Discrimination between Steady-State Kinetic Models of the Mechanism of Action of Yeast Glyoxalase I†

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ABSTRACT: The determination of the best model of the steady-state kinetics of yeast glyoxalase I was approached by statistical methods. Linear and nonlinear regressions were carried out to fit a number of sets of experimental data to each of several kinetic models. The basic problem was to discriminate between a one-substrate mechanism involving hemimercaptal and a two-substrate mechanism involving free methylglyoxal and free glutathione. Discrimination criteria are given to define the best fit. According to these criteria the kinetics of yeast glyoxalase I are better described by one model than by all other models tested. However, this particular model is ambiguous and can be interpreted either as a one-substrate mechanism, in which hemimercaptal is the substrate and glutathione a competitive inhibitor, or as a two-substrate mechanism (rapid equilibrium ordered), glutathione being the first substrate bound and methylglyoxal the last.

Glyoxalase I catalyzes the formation of *S*-lactoylglutathione from methylglyoxal and glutathione (Racker, 1951) and of similar thiolesters from other 2-ketoaldehydes and glutathione or glutathione-like thiols (Knox, 1960). The crucial step in the mechanism of action of the enzyme is the transfer of a hydride equivalent from C-1 to C-2 of the ketoaldehyde with concurrent thiol ester formation (Franzen, 1956; Rose, 1957). Steady-state kinetic studies have been carried out to support on the one hand a mechanism involving free methylglyoxal (M) and free glutathione (G) (Kermack and Matheson, 1957)

$$v = \frac{V[G][M]}{K + K_m^G[M] + K_m^M[G] + [G][M]} \quad (I)$$

or, on the other hand, a mechanism in which preformed hemimercaptal (A) of methylglyoxal and glutathione is the true substrate (Cliffe and Waley, 1961)

$$v = \frac{V[A]}{K_m + [A]} \quad (II)$$

In these equations V is the maximal velocity, K_m, K_m^G, and K_m^M are Michaelis constants for A, G, and M, respectively, and K is an additional constant. The first of these alternatives would imply an enzymatic reaction involving two substrates and the second a one-substrate reaction.

However, these investigators concentrated on the data supporting one of the mechanisms and did not rigorously exclude any of the alternative mechanisms. This approach contrasts with the well-known fact that kinetic studies have their strength in disproof of kinetic models and not in proof of one of the alternatives. The present investigation, therefore, was undertaken to see if any of the two basic mechanisms, *i.e.*, a one-substrate or a two-substrate mechanism, were definitely inferior as a description of the action of glyoxalase I.

A choice between the alternatives proposed could easily be made provided that experimental conditions could be realized under which methylglyoxal and glutathione were present in the absence of the hemimercaptal or *vice versa*. However, due to the rapid spontaneous reaction

$$G + M \rightleftharpoons A \quad (1)$$

all three species are present, and their concentrations at equilibrium are determined by

$$K_d = [G][M]/[A] \quad (2)$$

where K_d is the equilibrium constant for dissociation of A into G and M. The rival kinetic models are therefore not readily distinguishable, and in the present investigation an attempt was made to apply the procedure recently described (Bártfai and Mannervik, 1972) to the discrimination between steady state kinetic models of glyoxalase I.

Materials and Methods

Glyoxalase I (*S*-lactoylglutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) from yeast was purchased from Sigma and was used without further purification. Glutathione (Sigma) was dissolved in water and the solution was standardized enzymatically (Klotsch and Bergmeyer, 1965a) or according to Ellman (1959). Methylglyoxal (40% water solution) was obtained from Fluka and was distilled before use. The distillate was standardized enzymatically (Klotsch and Bergmeyer, 1965b) and subsequently diluted to 1 M stock solution to prevent polymerization. Human serum albumin was obtained from KABI, Stockholm.

Determination of the Reaction Catalyzed by Glyoxalase I. The basis of the assay of glyoxalase I activity was the determination of thiol ester formation. This was followed spectrophotometrically at 240 nm (Racker, 1951) on a Beckman DB-G spectrophotometer coupled to a W + W Electronic Model 3002 recorder. The reaction system of a final volume of 1 ml contained: 0.1 M sodium phosphate buffer (pH 6.8); variable concentrations of glutathione (0.4–8.4 mM) and methylglyoxal (0.6–12.2 mM); and 50 μl of enzyme (diluted 1:100 with buffer containing 0.1% human serum albumin). The reaction temperature was 30° and the velocity was expressed in absorbancy change at 240 nm (ΔA) per min.

