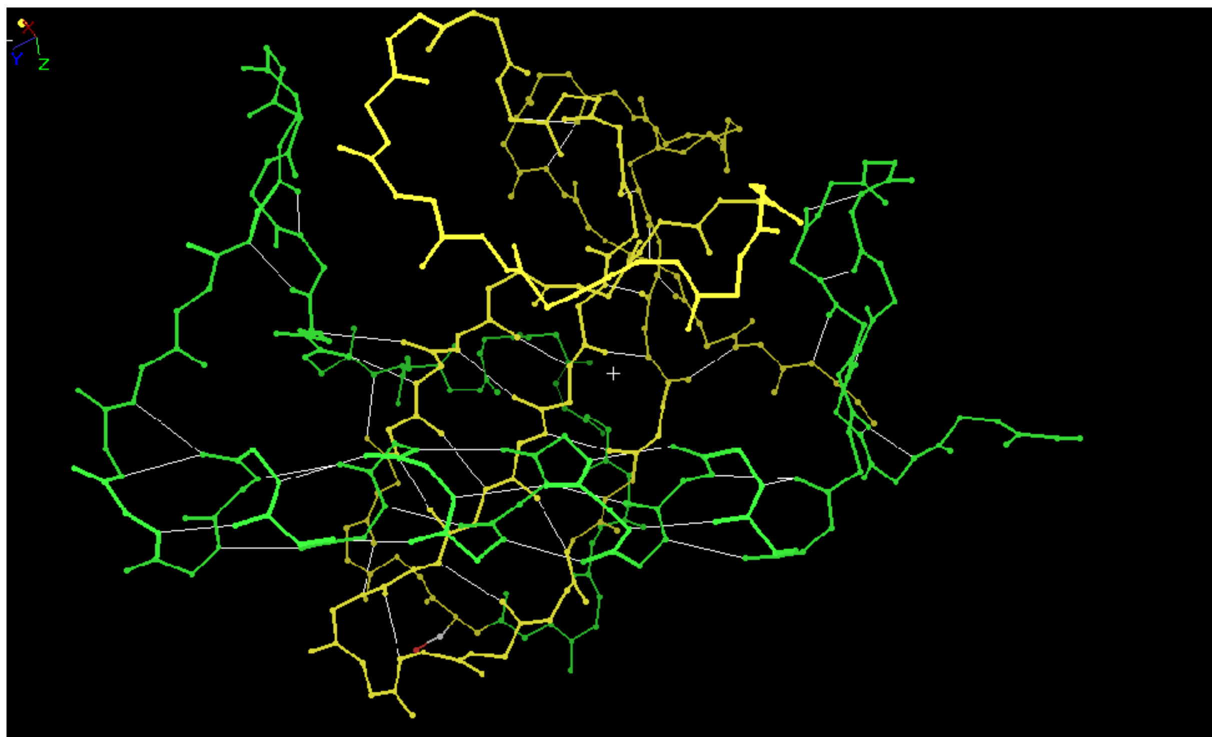
the imidazole ring of his57) so that on domain closure in the nutcracker operation the two are forced together driving the ser195 proton onto the histidine group mechanically distorting the subsrate and forcing rearrangement about its α-carbon. (Those rearrangements are illustrated by Fig. @ in Chapter @,Volume 1.)

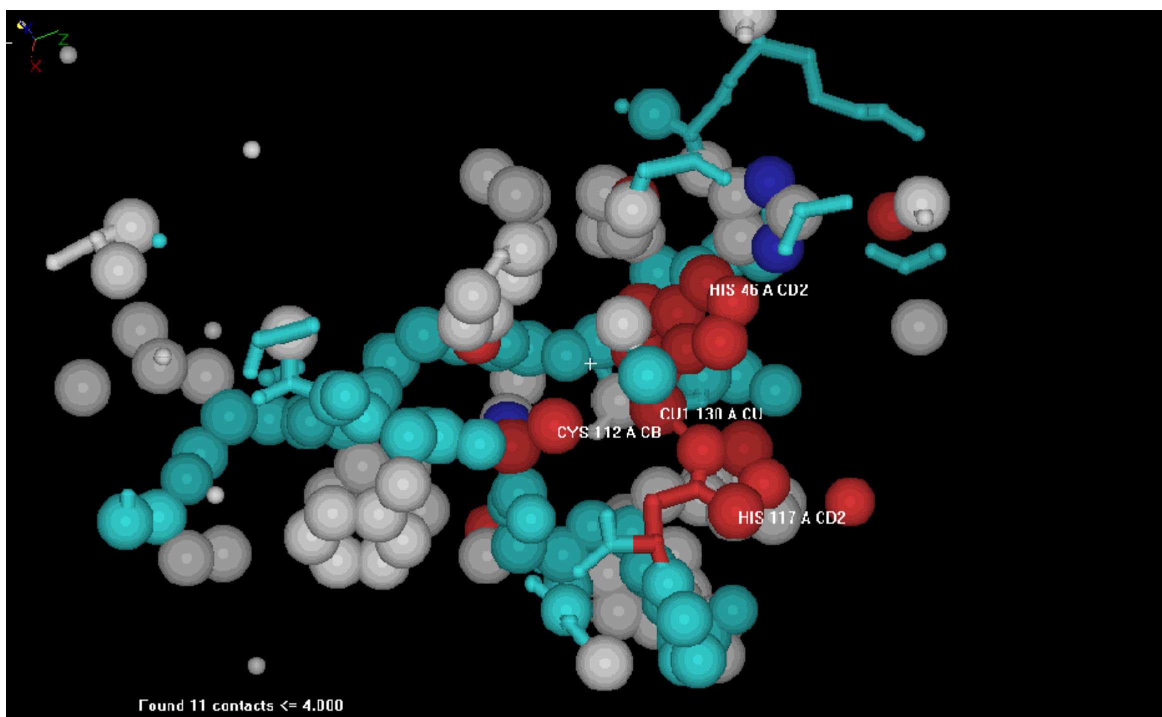
Chapter 9 part C second half for transmission (Brunori) Oct.2007

Ribonuclease T1



Half-sized atoms used in this figure show an unusual combination of knots, one three-strand large β-sheet knot and one helix knot with few low-B atoms. The catalytic residues, his 40 and glu58 are positioned to meet on domain closure and the catalytic domain pair is defined in the usual way but the difference knot structures reveal a different and apparently weaker nutcracker. The newer study from Saenger's group has a resolution of 1.5 and the B-factor values agrees with those reported in early studies (cf. Lumry, Biophysical Chemistry 101-102 (2002)83). This study was carried out to detect conformation changes in function but none were reported. As suggested by the even more complicated structure or pepsin the binding of specific ligands, in that case pepstatin, can produce major conformation changes throughout the molecules.5APR.pdb versus 6APR.pdb.). Major deviations from the simpler pattern are often found when the substrates are polymers and this may be another example to be added to HEW and ribnoculease A, both unusual in that respect.

