Chapter 3 (volume 2)

Conformation information from B factors: temperature and catalytic function.

The temperature dependence of B factors –

In addition to their description of the familiar thermal denaturation process of proteins, Lumry, Biltonen and Brandts in 1966 described a process with major enthalpy and entropy changes but without the heat-capacity change that Brandts had previously shown to be the predominate characteristic of the protein melting. Because the newly discovered process was initially difficult to detect, it become known as a "subtle change". Lumry and Biltonen in 1969 reviewed the several kinds of observables that reveal subtle changes and showed using chymotryptic catalysis that the potential-energy redistribution generated in enzyme subtle change accelerates catalysis by excitation of a pretransition state rather by lowering the classical transition state. The catalytic mechanism depends on mechanical rather than thermal excitation to accelerate the slowest step in the catalytic transformation. Thermal excitation demonstrated by most small-molecule rate processes has dominated thinking about rate processes in biology despite the obvious differences between rate processes of proteins and small molecules. As a result the popular "transition-state stabilization" hypothesis of enzymic catalysis has been based on thermal excitation regardless of the apparent advantages of mechanic effects managed and evolved by the packing of protein conformations. The dynamical consequence of the latter first described for enzymes more than fifty years ago are easily detectable in measurements of protein properties by optical rotation, proton exchange and several other common methods but it has been hidden from those protein scientists who have come to depend on x-ray-diffraction methods. The conceptual problem lies in the fact that the experimental errors of coordinates determined by the x-ray method are larger than the actual coordinate changes in function. The experimental errors are considerable larger than

the changes in bond length and angels determinable at even the highest refinement for protein crystals thus hiding the actual changes that take place. Fortunately for the future the essential quantitative information can be easily extracted from the Debye-Waller factors describing the x-ray scattering. These factors known in protein structure study as "temperature factors "or B factors are tabulated for most diffraction studies in the Protein Databank. They give the mean square deviation of an atom from the perfect lattice which is usually much less than 0.3Å and thus about a third of the most accurate values of the coordinates. Rates of proton exchange between solvent water and the amide sites on the protein backbone are an import because they depend on average on the same average fluctuations in free volume but entirely independent of diffraction data. As a consequence Gregory, Rosenberg and coworkers found in the exchange data that many familiar proteins have only three kinds of substructure. It then became possible to give the changes in B factors structural descriptions. That has been done for many proteins stable in the mesothermal range defined pragmatically in terms of melting temperatures as above 280K and below 354K. This range applies for proteins from bacteria, eukaryotic organisms and *archaea* proteins because the characteristic temperature of 354K found in protein behavior it is a water property rather than a solute property. It is dependent on pressure so archaea have folded stability at the high temperatures prevailing in deep ocean vents. 354K is not an accident of evolution but rather the temperature at which the amphiphiles and water mix at constant enthalpy and free energy at 1 atm pressure. It is critical hydraton makes a major contribution to protein stability below 354K becoming unstabilizing above.

The major variable available for protein evolution and perhaps the only one is free volume, that is, the arrangement of the amino-acid sequence of a protein to so that on folding the local characteristics necessary for success in folding and function are found. Such packing in the large, relatively soft protein molecules provides no way to enhance the familiar thermal activation of rate processes ubiquitous in small-molecules but it makes possible by packing adjustments with neighboring atoms the

versatile manipulation of force vectors both amplitudes and directions, In practice in protein research neither thermal not mechanical possibilities have been noticed so residues sequences have been the sole focus of attention. An obvious but entirely misleading consequence is the idea that the x-ray pictures of proteins must ultimately reveal protein mechanisms. Sequence variations are thus the primary tool. However, packing considerations make that dangerously unreliable. In the first place the properties of a given kind of residue in a mechanical machine depend in important ways on the neighboring residues thus different to a greater or lesser degree on position quite different for a given kind of residue in each position in a folded protein. As a result residue sequence is not tightly conserved in a protein family even in the most stable substructures. The atoms forming the functional pair of knots in enzymes are palindromically related as determined by packing but with no obvious basis in sequence similarity. This leaves the B factors as quantitative measures of packing the single essential tool for the protein scientist. It has never been established that seqennce in itself could be converted quantitatively into accurate strudture and mechanism and recent successes in genome sequences further discourages any but very sophisticated use of sequence data.

