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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 described a process with major enthalpy and entropy changes but without the heat-capacity change Brandts had previously shown to be characteristic of thermal melting. The latter process was often difficult to detect because of the latter characteristic and had become known as a "subtle change". Lumry and Biltonen then 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 is used to excite a pretransition state rather than to lower the transition state. The catalytic mechanism depends on the mechanical excitation rather then the thermal activation demonstrated by most small-molecule rate processes and generally forming the basis of the popular "transition-state stabilization" hypothesis of enzymic catalysis.

Changes in conformation expected for subtle changes were not obviously revealed by coordinate information from x-ray-diffraction experiments carried out in the next few years and had the effect then as now to deemphasize both subtle changes and the mechanical mechanism of enzymic catalysis. This result responsible for so many wasted careers and so much wasted support money is due to the large errors in the most precise coordinates that can be extracted from diffraction data. The experimental errors are considerable larger than the changes in bond length and angels determinable at even the highest possible refinement for protein crystals and as a result hide the actual changes that take place in enzymic catalysis as in most other physiological functions of proteins even muscle function. 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 which is in turn 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 give somewhat different information Gregory and coworkers found with such data that familiar proteins have two and perhaps three kinds of substructure so it then became possible to give the changes in B factors structural description. That has been done for many proteins stable in the mesothermal range defined pragmatically in terms of thermal stability as being above 280K and below 354K but conformational changes supporting physiological function have not been completely resolved from those produced by temperature change even in this range. It is currently thought that the original forms of living things existed at extreme temperatures such as those the archaea in the hot deep sea vents and perhaps at extreme acid or salt concentration. Proteins stable and functional at such extremes are called extremophiles. It is desirable to explain the extreme properties in terms of the substructures and the construction and mechanisms of the mesothermal proteins. In particular one can begin with the extreme enzymes since all the many mesophilic enzymes appear to be constructed to have the same set of features supporting a single catalytic mechanism. We first examine the role of temperature necessary not only for the extremophiles study but also necessary to separate such effects from those occurring during catalysis, etc.

Tilton, Dervan and Petsko carried out x-ray-diffraction studies of ribonuclease A free or ligation and residue exchanges at nine temperatures from 98K to 320K. Gregory has already analyzed these valuable data in terms of the substructures and it is now possible to improve the reliability of his analysis with newer information about the precision of the B factors. Ribonuclease A is not the best choice for this examination because is does not have complete C-2 symmetry in its knot B-factor palindrome. This groups it with T-4 lysozyme and T-1 nuclease which have even larger deviations but ones that suggest accommodation to large polymeric substrates

rather than critical tests of the good dynamic balance found in most enzymes. However the bovine ribonuclease A has been very extensively studied with a rare study of temperature dependence by Tilton from which the rough quantitative comparison is given in Table 1.

Table 1. B-factors for bovine ribonuclease A at several temperatures.

Temp K	PDB file	MEAN (all)	Ö (all)	Mean (114)	Ö (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 the usual two functional domains but 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 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. This is not always the case at some of the temperatures of the table but that may reflect the experimental error. The study is ten years old and was not carried out so far as can be deduced to optimize atom B factors. One can see that the results in some columns suggest error rather than intrinsic construction feature.

Columns three and four are 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 12 % of their total 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.

There are several patterns of temperature dependence that might appear. From 320K to 280K matrix contraction is to be expected for the ligand-free 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 variance of standard deviation of those values with which some potentially useful deductions can be made as follows:

