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Molecular Symmetry and Metastable States of Enzymes Exhibiting Half-of-the-Sites Reactivity†

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ABSTRACT: Half-of-the-sites reactivity in oligomeric enzymes has generally been accepted as evidence for structural asymmetry between subunits. However, we show that the symmetric two-state allosteric model [Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118] is quantitatively consistent with half-of-the-sites reactivity data for several hexameric and tetrameric enzymes. Specifically, the time courses for both the modification and the inactivation of glutamate dehydrogenase by glutamyl α-chloromethyl ketone and uridine diphosphoglucose dehydrogenase by 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid are fit with just five parameters for each enzyme–modifier pair. In the case of glyceraldehyde-3-phosphate dehydrogenase, the time courses for modification of the yeast enzyme by iodoacetic acid and the rabbit-muscle enzyme by 3,3,3-trifluorobromoacetone are fit with the same model, and parameter values from these fits are used to generate theoretical inactivation curves which are found to agree well with the experimentally measured inactivation. We conclude that half-of-the-sites reactivity, if it is not an artifact of residual heterogeneity, could be a kinetic phenomenon related to metastability of partially modified states of a symmetric oligomer and that asymmetry between subunits should therefore not necessarily be inferred from such behavior. If similar metastability occurs in substrate binding, it may play a significant role in mechanisms of catalysis and control. In such cases, the virtual inaccessibility of the substrate binding equilibrium would preclude conventional quasi-equilibrium models for the enzyme kinetics.

The term "half-of-the-sites reactivity" encompasses several different phenomena observed in some oligomeric enzymes. One of these is biphasic kinetics for ligand binding or active site modification. For example, in the alkylation of yeast glyceraldehyde-3-phosphate dehydrogenase with iodoacetate or iodoacetamide, the first 2 equiv react much faster than the third and fourth (Stallcup & Koshland, 1973a,b). A biphasic time course is also observed for the incorporation of radioactivity from tritiated L-glutamyl α-chloromethyl ketone into the six subunits of bovine liver glutamate dehydrogenase (Rasool et al., 1976).

In extreme cases, the last reaction steps are so slow that complete ligand association or chemical modification is not observed at all. For example, in the absence of carbamoyl phosphate, succinate has been found to bind to only three of the six catalytic subunits of aspartate transcarbamoylase from *Escherichia coli* (Suter & Rosenbusch, 1976). Similarly, the maximum number of molecules of the inducer isopropyl β-D-thiogalactoside bound per tetramer of the *lac* repressor of *E. coli* is found to range from 2.3 to 4.0 depending on the pH, temperature, and anti-inducer concentration (Oshima et al., 1974).

Another facet of half-of-the-sites reactivity is the observation that some enzymes may be rendered completely inactive by modification of only half of the subunits. For example, rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase shows no catalytic activity when modified by β-(2-furyl)acryloyl phosphate at only two of the four subunits (MacQuarrie & Bernhard, 1971). The yeast enzyme shows no catalytic activity when modified by *p,p'*-difluoro-*m,m'*-dinitrophenyl sulfone at only two of the four subunits (Givol, 1969). Similarly, the activity of cytidine triphosphate synthetase is abolished by the binding of 6-diazo-5-oxonorleucine to only two of the four subunits (Levitzki et al., 1971).

The functional significance of half-of-the-sites reactivity remains unclear, and attention has focused on its structural implications [for a summary see Levitzki (1978)]. It is generally accepted that half-of-the-sites reactivity requires asymmetry among the enzyme subunits. The concerted allosteric mechanism of Monod et al. (1965) has been excluded because its symmetric character is known to produce only positive cooperativity in homotropic ligand binding *equilibria*. In the case of yeast glyceraldehyde-3-phosphate dehydrogenase, it has been shown that the apparent asymmetry does not preexist chemical modification and it was therefore inferred that the presumed asymmetry is allosterically induced by chemical

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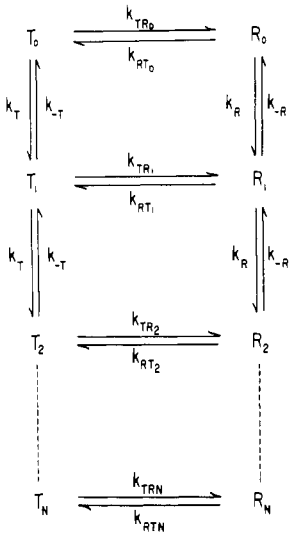

FIGURE 1: Reaction scheme for modification of an oligomer of N subunits in the two-state model. Notation is defined in the text.

modification (Stallcup & Koshland, 1973a).

