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A DIFFERENTIAL LABELING MODEL FOR DETERMINING THE NUMBER OF CATALYTICALLY ESSENTIAL CARBOXYL GROUPS IN FUMARASE

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We have developed a mathematical model of the nonideal case in which enzymatic activity changes may also result from modification of non-essential groups. As an illustration of this method, the number of essential carboxyl groups in pig heart fumarase (L-malate hydro-lyase, EC 4.2.1.2) was determined by the differential labeling technique. Enzymatic activity was related to the number of modified carboxyl groups according to the model and the results were compatible with the existence of two essential carboxyl groups in fumarase.

Introduction

Enzyme structure-function relationships have been elucidated by several approaches [1–3]. Chemical modification techniques have been employed in attempts to identify amino acid residues essential for catalytic activity [4]. One approach, differential labeling, can facilitate the labeling of active sites with group-specific reagents to identify reactive groups which are associated with activity [5]. A general labeling is first done in the presence of an active site protecting agent. Then after its removal, the labeling is repeated. If enzymatic activity is retained following modification in the presence of a protecting agent but is lost in its absence, it may be assumed that active site protection was achieved. In such cases, it may be possible to determine the number of groups the modification of which is responsible for the inactivation. However, designation of such amino acids as catalytically essential can be misleading if protection is also conferred upon residues outside the active site by a structural change accompanying the interaction of the enzyme with substrate. Quantitative determination of the number of essential residues by differential labeling can be performed only when substrate, inhibitor, or ligand protection of essential residues is observed. Another limitation of differential labeling is that covalent modification of non-essential groups and denaturation may result in activity loss.

Theory

According to Tsou [6], the chemical modification of any one of the catalytically essential amino acids in a protein leads to complete loss of activity, and the fraction of the activity remaining, α, after partial modification is given by Eqn. 1, where X_e equals the fraction of unmodified essential groups and i equals the number of essential groups of a particular type.

$$\alpha = X_e^i \tag{1}$$

The value of i is found from the plot of X_e vs. $\alpha^{1/i}$ which gives the best straight line. Eqn. 1 implies that an enzyme would remain completely active even if all the non-essential groups of a particular type were modified. Such an occurrence would be unlikely considering the inevitable changes in protein conforma-

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tion produced by alteration of amino acid side chains. In order to determine the actual number of essential groups for the nonideal case, we define a time-dependent function, $g(t)$, which one can determine experimentally by shielding the essential groups from the modifying reagent (usually by protection with effective inhibitors or substrates as in differential labeling) and assaying the enzymatic activity remaining after the effects of modification of non-essential groups and denaturation processes. The fractional reduction of activity due to secondary losses is simply equal to $1-g(t)$, and

$$1 - g(t) = \frac{\alpha(t)_c - \alpha(t)_{obs}}{\alpha(t)_c} \tag{2}$$

where $\alpha(t)_{obs}$ represents the residual activity observed after modification without active site protection. The compensated activity, $\alpha(t)_c$, represents the residual activity that would have resulted due to modification of only essential residues. Rearrangement and substitution of Eqn. 2 into Eqn. 1 leads to Eqn. 3:

$$\alpha(t)_c = \frac{\alpha(t)_{obs}}{g(t)} = X_e(t)^i \tag{3}$$

Eqn. 3 transforms data obtained in the nonideal situation into 'ideal' data.

Materials and Methods

Materials. Pig heart fumarase (EC 4.2.1.2) was purchased from Calbiochem. Bromomesaconic acid was synthesized according to a published procedure [8].

