STUDIES IN MODERN THERMODYNAMICS 1

BIOCHEMICAL THERMO-DYNAMICS

M.N. JONES
(EDITOR)

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EDITOR'S PREFACE

In recent years there has been a considerable increase in the application of thermodynamic techniques to biochemical and biological problems. The objective of this book is to present in a single volume accounts of some of these thermodynamic studies. The topics range from work on simple molecules of biochemical importance and macromolecules of biological origin to cellular systemsand metabolism. It should be noted that at the present time not all these aspects of biochemical thermodynamics have reached comparable levels of development thus work on cellular systemsis in its infancy compared with the degrees of sophistication which can be achieved with small molecule systems. Similarly of the important biological macromolecules, proteinsand nucleic acids have received more attention than polysaccharides.

My initial vision of this volume consisted of a spectrum of chapters commencing with a rigorous treatment of the simple molecules and progressing to the more complex problems of specialized structures and concluding with an appraisal of the application of thermodynamics in metabolism. I am greatly indebted to all the contributors who have willingly given their time and effort to make this vision a reality and who have cooperated with me so readily. I thank Dr. H.A. Skinner of the Department of Chemistry at Manchester for his encouragement and also for his assistance with the editing of Chapter 3, Miss Margaret Barber for typing the first drafts of Chapters 1, 6 and 10 and for general secretarial assistance and Mr. Richard Littlemore for photographic work.

Finally it is a pleasure to acknowledge the vital part played by Mrs. Irma Farnsworth who undertook the difficult task of producing the camera-ready copy; her help and great attention to detail has been invaluable, she brought to my notice many inconsistencies in the text which otherwise would have been overlooked.

Despite every effort to produce a perfect copy I take full responsibility for any remaining errors in the text. I regret that while being well aware of the recommendations for measurement and presentation of biochemical equlibrium data prepared by the Interunion Commission on Biothermodynamics (Biochemical Journal (1977) 163, 1), as a consequence of contributors reproducing material from original sources it has not always been possible to use exclusively SI units throughout the text. For these shortcomings I beg the readers indulgence.

Manchester June 1978 Malcolm N. Jones

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THE SCOPE OF THERMODYNAMICS IN BIOCHEMISTRY

CHAPTER 1

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1. INTRODUCTION

Biochemistry may be defined as the study of living organisms at the molecular level. It is concerned with the chemical composition and structure of the molecules and ions of which living matter is composed and the way in which these molecular species are synthesised, arranged in the organism to form structural entities and the way in which they can react to maintain the integrity of the organelles, cells and tissues of the many millions of living species which have evolved. It is a relatively modern science since understandably some of the major developments in the subject have depended heavily on prior progress in pure chemistry, biology and physics. An obvious example of this is the application of X-ray diffraction to determine the structure of proteins and nucleic acids. Developments in X-ray diffraction and methods of isolating and characterising macromolecules from living organisms being essential prerequisites.

Classical thermodynamics is concerned with the conversion of one kind of energy into another and with the relationships between experimental quantities pertaining to systems at equilibrium. It forms the basis for quantifying the effects of temperature, pressure and concentration on the positions of chemical equilibria. It is not concerned with the rate at which processes occur. Time has no place in classical thermodynamic considerations but is introduced under the separate heading of 'non-equilibrium' or 'irreversible' thermodynamics. Furthermore, thermodynamic relationships are independent of assumptions regarding the molecular nature of matter and in a rigorous sense tell us nothing about the mechanisms of chemical or biochemical reactions but only the changes which occur on going from initial to the final states. Nevertheless in the hands of physical chemists the concepts of Gibbs free energy, enthalpy and entropy applied to individual atomic and molecular species in precisely defined standard states have played and continue to play key roles in the understanding of the energetics of chemical reactions and other physico-chemical processes.

Living systems are dynamic in nature consisting of a multitude of interrelated and interdependent biochemical reactions which are not in general at equilibrium but more often are in a steady state. If we consider a particular organism such