We have previously suggested that half-of-the-sites reactivity in glyceraldehyde-3-phosphate dehydrogenase may arise from shifts in the equilibrium between symmetric quaternary states (Herzfeld & Schlesinger, 1975). Here we demonstrate quantitatively that the concerted allosteric mechanism of Monod et al. (1965) can fit the half-of-the-sites reactivity data for several hexameric and tetrameric enzymes. We find that half-of-the sites behavior can occur in a symmetric oligomer due to metastability of the partially modified state.

The Model

The model used here is a kinetic version of the concerted allosteric model of Monod et al. (1965), which has previously been applied to hemoglobin (Hopfield et al., 1971; Bansil et al., 1976). It is assumed that the oligomer may exist in two relevant quaternary conformations, conventionally labeled T and R, in each of which the subunits are all equivalent and noninteracting. Thus, the reactivity of a subunit depends only on the quaternary conformation of the oligomer and is independent of the chemical state of the other subunits. If we denote oligomers in the T and R quaternary conformations, with i subunits modified, by T_i and R_i, respectively, then the reaction scheme for oligomers of N subunits may be represented diagrammatically as in Figure 1 and is described by the differential equations

$$dT_i/dt = (N - i + 1)[M]k_T T_{i-1} + (i + 1)k_{-T}T_{i+1} - \{(N - i)[M]k_T + ik_{-T}\}T_i + k_{RTi}R_i - k_{TRi}T_i \quad (1a)$$

$$dR_i/dt = (N - i + 1)[M]k_R R_{i-1} + (i + 1)k_{-R}R_{i+1} - \{(N - i)[M]k_R + ik_{-R}\}R_i + k_{TRi}T_i - k_{RTi}R_i \quad (1b)$$

for $i = 0, ..., N$, and $T_i = 0$ and $R_i = 0$ when $i < 0$ or $i > N$. In these equations, [M] is the free concentration of modifying reagent. $k_{\pm R}$ and $k_{\pm T}$ are the intrinsic forward and backward rate constants for modification of a subunit when the oligomer is in the R and T forms, respectively. The rates for conversion of R_i to T_i and vice versa are k_{RTi} and k_{TRi}, respectively, and are related to the equilibrium parameters L and c of Monod et al. (1965) by

$$k_{RTi}/k_{TRi} = Lc^i \quad (2)$$

where

$$L = k_{RT0}/k_{TR0} \quad (3)$$

and

$$c = \frac{k_T/k_{-T}}{k_R/k_{-R}} \quad (4)$$

If k_{RTi} and k_{TRi} are large compared to $[M]k_R$, $[M]k_T$, k_{-R}, and k_{-T}, then the quaternary equilibrium is established rapidly compared to the rate of the subunit reactions, and we may assume that

$$T_i/R_i = Lc^i \quad (5)$$

at all times. Using S_i to represent the total population of oligomers with i subunits modified, so that

$$S_i = R_i + T_i \quad (6a)$$

$$R_i = S_i/(Lc^i + 1) \quad (6b)$$

$$T_i = S_i Lc^i/(Lc^i + 1) \quad (6c)$$

we can reduce the two sets of differential equations, (1a) and (1b), to a single set

$$dS_i/dt = (N - i + 1)[M]k_i S_{i-1} + (i + 1)k_{-(i+1)}S_{i+1} - \{(N - i)[M]k_{i+1} + ik_{-i}\}S_i \quad (7a)$$

where the intrinsic rate constants are

$$k_i = (k_T Lc^{i-1} + k_R)/(Lc^{i-1} + 1) \quad (7b)$$

$$k_{-i} = (k_{-T}Lc^i + k_{-R})/(Lc^i + 1) \quad (7c)$$

for $i = 1, ..., N$ and $k_{\pm i} = 0$ for $i < 1$ or $i > N$.

