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Comparison of Glyoxalase I purified from Yeast (*Saccharomyces cerevisiae*) with the Enzyme from Mammalian Sources

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Glyoxalase I from yeast (*Saccharomyces cerevisiae*) purified by affinity chromatography on *S*-hexylglutathione–Sepharose 6B was characterized and compared with the enzyme from rat liver, pig erythrocytes and human erythrocytes. The molecular weight of glyoxalase I from yeast was, like the enzyme from *Rhodospirillum rubrum* and *Escherichia coli*, significantly less (approx. 32000) than that of the enzyme from mammals (approx. 46000). The yeast enzyme is a monomer, whereas the mammalian enzymes are composed of two very similar or identical subunits. The enzymes contain 1 Zn atom per subunit. The isoelectric points (at 4°C) for the yeast and mammalian enzymes are at pH 7.0 and 4.8 respectively; tryptic-peptide 'maps' display corresponding dissimilarities in structure. These and some additional data indicate that the microbial and the mammalian enzymes may have separate evolutionary origins. The similarities demonstrated in mechanistic and kinetic properties, on the other hand, indicate convergent evolution. The $k_{cat.}$ and K_m values for the yeast enzyme were both higher than those for the enzyme from the mammalian sources with the hemimercaptal adduct of methylglyoxal or phenylglyoxal as the varied substrate and free glutathione at a constant and physiological concentration (2mM). Glyoxalase I from all sources investigated had a $k_{cat.}/K_m$ value near $10^7 s^{-1} \cdot M^{-1}$, which is close to the theoretical diffusion-controlled rate of enzyme–substrate association. The initial-velocity data show non-Michaelian rate saturation and apparent non-linear inhibition by free glutathione for both yeast and mammalian enzyme. This rate behaviour may have physiological importance, since it counteracts the effects of fluctuations in total glutathione concentrations on the glyoxalase I-dependent metabolism of 2-oxoaldehydes.

Glyoxalase I (EC 4.4.1.5) catalyses the transformation of 2-oxoaldehydes to *S*-(2-hydroxyacyl)-glutathione derivatives. The significance of such reactions is far from clear, but it is evident that they represent a detoxification mechanism that inactivates the electrophilic 2-oxoaldehydes. Many compounds, aliphatic as well as aromatic, containing the dicarbonyl function have been tested and found to serve as substrates for the enzyme. However, it is not known which compounds should be regarded as natural substrates for the enzyme. Methylglyoxal is formed from dihydroxyacetone phosphate by the action of methylglyoxal synthase, which has been identified and characterized in *Escherichia coli* (Hopper & Cooper, 1972), *Pseudomonas saccharophila* (Cooper, 1974) and *Proteus vulgaris* (Tsai & Gracy, 1976). The enzyme is present in various enterobacteria and closely related species, but no activity has been found in human and rat tissues (or any of a variety of vertebrates, invertebrates, yeast, fungi and algae) (Yuan *et al.*, 1978). It has consequently been suggested that glyoxalase I may have as one of its functions the protection of mammals from toxic substances produced by the enterobacteria in the intestinal flora (Aronsson & Mannervik, 1977). A challenging theory concerning a possible function of glyoxalase I in the control of cell proliferation has been put forward by Szent-Györgyi (1976, and papers cited therein). The essence of the theory is that methylglyoxal acts as an endogenous inhibitor of mitosis and that glyoxalase I promotes cell division by degrading methylglyoxal. However, the enzyme is not only present in various organs of mammals, but is also highly active in simpler organisms. The functions indicated above for a multicellular organism cannot be expressed in the same manner in a unicellular species such as yeast or a bacterium. We therefore decided to study the structural and catalytic properties of glyoxalase I from yeast (*Saccharomyces cerevisiae*) and make a comparison with the data for the mammalian enzymes in order to arrive at a better understanding of the importance of

the enzyme in biological systems. A comprehensive characterization of glyoxalase I from yeast has not been published. It is also important to clarify the difference in structure and function between the enzyme from yeast and from mammals in view of the research on inhibitors of glyoxalase I as anticancer agents (Vince & Daluge, 1971; Vince *et al.*, 1971), which has largely relied on assays on the yeast enzyme.