Still to be established is the relationship of the conformational processes to physiological function and particularly to enzymic catalysis. B factors are essential to that undertaking. These show that proteins do not have a homogeneous construction. Two major substructures make function possible one for thermal and genetic stability the other for the subtle-change process providing the mechanism of catalysis. A first step is to separate the effects of temperature from those produced by steps in the catalytic process. This paper is a contribution to that undertaking developed using the x-ray diffraction data for ligand-free ribonuclease A reported by Tilton, Dervan and Petsko for nine temperatures from 98K to 320K. Gregory has already analyzed these valuable data in terms of the substructures (R. Gregory in “Protein-solvent interactions” ed. Gregory, Dekker, New Youk (1995) 191: and in “Role of water in foods” ed. D. Reid, ISOPOW Meeting, Santa Rosa, CA 1996 published by Chapman and Hall, (1997)) and it is now

possible to make that analysis quantitative by manipulation of the atomic B factors tabulated in the Protein Database. The procedure used in constructing Table 1 involves conversion of the atom B factors for all atoms to mean square displacements from idea lattice positions followed by conversion of those to mean displacements and volumes available for the center of each atom. The root mean square and its formal cube The B data were computed by Tilton et al on the assumption of isotropic scattering. The final step in our analysis is to sort the mean square values according to increasing size from which list the smallest 12% of the atoms were selected as those in the knot substructures. The remaining atoms can be assumed to form the matrix substructures estimated to be 80 plus percentage of the total residues at least for mesophilic proteins. The justification for this separation into two substructures is Pohl's compensation plot for melting rate data (discussed in other places). The data computed for the rate of denaturation of ribonuclease A falls on this plot as do the activation enthalpy-entropy pairs for most and possibly all mesophile proteins. Though still falling on the plot, the values of these quantities for ribonuclease A are somewhat larger than those for most of the other proteins as are those for bovine plasma trypsin inhibitor. We have suggested that this is a consequence of their large number of disulfide bonds. This explanation for the high melting temperature, 380K as opposed to 354K, has not yet supported by other kinds of data. Data for proteins from bacteria and eukaryote proteins fall on the Pohl plot with high precision. The data for proteins from archaea are not sufficient at this time to test a similar conclusion.

Recently Teeter and coworkers carried out a similar study of the temperature dependence of ht B factors for crambin over the limited range from 240K down to 140K with a goal of explaining the ubiquitous cooperative process just below 200K. Many proteins have been found to have this process and the B factors for crambin show a decrease as temperature decreases above the critical temperature and constant below. Crambin is not an enzyme and despite the high resolution of the crambin studies it is not possible to distinguish its substructures It apparently has only one functional domain but the knot B-factor pattern is not obvious although an imperfect palindrome in the low-

B atoms is not excluded. In other single-domain structures such as bovine pancreatic trypsin inhibitor this distinction is possible but there is no knot palindrome.

Importance of B factors. It is not surprising that so muich of research on proteins is based on their relationship to reliable facts from small-molecule chemistry but nevertheless the analogy is not close and the assumption that it is continues to impede attempts to understand protein structure and function. Among the most serious is that of the diffraction studies because the comparison usually leads to false assumptions. Small–molecules are usually so hard that diffraction patterns are preserved over large numbers of molecules so that molecule scattering parameters can be determined with high accuracy, even over determined. Atom position can be determined to vibrational amplutudes. .Protein crystals are entirely different not primarily because intermolecular order is much worse but because of heterogeneity within the molecules including both because of large variations in atom positions in the large, soft substructures and local variations in hydration. Protein crystals are sost as a result but not weak as usually assumed. The inter-protein lattice is strong enough to minimize lattice disorder among proteins but variations in atom positions tend to be smaller than the over4all accuracy of the method. On the other hand the precision which is long-range can be very high. Accuracy limitations occur in the Cartesian positions of the atoms because of the jitter; precision is high in B values simply because the averages are mean-square positions averages that eliminate or greatly reduce that jitter. The positional jitter is reduced to a small scale factor. The problems is due to different averaging procedures in using the diffraction data. Although that is a reliable empirical result, its explanation has not yet been given. The jitter as is affects the Cartesian positions large with respect to the intrinsic errors of the diffraction methods as applied to wet protein crystals so molecular details revealed by B factors are lost in the Cartesian coordinates. However, by B-factor comparisons atom by atom between the two cusps of an accurate palindrome the major physical parameters of a protein structure can be estimated with useful accuracy. Relative to the accuracy of their determination from diffraction data B-factors undergo major changes with change of temperature, change of pressure, change of solvent and solvent components, ligation, post-translational modification and change of residue composition. The averaging over the two cusps gives the B factors high precision even though their palindromic relationship is rarely exact at least in enzymes.. The scale factors as among the members of a protein family are large but are minimized

by this comparison procedue. With this method of comparison root-mean square differences are found to be about 0.05Å which assuming an isotropic model is the average displacement of a knot atom from its ideal lattice position. It is a good estimate of the mean displacement of a knot atom in the cage of its neighbors; expanded matrices have greater root mean square separations decreasing with contraction sometimes to knot-atom B-values. Becaise of the approximation C-2 symmetry resulting from the palindrome, the B factors form approximately ellipsoidal copies of the overall structure nested about the loci as determined by the knots cusps. Since expanded matrices are mechanically transparent, increased pressure has little effect on the shell dimensions in contrast to the enhanced thermodynamic stability of the solid knots that describe the phase boundary for pressure change. In deep sea vents high pressures counterbalance the high temperatures (Chapter 9C on extremophiles).

