Chapter 5 Volume 2

Operation of enzymes-"The nutcracker mechanism"*

Fortunately Dorovska-Taran and Martnek with their respective coworkers measured the steady-state rate parameters with chymotrypsin for a congener series of methyl esters of amino acids substrates. The results were consolidated into complete profiles of the activation thermodynamic quantities as diagrammed in Fig. 5-1.

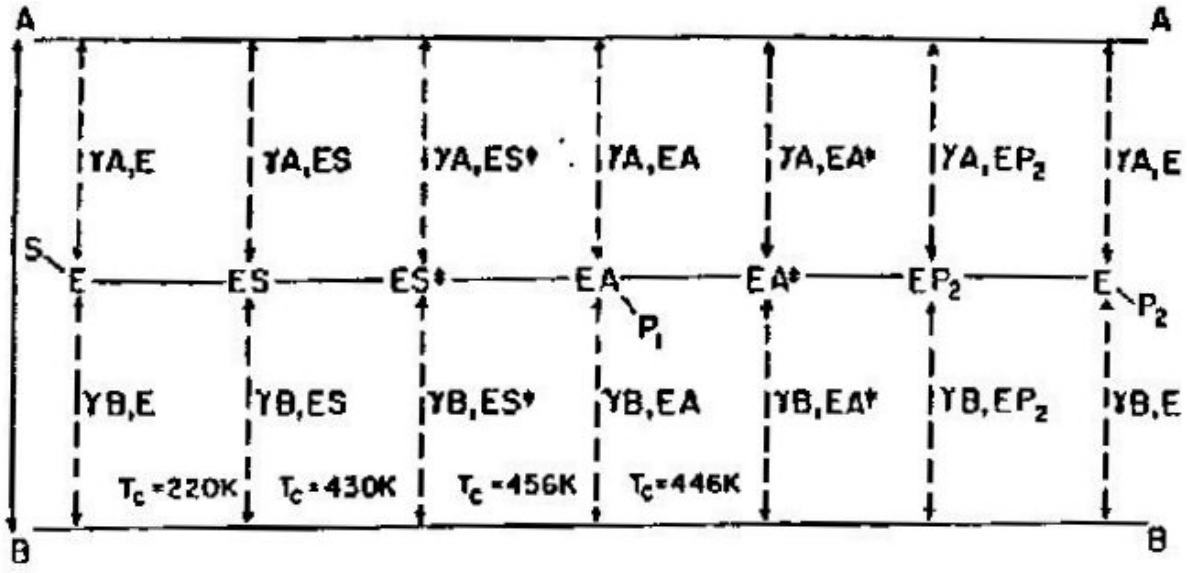


Fig.5-1. This figure explains the analysis of the congener series of N-acetyl-L-amino-acid ethyl esters for chymotrypsin- data from Dorovska-Taran and Martinek and coworkers. The Hartley-Kilby mechanism for steady-state rate includes the single primary-bonded ester of the substrate acid group. The A row describes the contributions from the intrinsic protein common to all substrates. The bottom row includes only those contributions from the substrate side chains. That series of amino-acid methyl esters demonstrated linear enthalpy-entropy compensation behavior for each set of parameters. The compensation temperatures for each step are listed as Tc values. It is apparent from the latter that each step involves a change in matrix conformation. The exception is the first association of substrate and enzyme. The G, H and S parameters appropriate to each step are described in the following figures and provide a quantitative description of nutcracker operation. * *The details of the procedures and conditions were first given by Lumry in Study of enzymes, ed. S. Kuby for*

CRC press 1991 (volume II, page 3 reproduced in the Utility folder of the Protein Primer web site)

. The following figures contain data for only two of these substrates: N-acetyl-α-aminobutyric acid methyl ester with low turn-over rate and N-acetyl-L-phenylalaninemethylester which has a catalysis rate seven orders of magnitude greater. The formal reaction mechanism is that of Hartley and Kilby including the intermediate covalent species between acid groups of the substrate and serine 195. The rate of the off-acylation step back to reactants was evaluated by varying methanol concentration the dependence of each rate parameter on residue side chain could be estimated by assuming the glycine substrate to depend only on the intrinsic contribution from the matrix contribution. That separation often suspected was finally verified in these studies.