Materials and Methods

All chemicals were standard commercial products of highest purity. Glyoxalase I was purified from rat liver (Marmstål & Mannervik, 1979), pig erythrocytes (Aronsson & Mannervik, 1977) and human erythrocytes (Aronsson *et al.*, 1979) as described in the references. The human enzyme exists as three separable isoenzymes with very similar properties (Aronsson *et al.*, 1979). The only known differences between them are in their chromatographic and electrophoretic properties, which make possible their separation. Even the amino acid compositions in hydrolysates of the three forms are not significantly different. In the present paper, data for form 1 of human glyoxalase I have been used. Protein concentrations were determined by the method of Kalckar (1947).

Purification of glyoxalase I from yeast

Glyoxalase I from yeast was obtained from Boehringer, Mannheim, Germany. A sample containing 50mg of protein was dialysed at 4°C against 10mM-Tris/HCl, pH7.8, overnight. All the subsequent handling of the enzyme during the purification was performed at 4°C.

Isoelectric focusing. The dialysed enzyme (20ml) was applied to a 110ml column (model 8101; LKB Produkter) containing a sucrose density gradient (0–47%, w/v) and ampholytes (1% Ampholine no. 1809-126, pH5–8). The focusing was performed according to the instructions of the manufacturer. After 48h the contents of the column were collected in 1ml fractions. The glyoxalase-I-containing fractions were pooled (43ml).

Affinity chromatography. The enzyme recovered after the isoelectric focusing was applied to a column (1cm×25cm) containing an adsorbent prepared by coupling *S*-hexylglutathione to epoxy-activated Sepharose 6B (see Aronsson *et al.*, 1978). The gel bed was previously equilibrated with 10mM-Tris/HCl, pH7.8, and, after application of the enzyme, it was washed with 25ml of 0.2M-NaCl dissolved in the same buffer. Glyoxalase I was eluted with a mixture of 3mM-*S*-hexylglutathione and 5mM reduced glutathione in 10mM-Tris/HCl, pH7.8. The fractions containing glyoxalase I were pooled (12ml).

Chromatography on Sephadex G-100. The pooled material from the affinity chromatography was chromatographed on a column (4cm×43cm) containing Sephadex G-100 (fine grade) equilibrated with 10mM-Tris/HCl, pH7.8. The enzyme was eluted with the same buffer and was recovered in 91ml of the effluent. For storage the enzyme was concentrated to about 10ml by use of a Millipore immersible molecular separator.

Analytical methods

The activity of glyoxalase I was assayed spectrophotometrically at 240nm (Racker, 1951) in a system (30°C) containing in 1ml: 1.0mM reduced glutathione, 2.0mM-methylglyoxal and 0.05M-sodium phosphate, pH7.0. A somewhat different assay system (0.66mM reduced glutathione, 2.0mM-methylglyoxal and 25mM-imidazole/HCl, pH7.0) has previously been used (Aronsson & Mannervik, 1977); the activities determined in the two systems were the same. The steady-state kinetic studies were carried out in the standard assay system with fixed concentrations of free glutathione (or hemimercaptal) and various concentrations of the 2-oxoaldehyde and hemimercaptal (or free glutathione). The equilibrium constants used for the dissociation of hemimercaptal to glutathione and 2-oxoaldehyde were 3.0 and 0.6mM for methylglyoxal and phenylglyoxal respectively (Vander Jagt *et al.*, 1972). The kinetic data were treated as described by Mannervik *et al.* (1973) and analysed by non-linear regression (Bartfai & Mannervik, 1972; Mannervik & Bartfai, 1973).

