Isoergonic Cooperativity in Glutamate Dehydrogenase Complexes: A New Form of Allostery†

Harvey F. Fisher* and Jon Tally

Laboratory of Molecular Biochemistry, VA Medical Center, Kansas City, Missouri 64128, and Department of Biochemistry and Molecular Biology, University of Kansas Medical Center

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The concept of cooperativity in biological systems has been conventionally expressed exclusively in free energy terms (Ackers et al., 1992; Monod et al., 1965; Perutz, 1989; Ho, 1992). Typically, the binding of a specific ligand to a multi-subunit protein is described as positively or negatively cooperative if the successive binding constants defining the process are found to differ substantially, usually by 1 or more orders of magnitude. We have observed what we believe to be a new class of such behavior, one in which the sequential binding steps have nearly identical observed binding constants but are characterized by large differences and even changes in sign of the $\Delta H°$ values which accompany them.[1] Here we present these experimental findings and develop a theory of "isoergonic cooperativity" to account for this class of behavior.

The phenomenological basis for the concept of isoergonic cooperativity is provided in Figure 1, which shows the results of isothermal calorimetric titrations of bovine liver glutamate dehydrogenase with ADP (E−ADP), with NADPH (E−R), and with NADPH in the presence of L-glutamate (E−G−R) using a Microcal Omega titration calorimeter. Each point represents the heat $(\Delta q)/[L_T]$ produced for an *incremental* addition of ligand ($\Delta[L_T]$) plotted *vs* the accumulated total ligand concentration ($[L_T]$) divided by $[M_T]$, the molar concentration of macromolecular binding sites. This form of plot, using sufficiently small incremental additions of ligand, is essentially the first derivative ($dq/d[L_T]$) of a conventional binding plot, in which the total accumulated signal is plotted *vs* the *total accumulated* ligand concentration. Introduced by Wiseman et al. (1989) and discussed in some detail by Fisher and Singh (1995), it is used to take advantage of the high degree of sensitivity of the Omega calorimeter. The theoretical behavior of such a plot for the general case of an oligomeric protein expressed as $dq/d[L_T]$ *vs* $[L_T]/[M_T]$ where q is the observed heat of any given value of $[L_T]$, and where the value of q is given by eq 1:[2]

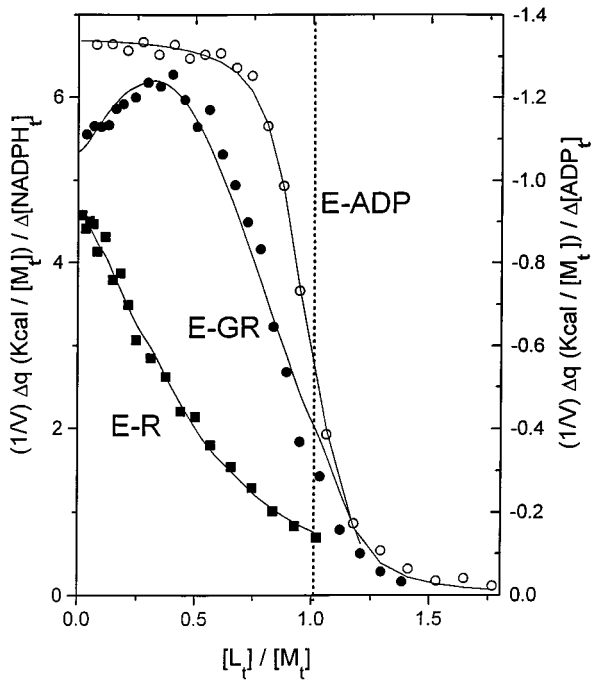

FIGURE 1: Isothermal titrations of bovine liver glutamate dehydrogenase, with ADP (right-hand ordinate), with NADPH, and with NADPH (left-hand ordinate in the presence of 50 mM L-glutamate at 4 °C in 0.1 M phosphate buffer, pH 7.6, experimental points; (—) Fit according to a three-step interactive equation of Wiseman et al. (1989). The titrations were carried out on a Microcal Omega isothermal titration calorimeter.

$$q/[M_T] = \{(\Delta H_1)K_1[L] + (\Delta H_1 + \Delta H_2)K_1K_2[L]^2 + (\Delta H_1 + \Delta H_2 \ldots + \Delta H_n)K_1K_2 \ldots K_n[L]^n\}/\{1 + K_1[L] + K_1K_2[L]^2 + K_1K_2 \ldots K_n[L]^n\} \quad (1)$$

where ΔH_n is the molar enthalpy of binding to each site and K_n is the binding constant (M^{-1}) for each individual binding site.