The Hartley-Kilby formalism does no more than conserve the protein in all its forms and in this case also shows the formation of the primary-bonded intermediate important because it suggests a two-stroke mechanism. There are, however, major differences on comparison with its application to the standard Briggs-Haldane mechanism particularly in comparing the pretransition state, ES± in the nutcracker mechanism with the ES compound and transition state in the conventional mechanism. The difference is established by the compensation temperatures all of which except 220K for simple association show involvement of the matrix expansion-contraction process. Although the transition state can be the state of highest free energy along the best reaction coordinate, that need not be the case. If ES± lies higher than the transition state, the latter is neither known nor necessary. If it lies lower, the deficiency is made up with the usual thermal activation. Then the deficiency is equal to the experimental activation enthalpy varying with the free energy requirement for passage through the transition state but not large with good substrates. Without these distinctions the experimental parameters can be very misleading. The steady-state model with back reaction from products assumed negligible on extension of the Hartley-Kilby formalism to include the extra step is adequate to include the simple association step forming ES and the excitation of the latter to ES± thus: $$\left(\frac{d[P]}{dt}\right)=\frac{k_D e[S]\left(\frac{k_1 k_2}{k_{-1}k_{-2}}\right)}{1+\left(\frac{k_1}{k_{-1}}+\frac{k_1 k_2}{k_{-1}k_{-2}}\right)[S]}$$

The results of the separation are shown in the H and TS plots for D (dependent on sidechain) and I (independent of side chain). The major deductions are those for G shown in Fig. 5-

2 the larger variations for the phenylalanyl substrate. The substrate-depended parameters are labeled D; those intrinsic to the enzyme I. Fig. 5-3 are the corresponding enthalpy and entropy profiles.

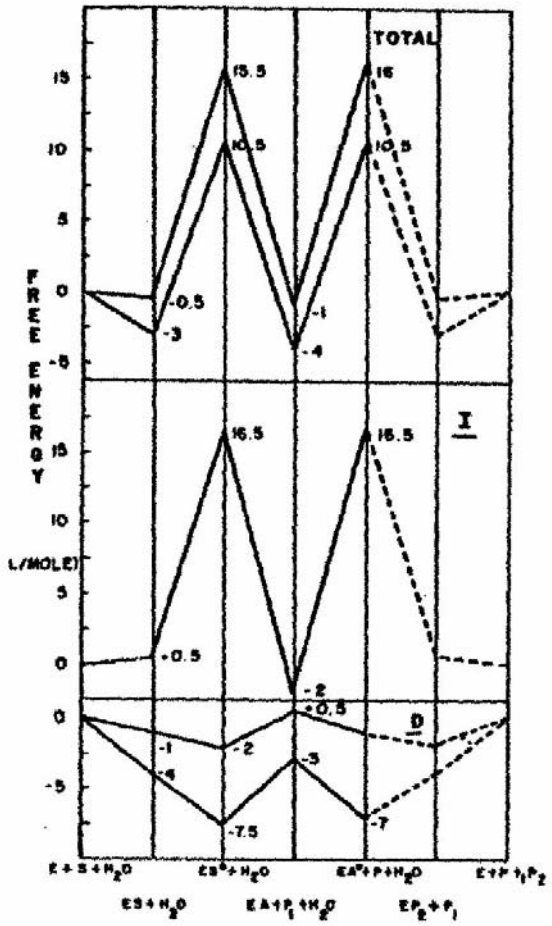


Fig.5-2 Free-energy profile for two substrates of α-chymotrypsin: N-acetyl-L-aminobutyric acid and N-acetyl-L-phenylalanine methyl esters. Substrate dependent contributions marked D and intrinsic protein contributions marked N (pH-7.5)

