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EVALUATION OF ENZYME SYSTEMS AND THEIR REGULATION

THE INAPPLICABILITY OF IRREVERSIBLE THERMODYNAMICS

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Summary

The formalism called irreversible thermodynamics has been examined for its applicability to the description of enzymic reactions. The basic assumption of this formalism is that the net flux through a reaction is related to the free energy change, a relationship which is usually assumed to be linear. These assumptions are shown to be approximately true for the trivial case of an enzyme reaction within 0.8 kJ/mol (0.2 kcal/mol) of equilibrium in the absence of changes in any regulatory parameters (such as inhibitor, activator, or enzyme concentration). For all other reaction conditions the net flux is not related to the free energy change and in special cases for which an apparent relationship is seen, it is not linear. Thus, application of the formalism of irreversible thermodynamics gives rise to qualitatively and quantitatively erroneous results and conclusions.

Since most regulatory enzymic reactions are far from equilibrium, and the net reaction rate of such reactions is regulated by changes in inhibitors, activators and/or enzyme concentration, the formalism of irreversible thermodynamics is in general neither applicable nor useful in understanding the behavior of biological reaction systems.

Introduction

In recent years there has been an increasing number of publications in which irreversible thermodynamics is used to describe the function of metabolic processes. Some authors (see for example Refs. 1—3) have suggested that this is the only applicable thermodynamic formalism for metabolic processes, while

we [4,5] have considered that the assumptions used in development of the formalism are excessively restrictive, making it essentially unusable for biological systems.

Debates over the theoretical basis of the current formalism of irreversible thermodynamics are usually phrased in mathematical equations and rely heavily on arguments concerning the definition and properties of thermodynamic functions. The proof of any theoretical treatment is the ability of the equations to fit real experimental data and to predict the behavior of the system under new and untested conditions. In the present paper, the basic predictions of irreversible thermodynamics are compared with the experimentally established kinetics of enzyme systems. From these comparisons it is possible to draw conclusions about the applicability of the equations of irreversible thermodynamics.

Rationale

Irreversible thermodynamics is a formalism developed in an attempt to relate reaction kinetics (rate) to reaction thermodynamics [6—9]. Fundamental to the formalism of irreversible thermodynamics is the assumption that the flow (flux) through a system is related to the force (negative free energy change) associated with the movement of reactants through the system [6—9]. This is usually assumed to be a relationship of the form

$$J_A = L_A(-\Delta\mu_A) = L_A A \tag{1}$$

where the flow (J_A) through the reaction (net product formation) is equal to a proportionality constant (L_A) times the change in chemical potential (free energy change), the latter term usually called the reaction affinity. Assuming that Eqn. 1 is correct, analysis can be extended to coupled reactions by linear combination of the forces of the reactions. The resulting equations are linear differential equations and, as such, can be readily solved.

All of the formalism of irreversible thermodynamics is based on the assumption that flow and force are related and on Eqn. 1. We will therefore test both this assumption and Eqn. 1 for applicability to enzyme kinetics. If these assumptions are both correct, plots of the reaction rate (v) against the concentration of any affector of the rate should give parallel plots of the calculated ΔG against the concentration of the affector. This may also be expressed:

$$\frac{J_A}{\Delta\mu_A} = \frac{v}{\Delta G} = L_A \tag{2}$$

and for each analysis the calculated ratio of the reaction rate to ΔG($v/\Delta G$) is also plotted in the figures. This ratio should have a constant value if Eqn. 1 is correct.

The test system

In order to test the applicability of Eqn. 1 to enzyme kinetics, a model system has been selected for which elementary enzyme kinetics and regulation can be expressed. Classical Michaelis-Menten kinetics will be used to describe the

behavior of the general reaction:

$$E + S \rightleftharpoons ES \rightarrow E + P$$

where the enzyme (E) has a maximal rate (V) of 1 unit/unit enzyme, a K_m for substrate (S) of $1 \cdot 10^{-3}$ M, a K_i for product (P) of $1 \cdot 10^{-3}$ M and an equilibrium constant, [P]/[S], of 10^4. Regulation of enzymic catalysis (a) by product inhibition, (b) by an allosteric activator (A) and (c) by an allosteric inhibitor (I) will be considered. For simplicity these will be assumed to be noncooperative phenomena. The allosteric activator is assumed to have a dissociation constant (K_d) of $1 \cdot 10^{-3}$ M and an activation factor (increase in V) of 10; the allosteric inhibitor is assumed to have a dissociation constant (K_i) of $1 \cdot 10^{-3}$ M and to be capable of complete inhibition.