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* Address correspondence to this author at Research Service, VA Medical Center, 4801 Linwood Blvd., Kansas City, MO 64128. Tel: (816) 861-4700 ext. 7156. FAX: (816) 861-1110.

[1] More precisely, we define the term "isoergonic cooperativity" as a case where the successive steps in the binding of a specific ligand to a homotropic oligomer have free energies of binding which differ among themselves by *less* than 1 kcal mol^{-1} (K_b values differing by a factor of less than 5), but whose corresponding enthalpies of binding differ among themselves by more than 7 kcal mol^{-1}.

[2] The analytical form of eq 1 provided by Wiseman et al. (1989) for the single-site binding case is

$$\frac{1}{V}(dq/d[L_T]) = \Delta H\left({}^1/_2 + \frac{1 - (1 + r)/2 - L_r/2}{(L_r^2 - 2L_r(1 - r) + (1 + r)^2)^{1/2}}\right)$$

where $r = 1/K_B[M_T]$, K_B is the ligand binding constant, and $[M_T]$ is the total molar concentration of the protein; $L_r = [L_T]/[M_T]$ and $[L_T]$ is the total molar ligand concentration. V is the cell volume in liters, H is molar binding enthalpy, and q is the heat developed expressed as calories/liter. The corresponding differential form for more complex cases such as that of eq 1 can be derived in the same fashion. As such equations are quite cumbersome, it is common practice to differentiate equations such as eq 1 numerically by computer using programs such as Origin, and we have done so in the work presented here using the Microcal Origin program. This equation, while quite rigorous and diagnostically useful, has a number of properties which may not be intuitively obvious from the algebraic expression. A detailed discussion of this equation and of its related forms for more complex cases will be provided elsewhere [Fisher, H. F. *Methods in Enzymology* (Ackers, G. K., and Johnson, M. L., Eds.) (to be published)].

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Table 1: Binding Constants ($\times 10^4$, in M^{-1}) Calculated from Single and Multiple Interacting Site Analysis[a]

complex	K_1	K_2	K_3
EGR	8.8 ± 1	8.1 ± 1	8.8 ± 1
ER	0.4 ± 0.05	0.4 ± 0.06	0.5 ± 0.1
EADP	45 ± 3		

[a] Calculations were carried out using the Microcal Origin ITC analysis software based on theory described in Wiseman et al. (1989). K_1, K_2, and K_3 values are those of the microconstants.

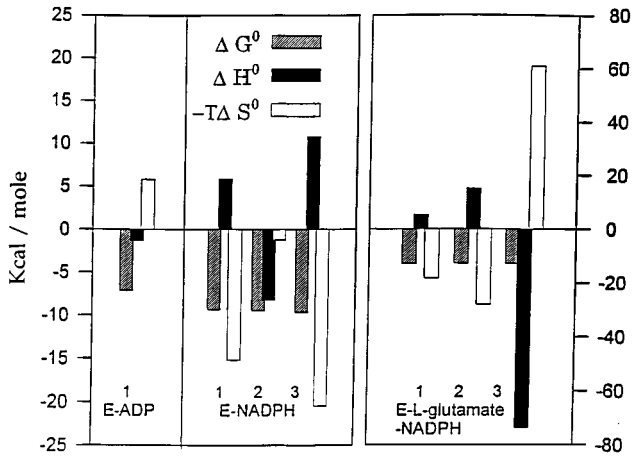
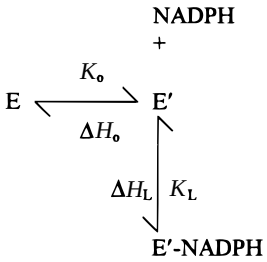


FIGURE 2: Thermodynamic parameters for the independent single site binding of the ADP complex and of the three successive sites for the NADPH and the L-glutamate−NADPH complexes of bovine liver glutamate dehydrogenase calculated from the fitted curves shown in Figure 1.

