

Chapter 4. Matrix “breathing” in physiological functions

Matrices and knots have quite different constructions and functions. Knots by fixing the lengths and positions of loops in the peptide chain maintain scaffolding but they have other functions of which the most important is the determination of thermodynamic stability. On the other hand the much larger group of exchange sites falling in the middle peak (peak II) of the proton-exchange rate and B-factor distribution functions is constructed to provide the mechanisms of the physiological processes supported by the protein. Such structures are adjustable by changes in the DNA for modulation of the static and dynamical detail of mechanism producing major or minor changes in functional behavior within a single protein family. In 1965 on the basis of changes in several properties of chymotrypsin and chymotrypsinogen on interactions with substrates and inhibitors Lumry and Biltonen postulated as the mechanical device in chymotryptic catalysis an expansion-contraction process they called a “subtle change”. Well before x-ray-diffraction studies the behavior of a number of conformationally sensitive variables made it obvious that catalytic function was closely correlated with expansion and contraction of major fractions of the total protein. These included substates detected through changes in catalytic parameters, optical rotation and circular dichroism, fluorescence, uv spectra, proton exchange rate distributions all under modulation by interaction with substrates and inhibitors in association complexes as well as real primary bond interaction. The data were mostly obtained by students in my laboratory but that was supplemented by work from the group of George ? at Cornell. When the substructures were discovered, subtle changes were found to be free-volume changes in matrices with little change in knots. Expansion-contraction processes of proteins often under the name “breathing” have been much discussed. In enzymes these subtle changes are modulated in amplitude and temporal behavior

by substrate binding, specific ligand binding, temperature, pressure, solvent composition, hydration level and association with surfaces and macromolecules. Proton-exchange behavior and B-factor changes show that oscillations occur between limits that can be called *liquidlike and glassy* or *expanded and contracted*. The limits vary with the type and strength of the perturbation. Expanded matrices as found in substrate-free enzymes are liquidlike in the sense that the free-volume is mobile and sufficient in amount to allow local atom fluctuations. Because of the high degree of cooperation produced in successful evolution there is no communal free volume and the contraction process is essentially a first-order phase change of a cooperative unit consisting of all or nearly all of the matrices. Contraction is uniform along radii extending from the knots to produce spherical or elliptical shells of constant B. The condensed species are glasslike in the sense that local motion is suppressed by the loss of free volume but the glassing behavior is not random but rather a general cooperative process made possible only by the selection of residues encoded in DNA.

Since there does not appear to be a source of periodic control, the process probably a free-running oscillator with no single fixed frequency. Direct measurements of the average frequency using dielectric relaxation of substrate-free chymotrypsin against its tosyl derivative as a frozen control demonstrated a range about 10^9 sec.^{-1} . This is the means by which catalysts for proton exchange reach the exchangeable sites and it allows permeation through the matrices of small molecules like methanol. The amount of water held in the matrices even at full expansion does not appear to be large but its determination has so far resisted direct measurement. The process is not detectable with the smallest coordinate errors of protein x-ray diffraction and it is still imperfectly characterized but that frequency range is consistent with estimates from fluorescence and EPR experiments and probably with proton-exchange rates. Several varieties of evidence have established that with familiar mesothermal

proteins proton exchange at matrix sites occurs without major unfolding at temperatures below 55°C. For example, exchange at matrix sites remains rapid at 10% of normal hydration. At ambient temperatures matrices even at that low hydration have high motility. The motility is due at least in part to water molecules freely dissolved in the protein acting as a plasticizer. The number of such freely dissolved internal hydration has not been established but may not be large. Schrier and Almog using calorimetry found only 6 water molecules in fully hydrated ribonuclease A to be bound with significant enthalpy changes. Under conditions in which cooperative melting to bubble states does not occur exchange rates depend on catalyst permeation rates and local thermal fluctuations so permeability is high at extreme expansion and very low in the fully contracted state. Bertil and Halle find in ribonuclease A that fixed water molecules, that is, with high occupancy and thus detectable in x-ray-diffraction have a residence time of 8ns at 26°C with an activation energy of 10 kcal/mole. Their release does not require any significant partial unfolding. In this study as in many others the volume of the bubble product in thermal denaturation was found to be no more than 50% larger than that of the native species. This rapid exchange of high-occupancy water and the even more rapid exchange of proton-exchange catalysts is also important in showing that protein compressibility is not that of matrices as single substructures but rather that of the peptide groups themselves. In other words the phase boundary for the application of pressure is at the surface of the polypeptide and not at the interface between protein and solvent.

