Direct Demonstration by ^{1}H 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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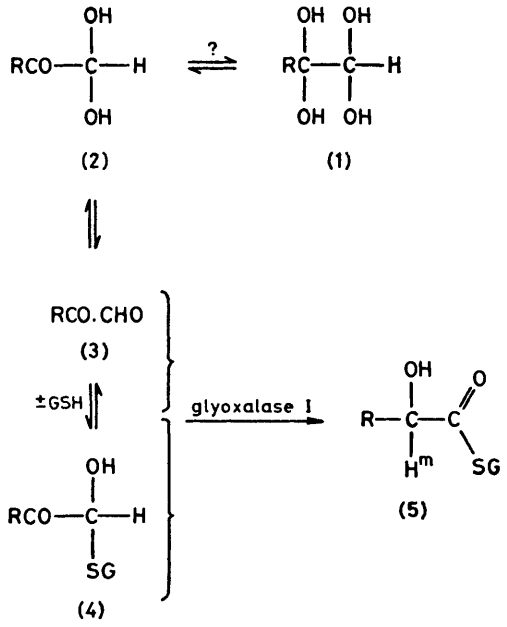
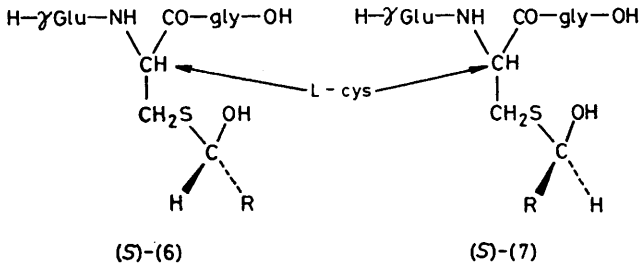
Summary Addition of yeast glyoxalase I at pD 4·4 leads to selective disappearance of one of the diastereotopic methine proton signals of the phenylglyoxal–glutathione hemithioacetal, the diastereomer with the lower field signal reacting preferentially with the enzyme, which directly establishes asymmetric 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).



SCHEME

Ekwall and Mannervik[2] 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) and yeast glyoxalase I 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 GSH.[4] For the 1-substrate mechanism[5] the substrate is hemithioacetal (**4**). In this case, it has been suggested[3] 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, *viz.*, a branching, alternative 1- and 2-substrate 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 ^{1}H n.m.r. analysis of the substrate [*i.e.* an equilibrated mixture of phenylglyoxal (Sigma) and GSH (Sigma)]. The 100 MHz ^{1}H spectra of the components and the mixture in D_2O solution are shown in the Figure. Only the monodeuteriate (**2**) was detectable for phenylglyoxal.[7] Under the concentration, pD (= pH_{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 H^u and H^d, respectively. Addition of various amounts of yeast glyoxalase I to the equilibrated hemithioacetal–aldehyde–GSH system led to progressive changes in intensity of H^u and H^d as well as the gradual appearance of the methine (H^m) signal of the mandeloyl product. A typical result is shown in the inset to the Figure. When the glyoxalase I concentration was sufficiently high, H^d was found to decrease more rapidly than H^u. Ultimately, the peak heights of H^u and H^d became