It can be seen from Figure 1, that the binding of ADP to bovine liver glutamate dehydrogenase does fit the derivative form of eq 1 for independent sites quite well, providing a value of $n = 0.94$. The binding of NADPH to the free enzyme and to its enzyme−L-glutamate complex, however, deviates significantly from this simple form, indicating substantial interactions between at least three of the individual binding sites of this hexameric protein.

The solid lines through the E-R and E-GR points in Figure 1 represent the best fit to the derivative form of eq 1 for three interacting sites. The individual values of the binding constants, K_1, K_2, and K_3, for each of these complexes are given in Table 1. The thermodynamic parameters calculated from the relationships, $\Delta G = -RT \ln K$ and $\Delta S = (\Delta H - \Delta G)/T$, are shown graphically in Figure 2.[3] It can be seen that the three sequential binding constant values are nearly identical for the formation of the binary and ternary complexes, while the corresponding ΔH and ΔS values change significantly in both magnitude and sign. Previously published spectrophotometric titrations and gel-filtration binding studies of both of these systems showed no evidence of any significant degree of inter-subunit cooperativity (Colen et al., 1974; Fisher et al., 1986; Engel & Dalziel, 1969). Thus, the data shown in Figure 1 appear to demonstrate the existence of a new class of homotropic cooperative behavior involving systems in which subsequent binding steps exhibit very different enthalpic properties, with only minor differences between their successive binding constants. In such cases, the cooperativity will not be evident from conventional binding studies which track only the *number* of ligand molecules bound to a macromolecule. We designate such behavior by the term "isoergonic cooperativity."

A clue to the nature of the phenomena shown in Figure 1 is provided by our finding that the enthalpy of the reaction E + NADPH ⇌ E−NADPH for this enzyme exhibits a striking nonlinear dependence on temperature (Fisher et al., 1981). The data were shown to be consistent with a reaction of the form



(2)

where

$$K_o = \frac{[E']}{[E]} \text{ and } K_L = \frac{[E{-}NADPH]}{[E]\,[NADPH]}$$

and where "E" and "E′" represent different conformations of an enzyme and where NADPH can bind only to the E′ form.

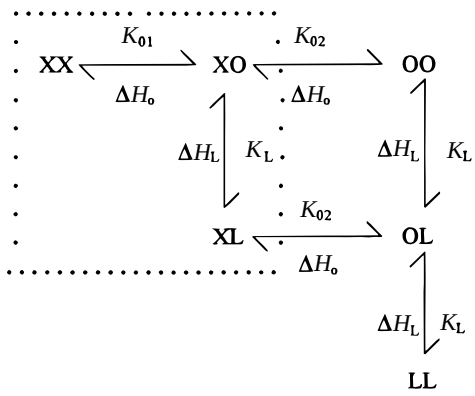
The dependence of the observed ΔH for such a system at saturating concentrations of the ligand is

$$\Delta H = \Delta H_L + \frac{\Delta H_o}{1 + K_o} \quad (3)$$

where $K_o = \exp[\Delta H_o(T - T_o)/RTT_o]$, and T_o is that temperature at which $K_o = 1$ and $\Delta G_o = 0$.

As noted by Fisher et al. (1981), the behavior of such a system can in itself generate a substantial negative ΔC_p phenomenon.

Extending this model to a system of two identical subunits leads to the following scheme:



(4)

where the dotted line encloses the basic element shown in eq 2.[4]

[3] The value of ΔH_1 shown in Figure 2 represents the properties of the binding of the first ligand molecule to an enzyme binding site, since it is calculated from the limit of $d(\Delta H)/d[L]$ as $L \rightarrow 0$. However, ΔH_2 and ΔH_3 represent only the subsequent phenomenological behavior of the system as a whole and cannot properly be assigned specifically to individual sites.

