

DIRECT DEMONSTRATION BY ¹H N.M.R. SPECTROMETRY OF THE STEREOSELECTIVITY OF YEAST GLYOXALASE I TOWARDS THE DIASTEREOMERIC FORMS OF THE α-KETOALDEHYDE-GLUTATHIONE HEMITHIOACETAL*

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Summary

Addition of yeast glyoxalase I at pD 4.4 leads to selective disappearance of one of the diastereotopic methine proton signals of the phenylglyoxal-glutathione hemithioacetal, the diastereomer with the lower field signal reacting preferentially with the enzyme, which directly establishes asymmetric binding of the hemithioacetal carbon region as the origin of the stereospecificity of the glyoxalase I reaction to form (S)-D-mandeloylglutathione.

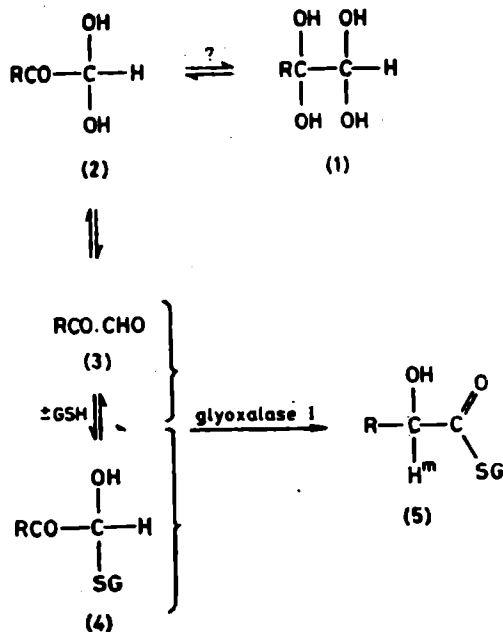
Glyoxalase I (EC 4.4.1.5) catalyses the conversion of glutathione (GSH) and α-ketoaldehydes into the glutathione thiol ester of the corresponding α-hydroxy-acid. It has been recognised for some time that α-ketoaldehydes (which exist largely as hydrated forms) and GSH react non-enzymically to form hemithioacetals(1) (Scheme).

Ekwall and Mannervik² have shown definitively that the product of the glyoxalase I reaction (yeast and pig erythrocyte enzymes) is (S)-D-lactoylglutathione (5; R=Me). With phenylglyoxal (3; R=Ph), the product is (S)-D-mandeloylglutathione. 3. In contrast, the nature of the substrate has remained in dispute. Three basic mechanisms have been proposed for yeast (and mammalian) glyoxalase. I. In the 2-substrate mechanism, the substrates are the free aldehyde form of the α-ketoaldehyde (3) and GSH⁴. For the 1-substrate mechanism⁵ the substrate is hemithioacetal (4). In this case, it has been suggested³ that glyoxalase I would presumably act selectively on one of the two diastereomeric hemithioacetals [(6) or (7)]. The third mechanistic possibility is a hybrid route, *v.z.*, a branching, alternative 1- and 2- substrate

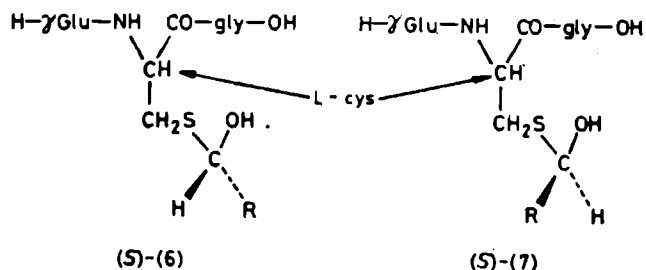
* Reprinted from: J.C.S, chem Comm., 1981.

mechanism in which the 1-substrate is the hemithioacetal and the first of the 2-substrates is GSH, with the α -ketoaldehyde the second. 6

We have approached the question of the origin of the product stereochemistry and the nature of the substrate for yeast glyoxalase I (Sigma, Grade X, lyophilised solid) using high-resolution ^1H n.m.r. analysis of the substrate [i.e. an equilibrated mixture of phenylglyoxal