Azurin



This common oxidation-reduction protein illustrates how the nutcracker activates a metal ion, usually copper but zinc is also familiar to a reactive valence state by mechanical distortion. The knot atoms from the two catalytic domains are blue forming the edges of the cleft in which the metal ion is held by his46, his1217 and cys1 12. domain closure raises the zero energy level of the metal-ion complex The knot B values are unusually high and the β-sheet ladder forming the knots contains only one side of the H-bonded ladders. However the ladders exist suggesting their H bonds are shortened and strengthen during matrix contraction to provide potential energy for nutcracker operation from knots. That has not been obviously suggested in other enzymes although some knot contraction is demonstrated by decreasing B factors in some enzymes; e.g. α-lyctic protease (Bone et al).. This diffraction study of azurin is not definitive because it does not give accurate information about the degree of matrix contraction. Earlier diffraction studies carried out without sulfate ion and

PEG may supply the necessary information The protein is relatively soft and some diffraction studies suggest that under some conditions the two functional domains may swing apart on replacing protein ligands to metal ion on one side by non-protein ligands. Sullivan was able to study ligand replacements by breaking the ligation of heme iron to threonine 80 in cytochrome C and could change the potential of the iron ion by several hundred millivolts. Similar changes could be produced by adding ethanol to the supporting medium and later with Lumry, Solbakken and Reyerson changed the number of unpaired iron electrons by progressive dehydration. In the latter study (reproduced in the Uti9lies folder of the Protein Primer, the various ways protein conformations can control the electronic properties in metal proteins were tabulated.Carboxypeptidase A is constructed in the same way as azurin but using zinc ion. (Chapter @, Volume 1 and chapter 7, volume 2). In Azurin the nutcracker greatly increases the oxidation-reduction rate with legends attached to the protein and is much used by Gray and coworkers to catalogue the rates of these processes. Carboxypeptidase A is a hydrolytic end peptidase depending on transient transfer of a proton from a water ligand to the excited metal ion thus releasing the hydroxyl ion as the direct catalyst.

Moffatt and coworkers with very high resolution diffraction studies of heme proteins very recently revealed using position coordinates rather than B values the long suspected rearrangements of proximal and distal imidazoles in intermediate conformational states (Takashima and Lumry, J.Am.Chem.Soc. 1958) and there are many later studies of these soft proteins suggesting matrix contraction in function for the four-chain assemblies as well as individual chains in those assemblies. However evidence relating matrix contraction to chemical function are limited for carboxypeptidase A by inadequate diffraction data and in azurin by use of sulfate, PEG and other cosolvents which improve crystal quality but hide the extent of matrix contraction As a consequence nutcracker operation is now suppored for

Carboxypeptidase A and azurin only by non-crystallographic data (Chapter 7, volume 2) and their nutcracker construction. A further criticism relevant here is that often diffraction studies are designed with the idea that single or even a few SDM changes will give useful clues to catalytic mechanism.

The central feature clearly revealed by B factors in many enzymes and suggested by the metal enzymes just discussed is the use of potential energy to produce the reaction rates necessary for physiological processes and probably dependent on temporary forced distortion away from stable states and lowest free energy. The versatility with which this features has been applied successfully in evolution seems quite remarkable but given enough time and sufficiently rapid mutation rates, it does not seem quite so remarkable. Furthermore given such more recent data such as those for photosystem II in photosynthesis by Wang, Lin, Allen, Williams, Blankert, Laser and Woodbury (Science 316 (2007) 747) suggesting that free-volume experiments may be the only vehicle available for evolution. (see caption Fig. @) The enzymes operating under extreme environmental condition, the "extremophiles", are discussed in Chapter 12 of this volume and like those on multistroke enzymes with implications for the evolution of such complex organelles. Volume 3 contains a variety of examples extending the data and analysis in the Protein Database. Some protein families have such different quantitative features to require special descriptions. The myoglobin-fold proteins are very soft with large knots but unusually high B factors. These proteins are soft and display many conformers in contrast to the enzymes which are strong showing only Arrhenius behavior. The immune globulins form another unusual family with unusual properties not yet clearly described. Their behavior remains a major puzzle both because of the small number of diffraction studies reported and but most use crystallizing agents that hide mechanistic details by contracting matrices.

Extremophiles

Enzymes operation outside usual ambient conditions are called extremophiles. The varieties as reported in Wikapedia are the following:

- Acidophile: An organism with an optimum pH level at or below pH 3.
- Alkaliphile: An organism with optimal growth at pH levels of 9 or above.
- Barophile: Bacteria which live in environments characterized by high gas or liquid pressure; synonymous with piezophile.
- Endolith: An organism that lives in microscopic spaces within rocks, such as pores between aggregate grains. These may also be called cryptoendoliths. This term also includes organisms populating fissures, aquifers, and faults filled with groundwater in the deep subsurface.
- Halophile: An organism requiring at least 2M of salt, NaCl, for growth.
- Hyperthermophile: An organism that can thrive at temperatures between 80-12 1 °C, such as those found in hydrothermal systems.
- Hypolith: An organism that lives inside rocks in cold deserts.
- Lithoautotroph: An organism (usually bacteria) whose sole source of carbon is carbon dioxide and exergonic inorganic oxidation (chemolithotrophs) such as *Nitrosomonas europaea.* These organisms are capable of deriving energy from reduced mineral compounds like pyrites, and are active in geochemical cycling and the weathering of parent bedrock to form soil.
- Metalotolerant: capable of tolerating high levels of dissolved heavy metals in solution, such as copper, cadmium, arsenic, and zinc.
- Oligotroph: An organism capable of growth in nutritionally limited environments.
- Osmophile: An organism capable of growth in environments with a high sugar concentration.
- Piezophile: An organism that lives optimally at high hydrostatic pressure. See also Barophile. Common in the deep terrestrial subsurface, as well as in oceanic trenches.
- Polyextremophile: An organism that qualifies as an extremophile under more than one category.
- Psychrophile/Cryophile: An organism that grows better at temperatures of 15 °C or lower. Common in cold soils, permafrost, polar ice, cold ocean water, and in/under alpine snowpack.
- Radioresistant: resistant to high levels of ionizing radiation, most commonly ultraviolet radiation but also includes organisms capable of resisting nuclear radiation.
- Thermophile: An organism that can thrive at temperatures between 60-80 °C.
- Xerophile: An organism that can grow in extremely dry, desiccating conditions. This type is exemplified by the soil microbes of the Atacama Desert.

The detailed information necessary to explain most of the classes is still not available.; despite the popularity and volume of protein diffraction studies, their number and design are still indequate for any one of the categories. Of those the

hyperthermophiles have attracted the most attention in part because most are archaea and thus still a novelty. The nutcracker mechanism is the same for bacteria and archaea so it can be utilized in attempts to explain extremophiles from both and its use does rationalize experimental data for some classes at the level of a first approximation. The nutcracker can be tuned for a particular physiological use in two ways. The first is by change in residues as has been discussed. The second is by adjustment through changes in composition at the interface as discussed in chapter 6, volume 2. and further elaborated in the following section.

"Tuning"

Tuning is a very broad term for changes in protein behavior resulting from residue change or modification of interface. Often it is not obviously related to change in diffraction pictures either because of their low precision or because the experimental designs artificially lower the B factors. However tuning results can be detected usually with both reliability and precision from protein behavior in free solution or normal crystals. Tuning is usually manifest through matrix behavior and was reviewed in that connection by Lumry and Biltonen in 1969 well before the "subtle change " conformation changes of proteins were well known and since by Gregory and Lumry in "Protein-solvent interactions" (R. Gregory, editor, Dekker 199. It is mostly achieved by local changes such as hydration, acid-base ionization and so on in the protein surface but there larger possibilities over large surface sections. These are seldom recognized in protein research despite much concern for extended structures like membranes. Because of their size proteins can be treated as a simple solute or as a separate phase, the latter thought rarely done being preferable. It is expected from the behavior of other kinds of particles of the same size that the fluctuations in volume and shape around some average values are large which in turn suggests a distribution of matrix sizes producing a distribution of available potential

energy which possible major importance in constructing theoretical treatment of enzyme rates. However the knots though small are very strong as demonstrated by the small atom-atom separations and that may diminish the various fluctuations. The apparent softness of proteins crystal due to interstitial water and soft matrices does not overwhelm the rigid geometry of the knots; fully dry crystals can resist hammer blows. At present these are sophistries but they must be accommodated in bringing theories of enzyme rate processes up to data. The related behavior are the cooperative fluctuations due to coupling of surface groups to each other over larger groups and larger distances and association with other macromolecules simple or in multi-enzyme structures, membrane assemblies and so on. The topic applies to cooperation at surfaces and has been treated under the name "Helfrich @ effect" but thus far with few deductions and estimates for proteins.

There is evidence that evolution uses the known varieties of turning to reach extreme conditions and there may be more not yet obvious. For example, the diffraction studies of crambin by Teeter and coworkers show clathrate water cages about groups at the surface but the significance of those cages becomes clear only after understanding the stability of the clathrate construction about structure-making cosolutes as described in Chapter 5 of this volume using new data from Koga et al. Such details are not likely to be appreciated for some time but the way in which they alter protein behavior is obvious for most of the extremophiles.

A class in which tuning may be particularly simple to understand although not a simple product of linear evolution is the aciodophiles represented by the pepsins. Rhizopepsin has 21 aspartate residues forming a shell about the protein mapping the knots (Lumry in Protein-solvent interactions, ed. R. Gregory, Dekker, Chapter 1, figure @) There is one aspartate attached to the polypeptides just inside each loop of the main chain so in the acid operating conditions the shell buffers the pH to follow

closely the contraction and expansion of the matrices so that they behave as they would at neutral pH values. Some pepsins use glutamate residues as well as aspartates and it is probable that the device explains using many lysines for buffering at alkaline pH values. But evolution has had to follow a path with many turnings to find and position the acid and base groups as shown by the a large insertion in a central region of the pepsins rather than at the ends thus impairing C-2 symmetry in order to get the loop turns and thus the aspartates correctly positioned. Recent diffraction pictures suggest the pepsin conformation is drastically altered on binding the strong inhibitor, pep statin.

The acidic and basic amino-acids have somewhat special properties because of their ionization ability and may be particularly useful in translating the gene into proteins. The major problem in explaining the extremophiles is the same restricting all scientific attempts to exploit the genome; the molecular path translating genes into proteins is still unknown. The concept of gene seems to be well known for the nucleic-acid polymers but not at all for proteins. A typical example is the reported finding that in a single organism as many as 40 genes contribute to the high-temperature stability. In Volume 3 it is shown that the knots of a protein have conserved free-volume descriptions over the species variants but no residue conservation but the basis of that in the utilization of the information in the gene is similar to the multi-gene connection to an extremophiles and it brings the search for real explanations for the extremophiles to a quick halt. Nevertheless most of the enzyme classes in the table appear to manifest some feature of tuning by residue change or interface selection of their nutcracker mechanisms or most likely both.

The major adaptation for most of the extremophiles listed in Table. @ is to change the parameters of the matrix contraction including both quantitative and qualitative domain closure and its triggering. Knots are not very sensitive to such

modifications of the matrices as shown by diffractions studies especially those of ribonuclease a by Tilton et al who ranged from 320K to 75K (chapter 3 of this volume) and there are others cooling to 100K to shown by decreasing B factors the contraction produced by freezing out vibrational and librational degrees of freedom. Knots are unstable outside the temperature range evolution has found acceptable for protein stability although thermal stability of some hyperthermophiles often exceeds the 354K upper limit for reasons not yet fully understood. .However in general both knots and matrices lose their thermodynamic stability at the lower temperatures where hydration of structure-making solutes changes from favorable to unfavorable.

