Chapter 2 fundamental tools

There are four little appreciated areas of newer physical chemistry essential for protein research. These are I The discovery by Benzinger of a ubiquitous error in the use of thermodynamics; II The discovery primarily by Walrafen that water has two chemical species; III The use of extrathermodynamic approximations for the treatment of non-stoichiometric systems and as partial replacement for rigorous thermodynamic treatments. IV The varieties of catalytic mechanisms rarely contain those depending on mechanical rate activation. Two enzyme examples are discussed as examples of a single mechanism that appears to be common to all enzymes. These are most fully - theoretical tools are particularly useful for research on biological systems because the latter are often adequately approximated by liner models. There are linear response behavior and mean-field potential functions and they are considered in a separate chapter.

I Errors in the use of thermodynamics (Benzinger 1967, 1971 Reconsidered by Lumry, Biophysical Chemistry @)

The errors--Matrices depend for their structures and functions in physiological process on knots and share some unusual features but not others. In particular nature has discovered thermodynamic features apparently of special importance in both but neither well known or well appreciate in chemistry. These are implicit in Carnot's discussion of heat engines but were made explicit only in 1967 by Benzinger. Two Centuries ago Carnot developed an expression for the the efficiency of the conversion of heat to work in a reversible heat engine. Most attempts to explain or explicated entropy depend on that work and very little progress beyond it has been made but following Benzinger some important clarification can be made and it shows that the classical application of thermodynamics to processes carried out at constant temperature is quantitatively applicable only to pure crystalline reactants and products. Thus starting with Carnot's efficiency expression $dw = dq\left[\frac{T_h - T_l}{T_h}\right]$ in which w is work and q is heat. Carnot applied this to pressure-volume work but it is general for the case under consideration which is that at which the two thermal reservoirs have the same temperature. With chemical, gravitational or electrical work the reservoirs are the system itself plus the surroundings acting as a constant-

temperature thermostat so if the two temperatures are equal and remain so during the process of the system any heat produced as by product cannot be used to do work. Benzinger developed this generalization in 1967 very long after Carnot. His major paper on the subject published in Nature in 1971 is often cited but rarely understood because the historical statement of entropy also arising from Carnot is incomplete. That can be simply illustrated by paraphrasing and argument from Biophysical Chemistry (105 (2002) 609 and see Utility folder on this web site) as follows.

The system is closed, contains only single species α initially and only β in the final state. Both are crystalline from the temperature of interest T down to 0K. For the constant volume case the Helmholtz free energy A is the center of the development. The internal energy of a system at 0K is determined calorimetrically from constant-volume heat-capacity data measured as a function of T down to 0K. The entropy expression is obtained by integration $\int_0^T \frac{C_v(T')}{T'} dT' = S(T)$ and since $\left(\frac{\partial A}{\partial T}\right)_v = -S(T)$, the thermodynamic "box" relating the change in Helmholtz work for the isothermal process $\alpha \leftrightarrow \beta$ at T to the energy change at 0K, $E_{0,\beta}(0) - E_{0,\alpha}(0)$, is eq. 4-3-1.

$$\begin{array}{ccc} \alpha(T) & \xleftrightarrow{\Delta A(T)} & \beta(T) \\ \downarrow & & \uparrow \\ -\int_T^0 S_\alpha(T')dT' & & -\int_0^T S_\beta(T')dT' \\ \downarrow & & \uparrow \\ \alpha(0) & \xleftrightarrow{\Delta E_0(0)} & \beta(0) \end{array} \qquad (4\text{-}3\text{-}1)$$

The overall expression for ΔA (T) = $(A_\beta - A_\alpha)$ is then eq. (4-3-2)

$$\Delta A(T) = \{\Delta E_0(0)\} - \left\{\int_0^T \Delta S(T')dT'\right\} \qquad (4\text{-}3\text{-}2)$$

This completes the derivation since it establishes that to make the expression for ΔA (T) in eq. (4-3-2) consistent with the conventional definition of A an integral

$\Delta Q(T) = \int_0^T \Delta C_V(T')dT'$ must be added and subtracted from the right side of eq. (4-3-2) to give eq. (4-3-3).

$$\Delta A(T) = \{\Delta E_0(0) + \Delta Q(T)\} - \left\{\int_0^T \Delta S(T')dT' + T\frac{\Delta Q(T)}{T}\right\} \qquad (4\text{-}3\text{-}3)$$

$$\{\ \Delta U(T)\ \} \quad - \qquad \{\ T\,\Delta S(T)\ \}$$

In the latter ΔE_0 (0) is the difference between the energies of the first eigenstates and ΔQ (T) is the difference between the mean energy at T and ΔE_0 (0). It is an average over fluctuations in energy and usually called the heat. That term is used in different ways and might be confusing. It is not a state function in irreversible processes and although our discussions apply only to reversible processes we shall call it the "thermal energy". The sum of the change in potential energy and the zero-point vibrational energies are directly related to the work. Those in thermal energy and entropy have no connection with work changes. Any engine doing work at constant temperature exchanges thermal energy and thermal entropy with the thermostats but the Carnot heat-engine efficiency factor establishes that thermal energy cannot be converted to work when both thermal reservoirs are at the same temperature.

A simple but general statistical-mechanical derivation **Error! Bookmark not defined.** is easily developed with the Helmholtz (free) energy expressed in terms of the constant-

$$A = -\kappa T \ln \sum_i^\infty e^{-E_i/\kappa T} = E_0 - \kappa T \ln \sum_i^\infty e^{-(E_i - E_0)/\kappa T} \qquad (4\text{-}3\text{-}4)$$

The partition function $p.f. = \left(\sum_i^\infty e^{-(E_{i0})/\kappa T}\right)$.

$$\left(\frac{\partial(A/T)}{\partial(1/T)}\right)_V = U = E_0 + (p.f.)^{-1}\sum_i (E_i - E_0)e^{-E_i/\kappa T} \qquad (4\text{-}3\text{-}5) \text{ and}$$

$$\left(\frac{\partial A}{\partial T}\right)_V = -S = -\kappa \ln \sum_i e^{-(E_i - E_0)/\kappa T} + (p.f.T)^{-1}\sum_i (E_i - E_0)e^{-E_i/\kappa T} \qquad (4\text{-}3\text{-}6)$$

The last term in each is the average energy fluctuation conventionally called heat and heat entropy, respectively but labeled thermal quantities here.. On reforming A from $A = U - TS$ the heat terms cancel: to yield

$A(T) = E_0(T) - \kappa T \ln \sum_i e^{-(E_i - E_0)/\kappa T}$ in which E_0 is the potential energy plus the zero-point vibrational energies and the sum term is the degeneracy weighted for T. The thermal terms have disappeared. Prior to Benzinger's 1967 and 1971 papers their disappearance by mutual cancellation had not been obvious obscured by a historical error in defining entropy as a single quantity rather than the sum of the degeneracy term and the thermal-energy term. The internal energy and enthalpy always have been expressed as a sum of potential energy plus thermal energy because of first-law conservation but they are very dissimilar the heat being at maximum entropy at the prevailing temperature and the potential energy having only the degeneracy entropy arising from the zero-point vibrational modes. Thermal energy is random kinetic energy totally disordered and potential energy is not disordered at all.