SCHEME



(Sigma) and GSH (Sigma)]. The 100 MHz ^1H spectra of the components and the mixture in D_2O solution are shown in the Figure. Only the monodeuteriate (2) was detectable for phenylglyoxal. Under the concentration, pD ($=\text{pH}_{\text{meas}} + 0.4$), and temperature conditions described in the legend to the Figure, the diastereotopic protons of (6) and (7) are clearly resolved, although not assignable from the spectrum in

absolute terms. At higher pD (ca. 6) or concentration the pair of lines broadens extensively, presumably because of an increased exchange rate for the diastereomers. Glyoxalase I suffered no detectable activity loss over 17 min incubation at pD 4.73 or pD 4.51, although at pD 4.31 there was some 10% loss after 4 min, 12 at 17 min, and 14 after 30 min, but < 1% loss after 20 s.

We have called the upfield and downfield diastereotopic proton resonances Hu and Hd, respectively. Addition of various amounts of yeast glyoxalase I to the equilibrated hemithioacetal-aldehyde-GSH system led to progressive changes in intensity of Hu and Hd as well as the gradual appearance of the methine (Hm) signal of the man-

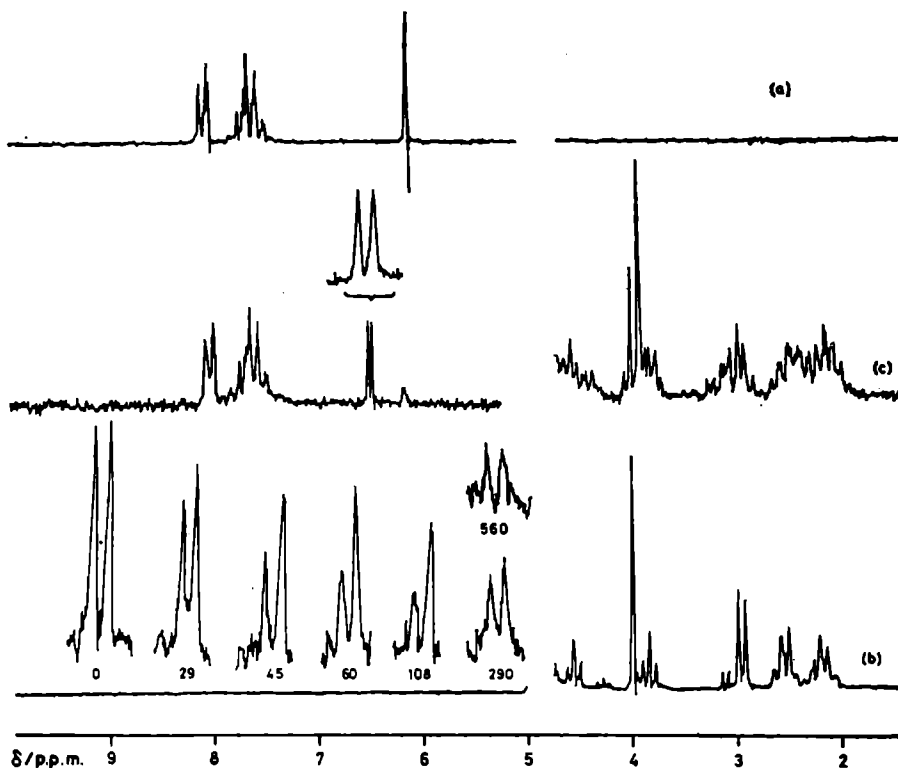


Figure. 100 MHz Spectra in D₂O solution pD (4.4) of (a) PhCOCHO, (b) glutathione, and (c) an equilibrium mixture of glutathione and PhCOCHO. The inset to (c) shows expansion of the diastereotopic hemithioacetal protons, and the time-dependent changes in these resonances (Hu and Hd) in D₂O at pD=4.4 and 35 °C ([GSH]₀=0.207 M, [phenylglyoxal]₀ = 0.190 M) upon addition of 80 μ l of a yeast glyoxalase I solution (Sigma, Grade X, 500 units/ml of D₂O) to 0.5 ml of equilibrated substrate solution. The times (in seconds) at which the spectra were measured are recorded as the numbers below the appropriate peaks.

