, E. and Mannervik, B. (1985) Comp. Biochem. Physiol. **82B**, 625–638
13 Racker, E. (1951) J. Biol. Chem. **190**, 685–696
14 Ranganathan, S., Walsh, E. S., Godwin, A. K. and Tew, K. D. (1993) J. Biol. Chem. **268**, 5661–5667
15 Kim, N.-S., Umezawa, Y., Ohmura, S. and Kato, S. (1993) J. Biol. Chem. **268**, 11217–11221
16 Aronsson, A.-C., Tibbelin, G. and Mannervik, B. (1979) Anal. Biochem. **92**, 390–393
17 Kim, N.-S., Sekine, S., Kiuchi, N. and Kato, S. (1995) J. Biochem. (Tokyo) **117**, 359–361
18 Ridderström, M. and Mannervik, B. (1996) Biochem. J. **314**, 463–467
19 Aronsson, A.-C., Marmstål, E. and Mannervik, B. (1978) Biochem. Biophys. Res. Commun. **81**, 1235–1240
20 Cameron, A. D., Olin, B., Ridderström, M., Mannervik, B. and Jones, T. A. (1997) EMBO J. **16**, 3386–3395
21 Sellin, S., Eriksson, L. E. G. and Mannervik, B. (1982) Biochemistry **21**, 4850–4857
22 Espartero, J., Sánchez-Aguayo, I. and Pardo, J. M. (1995) Plant. Mol. Biol. **29**, 1223–1233
23 Inoue, Y. and Kimura, A. (1996) J. Biol. Chem. **271**, 25958–25965
24 Lu, T., Creighton, D. J., Antoine, M., Fenselau, C. and Lovett, P. S. (1994) Gene **150**, 93–96
25 Ridderström, M. and Mannervik, B. (1996) Biochem. J. **316**, 1005–1006
26 Kellum, M. W., Oray, B. and Norton, S. J. (1978) Anal. Biochem. **85**, 586–590

27 Vince, R., Daluge, S. and Wadd, W. B. (1971) J. Med. Chem. **14**, 402–404
28 Björnestedt, R., Widersten, M., Board, P. G. and Mannervik, B. (1992) Biochem. J. **282**, 505–510
29 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
30 Vander Jagt, D. L., Han, L.-P. B. and Lehman, C. H. (1972) Biochemistry **11**, 3735–3740
31 Bardsley, W. G., McGinlay, P. B. and Roig, M. G. (1989) J. Theor. Biol. **139**, 85–102
32 Aronsson, A.-C. and Mannervik, B. (1977) Biochem. J. **165**, 503–509

Received 16 April 1997/7 July 1997; accepted 30 July 1997