Results

Substrate concentration dependence for simple Michaelis-Menten kinetics. The substrate concentration dependence of the rate of the enzymic reaction has been calculated assuming three different concentrations of product and Michaelis-Menten kinetics (Fig. 1). The concentrations of product were selected to give the calculated free energy change (ΔG) a reasonable value. If initial conditions were selected for which [P] is zero, the calculated free energy change would be infinite while the rate of reaction was finite and dependent on substrate concentration. Thus, it is mathematically impossible for Eqn. 1 to describe enzyme kinetics when the product concentration is zero and a significant concentration of product must be present in order to enable Eqn. 1 to be relevant to enzyme kinetics. Having assumed concentrations of $1 \cdot 10^{-6}$ M, $1 \cdot 10^{-3}$ M and $1 \cdot 10^{-1}$ M for the product, the concentration of substrate was varied from $1 \cdot 10^{-5}$ M to 1 M and the calculated rate of reaction and free energy change plotted against the logarithm of substrate concentration (Fig. 1a). The use of a logarithmic scale allows a wide range of substrate concentrations to be examined and the free energy change is a straight line. The plot of v against log [S] is a continuously curved function and is different for each value of [P].

The ratio $v/\Delta G$ has been calculated and is plotted against log [S] in Fig. 1b. It is apparent that $v/\Delta G$ is not constant for any concentration of substrate but is a continuous variable ranging from less than 10^{-4} to greater than 10^{-1}. Larger changes in $v/\Delta G$ are observed for other concentrations of product or substrate. Most important in the current evaluation is the absence of a region for which $v/\Delta G$ is independent of [S].

Dependence of the rate and free energy change of enzymic reactions on enzyme concentration. In describing the rate of an enzymatic reaction it is important to have a measure of the concentration of enzyme present in the system, particularly as modulation of active enzyme concentration in an important physiological control mechanism. When all other conditions are held constant, the rate of reaction is generally proportional to enzyme concentration, assuming no interactions among enzyme units (Fig. 2a). The free energy change for a reaction is independent of the concentration of catalyst (enzyme), being dependent only on the activities of substrate and product. Thus, if the