Exchange at knot sites in mesophile families is characterized by enthalpy-entropy compensation with a compensation temperature near 354K and large activation enthalpies. For the mesothermal proteins this temperature is characteristic of the rate of knot disruption in denaturation. Morozov and

Morozov found that the Young's modulus becomes negligible at that temperature showing that the stress on the knots from the matrices just balances knot strength at that temperature. True hyperthermal proteins such as the extremeophiles from archaea are quantitatively different but as is discussed in a later chapter, their extreme knot properties may be due to the same electrostatic construction principles applied to larger or stronger knots. Covalency in knot hydrogen bonds suggested by the BPTI knot and the extreme strength of the antiparallel β sheet units of the dragline spider silks may be receiving experimental support from the through-bond coupling constants in protein peptide-peptide hydrogen bonds as determined for example by Cornilescu et al. However, disagreements among computation studies and experimental results confused by current enthusiasm for low-barrier hydrogen bonds as a potential basis of enzymic catalysis continue to divert research from more profitable directions.(cf. section on enzyme mechanisms.).

The stress-strain relationship measured by the Young's modulus may have an important contribution from the B-shell water, Kuntz' non-freezing water, due to the decrease in stabilization of native state resulting from that shell as temperature increases. Morozov and Morozov found that urea as cosolvent decreased the Young's modulus. However urea also stabilizes the bubble state and thus should increase the stress on the knots by raising the free-energy change in expansion from transition state to bubble state..

Fig.9. illustrates the extreme contraction of rhizopepsin triggered by binding of one pepstatin molecule. Triggering is the updated version of the ancient "lock and key" proposal for enzymic catalysis. The key appears to regulate the entropy change in contraction so the lock is the entire matrix. The wide range of specificity in substrate and ligand binding requires locks with many tumblers. Matrices fulfill that requirement but do much more by using

conformation rearrangements to make the locks dynamic rather than steric. Note in the figure that the knot B factors are little changed and contraction of matrix free volume is about 50%, probably close to the maximum allowed in contraction. Cooling to 100K (Tilton and Petsko) produces also about 50% contraction in B values but most of that is the result of freezing out vibrational excitation. B reduction in matrices tends to be symmetrical along radii based on origins in the knots thus producing smoothly maximum contraction properties like those of the knots.

Each knot has associated a matrix but the high degree of C-2 symmetry found in the knots of matched domains is not found in the matrices of enzymes. When a protein is a collection of loops tethered to knots well separated along the polypeptide, the ends of the loops, usually the atoms with highest B factors, tend to copy the knot palindrome. Pepsin is an example in which electrostatic repulsion among the many aspartate acid groups necessary for stability at low pH is minimized by placing those residues at the ends of the loops. The 21 aspartate residues of rhizopepsin are bound just before the outer end of the loops generated by the knots so mimic the palindrome of the knots. This is expected for the pepsins which are active in highly acid conditions but it also suggests a general pattern to which acid and base residues may approach.

The most surprising feature of matrices is the semi-spherical contraction process that reduces matrix free volumes to uniform small values. At the contraction limit, about 60% of the total in the expanded state the B values are as low as those of knot atoms. Low-temperature measurements show the vibrational contributions to the B values in the expanded states to be about 50%. The contracted species are glasslike but contraction in matrices is highly coordinated to be inversely proportional to the local B value in the expanded state. Selection of matrix residues for the process produces in single or paired

matrices of enzymes phaselike behavior very similar to a first-order phase transition. The expansion-contraction processes are coordinated to simulate a single harmonic mode, a dynamical feature ubiquitous in enzymes and thus apparently essential for enzyme functions. The behavior is not intrinsic to polypeptides but is rather produced by evolutionary selection.

Although the coordinate changes in the process are not detectable at the precision of x-ray-diffraction and nmr methodologies, the B factors from the former and the proton-exchange rates from the latter measure the process with considerable accuracy as is shown below. As packing improves with increasing loss of free volume the amide I band of the α -peptide groups is progressively changed to produce the circular dichroism in the peptide bands near 209nm. The ellipticities are very large, of the order of kilodegrees, because most of the many matrix peptide chromophores are perturbed. CD spectra once critical in the detection of the compression process have become an important supplement to B factors though this use has not yet been much developed. Like the Young's modulus the deep uv CD loses strength with increasing temperatures. Alexander et al found the large negative ellipticity at 221nm with the streptococcus G protein to fall to a low plateau value by 354K in tandem with the Young's modulus both reflecting loss of knot strength with increasing stress from the matrices.

