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ON THERMAL TRANSITIONS IN BIOLOGICAL MACROMOLECULES

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The thermal transitions shown by macromolecules are to be understood as an allosteric phenomenon. They can be dealt with in terms of the same linkage principles as those governing the binding of a chemical ligand. This provides a basis for analyzing the observed differences between the reversible heat denaturation of proteins and the melting of nucleic acids. It also adds to our understanding of cooperativity and heterotropic linkage.

1. Introduction

In any interpretation of thermal transitions of macromolecules, whether they be those involved in the reversible denaturation of a protein [1] or the melting of a nucleic acid [2], there are two things to bear in mind. The first is the complete formal equivalence of the physical and chemical variables as they occur in the group of linkage potentials which govern the system and of which the most widely useful one is the binding potential Π [3,4]. Thus $\overline{S}$ and $\overline{V}$, the entropy and volume per unit of macromolecule, correspond exactly to $\overline{X}$, the amount of a ligand X, also per unit of macromolecule. Similarly each of the intensive variables, T or p, corresponds exactly to a chemical potential, μ_X. As a result of this a curve of entropy (or as we shall presently see, enthalpy) as a function of T corresponds exactly to a binding curve of $\overline{X}$ as a function of μ_X [5]. Indeed if we like we may think of entropy (or volume) as a ligand of chemical potential T (or p).

The second thing to bear in mind is the fact that we are dealing with an allosteric system [6] in which the reference component, the macromolecule, exists in several different states, or conformations, which are at all times in equilibrium with one another, the equilibrium being under the control of the various ligands, in the above extended sense of the term. The result of this is that the binding potential Π may be expressed in a particularly simple form, which, although not truly exact, should nevertheless be valid to a high degree of approximation. This may be developed as follows.

2. The generalized binding potential for an allosteric system

Suppose that the macromolecule exists in r different conformations and let Ω represent any one of the extensive quantities (S, V, or ligand X) associated with the system. The total amount of $\overline{\Omega}$ "bound" per unit of macromolecule is given by

$$\overline{\Omega} = \frac{(\nu_1\overline{\Omega}_1 + \nu_2\overline{\Omega}_2 + \ldots \nu_r\overline{\Omega}_r)}{\nu_1 + \nu_2 + \ldots + \nu_r}, \qquad (1)$$

where $\overline{\Omega}_i$ is the amount of Ω "bound" per unit of macromolecule in conformation i and ν_i is the fraction of the macromolecule present in that conformation. The

ratio ν_i/ν_1 is, subject to multiplication by a ratio of activity coefficients which may be expected to be close to unity, the same as the equilibrium constant L_i for the transition of the macromolecule from conformation 1 to conformation i. Consequently L_i, expressed in terms of the binding potentials of the two conformations involved, may be written as

$$L_i = L_{i0} \exp[(\Pi_i - \Pi_1)/RT]$$

$$= \frac{\nu_{i0}}{\nu_{10}} \exp[(\Pi_i - \Pi_1)/RT], \qquad (2)$$

where subscript 0 refers to a standard state defined in terms of the various intensive variables T, p, or μ, which we denote collectively by ω [6]. In accordance with properties of the binding potential it is always true that

$$\overline{\Omega} = \partial\Pi/\partial\omega$$

and at the same time for each conformation:

$$\overline{\Omega}_i = \partial\Pi_i/\partial\omega, \qquad (3)$$

It follows directly that for the allosteric system:

$$\partial\Pi/\partial\omega = [\nu_{10} \exp(\Pi_1/RT)\, \partial\Pi_1/\partial\omega + \ldots + \nu_{r0} \exp(\Pi_r/RT)\, \partial\Pi_i/\partial\omega]$$

$$\times [\nu_{10} \exp(\Pi_1/RT) + \ldots + \nu_{r0} \exp(\Pi_r/RT)]^{-1}, \qquad (4)$$

from which we obtain

$$\Pi = RT \ln[\nu_{10} \exp(\Pi_1/RT) + \ldots + \nu_{r0} \exp(\Pi_r/RT)]. \qquad (5)$$

This equation has been frequently employed in the past [6] in dealing with ligand binding at constant temperature and pressure.

