Role of coupling entropy in establishing the nature and magnitude of allosteric response

(enzyme regulation/protein dynamics/ligand binding/phosphofructokinase/isocitrate dehydrogenase)

GREGORY D. REINHART*, SHARON B. HARTLEIP, AND MARINA M. SYMCOX

Department of Chemistry, University of Oklahoma, Norman, OK 73019

Communicated by Gregorio Weber, March 6, 1989

ABSTRACT The coupling free energy between an allosteric ligand and a substrate, ΔG_{ax} , is an explicit measure of the nature as well as the magnitude of impact that an allosteric ligand has on the binding of the substrate ligand to the enzyme, with positive values indicating inhibition and negative values indicating activation. By measuring the variation with temperature of the coupling free energy between the allosteric ligand and the substrate, it is possible to determine the enthalpic and entropic components that give rise to the coupling free energy. We have performed this analysis on two different K-type allosteric systems: the allosteric inhibition of rat liver phosphofructokinase by MgATP, and the allosteric activation of beef heart NAD⁺-dependent isocitrate dehydrogenase by ADP. In both cases the coupling free energy arises as the net result of opposing enthalpic and entropic components, with the coupling enthalpy (ΔH_{ax}) favoring activation and the coupling entropy (ΔS_{ax}) favoring inhibition. For phosphofructokinase at 25°C, the absolute value of $T\Delta S_{ax}$ is greater than the absolute value of ΔH_{ax} , and net inhibition of rat liver phosphofructokinase by MgATP is realized. For isocitrate dehydrogenase, ΔH_{ax} dominates; however, the net activation is substantially mitigated by the magnitude of $T\Delta S_{ax}$. Hence, the coupling entropy plays an important role in establishing both the nature and magnitude of the allosteric response. We hypothesize that the negative coupling entropy arises from the particular constraint placed upon the internal dynamical properties of the enzyme by the simultaneous binding of both allosteric and substrate ligands.

Upon binding, allosteric ligands can affect enzymatic activity by altering the maximal rate of turnover (V-type effect) and/or by altering the apparent affinity displayed by the enzyme for its substrate (K-type effect) (1). Traditionally, the behavior of enzymes subject to K-type allosteric regulation has been evaluated on the basis of the two-state models proposed by either Monod *et al.* (1) or Koshland *et al.* (2). These models presume that allosteric activators and inhibitors differ greatly in their respective effects on enzyme conformation. Activators are thought to induce or stabilize a structure that is comparable to the one stabilized by the substrate itself and therefore more correctly conforms to the geometry required for facile substrate binding. Inhibitors, on the other hand, distort the substrate binding domain so that little or no interaction between the substrate and enzyme is possible after binding of the inhibitor (3). The variation of enzyme activity imposed by various substrate and effector concentrations is then explained in terms of competing equilibria between these two functional states.

We prefer to not limit our interpretation to this traditional view and have instead utilized an approach that considers the various ligated forms as distinct species without regard for the nature of the allosteric effect (4). Consequently, a mini-

mum of four species must be considered: free enzyme, enzyme with substrate bound, enzyme with allosteric ligand bound, and enzyme with both ligands bound. It is the relative population of these four species, determined by the relative rates of their interconversion, that dictates the enzyme's response to substrate and allosteric ligand concentration.

The simplest such initial velocity scheme, pertaining to a monomeric enzyme with a single substrate and a single effector binding site, is represented by the following scheme:

Scheme I.

in which E, X, and A represent free enzyme, allosteric ligand, and substrate respectively, and E-A, X-E, and X-E-A represent enzyme with substrate bound, with allosteric ligand bound, and with both substrate and allosteric ligand bound, respectively. This scheme applies regardless of whether X is an inhibitor or activator. Although most allosteric enzymes are actually oligomeric and subject to multiple substrate and allosteric ligand interactions, we have found empirically that Scheme I often provides a good basis for evaluating the changes in the average substrate affinity brought about by the allosteric ligand (5, 6).