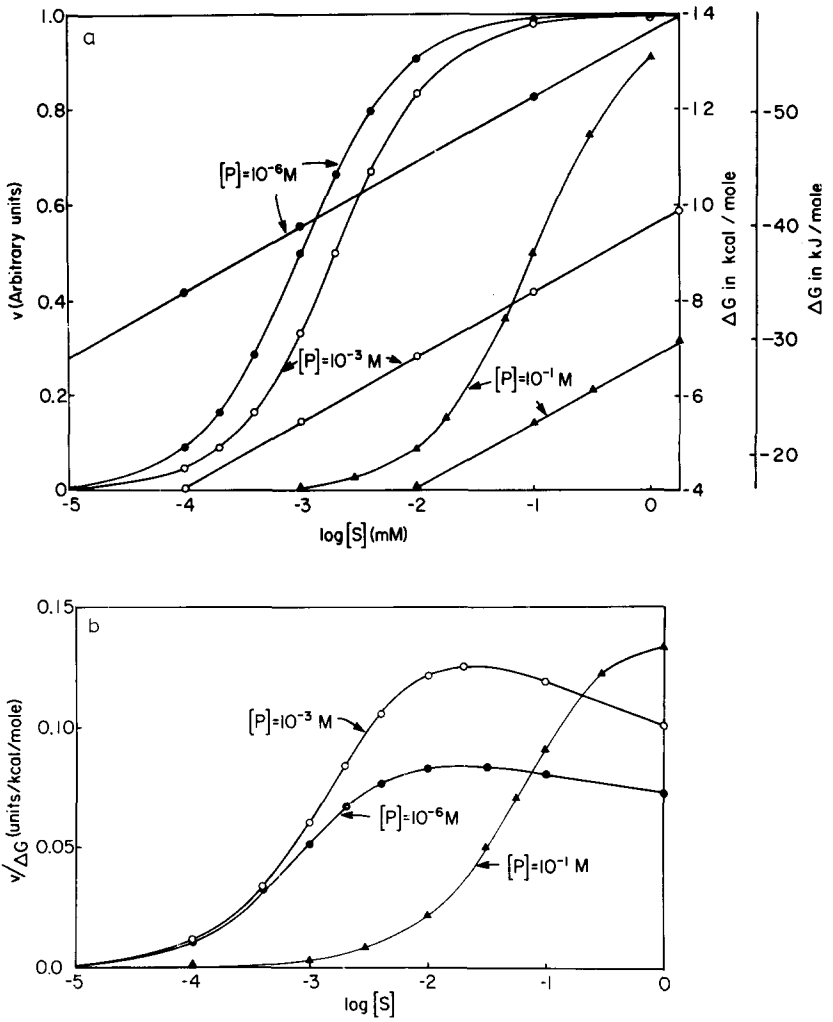

Fig. 1. (a) Dependence of the rate of enzymatic reaction on the concentration of substrate at different product concentrations. The rate and free energy change of reaction has been calculated as described in the text for three different concentrations of product, $1 \cdot 10^{-6}$ M, $1 \cdot 10^{-3}$ M and $1 \cdot 10^{-1}$ M and are plotted against the logarithm of the substrate concentration (abscissa). (b) The relationship of the reaction rate (v) to the free energy change (ΔG) of the reaction. The values of v and ΔG calculated for Fig. 1a were used to calculate the ratio of $v/-\Delta G$ and this was plotted against the logarithm [S].

concentrations of substrate and product are held constant, the rate of the reaction may be increased or decreased by adding or detecting enzyme with no change in ΔG.

When the calculated values of $v/-\Delta G$ are plotted against enzyme concentration (Fig. 2b) the values increase as the enzyme concentration increases, since ΔG is a constant. Thus, $v/-\Delta G$ ranges from essentially zero in the absence of enzyme (many biological reactions occur in the absence of enzyme at less than 10^{-5} of the biological rate) to that observed in the presence of physiological amounts of enzyme.

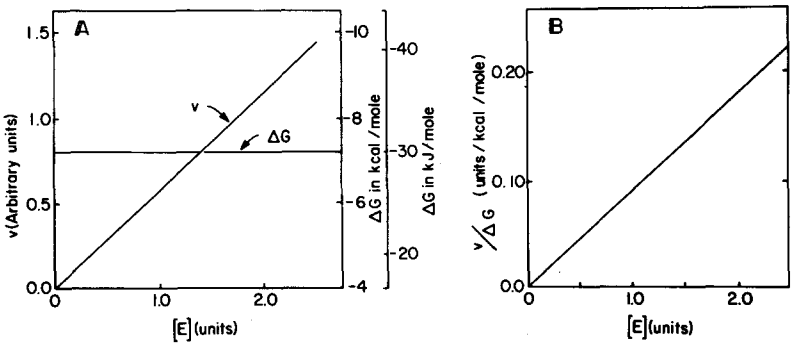

Fig. 2. The dependence of the rate and free energy change of an enzymatic reaction on the enzyme concentration. In Fig. 2a the calculated rate of reaction (left ordinate) and the ΔG (right ordinate) are plotted against enzyme concentration (abscissa). The selected reaction conditions were $2 \cdot 10^{-3}$ M substrate and $1 \cdot 10^{-4}$ M product but similar results are observed for any other conditions of constant [S] and [P]. In Fig. 2b the calculated ratio $v/-\Delta G$, is plotted against enzyme concentration.