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Data Processing. Sets of kinetic data were fitted by the least-squares method to all of the rate equations considered in the analysis. Computer programs were written in Fortran essentially as described by Cleland (1967) and consisted of an initial primary estimation of parameter values in linearized models followed by nonlinear regression based on the steepest descent method. Input data are initial velocities, total concentrations of methylglyoxal and glutathione, respectively, and the assumed value (2.0 mM) of the dissociation constant, K_d, for the equilibrium between hemimercaptal, glutathione and methylglyoxal.

Equilibrium Constant. The equilibrium constant, K_d, for the dissociation of hemimercaptal into methylglyoxal and glutathione is fundamental in the calculation of the actual concentrations of reactants in the kinetic experiments. A value of 2.0 mM (Cliffe and Waley, 1961; Ekwall, 1971) was used in the discrimination procedure. It was established that changes of K_d affected Q^2 (*cf.* Theory) only slightly, but it is nonetheless clear that use of an erroneous value of K_d deforms the data in a nonlinear manner. When the best model had been selected, it was attempted to refine the value of K_d, using K_d as an additional parameter, but no value significantly better than 2.0 mM could be obtained.

Theory

To discriminate between rival steady-state kinetic models of a particular enzymatic reaction, initial velocity data are required from large concentration ranges of reactants (*i.e.*, substrates, inhibitors, etc.). The data are often examined by means of linear plots, or to an increasing extent treated by computers (Cleland, 1963, 1967; Garfinkel *et al.*, 1970; Swann, 1969). Computers for regression analysis not only make possible the handling of a vast amount of data but also help to choose the best fitting model by calculation of quantities used for statistical considerations (*cf.* Haarhoff, 1969; Arihood and Trowbridge, 1970; Bártfai and Mannervik, 1972). It should be pointed out, however, that the use of statistics does not enrich the information content of the experiments, but only assists in extracting the maximal amount of information from the experimental data. In other words, the design of the experiments ultimately limits what can be learnt of the system investigated.

The regression (optimization) programs are written to search for a given model (j) a set of parameter values minimizing the sum of squares (Q^2)

$$Q_j^2 = \sum_{i=1}^{n}(v_i - \hat{v}_{ij})^2/(n - p_j) \qquad (3)$$

where v_i and $\hat{v}_{ij}$ are the measured and predicted velocity values in the ith experimental point, n is the number of measurements, and p_j the number of parameters included in model j.

A summary of the criteria used for discrimination between rival kinetic models (Bártfai and Mannervik, 1972) is given below.

Criteria for Examining the Adequacy of Models. The steady-state models considered in the present paper can, in the absence of products, be linearized by inversion, *e.g.*

$$v_i = \frac{V[S]_i}{K_m + [S]_i} \curvearrowright \frac{1}{v_i} = \frac{1}{V} + \frac{K_m}{V}\frac{1}{[S]_i} \qquad (4)$$

or in vector form $\mathbf{a} = \mathbf{BK}^*$, where v_i is the initial velocity corresponding to the substrate concentrations $[S]_i$, V the maximal velocity, K_m the Michaelis constant, $\mathbf{a}$ the vector of inverted velocities, $\mathbf{B}$ a matrix of $\{1, 1/[S]_i\}$ rows, and $\mathbf{K}^* = \{1/V, K_m/V\}$, the vector of parameters. Now eq 5

$$\mathbf{K}^* = (\mathbf{B}^T\mathbf{B})^{-1}\mathbf{B}^T\mathbf{a} \qquad (5)$$

is a solution of the linear regression problem if the matrix $(\mathbf{B}^T\mathbf{B})$ can be inverted (Draper and Smith, 1966). Failure of inversion can be due to singularity of the matrix, which implies that the model is overdetermined. *Criterion A: Models with singular matrices* $(\mathbf{B}^T\mathbf{B})$ *are considered unsatisfactory.*

The solution of eq 5, used as primary estimates of the parameters, is usually sufficiently accurate to give rapid convergence with most nonlinear optimization programs. Only models giving convergence can be considered compatible with the data. *Criterion B: Models which do not give convergence in the nonlinear regression step with any of several sets of primary estimates are rejected.*

The significance of the parameter values obtained can be tested by the statistical t test.