[4] This operation effectively splits the apparent independent binding constant K_B into the product of two factors: (1) an intrinsic K_L (common to all subunits) and (2) a factor $K_o/(K_o + 1)$. This factor, which contains the protein isomerization constant, can have different values over the range of 0−1 in various subunits.

To establish the minimum conditions required for eq 4 to exhibit the phenomena shown in Figure 1, we impose the following physically oversimplistic conditions on the equation: we assume that each subunit may occupy one of two states: a low-enthalpy closed form (X) or a high enthalpy open form (O). The ligand, L, cannot bind to any closed site (X) but can bind to any open form equally well with the same binding constant K_L and the same binding enthalpy ΔH_L. The enthalpy of the X ⇌ O transition, ΔH_o, is assumed to be identical for all transitions. The only variable parameters in the system are the equilibrium constants of the X ⇌ O isomerization step, which may have the values K_{01} or K_{02}, respectively, depending only on the number of open states on the other subunits. It should be noted that in our assumed model this value is unaffected by the binding of L. The dependence of q on L_T for the two subunit case is[5]

$$\frac{1}{V}\left(\frac{q}{[M_T]}\right) = \{[(\Delta H_o)K_{01} + (2\Delta H_o)K_{01}K_{02} + (\Delta H_o + \Delta H_L)K_{01}K_L[L] + (2\Delta H_o + \Delta H_L)K_{01}K_{02}K_L[L] + (2\Delta H_o + 2\Delta H_L)K_L^2K_{01}K_{02}[L]^2] - [(\Delta H_o)K_{01} + (2\Delta H_o)K_{01}K_{02}]\}/\{1 + K_{01} + K_{01}K_{02} + K_LK_{01}[L] + K_{01}K_{02}K_L[L] + K_{01}K_{02}K_L^2[L]^2\} \quad (5)$$

where the parameters are defined as in eq 4.

Equations 3 and 4 can of course be easily extended to include a larger number of subunits, but the expressions become too cumbersome to write explicitly beyond $n = 3$. The behavior of the differential form of eq 4 for the case of a symmetrical trimer, assuming $\Delta H_o = 22$ kcal mol^{-1} and $\Delta H_L = -16$ kcal/mol^{-1} is shown in Figure 3 for three different sets of K_{01}, K_{02}, and K_{03} values. It can be seen that these simulated curves resemble the experimental data shown in Figure 1 both in their quantitative as well as their qualitative behavior. While the values of K_{01}, K_{02}, and K_{03} have been arbitrarily manipulated to provide the phenomena of Figure 1, the values chosen for ΔH_o, ΔH_L, and K_L are based on estimates from previous experimental work on the formation of these complexes (Fisher, 1988; Fisher et al., 1986), and the close agreement between experiment and theory is unlikely to be fortuitous.

It is clear that this very simple model, in which the equilibrium between open and closed states of any given subunit is affected only by the state of its neighboring subunits, is quite competent to account for the experimentally observed phenomena. The essential feature which appears to be responsible for this unusual type of cooperativity is the obligatory linkage between the highly exothermic ligand binding process and the highly endothermic process required to provide the open site at which that binding can occur. Thus, changes in the overall free energy of ligand binding in a multisubunit system may be largely damped by enthalpy−entropy compensation in the two-step process defined in eq 1, but the substantial enthalpic variations observed can still occur.

[5] The differential form of eq 5 for the single binding site is

$$\frac{1}{V}\frac{dq}{dL_T} = \left(\Delta H_L + \frac{\Delta H_o}{1 + K_o}\right)\left({}^1/_2 + \frac{1 - X_R - r}{2\sqrt{(X_R + r + 1)^2 - 4X_R}}\right)$$

where $X_R = [L_T]/[M_T]$ and $r = (1 + K_o)/(K_oK_L[M_T])$.

