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CONTENTS

A Unifying Model of the Thermodynamics of Formation of Dehydrogenase–Ligand Complexes ........................................ 1
  Harvey F. Fisher

Sorbitol Dehydrogenase .................................................. 47
  Jonathan Jeffery and Hans Jornvall

Molecular Size Determination of Enzymes by Radiation Inactivation ......................................................... 107
  Ellis S. Kempner

Calcineurin ................................................................. 149
  C. B. Klee, G. F. Draetta, and M. J. Hubbard

The Behavior and Significance of Slow-Binding Enzyme Inhibitors ............................................................... 201
  John F. Morrison and Christopher T. Walsh

ADP-Ribosylation of Guanyl Nucleotide-Binding Regulatory Proteins by Bacterial Toxins ......................... 303
  Joel Moss and Martha Vaughan

Kinetics of Substrate Reaction During Irreversible Modification of Enzyme Activity .................................. 381
  C. L. Tsou

The Dynamics of DNA Polymerase-Catalyzed Reactions .... 437
  Valerie Mizrahi and Stephen J. Benkovic

Author Index ............................................................. 459

Subject Index .......................................................... 501

Cumulative Indexes Vols. 1–61 ........................................ 509
A UNIFYING MODEL OF THE THERMODYNAMICS OF FORMATION OF DEHYDROGENASE-LIGAND COMPLEXES

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CONTENTS

I. Introduction
   A. Ligand Binding and Catalysis
   B. Scope
   C. Ligand-Induced Protein Changes
   D. Thermodynamic Parameters

II. Fundamental Concepts
   A. Levels of Enthalpic Measurements
   B. Interaction Parameters

III. The Problem
   A. The Phenomena
      1. Thermodynamic Parameters of the Formation of L-Glutamate Dehydrogenase-Ternary Complexes
      2. Thermodynamic Parameters for Individual Steps in the Formation of Other Pyridine Nucleotide Dehydrogenase Complexes
      3. Phenomenological Patterns
   B. Specific Questions

IV. The Answer
   A. The Development of the Model
      1. An Initial Clue
      2. A Paradox
      3. The Resolution
   B. The Model
      1. Description
      2. General Properties and Predictions
   C. Answers to the Specific Questions Posed

V. Evidence in Support of the Theory
   A. $T_0$ Values from the Temperature Dependence of $\Delta H$
   B. Isothermal Measurements—Interaction Parameters
   C. Thermal Stability
I. Introduction

A. Ligand Binding and Catalysis

The notion that ligand–protein binding energy can be utilized in subsequent catalytic steps in an enzyme-catalyzed reaction is a widely held view. The concept of coupling of interaction energy to produce specific biological functions was expressed by Lumry in 1959 (1). Elegant thermodynamic formulations of such processes have been developed by Wyman (2) and Weber (3), and applied to enzymatic catalysis by Jencks (4) in a well-known review under the sobriquet of the "Circe effect." Without in anyway wishing to artificially separate binding and catalytic processes (for to do so would be counter to the concept itself) we may yet consider two separate aspects of the problem: (a) the concept that ligand binding may induce certain energetically significant changes in a protein molecule; and (b) the concept that such changes may in some manner provide the driving force for catalysis. In the work described here, we deal only with the first of these two aspects.

B. Scope

Calorimetric studies on the temperature dependence of the $\Delta H^\circ$ of formation of a number of enzyme–coenzyme complexes have uncovered a group of interesting and seemingly related, but quite puzzling phenomena. These phenomena include the anomalous energetic parameters of the glutamate dehydrogenase and liver alcohol dehydrogenase—reduced coenzyme binary complexes; the large negative enthalpies of interaction and heat capacities of interaction for both positively and negatively interacting systems; the wide variations in heat capacities among the various dehydrogenase com-
plexes; and the dramatic effects of the formation of such complexes on the thermal stabilities of the enzymes. I propose here a simple experimentally testable theory that accounts for all of these diverse phenomena in a particularly simple manner, and which indeed appears to actually force the observed behavior.

This proposed model assumes only that the enzyme can exist in either of two physically distinguishable states; that the $\Delta H^o$ between these two states is very large, is temperature independent, and is the same for all forms of any given enzyme (free enzyme and enzyme in any binary or ternary complex); and that the $\Delta G^o$'s of the two-state equilibria differ from one enzyme form to another.

The sections that follow describe the development of the model from the experimental evidence; explore its properties, examine its ability to account for known facts, and predict properties of less studied systems; describe independent evidence in support of the enzyme interconversion process that forms its basis; and speculate on the physical nature of that process.

C. LIGAND-INDUCED PROTEIN CHANGES

To introduce this subject we can hardly do better than to simply restate the views of Gregorio Weber (3).