Major complications can arise from the variability of the B factors for several reasons: They have very high precision at least 0.05Å in the mean atomic radii so that corresponding atoms in the two domains of knots are matched with errors not much larger than those of primary-bond lengths in small molecules. The requirements for successful protein evolution are set by the high resolution consistent with the deductions of Pauling and Coryell from immune-globulin specificity. This is obviously a functional requirement for domain matching in enzymes but in comparing B values from different studies of the same protein the variation is somewhat larger. The reasons are only partially known and need to be understood if the quantitative information inherent in that precision is to be extracted. Most of the total changes in protein conformation in enzymic catalysis are less than 0.4 Å which is large with respect to interatomic distances from B factors but small with respect to coordinate errors in diffraction studies. Thus it is the B factors and only the B factors that make possible quantitative explanations for enzymic catalysis. But the major error has arisen in assuming not only high accuracy for Cartesian coordinate values but also that the B factors are constant regardless of the experimental conditions, the other members of a protein family and so on. The actual variations are large often exceeding the range of

corresponding range of the parameters not only those of dispersion potential functions but also parameters of hydrogen bonds. These errors are inconsistent with the small values usually reported for coordinates. Computer modeling os proteins is very popular but without some accurate values for coordinates perhaps at least as unreliable as the coordinate values from high-resolution diffraction studies. There are no true position values with which to compute true standard deviations for proteins.

Statistical parameters given in Table 1 were computed for ribonuclease A (bovine) from the diffraction results reported by Tilton et al As just noted they had no absolute values but there are significant adjustments for statistical variation in the variances resulting from the cancellation of scale factors in the standard deviation.. Their data do not yield the highest possible precision even in the standard deviatios but they do describe variations in structural patterns with useful precision. The study did not distinguish between knot and matrix substructures.

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Table 1 B factors for bovine ribonuclese A

Temp K	PDB file	MEAN (all)	O (all)	Mean (114)	O (114)	MEAN(remain)	
98	1RAT	6.62	8.55	0.5	0	6.62	8.55
130	2RAT	7.16	9.4	0.86	0.34	8.0	9.71
160	3RAT	6.31	5.12	1.86	0.61	6.31	5.12
180	4RAT	8.30	8.06	2.30	0.74	9.12	8.25
220	5RAT	9.78	10.13	1.58	0.78	10.9	10.13
240	6RAT	12.35	8.58	5.83	0.74	13.24	8.73
260	7RAT	13.65	9.09	4.58	1.31	14.88	8.99
300	8RAT	13.74	11.70	4.31	1.05	15.03	11.90
320	9RAT	15.67	12.78	2.41	1.36	17.45	12.60

The knot B-factor palindrome has parts from each of the usual two functional domains common to enzymes but they are somewhat less exactly matched because the two functional groups are somewhat displaced in order to position correctly the chemical reacting pair bridging the domains. With this protein that inexact matching seems to be required because of the difficulty in incorporating long RNA substrates properly in the nutcracker. When the B factors are sorted and then plotted against the atom numbers or residue numbers it can be seen the ladders of increasing B factors are identical in the two domains within error (Fig. 1). This is not always the case at some of the temperatures of the table but that may reflect the experimental error. The B factors were not optimized in the experiments and the results in some columns suggest experimental error rather than intrinsic construction features. Within experimental error the knots have constant B values from 240K down to 160K The matrices as shown in the last two columns of the table are less constant above 240K but more so from 240K to 300K This is the extent of the agreement with the crambin studies of Teeter and coworkers.

Columns three and four list the means and the standard deviations of the B factors of all the atoms. Columns five and six are for the knot atoms deduced as 114 from the fact that Pohl and since then others have found this protein to lie on Pohl's compensation plot. We have shown elsewhere that that plot establishes that mesophiles have a minimum of 12 % of their total non-proton atom count in the knots.

Columns seven and eight apply to the total of atoms minus 114 and thus are good approximations for the matrices.

In Table 1 and Fig. 1 several patterns of temperature dependence are suggested. From 320K to 280K matrix contraction is to be expected for the ligandfree wild types. Then down to about 220K there is a very common further contraction much studied but still to be fully explained. Finally on down toward 0K the remaining irrational excitation disappears. All of these changes are suggested by the data in the Table but not in easily and reliably interpretable ways. The statistical tools consist of the mean values for the atom B values and the standard deviation of those values with which some potentially useful deductions can be made as follows:using the @ data.