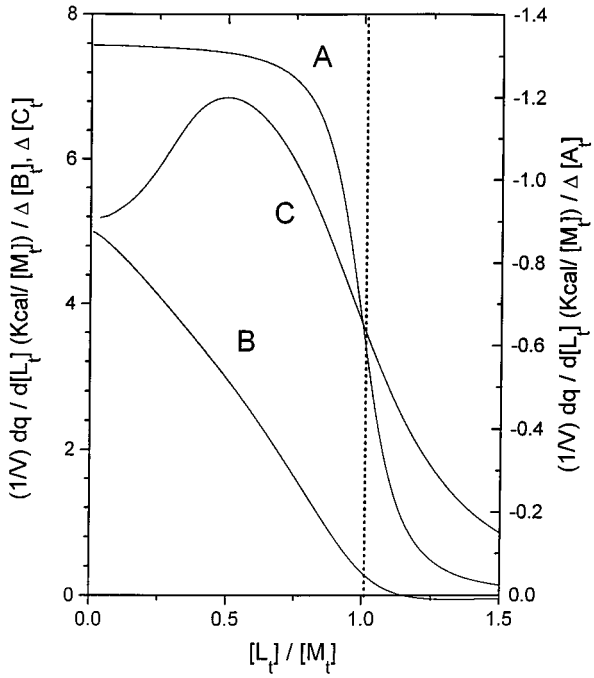


FIGURE 3: Simulations of the isothermal titrations shown in Figure 1 calculated according to a three-step version of eq 4. The line representations are analogous to those defined in Figure 1. Assumed values are as follows: (A) $K_{01} = K_{02} = K_{03} = 0.5$, $K_L = 20$, $\Delta H_L = -16\,000$ $\Delta H_o = 22\,000$; (B) $K_{01} = 0.15$, $K_{02} = 0.20$, $K_{03} = 0.5$, $K_L = 5$, $\Delta H_o^\circ = 22\,000$, $\Delta H_L = -16\,000$; (C) $K_{01} = 0.05$, $K_{02} = 0.01$, $K_{03} = 0.01$, $K_L = 80$, $\Delta H_o^\circ = 22\,000$, $\Delta H_L = -14\,000$ (K values in units of 10^4 M^{-1}; ΔH values in units of kcal mol^{-1}).

The existence of the isoergonic cooperative phenomena we described here is supported by a number of phenomenological observations which provide a somewhat more fundamental level for their interpretation: (1) It accounts for the large nonlinear ΔC_p effects and for the striking differences in those effects among the various complexes that we have reported. (2) It is supported by the very large differences between the thermal stability of the two complexes shown in Figure 1 whose "melting points" differ by 50 °C (Fisher, 1988). (3) It explains the long-controversial claim of kinetic cooperativity of this enzyme by Engel (1969), who observed substantial changes in K_m over a 10 000-fold concentration range, while finding no evidence of non-hyperbolic behavior over any discrete concentration range. (4) Its strongest support comes from X-ray crystal structures of the closely analogous *C. symbiosum* form of the enzyme, which show that some forms of the enzyme exist in a conformation in which the active site cleft is decidedly open, while other complexes of that enzyme crystallize in a completely closed form (Baker et al., 1992). (5) We have shown elsewhere that measurements of the protein component of the free-energies of various reactive intermediates along the reaction coordinate of the enzyme-catalyzed reaction support the Lumry concept of catalysis by the transduction of ligand-binding energy into a catalytic driving force (Fisher & Singh, 1991). In our original concept, that compensated free-energy transduction was envisioned as occurring internally within each subunit. The results presented here now suggest that the energy of the binding of a ligand to one subunit of the hexamer must be transduced to provide a driving force for a chemical event occurring on an adjacent subunit. This notion may provide at least a partial answer to the puzzling question of the apparent

necessity of a multi-subunit structure for the whole class of pyridine-nucleotide dehydrogenases, for which (with the single exception of glyceraldehyde dehydrogenase) no evidence of inter-subunit cooperativity has previously been observed.

The work described here provides direct evidence for only a single case of the phenomenon of isoergonic cooperativity. However, we have previously cited ample evidence which showed that, with the single assumption of a highly enthalpic two-state transition of a fixed ΔH but a variable ΔG, one can account quantitatively for the large variations in the thermodynamic properties of the pyridine-dehydrogenases as a class. Since that is precisely the thermodynamic behavior which constitutes the basis of the specific theorem proposed here, it would be surprising if isoergonic cooperativity were not found to occur generally in this class of enzymes, and probably among other oligomers. The approach described here affords a simple experimental means of testing that theorem.

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