Disagreement between calorimetric and van't Hoff enthalpies of assembly of protein supramolecular structures

(thermodynamics/polymerization/self-assembly/microtubules)

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ABSTRACT  The effect of temperature on the extent of association of self-assembling protein polymers is expressed mathematically in terms of the van’t Hoff enthalpy of polymerization, $\Delta H_{v.H}$. This quantity has been experimentally defined in two ways—from the respective temperature derivatives of the critical polymerization concentration and of the fractional conversion of protein into polymer. These two definitions are shown not to be identical, except in certain limits. In terms of both definitions, it is shown that $\Delta H_{v.H}$ depends not only upon the enthalpy changes but also upon the corresponding equilibrium constants for the various equilibria involved in polymer formation. This has two consequences: (i) large $\Delta H_{v.H}$ values may result from reactions having small calorimetric enthalpy changes; and (ii) $\Delta H_{v.H}$ can depend strongly on temperature. These considerations are applied to two systems for which there exist considerable experimental data—namely, hemoglobin $S$ and tubulin. The large discrepancy between the calorimetric and van’t Hoff enthalpies for the polymerization of tubulin is shown to be explicable in terms of these considerations.

Various biologically important self-assembling systems composed of protein subunits have been investigated in vitro, utilizing temperature to regulate assembly. The parameter expressing the effect of temperature on the extent of assembly is the van’t Hoff enthalpy of polymerization, $\Delta H_{v.H}$. This quantity has been reported for a number of systems (1–7). In two of these systems, tubulin (1–3) and bacterial flagellin (4, 5), there exists a large discrepancy between $\Delta H_{v.H}$ and the calorimetrically measured enthalpy change, $\Delta H_{cal}$. In the hemoglobin $S$ system, on the other hand, $\Delta H_{cal} \approx \Delta H_{v.H}$. Several difficulties in particular attend the interpretation of van’t Hoff data in such complex systems as these. The size of the cooperative unit to which the "mole" refers is generally unknown in the van’t Hoff procedure. Moreover, $\Delta H_{v.H}$ may be sensitive to the mechanism in multistep transitions (8). Furthermore, $\Delta H_{v.H}$ itself is defined in at least two different ways that depend on the temperature derivatives of the critical polymerization concentration and the fractional conversion of protein to polymer, respectively. Some self-assembling systems appear to be more temperature sensitive than others (e.g., large values for $\Delta H_{v.H}$ (>20 kcal/mol) have been reported for tubulin (1), while those reported for hemoglobin $S$ are relatively small (<5 kcal/mol) (6, 7)). A frequent feature of these results for $\Delta H_{v.H}$ is their own strong negative temperature dependence, values of $\Delta C_p \equiv d\Delta H_{v.H}/dT$ on the order of −0.1 kcal/mole-cudegree not being unusual (1, 6).

This paper treats the effect of temperature on model systems representing two physically distinct situations: (i) noncooperative stepwise association of monomer units, and (ii) cooperative addition of monomer units to thermodynamically unstable nuclei.

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METHODS OF DETERMINING $\Delta H_{v.H}$.

Measurements based on the critical concentration

Self-assembling systems display, with varying degrees of sharpness, a critical concentration of total protein ($C_*$), below which no polymer forms and above which polymer forms under appropriate conditions (9). In such systems, $\Delta H_{v.H}$ is frequently defined from the effect of temperature on $C_*$. These measurements presume that the standard free energy of polymerization is given by:

$$\Delta G = RT \ln C^*$$  \[1\]

and, hence that:

$$\Delta H_{v.H} = -T^2 \left( \frac{\partial (\Delta G^*/T)}{\partial T} \right) = -RT^2 \frac{\partial \ln C^*}{\partial T}.$$  \[2\]

The critical concentration may be determined experimentally as follows. One measures some observable property (e.g., turbidity), $Q$, that is assumed to be proportional to the mass of polymerized material (a practical distinction between "polymer" and smaller oligomers is made if the measurement is insensitive to aggregates smaller than a certain size). This quantity, $Q$, is then plotted against the total concentration of protein, $C$ (Fig. 1). In relatively noncooperative systems wherein the transition to polymer is not sharp, the extrapolation of $Q$ back to the $C$ axis is uncertain. The determination of the critical concentrations may therefore involve substantial scatter [Gaskin et al. (1) estimated the errors in their measurements of $C_*$ for tubulin to be on the order of 30%]. It can readily be appreciated that this procedure for determining $\Delta H_{v.H}$ is laborious because measurements of $Q$ over a wide range of temperatures and concentrations are necessary.