Modification of carboxyl groups in native fumarase. 5 ml pig heart fumarase solution (3 mg/ml) were added to 5 ml 0.2 M taurine solution adjusted to pH 7.0. Solid 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide · HCl (EDC) was added to a final concentration of 0.1 M, and the mixture was stirred at room temperature and maintained at pH 7.0. Aliquots (0.5 ml) of the mixture were withdrawn at regular intervals; a portion (20 µl) was assayed for residual activity by the method of Kanarek and Hill [9]. The remaining portion was added to 0.5 ml 2 N sodium acetate solution (pH 4.0) to quench the reaction. The solution was then dialyzed several times (2 h each) in 2 l 10% acetic acid solution. Afterwards, the samples were lyophilized and then hydrolyzed with 6 N HCl for 24 h for amino acid determination.

Modification of carboxyls in mesaconyl-fumarase. Fumarase was rapidly inactivated with bromomesaconate (1 mM) in 0.01 M sodium phosphate buffer (pH 7.3) at 25°C as described by Laursen et al. [10]. After dialysis to remove excess bromomesaconate, mesaconyl-fumarase (5 ml, 3 mg/ml) was treated with EDC and taurine as described for native fumarase. Aliquots (0.5 ml) were withdrawn at various time intervals, and 10 µl 2-mercaptoethanol were added to each. The solutions were allowed to stand for 24 h at 4°C before assaying for activity at 25°C.

Amino acid analyses. A Durrum D-500 amino acid analyzer was employed. The column (0.9 × 30 cm) consisted of Durrum DC-6A resin. Bed temperature of 45°C was changed at 20 min to 65°C. The Durrum Pico-Buffer (three buffer solutions plus regenerant) was used with a flow of 70 ml/h. Amino acid peaks were detected with ninhydrin at 570 nm. The amount of taurine in each sample was determined on the amino acid chromatogram by comparison with the relative amount of valine found in the hydrolysate of the modified protein. Valine was chosen since it eluted as a single well resolved peak. Triplicate analyses were performed for each hydrolyzed sample and repeated measurements of the taurine/valine ratios were of the order of ±1.5%.

Data analysis. The real number of catalytically essential residues of a particular type, i_0, can be determined by finding which integral value of i in the plot of α versus X_e^i produces the best straight line. When i becomes equal to i_0, a straight line must result in the plot due to the inherent curvature of the $\alpha = X_0^i$ equation. The curvature of this equation can be demonstrated as follows.

Taking the ith root of both sides of the $\alpha = X_0^i$ equation results in Eqn. 4:

$$\alpha^{1/i} = X^{i_0/i} \tag{4}$$

The second derivative of the above equation with respect to X can be represented as follows:

$$\frac{d^2(\alpha^{1/i})}{dX^2} = \left[\frac{i_0}{i}\right]\left[\frac{i_0}{i} - 1\right][X^{i_0/i-2}] \tag{5}$$

Since the first and third terms of Eqn. 5 are always positive, the most significant factor affecting sign change is the second term. When $i < i_0$, the second derivative of $\alpha^{1/i}$ is postive; when $i > i_0$, the second derivative is negative. A function, $f(x)$, is defined as being strictly convex when $f''(x)$ is less than zero, and strictly concave when $f''(x)$ is greater than zero. If $f''(x)$ equals zero, then the function $f(x)$ is a straight line. Thus, from Eqn. 5, a family of convex curves would be generated when values of i greater than i_0 are plotted in $\alpha^{1/i}$ vs. X plots, and similarly, a family of concave curves would result when $i < i_0$ values are plotted. When $i = i_0$, the plot of $\alpha^{1/i}$ vs. X would result in a straight line.