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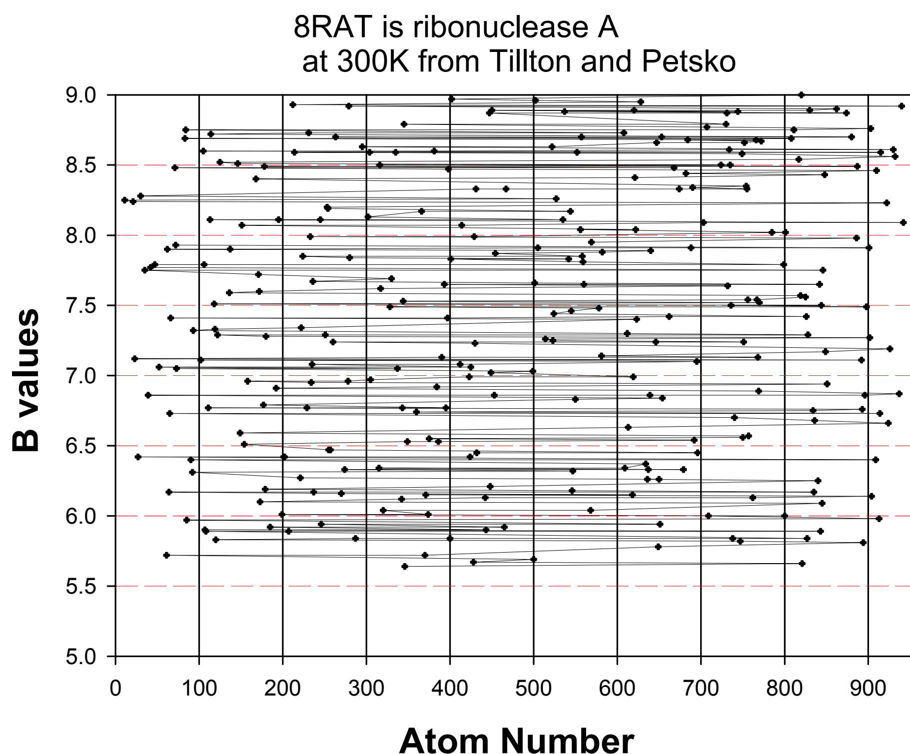

Fig. B factors are sorted from lowest to highest, then plotted against atom number to show the high precision with which the two functional domains are matched in free volume well into the matrices. Atom pairs for knots are matched to 0.05 Ǻ and the matrices of the two catalytic domains are well matched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

The knots are indicated by decline in their mean values with temperature to be somewhat less hard than previously reported. This behavior for knot pairs is also demonstrated in the constant-temperature study by Bone et al on α-lyctic protease with a series of boronic acid acyl ligands with side chains corresponding to ester substrates. However only the catalytically most effective side chains produce much compression e.g.;phenylalanyl. RL `Insert here the rnas a pair` The ligand-free RNase A knot B values are constant within error above 220K and the standard deviation is constant up to 240K indicating nearly constant free volume distribution in the knot free volumes as is expected with hard structures. Above 240K it is again constant at a somewhat larger value. These observations as well as those on

the matix gain some 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 fluctuation is indicated for the Rnase A knots.

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: 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 these also are beginning to reveal simple relationships to other structure parameters.

Because unconstrained enzyme matrices not liganded with substrates or competitive inhibitors oscillate between open and closed position with periods of 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 examination of some such data indicates that free-volume matching patterns are as preserved in three dimensions as in one.

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

Temp. K	Mean radius -matrix Å	Mean volume- matrix Å^3	Mean volume- knot Å^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 nmr methods. There is no way to establish the accuracy with either method since 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 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 to more compact form and probably a major change in interaction with water on cooling below about 200K (reviewed by Gregory in his papers on the Protein Primer web site) so cooling effects are not limited to vibrational quenching. However in general 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 product major interactions between the catalytic domains often including over closure as is the case with pepsins. In the past the rhizopepsin study of Davies and coworkers has been used as an example because the protein with pepstatin bound was carefully compared 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 that does confirm the deduction that the larger the original atomic B factor, the greater the reduction for that atom with pepstatin bound. Pepstatin has only 46 non-hydrogen atoms and Zundel and coworkers using infra-red spectroscopy were able to establish that the interaction between the two aspartate groups that participate in the catalytic process actually drove a proton across the inter-domain hydrogen bond from on domain to the other. Less effective ligands had been shown previously to produce somewhat smaller average 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. 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. It achieves behavior in the acid gut because there is a shell of aspartate and glutamate groups located in exactly the same way at the outer end of each polypeptide loop. That construction seems to have been difficult to find in evolution because pepsin has several anomalies most of which are discussed elsewhere. More careful examination has shown that some parts of the knot also contract and the contraction can be large as shown in Fig. 4 but that the matrices is larger. The comparison illustrated in Fig. 3 shown that not only much larger reduction if matrix B factors but also that the standard deviation in those factors is much smaller than in the free enzyme. 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 importance of circular dichroism in following this process has been detailed recently be Akasaka and coworkers studying the thermal denaturation of Tamura (Kimura, Takahara and Akasaka, Biochemistry 30 (1991) 11307.)