Measurements based on fractional conversion of protein to polymer

This procedure has been dealt with at length for nonpolymerizing systems (10) but will be discussed here briefly. The major assumption is again that $Q(T) \propto$ the mass of polymerized material at any temperature, $T$. The fractional conversion of protein to polymer is then given by:

$$f(T) = \frac{Q(T) - Q_0}{Q_1 - Q_0}$$  \[3\]

and extrapolations must again be made (Fig. 2). An equilibrium constant is then defined by:

$$K(T) = \frac{f(T)}{1 - f(T)},$$  \[4\]

and, since $\Delta H = RT^2 \frac{\partial \ln K}{\partial T}$, we find

$$\Delta H_{v.H} = \frac{RT^2}{f(1 - f)}.$$  \[5\]
which determines $\Delta H^1_{\text{H}}$ at any chosen temperature or degree of conversion. For example, at the point at which one-half of the protein is converted to polymer,

$$\Delta H^1_{\text{H}} = 4RT^2\alpha_p \left( \frac{\partial f}{\partial T} \right)_{0.5}$$

This procedure determines $\Delta H^1_{\text{H}}$ from $\partial f/\partial T$ at any given value of $C$; thus, one expects that $\Delta H^1_{\text{H}}$ will also generally depend on $C$, in contrast with $\Delta H^C_{\text{H}}$. We turn now to the models.

**MODELS**

1. Isodesmic case

This case involves sequential monomeric addition, the equilibrium constant, $K$, being the same for each step:

$$A_{i-1} + A \rightleftharpoons A_i \rightarrow \sum_{i=1}^{n} i[A_i] = [A](1 - K[A])^{-2}. \quad [6]$$

The total protein concentration, $C$, is a constant, independent of temperature, $T$, and is related to the concentration of free monomer, $A$, by the relationship:

$$C = \sum_{i=1}^{n} i[A_i] = |A|(1 - K[A])^{-2}. \quad [7]$$

Defining all aggregates of size $> n$ to be polymers, we find the polymer concentration, $P(A)$, to be:

$$P(A) = \sum_{i=n+1}^{\infty} i[A_i] = C[(n + 1)(K[A])^n - n(K[A])^{n+1}] \quad [8]$$

The concentration of nuclei is $N(A) = C - P(A)$.

The fractional conversion of protein into polymer is then

$$f = \frac{P(A)}{C} = (n + 1)(K[A])^n - n(K[A])^{n+1}. \quad [9]$$

Because this system displays no cooperativity, the transition from nonpolymer to polymer is not sharp (Fig. 3). Therefore, the choice as to what constitutes a polymer amounts to a definition. However, a choice for $n$ can nonetheless be made and the sums formally evaluated.

We shall evaluate $\Delta H_{\text{H}}$ by each of the two methods referred to previously. We begin with the method based on $C^*$. It is convenient to introduce the dimensionless variables $A = KA$, $\Theta = KC$, $P = KP$ and $N = KN$.

From Eqs. 7 and 8,

$$P(A) = \frac{A^{n+1}}{(1 - A)^2} \ln(1 - A) + 1$$

and

$$\Theta(A) = \frac{A}{(1 - A)^{3/2}}. \quad [10]$$

We may invert the above equation to obtain

$$A = 1 + (2\Theta)^{-1} - \frac{1}{2} \left\{ \frac{4}{\Theta} + \frac{1}{\Theta^2} \right\}^{1/2}. \quad [11]$$

Substituting Eq. 11 into Eq. 9 gives $f = f(\Theta)$. For any integral

**FIG. 1.** Determination of the critical concentration, $C^*$, from a plot of equilibrium polymer concentration, $P$ (or an observable, $Q \propto P$), versus total protein concentration, $C$, for an arbitrary system. At some specified degree of polymerization, the curve is extrapolated back to the $C$ axis. At the point ($P_{0.5}, C_{0.5}$) shown above, half of the protein is polymerized and $C^* = C_{0.5} - P_{0.5} (\partial P/\partial C)_{0.5}$. The temperature derivative of $C^*$ gives $\Delta H^1_{\text{H}}$ (see text). Inset. $P$ versus $C$ for an indefinitely cooperative system. For $C < C^*$, no polymer can exist and all protein is in the form of nuclei, $N (N = C)$. As $C$ increases beyond $C^*$, the additional protein goes into the polymeric form and $P = C - C^*$.