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as a cell as a thermodynamic system then we find immediately that our system is, in rigorous thermodynamic terms, 'open'; it is exchanging matter and energy with its surroundings. Open non-equilibrium systems are the province of irreversible thermodynamics and at present the applications of this science to biochemistry have not been very numerous. These observations may lead one to the view that classical thermodynamics might have rather a limited role to play in biochemistry and in fact this view has been expressed in the literature (Banks, 1969). However it is noteworthy that of necessity the vast majority of 'chemical' reactions which are carried out are not under conditions of constant temperature and pressure nor are they maintained in a state of equilibrium and yet thermodynamics has become an invaluable tool in pure chemistry. Likewise in biochemistry there has developed a field concerned with the study of biochemically important processes by the application of classical thermodynamics. Such studies discussed in detail in subsequent chapters range from the thermodynamics of small molecules of biochemical significance (Chapter 2), studies of the important macromolecules in living systems such as proteins (Chapter 3), nucleic acids (Chapter 4), polysaccharides (Chapter 5) to subcellular organelles such as membranes (Chapter 6). Cellular systems are the subject of Chapters 8 and 9 and the over all thermodynamics of metabolism is dealt with in Chapter 11. Analyses of the complexity of the all important binding of oxygen to haemoglobin and the thermodynamic aspects of muscle contraction are the sujects of Chapters 7 and 10 respectively.

2. THE THERMODYNAMIC APPROACH

It is not the purpose of this introductory chapter to go into the exhaustive detail of either the basis of classical thermodynamics nor of its numerous applications in biochemistry which are dealt with in the succeeding chapters. It is of interest however to briefly outline the general thermodynamic approach and to draw attention to some significant developments in experimental techniques and to some of the particular difficulties associated with biochemical problems.

In broad terms the thermodynamic description of a process involves the determination of the Gibbs free energy change ΔG, the enthalpy change, ΔH, and the entropy change, ΔS, for the process under specified conditions. The use of Gibbs free energy is appropriate under conditions of constant temperature and pressure since under these conditions if a process occurs spontaneously (i.e. irreversibly) then ΔG will be negative. This is the basis for assessing the thermodynamic feasibility of a process at constant temperature and pressure. A reaction in which the free energy change is large and negative will, assuming it is kinetically feasible, have an equilibrium on the side of the products. The equilibrium will favour the reactants if the free energy change is large and positive. For a system at equilibrium $\Delta G = 0$. The calculation of ΔG for a particular process gener-

ally involves the measurement of concentrations under given conditions and the application of an equation of the form

$$\Delta G = \Delta G^{o} + RT \ln \frac{\Pi C_p}{\Pi C_r} \qquad (1)$$

in which ΠC_p and ΠC_r refer to the products of the concentrations of 'products' and 'reactants' respectively, each raised to the appropriate power as required by the stoichiometric equation for the process. Eq.(1) assumes the reactants and products behave ideally, if this is not so then activity coefficients must be introduced. When the system is in equilibrium $\Delta G = 0$ and hence

$$\Delta G^{o} = -RT \ln \left(\frac{\Pi C_p}{\Pi C_r} \right)_{eq} = - RT \ln K \qquad (2)$$

where the concentrations are those pertaining to the equilibrium state from which the equilibrium constant K can be calculated. ΔG^{o} refers to the Gibbs free energy change for the formation of the products in their standard states from reactant in their standard states. For condensed systems such as solutions the standard state depends on the concentration-scale adopted. Conventionally, a one molar hypothetical ideal solution is often used. Measurement of the equilibrium constant K for any given process thus gives us directly a value of ΔG^{o}. Since ΔG^{o} is related to the corresponding standard enthalpy and entropy changes by the equation

$$\Delta G^{o} = \Delta H^{o} - T\Delta S^{o} \qquad (3)$$

measurement of either ΔH^{o} or ΔS^{o} enables all three parameters to be obtained. ΔH^{o} is the quantity readily amenable to experimental measurement either directly by a calorimetric method or indirectly from the temperature coefficient of the equilibrium constant by use of the Gibbs-Helmholtz equation

$$\Delta H^{o} = \left\{ \frac{\partial \frac{\Delta G}{T}}{\partial \frac{1}{T}} \right\}_P = R \left\{ \frac{\partial \ln K}{\partial 1/T} \right\}_P \qquad (4)$$

For many systems particularly biological ones application of eq.(4) to give the so-called van't Hoff enthalpy is hampered by the limited temperature range available over which K can be measured. This problem is discussed in more detail in Chapters 2 and 3. For example, for processes involving proteins in aqueous solution which are susceptible to denaturation at temperatures much above the physiological temperature a working range of $\sim 0\text{-}50^{o}$ is all that can be utilized.

This severely limits the precision with which ΔH^{o} can be obtained by use of eq. (4). For this reason direct calorimetric measurements are often to be preferred for the determination of enthalpy changes.