Dependence of the rate and the free energy change of the reaction on product concentration. When the substrate and enzyme concentrations are held constant the rate of reaction is dependent on the concentration of product (Fig. 3a). The rate of reaction at low product concentrations approached that for the absence of product and inhibition is observed only when the substrate concentration and product concentration are in the appropriate ratio. In Fig. 3a the reaction rate and free energy change are plotted on the ordinate with the logarithm of the ratio [P]/[S] on the abscissa. ΔG is a function only of [P]/[S] and is independent of the actual value of [S]. Thus the plotted data form a single straight line. The reaction rate, in contrast, is different for each concentration of substrate and a family of curves is generated. In Fig. 3a, the curves for four concentrations of substrate ($2 \cdot 10^{-4}$ M, $5 \cdot 10^{-4}$ M, $2 \cdot 10^{-3}$ M and $6 \cdot 10^{-3}$ M) are presented as examples.

The plots for the rate of reaction against log [P]/[S] shows v to be a strongly nonlinear function of [P]/[S] and therefore of ΔG. Also important is the observation that the maximum slopes of these curves are dependent on the concentration of substrate, being higher with higher substrate levels. When $v/-\Delta G$ is plotted against log [P]/[S] (Fig. 3b), a completely different curve is obtained for each concentration of substrate. The value of $v/-\Delta G$ first increases with increasing [P]/[S], goes through a maximum and then decreases. There is no region of [P]/[S] for which $v/-\Delta G$ is independent of substrate concentration. In general, for any value of [P]/[S], $v/-\Delta G$ increases with increasing substrate concentrations.

The influence of allosteric activators and inhibitors on the rate and free energy change of the reaction. The effect of enzyme inhibitors is illustrated using an allosteric inhibitor with a dissociation constant of $1 \cdot 10^{-3}$ M. The substrate concentration was assumed to be $2 \cdot 10^{-3}$ M and the product $1 \cdot 10^{-4}$ M. The reaction rate was then calculated as a function of the inhibitor concentration and presented in Fig. 4a. The reaction rate in the absence of inhibitor was 0.623 units and this fell to very low values as [I] increased. The abscissa in Fig. 4 was chosen as logarithm $[I]/K_i$ in order for the analysis to be independent of the absolute value of [I] and K_i.