The principles of thermodynamic linkage (7–11) will aid in understanding the mechanism by which an allosteric ligand effects its influence (4–6, 12) provided that either the substrate or the allosteric ligand achieves binding equilibrium in the steady state. Thermodynamic linkage refers to the fact that the influence that the binding of X has on the affinity of E for A must be equal to the influence that the binding of A has on the affinity of E for X. In free-energy terms, this mutual interaction is defined to be the coupling free energy between A and X, ΔG_{ax} (10, 11), which is given by:

$$\Delta G_{ax} = \Delta G_{a/x} - \Delta G_a = \Delta G_{x/a} - \Delta G_x, \quad [1]$$

where ΔG_a and ΔG_x equal the standard free energy of binding A and X to E, respectively. $\Delta G_{a/x}$ and $\Delta G_{x/a}$ equal the standard free energy of binding of A and X to X-E and E-A, respectively. If X antagonizes the binding of A, then $\Delta G_{ax} > 0$; and if X facilitates the binding of A, then $\Delta G_{ax} < 0$.

The allosteric inhibition of rat liver phosphofructokinase by MgATP and the allosteric activation of beef heart mitochondrial NAD⁺-dependent isocitrate dehydrogenase by

ADP can be evaluated by this approach. The maximal rate of turnover for each of these enzymes is unaffected by these allosteric ligands (13, 14), and so each exclusively exhibits a K-type response. We have measured the effects of varying temperature on the apparent coupling free energy between MgATP and fructose 6-phosphate (Fru-6-P) on rat liver phosphofructokinase and between ADP and magnesium-isocitrate complex on beef heart mitochondrial isocitrate dehydrogenase in an effort to ascertain the thermodynamic basis for the mutual interaction displayed by these substrate-allosteric ligand pairs. In this report we present these results and discuss what we believe to be the interesting conceptual ramifications that they suggest.

MATERIALS AND METHODS

Rat liver phosphofructokinase was purified from male rats, obtained from Holtzmann (Madison, WI), as previously described (13). Beef heart mitochondrial NAD⁺-dependent isocitrate dehydrogenase was purified by the method of Giorgio *et al.* (15). The coupling enzymes aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase were obtained as ammonium sulfate suspensions from Boehringer Mannheim Biochemicals, and dialyzed extensively prior to use against a buffer consisting of 25 mM 4-morpholinepropanesulfonic acid (Mops)/KOH, 50 mM KCl, 2.5 mM MgCl₂, and 50 μ M EDTA at pH 7. ATP, ADP, and isocitrate (sodium salts); Fru-6-P (potassium salt); Mops and 2-(N-cyclohexylamino)ethanesulfonic acid (Ches) (free acids); NAD⁺; NADH; and dithiothreitol were obtained from Sigma. All other chemicals were reagent grade, and deionized distilled water was used throughout.

Phosphofructokinase assays were performed by adding 10 μ l of an appropriately diluted stock solution of rat liver phosphofructokinase to a 1.0-ml assay mixture adjusted to pH 9.0 at the indicated temperature and containing 50 mM Ches/KOH, 100 mM KCl, 5 mM MgCl₂, 100 μ M EDTA, 0.2 mM NADH, 250 μ g of aldolase, 50 μ g of glycerol-3-phosphate dehydrogenase, 5 μ g of triosephosphate isomerase, and variable concentrations of MgCl₂ and ATP so that the assays contained a total MgCl₂ concentration that was 5 mM in excess of the ATP concentration. Steady-state rates of NADH oxidation, observed after the completion of the slow pre-steady-state transients (13), were determined by measuring the slope of a strip chart-recorder tracing of absorbance at 340 nm as a function of time. Substrate concentration at the point of rate determination was never allowed to have decreased from the initial concentration by more than 50 μ M at high substrate concentrations (>1 mM) or 5% at low substrate concentrations (<1 mM). Assays were performed at pH 9 to avoid complications in the interpretation of the results arising from compensatory changes in the degree of protonation (16) that might occur at a pH near or below the regulatory pK_a of 8.1 (5).