deloyl product. A typical result is shown in the inset to the Figure. When the glyoxalase I concentration was sufficiently high, Hd was found to decrease more rapidly than Hu. Ultimately, the peak heights of Hu and Hd became ca equal and thereafter decreased in parallel. Thus, yeast glyoxalase I preferentially reacts with the diastereomeric form of the hemithioacetal† corresponding to Hd. At low enzyme concentrations the enzymic removal of substrate is slower than the rate of equilibration, of (6) and (7), and the selectivity for Hd appears to decrease. Hu and Hd become of equal intensity quite rapidly and decrease in parallel for most of the reaction time-course. The change in rate-determining step, during the course of such an n.m.r. run, from enzyme-catalysed diminution of Hd to equilibration of (6) and (7) is presumably caused by the ca. hyperbolic decrease in enzyme velocity as the substrate level is depleted through the K_m as the reaction proceeds. † In all cases studied, the mandeloyl product appeared smoothly as the hemithioacetal (Hu + Hd) was removed. The decrease in (Hu + Hd) and the increase in Hm approximately reflect one another's time courses if plotted out. As anticipated, the sum of the peak intensities of Hu, Hd, and Hm remains ca. constant during the course of a run, indicating the internal consistency of the experiment.

As the initial n.m.r. experiments were recorded at pD 4.4 at 29, 45, and 60 seconds, very little, if any, enzyme activity would be lost under these conditions. However, the diastereomeric selectivity is quite clear at this stage of the reaction, before any complications from acidic deactivation of glyoxalase I can occur.

† If one assumes that the mechanism involves a *cis*-enediol intermediate or transition-state (S. S. Hall, A. M. Doweiko, and F. Jordan, *J. Am. Chem. Soc.*, 1978, 100, 5934), then one can reasonably postulate that the Hd resonance corresponds to diastereomer (6) as the product has the D-configuration. Assignment is not possible if the mechanism is a hydride transfer (V. Franzen, *Chem. Ber.* 1956, 89, 1020; I. A. Rose, *Biochim Biophys. Acta*, 1957, 25, 214).

† A referee has suggested that this appears to imply a K_m 0.1 M for phenylglyoxal, a rather high value. However these data involve a hyperbolic enzyme velocity change and uncertainty in the rate of equilibration of the diastereomers, both of which make it difficult to derive meaningful K_m values from them.

In summary, these results show directly the formation of the (S)-mandeloyl thiolester of glutathione by selective glyoxalase I (yeast)-catalysed reaction of one of the diastereomers of the hemithioacetal adduct of GSH and phenylglyoxal. The experiments also indicate that the most likely§ origin of the product stereochemistry is an asymmetric hemithioacetal binding site on glyoxalase I, which selects one of the diastereomers for subsequent reaction.

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- 1 M. Jowett and J. H. Quastel, *Biochem J.*, 1933, 27, 486; M. P. Shubert, *J. Biol. Chem.*, 1935, 111, 681.
- 2 K. Ekwall and B. Mannervik, *Biochim. Biophys. Acta*, 1973 297, 297.
- 3 D.L. Vander Jagt, E. Baub, J. A. Krohn, and L.-P. B. Han, *Biochemistry*, 1975, 14, 8669.
- 4 W. O. Kermack and N. A. Matheson, *Biochem. J.*, 1957, 65, 48.
- 5 E. E. Cliffe and S. G. Waley, *Biochem. J.*, 1961, 79, 475.
- 6 B. Mannervik, B. Gorna-Hall, and T. Bartfai, *Eur. J. Biochem.*, 1973, 37, 270.
- 7 This is further supported by the ¹³C n.m.r. spectrum of phenylglyoxal in D₂O, C. Brown, K.T. Douglas, and D.O. Smith, unpublished observations.

§ Given the common enantiomerically-selective binding sites of many enzymes (e.g. the L-configuration binding peptidases) this suggested absolute requirement for only one of the hemimercaptal diastereomers is reasonable. However, in the absence of extensive quantitative analysis a differential reactivity of both Hu and Hd with the enzyme cannot be excluded rigorously.