The criterion we employed for determining the real i_0 value involved measuring the curvature of the $i = 1, 2, 3, 4$, etc., lines in the $\alpha^{1/i}$ vs. X plot. These lines should either be convex ($i > i_0$) or concave ($i < i_0$). Only when $i = i_0$, does a straight line result (vide supra). A simple computer performed test was devised. Given a set of data points P(1), P(2) . . . P(N), a straight line is drawn from point P(1) to P(3). Point P(2) is determined to lie above or below the line. If P(2) lies above, then +1 is added to a counter. A−1 is added if P(2) lies below. Next, a second line connecting P(2) to P(4) is drawn; the position, above or below this line of P(3) is determined and a +1 or −1 is added to the counter. This process is repeated until the last line L(N−2, N) is drawn and the position of P(N−1) is determined. If the final value of the counter is positive or zero, then the curve represented by the set of data points is convex; if it is negative, then the curve is concave. Each curve (starting from $i = 1$ curve) is then essentially labeled as being either concave or convex. When the counter value jumps from a negative to a positive value for the particular i and $i + 1$ curves (a change from concavity to convexity has occurred), the computer automatically reserves the i and $i + 1$ curves. The linear least-squares residual test is then used to make the final choice as to which of these two curves is a better straight line.

Results and Discussion

As an illustration of the proposed method, we have investigated the catalytic action of fumarase, a Kreb's citric acid cycle enzyme which catalyzes the reversible hydration of fumarate to L-malate. A kinetic study [11] has implicated an essential carboxyl group at the active center of fumarase. In addition, results of other kinetic studies, sequence analysis and chemical modification procedures indicate that at least one essential glutamyl residue is responsible for catalytic activity (R.C. Seid, Jr., unpublished data). The possible involvement of more than one essential carboxyl group in fumarase is consistent with data indicating the involvement of two carboxyl groups essential for catalytic activity in enzymes such as lysozyme [12] and pepsin [7].

Preliminary observations have shown that fumarase protected with mesotartrate, a potent competitive inhibitor [13], loses 80% of its initial enzymatic activity when its carboxyl groups are modified with a carbodiimide-nucleophile reagent under mild conditions [14]. This rapid inactivation has been correlated to non-essential carboxyl groups being modified, since the essential carboxyl(s) protected by the competitive inhibitor remains intact. Based on this experimental observation, fumarase is an excellent example to illustrate the nonideal case model for determining the number of essential carboxyls.

Furthermore, differential labeling of fumarase carboxyls is facilitated by the existence of an active site protecting agent, bromomesaconic acid [8,10]. Treatment of inactive mesaconyl-fumarase with sulfhydryl reagents such as 2-mercaptoethanol results in quantitative displacement of the mesaconyl group from the active center and complete recovery of enzyme activity [14].

The differential labeling technique was employed to determine the $g(t)$ curve during the modification of fumarase carboxyl groups as outlined in Fig. 1. First, the enzyme active site was protected with bromomesaconic acid, then the mesaconyl-fumarase was treated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and taurine to modify the non-essential carboxyls. After different time intervals, 2-mercaptoethanol was added to remove the protecting agent from the essential carboxyl group(s). Assay of enzymatic activity at this stage provided the $g(t)$ curve. To obtain the $\alpha(t)_{obs}$ curve, native fumarase was treated with EDC and taurine.

Fig. 2 presents the $g(t)$ curve, obtained by measuring residual activity of bromomesaconic acid-protected fumarase with respect to reaction time. The