When the free concentration of modifier is constant, as is the case when the enzyme concentration is relatively small, the differential equations are linear. Furthermore, if the modification reaction is essentially irreversible (k_{-i} negligibly small for all i), simple analytical solutions may be obtained for the S_i as functions of time (Capellos & Bielski, 1972). The time dependence of the fraction of sites modified is then

$$B = \frac{\sum_{i=0}^{N} iS_i}{N\sum_{i=0}^{N} S_i} \quad (8)$$

Since the model assumes no direct interactions between subunits, the catalytic activity of an oligomer depends only on the number of sites unmodified and the quaternary conformation. If we assume that substrate binding does not significantly shift the quaternary equilibrium influenced by the modification reaction, then the activity of a partially modified collection of oligomers is

$$A = \sum_{i=0}^{N}(N - i)R_i A_R + \sum_{i=0}^{N}(N - i)T_i A_T \quad (9)$$

and the fractional activity compared to the unmodified state is

$$A/A_0 = \frac{\sum_{i=0}^{N}(N - i)S_i(Lc^i A_T + A_R)/(Lc^i + 1)}{\sum_{i=0}^{N} NS_i(LA_T + A_R)/(L + 1)} = \frac{\sum_{i=0}^{N}(N - i)S_i(Lc^i + a)/(Lc^i + 1)}{\sum_{i=0}^{N} NS_i(L + a)/(L + 1)} \quad (10)$$

where $a = A_R/A_T$ and A_R and A_T are the activities of an

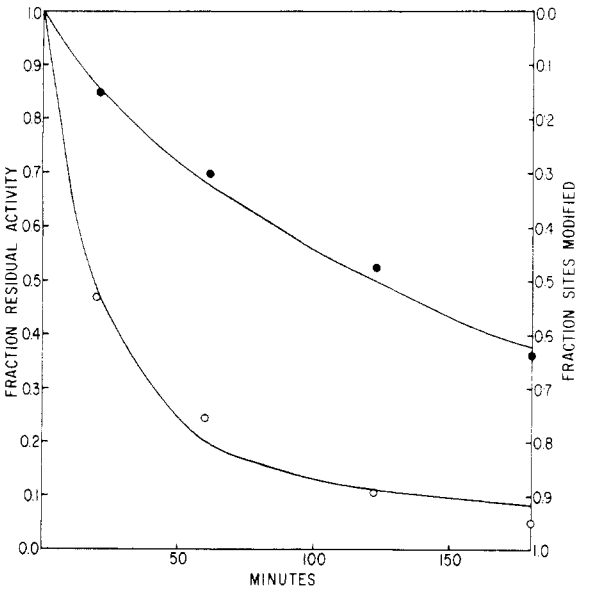

FIGURE 2: Reaction of bovine liver glutamate dehydrogenase with 6 mM L-[^{3}H]glutamyl α-chloromethyl ketone at 30 °C in 0.05 M sodium phosphate buffer at pH 6.2. Data of Rasool et al. (1976; Figure 4): filled circles and right-hand ordinate represent the fraction of subunits modified as measured by the incorporation of tritium; open circles and left-hand ordinate represent the fraction remaining of the original activity of the unmodified enzyme. The curves represent the theoretical fractional modification and activity for the parameter values $L = 5.24 \times 10^4$, $c = 1.92 \times 10^{-6}$, $k_T = 1.43$ M^{-1} min^{-1}, $k_R = 0.793$ M^{-1} min^{-1}, and $a = 0.210$. Equally good fits (i.e., with the same sum of the squares of the errors) are obtained with other sets of parameter values, such as $L = 40.2$, $c = 2.40 \times 10^{-3}$, $k_T = 1.44$ M^{-1} min^{-1}, $k_R = 0.794$ M^{-1} min^{-1}, and $a = 0.206$.

unmodified subunit when the oligomer is in the R and T conformation, respectively. Note that the value of a is specific to the modifier, as well as to the enzyme, since the conformation stabilized by one modifier need not be identical with that stabilized by another.