**FIG. 2.** Determination, at some specified degree of polymerization, of $\partial Q/\partial T$, in which $Q$ is an observable property proportional to polymer concentration. Because the fractional degree of polymerization, $f$, equals $(Q(T) - Q_0)/(Q_1 - Q_0)$, one may evaluate $\Delta H^1_{\text{H}} = RT^2/[(1 - f) (\partial Q/\partial T)]$. Note that high- and low-temperature extrapolations of $Q$ must be made to the temperature region at which the slope is measured.
value of \( n \), we can solve the resulting equation to obtain values of \( \mathcal{A} \) or \( \mathcal{C} \) corresponding to any fractional value of \( f \). We designate these values corresponding to \( f = 0.5 \) by \( \mathcal{A}_{0.5} \) and \( \mathcal{C}_{0.5} \) in Table 1. Now we see from Fig. 1 that:

\[
\mathcal{A}(*) = \mathcal{C}_{0.5} - \mathcal{P}_{0.5} \left( \frac{\partial \mathcal{P}}{\partial \mathcal{C}} \right)^{-1} \]  

[12]

Next we note that

\[
\frac{\partial \mathcal{P}}{\partial \mathcal{C}} = \frac{\partial \mathcal{P}(\mathcal{A})}{\partial \mathcal{A}} \left( \frac{\partial \mathcal{C}(\mathcal{A})}{\partial \mathcal{A}} \right)^{-1}. \]  

[13]

\[
\frac{\mathcal{A}_n}{1 + \mathcal{A}} = \mathcal{A}(n + 1)^2 + \mathcal{A}(1 - 2n)(n + 1)^2 + n^2 \mathcal{A}^2. \]  

[14]

The alternative procedure is to calculate \( \Delta H_{\nu, \mathcal{H}} \) from the effect of temperature on \( f \). We see that

\[
\frac{\partial f}{\partial \mathcal{A}} = n(n + 1) \mathcal{A}^{n-1} (1 - \mathcal{A}), \]  

[17]

and from Eq. 11,

\[
\frac{\partial \mathcal{A}}{\partial \mathcal{C}} = -\frac{1}{2} \left[ 1 - \frac{(2 + 1) \mathcal{C}}{(4 \mathcal{C} + 1) \mathcal{C}^2} \right], \]  

[18]

From the relation

\[
\Delta H_{\nu, \mathcal{H}} = 4R \mathcal{A}_{0.5} \left( \frac{\partial f}{\partial \mathcal{C}} \right)_{0.5}, \]  

we have

\[
\frac{\Delta H_{\nu, \mathcal{H}}^c}{\Delta H_{K}} = 2n(n + 1) \mathcal{A}^{n-1} (1 - \mathcal{A}_{0.5}) \mathcal{C}_{0.5} \times \left[ -1 + \frac{2 + \mathcal{C}_{0.5}^2}{[4 \mathcal{C}_{0.5} + 1] \mathcal{C}_{0.5}^2} \right]. \]  

[19]

It can be seen from Table 1 that \( \Delta H_{\nu, \mathcal{H}}^c \) calculated in this way can differ from \( \Delta H_{\nu, \mathcal{H}} = \Delta H_{K} \) by at most \( \sim 10\% \), depending on the value of \( n \).

2. Nucleated polymerization

Some self-assembling protein systems display two features of strongly cooperative association—namely, a critical concentration of total protein, and a kinetic lag time during which no fully developed polymer can be detected. These two characteristics can be explained in terms of a model of nucleated polymerization (9, 11), according to which subunits associate to form unstable oligomers or "nuclei" that are themselves capable of undergoing further association to form stable polymers only if they exceed a certain critical size, \( n \). The critical protein concentration therefore simply reflects the minimal amount of total protein required by mass action to force the aggregation through a series of \( n \) thermodynamically unfavorable nucleation steps. This process occurs during the kinetic lag period. For an idealized system displaying infinite cooperativity, a plot of polymer and nuclei concentrations (\( P \) and \( N \), respectively) at various concentrations of total protein, \( C \), is given in Fig. 1 inset. It may be seen that, as \( C \) increases, all of the protein initially goes into the form of nuclei. Once \( C \) exceeds \( C^* \), polymer increases linearly with \( C \), while the nuclei concentration remains at the critical value. The model discussed here approaches this behavior to any arbitrarily chosen degree, for appropriate values of the parameters, which are: \( \sigma \), the equilibrium constant for nucleation; \( K \), the equilibrium constant for propagation (\( K \gg \sigma \)); and \( n \), the size of the critical aggregate. The relevant equilibria are therefore:

**Nucleation**

\[
A + \mathcal{A} \xrightarrow{\sigma} \mathcal{A}_2 \quad [A_2] = \sigma[A]^2 \]

\[
A_2 + \mathcal{A} \xrightarrow{\sigma} \mathcal{A}_3 \quad [A_3] = (\sigma[A])^3/\sigma \]

\[
\vdots \]

\[
A_{n-1} + \mathcal{A} \xrightarrow{\sigma} \mathcal{A}_n \quad [A_n] = (\sigma[A])^n/\sigma \]