It is nearly forty years since Benzinger first published his discovery. Frank was the first reviewer to understand the importance of the discovery. He called those parts of H or U and S contributing to a change in free energy whether A or G "motive" parts after Carnot. Benzinger's formulation has several consequences of major importance as reviewed elsewhere (Biophysical Chemistry 105(2002) 609).. Chief among these is the revelation that some much used applications of thermodynamics cannot produce rigorous quantitative results Thus for application to experiments carried out at constant temperature some essential information is not experimentally available unless the reactants and products are pure crystalline materials which is assumed for the first development here. As shown in Fig. (4-3-7) also taken from Biophysical Chemistry and also copies in the Utilities folder on this web site, the division of latent heat in phase changes is not possible experimentally so the heat-capacity summation through a phase change to 0K cannot produce results

appropriate to any experimental temperature**.** In all phase changes including the first-order changes in protein matrices on which their function depends the motive parts undergo large changes that cannot be estimated with any accuracy. The separation can be estimated for very simple systems by theory but the larger thermal errors occur with soft materials like polymers and proteins for which accurate computations are rarely possible. This is illustrated with the following example:

The major consequences of Benzinger's discovery--

1. Inapplicability of thermodynamics to processes at constant temperature-If there is a change in state, as $\alpha(T') \leftrightarrow \alpha'(T')$ in the diagrammed process below, the two cooling steps apply to two different chemical systems, α at temperatures above T' and $\alpha'(T')$ down to 0K.

$$
\begin{array}{ccc}
\alpha(T) & \xleftarrow{\Delta A_{\alpha\beta}(T)} & \beta(T) \\
\downarrow & & \uparrow \\
-\int_{T}^{T'} S_{\alpha}(T')dT' & & -\int_{0}^{T} S_{\beta}(T')dT' \\
\downarrow & & \uparrow \\
\alpha(T') & \xleftarrow{\Delta G_{\alpha\alpha'}(T')} & \alpha'(T') \quad \uparrow \\
 & & \downarrow \quad \uparrow \\
 & & -\int_{T'}^{0} S_{\alpha'}(T)dT \quad \uparrow \\
 & & \downarrow \quad \uparrow \\
 & & \alpha'(0) \xleftarrow{\Delta E(0)_{\alpha'\beta}} \beta(0)
\end{array}
\qquad (4\text{-}3\text{-}7
$$

In this thermodynamic box the $\alpha \leftrightarrow \alpha'$ process is the solidification of liquid α at its melting point T'. The standard free energy change is zero As usual $\Delta H_{\alpha\alpha',t} = T'\Delta S_{\alpha\alpha',t}$ and at the melting temperature $\Delta H_{\alpha\alpha',m} = T'\Delta S_{\alpha\alpha',m}$ but neither side can be evaluated.

Further cooling to 0K will yield $E_{\alpha'}(0)$ but that applies to the frozen system at 0K and not to the liquid system at T. As with any chemical change, any phase change is a change in Hamiltonian, which in turn always causes changes in the values of motive parts. Melting and evaporation have large motive contributions because many

vibrational modes change in number and in characteristic frequency. As a result phase changes eliminate the calorimetric approach to the separation of motive and thermal quantitites in A and G.

Explanation As is generally realized in discussions of entropy, the driving force is the increase in disorder called heat. In our part of the universe spontaneous changes produce and are driven by entropy increase as potential energy is converted to thermal energy. At 0K entropy and enthalpy have their lowest values. As temperature rises spontaneous processes can and do occur using up the potential energy and thus the ability of a system to do work. The bookkeeping is done with Helmholtz free energy at constant volume:

$$A(T) = E_0(T) - \kappa T \ln \sum_i e^{-(E_i - E_0)/\kappa T}$$

negative initial value becoming positive due the increase in degeneracy resulting from an average decrease in the energy quanta.

Planck perhaps anticipating Boltzmann and entropy divided the free-energy expression by T thus converting the potential energy to potential entropy and heat to heat entropy. If there is a lowest temperature, the third law is a direct consequence to overwhelm the divergence of Planck's function to infinity at 0K. The conversion of potential entropy to heat entropy and degeneracy entropy alters the free energy only through the second term since the heat entropy is canceled by the heat enthalpy divided by T. As irreversible change takes place, the local part of the universe becomes increasingly hot apparently reducing the degeneracy to a simple sum over states at infinite temperatures perhaps to be reversed by black-hole action elsewhere in the universe.

2 Although free energy Helmholtz or Gibbs remains a reliable source of information about a process, Enthalpy, internal energy, entropy and volume are not only not reliable but as shown in the preceding paragraphs for most

processes carried out at constant temperature, their heat and motive parts cannot be evaluated. Very little of conventional thermodynamics provides reliable information although the heat capacity and higher T and P derivatives of H and U can be used unless they contain "between-states" terms with different motive enthalpies or energies. The latter can be estimated from theory for simple systems but it is for complicated systems containing polymers, proteins and other biological macromolecules that reliable motive information is most necessary. Enthalpy-entropy compensation behavior fills this gap to a small but often essential degree as shown later in this chapter.

The enthalpy or internal energy change in a process has its heat term equal to the thermal entropy term in the ratio of the mean experimental temperature so the total enthalpy or internal energy change and the total entropy in any constant-temperature process are always related. The degree to which they parallel each other is greater the larger the heat terms relative to the motive terms. The relationship is now obvious but prior to Benzinger's discovery was usually thought to have a chemical rather than a thermodynamic basis.

3. An important procedure that remains unchanged is the much-used test for dominance of entropy versus entropy in determining a free energy change is not invalidated. The relationship between the conventional test ratio $\frac{\Delta H}{T\Delta S}$ and the correct one $\frac{\Delta H_m}{T\Delta S_m}$ is

$$\frac{\Delta H}{T\Delta S} = \frac{\frac{\Delta H_m}{T\Delta S_m} + \frac{\Delta S_t}{\Delta S_m}}{1 + \frac{\Delta S_t}{\Delta S_m}}. \quad (12)$$

.The two ratios are not equal but their corresponding inequalities are the same since adding a quantity to both sides of an inequality does not change the inequality. The test for two-state behavior in protein melting discovered by Brandts also retains

reliability. It depends on the ratio of enthalpy change determined by calorimetry to enthalpy change from application of the van't Hoff equation. The ratios should have value 1 within error and usually does. Both sources give the total enthalpy change which is not the enthalpy change contributing to the free energy change so the test is reliable even though the division of enthalpy change into motive and thermal parts is not. The latter division can be and usually is large in protein melting but fortunately small in the melting rate process and in the matrix expansion-contraction process powering the nutcracker catalytic operation. That is a result of evolutionary successes forcing the heat capacity changes in both processes to be zero.

4. Transfer of vibrational or librational energy between molecules or systems at constant temperature cannot take place unless there is a change in the electronic Hamiltonian of each system .Although vibrational zero-point energies and concentration factors in entropy are motive quantities, all excited states are thermal energy and useless for free-energy exchange at constant temperature. This is the criterion that defines thermodynamic microstates and it does not appear to have been known prior to Benzinger's discovery and forty years later is still not found in textbooks.