But it will be seen and should be emphasized that it applies equally to all the "ligands" and that Ω may be identified indifferently with entropy, volume, or any one of the chemical ligands. At the same time, however, and in contrast to this, it should also be emphasized that the form of the binding potential Π will be different for the different "ligands". In the case of a chemical ligand X, Π_i can be expressed, with a high degree of approximation, in terms of a polynomial, known as the binding polynomial [3], in the activity x of the ligand:

$$\Pi_i = RT \ln(1 + K_{i1}x + K_{i2}x^2 + \ldots + K_{it}x^t)$$

$$\equiv RT \ln P_i,$$

where any K_{ij} is the overall equilibrium constant for the combination of the macromolecule in conformation i with j molecules of ligand and t is the total number of binding sites in the macromolecule ‡. Since the logarithm and exponential are inverse functions the result is that the binding potential for the whole system can be expressed in the same way:

$$\Pi = RT \ln(\nu_{10}P_1 + \nu_{20}P_2 + \ldots + \nu_{r0}P_r)$$

$$= RT \ln P. \qquad (6)$$

In the case of entropy and volume no such simplifying logarithmic expression for Π in terms of T or p exists and the situation is much more complicated.

3. Relations applicable to thermal transitions

In the study of thermal transitions what is usually measured is $\overline{C}_p$, the heat capacity per unit (gram or mole) at constant pressure, as a function of temperature. Since $\overline{C}_p = T\partial\overline{S}/\partial T$ it is clear that in this case entropy is playing the role of "ligand". In order to obtain an expression for this we go back to eq. (1), which, assuming for the moment that there are only two conformations, we write as

$$\overline{S} = \frac{\nu_1\overline{S}_1 + \nu_2\overline{S}_2}{\nu_1 + \nu_2} = \overline{S}_1 + \frac{L(\overline{S}_1 + \Delta\overline{S})}{1 + L}. \qquad (7)$$

As pointed out above, the dimensionless ratio $L = \nu_2/\nu_1$ is the equilibrium constant for the transition of the macromolecule from conformation 1 to conformation 2. As such it is subject to the van 't Hoff relation

‡ It is assumed here that the *activities* (chemical potentials) of all the other ligands are held constant. In this case Π is equal to minus the chemical potential of the macromolecule. If the *amounts* of certain of the other ligands are substituted for their chemical potentials, Π is replaced by a different function ψ which is another member of the group of linkage potentials applicable to the system [4]. Although the new function ψ is not equal to minus the chemical potential of the macromolecule, it is still true that $\partial\psi/\partial\mu_X = \overline{X}$.

$$\partial \ln L/\partial T = \Delta\bar{H}/RT^2, \tag{8}$$

where $\Delta\bar{H}$ is the heat of the reaction ≒. Differentiation of eq. (7) with respect to T, which we denote by a dot, gives

$$\dot{\bar{S}} = \dot{\bar{S}}_1 + \frac{L\Delta\bar{S}}{(1+L)^2} + \frac{\dot{L}}{1+L}\Delta\bar{S}$$

$$= \dot{\bar{S}}_1 + \frac{\dot{L}}{L}\frac{L}{(1+L)^2}\Delta\bar{S} + \frac{L}{1+L}\Delta\dot{\bar{S}}. \tag{9}$$

From this, by introducing the relations

$$\dot{\bar{S}} = \bar{C}_p/T, \quad \dot{L}/L = \Delta\bar{H}/RT^2, \quad \Delta H = T\Delta S,$$

we obtain at once

$$\bar{C}_p = \bar{C}_{p1} + \frac{L}{(1+L)^2}\frac{(\Delta\bar{H})^2}{RT^2} + \frac{L}{1+L}\Delta\bar{C}_p. \tag{10}$$