**Propagation**

\[
A_n + \mathcal{A} \xrightarrow{K} \mathcal{A}_{n+1} \quad (A_{n+1}) = K[A_{n+1}][A] \]

\[
\vdots \]

\[
A_{i-1} + \mathcal{A} \xrightarrow{K} A_i \quad (A_i) = K[A_{i-1}][A] \]  

[20]

As in the isodesmic case, the appropriate summations can be done to express \( N, P, C, \) and \( f \) as functions of concentration of free monomer, \( [A] \). In terms of the previously defined reduced variables, we find:

\[
N(\mathcal{A}) = \mathcal{A} \left( 1 - (n + 1) \left( \frac{\sigma}{K} \mathcal{A} \right)^n + n \left( \frac{\sigma}{K} \mathcal{A} \right)^{n+1} \right) \]  

[21]

\[
\sim (1 - \mathcal{A} \sigma/K)^{-2}, \quad (\sigma/K)^n \ll 1, \]  

[22]

\[
P(\mathcal{A}) = \left( \frac{\sigma}{K} \right)^{n-1} \mathcal{A}^{n+1} \left[ 1 + n(1 - \mathcal{A}) \right] (\mathcal{A} < 1), \]  

\[
\mathcal{E}(\mathcal{A}) = N(\mathcal{A}) + P(\mathcal{A}), \]  

[23]

\[
f = \frac{\mathcal{P}(\mathcal{A})}{\mathcal{E}(\mathcal{A})}. \]  

[24]

A plot of \( f \) versus \( \mathcal{E} \) for various values of \( n \) is shown in Fig. 3. The critical concentration is evaluated as follows. We note from Eq. 23 that \( \mathcal{P}(\mathcal{A}) \) possesses a singularity at \( \mathcal{A} = 1 \). Thus, the maximal value \( N(\mathcal{A}) \) can take \( (\mathcal{E}^*) \) is just

\[
\mathcal{E}^* = N(1) \sim (1 - \sigma/K)^{-2} \]  

[26]

or

\[
C^* = K^{-1}(1 - \sigma/K)^{-2} \]  

[27]
Eq. 30 shows that the concentration dependence of $\Delta H^{\dagger}_{\text{Hi}}$ for this model is that $\Delta H^{\dagger}_{\text{Hi}} = \Delta H_{\text{cal}}^{C^*}$ only if $T$ and $C$ are high enough that $C^*/C \ll 1$. Moreover, $f$ increases as $C^*$ decreases (i.e., as $K$ increases, or as $\sigma$ decreases). An increase in temperature will therefore result in polymer formation if chain propagation is endothermic or if nucleation is exothermic.

**COMPARISON WITH EXPERIMENT**

**Hemoglobin S gelation**

Ross et al. (6), in their extensive study of this system, have shown that the kinetic delay time of temperature-induced gelation depends on a high power of the protein concentration. They have interpreted their data in terms of a nucleated polymerization mechanism (case 2) and observed that $\Delta H_{\text{cal}} \sim \Delta H^{C^*}_{\text{Hi}}$ over a range of temperatures. However, as shown earlier, $\Delta H_{\text{Hi}}$ should not generally $\sim \Delta H_{\text{cal}}$ unless (i) $\Delta H_{\text{cal}} \sim \Delta H_K$ or (ii) $\sigma/k < 1$. If nuclei are present only in vanishingly small concentrations, $\Delta H_K$ is difficult to measure calorimetrically. However, the fact that intermediates have not been detected by ultracentrifugation (12) suggests that $\sigma/K$ may indeed be $\ll 1$.

**Tubulin polymerization**

The value for $\Delta H^{C^*}_{\text{Hi}}$ for this system has been reported to be 21 kcal/mol (1). Alternatively, a value of about 25 kcal/mol, depending on the concentration, may be calculated for $\Delta H^{C^*}_{\text{Hi}}$ from the data of ref. 3. Both values are much larger than the recently reported (18) calorimetric value for $\Delta H_K$ of 0 ± 1 kcal/mol. Tubulin appears to be a more complicated system than hemoglobin S for several reasons: (i) there exists for tubulin a variety of microtubule-associated proteins whose function has not yet been completely established (14–16); (ii) stable oligomers of tubulin which may be reaction intermediates are known to exist (17, 18); (iii) the role of nucleotides and possibly of nucleotide hydrolysis is still uncertain (13, 19). Even if the answers to these questions were known, realistic models would involve too many adjustable parameters to permit unambiguous interpretations of the available thermodynamic data. A few conclusions can be drawn, however.