In a classic paper on "Energetics of Ligand Binding to Proteins" Weber has pointed out that: (a) the study of the interactions of proteins and small ligands provides a means of relating biological function to fundamental physical chemical principles; (b) structural information must be accompanied by corresponding energetic studies if it is to lead to appropriate physical interpretation; (c) the binding of a ligand to a protein cannot be considered as an event apart from the changes in that protein induced by that binding; (d) "we never deal with the binding of a single ligand to a protein"; and (e) therefore, it is impossible for the binding of two ligands to the same protein to be really independent. On this basis, Weber has developed a unified approach to the free energy coupling of multiple binding of ligands to proteins based on the earlier linkage theory of Wyman (2).

D. THERMODYNAMIC PARAMETERS

Changes in free energy, enthalpy, and entropy each contribute a unique kind of information about ligand binding and each has its own inherent limitations. The overwhelming majority of experimental
thermodynamic studies on ligand interactions have been limited to free energy measurements, and the major theoretical treatments on the subject (such as those of Weber and of Jencks) are based on free energy changes alone. Valuable as this approach has been, there are some decided limitations to interpretations based on this one parameter. Rajender and Lumry (5) have pointed out that $\Delta G^\circ$ values are frequently quite insensitive to conformational changes because of compensation between the $\Delta H^\circ$ and $T\Delta S^\circ$ terms. Eftink and Biltonen have discussed a number of cases in which enthalpy measurements reveal such otherwise obscured mechanistic detail (6). Finally, Edsall and Gutfreund (7) have called attention to the fact that in reactions that involve substantial $\Delta S^\circ$ components, $\Delta G^\circ$ is a very temperature dependent parameter and can even change its algebraic sign over a course of a few degrees.

Supplementing $\Delta G^\circ$ measurements with corresponding $\Delta H^\circ$ measurements (aside from providing $\Delta S^\circ$ values) can yield temperature independent $\Delta G^\circ$ values. In favorable cases enthalpy measurements can separate complex reaction processes into high and low enthalpic steps. Finally, the use of two parameters to characterize the nature of a resolved reaction step rather than relying on a single rather insensitive parameter may be of some advantage,* and it is from such enthalpic measurements that the theory presented here has evolved.

Aside from the enthalpic measurements, a second type of phenomenon—protein thermal stability and the sensitivity of that stability to enzyme–ligand complex formation—will engage our attention. In a very recent report by the National Academy of Sciences, a committee of experts (chaired by Frederic Richards) assessed the

* It is this use of enthalpy values that has been heavily criticized by the physical organic chemists. It is quite true, as they frequently point out, that large $\Delta H^\circ$’s most frequently arise from changes in hydration. This renders them quite useless in interpreting phenomena observed in enzyme binding studies in terms of fundamental physical organic theory. Hydration itself, however, is hardly a willy-nilly affair; each type of ligand–enzyme interaction has its own characteristic hydration pattern and thus generates its own characteristic $\Delta H^\circ$–$\Delta S^\circ$ parameters. As with any other experimental signal, when the phenomena remain small, as they do in many cases, interpretation may be impossible. But in cases where strikingly large signals happen to occur, or can be discerned by appropriate dissection, some definitive information about the nature of the interactions may be learned. It is just such a situation that presents itself in the study of ligand binding to pyridine nucleotide dehydrogenases.
most challenging questions that now lie at the interface between chemistry and biology (8). They concluded that the protein chain "folding problem" is foremost among these, and that the question of protein stability represents a facet of that problem. Noting that the thermodynamic stability of proteins is quite marginal at best and is, therefore, very sensitive to small changes, the panel concluded that "the ability to predict [protein] stability is a stringent, but elusive test of theoretical understanding" and one that indeed has some immediate practical applications. Such phenomena will play an important role in the matters that follow here.

II. Fundamental Concepts

A. LEVELS OF ENTHALPIC MEASUREMENTS

The data we discuss will frequently be expressed in such terms as temperature-dependent (or independent) $\Delta H^\circ$'s and temperature dependent (or independent) $\Delta C_P^\circ$'s. Thermodynamic parameters such as these involve a number of levels of differentiation, and that number of levels itself depends on the nature of the experimental method of measurement employed. In Fig. 1 we provide a scheme delineating these relationships. It can be seen that the phenomena involved cover five levels of differentiation, and that the three general approaches differ among each other over three levels of differentiation. The van't Hoff plot, which involves the dependence of an equilibrium constant on temperature, can provide quantitative parameters only for cases I and II. Using the most accurate equilibrium constant measurements available, significant curvature of the line from such a plot will only be apparent when the $\Delta C_P^\circ$ of the reaction exceeds 200 cal K$^{-1}$ mol$^{-1}$. Thus, while a van't Hoff plot may at best provide some indication of the existence of cases 3 through 5, it cannot possibly distinguish between them. The two calorimetric approaches, which measure enthalpies directly at one and two higher levels of differentiation, respectively, can distinguish between the latter four levels of phenomena. The phenomena that concerns us here involve levels 3 through 5, and necessarily require direct calorimetric measurements. The theoretical basis of the individual curves indicated in the figure will be discussed in a later section; here we wish only to distinguish between the levels of phenomena that may be observed.
B. INTERACTION PARAMETERS

Returning to Weber’s expression of interaction energies, or “free energy linkage functions,” as he has termed them, while we find it convenient to use a slightly different formalism here and will require an extension of those concepts to other thermodynamic parameters, our expression of interaction energies does not differ from Weber’s in any fundamental way.