The hyperthermophiles are catalytically active at the higher temperatures in comparison with the enzyme with the same substrate selectivity but stable at temperatures as high as 354K. On the other hand hyperthermophiles grow successfully at temperatures over 373K because they have higher thermal stability against denaturation. Chen and Lumry thought this a manifestation of different temperatures for nutcracker operation thus at low temperature nutcracker operation is inefficient because the nutcracker is closed most of the time with high thermal stability butl low catalytic efficiency. At high temperatures the nutcracker is open and catalytically active.. They tested this idea by computing the mean and standard deviation of the B values for a hyperthermal enzyme and its lower-temperature analog. In most pairs the mean was lower for the hyperthermophiles at lower temperature rising to similar values at its operating temperature.. Furthermore the standard deviation of B for the hyperthermophiles at the lower temperature is usually about the same for knot and matrices as expected for the closed nutcracker. This B-values behavior is to be expected on the basis of rate and stability data previously reported.. That matrix contraction of the hyperthermophiles at the lower temperature

to knot B values is as severe as we have found at 298K but there is a much-studied transition for many mezothermal proteins in the range 200-180K which appears to be matrix collapse (reviewed by Gregory). B values in the cold region change rapidly on cooling not only because of the collapse but also because of progressive freezing out of vibrational modes. also much studied. The statistical assessment of B values given in Chapter 3 of volume 2 is consistent with the behavior found for many hyperthermophiles bur does not agree for others so there must be more than one class of hyperthermal enzymes having in common only their defining characteristic of being able to grow at 373K That common stability against thermal denaturation requires another explanation but may be due to the same kinds of tuning but not yet in more than guesswork. The average time for domain closure can be altered by residue changes but so can the high-temperature folded stability and both by interface construction.. Chen has examined the residue differences and B factors finding some patterns relevant to these puzzles (C-H.Chen and Lumry, To be published in 2007).

Enzymes that operate well below ambient temperatures are called @. The explanation is the same but their high-temperature region appears to be that in which the matrix contraction for nutcracker function is slow and becomes rapid only at temperatures well below 354K what of their thermal stability? salt and other dependent on media not clear and must be deferred to the full paper by Chen and Lumry. Pepsin adaptation appears simple but also has presented evooution problems-explains. Not clear whether ot not all extremophiles can be explained by tuning nutcracker.

Strong and specific grasping using the matrix potential energy.

Attachment for strength like the triggering of nutcracker closure by binding a specific substrate depends on small enthalpy and entropy changes favoring the closed form over the open form. This two-state process by which potential energy is shifted from

matrix to substrate accompanied by without major coverall changes in G, H and S but activating the nutcracker. With large or even small changes in tuning it can be modified for some other important psychological needs and it is unlikely to have escaped evolutionary experiments. One such is suggested by the strong binding of biotin by Streptavidin and the G protein from streptococcus suggests how domain closure makes high-affinity associations also highly specific. The G protein joins the virus to a specific immune protein and when the specificity requirements for both are met, matrix contraction converts the potential energy released in their association into lost in free energy thus adding stability to the association. The specificity matches the immune and virus proteins via the near palindrome of the G protein matching matrix contraction to the participants. . The matrices stay closed in their solid form during the association and in this kind of application lowest free energy may be the only test for survival value

Matrix contraction and knot contraction as potential-energy sources may play essential roles in many physiological processes. The G protein from streptococcus (Fig. @) is an illustrative example of the modification of physiological function by adjustment of the nutcracker without the chemical consequences of complete catalysis. Consider an open hand attempting to grasp a ball. There can be some contact specificity in the way the open hand can grasp the ball but minor compared with those taking place when the hand closes about the ball thus locking the association using the overall decrease in potential energy.. Unlike the enzyme mechanism there is no subsequent chemical change allowing the matrices to return to their expanded state. Reversal by dissociation can be fast or slow consistent with the evolutionary advantage. The G protein is an especially illuminating example because the properties of the association between the bacteria and a specific immune protein can be seen to depend on domain closure just as with the enzyme nutcracker. And the fan construction allowing symmetric matrix contraction has a near perfect palindrome

pattern in both knots and matrices. The stability of the binding of biotin by Streptavidin also depends on specific potential-energy management but using only a single domain as can be tested using its B factors. At a much more advance level the "sucker fish" *remora* may well use no more complicated mechanism and as already mentioned, evolution may not have found an alternative path.

This grasping mechanism provides a general explanation of the specificity of most successful pharmaceuticals being a more important factor the lower the effective concentration. In most applications domain closure offsets the increase in dilution entropy. Without B factors the potential-energy device is revealed only marginally with the consequence that the classical "lock and key" model lacking any sophisticated description for the lock continues to dominate pharmacological research. Molecules fitting into protein holes in the specific way must trigger domain closure as would be easily seen using the B factors were the appropriate diffraction studies completed. Double-cusp B-factor plots just like that for the G protein have been found without exception in some hundreds of protein for which high precision B factors are tabulated in the Protein Databank and it is perhaps surprising but it is also very important that the positional coordinate as they are routinely extracted from diffraction data reveal none of the detail found in the B factors. It does not seem to be clear why those have so much higher precision since the theory of scattering relates them directly to the mean square displacement of the atoms from their ideal lattice positions.

There are some other untested assumption that gained firm foothold with the emergence of diffraction methods for protein research: residue sequence is conserved, protein construction is isotropic, structure information from diffraction experiments is reliable and excludes conformation changes, folded stability depends on van der Waals association of non-polar side chains, poor solubility of hydrophobes in water is due to unfavorable entropy change.. Firm belief that the above are correct has

discouraged any testing.-psychology triumphs chemistry. A few efforts to avoid B factors are very clever. In particular Bahar assuming that small atom separations are associated with high-frequency vibrational modes, has constructed using diffraction coordinate data regions with different dynamical characteristics. She has not been alone in closing in on the nutcracker mechanism but still hesitates to get the structural parameters correct using B factors.