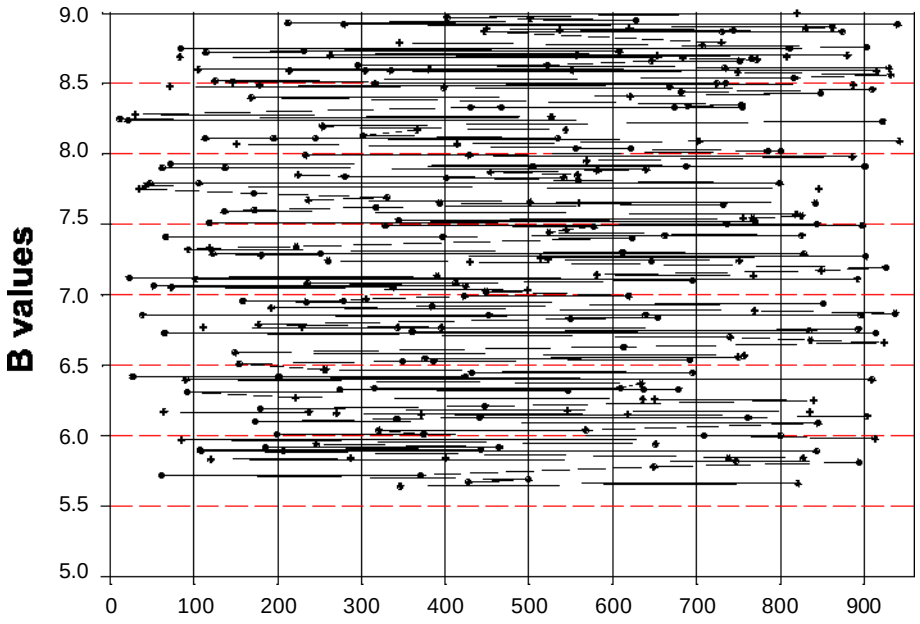


Fig. 1 factors are sorted from lowest to highest, then plotted against atom number to show the high precision with which the two functional domains are matched in free volume well into the matrices. Atom pairs for knots are matched to 0.05 Á and the matrices of the two catalytic domains are well matched though not so well and somewhat variable depending on the specific function though only in the ligand-free fully expanded forms.

As is indicated by decline in their mean values with temperature, the knots can be more temperature dependent than previously reported n the Protein Primer, vol. 1. This behavior for knot pairs is also demonstrated in the constant-temperature study by Bone et al on α-lyctic protease acylated with a series of boronic acid acyl ligands with side chains corresponding to a common set of ester substrates. However only the catalytically most effective side chains produce much compression, e.g. .;phenylalanyl. Specific substrates and inhibitors of enzymes generally produce larger domain closure the more effective they are. That can be judged by rates or overshoot and inhibitor effectiveness but not many suitable examples have yet appeared in the PDB. That is because the accurate comparisons of enzymes in open and closed from are rare and will be until the very important difference is more appreciated. Those comparisons are the essential to any study of catalytic mechanism.

The B values of the knot atoms in ligand-free RNase A are constant within error above 220K and the standard deviation is constant up to 240K indicating nearly constant free volume distribution as is expected with hard structures. Above 240K knot free volume is again constant but at a somewhat larger value. These observations as well as those on the matix gain clarification from Gregory's study of the effects of hydration with temperature variations and his reviews of the matrix contraction process (see his articles in the Utilities folder of the Protein Primer web site). From 130K down contraction with almost no further contraction is indicated for the Rnase A knots and matrices. The RNase A matices show consistent increase in both mean and standard deviation with increasing temperature but the latter though large does not show much change in hardness. Thus even at 98K matrices are soft

with large standard deviations nearly constant up to at least 260K but the matrix means show regular expansion over the entire temperature range.. The amount of change in volume can be estimated from the relationship between R and the man square displacement of an atom from it ideal lattice position using the relationship between B value and root mean for the isotropic scattering assumption since the mean square is 1Å for B=70. In time as the data improve much more information of matrix contraction and organization will be extracted using the three moments of the scattering ellipsoid These have appeared to be too complicated for analysis at this time but some simple relationships to other structure parameters are already beginning to appear.

Because unconstrained enzyme matrices not liganded with substrates or competitive inhibitors oscillate between open and closed position with random periods averaging about 1ns at ordinary temperatures, the B values and especially those for matrices are averages which underestimate the free volume of the free protein.

The isotropic model yields the matrix data given in Table 2. It is noteworthy that the volume and positional changes from B factors even now have a precision better than 0.1 Å. Figure 1 is particularly useful in testing for the precision in C-2 matching of the functional domains. As already noted the precision attainted in protein evolution is remarkable. These "symmetry plots" for the temperature data of Tilton et al show matching to a fraction of an angstrom at most temperatures with a few deviations at some temperatures that contain additional information for more detailed analysis as the anisotropy data improve and accumulate. Preliminary examinations of some such data suggest that free-volume matching patterns are as preserved in three dimensions as in one.

Table 2 Estimated mean atomic radii and volumes using B values from (Table 1)

Temp. K	Mean radius -matrix Á	Mean volume- matrix Á^3	Mean volume- knot Á^3
98	0.28	0.09	0.003
130	0.32	0.135	0.005
160	0.28	0.095	0.015
180	0.34	0.19	0.021
220	0.37	0.22	0.012
240	0.41	0.29	0.084
260	0.43	0.34	0.060
300	0.44	0.35	0.054
320	0.47	0.44	0.022

Hydrogen atoms are not included in the averaging nor in anisotropy.