Molecular weights were determined by the method of Siegel & Monty (1966) by using the following reference proteins (with Stokes radii in parentheses): glutathione reductase (4.3nm), bovine serum albumin (3.5nm) and horse heart cytochrome *c* (1.0nm). For the estimation of the sedimentation coefficient, catalase ($s_{20,w} = 11.3$S) was used as a reference.

Isoelectric focusing for determination of isoelectric points was carried out as described above for the purification of glyoxalase I from yeast; the pH of eluted fractions was determined at 4°C.

Free thiol groups of glyoxalase I were determined as described by Glazer *et al.* (1975).

Peptide 'maps' of tryptic digests were made on paper by two-dimensional electrophoresis–chromatography (Marmstål & Mannervik, 1978).

Results

Purification of yeast glyoxalase I

The commercial enzyme, stabilized against denaturation by addition of bovine serum albumin, was purified for subsequent characterization. Table 1 summarizes the results of the purification. The purified enzyme was homogeneous in the electrophoretic systems *a*, *b* and *c* cited in Aronsson &

Table 1. *Purification of glyoxalase I from yeast*

Step	Volume (ml)	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min per mg)	Yield (%)	Purification factor
Commercial preparation	10	10000	50	200	100	1
Isoelectric focusing	43	7620	—*	—*	76	—*
S-Hexylglutathione–Sepharose 6B	12	6090	5.7	1070	61	5.4
Sephadex G-100	91	5080	4.5	1130	51	5.7

* The protein concentration was not determined owing to interference by ampholytes.

Table 2. *Physical properties of glyoxalase I from various sources*

Source	Molecular weight	Subunits (no.)	Stokes radius (nm)	$s_{20,w}$ (S)	Isoelectric point (pH)
Microbial					
Yeast (*Saccharomyces cerevisiae*)	32000	1	2.1	3.6	7.0
Escherichia coli	34000*				
Rhodospirillum rubrum	31000*				
Mammalian					
Rat liver	46000	2	2.8	4.0	4.7
Pig erythrocytes	41000	2	2.6	3.6	4.8
Human erythrocytes	46000	2	2.8	4.0	4.8

* Molecular weights estimated by gel filtration only (Andrews, 1970). The additional properties could not be determined owing to lack of material.

Mannervik (1977). It was previously demonstrated that glyoxalase I prepared from fresh yeast cells has the same molecular properties as the commercial product (Marmstål & Mannervik, 1978). The specific activity of the homogeneous yeast enzyme (approx. 1100 μmol/min per mg of protein) is comparable with the specific activities of the mammalian enzymes studied in our laboratory. The latter include glyoxalase I from pig erythrocytes (Aronsson & Mannervik, 1977), rat liver (Marmstål & Mannervik, 1979) and human erythrocytes (Aronsson *et al.*, 1979), and their specific activities are all about 1000 μmol/min per mg. Higher values reported in the literature have almost invariably been estimated with preparations containing very small amounts of protein, and difficulties in the protein determination may have resulted in erroneous values.

Physical properties of glyoxalase I

Table 2 gives physical data for glyoxalase I from various sources. In addition to the homogeneous enzymes, two crude preparations of microbial glyoxalase I were examined. The low activities in the cell extracts made purification difficult, and only estimates of molecular weights are given for these enzymes. The data for the bacterial enzymes are very similar to those for the yeast enzyme and, by analogy, it seems reasonable to suggest that all three microbial enzymes examined are monomeric. The molecular weights reported for the mammalian enzymes in Table 2 are 10–20% lower than those previously published (Aronsson & Mannervik, 1977; Marmstål & Mannervik, 1979; Aronsson *et al.*, 1979). The previous values were based on gel filtration only (Andrews, 1970), whereas the new values are calculated on the basis of estimated Stokes radii and sedimentation coefficients as described by Siegel & Monty (1966). Previously we found that the molecular weight of the dimeric (and prolate) molecule of glutathione reductase was similarly overestimated when determined by gel filtration only (Boggaram *et al.*, 1978; I. Carlberg & B. Mannervik, unpublished work). The estimate of the molecular weight of the monomeric yeast enzyme, however, was the same by both methods, and if it is assumed that the bacterial enzymes are also monomeric their molecular weights reported in Table 2 should be similarly reliable. The differences in molecular weights for the mammalian enzymes obtained by different physical methods suggest that, like glutathione reductase (Boggaram *et al.*, 1978), mammalian glyoxalase I may have an elongated molecular shape. The lack of a corresponding difference for the yeast enzyme implies that