Figure 1. Levels of enthalpic measurement.
The problem is best stated in terms of the classic "thermodynamic square":

\[
\begin{array}{c}
A + EB \xrightarrow{3} EAB \\
A + E \xrightarrow{1} EA \\
B + B
\end{array}
\]

where equilibrium constants \( K_1, K_2, K_3, \) and \( K_4 \) are assigned to the corresponding numbered steps, and \( \Delta G_n = -RT \ln K_n \) are again defined for each step.

We define an interaction parameter \( \Delta G_1 \) as the difference between the free energy of formation of the ternary complex from \( E, A, \) and \( B \) and that of the sum of the free energies of formation of the two binary complexes:

\[
\Delta G_1^o = \Delta G_1^o + \Delta G_4^o - (\Delta G_1^o + \Delta G_2^o) \tag{2}
\]

Since

\[
\Delta G_1^o + \Delta G_2^o = \Delta G_2^o + \Delta G_3^o \tag{3}
\]

\[
\Delta G_1^o = \Delta G_4^o - \Delta G_2^o = \Delta G_3^o - \Delta G_1^o
\]

\[
= -RT \ln \frac{K_4}{K_2} = -RT \ln \frac{K_3}{K_1} \tag{4}
\]

In other words, the \( \Delta G_1^o \) expresses the difference in the free energy of \( A \) binding to the \( EB \) complex over that of \( A \) binding to free \( E \). Of course, the effect of the presence of bound \( B \) on the binding of \( A \) must be identical in sign and magnitude to that of the presence of bound \( A \) on the binding of \( B \). It should be noted that \( \Delta G_1^o \) may be either positive or negative depending on whether \( A \) and \( B \) mutually decrease or increase each other's affinity for the enzyme.

In a previous paper (9) we pointed out that equivalent interaction parameters for other thermodynamic parameters can be derived in an analogous manner. Thus, we define \( \Delta H_1^o = \Delta H_2^o - \Delta H_2^o \) and go on to define \( \Delta S_1^o \) and \( \Delta C_{pl} = \Delta(\Delta H_1^o/dT) \). We find these to be very useful functions.
III. The Problem

A. THE PHENOMENA

1. Thermodynamic Parameters of the Formation of l-Glutamate Dehydrogenase-Ternary Complexes

The complete thermodynamic characterization of the formation of a ternary complex requires the determination of $\Delta G^\circ$, $\Delta H^\circ$, $\Delta S^\circ$, and $\Delta C_p$ of each of the four steps of its thermodynamic square [Eq. (1)]. The determination of these parameters for any pair of opposing sides of the square suffice for the calculation of the set of interaction parameters, as shown by Eq. (4). Since the interaction parameters alone are free of the intrinsic binding energies of individual ligands, we find them to be the most easily interpretable functions for comparing different ternary complexes. The remaining measurements of individual steps are, however, quite useful in themselves in comparing corresponding steps, such as, for example, the enzyme-enzyme binary complexes of a variety of pyridine-nucleotide dehydrogenases. An example of such a complete set of measurements for a typical ternary complex is shown in Fig. 2.* The $\Delta C_p$ profile (calculated from the $\Delta H^\circ$'s measured at 25 and 15°C) is shown in the accompanying panel. Interaction parameters calculated for both a positively and a negatively interacting system, are shown in Fig. 3. Interaction parameters calculated in this fashion for a number of glutamate dehydrogenase ternary complexes are listed in Table I.

Eftink and Biltonen (6) and Hinz (14) have recently reviewed the thermodynamics of protein-ligand interactions; Beaudette and Langerman have reviewed the more limited field of the thermodynamics of nucleotide binding to proteins (15); while Subramanian has reviewed the specific area that is of primary interest here, the thermodynamics of pyridine nucleotide dehydrogenases and their reactions (16).