Conformational consequences of tuning have been reviewed in chapters 1 and 2, volume 2 and more recently updated by Gregory (http://kent.edu.chemistry/moleimage)). The papers of Ng and Rosenberg illustrate that perturbations of matrices by change of solvent produce major changes in enzymic rates and matrix proton-exchange rates. An inverse picture appears in the studies of the hydrolysis of furoyl chymotrypsin in which Huang and Bolen show that intermediate states in substrate hydrolysis alter thermodynamic stability. Gray and others examine the variations in electron-migration rates in proteins as those depend on residue substitutions and positions of redox centers use the term tuning and it has been used in this volume to cover hydration effects on protein melting and on quantitative changes in enzymic rate parameters. The objectives are to relate the matrix processes in their several different applications through manipulations of their potential-energy .devices. The obvious categories are the tailoring of functional parameters as achieved in evolution, the storage of potential energy to explain the unique features of protein function as they depend on the three substructures. In contrast to the electron-based chemistry of small molecules protein mechanisms are largely mechanical. That is still very little appreciated. Some studies relevant to the central role of matrix conformation changes driving domain-closure are the following:

Lumry, Solbakken, Sullivan and Reyerson (J.Phys. Chem. 1961) rreporting on the large changes in functional parameters and magnetic susceptibility of cytochrome C

produced by simple changes in hydration. Nothing similar was found with trypsin. By hydration changes even electron pairing could be changed to states of intermediate pairing on recently found in small molecules.

H. Parker studied the ellipticity and electronic spectra of chymotrypsin aceylated with hydrocinnamic acid to find major reversible spectral changes` occurring on melting and resolidifidation. The ellipticity at 206nm is particularly sensitive to domain closure as also found byAkasaka et al but interpreted differently (ref@.).

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Phosphate ion and sulfate ions among others in post expression modifications often produce major changes in protein parameters as discussed in chapter 6 of volume 2. Nature has used them to stabilize protein conformations. Single ligation with these ions can often lock conformation to play a role in physiological function illustrating the utility of post translational modifications. Of similar importance they can drive motor; even single protons can provide the effectors as in flagella rotator motors. Protein motors require a source of force but also changes in structure to generate work from force. Any change in structure whether spontaneous within a protein or a result of change in ligation including hydration has associated conformation changes which must be taken into account in describing and explaiing protein reactions. Haurowitz' first suggested a conformational mechanism for the linkage among oxygenation sites if hemoglobin but the "induced-fit" version proposed by Koshland for enzymes became more popular and remains so to this day more or less as a historical accident arising from the early success of diffraction methods for proteins. Induced fit as it is still described is a relaxation process in which the electronic properties of normal small molecules cooperate by spatial relaxation to greatly enhance the aggregate of their small-molecule contributions to catalysis. It dies bit include mechanical force and thus excludes potential energy possibilities arising from the selection of useful construction details in protein evolution. The alternate suggestion from Eyring, Lumry and Spikes appearing at the same time depends on

such force generation in catalysis as well as other physiological processes including varieties of protein motors. Its essential feature is the use of potential energy and the corresponding motive entropy in those processes but not to approach equilibria by minimization of free energy. Heat and heat entropy are sufficient for the electronic rearrangements of most small-molecules reactions but crude and difficult to adjust in useful ways. The thermodynamic distinction between small-molecule chemistry and processes susceptible to modification by evolution is one of rates and specificity. In the biosphere the criterion for success if survival. In small-molecule the only test ius the reduction in free energy. The unifying feature of enzymes is the C-2 symmetry that results from division of the whole protein into matched pairs usually but not necessarily having the palindrome pattern. There is no thermodynamic basis for such selection nor for the fact that protein evolution has been possible only because of accidental discovery of devices resulting from random manipulation of building blocks also having no thermodynamic direction and no thermodynamic consequence. (Chapter 1. "Protein-solvent interactions", ed. R. Gregory for Dekker 1994) and in volume 1 of this treatise).

The devices thus far detected all have a mechanical feature as illustrated in particular by the catalytic mechanism (Chapter 7, volume 2). The chemical side of life is the same as the chemical side of small-molecule chemistry and must involve the same kinds of thermodynamic changes which have depend on changes in chemical free energy so it now is obvious that the use of mechanical rather than chemical potential energy is what evolution has found to control and adjust chemical change. Other tuning adjustments whether directly or indirectly consequences of residue change are exploiting to achieve further direction and further control but usually thought their modulation of the conformation changes that generate the mechanical forces. Figure @ illustrates the versatile balancing of chemical change by mechanical change to form an isoergonic conformation coordinate that permits the potential

energy fluctuations of nutcracker mechanisms. The figure describes a formal mechanism for evolution of a new physiological feature such as a new eye or an arm for a blind watch maker.

The bottom reaction profile is a crude description of the mechanical-chemical balance actually achieved over a short series of what might be called events. Each might be an enzyme event. In such a nutcracker mechanism contact between enzyme and substrate triggers domain closure activating the nutcracker and chemical change in the substrate. The enzyme relaxes to its initial state so all chemical change takes place in the substrate. The number of events in a series can be large as suggested by the multi-stoke mechanism of glutamate reductase and the four-chain hemoglobins. The ordering and coordination of the events must be consistent with the chemistry but the evolution may have been chaotic. And many such conformation patterns interact to provide the full description of the organism. It is likely that it is those patterns rather than descriptions of individual enzymes that define the genome.. What has been added to the original mechanical idea, the "rack" now the "nutcracker", is the use of the interface to modulate conformation change and provide fine adjustments to the enthalpy and entropy changes in domain closure.. Notice that the ions, phosphate, sulfate, proton, are just the ions that make the largest changes on change in hydration as a result of the shifts in clathrate equilibria.as they move from pure clathrate to dry ligation to chemical combination at the protein surface. Sulfate ion may be quantitatively most effective since is forms the strongest and most complete clathrates but phosphate ions may be the most versatile of the group. The operation of the Boyer-Walker phosphorylase motor illustrate these changes not just for the ADP-ATP system but probably for all clathrate-conformation dependent interface interactions. Recent publications allow quantitative dissection of the phophorylase mechanism.