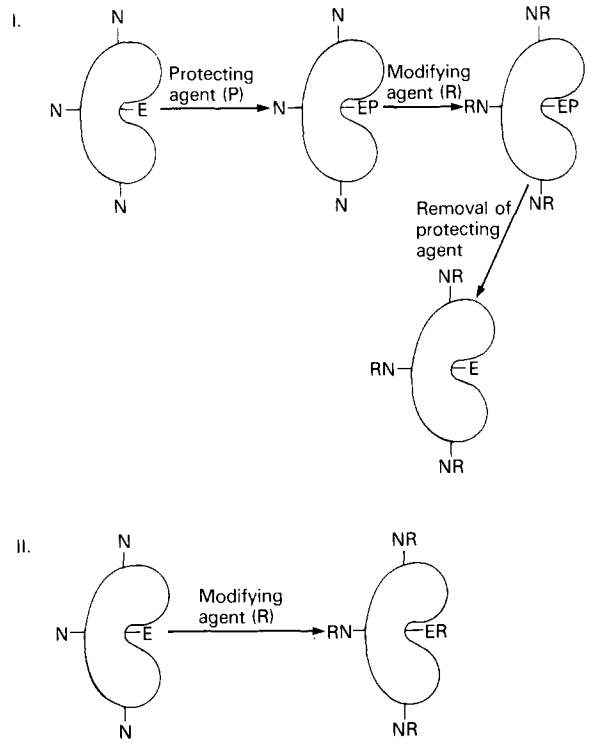
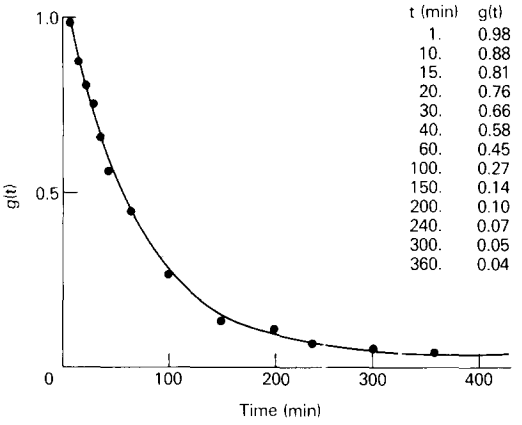


Fig. 1. Schematic illustration of experimental procedure. The catalytically essential residues are represented as E, and other reactive sites are represented as N. (I) Chemical modifications leading to the determination of $g(t)$, activity remaining after the effects of modification of non-essential groups and denaturation. (II) Chemical modifications leading to the determination of $\alpha(t)_{obs}$, residual activity observed after modification without active site protection. For the experiment with fumarase, P is bromomesaconic acid, R is taurine and the carbodiimide EDC, and removal of the protecting agent is by treatment with 2-mercaptoethanol.



t (min)	g(t)
1.	0.98
10.	0.88
15.	0.81
20.	0.76
30.	0.66
40.	0.58
60.	0.45
100.	0.27
150.	0.14
200.	0.10
240.	0.07
300.	0.05
360.	0.04

Fig. 2. The $g(t)$ curve for the carboxyl group modification of mesaconyl-fumarase. The table in the figure gives the residual activity measured at reaction time, t. ———, theoretical value; •, experimental value.

experimental points fit reasonably well with the theoretical curve, $g(t) = 0.96\ e^{-0.014t} + 0.04$, obtained by treating the experimental data to a least-squares fit for exponential curves. The $g(t)$ curve represents time decay of activity due to the effects of modification of non-essential groups and activity loss due to protein denaturation. The theoretical curve, along with the experimental points, is plotted.

The experimentally observed activity, α_{obs}, the number of modified carboxyls per subunit, m, the reaction time, t, and the compensated activity obtained by the ratio of $\alpha(t)_{obs}$ to $g(t)$ (Eqn. 3) are given in Table I. The number of carboxyl groups was measured by the amount of taurine incorporated per subunit. Taurine was selected as the nucleophile because the net charge would be preserved after modifications of the carboxyl groups and also because is is easily identifiable in the amino acid chromatogram.

In Fig. 3, the plots of $\alpha^{1/i}$ vs. m for the $i = 1, 2, 3, 4$ and 5 lines are shown. The experimental points lie reasonably well on the theoretical curves derived from Eqn. 6 which is based on the $i = 2$ lines.

$$\alpha^{1/i} = (-0.01\ m + 1.1)^{2/i} \qquad (6)$$

It is evident that for i values greater than 2, convex curves are produced; and for $i = 1$, a concave curve results. The curvature test calculates the counter values of −8, −2 and +14 for the $i = 1, 2$ and 3 lines, respectively. Thus, the two lines considered for the linear least-squares test are the $i = 2$ (concave) and the $i = 3$ (convex) curves. The total residues (deviation from straight lines) are 0.0001186 and 0.0002798, respectively, for the $i = 2$ and $i = 3$ cases. The best straight line among the set of convex curves is the $i = 2$ line. Thus, there are apparently two essential carboxyl groups in fumarase. The curves ($i = 1, 2, 3, 4$ and 5) in Fig. 3 also intersect the residual activity line of 1 at approx. $m = 1$, indicating that there is one rapidly modified non-essential group according to Eqn. 4 of Ref. 6.

