Optimized heterologous expression of the human zinc enzyme glyoxalase I

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DNA coding for human glyoxalase I was isolated from a HeLa cell cDNA library by means of PCR. The deduced amino acid sequence differs from previously isolated sequences in that a glutamic acid replaces an alanine in position 111. This variant cDNA may represent the more acidic isoform of glyoxalase I originally identified at the protein level. An expression clone was constructed for high-level production of glyoxalase I in *Escherichia coli*. For optimal yield of the recombinant protein, silent random mutations were introduced in the cDNA coding region. Antisera against human glyoxalase I were used to select a high-level expression clone. This clone afforded 60 mg of purified enzyme per litre of culture medium. Addition of a zinc salt to the culture medium was essential to obtain an active enzyme and a stoicheiometric metal content. The functional characterization of the recombinant enzyme included determination of kinetic constants for methylglyoxal, phenylglyoxal and *p*-phenylphenylglyoxal, as well as inhibition studies. The kinetic properties of recombinant glyoxalase I were indistinguishable from those of the enzyme purified from human tissues.

INTRODUCTION

Glyoxalase I (EC 4.4.1.5) and glyoxalase II (EC 3.1.2.6) [1] are the enzyme components of the glyoxalase system [2,3]. Glyoxalase I is an isomerase that catalyses the formation of *S*-D-lactoylglutathione from the hemimercaptal adduct that forms spontaneously between methylglyoxal and reduced GSH [4]. *S*-D-lactoylglutathione is in turn hydrolysed by glyoxalase II to D-lactic acid and GSH. The physiological substrate methylglyoxal is mainly produced from dihydroxyacetone phosphate and glyceraldehyde 3-phosphate in glycolysis [5], but the substrate can be formed also from aminoacetone and hydroxyacetone in the catabolism of threonine and acetone [6,7].

Judging from its ubiquity, glyoxalase I has an important role in the cell. It has been found in a wide variety of species, such as mammals, prokaryotes and plants [2,8,9]. The enzyme is expressed in most tissues [10,11]. Glyoxalase I is also present in many tumour cell lines, in which its concentration often is up-regulated [12]. Even though the biological role of the enzyme remains unclear, the enzyme activity is relevant to diabetes, since accumulation of methylglyoxal is believed to give rise to toxicity resulting in nephropathy, retinopathy and neuropathy [13]. Another area of current interest is the design of efficent inhibitors for cancer therapy [14].

Glyoxalase I has been purified from mammalian tissues [2,15], yeast [15], bacteria and plants [9]. The molecular mass of the enzyme from the different sources varies between 20 and 48 kDa [3]. The enzyme from yeast and bacteria appears to be monomeric, while the human enzyme is a dimer of 21 kDa subunits. Each subunit of the mammalian enzyme has a Zn^{2+} bound at the active site [16]. Other divalent ions such as Mg^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} have been substituted for Zn^{2+}, but the metal-ion-substituted enzyme generally has lower catalytic activity as well as lower affinity for the substrate [17,18]. Human glyoxalase I is an acidic protein with an isoelectric point of 4.8–5.1. It exists as homo- and hetero-dimers of two allelic subunit variants, which differ in charge [19]. A human cDNA clone from colon [20] and another from U937 cells [21] have been isolated. The sequence was found to be very similar to that of a bacterial glyoxalase I sequence from *Pseudomonas putida* [22].

In the present paper, cDNA for glyoxalase I has been isolated via PCR of cDNA derived from HeLa cells. A high-level-expression clone has been constructed in which the codon usage in the 5′ coding region of the cDNA has been optimized for expression in *Escherichia coli*. Thus a recombinant protein with full catalytic activity has been obtained in high yield.

MATERIALS AND METHODS

Materials

Oligonucleotides were obtained from Operon Technologies (Alameda, CA, U.S.A.). Restriction enzymes and other enzymes for recombinant DNA work were purchased from Boehringer Mannheim (Mannheim, Germany). [α-^{32}P]CTP was from Amersham International (Amersham, Bucks., U.K.). The phagemid vector pGEM-3zf(+) and restriction enzymes were obtained from Promega (Madison, WI, U.S.A.). The expression vector pKK223-3 was obtained from Pharmacia Biotech (Uppsala, Sweden). Methylglyoxal and glutathione were bought from Sigma (St. Louis, MO, U.S.A). Phenylglyoxal was obtained from Aldrich (St. Louis, MO, U.S.A.). *p*-Phenylphenylglyoxal was a generous gift from Dr. David L. Vander Jagt (University of New Mexico, School of Medicine, Albuquerque, NM, U.S.A.). *S*-(*p*-Bromobenzyl)glutathione was synthesized as described previously [23]. Other chemicals were commercial products of high purity.

cDNA synthesis and PCR

RNA was prepared from HeLa cells, and first-strand cDNA synthesis was carried out as described previously [24]. cDNA (50 ng) was used for amplification with the 5′ primer GIAEx, 5′-

Abbrevation used: IPTG, isopropyl β-D-thiogalactoside.