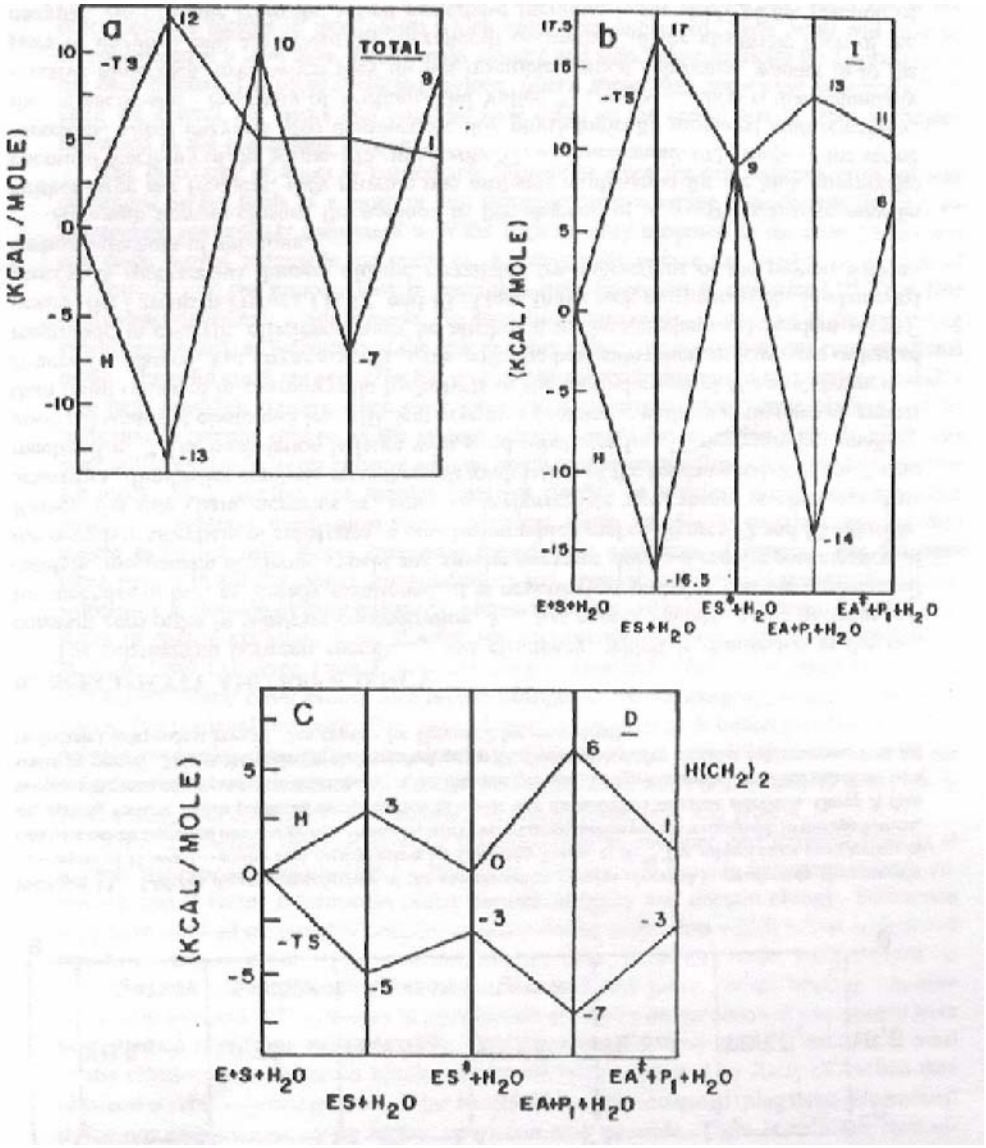
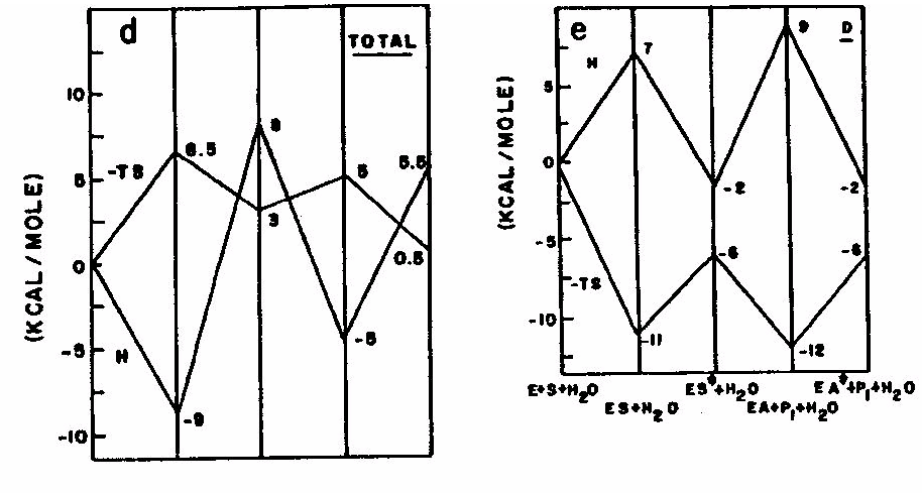


Fig. 5-4 Corresponding H and S profiles 1 (a and b for butyryl substrate, c and e for phenylalanyl substrate.