Enzymes do no net work in catalysis but there is no reason to believe that nature once having found a device to generate force and do work has not exploited the matrix expansion-contraction process wherever it may be applicable. Muscle is a likely example and so are the many protein motors of which the Boyer-Walker phosphorylase assembly is among the most informative. It has an important but not unique role in its ability to interconvert mechanical and chemical free energy in either direction and in amounts limited only by the folded stability of the several proteins in the assembly. As an academic illustration is has importance in showing the matrix process which is often hidden in poor x-ray-diffraction precision similarly since the degree of rotation of the core against the ligation sites, it provides a means to estimate the actually force and work done at each step of the rotation process. Baylis-Scanlon, Al-Shawn, Li and Nakamoto (Biochemistry 2007, 46, 8785) in addition find the rates of phosphorylation and hydrolysis to be equal under many experimental conditions consistent with the uses of the motor in both directions for converting mechanical work to chemical work and vice versa. These authors proved an updated proposal relating the work to hydration of ATP, ADP and Pi . The analogy with the detail found in the work done by muscle is consistent with the hypothesis that nature never abandons a device once found useful Most studies of this central motor and others like it are unfamiliar with the properties of water responsible for the major contributions from hydration and surface ligation chapters 4, 5 and 6 of volume 2.. Among those the new descriptions of pure water and aqueous mixtures have central importance because they reveal much larger thermodynamic contributions than have been suspected.

Izbicka and Bolen using a methodology perfected by Kaiser and Bohlen have moved deeper toward an understanding of the subtlties evolution has found to manage energy and free energy. They compared a six-memberd sultone substrate for chymotrypsin with its five-membered analogue, the former having neglible, the latter

with 23 kcal/M of ring strain potential energy. The steady-state kinetics of the two were qualitatively very similar and in particular in both the open-ring, acyl-enzyme covalent intermediate with the enzyme was reversible to the original molecular species. The protein was thus able to conserve the ring-strain potential energy in the covalent species of the five-membered substrate ion as potential energy available to reform the original five-membered ring form.. In related studies by? and Bohlen on the tosyl substrates the argument was advanced that these energy manipulations required direct participation of the primary-bond energy of substrates into catalytic activation. Subsequent work summarized in this chapter suggests that instead opening the smaller-ring substrate drove the contracted matrices into the expanded configuration. That alternative has not yet been fully tested and suggests the importance of such more-sophisticated experiments.

Currently to get enzyme research on track conformational and surface features must replace static structural descriptions elaborated using any residue-conservation idea. factors must be incorporated was the first to include conformation changes .Structural variations necessary to facilitate physiological function are adjustments in chemistry and mechanical parameters one as important as the other. The phophorylase complex protein assembly for fusion of ADP and phosphate ion discovered by Boyer is an important bridge between chemical manipulation of energy in biological processes and manipulation of mechanical work. The mechanically driven rotation of the protein assembly forcing phosphorylation of ADP can be driven backwards by hydrolysis of ATP: chemistry to work in hydrolysis in one direction and work to chemistry in the other. Such a coupling device providing operation at near constant free energy is obviously essential for evolution to have advanced through fusion of chemistry and mechanical work The ADP-ATP couple is not the only device for such essential interchanges but it is the one most often found .Muscle studies suggest that domain closure is triggered by initial binding of ATP

releasing the terminal phosphate group but not the associated free energy. The latter is then released on dissociation of ADP.to restore the potential-energy in the storage device. The mechanical work done by muscle by conversion of the chemical work stored as free energy in ATP is probably a direct consequence of matrix contraction. So far as we now know that is the only way nature has found by which proteins can generate mechanical force.

Higher level evolution

If the deduction that proteins evolve to play useful roles in life only by cooperative testing in which changes in one residue depend on changes at most other positions well beyond the immediate neighbors, is correct, mutations rates must be large even for more than four million years of since the first successful of some essential mechanisms were found. That success that most evolution has depended on the high mutation rates of bacteria and archaea.a suggestion that seems to be consistent with latest ideas of evolution scientists about the "tree of life". It also supports the tentative conclusions that bacteria and archaea have learned to accommodate to the two-states of liquid water and the behavior of aqueous mixtures with structure-making and structure-breaking solutes. Equally important then is the supposition that potential-energy has replaced or supplemented heat as the device for accelerating and tailoring chemical reactions. The latter deduction is strongly favored by the success of deductions from its use, rather than residue conservation and the failure to find devices depending on chemistry, that is, catalysis by electron tricks not depending on conformation changes. The complexity apparent in physiological mechanisms must then be no more than can be attained by management of atom free volumes. DNA and RNA seem to rest on that limited basis so their connection to protein synthesis is similarly limited but one step more complicated than children's blocks. With so many blocks designing a new enzyme is not a promising undertaking. However the variety of successful residue exchanges illustrated by the knots of the