5. Rate processes adequately treated by Absolute rate theory or Kramers rate theory are subject to the same restrictions on total activation enthalpy and entropy as normal equilibrium processes. Item 1 in this list applies. Activation free energies are reliable but not equal to true thermodynamic free energies because the degree of freedom including the reaction coordinate has been used for the time-dependence. The Eyring-Leffler-Hammond "principle"[i] for comparison between activation free energy in a rate process and the standard free energy change to estimate progression along the reaction coordinate is not jeopardized so long as it is applied to free energy. Because the enthalpy has no quantitative relationship to the free energy, it cannot be used as a substitute.

6. Tables of bond energies computed using total enthalpy changes cannot be accurate since they contain thermal parts that have no relevance for the potential energy that determines bond strength. However, bond-rupture quantities computed from gas-phase enthalpy data have small errors from equating total change to motive enthalpy change.

7. Benzinger's discovery provides a general explanation and formal structure for the extrathermodynamic relationships known as linear-free-energy behavior and its associated enthalpy-entropy compensation relationship. The developments are given below. In addition the heat capacity and other first and higher derivatives of enthalpy sometimes provide a way to estimate important quantities Benzinger has shown to be unavailable through rigorous application of thermodynamics. The methods for estimation are given in the chapter on Compensation phenomena with applications of compensation analysis. (Chapter. 6, volume 2).

II The two macro states of pure water

One hundred years after Roentgen suggested that the special behavior of liquid water could be explained if it existed in two chemically different species Walrafen and coworkers in 1983 using greatly improved quantitative Raman spectroscopy proved him correct.. They found a single isosbestic point as had Worley and Klotz using infra-red spectroscopy in 1968.. The two groups established sharp isosbestic points at three frequencies from which the standard enthalpy difference between the two states was found to be 10.5 ± 0.5 kj/mole of formula weight. The standard entropy change depends on the model but assuming 1:1 interconversion stoichiometry it is about 28.2 j/Kmole of formula weight. Walrafen et al[ii] cited ten reports of that enthalpy value obtained from six different kinds of experiments and there are three additional confirmations the highly accurate heat-capacity data. Thus with the constant-pressure heat capacities Benson and Seibert[iii] and Stey[iv] found two states and only two states separated at 298 K and 1 atm by 9.6 kj/mole of cooperative unit for the enthalpy

difference and Chen[v] with the constant-volume data found 9.6 kj/mole of cooperative unit for the internal-energy difference consistent with the higher PV difference reported by Walrafen for the enthalpy. Stey and Chen also showed that mercury, benzene, methanol and ethanol have single-peak probability density distribution functions for enthalpy and internal-energy, respectively. Thus far among ordinary pure liquids only water has been found to have more than one macrostate and it is sharply limited to two. In pure water the second predominates only from 277K down into the supercooled region but as shown in Chapter 4, it is the basis of the solubility behavior of amphiphiles at higher temperatures as well.

The higher temperature state H is now generally thought to be the "random connectivity" state stabilized by the entropy arising from the bending of hydrogen bonds first proposed by Pople. The lower-density state, L, is a cooperative cluster produced by inductive electron rearrangements producing a dismutation among the hydrogen bonds of the cluster. The enthalpy and entropy changes in the conversion of the higher-temperature water state to the lower cited above agree with those obtained from several other kinds of experiments using the two-state model for water. The stoichiometry has not been established but based on the suggestion by Frank that hydrazine is "inhibited water' , that is, water without the special solubility properties detailed in Chapter 4, the estimate based on the heat capacity data of Oguni and Angell by Lumry et al is four or five water molecules.

The standard chemical potentials of the two species at 1atm are equal at 285K so from slightly above that temperature down into the supercooled region, there is a mixing term to complicate analysis of the thermodynamic quantities. Lumry and Rajender reviewing the literature on linear enthalpy-entropy compensation attributed most examples with compensation temperatures near that experimental value to the mixing term but that is not always the case as discussed in the next section.

The term ***hydrophobic hydration*** has become ubiquitous in discussions of the thermodynamic features responsible for the stable folding and the catalytic feature of proteins. These developments have finally clarified not only pH but also the major role protein hydration plays in determining protein construction and enzyme function. The clarification shows that is it not the conflict between non-polar and polar interactions that detemines proteins behavior but that between clathrate formation and the structure beakers. In 1938 Russian workers showed that this distinction breaks down at 354K because the enthalpy change stabilizing protein folding becomes unfavorable at those temperatures. For proteins its is the favorable interactions of water via clathrate formation of all otherwise inadequately hydrated regions. In turn this complicated situation varies depends on ability to form clathrates a matter of size, shape, polar nature and competition from structure breakers. of groups. folding of a polypeptide into its native conformation in attempts to explain the path from ribosome to fully folded protein in terms of the familiar antagonism between polar non polar substance the former being water and the latter one of a very wide range of amphiphilic or totally non-polar species such as the perfect gases or methane. Kauzmann in 1959 suggested simple antagonism on the basis of solubility data from Edsall but Shinoda and then others (e.g..;Lumry, Battistel and Jolicoeur, Faraday Symposium on water 1983 Leicester) showed the suggestion to wrong. Instead as devolved from the work of Henry Frank, it is unfavorable enthalpy in such associations rather than unfavorable entropy. More recently Franks comparison of structure-breaking solutes such as hydrazine, hydrogen peroxide and urea with structure makers including the amphiphiles and hydrophones also including sulfate ion, the best of the structure-makers and the prorated have led to what amounts to a totally revision in the description of mixtures of structure makers of all kinds with mater. Structure makes of all kinds form water clathrates just like those found in the ice clathrates and depending on size the process uses up about half of the free water. The missing features have been the high stability of such compounds resulting from

small but critical chemical interaction and the dependence of clathrate size on the guest. The data on water clathrates of amphiphiles provide by Koga et al have clarified a long standing puzzle first discovered by Arnett and McKelvey thirty years ago. The clathrates stabilize the low-density cluster form of water which is in equilibrium with the higher-density form, thought to be a the random-connectivity form.

III <u>Enthalpy-entropy compensation behavior</u>-

Hammett popularized the concept of "linear free energy relationships" but the Bronstead analysis of acid and base dissociation was already well known. The relationships are extra-thermodynamic which means they cannot be treated rigorously by equilibrium thermodynamics. They are linear only to a rough limit set by the errors in the experimental data and many escape detection because of their non-linearity. Leffler finally pointed out that a more deceptive test is to check for linear enthalpy-entropy behavior now known as "compensation behavior" since he showed than any LFE has a companion linear compensation relationship much more likely to be linear since advancement along the free-energy coordinate does not destroy the linearity between H or U and S. Leffler and Grunwald explored the theoretical area in ground-breaking detail in 1963 (Rates and equilibria of organic reactions, J. Wiley and sons, New York 1963) under the rubric "isoequilibrium and isokinetic reactions" since updated by Grunwald in 2001 @) Otherwise the topics have largely suffered from aimless curiosity with minor profit and little attention to the fundamental basis in Benzinger's discovery. The latter provides on of the few thermodynamically sound classes of the generally extrathermodynamic phenomena and is first considered.