its molecule (and probably that of the bacterial enzymes) is more-or-less spherical.

A mol.wt. of 43000 for glyoxalase I from mouse liver was determined by ultracentrifugation (Kester & Norton, 1975). Other values for mammalian glyoxalase I were estimated by gel filtration: sheep liver, 45900 (Uotila & Koivusalo, 1975), rat erythrocytes and rat liver, 50000 (Han *et al.*, 1976), and rabbit liver, 42000 (Elango *et al.*, 1978). Thus the microbial glyoxalase I molecules (mol.wts. 31000–34000) are significantly smaller than the mammalian ones. Even more significantly, in all cases so far examined the mammalian enzyme molecules are dimeric, whereas the glyoxalase I molecule from the unicellular organisms (at least from yeast; Marmstål & Mannervik, 1978) appears to be a monomeric structure.

Another significant difference in the physical properties of glyoxalase I from yeast and from mammals was in their isoelectric points. The mammalian enzymes are acidic proteins, whereas the yeast enzyme is a neutral protein. This difference correlates well with differences in tryptic-peptide 'maps' reported below.

Chemical properties of glyoxalase I

The chemical characterization of the enzyme from different sources is summarized in Table 3. The amino acid compositions previously reported for the rat and pig enzymes have been corrected to values consistent with the new determinations of molecular weight. Differences in amounts of (Asx+Glx) relative to the basic amino acids between the yeast enzyme and the mammalian enzymes are not distinct enough to explain the differences in isoelectric points (Table 2). The proportion of Asx and Glx that corresponds to free acids has not been determined, but the high proportion of basic peptides in tryptic digests of the yeast enzyme (Fig. 1) indicates that the mammalian glyoxalase I contains more acidic and neutral peptides than the yeast enzyme because of a higher (Asp+Glu)/(Asn+Gln) ratio.

Table 3. *Chemical composition of glyoxalase I from yeast and from mammals*

The values reported here have been calculated per molecule of enzyme by using the molecular weights given in Table 2. The amino acid contents previously reported for rat liver (Marmstål & Mannervik, 1979) and pig erythrocytes (Aronsson & Mannervik, 1977) were higher owing to the previous overestimation of the molecular weights for these enzymes. Abbreviation: n.d., not determined.

Component	Yeast	Rat liver	Pig erythrocytes	Human erythrocytes
Asx*	30	39	48	56
Thr	14	23	20	23
Ser	20	19	24	24
Glx	30	55	37	41
Pro	15	21	23	27
Gly	26	16	27	31
Ala	10	39	26	28
Cys	15	22	8	10
Val	12	24	9	14
Met	2	5	7	11
Ile	14	12	22	20
Leu	20	38	36	39
Tyr	10	14	10	14
Phe	13	20	21	19
His	9	9	8	7
Lys	21	37	33	40
Trp	n.d.	n.d.	5	5
Arg	10	17	11	11
Free thiol groups per molecule	2	2	2	4
Tryptic peptides				
Max. expected	32	55	45	52
Found	30	26	24	27
Half-cystine-containing tryptic peptides				
Max. expected	15	22	8	10
Found†	n.d.	12	5	5
Zinc atoms per molecule	1	2	2	2

* Amino acid composition is given as residues/molecule.

† Peptides containing [^{14}C]carboxymethyl groups detected by radioautography.