In order to implement eq. (10) to determine $\bar{C}_p$ as a function of temperature, it is of course necessary to know both $\Delta\bar{H}$ and L as functions of temperature. If we neglect higher order effects involving changes of $\Delta\bar{C}_p$ with temperature we can write

$$\Delta\bar{H} = \Delta\bar{H}_0 + \Delta\bar{C}_p(T - T_0), \tag{11}$$

where T_0 is a reference temprature at which $\Delta\bar{H} = \Delta\bar{H}_0$. From this, on the basis of eq. (8) it follows that

$$\ln\frac{L}{L_0} = \frac{(\Delta\bar{H}_0 - T_0\Delta\bar{C}_p)(T - T_0)}{RTT_0} + \frac{\Delta\bar{C}_p}{R}\ln\frac{T}{T_0} \tag{12}$$

When these are more than two conformations (1 transition), we have only to take account of additional terms in eq. (1), which remains as our starting point. The algebra becomes a little more complicated but the procedure remains the same. For three conformations, eq. (10) then becomes

$$\bar{C}_p = \bar{C}_{p1} + \frac{L_{12}\Delta\bar{H}_{12}^2 + L_{13}\Delta\bar{H}_{13}^2 + L_{12}L_{13}\Delta\bar{H}_{23}^2}{RT^2(1+L_{12}+L_{13})^2}$$

$$+ \frac{L_{12}\Delta\bar{C}_{p12} + L_{13}\Delta\bar{C}_{p13}}{1+L_{12}+L_{13}}, \tag{13}$$

where L_{ij} is the equilibrium constant for the transition from the conformation i to the conformation j. For four,

$$\bar{C}_p = C_{p1} + (L_{12}\Delta\bar{H}_{12}^2 + L_{13}\Delta\bar{H}_{13}^2 + L_{14}\Delta\bar{H}_{14}^2$$

$$+ L_{12}L_{13}\Delta\bar{H}_{23}^2 + L_{12}L_{14}\Delta\bar{H}_{24}^2 + L_{13}L_{14}\Delta\bar{H}_{34}^2)$$

$$\times [RT^2(1+L_{12}+L_{13}+L_{14})^2]^{-1}$$

$$+ \frac{L_{12}\Delta\bar{C}_{p12} + L_{13}\Delta\bar{C}_{p13}}{1+L_{12}+L_{13}+L_{14}}. \tag{14}$$

And it is easy to generalize the expression to take case of any number of conformations. In all cases eq. (11) and (12), which give the various values of the $\Delta\bar{H}$'s and L's, are the same. We emphasize that in the treatment just given we are thinking of the system as an allosteric one, consisting of a single phase where the various transitions of the macromolecule involve no change of molecular weight. This analysis leads to essentially the same predictions as a previous one given by Friere and Biltonen involving a somewhat different approach using classical partition functions [7,8,9].

4. Type cases

As the temperature of the allosteric system is increased the macromolecule will pass through a series of conformational transitions just as it does when the chemical potential μ_x of a chemical ligand X is increased. Each of these transitions will be associated with an excess heat capacity (i.e. $\bar{C}_p - \bar{C}_{p1}$), and it is intuitively more or less obvious that a significant peak in the heat capacity versus temperature curve will occur only at points where the system is poised between two or more predominant conformations of roughly equal frequency. It is to be expected that the sharpness and height of a peak will increase with the dimensionless ratio $\Delta\bar{H}/RT$. This is born out by the equations and is illustrated by

≒ It may help to see the whole picture in a unified way to note that the van't Hoff equation can be obtained at once from eq. (2) as $\partial \ln L/\partial T = (1/RT)\Delta\bar{S} = (1/RT^2)\Delta\bar{H}$, just as can $\partial \ln L/\partial p = -\Delta\bar{V}/RT$ or $\partial \ln L/\partial\mu_x = \Delta\bar{X}/RT$. From this it will be seen that the analogues of eq. (10), given below, when p and μ_x are considered as the independent variables, are respectively $\bar{V} = \bar{V}_1 - L(1+L)^{-2}(\Delta\bar{V}/RT)^2 + L(1+L)^{-1}\Delta\bar{V}$ and $\bar{X} = \bar{X}_1 + L(1+L)^{-2}(\Delta\bar{X}/RT)^2 + L(1+L)^{-1}\Delta\bar{X}$, the dot now denoting either $\partial/\partial p$ pr $\partial/\partial\mu_x$.