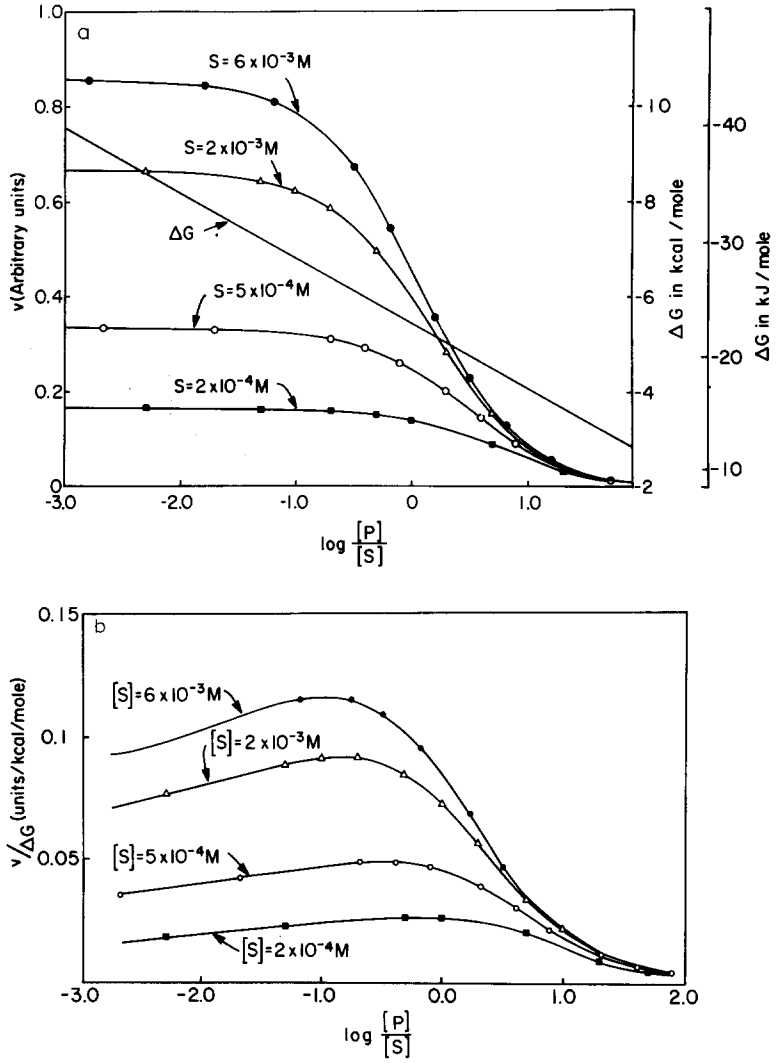

Fig. 3. The dependence of enzyme reaction rate, ΔG and $v/-\Delta G$ on the concentration of product. The enzyme reaction rate (left ordinate) and ΔG (right ordinate) were calculated as functions of product concentration for four different substrate concentrations. The results are plotted in Fig. 3a against logarithm [P]/[S] (abscissa) for convenience. The calculated values for ΔG are independent of the absolute concentrations of P and S, giving rise to a single straight line function. The calculated ratio $v/-\Delta G$ is plotted against logarithm [P]/[S] in Fig. 3b.

Activation of the enzyme by an allosteric activator (A) with an activation factor of 10 is also shown in Fig. 4a. The ordinate has been scaled down by 10-fold to display the new activity range (0.623 units in the absence of activator to 6.23 units with saturating concentrations of activator). As was the case for inhibitor, ΔG is independent of the concentration of activator and because [S] and [P] are held constant, ΔG is constant.

In Fig. 4b the value of $v/-\Delta G$ is plotted on the ordinate against either log $[I]/K_i$ and log $[A]/K_d$ on the ordinate. When the ordinate is -2.0 the values of