Equations 4-3-5 and 4-3-6 show that U and S share the thermal term with a consequence that when a thermal contribution is large relative to the motive contributions U and S will tend to parallel each other in related experiments. That the thermal terms are in ratio of the mean experimental temperature is the basis for a ubiquitous but well-hidden class in which the experimental compensation temperature from the linear slope of the enthalpy-entropy plot tends toward the mean

experimental temperature. The agreement between the two is often the test for this class.

As Lumry and Rajender suspected (Biopolymers 9 (1970) 1206), the two-microstates of water provide a second very common class. The equilibrium between the two chemical species of water with compensation temperatures near 285K because the two standard chemical potentials are equal at that temperature. The relationship responsible for the change in proportions of the two species produced by the advancement in the measured process is generally called "a linkage relationship" a term attributable to Wyman. Wyman and Gill and Wyman exploited grand partition functions to relate the several concentrations changing in this relationship but they can be also usefully treated with familiar thermodynamic quantities although the treatments are generally non-rigorous and the relationships neither stoichiometric nor accurately linear. As the proportions of the two water species are altered by a solute process they make a contribution to the total free-energy change at all temperatures except that at which the standard chemical potentials of the water species are equal. That temperature is equal to the compensation temperature.

If the linkage between solute process and water has no chemical features, it would be described by the simple equivalence of chemical potentials of the water species. That is, if the water species equilibrium was altered by van der Waals potentials and geometric changes in volume distribution. The chemical features on the other hand are those resulting from changes in the electronic Hamiltonians of the participating materials. This may have been appreciated prior to Benzinger's Nature paper in 1971 but it follows from the fact that only the motive parts contribute to free-energy changes and those vary only as a consequence of changes in the electronic Hamiltonians. Frank and Evans realized in1945 that the most solutes including all amphiphiles interact chemically with at least one of the pure-water species (cf. Chapter 4 part A Vol. 2 for details). It is shown in Chapter 4 that hydrophobic solutes and mixed polarity molecules called amphiphiles sequester large number of water

molecules forming with considerably cooperativity single-shell cages like those already familiar in ice. These are sufficiently stable compounds to tie up many water molecules depending on their size, planarity and competitive effectiveness against other solutes of the same kind or competitive amphiphiles. Thus in real water mixtures with structure makers there is very little normal water. Structure breakers like hydrazine and urea also reduce the amount of normal water by destroying the lower-temperature form of water from which the clathrates are formed. Call that species L.

Small amphiphiles and hydrophobic molecules favor pentagonal dodecahedral cages containing 20 water molecules reduced by the number of solute hydroxyl and amino groups able to substitute for water. Ethanol for examples (part 1 Chapter 4A) competes with other ethanol molecules until all have on average about 10 rather than 19 water molecules producing a sharp change in heat capacity at about 0.1 M ethanol. For convenience we assume an average n and neglect its dependence on cooperativity. Then

$$dG = \mu_H dn_H + \mu_L dn_H + \mu_{LC} dn_{LC} \qquad 2$$
$$\mu_{LC} = \mu_L + \mu_C$$

Only L reacts with the solute C in this model and

$\mu_H = \mu_L$ The total of water based on assumed unit stoichiometry is $n_H + n_L + n_{LC} = w$ 3

Using concentrations the simplest reaction scheme is

$$H + C \leftrightarrow HC \quad K_H = \frac{[HC]}{[H][C]}$$

$$L + C \leftrightarrow LC \quad K_L = \frac{[LC]}{[L][C]} \qquad 4$$

$$L \leftrightarrow H \quad K_W = \frac{[H]}{[D]} \quad [H]+[L]=W$$

$$K_{app} = \frac{([HC]+[LC])}{(([H]+[L]))[C]} \qquad = K_H + (K_L - K_H)\frac{[L]}{W} \qquad 5$$

with which the size of C can be varied; the stoichiometry of L and H with C can be varied as can the proportion of water in L or H form. An improved theory accommodates the averaging of solute-water species. Obviously much of the chemical information based on experiments in which water has been assumed to have only one homogeneous species have become unreliable. Note for example that conventional single-species acidic and basic constants for water are not acceptable because the hydrated proton is a highly hydrated amphiphile. That finally explains the peculiar ionization thermodynamics of simple acids and bases. and thus the compensation behavior originally familiar from the Brõnsted relationship.

The simple theory of linear enthalpy-entropy compensation behavior.

In the absence of chemical interaction as defined above interaction between the driver species and the driven species as defined by the choice of reaction series can be described by activity coefficients but we strart with an example in which the iteraction can be described by a linear-free-energy (LFE) expression following Hammett.

$$\Delta G_i = \Delta G_0 + f(i)g = \Delta H_0 - T\Delta S_0 + f(i)h - Tf(i)s$$

$$\Delta H_i = \Delta H_0 + f(i)h - Tg\left(\frac{\partial f}{\partial T}\right)_P \;;\Delta S_i = \Delta S_0 + f(i)s - g\left(\frac{\partial f}{\partial T}\right)_P$$

$$\Delta H_i = \Delta H_0 - T_C \Delta S_0 + T_C \Delta S_i + h\left(\frac{\partial f}{\partial T}\right)_P \left[\frac{(T_C - T)^2}{T}\right]$$

in which $T_C = \frac{h}{s}$, called "the compensation temperature" is eqial to the slope of the plot of experimental enthalpy change against experimental entropy change, call a "compensation plot".

The last equation must be expanded if the linkage factor ***f(i)*** is temperature dependent or T_C is well out of the range of experimental temperatures but in the latter case that is unlikely to be important since it is only a parameter and may be quite different from the temperature at which the actual experimental free-energy changes are zero. Note again that the phenomenon is not rigorous, that is, it is extrathermodynamic and reliable only if it is a close approximation to the temperature at which the actual chemical or physical change occurs with zero free-energy change. Obviously the experimental errors must be small relative to the deviation of the latter from linearity. The compensation temperature has physical significance only when the standard state is chosen to give enthalpy and free energy the same dimensions. Then it is relevant to a real but hypothetical experimental temperature at which the free-energy change is zero for all members of the group chosen to test for compensation behavior. With such choices and testing it becomes possible to estimate the contributions to overall free-energy, enthalpy, entropy and volume changes in the measured process.(Lumry in Biophysical chemistry 205 (2003) 545 [also copied into Utility folder on this web site}and see Winzor D on the severity of the errors possible in non-stoichiometric linked systems.).

Because of the way ***f(i)*** enters these equations, compensation plots are generally more linearly that the LFE relationship thus likely to reveal a systematic enthalpy-entropy relationship than the LFE approximation. The use of compensation plotting has become common only in recent years so many examples have been missed. In organic chemistry inductive effects are a common source as suggested by Hammett's

original LFE examples. Engberts and his students in particular have reported many examples of compensation behavior not all of the linear variety.

There are several bases for linearity but in general that characteristic requires only a single linkage relationship. The chosen series must be measured when the independent variables are chosen to minimize any other linkage connection such as pH with correction for buffer. As can be deduced from the development given above, two sources for the same measured process will produce deviations from linearity unless their associated T_C values are very similar. Benzinger's heat enthalpy-entropy relationship with rare exceptions provides a second basis for compensation behavior but the compensation temperature for the latter is usually equal within error to the mean experimental temperature to test for a true linkage relationship.