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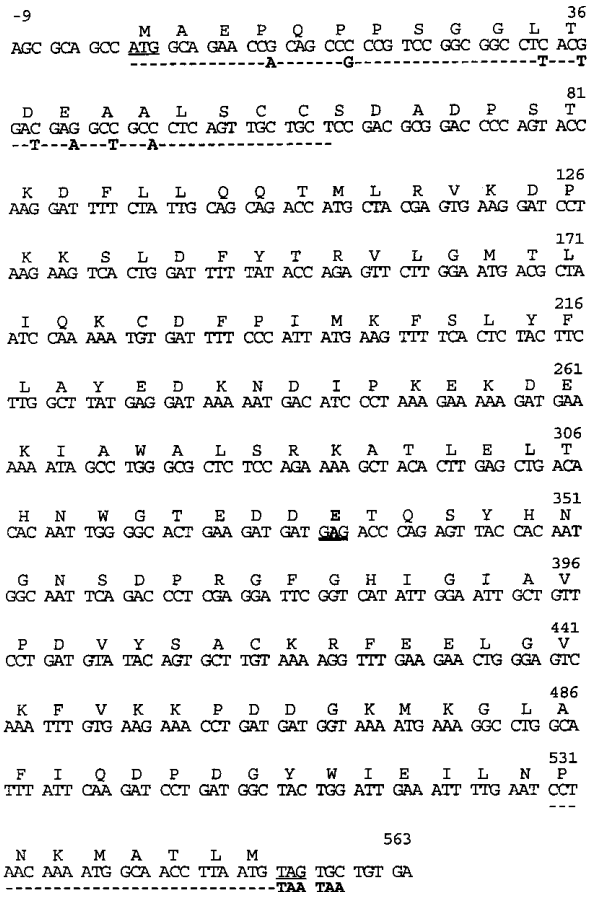

Figure 1 cDNA sequence encoding human glyoxalase I isolated from HeLa cells and the deduced amino acid sequence (above the codons)

Primers used for the PCR of the cDNA for the expression in *E. coli* are indicated with dashes. The novel nucleotides introduced in the expression clone are marked. Nucleotide position 332, which gives rise to glutamic acid instead of an alanine previously reported [20,21] for glyoxalase I sequences, is doubly underlined and presented in bold. The start and stop codons are underlined.

TTAGAATTCAGCGCAGCCATGGCAGAACACGC-3′, and the 3′ primer GIBEx, 5′-CAGCTGCAGTCACAGCACTACAT-3′. The primers were designed on the basis of previously published human glyoxalase I sequences [20,21], and recognition sites for endonucleases *Eco*RI and *Pst*I (underlined) were added at the 5′ and 3′ ends. PCR was carried out in a reaction system of 100 μl consisting of 10 mM Tris/HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, 200 μM dNTP, 0.8 μM each primer and 2.5 units of *Taq* DNA polymerase, with denaturation at 95 °C for 1 min, annealing at 58 °C for 2 min and elongation at 72 °C for 2 min for 30 cycles. The PCR product was digested with *Eco*RI and *Pst*I, inserted into the phagemid vector pGEM-3zf(+) and sequenced for verification [25].

Expression and purification

For optimal expression in *E. coli*, primers were designed by randomizing some of the silent positions in the first 15 codons following the start codon (Figure 1). Endonuclease recognition sites (underlined) for *Eco*RI and *Xba*I were included in the 5′ primer G1EX15A, 5′-TCTAGAATTCATGGCNGARCCDCARCCDCCDTCBGGYGGYCTBACYGAYGARGCDGCDCTCAGTTGCTGCT-3′. In the 3′ primer, GIEX2STOP, two TAA stop codons in tandem (doubly underlined) were substituted for TAG and endonuclease recognition sites (underlined) for *Pst*I and *Sal*I were introduced: 5′-TAA GTCGACTGCAGTTATTACATTAAGGTTGCCATTTTGTTAGG-3′. PCR was performed with denaturation at 95 °C for 1 min, annealing at 50 °C for 2 min and extension at 72 °C for 2 min for 30 cycles. The library of fragments with variant 5′ coding regions was inserted into the expression vector pKK223-3 after digestion with *Eco*RI and *Pst*I, and used for transformation of *E. coli* JM 103 by electroporation.