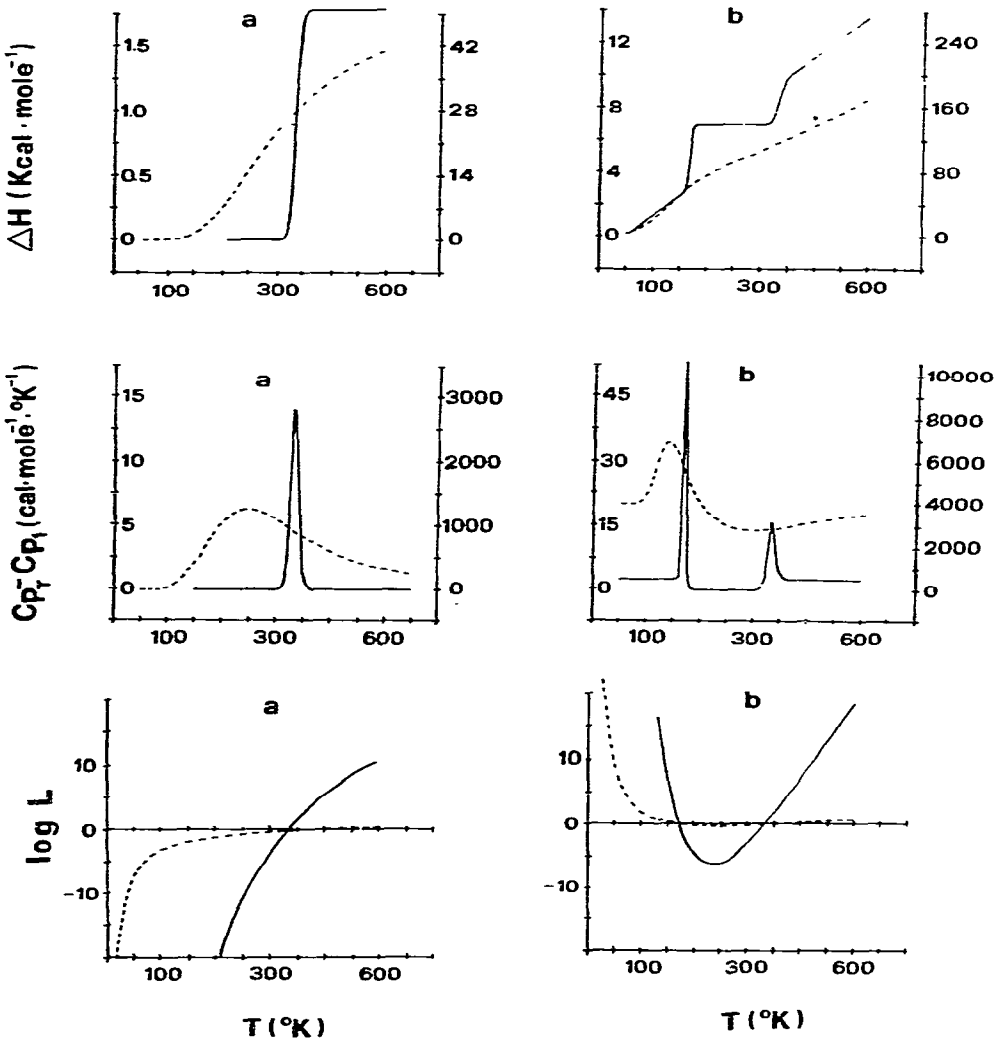

Fig. 1. Thermal transitions in a 2 state allosteric system calculated by eqs. (10), (11) and (12) using parameters given in table 1. Center row gives $\overline{C}_p - \overline{C}_{p1}$ versus T; top row, $\Delta\overline{H}$ versus T bottom row, $\log L$ versus T. (Note 200 fold difference of scale of ordinates between dashed and full curves.)

the various type cases shown in the following figures calculated from them. Another dimensionless ratio, namely $\Delta\overline{C}_p/\Delta\overline{H}/T = \Delta\overline{C}_p/\Delta\overline{S}$ also plays a role in determining the number of observable peaks.