Uses of compensation behavior

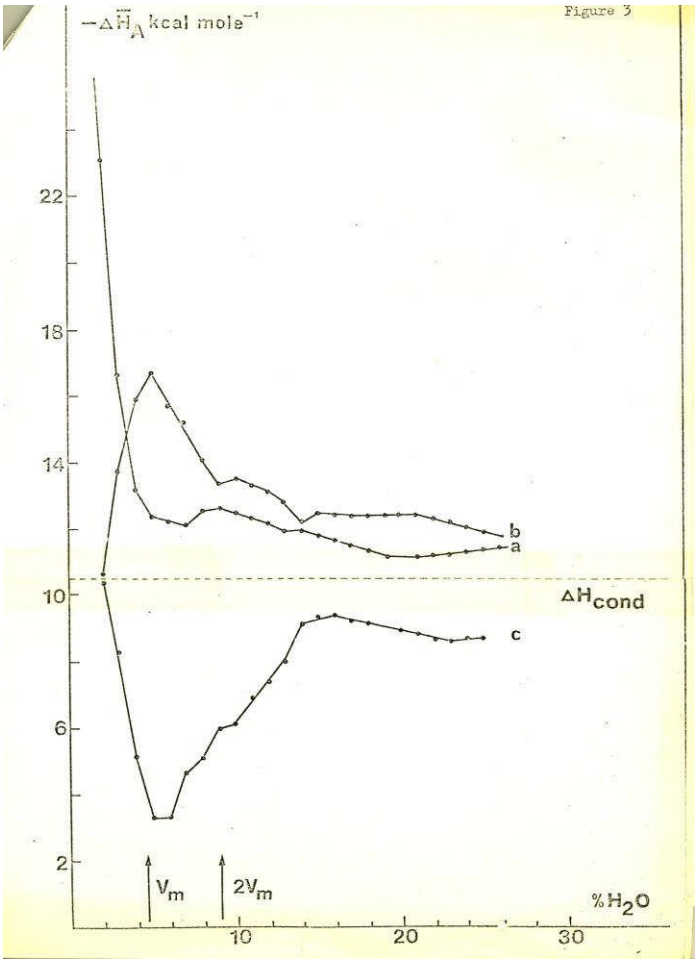
The enthalpy can be expanded in a temperature series in which the constant term is the potential energy and the other terms are the moments about the mean the description of energy fluctuations and thus a description of the heat at constant volume. For constant pressure those terms also include the volume fluctuations. In general it is not possible to evaluate the constant term for a process at constant temperature unless the heat change is zero. Then as already noted the enthalpy or volume changes are pure motive quantities and can be equated to the free-energy changes. In such cases the severe complications usually arising from Benzinger's discovery can be avoided but zero heat-capacity change is very rare in randomly constructred processes such as dominate small-molecule chemistry. Fortunately important protein processes require zero heat-capacity change to be useful and evolution has been surprisingly successful in finding the necessary construction and constraints.

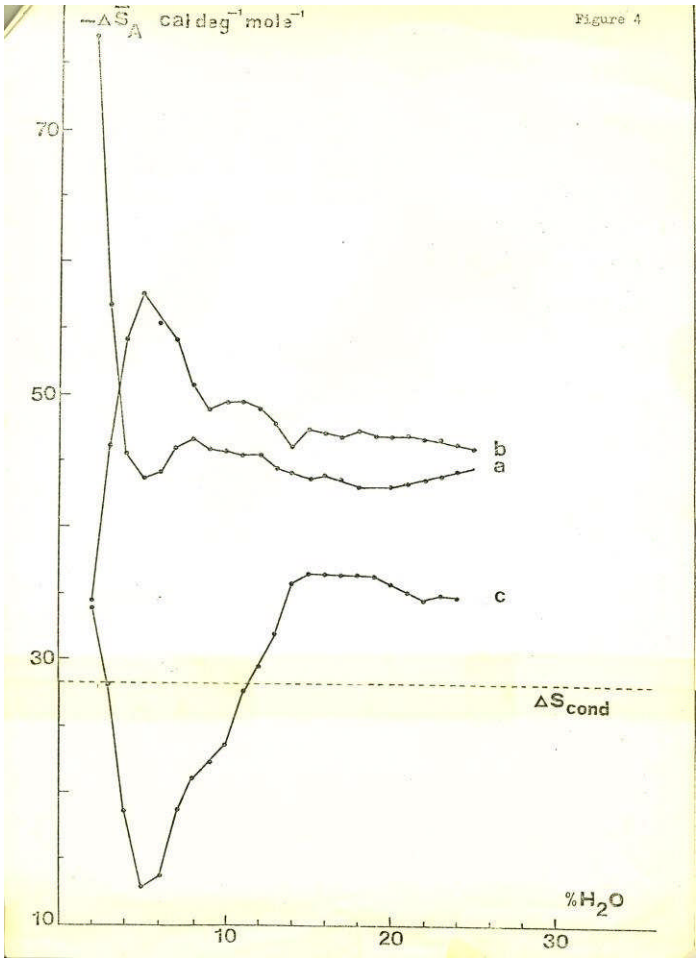
In general one must live with non-zero heat changes. The formal treatment is simple and applicable to data from appropriately selected series such as the congener series of inhibitors of an enzyme. It applies to non-stoichiometry coupling of a

common measures process and one independent variable, other independent variables being held constant. The treatment of protein hydration by Luscher and coworkers is particularly complete testing for statistical sources of compensatioin behavior that have jeapordized use of such behavior for many years.In 1976 Krug. Hunter and Grieger published a set of tests for such behavior almost immediately applied by Lüscher and now often applied. They do not exclude the fact that compensation behavior is extra-thermodynamic but they do provide confidence that it is not a statistical artifact.

Two papers by Lüscher and coworkers are particularly important going well beyond thorough exemplification of the use of such data because they also reveal the several complications that have attended attempts to extract reliable information about protein hydration from thermodynamic measurements. They found those quantities to be very different depending on experimental temperature and degree of

hydration and could characterize each of the four main regions of behavior by its own compensation temperature.





These two figures are copied from a manuscript by Luscher and Ruegg which was intended for Biopolymers in 1977. Subsequent papers in Biochimica et Biophysica Acta given above present the same data in a way which is less useful for this chapter. The top figure shows the enthalpy changes resulting from progressive addition of water at three different temperature ranges. The lower figure plots the corresponding entropy changes.

Compensation analysis is applicable for series of related processes all carried out at the same temperature and pressure. As shown in the two figures above, application to sets of data for a given series is much more informative when several temperatures are used and the same applies to the use of constant pressure data for volume information. Three temperature ranges are represented in these figures from Luscher

and Ruegg making it possible to answer several questions about the entire series in this case variations in amount of hydration water:

1. Do that data demonstrate linear enthalpy-entropy compensation behavior?

2. How many linear compensation patterns are demonstrated and is each an intrinsic consequence of the chemistry or physics of the series or a statistical consequence of the choice of members of the series. Data of inadequate precision can also be the source but the method of Krug, Hunter and Geiger or similar alternatives required to test for a statistical basis can also be used to determine whether the necessary level of precision obtains in any case.