Phosphorescence at room temperature is a consequence of chromophores rigidly fixed in knots thus limiting internal conversion. so in expanded states only knot chromophores emit. Contraction of matrices freezes out motion of aromatic chromophores to produce additional phosphorescence. Fluorescence from knot chromophores also has fixed characteristics while that from chromophores in matrices can be influenced by contraction. Substrates are bound in matrices. With chymotrypsin, for example, protein difference spectra

in the near ultraviolet are different for each non-bonded inhibitor and each acyl derivative. The changes are small but so varied as to indicate much freedom in adjustment of conformation to bound molecule. Much larger changes can be demonstrated by denaturation of the primary-bonded complex of an inhibitor and the protein. Thus the uv spectrum of the hydrocinnamoyl ester compound with serine 195 of chymotrypsin is very similar to that of model compound between the acid group and histidine as can be shown reversibly on heat or urea denaturation of the complex to release the true model spectrum, that of the unperturbed ester. The catalytic domains of the trypsin family of serine proteases are forced together by contraction of their matrices to complete a single non-peptide hydrogen bond and according to Zundel to drive the bonding proton from one domain to the other. This deprotonates serine 195 making it a very strong base during the lifetime of a contraction. Considerable distortion of the reacting assembly is produced by this increase in its potential energy. Carey and Tonge using near infra-red raman spectroscopy have followed the primary bond changes producing such ultra-violet spectral changes. Recently other methods have been used to follow these primary bond changes in substrate and protein. Zundel using infra-red spectra follows migration of the proton between the two catalytic domains in both static and dynamic changes to provide considerable clarification of current confusion about low-barrier hydrogen bonds. Often as in trypsin and pepsin catalysis such bonds are formed as the domains are forced together by matrix contraction. They do not exist in the expanded enzyme making the search very frustrating for those unfamiliar with the conformational dynamics of enzymic catalysis. Compression changes the proton donating properties of the specific catalytic residues and at the same time distorts the substrate toward the transition state for the final bond-rearrangement. The catalytic process is discussed in the Conclusions section.

Tosylation of Ser 195 in chymotrypsin freezes in the contracted state and changes properties as general as the hydration. Lüscher and coworkers found that the tosylation increases the amount of B-shell water by 50 molecules per molecule of protein and demonstrates enthalpy-entropy compensation with T_C of 290K, characteristic of attachment of water to a passive surface. On the other hand hydration of the non-tosylated protein demonstrates compensation behavior with T_C equal to 433K, the usual matrix value associated with the expansion-contraction process. Hydration relaxes the free enzyme into its normal expanded state but has no such effect on the species locked into the contracted state. Gregory list several examples of such behavior.

The motility of catalyts (hydroxyl ions, protons and water) for exchange of peptide protons with protons in solvent varies with the degree of expansion but Woodward and Rosenberg found that regardless of kind of catalysis, temperature and pH the rank order of the proton exchange rates at matrix sites is preserved. This homogeneity is further confirmed by finding that the activation enthalpies and entropies for exchange at matrix sites fall on a single enthalpy-entropy compensation plot different from that for knot sites. Rank-order itself guarantees compensation behavior but not the observed linear compensation behavior. For matrices the compensation temperature using activation enthalpy and entropy values from proton-exchange rates from peak II is about 480K Values between 420-480K have proved to be a reliable indication of matrix participation. The characteristic temperature for proton-exchange from knot sites, 354K, is very common indicator of knot participation. It is less variable thus quite reliable as an indication of knot participation. Data for the several quantifiable steps in the chymotryptic catalysis of a congener family of ester substrates reveal compensation behavior with T_C values near 450K again indicating participation of the matrices. Specific inhibitors are bound with still greater conformation changes characteristically demonstrating compensation

temperatures near 290K. Although enthalpy-entropy compensation behavior does not rest on rigorous thermodynamics, it is the most useful source of quantitative information when processes are not coupled in an obligatory way as is the general situation in biology. More detail is given in the chapter on chymotryptic catalysis.

Compensation behavior in rates of melting and overall denaturation of mesophile proteins establishes that most are related through a residue-number index. Specifically when activation and equilibrium thermodynamic quantities for thermal denaturation are normalized to the number of residues, the proteins are all the same. This remarkable finding was revealed in data reported by Pohl and by Murphy and coworkers. As shown in a later section deviations from this high degree of convergent evolution are small and due to minor differences in interaction between denatured protein and solvent.

The expansion-contraction process is ubiquitous in enzymes and widespread throughout the protein kingdom as indicated by its close association with the C-2 symmetry characteristic of domain pairing as also discussed below. The process is accompanied by major changes in x-ray scattering that Singh, Bone and Huber attributed to a first-order phase change with characteristics only weakly dependent on temperature and methanol used as a crysolvent. According to Gregory the temperature range in which this was observed identifies it with the matrix expansion-contraction process. Then since the scattering data give roughly a change from 10 to 90% of the process in 5 degrees, application of the van't Hoff equation produces an unreliable estimate of 40 kcal/mole for the standard enthalpy change. Although the process was studied in wet crystals that suppressed cold denaturation, the presence of large concentrations of methanol disperses the Kuntz hydration water and introduced some uncertainty as to the quantitative analysis of the data. The protein was

trypsinogen rather than trypsin but the B factors show major differences between the two only in the small unstructured regions of the zymogen. The estimate appears to be the best available at this time. The corresponding entropy decrease at 215K, the transition midpoint, is about 185 cal.MK These numbers are quite significant and must produce major differences in calorimetric studies of thermal denaturation when contracted forms are compared with expanded forms of a protein.