1. Even neglecting complicated reaction mechanisms, there need be no discrepancy between the experimental values for $\Delta H_{\text{Hi}}$ and $\Delta H_{\text{cal}}$. To illustrate this point, we consider model 2 and take $\Delta H_K = 0$ and $\sigma/K = 0.8$. From Eq. 28 we could have $\Delta H_{\text{Hi}} \sim 20$ kcal/mol $= -\Delta H_{\text{cal}}(2\sigma/K)/(1 - \sigma/K)$ which gives $\Delta H_{\text{Hi}} \sim -8.5$ kcal/mol. Considering that the protein solutions are very dilute on a molar basis, because of the high molecular weight of the tubulin dimer, such a small value for $\Delta H_{\text{Hi}}$ would have been difficult to detect by scanning calorimetry (13). A similar conclusion follows from models based on thermally induced conformational changes responsible for “activation” of monomers or intermediates (9).

2. There is no thermodynamic necessity for polymerization-linked GTP hydrolysis, because nucleotides could facilitate assembly allosterically (19). Indeed, if the nucleotides regulated assembly and possessed sizeable binding enthalpies, this alone would be sufficient to account for the effect of temperature on assembly.

3. Because $\Delta H_K \sim 0$, the chain-propagating step (which presumably accounts for the bulk of polymer formation) is entropically driven. Thus, we see that comparatively small enthalpy changes, because they may be magnified by potentially large equilibrium constants, can be sufficient to account for the strong temperature dependence of this system. It is conceivable that, when the factors governing the in vivo regulation of assembly are known, a similar situation may per-
tain—i.e., the effect of a regulator on \( f \) or \( C^* \) may be large not because of a large difference in chemical potential between bound and unbound regulator per se, but because such differences in chemical potential as do exist are magnified by the pertinent equilibrium constants.

**DISCUSSION**

Although the preceding considerations clearly establish the need for taking the mechanism and type of experiment into consideration in interpreting van't Hoff measurements, there are problems in applying such analyses to real polymerizing systems. Some systems exhibit hysteresis (1, 4); accordingly, the transitions may not be thermally reversible and may involve states of metastable equilibrium. Calorimeters detect only overall enthalpy changes. Apart from the uncertainty resulting from the measurement of these usually small quantities, it may be impossible to measure enthalpy changes for separate steps of the overall reaction. These treatments assume solution ideality. This approximation may be acceptable for tubulin and flagellin, wherein protein concentrations are typically \( <1\%) \) by weight, but its consequence with concentrated solutions is unclear. Although \( \Delta H_{\text{cal}} = \Delta H_{\text{r}} \) for hemoglobin \( S \) when activities are neglected, it appears that \( \Delta H_{\text{r}} \gg \Delta H_{\text{cal}} \) taking activities into account (P. D. Ross, personal communication). As noted with tubulin, realistic models generally involve too many parameters to permit their specification uniquely. This is particularly true of systems characterized by length-regulating (20) and other factors (9).

The preceding considerations were applied only to tubulin and to hemoglobin \( S \). In spite of the availability of data for bacterial flagellin polymerization, this system was not discussed for several reasons: (i) the van't Hoff studies (21) were carried out at a temperature (\( 40^\circ \)) at which partial denaturation may have occurred (22); (ii) the two calorimetric studies disagree (4, 5), although this could be due to species differences; and (iii) the flagellar subunits may exist in a kinetically determined metastable state (4).

The formal extension of the preceding analyses to volume rather than enthalpy changes is straightforward and will not be discussed here. Interpretative difficulties must once again be anticipated, however, unless both the mechanism and the \( \Delta V \)s for the individual reaction steps can be determined. We have likewise omitted from consideration the multitude of in \( \text{vivo} \) microtubule studies (23–26). It is questionable whether these systems are sufficiently close to thermodynamic equilibrium for thermodynamic measurements to be meaningful. Apart from that, these studies typically focus on the effect of temperature or pressure on the birefringence measured at some point along the mitotic spindle, although it has not yet been established that the mitotic spindle is composed solely of tubulin. As observed by Hofrichter [quoted in Ross et al. (6)], to establish a direct relationship between the orientationally dependent birefringence and the mass of polymer requires an absolute calibration of birefringence with protein concentration. Such a direct calibration is impossible for an in \( \text{vivo} \) system; an indirect comparison with tubulin polymerized in \( \text{vitro} \) has apparently not yet been made.

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