Data Analysis

Bovine liver glutamate dehydrogenase has six identical subunits (Moon et al., 1972) with one ligand binding site per subunit. Figure 2 shows the data of Rasool et al. (1976) for the modification and inactivation of the enzyme by L-glutamyl α-chloromethyl ketone. We have fit these data with the above described model for $N = 6$, by varying the five parameters k_T, k_R, L, c, and a. As shown in Figure 2, the theory is able to fit the data quite well. The parameters k_T, k_R, and a are well determined, but a range of values of L and c will fit the data equally well. The requirements are that $Lc \simeq 0.1$ with $L \gg 1$ and $Lc^2 \ll 1$, so that the unmodified hexamer is essentially exclusively in the T conformation, the singly modified hexamer is ~9% in the T conformation, and the more completely modified hexamers are essentially exclusively in the R conformation.

Examination of the parameter values shows that in this model the biphasic modification kinetics results from the fact that $k_R < k_T$ although $c \ll 1$. From eq 4 we see that this implies that

$$k_{-R}/k_{-T} \ll k_R/k_T < 1$$

However, this prediction is difficult to verify experimentally. The disproportionality between the extent of modification and the degree of inactivation of the enzyme is seen to result from the fact that $a < 1$, so that as the R conformation is stabilized with increasing modification, even the unmodified subunits lose ~80% of their activity.

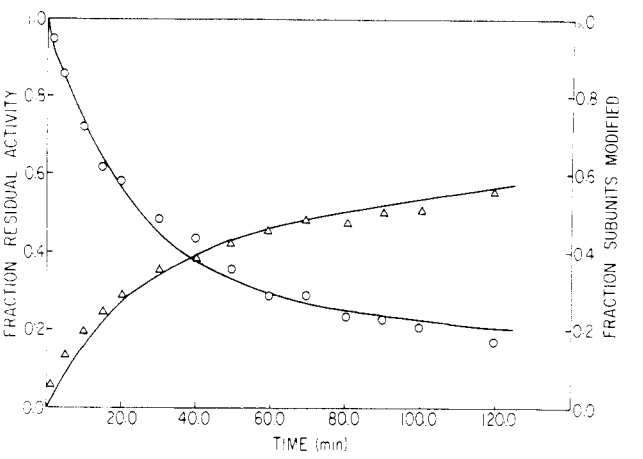

FIGURE 3: Reaction of bovine liver uridine diphosphoglucose dehydrogenase with 60 μM 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid at 25 °C in 0.1 M potassium phosphate buffer at pH 8.0. Triangles and circles represent fractional modification and activity, respectively, as measured by Franzen et al. (1980; Figure 1). The curves represent the theoretical fractional modification and activity for $L = 15.2$, $c = 0.20$, $k_T = 348.6$ M^{-1} min^{-1}, $k_R = 2.5$ M^{-1} min^{-1}, and $a = 0.393$.

Bovine liver uridine diphosphoglucose dehydrogenase also consists of six identical subunits. Figure 3 shows the data of Franzen et al. (1980) for the modification and inactivation of the enzyme by 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid. The figure also shows that for $N = 6$ our model is able to fit the data reasonably well. Again, the parameters are not all very well determined. The point, however, remains that the data can be explained without invoking molecular asymmetry by assuming that the modifier stabilizes a quaternary conformation of high affinity ($c < 1$) but slow reactivity ($k_R < k_T$) and low catalytic activity ($a < 1$).

Yeast glyceraldehyde-3-phosphate dehydrogenase consists of four identical subunits (Jones & Harris, 1972) with one ligand binding site per subunit. Figure 4a shows the data of Stallcup & Koshland (1973a) for the modification of the cysteine-149 residues by iodoacetic acid. We have fit these data with the foregoing model for $N = 4$, by varying the four parameters k_T, k_R, L, and c. As shown in Figure 4a, the theory is able to fit the data reasonably closely. Again, L and c individually are not well determined; the requirements for a good fit are that $L \gg 1$ and $Lc^2 \simeq 0.06$. Thus, the unmodified enzyme is essentially completely in the T conformation, but only ~6% of the doubly modified enzyme is in the T conformation, and the triply and quadruply modified enzyme are essentially exclusively in the R conformation. Again, $k_R < k_T$ so that modification occurs more slowly in the R state than in the T state and the rate of the modification reaction drops very rapidly as the number of sites modified increases beyond one. We have used the values of k_T, k_R, L, and c obtained by fitting the time course of modification in Figure 4a to calculate the extent of inactivation predicted by the model. Figure 4b shows that the theoretical inactivation profile obtained by assuming that $a = 0.15$ agrees closely with the measured inactivation.