The well delineated pattern of B contraction in the matrices is made complicated for analysis by the special features of the pepsins. They have a shell of aspartates and glutamate residues attached at the ends of the peptide loops roughly mimicking the knot palindrome and roughly spherical. The many loops required for this construction show the knots to be unusually complex with an added C-terminal stretch of about 50 peptides that supplement the normal knot is ways not yet obvious.

Whether or not this special feature is associated with the acid tolerance of the pepsins is also not yet obvious.

The large contraction of the knots is not consistent with deductions drawn previously and has major implications for the catalytic mechanism. It can now be related to the contraction found by Bone et al with alpha-lyctic protease ligated with boronic acid acyl analogs of ester 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, specifically to 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 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.).

Fig. 2 for Rhizopepsin computed from data of Davies and coworkers (2apr.pdb and 6apr.pdb)

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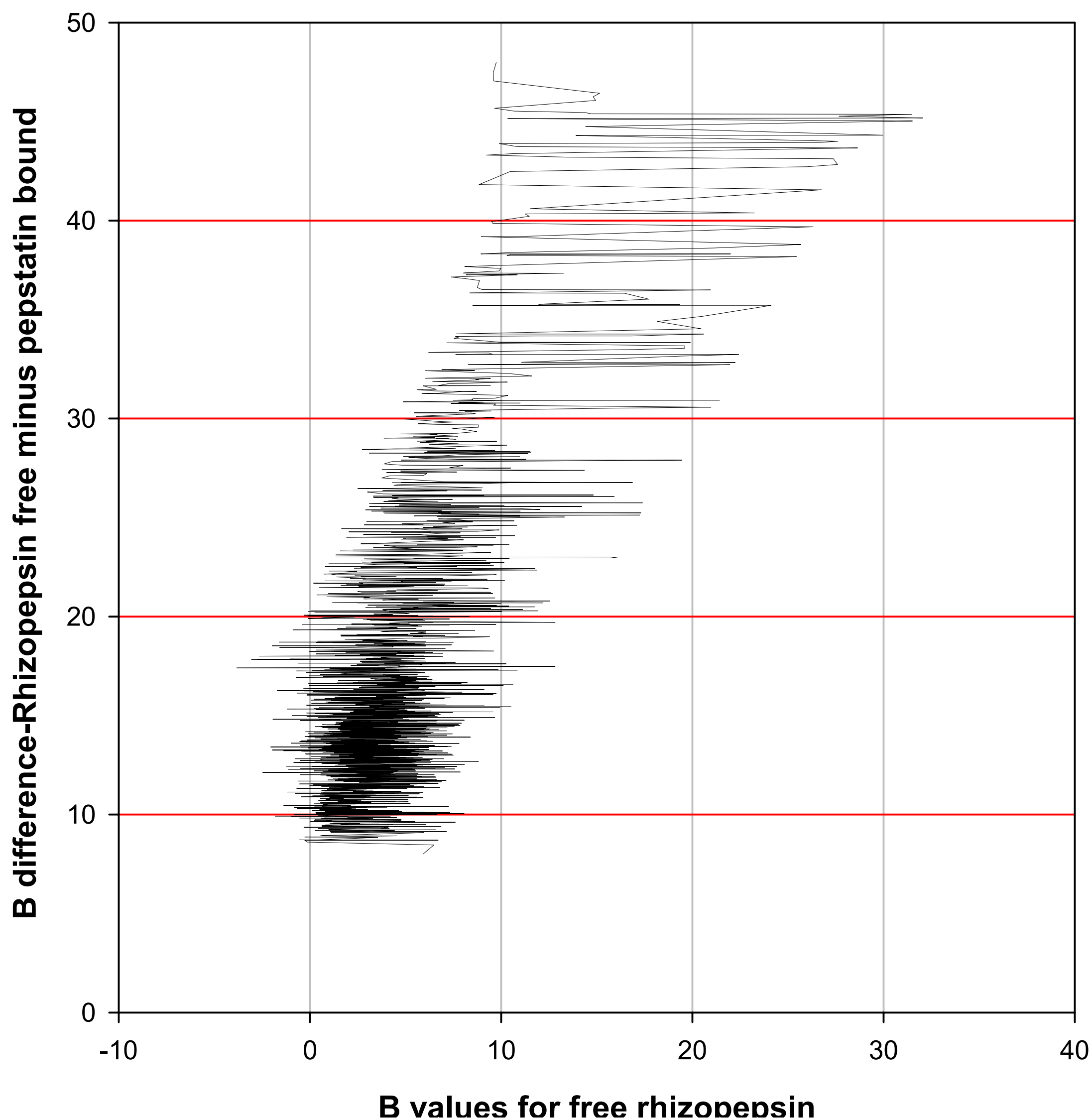

