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HYDROPHOBIC BINDING IS NOT AN INDEPENDENT STEREOCHEMICAL DETERMINANT IN THE YEAST GLYOXALASE I REACTION

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For yeast glyoxalase I, a stereospecific proton-transfer mechanism requires the formation of either a cis or a trans-enediol intermediate. Analogs of the two possible isomeric enediol intermediates, formed from the hemimercaptal due to phenylglyoxal and glutathione, have been synthesized in which the oxygen atoms of the enediol are replaced by protons. Both isomeric analogs are strong linear competitive inhibitors of the enzyme having nearly equal inhibition constants: K_i(cis) = 0.10 mM; K_i(trans) = 0.16 mM. This suggests that while hydrophobic interactions between substrate, enediol intermediate and enzyme may contribute significantly to binding, this type of interaction is not an independent stereochemical determinant of the reaction.

1. Introduction

The glutathione-dependent enzyme, yeast glyoxalase I, catalyzes the conversion of a variety of structurally different aromatic and aliphatic α-ketoaldehydes to their corresponding α-hydroxythioesters. The kinetically favored form of substrate is the hemimercaptal, formed in a preenzymic reaction between α-ketoaldehyde and glutathione [1]. The enzyme has been demonstrated to stereospecifically convert methylglyoxal and phenylglyoxal to S-D-lactoyl- and S-D-mandeloylglutathione, respectively [1,2].

The glyoxalase I reaction most likely involves the formation of an enediol intermediate, based on the recent observation that during the catalytic turnover of methylglyoxal in D_2O solvent there is significant deuterium incorporation into final product [3]. In this regard, the enzyme is similar to the aldose–ketose isomerases that are known to involve the formation of cis-enediol intermediates [4]. In an attempt to test for stereochemical conservatism between the glyoxalase I and the aldose–ketose isomerase reactions, preferential inhibition of glyoxalase I by cis- versus trans-S-phenethenyl-glutathione was tested for. These compounds are viewed as isomeric apolar analogs of the two possible isomeric enediol intermediates that could form from the hemimercaptal substrate due to glutathione and phenylglyoxal. Thus, differential inhibition should reflect apolar interactions leading to stabilization of one isomeric form of the enediol intermediate over the other.

2. Materials and methods

Yeast glyoxalase I was obtained from Sigma (type IV) and used without further purification. No indication of more than one form of the yeast enzyme has been observed [5]. Methylglyoxal was purified by vacuum distillation of the commercial, 40% aqueous solution (Aldrich) [6]. Contaminating lactic acid was removed from the distillate by filtration through Dowex-carbonate. Phenylglyoxal (Aldrich) was purified by vacuum distillation. Glutathione (Sigma) was >97% pure based on a sulfhydryl group assay using 4-pyridine disulfide.

2.1. Synthetic methods.

The synthetic route to S-phenethenylglutathione, alternately enriched in the cis and trans isomers, is based on Oswald's low temperature photochemical method