A large number of $\Delta G_1$ (or free energy linkage functions) have been reported for a wide variety of enzyme ternary complexes. Ef-

* The reader may note that what we have done here is simply to add a vertical coordinate (kcal mol$^{-1}$) to the thermodynamic square. This step produces a three-dimensional figure of the type portrayed by Jencks (4). Since we are plotting three parameters in addition to the $\Delta G^\circ$ parameter used in Jencks diagrams, we, in effect, “cut” the diagram vertically at the “E” coordinate and lay the plot flat, with the E parameters are duplicated at the right and left extremes of the profile.
Figure 2. Thermodynamic profiles for the formation of the E-NADPH-L-glutamate complex. It should be noted that $\Delta G^\circ$ and $\Delta H^\circ$ are plotted in the positive sense while $T\Delta S^\circ$ is plotted in the negative sense so that downward changes in parameters reflect increased binding tendencies. (a) 25°C; (b) 15°C; (c) $C_p$ over the range 15-25°C [from ref. (9)]. R = NADPH; G = L-glutamate.

tink and Biltonen, pointing out that $\Delta H^\circ_I$ and $\Delta S^\circ_I$ values are likely to vary over a much wider range than the -1 to -3 kcal mol$^{-1}$ customarily found for $\Delta G^\circ_I$ values, have noted that only a handful of such $\Delta H^\circ_I$ values have been reported for any enzyme complexes (6). They cite two examples of such studies from their own work—a $\Delta H^\circ_I$ of 40 kcal mol$^{-1}$ for the binding of Mg$^{2+}$ and phosphate to ATPase (17), and an entropy driven interaction between the binding of two protons to RNAse A in which $\Delta G^\circ_I = -4.6$ kcal mol$^{-1}$, $\Delta H^\circ_I = 0$, and $\Delta S^\circ_I = 16$ cal K$^{-1}$ mol$^{-1}$ (18). Valdes and Ackers (19) have measured $\Delta H^\circ_I$'s to study the linkage between oxygenation and subunit association of hemoglobin dimers.
Figure 3. Interaction parameters for the enzyme–NADPH–l-glutamate complex (a positive interaction) and for the enzyme–NADPH–ADP complex (a negative interaction). Data are from refs. (9) and (10).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$\Delta G^\circ$ (kcal mol$^{-1}$)</th>
<th>$\Delta H^\circ$ (kcal mol$^{-1}$)$^a$</th>
<th>$\Delta C_p^\circ$ (cal K$^{-1}$ mol$^{-1}$)$^a$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 l-Glutamate</td>
<td>-1.9</td>
<td>-7.2</td>
<td>-270</td>
<td>9</td>
</tr>
<tr>
<td>2 D-Glutamate</td>
<td>-2.9</td>
<td>-10.2</td>
<td>-330</td>
<td>9</td>
</tr>
<tr>
<td>3 Glutarate</td>
<td>+0.7</td>
<td>-10.9</td>
<td>-230</td>
<td>9</td>
</tr>
<tr>
<td>4 ADP</td>
<td>+1.0</td>
<td>-5.4</td>
<td>-40</td>
<td>10</td>
</tr>
<tr>
<td>5 AMP</td>
<td>+0.1</td>
<td>-2.0</td>
<td>+60</td>
<td>10</td>
</tr>
<tr>
<td>6 ATP</td>
<td>+0.5</td>
<td>-0.3</td>
<td>+200</td>
<td>10</td>
</tr>
<tr>
<td>7 Adenosine</td>
<td>+0.5</td>
<td>+0.8</td>
<td>+230</td>
<td>10</td>
</tr>
<tr>
<td>8 a-Ketoglutarate</td>
<td>-3.0</td>
<td>-16.4</td>
<td>-360</td>
<td>11</td>
</tr>
<tr>
<td>9 Oxalylglycine</td>
<td>-1.0</td>
<td>-15</td>
<td>-483</td>
<td>12</td>
</tr>
</tbody>
</table>

$^a$ These values have been recalculated from the data in the cited references using the more accurate values of $\Delta H_f^{298} = 2.3$ kcal mol$^{-1}$ and $\Delta H_f^{15} = 4.0$ kcal mol$^{-1}$ of ref. (13) for $E + R \rightleftharpoons ER$.  

10
A number of studies on proton ionization-linked ligand binding interactions that do include enthalpic interaction information include those of Ginsberg and co-workers on glutamine synthetase (20,21); a substantial literature on aspartate transcarbamoylase from the laboratories of Allewell and co-workers (22-24), Gerhart and Schachman (25), and Ginsberg and co-workers (26), and a report on tryptophan synthetase by Hinz and co-workers, which represents one of the very few cases in which $\Delta C_{pl}$'s are provided (27).

Regrettably, aside from the data on glutamate dehydrogenase complexes listed in Table I, such sets of interaction parameters for pyridine nucleotide dehydrogenase ternary complexes do not appear to be available.

2. Thermodynamic Parameters for Individual Steps in the Formation of Other Pyridine Nucleotide Complexes

Despite the lack of interaction parameter data for other pyridine nucleotide dehydrogenase complexes, a number of enthalpic measurements for individual steps in such systems have been reported, and those for the formation of dehydrogenase–coenzyme binary and ternary complexes are listed in Table II.