For the identification of clones expressing the desired protein, antiserum against human glyoxalase I [11] was used for immunoscreening on nitrocellulose filters [26].

Purification of protein was made from a 3 l culture grown in 20 % (w/v) tryptone, 15 % (w/v) yeast extract, 5 % (w/v) NaCl and 10 % (w/v) glycerol supplemented with 1 mM $ZnSO_4$. The bacteria were initially grown at 37 °C and after addition of $ZnSO_4$ were grown at 30 °C. When the culture had reached A_{600} 0.3, the expression was induced with 0.2 mM IPTG, and the bacteria were grown overnight. Cells were harvested by centrifugation, resuspended in 10 mM Tris/HCl (pH 7.8)/50 μM PMSF. Lysozyme (10 mg) was added, and the cells were incubated on ice for 1 h. The cells were disrupted by sonication for 3×20 s. The lysate was centrifuged for 60 min at 100000 ***g*** at 4 °C, and the supernatant fraction applied to a column (2 cm × 5 cm) of *S*-hexylglutathione affinity matrix equilibrated with buffer A (10 mM Tris/HCl, pH 7.8 and 0.1 mM DTT) [27]. The matrix was washed with buffer A supplemented with 0.2 M NaCl. The enzyme was eluted with 30 ml of 5 mM *S*-hexylglutathione in buffer A. The fractions containing glyoxalase I activity were pooled and dialysed against 10 mM Tris/HCl (pH 7.8)/0.1 mM DTT. The molecular mass of the protein was determined by SDS/12.5 % (w/v) PAGE [28], with silver staining for protein [29]. Isoelectric focusing was made in the interval pH 3.5–9.5 using precast gels from Pharmacia Biotech.

Metal determination

Recombinant glyoxalase I (50–100 nmol) was analysed by inductively coupled plasma atomic emission spectrometry after digestion in nitric acid. For the enzyme obtained from bacteria grown without $ZnSO_4$ supplementation, the Zn^{2+}, Ni^{2+} and Cu^{2+} content was also determined, by flame atomic absorption spectrometry.

Assay of glyoxalase I activity

Specific activity was determined spectrophotometrically with 2 mM methylglyoxal/2 mM GSH/0.1 M sodium phosphate (pH 7.0) at 30 °C in a 1 ml system [1]. The formation of the *S*-ester was followed at 240 nm. The absorption coefficient used was 3.37 $mM^{-1} \cdot cm^{-1}$ for the *S*-D-lactoylglutathione formation. A unit corresponds to 1 μmol of product formed per min.

Kinetic studies

Steady-state kinetic measurements with methylglyoxal, phenylglyoxal and *p*-phenylphenylglyoxal were made spectrophotometrically at 240 nm, 263 nm and 303 nm respectively [30]. The absorption coefficients used for the formation of the *S*-esters were 1.10 $mM^{-1} \cdot cm^{-1}$ for phenylglyoxal and 2.28 $mM^{-1} \cdot cm^{-1}$ for *p*-phenylphenylglyoxal [30]. The reaction system of 1 ml at 30 °C contained a constant concentration of free GSH at 0.1 mM and varying concentrations of the 2-oxoaldehyde in 0.1 M phosphate buffer, pH 7.0. The kinetic data were analysed as described previously [31], using the SIMFIT computer program

[32]. The equilibrium constants for methylglyoxal, phenylglyoxal and *p*-phenylphenylglyoxal used for calculation of the concentration of their corresponding hemimercaptal adducts were 3.0 mM, 0.60 mM and 0.89 mM [30].

Inhibition studies

Inhibition with *S*-(*p*-bromobenzyl)glutathione was followed at 240 nm with varying concentrations of methylglyoxal and 0.1 mM free GSH. IC_{50} values were determined for other inhibitors with 20 μM adduct formed from phenylglyoxal and glutathione; the concentration of free glutathione was kept at 0.1 mM.