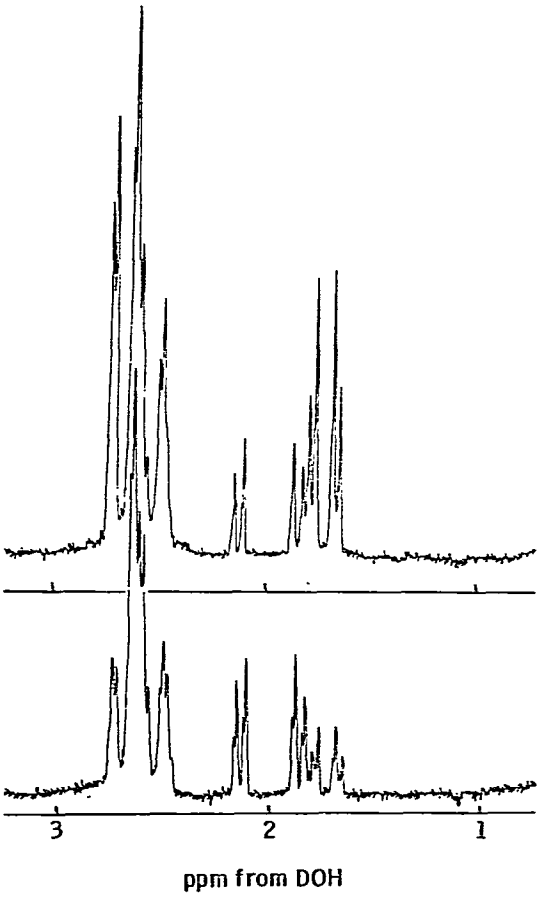

Fig. 1. Upfield portion of the 360 MHz proton spectra of samples of S-phenethenylglutathione, alternately enriched in the cis isomer (upper spectrum) and trans isomer (lower spectrum), showing the cis-vinyl proton resonances, δ = 1.657, 1.767 (J = 10.5 Hz), trans-vinyl proton resonances, δ = 1.833, 2.106 (J = 15 Hz) and aromatic ring proton resonances between δ = 2.45 and δ = 2.80 relative to DOH in D_2O: d_6-ethanol (2:3). Note that the absolute magnitude of chemical shifts reported from HOD are not highly reproducible. However, the *relative* chemical shifts of the cis and trans-vinyl proton resonances, which was of primary concern for calculating % cis and % trans isomer, are highly reproducible.

for preparing cis-enriched S-phenethenyl-mercaptans [7]. Cis-enriched S-phenethenylglutathione is prepared by irradiation of a solution of glutathione, 400 mg (1.56 mmoles), and phenyl acetylene, 3.58 ml (35.1 mmoles), in 6.9 ml water and 21.5 ml 95% ethanol with a high intensity Hanovia Lamp, positioned 4 cm from the reaction mixture. After the sulfhydryl group content decreases by ca. 60% (ca. 50–60 hrs.), solvent is removed from the reaction mixture under vacuum and the orange residue extracted with four 50 ml aliquots of water. The aqueous extract is neutralized to pH 6–7, placed on a Dowex-formate column (11 × 1.2 cm) and eluted with 400 ml of a 0–0.6 N linear formic acid gradient. Unreacted glutathione and S-phenethenyl glutathione elute at ca. 0.1 N and ca. 0.25 N formic acid respectively. The tubes containing product, uncontaminated with glutathione, are pooled and lyophylized to dryness to give 60 mg product: yield 15%. The product gave a single spot on Whatman 3 MM cellulose paper, R_f 0.74 (1-butanol: 90% formic acid: water = 100: 37.8:22.4). The NMR spectrum corresponded to that for the anticipated product. Isomerization of this material (ca. 70% cis) to the thermodynamically favorable trans-enriched product is accomplished by incubating the cis-enriched product (70 mg/ml) with 0.4 M benzenethiol at pH 7 in 75% ethanol–D_2O in a sealed NMR tube at 80°. The isomerization is monitored by following the cis-trans vinyl proton resonances on an A60 NMR spectrometer and is complete within 25–30 hrs. The isomerized product is rechromatographed on Dowex-formate, as described above, to afford a chromatographically pure product on Whatmann 3 MM cellulose paper: yield 30%.

Determination of the cis/trans isomer ratio in product is based on the relative integration ratios for the cis and trans vinyl proton resonances taken on a Bruker WH 360–180 NMR, fig. 1.

2.2. Inhibition kinetics

Inhibition studies were done under the assay conditions described by Vander Jagt, using his determined value for the dissociation constant of the hemimercaptal of methylglyoxal and glutathione, $K_{diss} = 3.0 \pm 0.5 \times 10^{-3}$ M [8]. Assay cuvettes (0.5 cm path length) contained phosphate buffer, 89.4 mM (pH7), and KCl, 100 mM, for a total ionic strength of 200 mM. Methylglyoxal and glutathione were introduced in proportions that kept the concentration of free glutathione at a constant 0.3 mM, independent of the concentration of hemimercaptal. Thirty minutes were allowed for the hemicaptal equilibrium to be established before introducing the enzyme into the cuvette and following appearance of product at 240 nm.