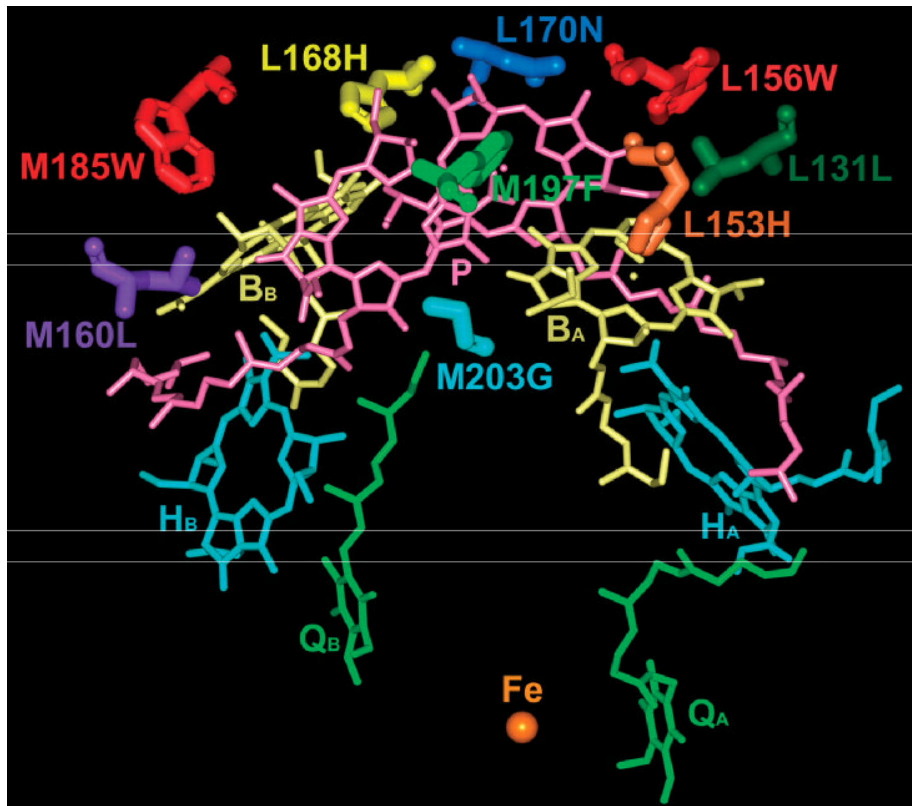
trypsin family (Volume 3) suggest that with much better understanding of the basics of free-volume arrangement the undertaking may not be impossible. Nature has set the rules of the game for us to learn and apply but even those will not be accessible without better B values. Two common mistakes are the belief that small-molecule chemistry is sufficient and that the force-work implications are expressed in large conformation changes. In fact, small-molecule chemistry shows that the larger the potential energy redistributions, the smaller the conformation changes.. In metal enzymes the perturbations of the ligand fields probably cannot be detected by coordinate data from diffraction studies though much bio-organic research has been carried out as though it can be.

Once nature found the conformation coordinate very sophisticated mechanisms became available as perhaps obvious from the caption of Fig.. @?. Enzyme matrices riding back and forth on that coordinate can be elaborated into multi-stroke versions of the nutcracker mechanism as so far elegantly illustrated by the unique success of. Fisher in developing the four-stroke mechanism for glutamate dehydrogenase. As suggested by the figure, the isoergonic exploitation of the conformation coordinate tailors a multistep chemical process in a single machine. More detail of that mechanism can be extracted using experiments designed to have a common feature, for enzymes usually the matrix contraction-expansion process driving the nutcracker. The example using data from Dorovska-Taran and Matinek and their respective coworkers is the family of ester substrates for chymotrypsin (data copied into chapter 7. That these substrates share the matrix process is revealed by the compensation temperatures for all but the first step (Fig. @) since these are all in 450K range. The reactions moves forward and backward along that coordinate. An addition feature in the figures is the inclusion of both the “on-acylation” step and the “off-acylation” step of this two-stroke mechanism. As illustrated by Fisher with glutamate dehydrogenase this occurs with a zero free-energy change in the enzyme

itself so that the quantitative changes in thermodynamic quantities at each step of the process can be estimated for each substrate. Compensation behavior is extra-thermodynamic and thus not-rigorously related to the thermodynamic laws but its use is essential in describing the mechanism of any enzyme (Lumry , Biophysical Chemistry 105 (2003) 454).

The multi-stoke nutcracker mechanism appears also to be demonstrated by the pyridoxyl-phosphate enzymes. In general multi-stroke machines seem to have required an increase in number of proteins to form cooperative snits so the four-chain hemoglobins are even more familiar example and aspartate transcarbamoylase with its combination of catalytic and regulatory protein units has become even still more thoroughly detailed. More recently the ADP-ATP phosphorylase motor of Boyer and Walker@ in directly relating the use of force to effect chemical change is even more illuminating.

The ubiquitins and proteosomes go well beyond limited catalysis to add cooperatively essential to multi-site biochemistry by exploiting the matrix process. The direction of progress in evolution toward more complex physiology become possible by potential-energy devices of proteins is apparent .and now Wang, Lin, Allen, Williams, Blankert, Laser and Woodbury (Science 316 (2007) 747)may have moved toward understanding complex organelles in finding that photosystem II of photosynthesis, the oxygen forming unit in green plants constructed with many proteins and many pigments may operate by the nutcracker mechanism. They found that its operation involves conformation changes as Shibata concluded on measuring the variation of grana volume with photosynthetic rate but the construction shown in their figure copied from Science, @ (last figure below), has C-2 symmetry and the other enzyme features making electron and proton migration from one large domain to the other in function possible..



That suggests that evolution may have had to depend on the volume management possibilities no greater than those of single enzymes. Some related potential deductions of current interest to evolutionary biologists are suggested by the knot-matrix substructures described in the following section.

How evolution is determined by the protein substructures

In chapter 7, volume 2, it is shown how the three substructures provide the common nutcracker mechanism and describe its operation Although simple to describe the evolution has been complicated particularly by devices evolved to limit normal chemical problems such as reversibility in enzymic catalysis. Here we suggest that the only vehicle available for protein evolution has been the arrangement of free volume. We do not find residue conservation even in single protein families to be more than the transient consequence of sharing of gene information. Instead we find

patterns of free-volume arrangement which are tightly conserved in protein families to preserve physiological function. Since the B factors directly measure average free-volume patterns, it is not surprising that the preservation of those patterns imposes severe constraints on evolution. Several of which become obvious once the whole-molecule B-factor patterns have been constructed The latter are easily constructed using the atom B factors now routinely tabulated with position data in repositories of which the Protein Databank is the largest. However, construction of the patterns to reveal the knot and matrix substructures has not become popular despite the availability of programs such as Molecular Image by D. McRee. Without those patterns it is not possible make any use of DNA information for protein construction but some useful deductions can be made using only the existence of the substructures. An important example is the controversy as to the relative importance of mutation versus selection. Mutation implies random discovery of a gene and selection means adaptation following initial discovery. The role of knots in function as thermodynamic lock and source of genetic stability is consistent with a random totally pragmatic discovery favorable in some way to survival. Further adaptation of knots is limited but does occur as shown by the knots of the trypsin family (volume 3, Protein Primer) demonstrating the basis of random selection in free-volume arrangement and not in residue sequence. In particular there is no simple relationship between the palindromic sequences of the two cusps of a knot although an early history of gene duplications may be sometimes involved. On the other hand matrices and surfaces demonstrate many variations, many from the migration of gene information rather than any dominant mutation. Matrices have to be adaptable for small and large changes in function, such as change from chymotrypsin to trypsin and within an enzyme family a change in substrate specificity or an improvement in efficiency. But note, these distinctions are unlikely to be obvious if the substructures are incorrectly described as by the current use of “hydrophobic cores”.