RESULTS

cDNA isolation and sequencing

Amplification of the cDNA prepared from HeLa cells with the primers specific for human glyoxalase I produced a fragment of 590 bp. The fragment was sequenced and found to be identical to the previously isolated sequences for human glyoxalase I [20,21] except in position 332 of the coding region, where an A was present instead of a C (Figure 1). This difference corresponds to a change from alanine to glutamic acid in the deduced amino acid sequence. The change (from C to A) in nucleotide position 332 created a recognition site for endonuclease *Alw*26I. In order to exclude the possibility that the difference was an artifact of the PCR amplification, other cDNA sequences of human glyoxalase I from different sources (the erythroleukemia cell line K562, liver and the hepatoma cell line Hep G2) were digested with *Alw*26I for discrimination between sequences. The *Alw*26I restriction site was present in all isolates. An undigested fragment, likely to represent the cDNA encoding the other isoform, was also detected (results not shown).

In addition, a number of glyoxalase I cDNA sequences from other human sources were cloned and analysed. Full-length sequences with the same base (A) in position 332 were obtained from cDNA libraries from HeLa cells, the hepatoma cell line Hep G2, and the erythroleukaemia cell line K562.

Expression and characterization of recombinant glyoxalase I

Heterologous expression was first tried with the 'wild-type' glyoxalase I cDNA ligated into the expression vector. Bacterial lysate was prepared and analysed for the presence of glyoxalase I by immunoblotting, after separation by SDS/PAGE. No glyoxalase I could be detected using antisera against the human enzyme [11]. The lysate was also further affinity purified using immobilized *S*-hexylglutathione, and analysed by SDS/PAGE and silver staining. The silver-stained gel did not show any trace of the protein.

In order to optimize the cDNA for expression in *E. coli*, primers were designed to contain codons more suitable for the bacterial host [33]. The cDNA sequence from HeLa cells was used as a template for PCR with primers containing silent mutations in codons of the 5′ region. The 'mutant' cDNA product from the PCR reaction was cloned into the expression vector pKK223-3 to yield a library of variant expression clones. Antisera against human glyoxalase I were used for the identification of clones with high expression levels. A positive clone was chosen for large-scale purification. Sequencing of its cDNA demonstrated that mutations were present in eight silent positions of the segment containing the first 15 codons (Figure 1).

The recombinant protein in a bacterial lysate was analysed by SDS/PAGE. A band with the apparent subunit M_r 21000 was present (Figure 2), which co-migrated with glyoxalase I affinity purified from human CHANG cells (cf. [12]). Immunoblot experiments with antisera against human glyoxalase I identified the protein band as glyoxalase I, in agreement with the bacterial colony screening used for the identification of high-level-expression clones. Purification of the fully active recombinant enzyme from a 3 l culture resulted in 180 mg of protein. The pure recombinant enzyme had an absorbance of 1.67 cm^{-1} at 280 nm at a concentration of 1 mg/ml, as determined by total amino acid analysis.

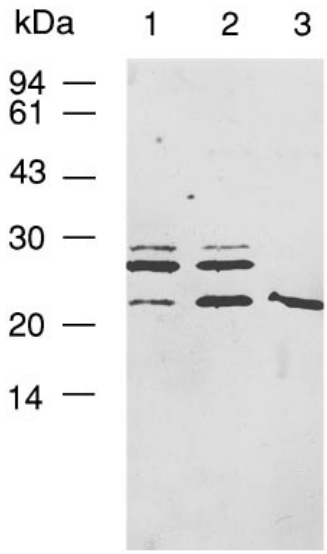

Figure 2 Silver-stained SDS/polyacrylamide gel of recombinant human glyoxalase I

Lane 1, affinity-purified (*S*-hexylglutathione matrix) lysate from CHANG cells. The bands correspond to glyoxalase I (21 kDa), glutathione transferase P1-1 (25 kDa) and glutathione transferase M1-1 (28 kDa) (cf. [12]). Lane 2, recombinant glyoxalase I co-migrates with glyoxalase I from the affinity-purified lysate from CHANG cells. Lane 3, affinity-purified recombinant glyoxalase I.

Amino acid sequence determination of the first 15 N-terminal amino acids of the recombinant protein revealed that the initiator methionine had been removed from the protein expressed in *E. coli*. The remainder of the sequence was identical to that deduced from the DNA sequence. The recombinant protein had an isoelectric point around 4.8–4.9, as determined by isoelectric focusing (results not shown).