Figure 5a shows the data of Bode et al. (1975) for the modification of rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase by 3,3,3-trifluorobromoacetone. Again, the theory for $N = 4$ is able to fit the data quite well. L and c, in this case, are such that the transition from the T to the R conformation is relatively gradual. The unmodified enzyme is nearly completely in the T conformation, the singly modified enzyme is ~80% in the T conformation, the doubly modified

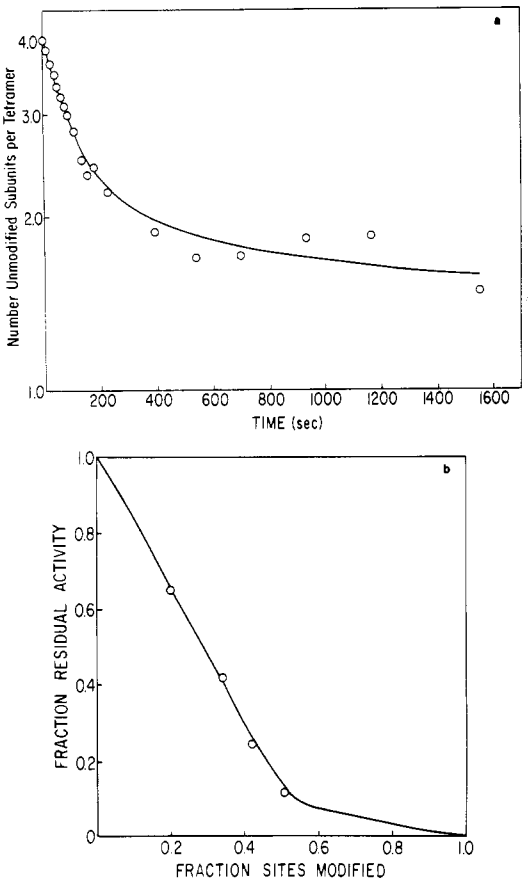

FIGURE 4: (a) Reaction of yeast glyceraldehyde-3-phosphate dehydrogenase (apoenzyme) with 1 mM [^{3}H]iodoacetate at pH 8.5. The circles represent the fraction of subunits modified as measured by Stallcup & Koshland (1973a; Figure 5). The curve represents the theoretical fractional modification for the parameter values L = 106.7, c = 0.0233, k_T = 4.20 M^{-1} s^{-1}, and k_R = 1.00 × 10^{-3} M^{-1} s^{-1}. (b) The predicted inactivation profile for modification of yeast glyceraldehyde-3-phosphate dehydrogenase by iodoacetate, based on the above parameter values for the fit of the time course of the reaction and the assumption that a = 0.15, is compared with the measurements of Stallcup & Koshland (1973a; Figure 3).