Regression without constraints may give negative parameter values in the absence of product inhibition, in conflict with the theory of steady-state kinetics. Such a result can be avoided by introduction of constraints on the parameters. Minimization of Q^2 in this case, however, will often result in a parameter value equal to one of the limit values, and further analysis will show redundancy of parameters (Swann, 1969). *Criterion C: Models giving unreasonable or unreliable parameter values are rejected.*

The residuals of model j, $(q_{ij} = v_i - \hat{v}_{ij})$, should be examined as functions of reactant concentrations and velocity values. *Criterion D: Models giving residuals which are not normally distributed or do not have zero mean are rejected.*

The sum of squares (Q^2) should be examined by means of the F test for pairs of models satisfactory according to criteria A–D. *Criterion E: The model giving a Q^2 value significantly smaller than the Q^2 values of all other satisfactory models is chosen as the best one.*

The goodness of fit in the finally accepted model can be examined by the F test using the quotient (R^2) of the sum of squares due to the regression and sum of squares about the mean (Draper and Smith, 1966).

Results

Model Fitting. To apply the criteria given in the Theory section, it is necessary to have available velocity data corresponding to a large range of reactant concentrations. Several experiments were carried out in the ranges of 0.6–12.2 and 0.4–8.4 mM total concentrations of methylglyoxal and glutathione, respectively, and the data sets obtained were individually fitted to all of the mathematical models considered in the discussion below.

The data set presented in Table I is given as an example. First the two main models, *i.e.*, the two-substrate (I) and the one-substrate models (II) were fitted. Model I did not give convergence, whereas model II did and gave reasonable parameter values (see Table II). However, the $\hat{v}$ values calculated were always lower than the measured values at low v values and *vice versa* at high v values. Now different inhibition patterns were fitted to test the possibility that any of A, G, and M were inhibitors in a one-substrate mechanism. The models included competitive inhibition by G (III) or M (IV) (*cf.*

TABLE I: Experimental Data Set.

Exptl Point (No.)	Hemimercaptal (mM)	Free Methylglyoxal (mM)	Free Glutathione (mM)	Initial Velocity (ΔA/min)
1	0.073	0.538	0.346	0.0136
2	0.147	1.076	0.272	0.0225
3	0.154	0.458	0.683	0.0240
4	0.220	2.260	0.199	0.0334
5	0.250	0.362	1.424	0.0349
6	0.270	0.953	0.567	0.0386
7	0.264	3.405	0.155	0.0394
8	0.320	0.292	2.191	0.0419
9	0.316	5.799	0.102	0.0442
10	0.369	11.860	0.049	0.0521
11	0.427	2.019	0.410	0.0560
12	0.466	0.757	1.208	0.0582
13	0.513	3.156	0.324	0.0663
14	0.607	0.616	1.904	0.0676
15	0.770	1.676	0.904	0.0815
16	1.040	1.406	1.471	0.0912
17	0.964	2.705	0.710	0.0936
18	1.189	4.926	0.485	0.1040
19	1.350	2.319	1.610	0.1056
20	1.440	1.066	2.745	0.1060
21	1.870	0.576	6.500	0.1189
22	1.400	10.830	0.274	0.1204
23	1.783	4.332	0.728	0.1212
24	1.942	1.727	2.243	0.1232
25	2.126	10.100	0.385	0.1424
26	2.795	3.320	1.390	0.1510
27	2.730	0.939	5.640	0.1520
28	3.428	8.802	0.757	0.2030

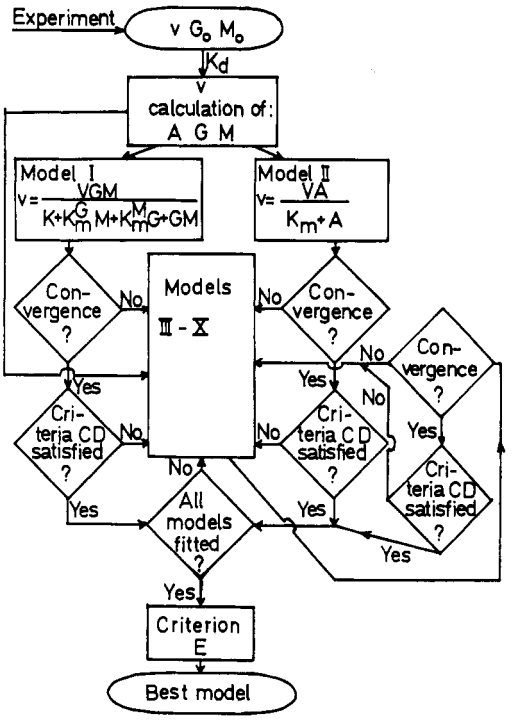

FIGURE 1: Flow chart of the discrimination procedure. The starting values are the measured velocities, v, and the total concentrations of glutathione, $[G_0]$, and methylglyoxal, $[M_0]$, respectively.