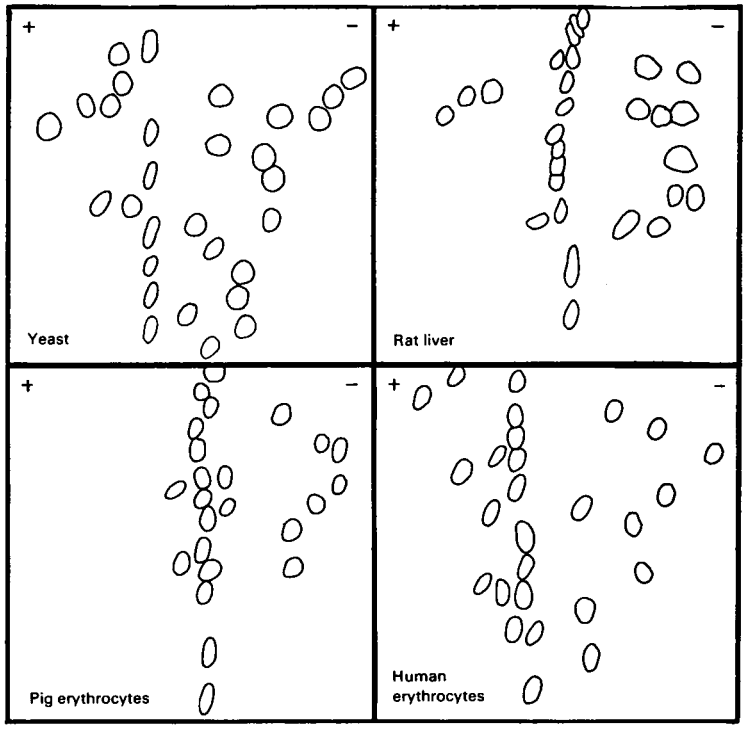

Fig. 1. *Tryptic-peptide 'maps' of glyoxalase I from yeast and mammalian sources*
The experimental procedures have previously been described (Marmstål & Mannnervik, 1978). Electrophoresis was carried out at pH 6.5 (horizontal direction; anode left, cathode right) followed by descending chromatography (vertical direction on the graphs) in butan-1-ol/acetic acid/water/pyridine (15:3:12:16, by vol.). The spots are ninhydrin-positive components of the tryptic digest.

The number of tryptic peptides of mammalian glyoxalase I identified in peptide 'maps' was about half the number expected for a monomer of the same molecular weight as that determined for the enzymes. This finding is consistent with the mammalian enzyme molecule being a dimer composed of two identical or very similar subunits. The number of tryptic peptides containing [^{14}C]carboxymethylated half-cystine residues was likewise only half of the number expected for a monomeric enzyme. These data thus strongly support the conclusion drawn from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, namely that mammalian glyoxalase I is a dimer composed of two probably identical subunits. The known exception is one of the isoenzymes of human glyoxalase I (form 2), which is believed to consist of two slightly different subunits (Aronsson *et al.*, 1979). The number of tryptic peptides of the yeast enzyme, on the other hand, is that expected for a monomeric enzyme. Theoretically it is possible to obtain the same number if the enzyme were composed of two non-identical subunits, but the failure to dissociate the enzyme molecule from yeast into subunits (Marmstål & Mannervik, 1978) eliminates this possibility.

There are two free thiol groups per molecule for all of the enzymes except the human one, which contains four thiol groups (Table 3). For mammalian glyoxalase I it appears that thiol groups have no direct role in the catalytic function (Mannervik *et al.*, 1975). However, the yeast enzyme is considerably more sensitive towards thiol-blocking reagents than are the mammalian enzymes (Ekwall & Mannervik, 1970), and the possibility of a role for thiol groups in the catalytic processes cannot be excluded for the yeast enzyme.