3. Phenomenological Patterns

Comparing the properties of the various glutamate dehydrogenase binary and ternary complexes with each other and with those of other closely related enzymes, we observe a number of striking patterns:

1. The formation of most pyridine nucleotide-linked dehydrogenase—NAD(P)H binary complexes are characterized by large negative $\Delta H^0$'s ($-7$ to $-15$ kcal mol$^{-1}$), almost equally large negative $\Delta S^0$'s ($-3$ to $-13$ eu) and variable, but frequently very substantial, negative $\Delta C^0$'s ($-200$ to $-750$ cal K$^{-1}$ mol$^{-1}$) (31). Those of beef liver glutamic dehydrogenase and horse liver alcohol dehydrogenase and (probably) octopine dehydrogenase, however, have negligible $\Delta H^0$'s ($0$ to $+2$ kcal mol$^{-1}$), and large positive $\Delta S^0$'s ($+20$ to $+30$ eu) and only very modest $\Delta C^0$'s at 20$^\circ$ ($-160$ and $-100$ cal K$^{-1}$ mol$^{-1}$, respectively). It may also be noted that the glutamate dehydrogenase-reduced coenzyme binary complex is about one to two orders of magnitude weaker than those of most
<table>
<thead>
<tr>
<th>Reactant</th>
<th>Ligand</th>
<th>$\Delta H^\circ$ (kcal mol$^{-1}$)</th>
<th>$\Delta S^\circ$ (cal K$^{-1}$ mol$^{-1}$)</th>
<th>$\Delta C_p$</th>
<th>Temperature Range (°C)</th>
<th>References</th>
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<tr>
<td>Bovine liver GDH</td>
<td>NADPH</td>
<td>+2.2$^b$</td>
<td>+30</td>
<td>$-70^b$</td>
<td>1–25</td>
<td>13</td>
</tr>
<tr>
<td>Mussel</td>
<td>ODH$^c$</td>
<td>NADH</td>
<td>0</td>
<td>$&lt;-90$</td>
<td>2–40</td>
<td>28</td>
</tr>
<tr>
<td>Mussel</td>
<td>ODH$^c$</td>
<td>NAD$^+$</td>
<td>0</td>
<td>$+16$</td>
<td>6–38</td>
<td>28</td>
</tr>
<tr>
<td>Horse liver</td>
<td>ADH</td>
<td>NADH</td>
<td>0$^d$</td>
<td>$-110^c$</td>
<td>6–35</td>
<td>29, 30</td>
</tr>
<tr>
<td>Horse liver</td>
<td>ADH</td>
<td>NAD$^+$</td>
<td>+1.0$^b$</td>
<td>+12</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td>ADH</td>
<td>NADH</td>
<td>$-9.5$</td>
<td>$-11$</td>
<td>$-270^{b,e}$</td>
<td>15–35</td>
</tr>
<tr>
<td>Yeast</td>
<td>ADH</td>
<td>NAD$^+$</td>
<td>$-9$</td>
<td>$-15$</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>Pig muscle</td>
<td>LDH</td>
<td>NADH</td>
<td>$-7.5^b$</td>
<td>$-2^b$</td>
<td>$-325$</td>
<td>10–35</td>
</tr>
<tr>
<td>Pig muscle</td>
<td>LDH</td>
<td>NAD$^+$</td>
<td>$-7$</td>
<td>$-7$</td>
<td>$-122$</td>
<td>34</td>
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<tr>
<td>Pig heart</td>
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<td>NADH</td>
<td>$-10.6$</td>
<td>$-11$</td>
<td>$-182$</td>
<td>10–35</td>
</tr>
<tr>
<td>Pig heart</td>
<td>LDH</td>
<td>NAD$^+$</td>
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<td>$-4$</td>
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<td>35</td>
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<td>Beef heart</td>
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<td>NADH</td>
<td>$-9.7$</td>
<td>$+3$</td>
<td></td>
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<tr>
<td>Beef heart</td>
<td>LDH</td>
<td>NAD$^+$</td>
<td>$-8.5$</td>
<td>$-14$</td>
<td></td>
<td>29, 31</td>
</tr>
<tr>
<td>Rabbit muscle</td>
<td>LDH</td>
<td>NADH</td>
<td>$-7.0$</td>
<td>$-2$</td>
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<tr>
<td>Source</td>
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<td>Enzyme 3</td>
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<tr>
<td>Rabbit muscle</td>
<td>LDH</td>
<td>NAD⁺</td>
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<tr>
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<td>m-MDH</td>
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<td></td>
<td></td>
<td>−12.1</td>
<td>29</td>
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<td>Pig heart</td>
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<td>29, 31</td>
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<tr>
<td>Pig heart</td>
<td>s-MDH</td>
<td>NADH</td>
<td></td>
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<td>36</td>
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<tr>
<td>Pig heart</td>
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<td>−290</td>
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<td>36</td>
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<tr>
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<td>GPDH</td>
<td>NAD⁺</td>
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<td>−522ᵇ</td>
<td>17ᵇ</td>
<td>5–40</td>
</tr>
<tr>
<td>Rabbit skeletal</td>
<td>GPDH</td>
<td>NAD⁺</td>
<td>−17.5ᶠ</td>
<td>−220ᶠ</td>
<td>−29ᶠ</td>
<td>5–25</td>
</tr>
</tbody>
</table>