The concentration of Fru-6-P required to produce half-maximal velocity at any given concentration of MgATP, $K_{1/2}$, was determined from a plot of steady-state rate versus the logarithm of the molar concentration of Fru-6-P (log[Fru-6-P]). Usually 8–10 assays were performed at varying molar concentrations of Fru-6-P to determine each $K_{1/2}$. The coupling parameter between Fru-6-P and MgATP, Q_{ax} , was then determined from these data as described below.

Isocitrate dehydrogenase was stored without loss in activity in a buffer containing 90 mM Mops/NaOH (pH 7.2), 0.8 M Li₂SO₄, and 1 mM dithiothreitol during each experiment (17). Assays of isocitrate dehydrogenase activity were performed by adding 10 μ l of this enzyme solution to a 1.0-ml solution containing 90 mM Mops/NaOH, 0.15 mM NAD⁺, 0.1 mM dithiothreitol, and variable total concentrations of isocitrate, ADP, and MgSO₄ adjusted to pH 7.4 at the indicated temperature. The magnesium-isocitrate complex

and uncomplexed ADP have been identified as the true substrate and the true allosteric activator, respectively, of isocitrate dehydrogenase (17). Concentrations of the magnesium-isocitrate complex and uncomplexed ADP were calculated from apparent stability constants for the Mg²⁺ complexation reactions measured at the experimental temperature by the arsenazo I method described by Gabriel *et al.* (18). Total concentrations of isocitrate and ADP were varied in a manner so as to maintain the concentration of free Mg²⁺ at 0.25 mM in all assays.

Initial rates of reaction were determined by measuring NADH formation at 340 nm as a function of time as monitored by a strip chart recorder. The $K_{1/2}$ for the magnesium-isocitrate complex at any given concentration of uncomplexed ADP was determined from the x-axis intercept of a Hill plot. Approximately 12 assays were performed at various magnesium-isocitrate concentrations to determine each $K_{1/2}$.

RESULTS

MgATP allosterically inhibits phosphofructokinase by increasing the concentration of its substrate, Fru-6-P, required to produce half-maximal velocity, $K_{1/2}$ (13). ADP allosterically activates isocitrate dehydrogenase by decreasing the $K_{1/2}$ for its substrate, magnesium-isocitrate complex (14). Each of these effects are saturable functions of the allosteric ligand concentration as shown in Fig. 1. We have defined the parameters K_a^o and K_a^∞ to be equal to $K_{1/2}$ in the absence of allosteric ligand and when the allosteric ligand is saturating, respectively. We further define the parameter Q_{ax} to be equal to the ratio of these two limiting values as follows:

$$Q_{ax} = \frac{K_a^o}{K_a^\infty} \quad [2]$$

Therefore, in the case of allosteric activation, $Q_{ax} > 1$, and for inhibition, $Q_{ax} < 1$.

Q_{ax} can be determined graphically as indicated in Fig. 1. Alternatively, the following equation, derived from Scheme I (4, 5), provides a good description of these data:

$$K_{1/2} = K_a^o \left(\frac{K_{ix}^o + [X]}{K_{ix}^o + Q_{ax}[X]} \right), \quad [3]$$

where [X] represents allosteric ligand concentration and K_{ix}^o is the apparent dissociation constant for X in the absence of substrate. Therefore, Q_{ax} can also be determined numerically via nonlinear regression to Eq. 3 as described (5). The solid curves in Fig. 1 represent the best fit of these data to this function, and the depicted value of log Q_{ax} was obtained from the regression analysis.

The initial velocity rate equation predicted by Scheme I is greatly simplified if the substrate achieves a binding equilibrium in the steady state (19–21). In that event, the Michaelis constant for substrate A, K_a , will equal a thermodynamic dissociation constant, denoted K_{ia} according to Cleland's notation (22). Consider the following simple kinetic scheme as it applies to both the top and bottom segments of Scheme I:

Scheme II.

In the general case:

$$K_a = \frac{k_2 + k_3}{k_1} = K_{ia} + \frac{k_3}{k_1} \quad [4]$$

The rapid equilibrium assumption is valid with respect to A if k_3/k_1 is negligible relative to K_{ia} .