Glyoxalase I from all sources examined contains zinc, and if the subunit structures of the enzymes are regarded as established the zinc content corresponds to one atom per subunit of enzyme. The difference between the yeast enzyme and the mammalian one is that the former cannot, by any known method, be re-activated with bivalent metal ions after inactivation of the enzyme with a chelator, whereas the mammalian enzymes can all be re-activated to various extents by several bivalent metal ions (Davis &

Table 4. *Catalytic properties of glyoxalase I from yeast and from mammals*
The data refer to the kinetics under steady-state conditions in the standard assay system. For determination of $k_{cat.}$ and K_m, the reactant concentrations were chosen to keep the concentration of free glutathione fixed at 2mM, whereas the concentration of the hemimercaptal adduct was varied. The parameter values were obtained by non-linear regression analysis; the $k_{cat.}$ values were based on the molecular weights given in Table 2.

Source of enzyme	Oxoaldehyde substrate	Molecular activity, $k_{cat.}$ (min^{-1})	K_m (mM)	Non-Michaelian rate-saturation curves	Apparent non-linear inhibition by glutathione
Yeast	Methylglyoxal	109000	0.53	+	+
	Phenylglyoxal	402000	0.21	+	+
Rat liver	Methylglyoxal	71000	0.14	+	+
	Phenylglyoxal	66000	0.06	+	+
Pig erythrocytes	Methylglyoxal	59000	0.12	+	+
	Phenylglyoxal	37000	0.10	+	+
Human erythrocytes	Methylglyoxal	68000	0.13	+	+
	Phenylglyoxal	64000	0.04	+	+

Williams, 1969; Mannervik *et al.*, 1972; Uotila & Koivusalo, 1975; Aronsson & Mannervik, 1977; Han *et al.*, 1977).

Catalytic properties of glyoxalase I

The molecular activity (turnover number) of glyoxalase I from yeast was somewhat higher (10.9×10^4 μmol/min per μmol of enzyme) than those of the mammalian enzymes ($5.9\times10^4-7.1\times10^4$) with methylglyoxal as substrate (Table 4). If both subunits of the mammalian enzymes are active, the turnover number per subunit is less than half of the activity of the monomeric yeast enzyme. Similar results were obtained with phenylglyoxal as substrate.

The ratio $k_{cat.}/K_m$ for the enzymes was in the range of $0.3\times10^7-3.2\times10^7 s^{-1}\cdot M^{-1}$ for the hemimercaptals of both methylglyoxal and phenylglyoxal as substrates. Even if the meaning of the quantity $k_{cat.}/K_m$ is not clear, especially when the Michaelis–Menten equation is only an approximation of the kinetics, the values determined for glyoxalase I place the enzyme among the most catalytically effective enzymes (cf. Fersht, 1977).

The steady-state kinetics were determined by using various hemimercaptal concentrations at a constant concentration of free glutathione (2mM) that corresponds to the value *in vivo* for many cells (Meister, 1975). The apparent Michaelis constants determined under these conditions show a small but clear difference between yeast and mammalian enzymes (Table 4). It is important to keep free glutathione at a fixed concentration, because the velocity of the reaction is dependent on both the hemimercaptal and the glutathione concentrations (Bartfai *et al.*, 1973). When wide ranges of reactant concentrations are examined, the steady-state kinetics are non-Michaelian for both yeast (Mannervik *et al.*, 1974) and mammalian glyoxalase I (Mannervik *et al.*, 1973; Marmstål & Mannervik, 1979), and furthermore show an apparent non-linear inhibition by glutathione. The finding that the yeast glyoxalase I is a monomer (Marmstål & Mannervik, 1978) excludes, for this enzyme, the possibility that the non-Michaelian kinetics result from co-operative subunit interactions.