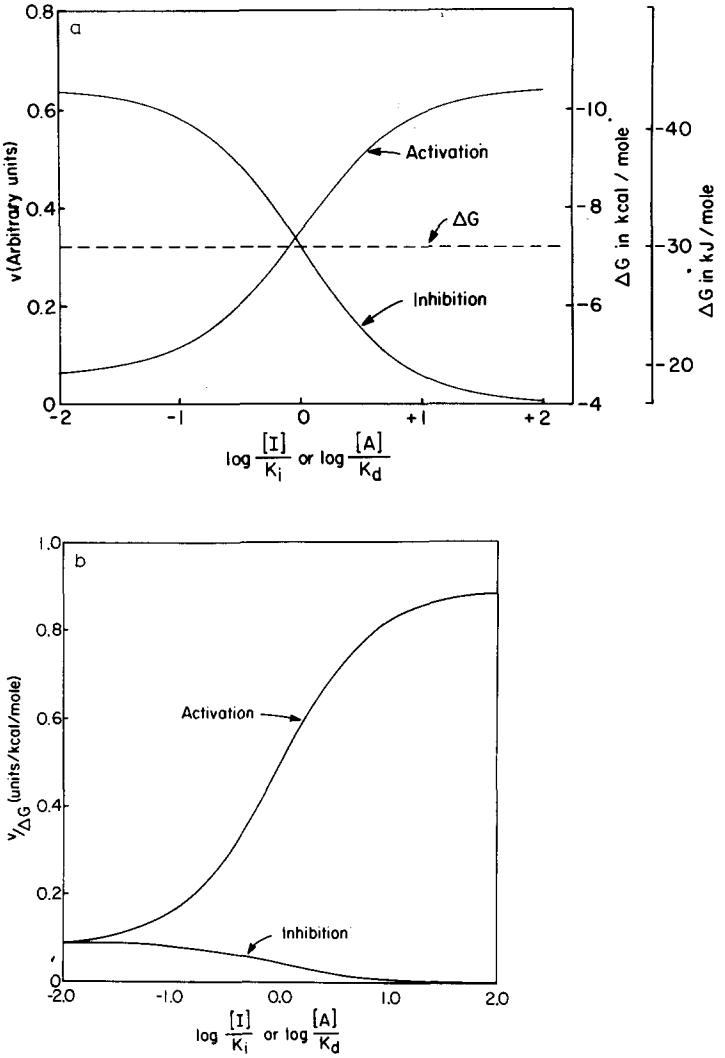

Fig. 4. The dependence of enzyme reaction rate, ΔG and $v/-\Delta G$ on concentration of activator (A) or inhibitor (I). The enzyme reaction rates and ΔG were calculated for a $2 \cdot 10^{-3}$ M substrate and $1 \cdot 10^{-4}$ M product. Activation was calculated for an allosteric activator with an activation factor of 10 and the data plotted with rate on the ordinate (scale should read 0—8 for activation) and logarithm $[A]/K_d$ on the abscissa. Inhibition was calculated for an allosteric inhibitor and the reaction rate plotted on the ordinate (0—0.8 scale) and logarithm $[I]/K_i$ on the abscissa. The calculated ΔG (right ordinate) is the same for both activation and inhibition. In Fig. 2b $v/-\Delta G$ is plotted against logarithm $[I]/K_i$ or logarithm $[A]/K_d$.

$v/-\Delta G$ are essentially equal for both activation and inhibition. At $[A]/K_d$ or $[I]/K_i$ values of 1.0 (abscissa = 0.0), $v/-\Delta G$ has decreased to 0.043 for inhibition and increased to 4.9 for activation giving a ratio of these values of 10^{-2}. By an $[A]/K_d$ or $[I]/K_i$ for 10, $v/-\Delta G$ has further decreased to approx. 0.008 for inhibition and increased to 8.2 for activation. This difference of 10^{-3} increases even more as the values of $[A]/K_d$ and $[I]/K_i$ increase.

Discussion

In the enzyme model system utilized in this paper it is clear that conditions for which the enzyme activity can be even approximated by Eqn. 1 are the exception rather than the rule. This arises from failure in the assumption that the reaction rate is linearly related to the free energy change. The failure is not surprising because enzymes are catalysts for metabolic reactions while the free energy change is dependent only on the activities of S and P and independent of catalytic function. Thus, ΔG is dependent only on the initial and final states of the system and is independent of the rate at which the change occurs. It follows therefore that any change in enzyme concentration, or activity per unit of enzyme (activation or inhibition) cannot have a direct consequence in the ΔG of the reaction. This conclusion is independent of how close the reaction is to equilibrium.

The failure of irreversible thermodynamics can be readily observed in real metabolic situations. In muscle the transition from rest to maximum work greatly increases the rate of ATP utilization but the increased flux is accompanied by a lower [ATP]/[ADP][P_i] (see for example Ref. 10) i.e., a less negative ΔG for ATP hydrolysis. In glycolysis, activation of the pathway (increased flux) is accompanied by a 'crossover' at phosphofructokinase in which the concentration of reactants (fructose 6-phosphate and ATP) decrease and the products (fructose 1,6-diphosphate and ADP) increase [11,12]. Thus, in glycolysis as in ATP utilization in muscle increased flux is associated with decreased driving force ($-\Delta G$ or A), opposite to the predictions of irreversible thermodynamics.

The applicability of irreversible thermodynamics

The basis for Eqn. 1 is found in the concept of microscopic reversibility, from which

$$\Delta G = RT \ln \frac{v_r}{v_f} \tag{3}$$

where R and T are the gas constant and absolute temperature, respectively, while v_r and v_f are the reverse and forward fluxes through the reaction. The net flux (v_n) is equal to $v_f - v_r$, and the equation may be written:

$$\Delta G = RT \ln \frac{v_f - v_n}{v_f} = RT \ln \left(1 - \frac{v_n}{v_f}\right) \tag{4}$$

The net flux through an enzymatic reaction will be proportional to ΔG if two important conditions are met.