The data given in Table 2 again demonstrate the high precision of the experimental B values and the reproducibility of the atomic average displacements and free volumes available to the center of mass of each atom. This precision is much greater than that of the experimental distance and angle parameters determined in the same studies and very much better than the estimates of the structural parameters using NMR methods but just how much is difficult to estimate since there is no way to establish the accuracy with either method. The absolute values of coordinates required for that computation are not known. Precision criteria are less limited because it has been found that any scale fluctuations cancel in comparing B values of the corresponding atom pairs in the knots in the two catalytic domains. In high resolution studies when the B factors are refined with the coordinates the intrinsic precision in this comparison has been found to be about one twentieth of an angstrom. The quality of the estimates from B factors is much higher than has been realized and as one result provides useful definitions of conformational changes in folding and in enzymic function. Even more useful estimates of the latter can be obtained by comparing the

changes in B parameters when an enzyme or other protein in free and unliganded state is converted to some functional state.

Changes occurring in physiological function.

As illustrated in the preceding section, the maximum change in B factors produced by changes in temperature or ionization can be estimated by extreme cooling to quench vibrational excitation. However, most proteins demonstrate a major transition on cooling below about 200K into a more compact form apparently due to major changes in interaction with water (reviewed by Gregory in his papers on the Protein Primer web site) so cooling effects are not limited to vibrational quenching. However of most interest are the changes in B factors not detectable in x-ray-coordinates produced by conformation changes arising from one or another of the physiological functions of the protein. They can be estimated by comparison of B values for a parent protein with those values as modified by binding specific ligands; specific inhibitors such as N-acetyl-L-tryptophan in Yapel's study of ligands for chymotrypsin, that produce major interaction between the catalytic domains often including over closure as is the case with pepsins with pepstatin. Before 1990 the rhizopepsin study of Davies and coworkers was used as an example such changes because the protein with pepstatin bound was carefully compared with the free protein at the same time in the same place by the same people. These conditions were expected to minimize differences in scaling of the two knots. The possibility for scale error is likely to be small but still a matter of concern as shown in Figure 2 constructed with the data from the Davies pepsin study. That figure shows that the larger the atom B factors in the free enzyme, the greater its contraction on pepstatin binding. Pepstatin has only 46 non-hydrogen atoms and Zundel and coworkers using infra-red spectroscopy demonstrated that the interaction between the two functional aspartate groups that participate in the catalytic process drives a proton across the inter-domain hydrogen bond between the aspartate groups thus from one of the

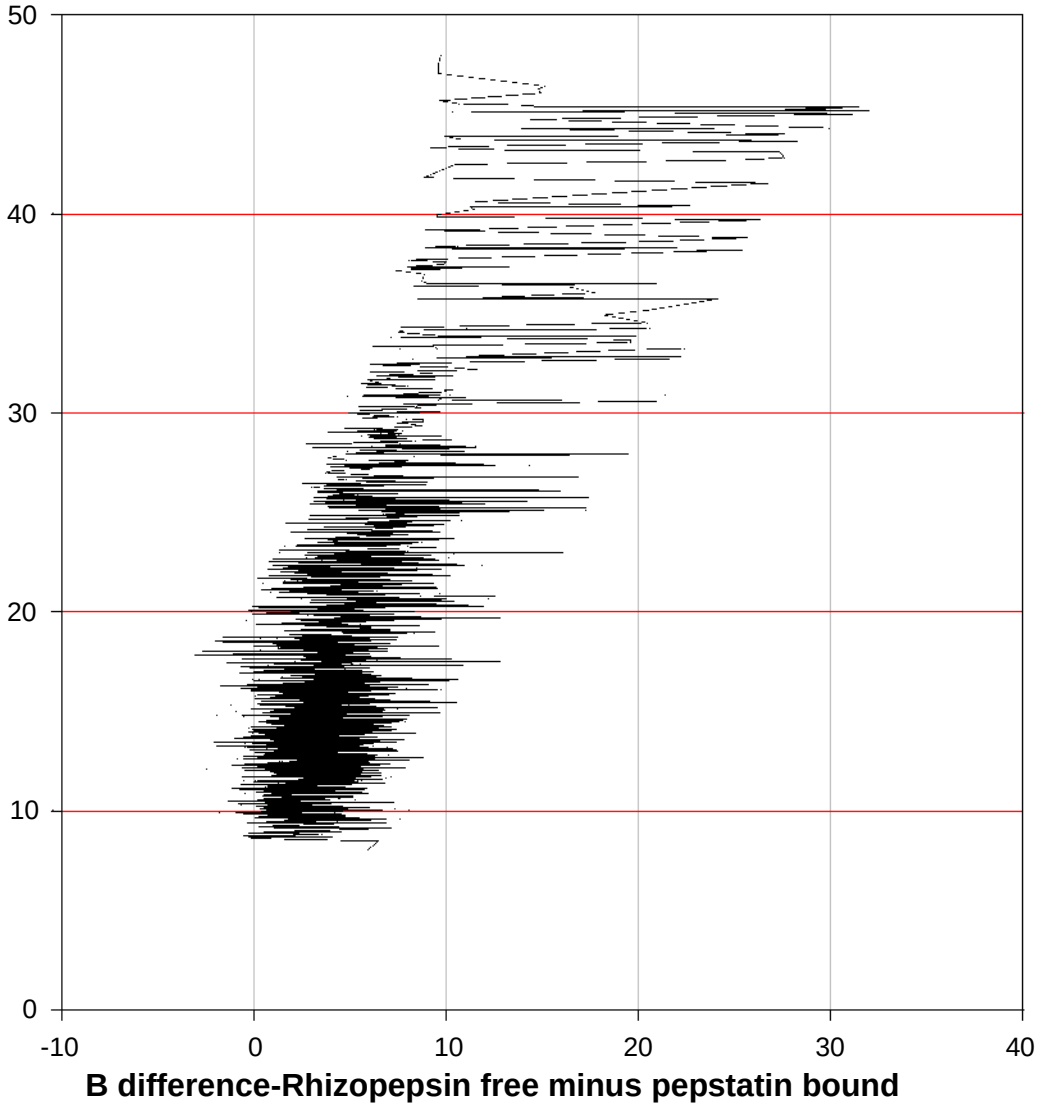
catalytic domains to the other. Less effective ligands used by other workers produce somewhat smaller average proton migration. This is largely due to loss of free-volume in the matrices as is shown by comparing Fig. 3a for the free enzyme with Fig. 3b with pepstatin bound.