Discussion

The structural characteristics show that glyoxalase I from yeast (and apparently from bacterial sources) has a molecular structure that differs in many respects from the mammalian enzymes (Tables 2 and 3). The microbial enzyme has only one subunit, whereas the mammalian enzymes have two subunits per molecule. The tryptic-peptide 'maps' (Fig. 1) show that the yeast enzyme is different from the enzyme from rat, pig and man. Likewise, the isoelectric points for yeast and mammalian enzymes are at pH7.0 and 4.8 respectively. Furthermore, the effects of thiol-blocking reagents show that glyoxalase I from yeast is significantly more sensitive to inactivation than is the enzyme from mammals (Ekwall & Mannervik, 1970; Mannervik *et al.*, 1975). Consequently, differences in the primary and quaternary structures of the enzyme from yeast and from mammals are pronounced and indicate lack of homology between the microbial and mammalian enzyme. On the other hand, the various data for the enzyme from mammalian sources are consistent with the assumption of a common evolutionary origin for the mammalian enzyme forms.

The chemical mechanism of the catalysis performed by glyoxalase I appears to be very similar for the yeast and mammalian enzymes. The stereochemistry of the product is the same for the two types of enzyme (Ekwall & Mannervik, 1973). In all sources zinc is present in a stoicheiometry of one atom per subunit

(Table 3, and Aronsson *et al.*, 1978). Furthermore, amino-group-blocking reagents have similar effects on the enzyme from all sources investigated (Mannervik *et al.*, 1975). The kinetics also show striking similarities in the deviations from Michaelian rate behaviour. Accordingly, it is probable that the chemical mechanism of the catalysis is similar for yeast and mammalian enzymes.

The steady-state kinetics of glyoxalase I have been found to depend on at least two components of the equilibrium system of 2-oxoaldehyde, glutathione and their hemimercaptal adduct (Bartfai *et al.*, 1973). This statement applies to both yeast and mammalian glyoxalase I. The rate behaviour can be interpreted as resulting from non-linear competitive inhibition between reduced glutathione and the hemimercaptal substrate. The origin of the non-linear inhibition and the non-hyperbolic rate-saturation curve (with respect to hemimercaptal) has not been explained definitively, but empirically the effects are clearly established for the enzyme from yeast as well as from mammals with both methylglyoxal and phenylglyoxal as dicarbonyl substrates (Marmstål & Mannervik, 1979). The demonstration of the same type of rate behaviour of glyoxalase I from yeast and from mammals, in spite of the distinct differences in molecular properties, indicates that the non-linear inhibition by glutathione may have a physiological function. In fact, the curve shapes of the kinetic data are such as to counteract changes in velocity which are due to fluctuations in the total concentration of reduced glutathione. Variations in the reduced-glutathione content in rat liver have been demonstrated which reflect the diurnal rhythm of food intake (Isaacs & Binkley, 1977), and decrease of glutathione concentration may occur after exposure of an organism to xenobiotics that are conjugated with glutathione (Chasseaud, 1976). A decreased glutathione content would lead to a lower concentration of hemimercaptal of a 2-oxoaldehyde, but the simultaneous decrease of free glutathione relieves the inhibition caused by glutathione. The non-linear dependence of the inhibition on glutathione concentration results in an optimal compensation of the lowered activity which is due to a decreased concentration of hemimercaptal. Consequently, it appears that glyoxalase I in both yeast and mammals has been designed to maintain an activity towards 2-oxoaldehydes that is insensitive to changes in glutathione concentration. This interpretation of the kinetic data may be a clue in the search for the physiological role of glyoxalase I in micro-organisms as well as in mammals.

The differences between glyoxalase I from yeast and from mammals are not limited to the differences in primary and quaternary structure discussed above. Also notable is the high sensitivity of the yeast enzyme to thiol-blocking agents and to reversible inhibition by *S*-(*p*-bromobenzyl)glutathione, in comparison with mammalian glyoxalase I (Mannervik, 1974). Whether or not the differences reflect different functions of the enzyme in yeast and in mammals cannot yet be decided. It is evident, however, that the evaluation of studies of compounds for potential use in chemotherapy of cancer may be seriously biased if performed on the yeast instead of the mammalian enzyme.

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