TABLE I

RESIDUAL ACTIVITY VS. NUMBER OF MODIFIED CARBOXYL GROUPS IN NATIVE FUMERASE

Each numerical value represents the arithmetic mean determined from triplicate amino acid analyses.

Activity observed (α_{obs})	No. modified groups (m)	Reaction time (min) (t)	Activity after compensation (α_c)
0.960	1.16	1	0.973
0.813	1.68	5	0.869
0.661	2.32	10	0.756
0.537	2.88	15	0.656
0.289	4.44	30	0.431
0.192	5.28	40	0.326
0.127	6.04	50	0.246
0.085	6.68	60	0.187
0.037	7.72	80	0.105
0.017	8.52	100	0.061
0.008	9.12	120	0.037
0.002	9.76	150	0.013

Differential labeling techniques used in conjunction with the theoretical model described here provide a method for determining the number of amino acid residues essential for catalytic activity. Ideally, complete analysis of an enzyme, carried out with several amino acid reagents of different specificity, could lead to the identification of the number of specific residues involved in catalysis. The model accounts for extraneous inactivation due to denaturation and modification of non-essential groups. In rare cases, chemical modification of non-essential groups may enhance activity. The model still applies since $g(t)$ describes any activity change caused by modifying non-essential groups.

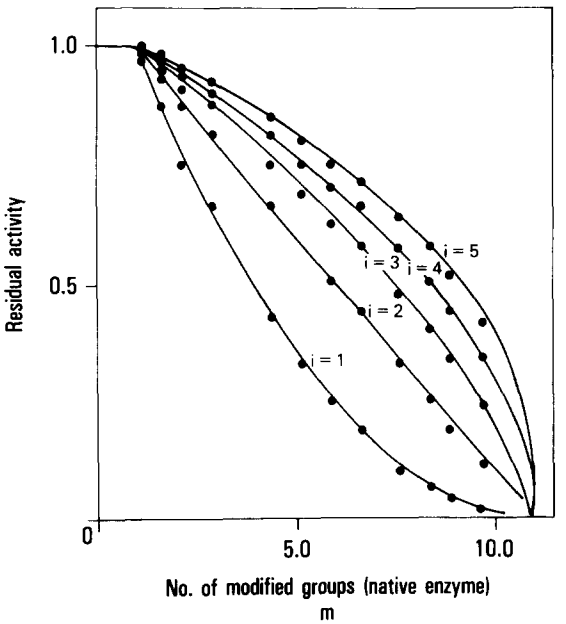


Fig. 3. Plot of $\alpha^{1/i}$ vs. m for the modification of fumarase by EDC and taurine. The lines for $i = 1$, 3, 4 and 5 are the theoretical curves based on Eqn. 6 for the $i = 2$ line.

Our model works for the simplest case. It is based on the assumption that complete loss in enzymatic activity results if any of the essential residues is modified by the inactivation reagent. Also, our model is tenable only if the reactivity of the non-essential groups is not appreciably altered when the protective ligand binds to enzyme molecule. In practice, the model can determine only the minimum number of essential groups. First, modification of one essential group could preclude the modification of another as a result of steric effects, though they can be minimized by avoiding bulky modifying reagents. Second, in general not all side chains of a particular type are susceptible to chemical modification. It is possible that the set of non-reacting groups contains one or more essential groups. These two factors would effect the $\alpha(t)_{obs}$ and could lead to a low value for i_0. Of course, additional chemical modification experiments and the careful choice of reagents would minimize the possibilities for underestimating the number of essential groups.

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