**Ternary Complexes**

<table>
<thead>
<tr>
<th>Source</th>
<th>Enzyme 1</th>
<th>Enzyme 2</th>
<th>Enzyme 3</th>
<th>Enzyme 4</th>
<th>pH</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Pig heart</td>
<td>LDH–NADH</td>
<td>Oxamate</td>
<td></td>
<td>−405</td>
<td>10–35</td>
<td>35</td>
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<tr>
<td>Pig heart</td>
<td>LDH–NAD</td>
<td>Oxalate</td>
<td></td>
<td>−340</td>
<td>10–35</td>
<td>35</td>
</tr>
<tr>
<td>Bovine liver</td>
<td>GDH–NADPH</td>
<td>L-Glutamate</td>
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<td>−370</td>
<td>15–25</td>
<td>9</td>
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<tr>
<td>Bovine liver</td>
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<td>D-Glutamate</td>
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<td>−450</td>
<td>15–25</td>
<td>9</td>
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<tr>
<td>Bovine liver</td>
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<td>α-Ketoglutarate</td>
<td>−17</td>
<td>−240</td>
<td>15–25</td>
<td>11</td>
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</tbody>
</table>

*a* Abbreviations: GDH = glutamate dehydrogenase; ODH = octapine dehydrogenase; ADH = alcohol dehydrogenase; LDH = lactic dehydrogenase; MDH = malic dehydrogenase; s = soluble; m = mitochondrial; GPDH = glyceraldehyde phosphate dehydrogenase.

ᵇ pH-dependent value.

ᶜ From van't Hoff equation measurements.

ᵈ pH-independent value.

ᵉ Unpublished results of S. Subramanian.

ᶠ Cooperative behavior.
pyridine nucleotide dehydrogenases (particularly the mammalian enzymes).

2. On the other hand, the ternary complexes of glutamate dehydrogenase are characterized by large $\Delta H^\circ$ values which are invariably negative without regard to the sign of the corresponding $\Delta G^\circ$ for that process (as can be seen in Fig. 3).

3. The $\Delta C_{pl}^\circ$ values for the ternary complexes of glutamate dehydrogenase are typically very large and negative in sign (as demonstrated in Fig. 3), a property shared by most pyridine nucleotide dehydrogenase complexes (35). However, we observe a few glutamate dehydrogenase—NADPH ternary complexes that show positive $\Delta C_{pl}^\circ$ values. This is rather puzzling since $\Delta C_{pl}^\circ$ values for the binding of any ligand to any protein are invariably negative in sign (6).

4. Finally, we note that bovine liver glutamate dehydrogenase and horse liver alcohol dehydrogenase, both of whose reduced coenzyme binary complexes show enthalpic and $\Delta C_{pl}^\circ$ anomalies as noted above, also show unusually high degrees of thermal stability. In the case of both enzymes, binary and ternary complex formation cause very substantial changes in the thermal stability of the protein. Grisolia was the first to point out that the thermal stability of glutamate dehydrogenase was actually decreased by the binding of its reduced coenzyme (39), and his observations have been confirmed by us in the course of the studies reported below. The free enzyme in phosphate buffer or in 0.1 M sulfate can be heated at $56^\circ$C for over a minute without substantial loss of activity. Indeed, that procedure is used in its purification. On the other hand, the glutamate dehydrogenase—NADPH complex is rapidly denatured at temperatures above $37^\circ$C. Each specific binary and ternary complex of this enzyme, in fact, appears to have its own special pattern of thermal stability. On the other hand, the high thermal stability of free horse liver alcohol dehydrogenase is further increased by the formation of its reduced coenzyme-binary complex (40).

B. SPECIFIC QUESTIONS

The considerations we have just described frame the problem to which we seek a unified and experimentally testable answer: (1) Why are the binary reduced coenzyme complexes of glutamate, liver alcohol, and octopine dehydrogenases energetically anomalous? (2) What is the cause of the large interaction parameters, and why are
FORMATION OF DEHYDROGENASE-LIGAND

\[ \Delta H_i^o \text{ and } \Delta C_p^o \text{ negative for both positively and negatively interacting systems?} \]

(3) What is the cause of the large negative \( \Delta C_p^o \)'s observed in the formation of pyridine nucleotide dehydrogenase complexes (4)? Why does the formation of coenzyme–dehydrogenase complexes dramatically change the thermal stability of the enzyme?