Table II); uncompetitive inhibition by G (V) or M (VI); noncompetitive inhibition by G (VII) or M (VIII); and inhibition by excess of A (IX). In addition the two-substrate Ping-Pong pattern (X) was tried. All these models gave convergence and the parameter values and Q (which is an estimate of σ) obtained are presented in Table II.

The models were fitted with several sets of data similar to that presented in Table I, and the results were the same (disregarding changes in numerical values) with the exception that model I in some cases gave convergence. However, in these fittings the value of K_m^G was not significantly different from zero as exemplified in Table III. It should be noted that all inhibition models except III gave one negative parameter value, which is in conflict with the kinetic theory.

Examination of the Results of Model Fitting. Models II, III, and X, which have only positive parameter values, were examined for goodness of fit by plotting v_i and $\hat{v}_{ij}$ against M, G, A, and v_i values and it was found that velocities calculated according to model III better depict the experimental values than do those of models II and X.

Models giving negative parameter values were also fitted by a constrained Gauss–Newton method, which resulted in parameter values equal to one of the limit values, *i.e.*, near zero or a very high positive value. This means that these models degenerated to the corresponding simpler equation (*e.g.*, II).

The goodness of fit as expressed by the R^2 value is also illustrated in Table II for models giving only positive parameter values in the regression.

As a result of replicate measurements over the whole range of reactant concentrations the experimental or pure error was estimated to be $\leq 5\%$ of the measured velocity values. The stability of the solution of the regression according to model III was examined by fitting velocity values randomly modified to simulate 5% error. Stability was demonstrated by the fact that convergence was obtained with modified data and that the Q^2 and parameter values were not significantly different from the original values.

Discussion

The analysis of the data presented shows how application of statistical criteria (Bártfai and Mannervik, 1972) can be useful in the discrimination between alternative kinetic models. The original problem was to discriminate between a one-substrate and a two-substrate mechanism as outlined in Figure 1. The result was that model II corresponding to a one-substrate mechanism was better than model I representing a two-substrate mechanism. The basis for this conclusion is that model I contained too many parameter values, as expressed by redundancy of one of the parameter values or lack of convergence. However, the fact that the sum of squares for model I, in cases when convergence was obtained, was smaller than the sum of squares for model II, indicated that a model better than II including three parameters could be derived. Furthermore, the residuals of model II were not normally distributed, being positive at low v values and negative at high values. It has to be noted that irrespective of the actual enzymatic mechanism three chemical species, namely, A, G, and M, are always present in the reaction mixture, and any of these which is not a substrate may affect the enzymatic

TABLE II: Model Fitting by Nonlinear Regression.

	Model	K_m (mM)	K_i (mM)	V (ΔA/min)	Q (ΔA/min)	R^2
I	$v = \frac{V[G][M]}{K + K_m^G[M] + K_m^M[G] + [G][M]}$	No convergence				
II	$v = \frac{V[A]}{K_m + [A]}$	1.70 ± 0.22		0.254 ± 0.017	0.0089	0.99
III	$v = \frac{V[A]}{K_m(1 + [G]/K_i) + [A]}$	1.77 ± 0.21	13.37 ± 0.14	0.274 ± 0.018	0.0076	1.00
IV	$v = \frac{V[A]}{K_m(1 + [M]/K_i) + [A]}$	1.32 ± 0.03	−42.13 ± 0.34	0.217 ± 0.004	0.0089	
V	$v = \frac{V[A]}{K_m + [A](1 + [G]/K_i)}$	0.300 ± 0.005	−3.07 ± 0.07	0.152 ± 0.007	0.0187	
VI	$v = \frac{V[A]}{K_m + [A](1 + [M]/K_i)}$	1.76 ± 0.23	−478 ± 0.40	0.272 ± 0.023	0.0029	
VII	$v = \frac{V[A]}{K_m(1 + [G]/K_{is}) + [A](1 + [G]/K_{ii})}$	1.57 ± 0.20	8.31 ± 5.61 (K_{is}) −28.32 ± 0.32 (K_{ii})	0.259 ± 0.020	0.0078	
VIII	$v = \frac{V[A]}{K_m(1 + [M]/K_{is}) + [A]/(1 + [M]/K_{ii})}$	1.15 ± 0.11	39.91 ± 30.15 (K_{is}) −28.44 ± 0.27 (K_{ii})	0.194 ± 0.012	0.0066	
IX	$v = \frac{V[A]}{K_m + [A] + [A]^2/K_i}$	0.630 ± 0.086	−6.97 ± 0.51	0.134 ± 0.009	0.0053	
X	$v = \frac{V[G][M]}{K_m^G[M] + K_m^M[G] + [G][M]}$	4.96 ± 4.59 (K_m^M) 1.37 ± 1.35 (K_m^G)		0.555 ± 0.415	0.0285	0.98

TABLE III: Parameter Values from Convergent Fittings of Data to Model I.