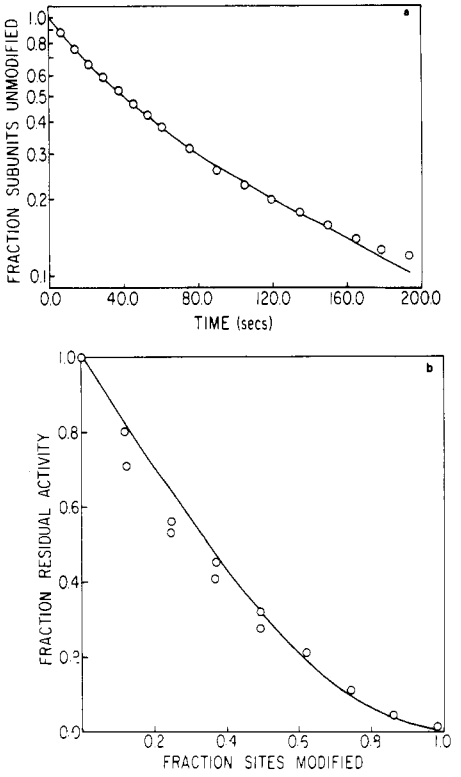

FIGURE 5: (a) Reaction of 36 μM rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase (holoenzyme) with 5.5 mM 3,3,3-trifluorobromoacetone, in the presence of 720 μM NAD^+, in 0.01 M ethylenediamine–0.1 M KCl–0.001 M EDTA (pH 7). The circles represent the fraction of subunits modified as measured by Bode et al. (1975; Figure 7). The curve represents the theoretical fractional modification for L = 28.33, c = 0.15, k_T = 3.83 M^{-1} s^{-1} and k_R = 1.26 M^{-1} s^{-1}. (b) The predicted inactivation profile, based on the above parameter values for the fit of the reaction kinetics and a = 0.15, is compared with the measurements of Bode et al. (1975; Figure 2a).

enzyme is ~40% in the T conformation, and the triply modified enzyme is still ~9% in the T conformation. Using the values of the four parameters k_T, k_R, L, and c from this fit, we find that the measured inactivation is closely reproduced by the model if a = 0.15, as shown in Figure 5b.

Discussion

Comparison with a General Kinetic Scheme. The significance of quantitative agreement between theory and experiment depends partially on the number of adjustable parameters involved. For irreversible chemical modification of an oligomer consisting of N subunits, assuming relatively fast conformational changes, the concerted allosteric model has four independently adjustable parameters (L, c, k_R, and k_T), and the conventional kinetic scheme has N (k_i for i = 1, ..., N). Clearly, for hexameric enzymes (N = 6) like glutamate dehydrogenase and uridine diphosphoglucose dehydrogenase, the concerted allosteric model is less malleable than the conventional kinetic scheme. However, even for a tetramer like glyceraldehyde-3-phosphate dehydrogenase, for which both models involve four independently adjustable parameters, the concerted allosteric model is less flexible than the conventional kinetic scheme. This is best illustrated by eq 7b which shows that in the concerted allosteric model there are certain constraints on the relative values that the apparent rate constants k_i may assume. In particular, the value of k_i must change monotonically between k_T and k_R, as i varies from 1 to N.

Consideration of the inactivation that accompanies modification highlights further the relative specificity of the concerted allosteric model. If it is assumed that the substrate binding does not influence the conformational equilibrium significantly, description of the inactivation by the concerted allosteric model calls for only one additional parameter, a. On the other hand, the conventional kinetic scheme would require $N - 1$ relative activity parameters because there is no molecular picture on which to base a more economical and physically meaningful choice of parameters.

Comparison with a Dimer Model. The simplest model of asymmetry regards the enzyme as consisting functionally of dimers. Thus, changes in one subunit are communicated across just one intersubunit contact domain. Equations 1 and 2 in Franzen et al. (1980) describe the time courses of modification and inactivation under such a model. Of the data considered in this paper, all but those for glutamate dehydrogenase can be fit equally well by these equations with three independently adjustable parameters. In the case of glutamate dehydrogenase, modification of just one of the six subunits by α-chloromethyl ketone reduces the activity to less than half; modification of two subunits reduces the activity to approximately one-fourth of its original value. Clearly these data are not explicable in terms of a dimer model.

In yeast glyceraldehyde-3-phosphate dehydrogenase, other data suggest that the dimer model is inadequate. For a variety of reagents, alkylation of just one-fourth of the subunits removes approximately three-fourths of the original activity (Stallcup & Koshland, 1973a). This requires that each of the

four subunits interacts with at least two others. In addition, Mockrin et al. (1975) found that, if they mixed tetracyanylated enzyme with unmodified enzyme, an overall decrease in specific activity occurred which was attributed to hybridization. However, hybridization should not alter the overall enzyme activity if, as the dimer model assumes, allosteric interactions occur only across the contact domain which is not broken during hybridization.