2.1 Calorimetry

It is an interesting historical fact that one of the earliest biochemical calorimetric experiments was done by Lavoisier in collaboration with the mathematician and astronomer Laplace. These experiments were inspired by the idea that respiration could possibly be likened to a combustion process so that the heat produced on exhalation of a known amount of carbon dioxide by an organism should be equal to that produced by oxidation of the corresponding amount of carbon. The experiments were done in 1782-83 and involved measuring the amount of ice melted and CO_2 produced by a guinea pig over a given time period (10 hrs) and comparing this with the heat produced over the same time period on combustion of the equivalent amount of carbon. The experiments did not however support the initial hypothesis, more heat was produced by the guinea pig for a given amount of exhaled carbon dioxide than was produced on combustion of the carbon. At the time Lavoisier suggested that the chilling effect of the ice increased the heat output of the guinea pig and modified his results accordingly, whereas the correct explanation which he later proposed is that the animal exhaled less carbon dioxide than would be predicted by the measured amount of inhaled oxygen (Culotta, 1972). These early experiments were the first steps into the field of the energetics of metabolism. The total energies involved in the experiments corresponded to those required to melt about 15 ozs (435g) of ice, which is of the order of 140 kJ or 14 kJ hr^{-1}.

The impetus to develop very sensitive calorimeters followed the discovery of radioactivity. Thus Pierre Curie developed a twin Dewar vessel 'microcalorimeter' in 1903 which he used to measure the heat output from radium bromide ($\sim$ 400 J g^{-1} hr^{-1}). The use of the twin system in which the process of interest is carried out in one vessel (the sample vessel) while unwanted heat effects are compensated for in an identical reference vessel was a feature of many subsequent designs of calorimeter and is still of great value. Over the years many designs of sensitive calorimeters were used, a very notable advance was made by Calvet who developed the twin conduction microcalorimeter which is commonly used today (Calvet, 1958). With this type of instrument it is possible to measure enthalpy changes of only a few mJ.

There is little doubt that the commercial development of microcalorimeters initially by Beckman based on the design of Benzinger (1965) and by LKB-Produckter based on the design of Wadsö (1968) have been of immense value in transferring the precise measurement of small enthalpy changes onto a more routine basis.

This has been of particular value in the area of biochemical microcalorimetry because of the limitations which are often imposed by the availability of many biological materials. The modern physical biochemist now has a wide choice of microcalorimeters which can be used to study the thermodynamics of biological systems (Reid, 1976). Nevertheless, the study of such systems often stretches the equipment to the limit of its sensitivity and also presents the biochemist with challenging problems of experiment control and design. To illustrate some of these problems it is useful to consider some specific examples.

2.2 Thermodynamics of binding to membrane-bound proteins

Studies on membrane systems are the subject of Chapter 6. The main purpose of this section is to illustrate some of the idiosyncrasies of thermodynamic measurements on biological systems. The choice of membrane protein-ligand interaction is partially a consequence of the authors interest in the subject and partially because the problem can perhaps be regarded as being fairly centrally placed in the spectrum of studies represented at one end by measurements on precisely defined systems consisting of completely characterised molecules and at the other end by whole organisms.

The 'lac operon' of E.coli has a special place in the main stream of biochemistry, since it represents the classical example of enzyme regulation and control. According to the model of Jacob and Monod (1961) the 'lac operon' consists of three closely linked genes which code for three enzymes, thiogalactoside transacetylase, β-galactosidase and the galactoside permease responsible for galactoside transport across the E.coli membrane - the so-called M-protein. The synthesis of the three enzymes is under the control of an operator gene which can be blocked with a repressor protein resulting in inhibition of transcription. In the presence of an external inducer which combines with the repressor protein, the operator gene is unblocked and enzyme synthesis can proceed. Amongst the various strains of E.coli are those designated as i^+ which in the absence of galactoside inducers have low levels of the three enzymes whereas as i^- strains normally have high levels of the enzyme but are not inducible.

The synthesised M-protein is incorporated into the cell membrane where it actually transports galactosides. Belaich et al. (1976) have investigated the thermodynamics of the binding of thiodigalactoside, TDG (D-galactopyransoyl-β-thiogalactopyranoside) to the M-protein using equilibrium dialysis and microcalorimetry. Equilibrium dialysis was used to measure the binding of TDG to the M-protein from which the equilibrium constant, K, and hence ΔG° for the reaction

$$M + TDG \rightleftharpoons M.TDG \qquad (5)$$

was obtained and ΔH was measured by microcalorimetry.

It would be impossible to determine either K or ΔH using suspensions of whole E. coli since clearly TDG could conceivably bind and react with other cellular proteins as well as the M-protein. Since the solubilization and purification of the M-protein has not yet been achieved the experiments were carried out on preparations of membrane vesicles which can be produced by either lysozyme-ethylenediaminetetraacetate treatment or sonication. Thus the environment of the M-protein is the bilayer membrane of the vesicles and from the nature of the preparation we might expect it would not be the only protein present in this form.