The simplest case (two conformations, one transition, $\Delta\overline{C}_p = 0$) is shown in fig. 1a. Here there is but one peak and the figure brings out the enormous sensitivity of both the height and sharpness of this peak to the ratio $\Delta\overline{H}_0/RT_0$. This results not only from the fact that $(\Delta\overline{H}/RT)$ enters eq. (10) raised to the second power, but also from the strong dependence of L on temperature. As $\Delta H/RT \to 1$ the peak becomes rapidly smaller and smaller and flatter and flatter, and it is interesting to see how the position of the maximum moves progressively away from the point where $L = 1$.

Fig. 1b shows the next simplest case (two conforma-

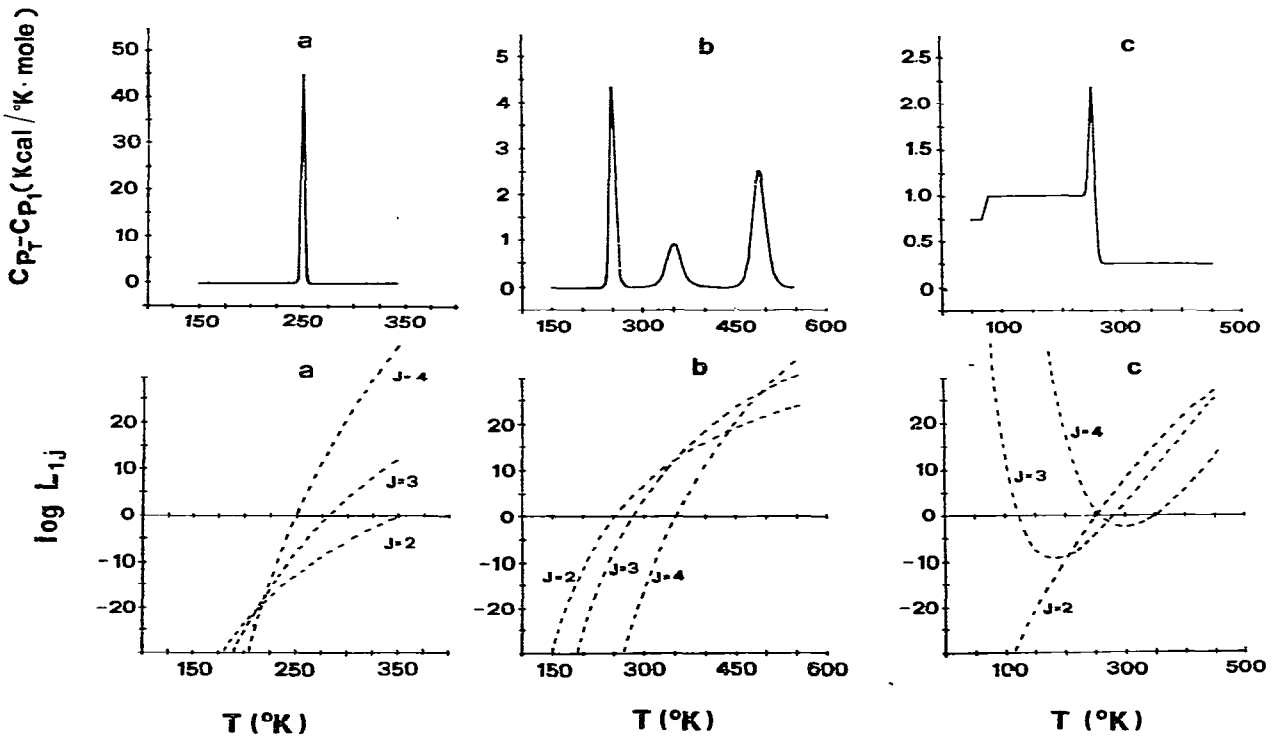

Fig. 2. Thermal transitions in a 4 state allosteric system calculated by eqs. (11), (12) and (14) using the parameters given in table 1. Upper row show $\overline{C}_p - \overline{C}_{p1}$ versus T; lower row, log L_{1j} versus T.

tions there are now *two* transtiions. The reason for this is apparent from the lower row of the figure (log L versus T). There are now *two* values of T for which $L = 1$. This results from the way in which $\Delta\overline{H}$ changes with temperature. (Note that it is arbitrary what temperature we choose at T_0). The figure further shows how, as $\Delta\overline{C}_p$ decreases in relation to $\Delta\overline{H}_0$ (more precisely, as the non dimensional ratio $\Delta\overline{C}_p/\Delta\overline{H}_0/T$ decreases), the two peaks move farther and farther apart. Under realistic physical conditions the span of experimentally accessible temperatures is not often likely to be large enough to include more than one peak (1).