3. If there are abrupt changes in slope as occurs in both figures, are they explicable and consistent with other information available? Generally this question can be answered by comparison of statistically reliable compensation temperatures (the slope of a plot of the enthalpy change for each member of the series plotted against its enthalpy change.) with compensation temperatures known from other experiments to be characteristic of one or more features of systems similar to those under investigation. Since the theoretical support for compensation analysis is extrathermodynamic, it is highly desirable that the data have a precision of 1 or 2%. Such precision in enzyme rate messurements is rare. Much of the prejudice surrounding the subject is a consequence of poor data precision.

4. Protein systems and water and aqueous mixtures have demonstrated a verity of compensation temperatures which often can be used to identify characteristic processes. Thus Gregory et al found the melting rate of proteins had 354K whch originally appeared to identify only the knots but in 1983 @ found it to be characteristic of the mixing of structure makers with water. Specifically the in enthalpy change in that process is negative below 354K and positive above. Compensation behavior with that compensation temperature identify the hydration of structure makers of which protein surfaces are only in example. Being stable below 354K and

unstable above. Similarly Gregory identified compensation temperatures in the range 420-470K as the characteristic of the contraction-expansion process of protein matrices. The identification is confined to protein matrices and appears in studies of most processes of those matrices including in enzymes the maximum rate of catalysis (usually called k_{cat}). The two species of pure water have equal mole fractions at 285K of 1 atm. pressure so any measured process which directly or indirectly changes that ratio can be made to demonstrate that compensation temperature Soft proteins like hemoglobin and Myoglobin allow continuous adjustment of conformation with compensation temperatures near 295K for ferric forms and 310K for ferrous forms. These temperatures reveal real chemical change in the linked processes with which they are associated and the compensation temperatures are only weakly dependent on experimental temperature.

Linear enthalpy-entropy compensation behavior exemplified by the above group makes contribution to the free energy change in the measured process. The compensation temperatures are only weakly dependent on temperature.
capacity change difference in that derivative To understand this it is necessary to discussed in sections 1 and 2, proteins have two major substructures one with 354K and the second about 460K Stoichiometric processes can demonstrate compensation and linear free energy behavior only over short ranges of an experimental series but most perhaps all mechanism in physiology are non-stoichiometric and it is found that compensation behavior often provides a way to extract useful information to somewhat offset the impotence of thermodynamics. Thermodynamics is not the problem. Here it is absence of exact stoichiometry but in the previous item it is the instrinic quantum mechanics responsible for the changes of state.

In the two figures from Luscher and Ruegg copies above the abscissa is volume of water in terms of the number of water molecules bound by chymotrypsin. The quantity labeled $\mathbf{v_m}$ is the protein surface area estimated by the equation of Brunauer, Emmett and Teller and estimate by Chothia et al as having about 350 acid-base

groups. The figures can be partitioned into four regions: low volume-low temperature T_C =310K ;:low volume-high temperature T_C =268K ; high volume-low temperature T_C =433K, and high volume-high temperature T_C =411K. The temperature range are a.) 10-20; b.) 25-40; c.) 20-25.i°C.: (The compensation temperatures are from Lutcher, Ruegg and Schindler. Biochim. Biophys. Acta 536 (1978) 27).

The isotherm a shows rapid change from 0 hydration to $\mathbf{v_m}$ in H and S followed by little change at higher hydration. The compensation temperature is not much higher than the range so the free energy change is relatively smll in region **a**. Much larger changes from 0 to $\mathbf{v_m}$ are found in regions **b** and **c** and they are roughly mirror imagines in H and S in both low and high hydration regions. revealing that the binding up to $\mathbf{v_m}$ is cooperative in forming a hydration feature which may or not be a complete inner shell. Complete formation is indicated by the abrupt change in slope in H and S at $\mathbf{v_m}$. From 0 to $\mathbf{v_m}$ in the low-volume regions the **b** and **c** enthalpy isotherms are inverted behavior which can now be attributed at least in part to the fact that at low temperature water moving from liquid phase to protein is mostly the L type and at higher temperature if the H type Recall that in pure water the two water species have equal standard chemical potentials at 12°C. The enthalpy change at higher temperature (**b curve)** suggests that the cluster species, L, is preferentially bound as appears to be consistent with the diffrerence in T_C values for the two temperature ranges.

The high T_C values at hydration levels above $\mathbf{v_m}$ indicate the existence of a second cooperative process and its T_C range around 430K is that found with protein matrices. That behavior indicates the onset of cooperativity in matrices which has also been detected by other methods. Proton-exchange at matrix sites is labilized at such low levels of hydration (estimated in these hydration studies as 70 out of 350 single water-binding sites).

Most of these observations are consistent with those published by Lutcher and coworkers as it has been possible to revise them using the existence or the two states

of water (chapter, 4, volume 2) and the diagnostic power of protein compensation temperatures.(Lumry, Chap.1 in R. Gregory, ed. "Protein-solvent interactions" Dekker and in volume 1, the Protein Primer). That of protein knots is 354K and since that does not appear at low volume in the hydration studies, any effect of hydration on knots is small and if present at all is concealed in the matrix behavior. Gregory has given a detailed integration of the several varieties of experimental data on which the analysis here is based (See R. Gregory in "Water in foods" paper in Utilities folder on the Protein Primer and at the URL given there.) Hydration thus takes place in at least two major cooperative processes the first of which provides enough free energy to compete formation of a protein able to support physiology and the second provides a molecular mechanism for that physiological undertaking. Enzymes illustrate these very well as discussed in chapters 4.5 and 8 of this volume. Apparently globular proteins can respond to constraints provided by their environments in normal functional ways after matrix relaxation has taken place. However in enzymes the normal catalytic mechanism can exist with only twice, something less than 20% hydration; 17% has been reported.

Luscher and Ruegg confirmed their deduction that hydration at higher temperatures and hydration greater than $\mathbf{v}_m$ in chymotrypsin is coupled to a conformation change in the protein by compressing the matrices thus freezing out the matrix expansion. They did this by acylating the protein with an inhibitor. The compensation temperature for the high-temperature-high-hydration region (see above figures) dropped 430K to the experimental temperature near 298K. In another important and closely related experiment Bolen and coworkers using a sultone substrate with high ring strain balanced the decrease in ring strain as the sultone ring opened on acylating the enzyme by the potential energy released by matrix contraction. As a result no potential energy was lost so the sultone ring was reformed on reversal of the process.

Varieties of catalysis

By definition a catalyst accelerates a process whether chemical or physical and in so doing it must return at the end of a catalytic cycle to its original state which is usually meant the same free energy of formation. If the final free energy were lower, the catalyst would soon be consumed. If higher, it has gained free energy from the reaction it catalyzed. Neither of the latter alternatives need be considered although in some complex, multi-stroke biological engineers transient sharing of free energy has been used to support or improve the changes in the reactant-product system for greater efficiency and greater specificity. Specificity here means sensitive in selecting reactants among groups of similar reactants and especially in biology is a major requirement. Some enzymes can accelerate a process involving a bond containing a proton to a different degree than a process in which the only difference is replacement of the proton by a deuteron or triton. A more familiar example is the retention of stereospecificity essential so as to fabricate proteins and other macromolecules from only one enantiomer. That is also a common feature of modern polymer synthesis since most properties of a given catalyst are different among syndiotactic, isotactic and atactic polymers. Success in producing nearly all common polymers has been the result of the discovery of ways to control stereospcificity as for example, the polyethylene and polypropylene polymers made possible with Ziegler-Natta catalysts.