Heterogeneity. Implicit in the foregoing is the assumption that the enzyme preparation is homogeneous. Slade has found that heterogeneity with respect to NAD affinity exists in yeast glyceraldehyde-3-phosphate dehydrogenase which is in other respects homogeneous (Gennis, 1976). On the other hand, the reordered alkylation experiment of Stallcup & Koshland (1973a) indicates that heterogeneity does not contribute to the half-of-the-sites reactivity that they observe in this enzyme. If heterogeneity occurs in any of the preparations considered here, the corresponding data must be reevaluated. Where heterogeneity exists, the kinetics of modification are expected to be multiphasic. Moreover, since the more active component is expected to also be the more rapidly modified component, the degree of inactivation would be expected to be disproportionate compared to the extent of modification. In the simplest case of two noncooperative species, the amount of species i which remains unmodified with time is

$$S_i(t) = S_i(0)\ \exp(-k_i t)$$

Thus, the fraction of sites unmodified is

$$1 - B = \frac{[S_1(0)/S_2(0)]\ \exp(-k_1 t) + \exp(-k_2 t)}{[S_1(0)/S_2(0)] + 1}$$

and the fractional activity remaining is

$$\frac{A}{A_0} = \frac{[S_1(0)/S_2(0)][a_1/a_2]\ \exp(-k_1 t) + \exp(-k_2 t)}{[S_1(0)/S_2(0)][a_1/a_2] + 1}$$

where a_i is the activity of species i. We have found that this simple four-parameter [k_1, k_2, $S_1(0)/S_2(0)$, and a_1/a_2] model fits all the data considered in this paper, including those for glutamate dehydrogenase, just as well as the four-parameter concerted model. Thus, the question of heterogeneity is central to the significance of half-of-the-sites behavior. If purified enzyme does not display half-of-the-sites reactivity, then the evidence for asymmetry vanishes. If purified enzyme does display half-of-the-sites reactivity but to a different extent than the heterogeneous samples, it is possible that new data may discriminate better between the various proposed models than do the currently available data.

Time Scales. In our model and in the others discussed above, it is assumed that the time scale for conformational changes is very different from that for the modification reaction. Specifically, we assume that interconversion between the R and T states is much faster than modification and that any other conformational changes that may occur are slow compared to the time scale of the experiments. This assumption does not seem unreasonable; for yeast glyceraldehyde-3-phosphate dehydrogenase, the conformational transition induced by NAD binding has forward and reverse rate constants of 0.18 and 5.5 s^{-1}, respectively, at 40 °C and pH 8.5 (Kirschner et al., 1966). These rates are much higher than k_T[M] and k_R[M] in Figure 4.

Even if conformational changes occurred at rates comparable to those for the modification reaction, biphasic kinetics and disproportionate inactivation would still be observed to the extent that the R conformation is induced by the modification. However, there would be some important differences. By increasing in the modifier concentration, the rate of modification could be accelerated, while the rate of conversion from T to R remains unchanged. Thus, at any point in the time course, the fraction of oligomers with i subunits modified which are in the T conformation will be higher and the reactivity of the oligomers will be higher. As a consequence, the apparent rate constants, k_i, $i = 1, ..., N$, for modification of successive subunits will depend on the modifier concentration used.

The distribution of modified subunits in the population of oligomers will also depend on the modifier concentration used. If modification is fast compared to the T to R transition, some oligomers will become fully modified; if the T to R transition is fast compared to modification, then complete modification of an oligomer will be less common. In an extreme case, for example, a collection of oligomers with half of the sites modified may contain equal numbers of fully modified and unmodified oligomers or may contain exclusively half-modified oligomers. In the former situation, the activity would be half the value for an unmodified sample; in the latter situation the residual activity would be less than half. Thus, in general, since the activity depends on the distribution of modified subunits in the population of oligomers, the inactivation profile will also depend on the modifier concentration used.

The above considerations are not specific to our model. In general, the common assumption that inactivation profiles and apparent rate constants are independent of modifier concentration presupposes that the conformational changes involved occur with time scales faster than the actual modification reaction.