Pepsin plays a major role in digestion in the gut where the pH is very acid. Optimum activity of an enzyme in such extreme conditions labels it an extremophiles.and its stability depends on a shell of aspartate and glutamate groups located just inside the ends of the polypeptide loops These placements very nearly copy the knot palindrome although there is no direct connection between them. Pepsin has an unusually large number of loops (Lumry, Chapter 1 Protein-solvent interactions. Ed. R. Gregory, Dekker 1995, Fig. 15) More careful examination has shown that some parts of the knot also contract; the contraction can be large as shown in Fig. 4 but that of the matrices is greater. Judging from cooling of other proteins, e.g. HEW lysozyme, the contraction produced by pepstatin is about the same as that produced by cooling the free protein as is to be expected from loss in atom free volumes. The changes with temperature and specific ligation measured in this way are consistent with the major changes in ellipticity near 206nm, a peptide absorption region. The very large ellipticity changes in function were first detected by Parker using chymotrypsin data in 1963. The values of thousands of degrees are produced by simultaneous compression of many peptide chromospheres. The use of circular dichroism in following denaturation has been detailed recently be Akasaka and coworkers studying the thermal denaturation of Tamura (Kimura, Takahara and Akasaka, Biochemistry 30 (1991) 11307.) They confirmed the wave lengths of the chronophers but as in most similar studies neglected the significance of the very large ellipticities.

The B values of the knot atoms of the pepsins are not much reduced by cooling or by binding pepstatin. However, Bone et al found with alpha-lyctic protease on conversion to the boronic-acid acyl analogs of ester substrates found considerable

knot contraction with those corresponding to the most efficient substrates. The matrix contraction is the expected subtle change that provides transient potential-energy pulses driving catalysis and as shown by the effects of pepstatin on pepsin, force contraction in the inter-domain H bond. The nutcracker thus activated also distorts substrates as indicated by observations on the serine proteases. Matrix contraction is very large in B-value terms but only a few tens of angstroms in bond contraction. The contraction generates a roughly spherical shell of contraction centered on the knots consistent with the orientation of the individual matrix residues from center toward periphery (as shown for the G protein of streptococcus in Biophysical Chemistry, 2002 reprinted in the Utilities folder of the Protein Primer WEB site.). That orientation is common in enzymes. It must have taken at least one billion years of evolution.

Fig. 2. The pattern ofcontraction produced by pepstatin bound to rhizopepsin computed from data of Davies and coworkers (2apr.pdb and 6apr.pdb) is shown below.



The new observation generated by the above figure is that the data do not go through 0 on the ordinate. That means the knots also contract on pepstatin binding and do so by almost ten units of difference between free protein and the pepstatin-carrying form. Most previous examinations of the scaling of B values in such comparisons has shown or suggested rather minor scale reduction on contraction. More comparisons from the PDB are necessary to resolve this major difference.

However note that to correct the figure for the scale difference only requires a downward shift along the ordinate reducing the maximum value of the contraction but not otherwise altering the pattern. Since the pattern describes the matrix contraction believed to drive the nutcracker mechanism, the latter behavior is encouraging but not convincing. The average contraction on pepstatin binding based on mean B of 17.07 for free enzyme and 12.67 with pepstatin bound so the volume change per non-hydrogen atom is 0.443-0.250=0.19 cubic angstroms This is the estimated free volume and for the free enzyme with 2417 non-hydrogen atoms it yields a contraction of 7 liters per mole of protein. The volume of one mole of this protein is about 26 liters so this estimate may be too large. Recall that the freezing of ethanol and msny other amphiphiles produces a 4% contraction. For the two enzyme species the B variance of 14.8 drops to 11.58 with pepstatin. Pepsin is unusual in its contraction behavior as indicated by the branching shown in Fig. 3 so these figures have limited quantitative general applicability.