With the G figure each step of the reaction can be described in its relationship to the thermodynamic changes. Formation of the major enzyme-substrate complex ES requires the initial casua.l contact almost at constant free energy followed by a major change to a pretransition state formed with large H decrease as the matrix contraction redistributes the potential energy initially stored in the expanded matrices to the chemically reacting assembly of substrate and functional groups of the protein, the so-called reaction center usually labeled active site. The latter is correct in one sense but ignores the matrix contraction actually driving catalysis. The entropy due to matrix contraction is also large but its TS change does not overwhelm the loss of potential energy transfer so the net free energy change is not only positive but large regardless of the substrate in the series. In these examples the further change from pretransition state to and through the classical transition state needs only a further small increase in free energy easily provided by conventional thermal excitation. The lifetime of the pretransition state is at least 1ns and probably more since it is determined not by matrix relaxation but rather by the time to pass through the transition state to the first product As shown in Fig. 5-1, in chymotrypsin that is not the free acid but rather the formation of the covalent compound formed between the hydroxyl group of ser 195 and the acid moiety of the substrate. The free acid is then released in the hydrolysis of that acyl enzyme in the second cycle of this engine. Many enzymic mechanisms appear to consist of several well-ordered contraction-expansion cycles utilizing the fundamental nutcracker device for potential-energy redistribution.

Evolution has found solutions to several problems to provide the reaction rates and specificities making life possible. Not the least of these is matrix construction to maximize the potential energy transferred at minimum entropy loss. Whether or not these changes are the best possible in an infinite evolution in our biosphere is not an easy question but given the successful discovery of DNA and the mechanical palindrome of the enzyme B factors, it might not be

surprising. The great enhancement in catalytic rate for the enzyme versus catalysis by homogeneous acid or base catalysis is due to the net free energy, the potential energy transferred less the correction for the temporary entropy decrease. Except for the first encounter the ratio of enthalpy to entropy change in each of the steps is about 463K consistent with the matrix expansion-contraction. This description not only gives confidence that the nutcracker model is correct but is also describes quantitatively how it works. The interaction between substrate and protein labeled D in this discussion if favorable to the rate and the larger the free energy lost in the association, the more favorable. Comparing the two substrates it can be seen that a large part of the specificity difference between them is a consequence of the relative difference in the decrease in that free energy. This supports the popular opinion that the better the binding, the better the catalysis and explains why. The term can be generalized insofar as all enzymes utilize the nutcracker mechanism.

Chymotryptic catalysis of ester substrates-

The observation that all enzymes examined for construction using B factors demonstrate the same set of structural features suggests that enzymes operate with the same mechanism. This would be a natural consequence of the discovery in evolution of one mechanism either very much more useful in rate and specificity or quite likely the only mechanism with the properties required for the successful evolution we find. Thus any one enzyme can be illustrate all and the obvioius choices are the serine proteases as more extensively studied than others. The classical formalism for these is the so-called Hartley-Kilby steady-state mechanism shown in Fig. 5-1 assumes the rectangular-hyperbolic formalism of Michaelis-Menten and Briggs-Haldane but it also applies to the nutcracker mechanism. Though formally identical the underlying physics and chemistry is very different one activated by thermal fluctuations, the other by force generated by matrix contraction. Both depend on reduction in free energy which Wyman described for the M-M mechanism as the "turning wheel" required because of the deviation, usually small, due to the irreversibility of the steady state. The nutcracker may depend on larger irreversibility. In fact, it is not obvious that the transition step of the latter does not exceed the requirements of the principle of reversibility required by the laws of thermodynamics for equilibria and also approximated in the steady state. However reversibility in the equivalence of the forward and backward rates has been established for many enzymic reactions implying that detailed balance is very closely approximated in the newer mechanism. Living processes require a compromise between thermodynamic efficiency and suitably rapid kinetics and it is likely that the best balance has been found already in evolution. That would mean that the balance

of entropy loss to potential energy transfer is optimal giving one possible explanation for the nearly ubiquitous compensation temperature of 353K for the protein melting rates.