Allosteric Effects of Substrate. In our model, we have assumed that substrate does not significantly shift the quaternary equilibrium influenced by the modifying reagent. Since the current data are compatible with this simplest approach, there was no point in considering any more complicated descriptions. However, some of the substrates of the enzymes considered here are known to induce conformational changes in the protein. To be consistent with our assumption, either the allosteric effect of the substrate must be much weaker than that of the modifier or the conformation stabilized by the substrate must be different from, and neutral with respect to, that stabilized by the modifier. On the other hand, the assumption may be wrong and the agreement with the data fortuitous. If the substrate does shift the conformational equilibrium influenced by the modifier [whether these be concerted (Monod et al., 1965) or sequential (Koshland et al., 1966) processes], then the measured inactivation of the enzyme should depend on the concentration of the substrate in the assay. Such an effect has not been reported. It seems to have generally been taken for granted that the inactivation profile should be independent of the substrate concentration in the assay. This assumes implicitly that substrate does not significantly influence the allosteric effects of modifier.

Metastability. The parameter values which fit the half-of-the-sites reactivity data indicate that although the R conformation is stabilized in the modified enzyme, the rate of modification is low for the R conformation. In an extreme case, the affinity of the modification reaction in the R conformation may be very high, so that at equilibrium the enzyme would be modified at all sites, but the rate of modification of the R conformation may be so slow that modification does not reach completion in an accessible period of time. Thus, the enzyme is trapped in a metastable partially modified state. Such metastable behavior can occur whenever positive equilibrium cooperativity is accompanied by negative kinetic co-

operativity, independent of whether the cooperativity occurs via a concerted (Monod et al., 1965) or a sequential (Koshland et al., 1966) mechanism.

Although high affinity combined with low reactivity may superficially seem contradictory, in fact, a low free energy for the modified state does not exclude a high free energy barrier for the reaction. For example, although the structure of the active site itself may be very favorable for modification in the R conformation, if the site is partially or completely sequestered from the bulk solution, the rate of reaction may be exceedingly slow. The potential weakness of the relationship between equilibrium constants and rate constants is illustrated by the contrast between the near equilibrium equivalence and the strong kinetic inequivalence of the α and β chains of hemoglobin (Gibson, 1973).

In this paper we have considered only the relatively simple situation in which a single chemical reacts slowly (relative to the rate of conformational change) and essentially irreversibly with the active site. Enzyme catalysis, on the other hand, typically involves rapid reversible association with several substrates. The states stabilized by one substrate may not be the same as those stabilized by others, and substrates may bind and dissociate in different combinations. To render these systems tractable, it is often assumed that some of these processes are approximately at equilibrium. Our results indicate that in certain circumstances the equilibrium state may be inaccessible and that metastable states could potentially play an important role in enzyme catalysis and control.

Concluding Remarks. The premise on which this paper is based is that negative kinetic cooperativity may occur with positive equilibrium cooperativity. From this it follows that (i) half-of-the-sites reactivity may occur without asymmetry between subunits and/or (ii) oligomeric proteins may exhibit metastability with respect to ligand binding that looks like negative equilibrium cooperativity. We have shown that the data for several systems can be accounted for quantitatively in this way. Thus, there is an amibiguity in the interpretation of these phenomena that has not been generally appreciated.

Resolving this ambiguity is difficult. Henis & Levitzki (1979) have proposed a method for differentiating sequential allosteric interactions from concerted allosteric interactions, in systems with positive equilibrium cooperativity, and from heterogeneity, in systems with apparent negative equilibrium cooperativity. Applied to data for rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase, the method indicates the presence of sequential allosteric interactions in the negatively cooperative binding of NAD (Henis & Levitzki, 1980). However, the method applies only to equilibrium behavior and cannot address the possibility of metastable behavior. In addition, even if the equilibrium requirement is satisfied, the method cannot exclude the possibility of *simultaneous* concerted allosteric interactions or heterogeneity. In any case, the relationship to half-of-the-sites reactivity, with which we are primarily concerned here, is unclear because both yeast glyceraldehyde-3-phosphate dehydrogenase, which binds NAD with purely positive cooperativity, and the rabbit-muscle enzyme, which binds NAD with weak apparent negative cooperativity (Scheek & Slater, 1978), exhibit half-of-the-sites reactivity. In fact, the phenomenon seems to be more pronounced in the positively cooperative yeast enzyme in that a variety of reagents reduce the activity of the yeast enzyme to $\sim$25% of its original value upon modification of just one of the four sites, whereas the activity of the rabbit-muscle enzyme modified at one site has in no case been reported to be less than $\sim$50% of its original value.

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