In fig. 2 we pass directly to the case where there are four conformations. In 2a and 2b all values of $\Delta\overline{C}_p$ are assumed to be small enough to be neglected; in 2c, on the other hand, appreciable values of $\Delta\overline{C}_p$ are introduced. These figures, especially 2a and 2b, where complications arising from $\Delta\overline{C}_p$ are eliminated, give an idea of what may be expected in the general case. A key to an understanding of the situation is again to be found in the lower row of figures, where values of the three independent L's are shown logarithmically (in accordance with eq. (12)) as a function of temperature. As would be expected, a significant peak is only observed where two or more of these curves (to which the abscissa axis is to be added) intersect at a point or at least within a very small span of temperature where $L \geqslant 1$ and then only provided no other curve lies above them. This means that the corresponding forms are the only significant ones present, and that they occur in approximately equal amounts, the whole system being poised at this point. It is easy to see that there will only be a sequence of peaks if there is a strong positive correlation between the values of the various L's and the magnitude of the corresponding heats. In the absence of any such correlation, which there is no *a priori* reason to expect, we may anticipate only one, or at most a very small number, of observable transition peaks. In the case of the proteins this anticipation is born out by the elegant measurements of Privalov, which show only a single peak [1].

Table 1
Parameters used for construction of figs. 1 and 2 on the basis of eqs. (10), (11), (12) (fig. 1) and eqs. (11), (12) (14) (fig. 2)

		Transitions	T_0 (°R)	L_0	$\Delta\bar{C}_p$ (cal/K mole)	$\Delta\bar{H}_0$ (cal/mole)	$\Delta C_p/\Delta H_0$ / T_0
Fig. 1a	Dashed line	1 → 2	333	1	0	2 000	0
	Full line	1 → 2	333	1	0	50 000	0
Fig. 1b	Dashed line	1 → 2	333	1	20	2 000	3.3
	Full line	1 → 2	333	1	500	50 000	3.3
Fig. 2a		1 → 2	280	1.5×10^{-8}	0	50 000	0
		1 → 3	280	1	0	80 000	0
		1 → 4	280	1.1×10^{14}	0	150 000	0
Fig. 2b		1 → 2	280	4.8×10^{4}	0	50 000	0
		1 → 3	280	1	0	80 000	0
		1 → 4	280	3.8×10^{-24}	0	150 000	0
Fig. 2c		1 → 2	280	1.0×10^{6}	250	50 000	1.5
		1 → 3	280	1	500	50 000	3.0
		1 → 4	280	1.2×10^{-2}	1000	50 000	6.0

In marked contrast to the proteins, the nucleic acids characteristically show a sequence of several peaks [2]. A tempting explanation of this would be to suppose that in these molecules different segments undergo conformational changes independently of one another, so that they may be treated, to a first approximation, as independent molecules. This indeed is the assumption employed by Privalov in the analysis of *all* his data [10]. There is an interesting analogy here between the dielectric dispersion observed in the proteins, which orient as rigid bodies, and various organic polymers, which show no such long relaxation times and where the polarizability is the sum of the polarizabilities of the various polar segments. Moreover it known from fluorescence depolarization experiments that local groups present in macromolecules commonly relax independently of one another. Also it is worth noting that when the energy involved is of the order of RT there will be no observable peak corresponding to a thermal transition. However, in view of the highly folded structures represented by the nucleic acids, it would still seem more proper to analyze the transition data in terms of the allosteric model discussed above.