IV. The Answer

A. THE DEVELOPMENT OF THE MODEL

1. An Initial Clue

An initial clue to the nature of the answer came from a fine-grained study of the temperature dependence of the enthalpy of formation of the glutamate dehydrogenase–NADPH complex. The data from our initial report on this study (13) are shown as the open circles in Fig. 4. The marked curvature of the dependence of \( \Delta H^o \) on temperature indicated that not only does the system have a large \( \Delta C_p^o \), but that this \( \Delta C_p^o \) is itself temperature dependent, approaching a value of 0 at low temperatures but reaching a value of \(-450\) cal \( K^{-1} \text{ mol}^{-1} \) at higher temperatures. In that earlier paper we were able to show that this large and very nonlinear temperature dependence could be quantitatively accounted for by assuming the existence of an "implicit" (hidden) step involving an equilibrium between two states of the free enzyme:

\[
L + K_0 \xrightarrow{K_1} EL
\]  

(4)

where \( E \) and \( E' \) are two forms of the enzyme. Since we are concerned here only with thermodynamic state functions and, therefore, only with equilibrium measurements, the results are of course independent of the pathway chosen. For simplicity, let us arbitrarily assume that the reaction takes place in two sequential steps:

\[
E \xrightarrow{K_0} E' \quad \text{where } K_0 = [E']/[E]
\]

(5)

\[
E' + L \xrightarrow{K_1} EL \quad \text{where } K_1 = [EL]/[E'] [L] \text{ and } \Delta H_0^o \text{ and } \Delta H_i^o \text{ refer to the two respective steps}
\]

(6)
Figure 4. The temperature dependence of the observed $\Delta H$ of formation of three complexes of glutamate dehydrogenase. All measurements were carried out in 0.1M phosphate buffer, pH 7.6 as described in ref. (1). The E-R curve is based on data previously published [Ref. (10)] above 7.7°C supplemented by five new points covering the range from 1.1 to 7°C.

If we are unaware of the existence of the enzyme isomerization step, and write the reaction simply as

$$E + L \rightleftharpoons EL$$

(7)

then (assuming that neither step of such a reaction has a finite $\Delta C_P^0$ in itself), the observed $\Delta H^0$ will be expressed by an equation described by Eftink and Biltonen (6) and by ourselves (13):

$$\Delta H(T) = \Delta H_1^0 + \frac{\Delta H_0^0}{1 + K_0}$$

(8)

where $K_0 = \exp[\Delta H_0^0(T - T_0)/RT_0]$ and $T_0$ is that temperature at which $K_0 = 1$ and $\Delta G_0^0 = 0$. The temperature dependence of the observed $\Delta H$ is shown in Fig. 5.

It can be seen from Fig. 5 that at any temperature very much higher than $T_0$, the free enzyme is almost entirely in the E' form, the observed $\Delta H = \Delta H_1^0$, and no $\Delta C_P^0$ is observed. On the other hand, at any temperature very much lower than $T_0$, the free enzyme
FORMATION OF DEHYDROGENASE-LIGAND

is almost completely in the E form and the overall reaction must now include both steps. The observed $\Delta H$, therefore, equals the sum of $\Delta H_1^0 + \Delta H_2^0$. Again, no $\Delta C_p$ would be observed. If the experimental temperature coincides with $T_0$ itself, the free enzyme is equally distributed between the two forms and $\Delta H = \Delta H_1^0 + \frac{1}{2} \Delta H_2^0$. Now a very substantial $\Delta C_p$ will be observed. It should be noted that when $T = T_0$, $\Delta H$ will appear to be a very nearly linear function of temperature over a range of about 40°. (The pronounced curvatures present in Fig. 5, are experimentally observable only when $T_0$ is close to 0°C or when it occurs in the 35 to 45°C range.)

The magnitude of the apparent $\Delta C_p$ generated by the effect of varying temperature on this implicit equilibrium is

$$\Delta C_p = \frac{\partial \Delta H_0}{\partial T} \frac{K_0(\Delta H_0^0)^2}{(1 + K_0)^2RT^2}$$  \hspace{1cm} (9)

This relationship is plotted for several assumed values of $\Delta H_0^0$ in Fig. 6. It can be seen from the figure that if a $\Delta C_p$ of 300 cal K$^{-1}$ mol$^{-1}$ is observed over a small temperature increment, it indicates the presence of an implicit step having a $\Delta H_0^0$ of 15 kcal mol$^{-1}$ or

Figure 5. The temperature dependence of the observed $\Delta H$ for a coupled, hidden two-state system $E \rightleftharpoons E' \rightleftharpoons EL$. It is assumed that [L] is present at saturating conditions.
any value above that number; it would be inconsistent with any \( \Delta H^\circ < 15 \text{ kcal mol}^{-1} \), however.