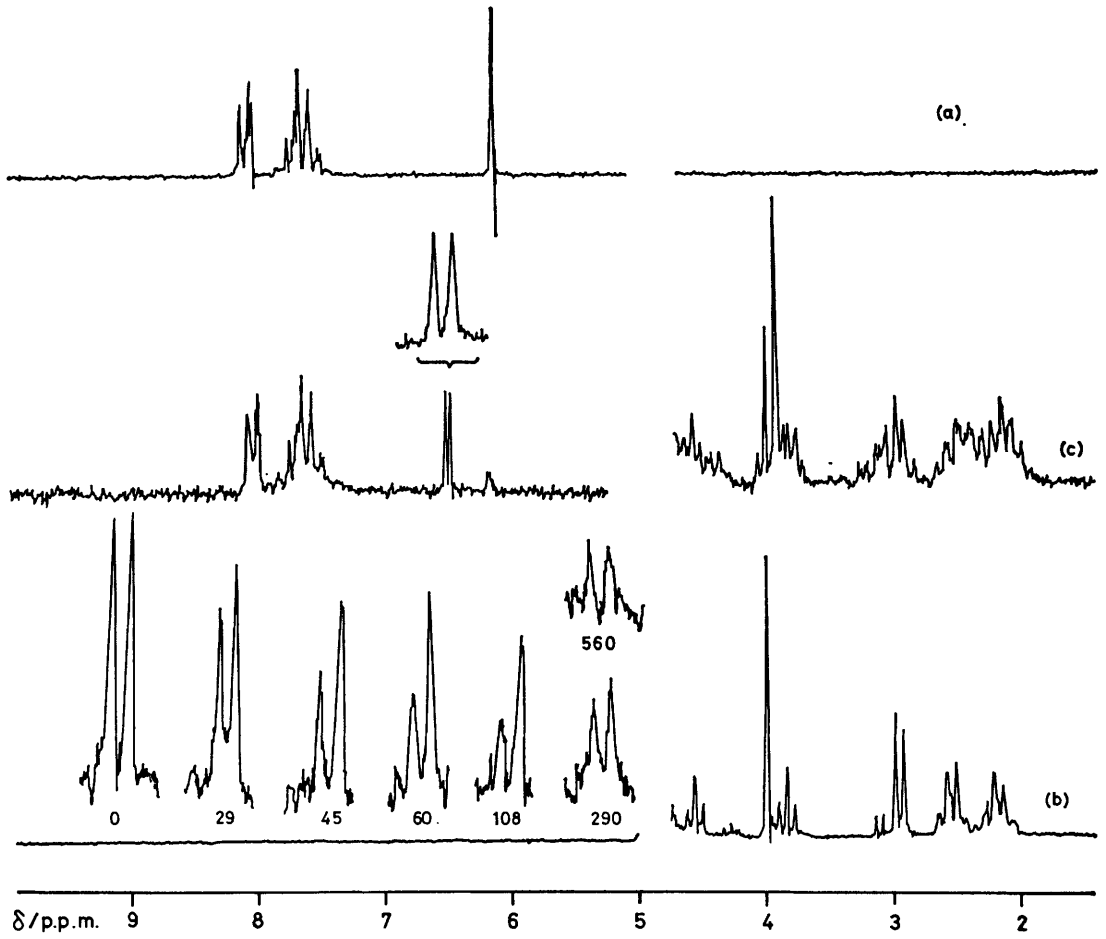


FIGURE. 100 MHz Spectra in D_2O solution (pD 4·4) of (a) PhCOCHO, (b) glutathione, and (c) an equilibrium mixture of glutathione and PhCOCHO. The inset to (c) shows expansion of the signals caused by the diastereotopic hemithioacetal protons, and the time-dependent changes in these resonances (H^u and H^d) in D_2O at pD = 4·4 and 35 °C ($[GSH]_0$ = 0·207 M, [phenylglyoxal]$_0$ = 0·190 M) upon addition of 80 μl of a yeast glyoxalase I solution (Sigma, Grade X, 500 units/ml of D_2O) 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.

ca. equal and thereafter decreased in parallel. Thus, yeast glyoxalase I preferentially reacts with the diastereomeric form of the hemithioacetal† corresponding to H^d. At low enzyme concentrations the enzymic removal of substrate is slower than the rate of equilibration of (**6**) and (**7**), and the selectivity for H^d appears to decrease. H^u and H^d 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 H^d 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 (H^u + H^d) was removed. The decrease in (H^u + H^d) and the increase in H^m approximately reflect one another's time courses if plotted out. As anticipated, the sum of the peak intensities of H^u, H^d, and H^m 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 de-activation of glyoxalase I can occur.

† If one assumes that the mechanism involves a *cis*-enediol intermediate or transition-state (S. S. Hall, A. M. Doweyko, and F. Jordan, *J. Am. Chem. Soc.*, 1978, **100**, 5934), then one can reasonably postulate that the H^d 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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§ 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 H^u and H^d with the enzyme cannot be excluded rigorously.

[1] M. Jowett and J. H. Quastel, *Biochem J.*, 1933, **27**, 486; M. P. Shubert, *J. Biol. Chem.*, 1935, **111**, 671.
[2] K. Ekwall and B. Mannervik, *Biochim. Biophys. Acta*, 1973, **297**, 297.
[3] D. L. VanderJagt, E. Daub, J. A. Krohn, and L.-P. B. Han, *Biochemistry*, 1975, **14**, 3669.
[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 ^{13}C n.m.r. spectrum of phenylglyoxal in D_2O, C. Brown, K. T. Douglas, and D. O. Smith, unpublished observations.