5. Cooperativity in thermal transitions

We have seen that a curve of entropy, or enthalpy, as a function of temperature is formally the same as a binding curve for a chemical ligand, $\bar{S}$ or $\bar{H}$ corresponding to $\bar{X}$, and T to μ_x. In the case of chemical binding, cooperativity, positive or negative, is represented by the difference between the slope of the actual binding curve and that of a reference curve, which is taken as the curve for a macromolecule which exists in only one conformation and in which the sites are all identical and independent. At any point of the curve, i.e. at any value of μ_x, where this difference is positive the cooperativity is positive; at any point where it is negative, the cooperativity is negative. In the intermediate case, where the curves are tangent, it is neutral. In just the same way, in the case of enthalpy cooperativity is represented by the difference between the slope of the actual $\bar{H}$ versus T curve and that of a reference curve. In this case the reference curve is that for a hypothetical macromolecule which exists in a single conformation and for which $\bar{C}_p$ is constant. In both cases, the geometrical concept of the tact invariant is useful [11].

In the study of chemical binding it is the binding curve itself, $\overline{X}$ versus μ_x, which is directly observed. In contrast, in the case of enthalpy binding, it is not the binding curve, but instead its first derivative, namely the curve of $\overline{C}_p$ versus T which is directly observed. This curve, however, provides an equally good, if not even better, criterion of cooperativity. It will be seen that any point where $\overline{C}_p$ is a maximum will be a point of inflection in the integral curve. At this point the cooperativity is locally neutral; to the left, where $d\overline{C}_p/dT$ is positive, it is positive; to the right, where $d\overline{C}_p/dT$ is negative, it is negative. In the long stretch between peaks, where $\overline{C}_p$ remains constant, cooperativity is again neutral and remains so. Thus it is really the third derivative of the binding potential with respect to temperature which is the true measure of cooperativity; and the same principle holds for pressure volume changes or for chemical binding when we substitute p or μ for T respectively. There is indeed complete correspondence of the three cases at a formal level. It should be realized, however, that from an experimental point of view there are significant differences. For example, in the case of chemical binding the value of μ can be varied between zero and what amounts, essentially, to infinity. In the case of enthalpy (or $\overline{C}_p$), the range of experimentally available temperatures is limited, and there is no such thing as saturation of the macromolecule with heat as there is with ligand. The same is true of volume and pressure ‡.

6. The effect of a chemical ligand

It is a matter of observation that in many cases the position and characteristics of the thermal transitions of a macromolecule are sensitive to the presence of various chemical ligands. This of course is just what would be expected on the basis of standard linkage theory [3]. It may be understood either, in detail, in terms of the effects of a ligand on the equilibrium (transition) constants L or, more globally, on the basis of the following overall linkage relations involving the enthalpy itself of which C_p is the temperature derivative.

‡. This general way of looking at cooperativity is revealing also in the case of imperfect solutions.

Introducing the binding potential Π, for which $d\Pi = \overline{S}dT - \overline{V}dp + \overline{X}d\mu_x$ we obtain at constant pressure

$$(1/T)(\partial\overline{H}/\partial\mu_x)_T = (\partial\overline{X}/\partial T)_{\mu_x}. \quad (15)$$

Here $(\partial\overline{H}/\partial\mu_x)_T$ refers to the enthalpy change as measured under conditions where the *chemical potential* (activity) of the ligand X is varied. From an operational point of view this may be a difficult condition to satisfy. In case it is preferred to have a relation in terms of $(\partial\overline{H}/\partial\overline{X})_T$, where the *amount* of the ligand is varied, we make use of another member of the group of linkage potentials namely $\psi = \psi(\overline{X}, T, p)$, for which $d\psi = -\mu_x d\overline{X} + \overline{S}dT - \overline{V}dp$. On the same basis as before this gives at constant pressure

$$(1/T)(\partial\overline{H}/\partial\overline{X})_T = -(\partial\mu_x/\partial T)_{\overline{X}}. \quad (16)$$

Either of these relations shows how the enthalpy, as measured calorimetrically, is affected by the ligand present. The right hand side can be obtained from the change in shape of the binding curve with temperature. This will in general represent the effect of temperature upon the various equilibrium constants of the allosteric linkage potentials. It is these linkage potentials which provide throughout the key to an understanding of the system in all its aspects.

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