The operating features of the nutcracker were first revealed by Yapel in his studies of the interactions between members of the N-acetyl-tryptophan family of substrates and inhibitors and chymotrypsin (elaborated initially by Lumry and Biltonen, Chapter 2 of Structure and stability, ed. Timasheff and Ashman, Dekker, 1969). Indole alone is sufficient to trigger domain closure and thus matrix contraction and potential energy redistribution. It is bound tightly and released slowly as is N-acetyl-L-tryptophan but N-acetyl-D-tryptophan although bound with nearly the same free-energy change is not a trigger. It is released with a rate constant five orders of magnitude larger. The large binding constant for the L form establishes that the stereochemistry is preserved by matrix contraction. Of the family N-acetyl-L-tryptophan ethyl ester is a substrate hydrolyzed with a high rate by this protein effected by contraction but producing little overcontraction up to the transition state for that process. The rate of the latter process is larger than that for insole or the L free acid as inhibitor and in this case does not reveal any major restriction. Poor substrates somewhat resembling N-acetyl-D-tryptophan ethyl ester are not bound, not hydrolyzed and do not trigger matrix contraction. As is shown in figs. 5-2 to 5-4 the aminobutyricacid methyl ester substrate is less rapidly hydrolyzed because the supplement from the sidechain is smaller. Overcontraction has been observed in other enzymes, notably pepsin which has a very similar nutcracker mechanism. It is a simple consequence of the contraction step and thus likely to be found with many enzymes. AThe variations in behavior are produced by very fine tailoring of the binding site so fine that it may not be easily detected with B factors and generally not detectable with coordinate values from diffraction studies.

The nutcracker is a very versatile device apparently the basis of all enzymic catalysis and as a device to interchange chemical and conformational free energy it may have needed only elaborations for specific functions. Its versatility depends on the delicate construction of the binding site as demonstrated by the binding of the members of the N-acetyl-tryptophan family described above. At the first level of selectivity the L-D stereo selectivity follows the Bergmann three-point rule by placing the major side-chain site in one domain or the other of the catalytic pair. Thus in pepsin and members of the trypsin-chymotrypsin family it is found in the C-terminal functional domain. The trigger sensitivity required for the high substrate specificity is not determined by direct interaction of substrate with the contact atoms at the binding site but rather as the matrix conformation responds to that local association. The same factors determine binding of the

substrates for carboxypeptidase A for example but the nutcracker function is different. In that metalloenzyme the ligands for the zinc ion are divided between the two functional domains. Domain closure forces readjustment of the lengths and angles of that complex ion apparently distorting the water ligand into the high potential energy of the pretransition state for attack on ester or amide substrate. Similar large ligand-field effects produced major changes in magnetic susceptibility of cytochrome C on varying hydration. To use coenzymes the coenzyme NADH for example is incorporated on one side of the inter-domain crevice so as to interact on matrix contraction with some group on the other side, often an acid or base group of the enzyme but there are no apparent limitations. In malate dehydrogenase for example the NADH bound in this way so that the redox end extends into the crevice where domain-closure activates the electron redistributions. These mechanisms are illustrated in Chapter. @ of volume 1.

ATP and ADP appear to yield complications in catalysis by as the N-acetyl-L-tryptophan methyl ester and its acid hydrolysis product suggesting the basis for the difference in number of phosphate groups. Then ATP depends on the nutcracker to active the third phosphate group during matrix contraction as a general device for converting conformational potential energy into chemical free energy. Somewhat similarly the ATP formation process from ADP discovered by Boyer and @ is probably driven by the same device for potential-energy redistribution found in the enzymes discussed in the preceding paragraphs forcing rotation in the multisubunit motor. Thus far, matrix contraction appears to be the only process necessary to interconvert conformational free energy and the chemical free energy of small molecules. Muscle mechanisms may depend on no more than a further elaboration of the enzyme conformational dynamics. The rotation of bacterial flagella is reversible depending only on the pH gradient; it may be a model for a way nature has found to import the principle of reversibility into biology.although it is not obvious why that might be necessary.