FIG. 1. (A) The logarithm of $K_{1/2}$ of Fru-6-P measured as a function of the logarithm of [MgATP] at 30°C for rat liver phosphofructokinase. (B) Logarithm of $K_{1/2}$ of magnesium-isocitrate complex measured as a function of the logarithm of [ADP] at 10°C for beef heart NAD⁺-dependent isocitrate dehydrogenase. Note the plateau approached at high concentrations of allosteric ligand in each case, signifying saturation of the allosteric effect. The coupling parameter Q_{ax} describes the maximum change that an allosteric ligand is able to make in $K_{1/2}$. The logarithm of Q_{ax} is equal to the difference between the two plateau values for $K_{1/2}$ as indicated in the figures by the arrow. The solid curves in each figure represent the best nonlinear regression fit of these data to Eq. 2 (described in the text), assuming constant relative error in the values of $K_{1/2}$ as discussed (6). The logarithm of Q_{ax} is negative for phosphofructokinase and positive for isocitrate dehydrogenase.

The turnover number, k_3 , for phosphofructokinase and isocitrate dehydrogenase can be calculated from their maximal specific activities and the molecular weights and is equal to 100 sec⁻¹ (13) and 80 sec⁻¹ (23, 24) at 25°C, respectively. The lowest $K_a^\dagger$ measured for phosphofructokinase at this temperature was approximately equal to 0.2 mM, and that for isocitrate dehydrogenase was 0.1 mM. Substituting these values into Eq. 4 implies that if $k_1 > 5 \times 10^6$ M⁻¹sec⁻¹ for phosphofructokinase and if $k_1 > 8 \times 10^6$ M⁻¹sec⁻¹ for isocitrate dehydrogenase, then the difference between K_a and K_{ia} would be less than 10%. The bimolecular rate constant pertaining to substrate binding, k_1 , rarely fails to exceed these

[†]Because both phosphofructokinase and isocitrate dehydrogenase display positive cooperativity in their respective substrate saturation profiles, the quantity measured, $K_{1/2}$, is not actually equal to K_a but rather is equal to the geometric mean of the individual K_a s pertaining to each individual substrate interaction on the respective oligomeric enzyme. For the purposes of the following discussion, we assume that the enzymes behave as monomeric allosteric systems with a single substrate interaction conforming to this average.

values in enzymes having comparable small molecular weight substrates (25). Consequently, we believe that the rapid equilibrium assumption is very likely to be valid with respect to the data presented in Figs. 1 and 2.

Assuming that to be the case, we may now write the equilibrium expressions to which K_a° , K_a^∞ , and Q_{ax} pertain:

$$K_a^\circ = \frac{[E][A]}{[E-A]}, \quad K_a^\infty = \frac{[X-E][A]}{[X-E-A]}, \quad Q_{ax} = \frac{[X-E-A][E]}{[X-E][E-A]} \quad [5]$$

Q_{ax} is the equilibrium form of ΔG_{ax} , the coupling free energy between A and X:

$$\Delta G_{ax} = -RT \ln(Q_{ax}). \quad [6]$$

According to Eqs. 2 and 6, the coupling free energy will be positive when X is an inhibitor and negative when X is an activator. Consequently, the sign and absolute value of ΔG_{ax} indicate the nature and magnitude, respectively, of the allosteric influence that X has upon binding.

The value of Q_{ax} was determined at several temperatures between 5°C and 45°C for both phosphofructokinase and isocitrate dehydrogenase. These results are summarized in Fig. 2 with a plot of $\log Q_{ax}$ versus reciprocal temperature expressed in degrees K. When plotted in this manner, the data for each enzyme are well described by a straight line with positive slope reflecting the fact that Q_{ax} increases with decreasing temperature.