Simple-acid-base catalysis-This is the most common variety and the simplest example of the class called "electron banking" since it depends on temporary borrowing or lending of a proton It is inefficient but often hard to avoid.

Charge-transfer is general version of electron banking often depending only on redistribution of electrons without complete transfer. Transition metal ions are especially effective because of the availability of low-lying excited states which can be used for charge redistribution. In organic molecules mixing of atomic species and

structure to produce electron displacement and bond strain provide a large part of orgainic chemistry. The electrons of the catalyst are arranged either permanently or transiently to destabilize the reactant or stabilize the product. The are then is to find electron rearrangement and effect it by chemical modification of the catalyst. That often involves positioning of the substrate and catalyst as well as the electron rearrangements. What ever the favorable combination the catalyst must be returned to its original state as the product leaves.

This catalytic mechanism is so familiar it was a natural direction to turn in early attempts to explain catalysis by enzymes and the most famous attempt is that of Pauling who suggested that enzymes effected catalysis by stabilizing the transition state. Thus the free energy of formation of the transition state somehow becomes more negative than that of the earlier combination of enzyme and substrate, Implicit in this idea is the replacement of the stabilizing free energy loss in the formation of the product. That also includes replacement of any free energy lost in formation of the enzyme-substrate compound formed before the transition state along the reaction coordinate. Those requirements necessary to return the catalyst to it initial state are quite demanding especially since they must be satisfied by any enzyme. They raise uncertainties about this "transition-state stabilization mechanism" which have not been adequately answered by experiment or theory but nevertheless have failed not for lack of trying but for lack of a more acceptable alternative. In particular a general kind of electron rearrangement process possible with all enzymes or an alternative way to destabilize the reactant and perhaps stabilize the product has not yet been found.

An obvious place to look is to the construction of the enzyme but that to be effective would require conformation changes in which the substrate is made unstable by mechanical stress tension or compression. Conformation changes might also localize potential energy for that purpose an alternative to the localization of thermal energy on which conventional Eyring rate theory depends. But protein conformation

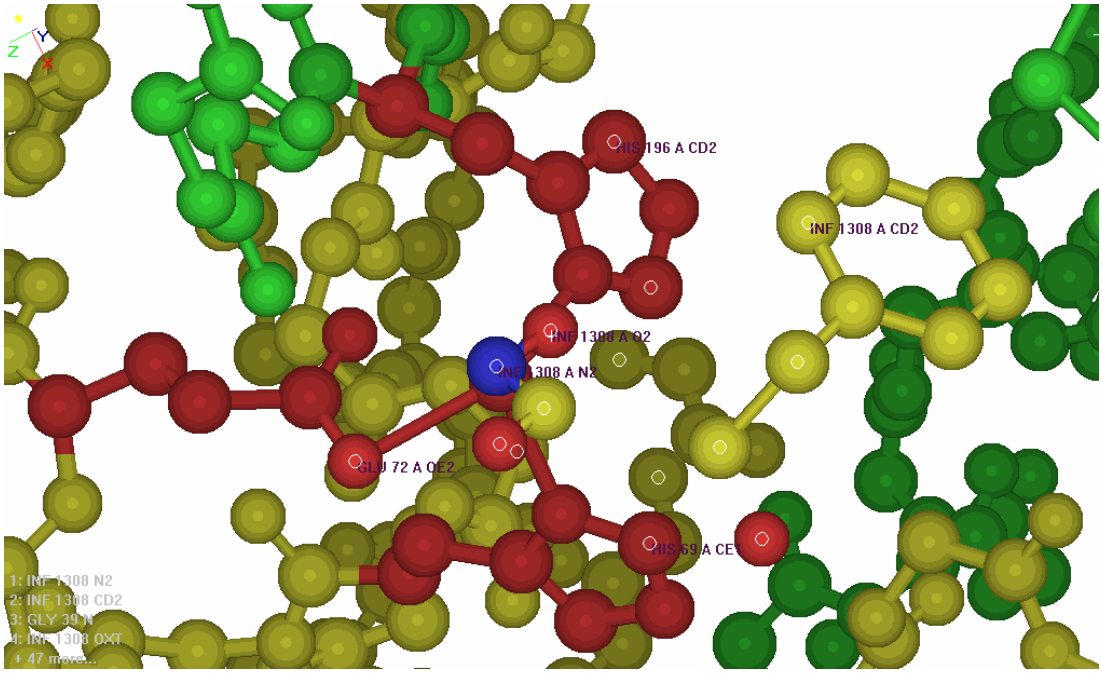
changes once quite popular and well established by a variety of expetimental data have not been detectable in x-ray-diffraction data so as the popularity of the latter increases that of protein conformation changes diminishes. This quantitative misconception has made a considerable fraction or protein research temporarily worthless since the necessary quantitative information is contained in the temperature factors determined as a byproduct in diffraction studies and tabulated with the bond and angle coordinates in the Protein Database. The errors in the latter are large relative to the their precison whereas those in the B factors are smaller than intrinsic errors. This discrepancy can be illustrate by plotting the latter again atom number for any high resolution protein diffraction study. In must such examples using enzymes the protein is shown to consist of two halves of equal mass in a pattern closely approximating a palindrome. That accounts for the rough C-2 rotation symmetry. The amino-acid residues do not reflect those patterns and aside from specific size, shape and charge distributions of the different kind or residues are used in protein evolution simply as building blocks. Aside from this entirely trial-and-error use which often incorrectly appears entirely random there is no residue conservation between the two halves and within protein families. Much like a group of monkeys with typewriters a group of children given enough time could duplicate any protein.

The two halves contain a pair of domains with free volume patterns approximately palindromic with rough dynamic matching. That construction was first apparently in the diffraction study of Myoglobin by Kendrew and Dickerson (Fig. @) completed well before the diffraction study of any enzymes illustrating the major importance of the C-2 symmetry in protein evolution. It is from this feature that the machinery by which the catalytic rates of enzymic catalysis have are effected by the use of potential energy to supplement or replace thermal activation. A particularly useful illustration is found in the work of Bone et al on α-lyctic protease.

Of the several studies of the aspartyl proteases measuring the effect of the strong inhibitor @ Zundel using infra-red spectroscopy was able to show a complete transfer

of the proton in the single inter-domain hydrogen bond between the catalytic domains of pepsin. Using the B factors this is shown to be due to major contraction of the nutcracker arrangement of the domains. The domains are in close contact only where they are hinged and iu the hydrogen bond across which the proton transfer occurs. That H bond has become famous recently as a low-barrier bond allowing some proton tunneling which has been seized on as a peculiarity of construction which might provide rate enhancement in a conformationally fixed enzyme. In fact because the conformation is not fixed varying degrees of domain closure make possible varying degrees of proton migration as has been found not only by Zundel and several other investigators of pepsin but also in other enzymes. Carey and coworkers using stimulate Raman spectroscopy have assembled a collection of similar examples. Since the B factors are already available in the ProteinDatabase, it is considerably easier to use them thus recovering the major information in the diffraction studies.