Summary

DNA is appreciated for its ability to preserve genetic information but we have shown elsewhere that this does not take the form of precise preservation of sequence. Rather it is the details of the free-volume arrangements that are maintained with great accuracy and precision so one must look to free-volume for evolutionary specificity. It has also been shown that there is no obligatory connection between small-molecule chemistry and the chemical transformation effected by proteins. All enzymes appear to function in the same way, as nutcrackers, just as they all manifest the same small set of construction devices. The details of that mechanism must be extracted from comparisons of free enzyme with the enzyme in various stages of the catalytic process usual produced by combination with substrate in its stages of conversion or by combination with specific inhibitors. Because most x-ray-diffraction studies have been focused on exchange of selected residues rather than B factors there are few sets of

data similar to those of Davies et al on rhizopepsin free and with pepstatin bound. Most diffraction studies reported in the Protein Data Bank are "engineered" exchanges of one or a few residues and these can produce major changes in conformation and mechanism hiding the true description of the free enzyme. Nevertheless in knots the largest changes might be expected by some exchanges have surprisingly little effects.

.. The major part of the information on mechanism of the serine proteases has been extracted from their acyl derivatives that came to attention in attempts to protect or reverse the effects of the irreversible DIP type of war gas. These did not involve residue exchanges and as a result produced unambiguous results. Steady progress in research on catalytic mechanisms is unlikely until more diffraction experiments of that type and of the rhizopepsin model without engineering are carried out.

Effects of solvent composition

The folded stability of proteins free of complications from ligands and residue exchanges depends very strongly on the surface hydration layer consisting primarily of partial Frank-Evans icebergs and as a result it is sensitive to structure breakers and structure makers as cosolvents. Species with contracted matrices are much less so. @@ Huber and Bone studied methanol effects as occuring in low-temperature studies to prevent freezing. Amphiphiles such as ethanol sometimes cause formation of mixed protein-alkanol micelles with compact structure but little relationship to normal protein conformations. There are several very different results on subzero cooling of proteins often due to the cryogenic cosolvent from matrix compaction to "cold denaturation" but in the usually stability temperature range most data on cosolvent effects are due to Timasheff and coworkers and Winzor and coworkers. These groups have interpreted their results using the thermodynamic formalism of excluded volume but the crambin studies of Teeter et al and the chemical interactions of structure breakers demonstrate that it is availability of free water as it controls the amount of normal free water by chemical sequestering that provides a more accurate picture.

Chapters 4 and 5 of volume 2. Structure breakers like urea then destroy the clathrate forming property normally dominated pure-water mixtures. Structure makers are effective not because they directly reduce the proportions of lower-density water; structure-makers use up water. Both are effective largely because they raise the free energy of the protein interface thus causing matrix contraction with enhanced thermal stability. Ligation as with specific substrate or inhibitor has a similar effect but produced directly rather than through changes at protein surfaces. This effect has been repeatedly illustrated using a strong inhibitor of pepsin, pepsinogen,.as shown by the following B-factor plots. Changes in knots are small but matrix contraction is extreme. Zundel has shown the latter to force the proton in the asp-asp H bond at which catalysis takes place from one of the functional domains to the other.