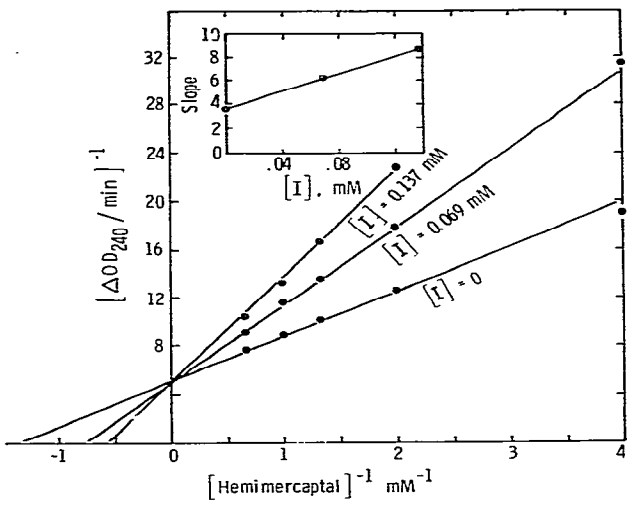

Fig. 2. Example of reciprocal plot of glyoxalase I activity versus concentration of hemimercaptal of methylglyoxal and glutathione at different fixed levels of S-phenethenylglutathione (I), 71.3 ± 2% cis isomer.

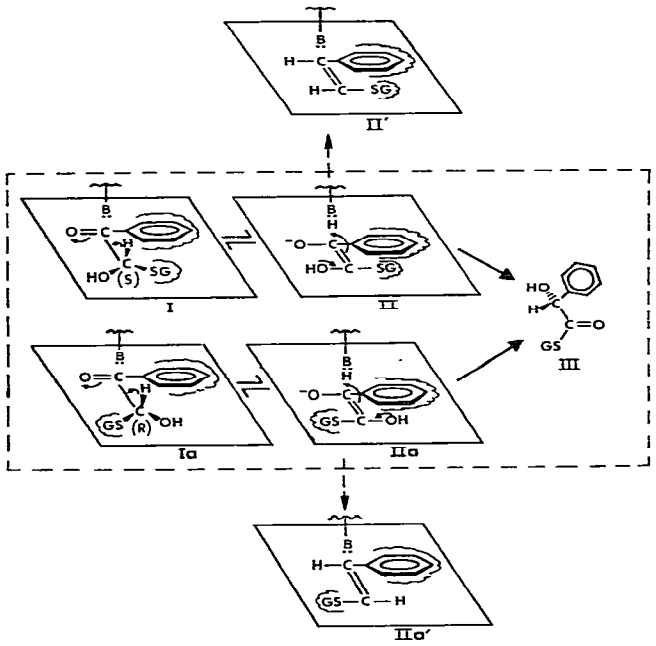

Fig. 3. Possible stereochemical routes in the glyoxalase I reaction (boxed in area) and possible stereospecific complexes between enzyme and cis-S- and trans-S-phenethenylglutathione.

3. Results

The preparations of cis-enriched and trans-enriched S-phenethenylglutathione, tested as inhibitors of yeast glyoxalase I, were determined to be 71.3 ± 2% cis isomer and 28.7 ± 1% cis isomer, respectively, by NMR spectroscopy, figure 1. Both preparations were demonstrated to give linear competitive inhibition with respect to substrate, fig. 2. In order to maximize precision in determining the relative degree of inhibition by the cis- and trans-enriched inhibitor preparations, five replicate measurements of enzyme activity were performed under identical conditions at a fixed substrate concentration near K_m (0.73 mM). Then the replicate activity measurements were repeated at a fixed inhibitor concentration sufficient to produce ca. 50% inhibition. The observed inhibition constants due to the cis- and trans-enriched inhibitor preparations were then calculated to be 0.114 ±0.015 mM and 0.136 ±0.013 mM respectively, using the equation for linear competitive inhibition. This same procedure has been used in the past to determine highly accurate inhibition constants due to aldehyde binding to papain [9]. Finally, the intrinsic inhibition constants due to the pure cis and pure trans isomers were calculated to be 0.10 ± 0.04 mM and 0.16 ± 0.06 mM respectively, by solving simultaneous forms of the following equation where K_i^{obs} = observed inhibition constant;

$$K_i^{obs} = (K_i^{cis}K_i^{trans})/(f_c K_i^{trans} + (1 - f_c)K_i^{cis}),$$

K_i^{cis} = intrinsic inhibition constant for the cis isomer; K_i^{trans} = intrinsic inhibition constant for the trans isomer and f_c = mole fraction of cis isomer in the preparation of S-phenethenylglutathione.