The new observation generated by the above figure is that the data do not go through 0 on the ordinate. That means the knots also contract on pepstatin binding and do so by almost ten units of difference between free protein and the pepstatin-carrying form. Most previous examinations of the scaling of 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 appears to be too large. Recall that the freezing of ethanol 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.

Relevant references with detail and scientific support are given in the Protein Primer web site http://www.chem.umn.edu/groups/lumry

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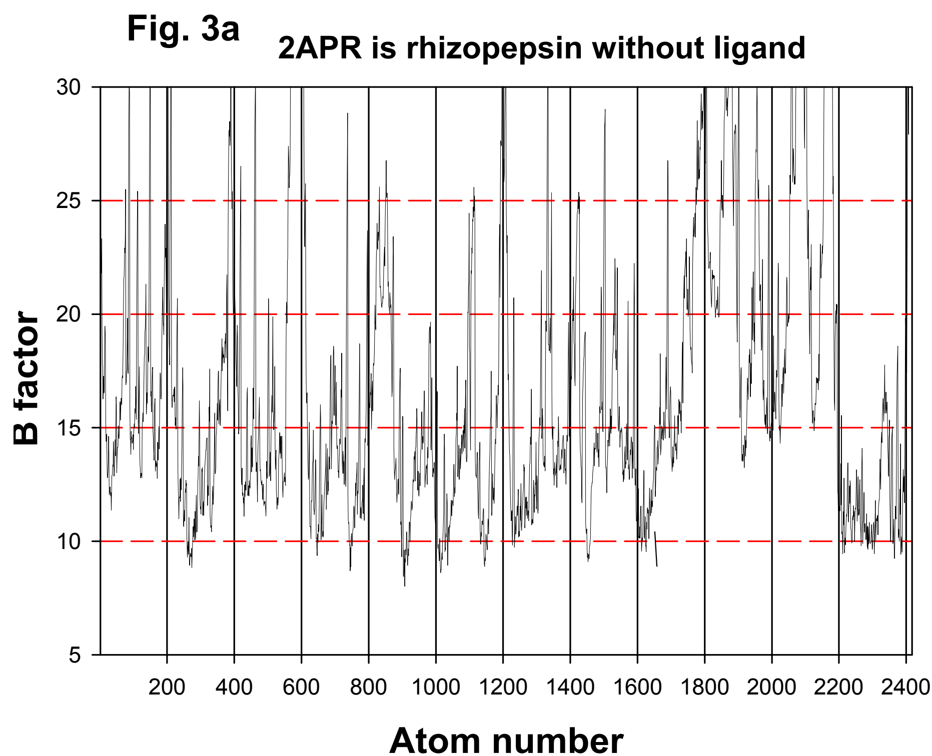