Eqs. 5 and 6 imply that Q_{ax} is the equilibrium constant and that ΔG_{ax} is the corresponding standard free energy for the following disproportionation reaction [as originally recognized by Weber (10, 11)]:

Therefore, the data presented in Fig. 2 are van't Hoff plots pertaining to this equilibrium, and one can determine from the slope and intercept of these plots the standard enthalpy change, ΔH_{ax} , and the standard entropy change, ΔS_{ax} , contributing to the coupling free energy (27):

$$\log Q_{ax} = -\left(\frac{\Delta H_{ax}}{2.3R}\right)\left(\frac{1}{T}\right) + \left(\frac{\Delta S_{ax}}{2.3R}\right). \quad [8]$$

FIG. 2. Variation with reciprocal temperature of the logarithm of Q_{ax} between ADP and magnesium-isocitrate on NAD⁺-dependent isocitrate dehydrogenase (●) and between MgATP and Fru-6-P on rat liver phosphofructokinase (■). Values of Q_{ax} were determined by nonlinear regression to Eq. 2 of data for each temperature comparable to that presented in Fig. 1, and the error bars in the figure indicate $\pm$ the standard errors of these determinations. The lines through each data set were determined by weighted linear regression (30).

Weighted linear regression analysis of these data indicates that for phosphofructokinase, $\Delta H_{ax} = -2.0 \pm 0.6$ kcal/mol and $\Delta S_{ax} = -12.1 \pm 2.0$ entropy units, whereas $\Delta H_{ax} = -10.0 \pm 0.2$ kcal/mol and $\Delta S_{ax} = -29.5 \pm 0.7$ entropy units for isocitrate dehydrogenase. Consequently, $T\Delta S_{ax} = -3.6$ kcal/mol and $\Delta G_{ax} = +1.6$ kcal/mol at 25°C for phosphofructokinase, with the positive value of ΔG_{ax} indicative of inhibition, and $T\Delta S_{ax} = -8.8$ kcal/mol and $\Delta G_{ax} = -1.2$ kcal/mol at 25°C for isocitrate dehydrogenase, with the negative value of ΔG_{ax} indicative of activation.

DISCUSSION

The disproportionation equilibrium presented in Eq. 7 serves to define the actions of a K-type allosteric ligand regardless of whether the ligand is an activator or an inhibitor. This equilibrium is established by the properties of X-E-A and E relative to those of X-E and E-A and thereby stands in contrast to more common evaluations of allosteric behavior that attempt to attribute allosteric effects to differences and/or similarities between the components appearing on either side of that equilibrium. For example, the properties of E-A and X-E are often considered to be substantially different when X is an inhibitor but virtually identical if X is an activator. Clearly such attempts do not address the central issue, which is to evaluate the extent of mutual binding antagonism or enhancement each ligand experiences with respect to the binding of the other. The equilibrium constant, Q_{ax} , and standard free energy, ΔG_{ax} , of the equilibrium depicted in Eq. 7 provide precisely this quantitation and, in so doing, provide an unambiguous characterization of the actions of both activating and inhibiting allosteric ligands.

The remarkable feature of the data presented herein is that for both allosteric systems examined, one an example of allosteric inhibition and the other an example of allosteric activation, ΔH_{ax} is negative and ΔS_{ax} is negative. Since $\Delta G_{ax} = \Delta H_{ax} - T\Delta S_{ax}$, this feature implies that *enthalpy is favoring activation and entropy is favoring inhibition in both cases*. Moreover, at 25°C the absolute value of $\Delta\Delta G_{ax}$ is less than the absolute value of either ΔH_{ax} or $T\Delta S_{ax}$. ΔH_{ax} and $T\Delta S_{ax}$, in other words, largely compensate one another, with only a small imbalance resulting in ΔG_{ax} and hence the allosteric effect.

This observation has several important ramifications. First of all, from this perspective, activation and inhibition are essentially related phenomena, differing only in the nature of this thermodynamic imbalance. Inhibitors and activators can each induce both activating and inhibiting characteristics. Secondly, because of the tenuous nature of this imbalance, large changes in the magnitude and even the nature of allosteric effects could be effected by fairly modest perturbations in ΔH_{ax} and/or ΔS_{ax} . For example, such a perturbation may account for the altered allosteric response induced in *Escherichia coli* phosphofructokinase by site-specific mutagenesis (28). Phosphoenolpyruvate changes from being a K-type inhibitor to being a K-type activator upon the mutation of a glutamate residue to an alanine residue in the effector binding site of the enzyme. It is easy to envision that such a change could produce an alteration in the relative magnitudes of ΔH_{ax} and ΔS_{ax} sufficient to change the sign of ΔG_{ax} .