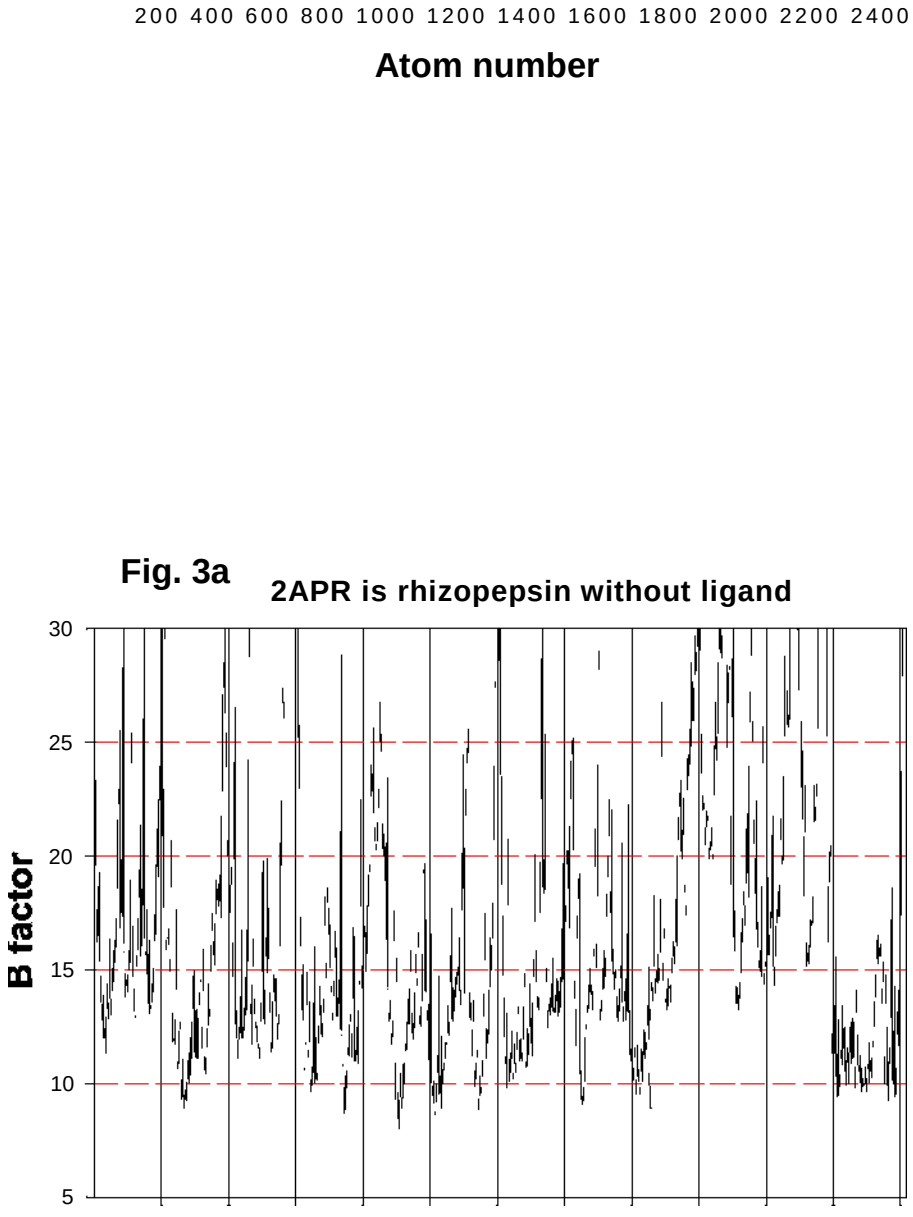


Fig. 3a **2APR is rhizopepsin without ligand**

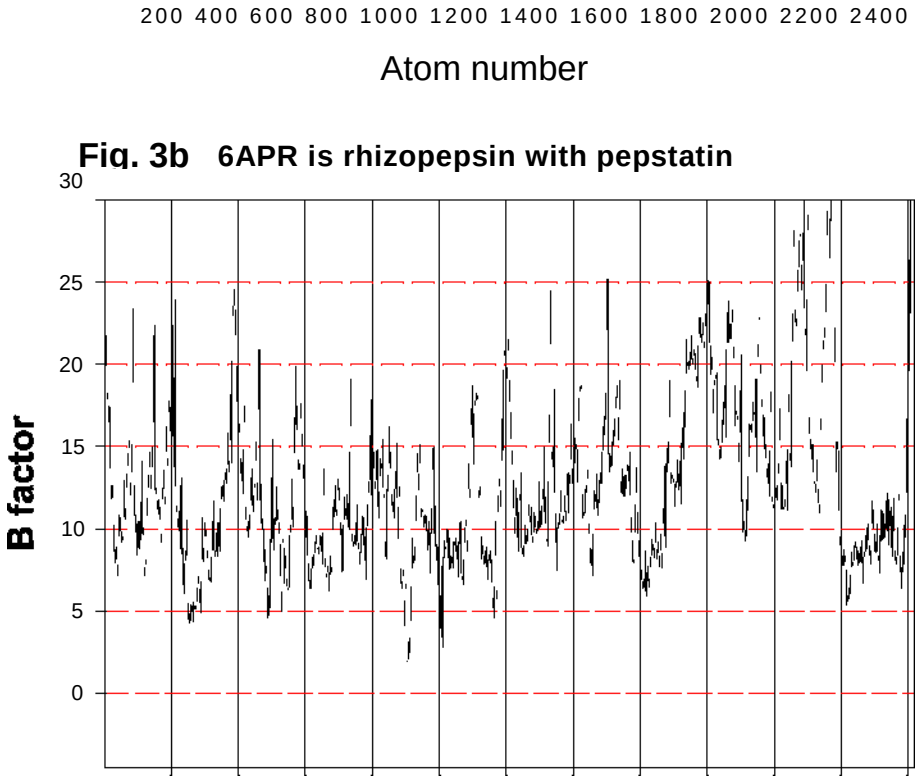


Fig. 3b 6APR is rhizopepsin with pepstatin

Fig. 3 Effect of pepstatin-Two figures for Rhizopepsin to show peculiar construction with displaced palindrome and added section of44 residues that block function and are cut off to allow rearrangement enabling function. Note that the knot palindrome exists before this activation. The aspartate residues participating chemically are 33 (atom 241) and 218 (atom 1613) in the numbering used by Davies et al. These residues are not parts of the knots but instead are positioned just as the chain leaves the knots. The knot palindrome is clearly defined in these figures only by the few atoms with lowest B values.

Note in this connection that protein-protein association allows proteins to communicate for physiological mechanisms and the communications is usually dependent on the free volume changes. Even in weak protein-protein interactions whether or not having physiological significance such associations can develop large stresses and deformations of the proteins involved. These are whole-molecule effects still little appreciated.