1. No change must occur in the 'catalytic activity' of the enzyme. The 'catalytic activity' in this case is defined as the reaction rate at a specified concentration of substrate and product and is a function of K_m, K_i, V, K_d and enzyme concentration. As may be seen from Eqn. 1, a change in L_A (catalytic activity) with no change in [P] and [S] (no change in ΔG) results in proportional changes in v_f and v_r (v_r/v_f = constant). Since $v_n = v_f - v_r$ this means that v_n changes with no change in ΔG.

2. The free energy change must be between 0 and ±0.8 kJ/mol. For reactions

at 25°C Eqn. 3 may be written

$$\Delta G = 1.36 \text{ kcal/mol} \log \frac{v_r}{v_f} \tag{5}$$

and the net flux is a linear function of ΔG only when v_n is a linear function of $\log v_r/v_f$. For the case in which the product concentration is constant (v_f is constant),

$$\Delta G = 1.36 \text{ kcal/mol} \left(\log \frac{\text{constant}}{v_f}\right),$$

and for the case in which the substrate concentration is constant,

$$\Delta G = 1.36 \text{ kcal/mol} \left(\log \frac{v_r}{\text{constant}}\right).$$

Taylor series expansion (or a table of logarithms) shows that linearity is approximated only when the ratio constant/v_f is between 1.1 and 0.9. This restricts ΔG to values between 0 and +0.8 kJ/mol (±0.2 kcal/mol).

Misconceptions concerning irreversible thermodynamics and their basis

The literature contains several papers in which the authors apply irreversible thermodynamics to biological systems including regulated enzymes [1—3,13—18]. These authors espouse a position which appears to be the result of a cumulative sequence of incorrect and conflicting assumptions. Assumptions made in derivation of the initial equations were later ignored and further analysis was based on additional assumptions which either conflicted with the initial ones or were simply inapplicable. Although it is beyond the scope of the present paper to examine in detail the errors involved in developing the formalism of irreversible thermodynamics as presented by some authors [1—3,13—18], it is useful to briefly discuss three points.

1. The requirement for an unchanged catalytic activity is completely unrecognized in most papers. As we have shown, this omission is disastrous.

2. The requirement for near equilibrium conditions, which we have discussed quantitatively above, has not been rigorously applied (for an exception see Ref. 17). Walz [1], for example, states "linearity between flows and forces holds only for 'relatively slow' processes, i.e. for processes not 'too far away' from equilibrium . . . " and then implies that essentially all irreversible reactions fall within this range, which is not true.

3. Logical errors have occurred in some attempts to extend the range of applicability of irreversible thermodynamics. It has been suggested [13,14] that Michaelis-Menten kinetic curves near the K_m for substrate can be approximated using a linear differential equation of the form used in irreversible thermodynamics and therefore irreversible thermodynamics is applicable. Fit by a mathematical equation does not imply, however, that the authors' physical interpretation of the mathematical symbols is correct.

As may be seen from Fig. 1: (1) $v/-\Delta G$ for a single value of [P] is a continuously variable function of [S] and does not have a constant value near [S] = K_m, and (2) the value of $v/-\Delta G$ is different for each different value of [P] ([S] held constant). Either of these observations is sufficient to prove that the

enzyme reaction rate is not a function of the free energy change (reaction affinity) of the type proposed for irreversible thermodynamics.

In conclusion

As we have discussed above, the conditions for the applicability of irreversible thermodynamics to biological systems are extremely restrictive. Close reading of the (current) literature reveals that in the majority of cases in which irreversible thermodynamics has been used to analyze biological systems, the formalism of irreversible thermodynamics is totally inapplicable due to violation of one or several of the conditions discussed above. As a result, the conclusions based on the incorrect analyses are wrong and, at worst, very misleading. In view of the conditions under which it is applicable, the formalism of irreversible thermodynamics has very limited use as a tool in analyzing metabolic reactions. Studies using the approach to understanding biological systems should be viewed with suspicion and scrutinized closely to see if the system studied obeys the conditions for which it is valid.

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