Specific activity and metal content of enzyme produced with and without $ZnSO_4$ in the culture medium

The recombinant protein was first obtained from bacteria grown at 37 °C without the addition of $ZnSO_4$ to the culture medium. Specific activity measured with methylglyoxal was 200 units/mg for the purified enzyme. This specific activity is only 1/6 of that expected for fully active glyoxalase I [19]. Analysis of the metal content of the recombinant enzyme showed that the dominating species were zinc, iron and calcium. However, in quantitative terms, the content of Zn^{2+} per subunit was only 0.18 mol/mol instead of 1.0 mol/mol, previously determined for the fully active enzyme from human erythrocytes [16]. Seven other elements were also present in lower concentrations. Among them were manganese, copper and magnesium.

When the protein was expressed in the presence of 1 mM $ZnSO_4$ and grown at 30 °C, the specific activity of the purified

Table 1 Kinetic constants for recombinant human glyoxalase I and enzyme purified from erythrocytes

The concentration of free GSH was maintained at 0.1 mM for all measurements. The data from erythrocyte glyoxalase I are from [15] and [34]; all values were measured at 30 °C and pH 7.0.

	Recombinant			Erythrocyte	
Substrate	K_m (μM)	$k_{cat.}$ (min^{-1})	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (min^{-1})
Methylglyoxal	71 ± 13	63000 ± 4000	2.2×10^7	71 ± 7*	68000
Phenylglyoxal	35 ± 5	53000 ± 3000	2.7×10^7	40	64000
p-Phenylphenylglyoxal	3.4 ± 0.2	20000 ± 2000	9.6×10^7		

Table 2 IC_{50} values for recombinant human glyoxalase I

An adduct concentration of 20 μM for phenylglyoxal was used. The concentration of free glutathione was kept at 0.1 mM.

Inhibitor	IC_{50} (μM)
Cibacron Brilliant Red	0.8
1,1′-Dibenzoylferrocene	1
9-Phenyl-2,3,7-trihydroxy-6-fluorone	2
6-Chloro-3-nitro-2*H*-chromene	3
Phloxine B	4
Fluorescein isothiocyanate, isomer 1	15

enzyme was increased to 1200 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. The stoicheiometry between Zn^{2+} and glyoxalase I subunits was determined as 1.0 mol/mol by metal analysis.

Kinetic properties of recombinant glyoxalase I

The K_m values for both methylglyoxal and phenylglyoxal were determined and were in accordance with data previously reported for the human enzyme [34]. K_m for methylglyoxal was 71 μM and K_m for phenylglyoxal was 34 μM (Table 1). $k_{cat.}/K_m$ values for methylglyoxal and phenylglyoxal were 2.2×10^7 $M^{-1} \cdot s^{-1}$ and 2.7×10^7 $M^{-1} \cdot s^{-1}$ respectively. *p*-Phenylphenylglyoxal, which had not been reported previously for the human enzyme, gave a K_m of 3.4 μM and a $k_{cat.}/K_m$ of 9.6×10^7 $M^{-1} \cdot s^{-1}$.

The potent inhibitor of glyoxalase I, *S*-(*p*-bromobenzyl) glutathione [34], was tested using methylglyoxal as substrate. The corresponding K_i value for the enzyme with the high specific activity was 83 ± 14 nM, in agreement with the published value [34]. All measurements were carried out with 0.1 mM concentration of free GSH, since it has been shown that GSH acts as a competitive inhibitor for glyoxalase I [35].

IC_{50} determinations were made for six new inhibitors (Table 2). The values were all in the μM range. The inhibitors with a partial structural similarity to the ene-diolate of the transition state, 9-phenyl-2,3,7-trihydroxy-6-fluorone and phloxine B, had IC_{50} values of 2 μM and 4 μM respectively.

DISCUSSION

For structural and physicochemical studies of human glyoxalase I, there is a need for large amounts of enzyme. In this work a high-level-expression clone of human glyoxalase I has been constructed. The recombinant enzyme has been characterized and has been shown to have functional properties indistinguishable from those of the enzyme isolated from human sources.

The partially randomized 5′ primer for optimal expression of glyoxalase I in *E. coli* was designed on the basis of the most frequently used codons for high-level expression in *E. coli* [33]. The mutations noted in the coding region of the clone affording large amounts of glyoxalase I indicated the importance of optimizing the DNA template for translation in the prokaryotic host, since the 'wild-type' cDNA used without changes did not express any detectable amount of the enzyme. It should be emphasized that the mutations used involve wobble positions in the altered codons and do not change the corresponding amino acid sequence of the protein. The approach of optimizing bacterial cDNA expression by introducing silent mutations in the 5′ coding region has previously been used for glutathione reductase [36,37] and glutathione transferases [38].