Enzyme construction includes the pair of catalytic functional domains each bearing one of the two knot cusps and each bearing one of the chemical groups that participate directly in function. Those groups may be part of the protein or part of a coenzyme including metal ions, NADH+, etc. and they are arranged to come into chemical contact but only on domain closure. They are positioned in the polypeptide close to the knot cusps but not in them so apparently among their several functions they act as anvils to apply the force to the chemically reacting assembly. This part of the total description of an enzyme appears to be ubiquitous although the T-1 nuclease illustrated above does not appear to conform and HEW lysozyme may have a minor deviation. Clearly the pattern indicates chemical changes in catalysis supplemented by the mechanical force developed in matrix conformation changes. Knots introduce a permanent and usually new feature from which sooner or later for some potential "advance" in evolution can follow. It seems likely that the fundamental advances require discovery of a new knot in which case they determine the survival values that test evolutionary experiments.

A second implication is that life depends on generation and regulation of mechanical force. If electron and proton banking on which most small-molecule catalysis depends is also important in enzymic catalysis, it remains an unsupported hypothesis of requires mechanical support to function-. A current example "low barrier hydrogen bonds" is a fairly obvious consequence of domain closure and it remains to be shown that nature has not extended the mechanical concept to RNA. Even the density pattern of t-RNA despite the small size suggests a knot-matrix construction. Stitching the residues together on the ribosome is another attractive mechanical possibility..

. The large sizes, precision in construction and versatility in matrix adaptation are ideal for mechanical experiments and a survey of the devices they support suggests that conformation changes and the resulting generations of force are essential for most. Thus, for example, how can the many pump motors with the major function of

repositioning atoms and molecule groups function without a source of force and what source other than matrix conformation change can be suggested? .The ATP phosphorylase enzyme assembly not only particularizes such a motor but also illustrates a major device nature has found to interconvert chemical and mechanical work, a necessary bridge from laboratory to biosphere.

At 354K the interaction between structure-makers like glycerol, methane oxygen and other cosolutes with non-polar parts changes dramatically as the enthalpy change in their association with water changes from negative to positive.. This usually means that the hydrophobic hydration of the solute ceases to be favorable. That is, it ceases to have a negative free-energy consequence because the clathrate structures formed with several water molecules are no longer favorable. The latter is a consequence of the difference between the two chemical species but neither those nor the clathrate property of liquid water are yet well known. The favorable clathrate formation is a major source of folded stability for proteins below 354K and it is replaced by an unstabilizing effect as temperature passes above 354K. The latter overwhelms the polar bonding within the protein and the folding becomes unstable. Nature uses several tricks to maintain folded stability at somewhat higher temperatures but has been relatively unsuccessful. A consequence is that most protein undergoes thermal denaturation at 354K That temperature dominates melting rates and melting thermodynamics almost as an accident although all of evolution is an accident. It is not a protein property but rather a water property and can be found in the compensation behavior exhibited by families of structure-making solutes in water when the total mole fraction of such solutes is not so large as to use up the free water. Its appearance in protein behavior as for, example in the food industry is a valuable clue to the properties of the water-protein interface particularly useful because the contribution to stability can be estimates and factored into the contributing group

interactions at the protein surface. In addition since those depend on the amount of water available for clathrate formation, electrolytes such as sodium chloride can modulate the interaction. Release of proton from a protein group to form H_3O^+ actual involves clathrate formation by a many as 20 water molecules depending on mole fraction of free water. That rather than the Eigen-DeMaeyer postulate of four water molecules appears to be the correct description of the proton in water.

Enthalpy change has two components, heat and potential energy. Of these only the latter contributes to the free-energy change which may mean that the only a small fraction of the enthalpy change actually measures the work change in a constant-temperature process. In general it is not possible to estimate that contribution using an experimental method, it the heat change is zero, the heat-capacity change is zero. This is not a common situation outside selection for biosphere processes but it is common in the physiologically important processes of enzymes and thus very useful. That has been illustrated in this chapter. In that connection it has been shown in Chapter 7 of this volume that the catalytic processes of enzymes must be exactly reversible.. It is not yet established that that reversibility as between expanded and contracted matrix states has overall lowest free energy. Proteins are metastable with respect to some unfolded and some polymolecular associated species and have been selected not for thermodynamic equilibria thus for low free energy but for their survival values. Such interesting questions remain to be considered. but of greater immediate importance is understanding and acceptance of the paradigm shift that is taking place in enzyme research.

The new paradigm for enzyme research- Kuhn's definition of a paradigm shift is perfectly exemplified in the discovery of the DNA double helix whuch unlike those forced by discovery of the first and second laws of thermodynamics took place within the few days required to publish the pictures. Detecting the protein paradigm requires a different one of Kuhn's rules. Specifically when research in a field has a fast

pace but real progress is limited, the cause is often an unrecognized paradigm. If that is the problem with enzyme research as seems probable, what is the missing paradigm? Sixty years ago Eyring and coworkers suggested that enzyme rate processes are facilitated by mechanical force much more than by heat . That is the essential feature of a missing paradigm and it subsequent full description showed it to be the critical one for enzymes. The major discovery followed from finding the unique usefulness of the temperature factors from protein diffraction studies. In particular it became possible to show that free-volume management rather than residue conservation that provides the paths of evolution.. However it was not until 1994 that the complete paradigm could be described (Lumry in "Protein-solvent interactions", Ed. R. Gregory, Dekker 1994, chapter 1) together with the devices discovered in evolution that have been required. Most of the latter are discussed in the Protein Primer but are otherwise little know and the paradigm shift itself remains little understood. The switch in orientation from stabilizing the transition state to destabilization of a pretransition state continues to provide a very high psychological barrier to research progress Viewed as a paradigm shift makes it less confusing..