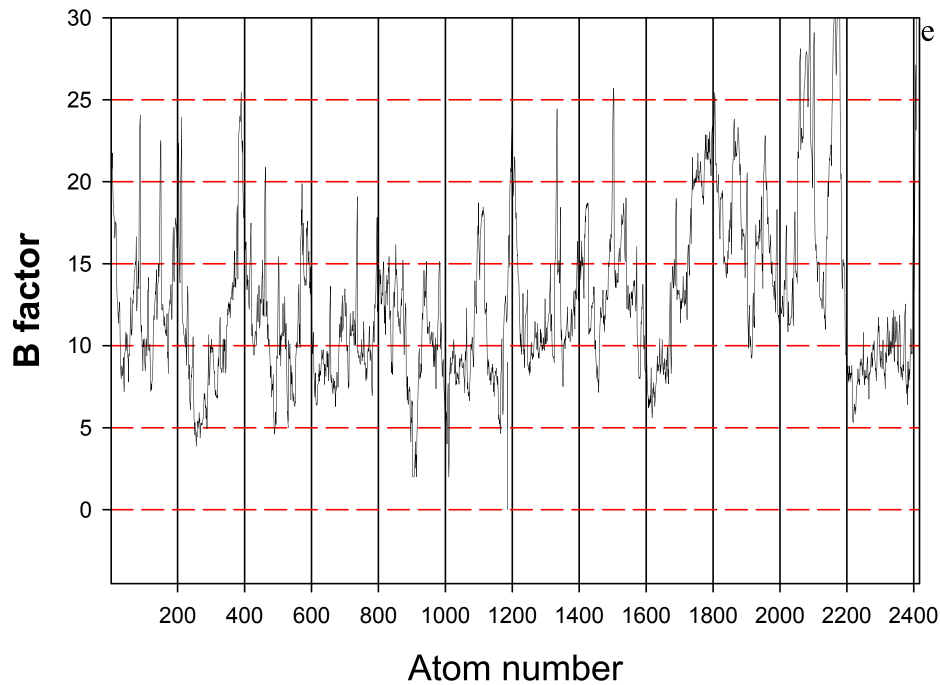
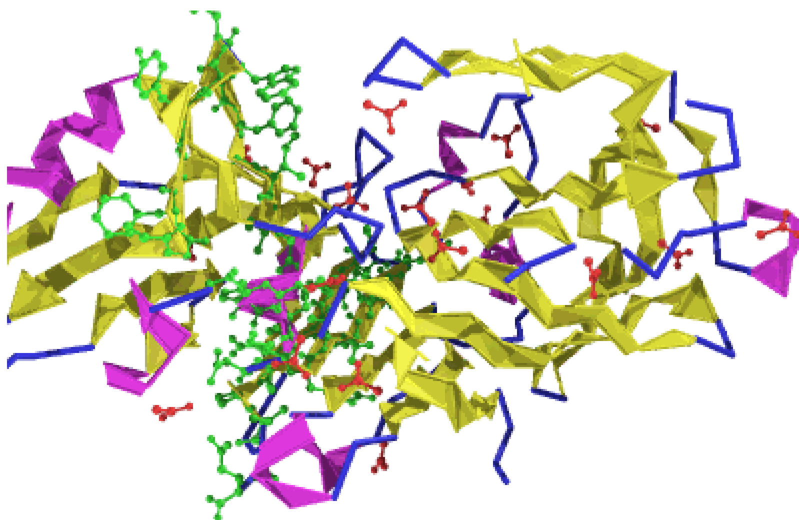


Fig. 3 Effect of pepstatin-Two figures for Rhizopepsin to show peculiar construction with displaced palindrome and added section of 44 residues that block function and are cut off to allow rearrangement enabling function. Note that the knot palindrome exists before this activation. The aspartate residues 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.



The green atoms form the removable part of pepsin and do not look like they are simply an add pm tp the C-terminal end of a simple protease like chymotrypsin but that is what they are.