From the chemical point of view the suspensions are very heterogeneous. It is thus of great importance to establish that the measurements being made correspond to the process represented by eq.(5).Choice of the correct control experiments is clearly essential. The measurement of the equilibrium constant by equilibrium dialysis involves equilibrating tritium labelled TDG against a membrane vesicle suspension; from the known total amount of TDG in the system and the measured free TDG concentration, $(TDG)_f$, the bound TDG concentration $(TDG)_b$ can be calculated and related to the equilibrium constant for the above reaction by the equation.

$$\bar{\nu} = \frac{[TDG]_b}{[M]_T} = \frac{n\,[TDG]_f}{K_d + [TDG]_f} \qquad (6)$$

where $\bar{\nu}$ is the average number of moles of TDG bound per mole of M-protein, n is the number of moles of binding sites per mole of M-protein and K_d is the dissociation constant for the complex M.TDG. Eq.(6) is based on the Adair equation for multiple equilibria involving n identical independent binding sites (Adair,1925).

Rearrangement of eq.(6) gives

$$\frac{1}{[\bar{TDG}]_b} = \frac{K_d}{n[M]_T} \cdot \frac{1}{[\bar{TDG}]_f} + \frac{1}{n[\bar{M}]_T} \qquad (7)$$

The double reciprocal plot based on eq.(7) gives K_d from the slope/intercept. The intercept gives the number of moles of binding sites per dm^3 of the membrane vesicle suspension if $[M]_T$ is in moles/dm^3. Since the molecular weight of the M-protein is not known $[M]_T$ cannot be calculated and n[M] must be expressed either as sites/dm^3 or sites/mg of membrane protein.

Returning now to the problem of suitable controls, since the level of M-protein in the i^+ strains of E.coli should be low in the absence of inducers it is possible to compare directly the binding of TDG to vesicles prepared from the membranes of induced and non-induced i^+ E.coli; isopropyl-β-thiogalactopyranoside

is a suitable inducer. Fig.1 shows the binding of TDG to three cell strains.

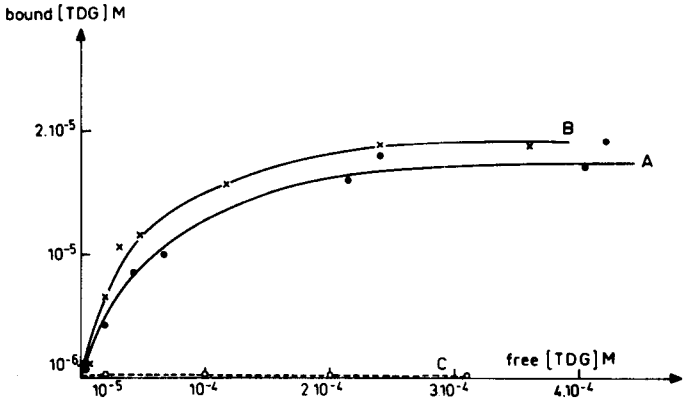

Fig.1 Binding of thiodigalactoside to membrane vesicles. Curve A *E.coli* (i^-), curve B *E.coli* ($i^{\mp}$) induced and curve C *E.coli* (i^+) not induced. (Belaich *et al.*, 1976). Reproduced by permission of the American Society of Biological Chemists, Inc. and the authors.

It can be seen from the curves that membranes derived from induced and constitutive strains (i^-) have binding sites which can be saturated while those derived from non-induced strains do not bind any significant amount of TDG. This is excellent confirmation that the binding process is concerned exclusively with the M-protein.

A further complication could arise if the binding observed corresponded only to binding to M-protein exposed on the outside of the membrane vesicles, however under the conditions of the experiments which included the addition of sodium azide to the buffer system and in the absence of an energy source it has been shown that the K_m for entry and exit of galactosides in cells are equal and hence it is safe to assume that the concentration of TDG is the same on the outside and inside of the vesicles.

Turning now to the measurement of ΔH for the binding process, the measurements can be made using a batch twin cell microcalorimeter by mixing a suspension of vesicles containing membrane protein with a TDG solution in one vessel and a similar suspension with buffer in the reference vessel. In principle if all the amounts are carefully adjusted the enthalpy of dilution of the vesicles in the two vessels should cancel and if the enthalpy of dilution of the TDG is measured in a separate experiment then the enthalpy of binding of the TDG can be calculated.

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