The N-terminal methionine was found by sequence analysis to be absent in the recombinant glyoxalase I. The enzyme isolated from human erythrocytes has a blocked N-terminus (M. Ridderström and B. Mannervik, unpublished work), but the nature of the modification is unknown. The removal of the N-terminal methionine has been observed also after expression of glyoxalase I from *Pseudomonas putida* in *E. coli* [22,39].

Human glyoxalase I has been purified from many sources and has been extensively characterized. Ion-exchange chromatography and electrophoresis have been used to demonstrate and separate these isoforms differing in charge from one another [19,40]. No differences among the isoenzymes are known in terms of enzymic activity or molecular mass. In tumour cell lines the different isoforms of the enzyme have been found to occur with distinct electrophoretic mobilities [41]. All three isoforms are expressed in K562 cells, while some other cell lines are homozygous for one of the two forms. It appears possible that the glutamic acid deduced for residue 111 in the present work represents the more acidic isoform, and alanine in the same position [20,21] the less acidic isoform. The enzyme is dimeric, and binary combinations of the two subunits differing in charge provide an obvious explanation for the occurrence of three isoenzymes. Glu-111 has been deduced from PCR-amplified glyoxalase I DNA from K562, HeLa and Hep G2 cells. Verification of the DNA sequence has been made by digestion with endonuclease *Alw*26I, which can distinguish between the alternative nucleotides in codon 111.

Glyoxalase I has the metal ion Zn^{2+} as cofactor [16]. The metal ion is of importance for the catalytic activity and for the structure of the enzyme. Replacement of the Zn^{2+} with other divalent ions alters the catalytic properties of the enzyme. For example, Mn^{2+}-, Co^{2+}-, and Ni^{2+}-substituted enzymes display lower K_m and $k_{cat.}$ values than does the native enzyme [17,18], whereas Cu^{2+} and Fe^{2+} do not give any measurable catalytic activity [42,43]. The purified recombinant glyoxalase I contains a considerable amount of iron, irrespective of whether the bacteria were grown in the presence or absence of $ZnSO_4$. The iron that is present in

the fully active Zn^{2+} enzyme is probably bound to metal-binding sites other than that of the active site.

Inhibition studies with *S*-(*p*-bromobenzyl)glutathione may distinguish among different metal-ion-substituted forms of glyoxalase I [34]. The affinity for the inhibitor is much higher for the Zn^{2+} enzyme than for the metal-substituted variants. Thus the kinetic data, together with the metal analysis, strongly indicate that the recombinant enzyme has the same functional properties as the enzyme purified from human tissues.

The substrate *p*-phenylphenylglyoxal was used for further kinetic characterization of glyoxalase I. The K_m value of this more hydrophobic substrate was 10-fold lower than that of phenylglyoxal. The $k_{cat.}$ value with *p*-phenylphenylglyoxal was about 22 % of the $k_{cat.}$ value for methylglyoxal, the most active substrate. The lower $k_{cat.}$ value may be due to the higher affinity for the more hydrophobic substrate, which could possibly decrease the rate of product release.

A number of organic compounds with different chemical structures were tested as novel inhibitors of recombinant glyoxalase I (Table 2). Previous studies have identified putative transition-state analogues mimicking the assumed ene-diolate in the mechanism of glyoxalase I [14,44]. Among the inhibitors in Table 2, 9-phenyl-2,3,7-trihydroxy-6-fluorone and phloxine B have structural similarity to ene-diolates, but other factors are obviously also important for the effectiveness of glyoxalase I inhibitors [45].

In summary, a high-level-expression clone for human glyoxalase I has been constructed that is suitable for production of the recombinant protein in *E. coli*. The physical and catalytic properties of recombinant glyoxalase I are indistinguishable from those of the enzyme purified from human erythrocytes, except for the N-terminal modification of the protein. With the availability of this expression clone, structural and functional studies will be facilitated. Crystals have been obtained from recombinant glyoxalase I (B. Olin, M. Ridderström and B. Mannervik, unpublished work) and make possible X-ray diffraction analysis.

Note added in proof (received 8 December 1995).

After completion of this study the Glu-111 variant of human glyoxylase I has been reported [46].

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