A third ramification of the compensation that exists between ΔH_{ax} and ΔS_{ax} comes from the fact that temperature, by virtue of its intrinsic effect on the relative contribution that ΔH_{ax} and ΔS_{ax} make to free energy, modifies the magnitude of the allosteric effect as our data have directly demonstrated. If the variation in Q_{ax} with temperature that we observed for each enzyme were to extend in similar fashion beyond the temperature range of this investigation, eventually $\log Q_{ax}$ would cross through 0 and change sign, thereby signifying a change in the nature of the allosteric effect as

well. Unfortunately, this cross-over temperature is not attainable experimentally in either of these systems, so this prediction has not yet been tested directly. At the very least, however, it is clear that temperature must be considered to be an important variable when attempting to extrapolate *in vitro* regulatory characteristics of an enzyme to *in vivo* behavior.

We are particularly intrigued by the major contribution that ΔS_{ax} makes to ΔG_{ax} . For example, it is evident that ΔS_{ax} , rather than ΔH_{ax} , is the principal determinant of the allosteric inhibition of phosphofructokinase by MgATP. ΔS_{ax} also substantially mitigates the impact of ΔH_{ax} in the case of isocitrate dehydrogenase, thus diminishing the magnitude of the resultant activation. If the participants in reaction 7 were static and equally solvated and merely differed in their three-dimensional conformations, one might expect ΔG_{ax} to equal ΔH_{ax} , in which case MgATP would actually activate phosphofructokinase, and ADP would be too potent an activator of isocitrate dehydrogenase to have any regulatory consequence of metabolic relevance. Clearly this view is not consistent with our data because ΔS_{ax} plays a major role in establishing the nature and magnitude of the allosteric effect in these enzymes.

The importance of ΔS_{ax} naturally leads to speculation as to its molecular origin. Varying degrees of freedom of the proteins, bound ligands, or solvent associated with the species participating in Eq. 7 all can potentially contribute to the negative entropy of that equilibrium. Of these possibilities we feel that solvent probably plays a lesser role, since Eq. 7 involves reactants and products containing the same total number of empty and filled binding sites and, importantly, does not contain free ligand. Moreover, the data shown in Fig. 2 are well fit by straight lines, indicating little if any change in heat capacity associated with the disproportionation reaction in Eq. 7 over the temperature range examined. Changes in heat capacity are usually associated with processes that involve changes in the degree of solvation (26).

To the extent that solvent effects do not dominate ΔS_{ax} , the protein-ligand species depicted in Eq. 7 must themselves differ in entropy content and, hence, the ability to interconvert between various configurational states or "substates," implying that *the dynamical properties of these protein species are germane to the establishment of the allosteric effect*.

Eftink *et al.* (16) have estimated that a typical unligated protein exists several kcal/mol above its energetic minimum at room temperature because of its internal dynamics. In addition, the binding of small molecular weight ligands will constrain these internal motions to a certain extent (26). Consequently, it is reasonable to expect that the dynamical properties, and hence entropy content, of these species will differ. To explain a negative ΔS_{ax} , the constraints imposed by both ligands in X-E-A must be greater than the sum of the constraints imposed by each ligand in X-E and E-A.

That allosteric effects might be transmitted via alterations in the dynamic characteristics of a protein rather than, or in addition to, time-averaged conformational changes has also been proposed by Cooper and Dryden (29), who have estimated on the basis of theoretical calculations that the magnitude of such effects can be more than sufficient to account for observed behavior. Interestingly, their calculations also predicted that a dynamic mechanism would lead to largely compensating ΔS_{ax} and ΔH_{ax} contributions to ΔG_{ax} such as we have observed.