4. Discussion

Glyoxalase I operates on an equilibrium mixture of two diasteriomeric forms of hemimercaptal substrate which differ in that the chirality at the hemimercaptal carbon is either R or S, fig. 3 (I, Ia). For the hemimercaptal due to phenylglyoxal and glutathione two possible stereochemical routes can be envisioned for the conversion to S-D-mandeloylglutathione (III), depending on whether the enzyme is stereospecific for the (S)-hemimercaptal (I → II → III) or the (R)-hemimer-

captal (Ia → IIa → III). In both pathways an active site base can be envisioned to catalyze proton transfer to the si face of the carbonyl group (I, Ia), since this is the face to which the transferred proton *must* add in order to generate III. The observation of intramolecular proton transfer between C-1 and C-2 requires that the transferred proton leave from and add to the same face of the enediol intermediate: II, IIa [10]. Thus, if the (S)-hemimercaptal is substrate, a cis-enediol intermediate will form (I → II); if the (R)-hemimercaptal is substrate, a trans-enediol intermediate will result (Ia → IIa).

Stereochemical constraints within the active site that could lead to preferential stabilization of a cis- or a trans-enediol intermediate could be due to directional polar interactions with the enediol oxygen atoms, steric restrictions within the active site, and/or localized hydrophobic binding with the aromatic ring of substrate. The possible importance of the latter interaction is suggested by observations that (a) various aromatic and aliphatic α-ketoaldehydes are good substrates for the enzyme [8,12,13] and (b) various S-aryl and S-alkyl substituted glutathione derivatives are strong competitive inhibitors of the enzyme, for which the inhibition constants decrease as the hydrophobicity of the substituents increase [11]. In principle, a syn or anti relationship between the binding sites for the aromatic ring and glutathione moieties could sufficiently constrain the stereochemical course of the enzymic reaction to involve either a cis- or a trans-enediol intermediate, fig. 1 (II, IIa). This should be reflected as differential inhibition by the isomeric S-phenethenylglutathione derivatives (II′, II′a).

On the other hand, the finding that both isomeric inhibitors bind almost equally well to yeast glyoxalase I, as indicated by the near equality of their inhibition constants, argues against such a highly constrained relationship between the aromatic ring and glutathione binding sites. The lack of discrimination in binding is *not* due to the absence of strong interactions between enzyme and the S-phenethenyl moiety of the inhibitors since their inhibition constants are substantially smaller than those for underivatized glutathione, ca. 5 mM, and S-methylglutathione, 8.3 mM [8,11]. The linear competitive nature of the inhibition by both isomers is fully consistent with binding to the active site, fig. 2.

The inability of the enzyme to discriminate between the isomeric inhibitors seems to be most readily attributed to a broad binding region for the aromatic ring of inhibitors rather than a flexible binding site for the glutathione moiety. Examination of molecular models indicates that in order to fit the aromatic ring of both cis and trans isomers into identical binding sites, drast conformational changes would be required to occur in the tripeptide backbone of bound inhibitor.

If the hydrophobic region in the active site can significantly contribute to binding substrate and enediol intermediate, as indicated by the tight binding of the isomeric inhibitors, yet is not sufficiently localized to independently constrain the stereochemical course of the enzymic reaction, then how does the enzyme solv the problem of stereospecific catalysis? Clearly, directional polar interactions between the active site and the oxygen atoms of substrate and enediol intermedia must play an *indispensible* role in this regard. In so far as the stereospecificity and the efficiency of enzymic catalysis are related [4], this conclusion most likely reflects the central role that such directional polar interactions play in catalysis, either due to polar amino acid side chains extending into the active site or perhaps due to the presence of an active site zinc ion [14

Acknowledgements

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