Equation (9) has a particularly interesting feature that will be of some importance for our further interpretation. The isomerization of a reactant form must produce a \( \Delta C_p^\circ \) that is negative in sign; the isomerization of a product form must produce a \( \Delta C_p^\circ \) that is positive in sign. These rules are completely independent of the algebraic sign of the \( \Delta H^\circ \) itself. Indeed the data of our earlier paper were fitted very closely by Eq. (3) with \( \Delta H_1^\circ = 4.73 \text{ kcal mol}^{-1} \), \( \Delta H_0^\circ = -20.1 \text{ kcal mol}^{-1} \), and \( T_0 = 43.6^\circ \text{C} \). This fit is shown by the solid line in the ER curve of Fig. 4. It may be noted that the original data from the earlier paper neither demanded the assumption of a two-state equilibrium for the E-NADPH binary complex nor, indeed, provided any evidence for the existence of such a complex.

Thus, we see that in certain circumstances an overall reaction may exhibit a very substantial experimentally measured \( \Delta C_p^\circ \), even though each individual step in that reaction has a real \( \Delta C_p^\circ \) of zero. The source of this fictitious \( \Delta C_p^\circ \) is worth examining. It arises, in fact, from an improper calculation of a difference in state functions. A \( \Delta H^\circ \) is properly equal to the heat release to the system during the conversion of 1 mol of reactant to 1 mol of product in a reversible manner. If the two-step nature of the reaction is known, and the
equilibrium constant for the isomerization step is available from other measurements, then the very obvious correction for the temperature dependence of the extent of the isomerization would be made; the two temperature dependent $\Delta H^\circ$'s would be calculated in the manner indicated by Eq. (8); and no $\Delta C_p$ would be reported. It is our ignorance of the poised equilibrium and the resulting assignment of a molar $\Delta H^\circ$ to a reaction involving a less than complete molar conversion which creates the $\Delta H^\circ_p$ phenomenon.

The concept that implicit equilibria can in themselves create observed $\Delta C_p^\circ$'s has been around for a long time. Hepler and co-workers, in developing general equations for such systems (41) points out that the idea itself was expressed by McCollum (42) in 1927 and discussed by Randall and Taylor in 1941 (43).* In more recent times Sturtevant has developed equations for the contributions of two-state processes to observed $\Delta H^\circ$'s and $\Delta C_p^\circ$'s (44) and included such effects as one of five possible mechanisms for explaining large $\Delta C_p^\circ$'s observed in protein systems (45). Prior to the demonstration of the fit of a "fine-grained" temperature dependence of an enzyme–ligand binding enthalpy shown in Fig. 4 (13), however, this class of explanation of the source of large $\Delta C_p^\circ$ effects in enzyme binding has not really received a great deal of attention. In view of this evidence, however, it is probably not going too far to state that whenever large $C_p$ effects are observed in enzyme–ligand binding reactions, they are very probably due solely to the presence of an unrecognized internal step that leads to an improper calculation of the $\Delta H^\circ$. In such cases, then, it is quite futile to seek an explanation that is based on hypothetical potential sources of $\Delta C_p^\circ$ effects themselves. It is more appropriate to consider the properties of the newly revealed implicit step as a basis for comparison with chemical bonding models. Equation (8) itself imposes some quite stringent requirements on the thermodynamic features necessary for the production of the phenomenon shown in Fig. 5. That equation requires that, first of all, the implicit step must have a very large positive $\Delta H^\circ$. Figure 7, however, demonstrates that for the phenomenon to be observed, a second requirement must be met: The $T_0$ must be "poised" in or near the 0 to 40°C temperature range. Of course, $T_0$ is essentially a free energy parameter, and it is clear from Eq. (8),

* We are grateful to Loren Hepler for bringing these papers to our attention.
that to be "poised" the reaction must have a $\Delta G^o$ that is close to zero in the 0 to 40°C range. Under these conditions, $\Delta H^o \approx T\Delta S^o$, and thus, the very large positive $\Delta H^o$ must be accompanied by a compensating very large positive $\Delta S^o$. Thus, in interpreting observed large negative temperature dependent $\Delta C_p$'s, we should be looking for reaction steps (or combinations of reaction steps) that could conceivably occur in or on the protein molecule, and that could be accompanied by $\Delta H^o$'s $> + 20$ kcal mol$^{-1}$ and $\Delta S^o$'s $> + 70$ cal K$^{-1}$ mol$^{-1}$.

2. A Paradox

The close agreement between theory and experiment and the existence of the enzyme isomerization that it implied, seemed to point to a reasonable explanation for the first question posed above and appeared to have at least some bearing on the remaining three.

To pursue this point further we have carried out studies analogous