It is of course inappropriate, without further investigation, to attribute to all regulatory enzymes the characteristics reported herein for phosphofructokinase and isocitrate dehydrogenase. In those enzymes to which these properties do pertain, however, an additional role for protein dynamics is suggested. Namely, the influence of ligands on the dynamical

properties of the protein–ligand matrix appear to play a vital role in establishing the magnitude and nature of the allosteric response by contributing to a coupling entropy that in large measure helps to determine the sign and absolute value of the coupling free energy.

We thank Dr. Gregorio Weber for his continued support and encouragement. G.D.R. is an Established Investigator of the American Heart Association. This investigation was funded by Research Grant GM 33216 and Biomedical Research Support Grant RR 07078 from the National Institutes of Health.

1. Monod, J., Wyman, J. & Changeux, J. P. (1965) *J. Mol. Biol.* **12**, 88–118.
2. Koshland, D. E., Jr., Nemethy, G. & Filmer, D. (1966) *Biochemistry* **5**, 365–385.
3. Koshland, D. E., Jr. (1970) *The Enzymes* (Academic, New York), 3rd Ed., Vol. 1, pp. 341–396.
4. Reinhart, G. D. (1983) *Arch. Biochem. Biophys.* **224**, 389–401.
5. Reinhart, G. D. (1985) *Biochemistry* **24**, 7166–7172.
6. Reinhart, G. D. & Hartleip, S. B. (1986) *Biochemistry* **25**, 7308–7313.
7. Wyman, J. (1948) *Adv. Prot. Chem.* **4**, 407–531.
8. Wyman, J. (1964) *Adv. Prot. Chem.* **19**, 223–286.
9. Wyman, J. (1967) *J. Am. Chem. Soc.* **89**, 2202–2218.
10. Weber, G. (1972) *Biochemistry* **11**, 864–878.
11. Weber, G. (1975) *Adv. Prot. Chem.* **29**, 1–83.
12. Reinhart, G. D. (1989) in *Fluorescent Biomolecules: Methodologies and Applications*, eds. Jameson, D. M. & Reinhart, G. D. (Plenum, New York), pp. 195–216.
13. Reinhart, G. D. & Lardy, H. A. (1980) *Biochemistry* **19**, 1477–1484.
14. Chen, R. F. & Plaut, G. W. E. (1963) *Biochemistry* **2**, 1023–1032.
15. Giorgio, N. A., Jr., Yip, A. T., Fleming, J. & Plaut, G. W. E. (1970) *J. Biol. Chem.* **245**, 5469–5477.
16. Eftink, M. R., Anusiem, A. C. & Biltonen, R. L. (1983) *Biochemistry* **22**, 3884–3896.
17. Plaut, G. W. E., Schramm, V. L. & Aogaichi, T. (1974) *J. Biol. Chem.* **249**, 1848–1856.
18. Gabriel, J. L., Aogaichi, T., Dearolf, C. R. & Plaut, G. W. E. (1983) *Anal. Lett.* **16**, 113–127.
19. Botts, J. & Morales, M. (1953) *Trans. Faraday Soc.* **49**, 696–707.
20. Alberty, R. A. & Bloomfield, V. (1963) *J. Biol. Chem.* **238**, 2804–2810.
21. Frieden, C. (1964) *J. Biol. Chem.* **239**, 3522–3531.
22. Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137.
23. Fan, C. C. & Plaut, G. W. E. (1974) *Biochemistry* **13**, 45–51.
24. Fan, C. C., Lin, J.-P. F. & Plaut, G. W. E. (1975) *J. Biol. Chem.* **250**, 2022–2027.
25. Fersht, A. (1985) *Enzyme Structure and Mechanism* (Freeman, New York), pp. 150–151.
26. Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2236–2240.
27. Van Holde, K. E. (1985) *Physical Biochemistry* (Prentice-Hall, Englewood Cliffs, NJ), 2nd Ed., pp. 56–57.
28. Lau, F. T.-K. & Fersht, A. R. (1987) *Nature (London)* **326**, 811–812.
29. Cooper, A. & Dryden, D. T. F. (1984) *Eur. Biophys. J.* **11**, 103–109.
30. Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences* (McGraw-Hill, New York), pp. 92–118.