The basic nutcracker construction makes possible the wide range of enzymic catalysis all of which depend on transient redistribution of potential energy. In the substrate-free enzyme the potential energy is stored in as expanded matrix substructures. It is the passive attachment of the substrate which triggers matrix contraction and when either substrate or product separtes from the protein, the matrices expand to their free states.. Metal enzymes use the nutcracker in a different way as can be illustrated by the activation of the zinc ion in carboxypeptidase A



As with the majority of enzymes there is a single polypeptide chain passing through each domain only once so the hinge in this protein is part of this chain Replacing the single inter-domain H bond of pepsin and trypsin is a single zinc ion chelated to an imidazole group from one chain (196) and from the second domain an imidazole group (69) plus a carboxyl groups of a glutamate residue(72) (Fig. above) The ion is held in this way exactly positioned between the two halves of the protein. Because the domains are connected by strong springs, matrix contraction forces the nutcracker to close on the chelated zinc plus non-covalently bound substrate. The matrix processes form a coordinated phase transitions stabilized at the contracted end which varies with the construction of the substrate. The potential energy transferred from the matrices to the chelation group raises the zero-point energy of the latter thus destabilizing the pretransition state from which the system can slide downhill through the true transition state to products. That process is usually assisted by some thermal supplement equal in amount to the experimental activation energy. The catalytic process depends on distortion of the ligands from the protein plus those from a single water molecule and it is the rupture of the latter resulting from the destabilization that cause peptide hydrolysis. In all enzymes is now appears that catalysis is predominately

a mechanical result of the transient redistribution of potential energy effect closure of the nutcracker. Exact reversibility of the protein process is required to prevent protein destruction. Though it is remarkable that nature had been able to find the matrix process for every enzyme, that search is only the beginning of the path to higher order physiological function.

Using the B factors for from diffraction study of free carboxypeptidase A and those for the same protein modified by the covalent binding of the acid moiety of a substrate to the zinc ion the degree of matrix and knot contraction in nutcracker operation can be estimated. The procedure as detailed in chapter 3 of this volume involves comparison of the mean B and its standard variation for the knots and matrices of the two proteins. The results for the free protein and that with a @ fragment are given in Table @. The C-2 symmetry can be established by similar comparisons of the B parameters of the two half molecules. Even with the errors of older diffraction reports the symmetry is precise often showing corresponding pairs of atom to have root mean square values equal within 0.05 Å. That error is a good estimate of the quality of protein conformations. Their fitting is so tight that the contraction to activate the nutcracker mechanism need be only a factor of two or three larger. (Biophysical Chemistry 101-102 (2002) 81)). It is not surprising that those changes are not detectable above the intrinsic errors of coordinate determination in diffraction measurements of proteins. Alternatively is essential for protein research that the volume data from B factors is so accurate.

Statistical parameters for carboxypeptidase A free of ligands (1yme.pdb) and @ as ligand replacing the water molecule of the zinc ion (1hdq.psb). As discussed in chapter 3 volume 2 of the Protein Primer, the mean and standard deviation of B contains important quantitative information. The mean of of the B values is an estimate of scaling differences essential for comparison of the experimental quality of a study measureing experimental differences between studies. The B values tabulated in all but the most recently reported studies in the Protein DataBank are computed

using the assumption that the electron scattering is isotropic. For a given kind of protein variation due to the more accurate ellipsoidal model are not likely to be important when comparing on study of the protein with another. However, preparation, solvent, equipment and operator variations can be significant. And here it is again necessary to point out that agents such as sulfate ion and polyethyleneglycol (PEG) usually used to improve crystallization by contraction of matices may vary from laboratory to laboratory and from one protein also cause some matrix contraction, The resulting errors are small relative to the intrinsic errors in the coordinates but large with respect to B-factor errors. However with better error control it may be possible to estimate the crystallization errors from the B-factor scaling.

Table 1 Mean B values fpr carboxypeptidase A
Unliganded (1YME) and accylated at zinc (1HDQ)

Statistics Mean values	Carboxypeptidase unliganded	Carboxypeptidase Acylated zinc ion
Overall B	16.8	16..2
Matrices B	20.2	17.6
Knots B	10.7	8,6
Overall B **S.D**	10.5	8.9
Matrices B **S.D**	10.8	9.0
Knots B **S.D.**	1.0	1.2

A related complication is the perturbing effects of methanol used to prevent freezing in many studies of temperature effects. Such studies are particularly useful in characterizing the matrix contraction process at low temperatures. Methanol like any amphiphile does prevent ice formation by sequestering water in strong clathrate structures as discussed in chapters 4 and 5 of this volume. Because hydration at protein interfaces plays a major role in determing the static and dynamic properties of matrices, methanol cosolvents produces unexpected complications. The matrix contraction-expansion process upon which enzymic catalysis depends is sensitive to temperature, extent of hydration and cosolvents as Gregory has shown with his studies of positron annihilation for measuring free-volume changes in proteins (see his papers in the Utilities folder on this web site). Huber, Bone and @ have used methanol to at low temperatures to effect progressive matrix closure. Our van't Hoff analysis of their data produces a rough estimate of 15 kcal for the total enthalpy change. Yapel found the same value for the amount of potential energy transfer from matrices to substrate system in studies of chymotrypsin kinetics at ambient temperatures. Ng and Rosenberg (.Biophysical Chemistry 39 1991), 57). have show direct quantitative correlation of proton-exchange rates for

matrices with catalytic rates using methanol perturbations of subtilisin. In a different kind of esperiment Bone A-lyctic protease: R. Bone, D.Frank, C. Kettner and D. Agard, Biochemistry, 28 (1989) 5925,760: R Bone, A. Fujishige, C. Kettner and D. Agard, ibid 30 (1991) 10388.

measured the correlation of catalytic rates for a congener series of ester substrates of α-lyctic protease with mean B values. They use boronic-acid acyl derivatives matching the ester series. They reported mean B values that decreased by as much as ten units with the most rapid. chymotrypsin turn-over rates increased

Even more useful information can be extracted from B factors using their tandard deviations for the knot and matrix substructures. Table 1 illustrates this utility for free and acyl carboxypeptidase A. Scaling differences between studies are eliminated by using the mean standar deviation, S.D, but the major advance in such use is the estimate of the extend of matrix contraction. For enzymes the the 12% of the atoms with lowest B factors are the knots. That was first found using Pohl's compensation plot for protein melting rates as a consequence of the zero activation heat capacities for those rates (Chapter 1, volume 1). The more closely the mean S.D. for matrices approaches that for the knots, the greater the advancement of the matrix contraction process. In on kind of hyperthermia enzyme the advancement although large at the normal operating temperatures, is very nearly complete at 298K. The same comparison can be used to determine the uniformity of the matrix contraction regardless of the protein and its operating conditions.

With these deductions about mean statistics the entries in table 1 give important details of the construction of carboxypeptidase A. In particular they show the degree of matrix contraction as well as the uniformity of the contrtaction produced by the binding of ligants..

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[i] **H. Eyring, R. Lumry and J. Spikes, @ Add Hammond and Leffler references**

[ii] **G. Walrafen. M. Fisher, M. Homabadi and W.-H. Yang, J.Chem. Phys.85 (1986) 6970f**

[iii] **S.Benson and E. Siebert J. Am. Chem. Soc. 114 (1992) 4269**

[iv] **G. Stey**

[v] **C-H. Chen. Chap.**