Data Set	K_m^G (mM)	K_m^M (mM)	K (mM^2)	V (ΔA/min)
1	0.013 ± 0.014	0.334 ± 0.048	1.11 ± 0.07	0.288 ± 0.007
2	−0.008 ± 0.018	0.210 ± 0.064	0.806 ± 0.094	0.217 ± 0.009

activity. Particularly relevant to this possibility is the structural similarity between A and G and the observations that *S*-substituted glutathione derivatives (Kermack and Matheson, 1957; Mannervik and Nise, 1969; Vince *et al.*, 1971; Górna *et al.*, 1972) and excessive glutathione (Cliffe and Waley, 1961) inhibit glyoxalase I. Consequently, a number of equations corresponding to reasonable inhibition patterns III–VIII were tested with M and G as inhibitors in the one-substrate model. Also the model corresponding to inhibition by excess of A (IX), which was better than model II for glyoxalase I from erythrocytes (Mannervik *et al.*, 1972), was considered. All inhibition models except III gave one negative parameter value and were therefore unsatisfactory. Equation III, on the other hand, fitted the data well and was superior to eq II according to criterion D. Furthermore, an experiment designed for discrimination between models II and III (*cf.* Bártfai and Mannervik, 1972), by utilizing lower concentrations of hemimercaptal in comparison with free glutathione, gave a significant difference according to criterion E (the Q values were 0.0103 and 0.0024 for models II and III, respectively; 30 experimental points; experiment carried out by Mrs. Górna). Model III was consequently the best model tested according to the one-substrate hypothesis.

An alternative way to obtain a rate equation better than I or II is to derive degenerate forms of the overdetermined two-substrate model I. Elimination of the constant term, K, in the denominator of I gives a Ping-Pong pattern (X), whereas elimination of $K_m^G[M]$ or $K_m^M[G]$ results in equations corresponding to rapid equilibrium ordered mechanisms having G or M as the first substrate, respectively. However, the latter equations are equivalent to the one-substrate models III and IV having G or M as competitive inhibitors, because $K_d[A]$ can according to eq 4 be substituted for [G][M] in these equations, *e.g.*

$$v = \frac{V[G][M]}{K + K_m^M[G] + [G][M]} = \frac{V[A]}{K_m(1 + [G]/K_i) + [A]} \quad (6)$$

where $K_m = K/K_d$ and $K_i = K/K_m^M$.

By the same substitution it can be seen that the equation obtained by deletion of both $K_m^G[M]$ and $K_m^M[G]$ is equivalent to II.

Thus, all degenerate forms of model I except model X are equivalent to one-substrate equations already tested. Model X, on the other hand, was unsatisfactory according to criterion C due to the uncertainty in the parameter values.

In conclusion, the best equation of all tested is III, which, however, can be interpreted either as a one-substrate or a two-substrate mechanism. According to the first alternative A is the substrate and G a competitive inhibitor, whereas according to the second alternative G is the first and M the second substrate in a rapid-equilibrium ordered mechanism.

It has not been possible to find a method to distinguish between the two alternative interpretations of model III by means of steady-state kinetic analysis. Cliffe and Waley (1961) reported as support for the one-substrate mechanism that the enzymatic activity was not proportional to the glyoxalase I concentration at high values of the latter, presumably due to dehydration of the hydrated form of methylglyoxal becoming rate limiting. This experimental result has also been obtained in our laboratory, but unfortunately this finding does not tell one mechanism from the other, because a two-substrate mechanism may also require methylglyoxal in its unhydrated form. Thus, the choice between the one-substrate and two-substrate mechanisms satisfying the steady-state kinetic data has to be based on transient kinetics. An attempt in this direction has been made by Davis and Williams (1969) and recently experiments with a stopped-flow spectrophotometer were initiated in our laboratory.

In conclusion, it can be stated that although the basic mechanism for glyoxalase I has not been delineated by the present analysis of the steady-state kinetics, it has nevertheless been possible to establish a rate law (model III) which is significantly better than all other kinetic models tested.

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