CROATICA CHEMICA ACTA CCACAA **65** (3) 779–788 (1992)

ISSN 0011-1643
UDC 547.96
CCA—2096

Original Scientific Paper

Reactive Intermediates and the Question of Concertedness in the Catalytic Power of Enzymes*

Richard L. Schowen and K. Barbara Schowen

Departments of Chemistry and Biochemistry, University of Kansas, Lawrence, Kansas 66045-0046, USA

Received March 23, 1992

Conditions derived from physical organic chemistry, which govern the circumstances under which the evolution of enzyme catalysis can lead to the conversion of an on-enzyme stepwise reaction into an on-enzyme concerted reaction, are presented and discussed.

INTRODUCTION

Professor Dionis Sunko has been a worldwide leader in defining the structures and properties of intermediate compounds and transition states in organic reactions in solution.[1] Biochemists and others who study the mechanisms of reactions catalyzed by enzymes find themselves especially grateful for the high level of precision in experiment and interpretation that Professor Sunko and his school have brought to their work, because their work frequently serves to define the chemical baseline and the structural, thermodynamic and kinetic context within which the evolution of enzyme catalytic power has taken place. In this paper, we propose to examine the question of the stability of reactive intermediates and their associated transition states, as affected by the structures of enzymes, and its relationship to whether enzymes are likely to evolve to convert stepwise reactions, involving reactive intermediates, into concerted reactions without intermediates.

CATALYTIC POWER AND MOLECULAR EVOLUTION[2]

Enzyme evolution is presumed to have taken place in the Darwinian manner, with the initial event being a random genetic mutation in the DNA code for the enzymic protein in the genome of the host organism. As a result of the mutation, the host organism begins the production of a new protein molecule, differing in at least one

* This paper is dedicated to Professor. Dionis E. Sunko on the occasion of his seventieth birthday.

amino-acid residue (and possibly in many residues) from the protein produced from the pre-mutation, or ancestral, DNA. If the new, mutant protein has properties that confer a selective advantage on the mutant organism, the organism will prosper reproductively and the population of its progeny will grow and eventually replace the population of the ancestral organism. The organism and its enzyme will be said to have evolved.

Many different kinds of properties of the mutant protein may serve to determine the qualitative and quantitative selective advantage or disadvantage it confers on its host organism. Among these are its catalytic power, for the greater the catalytic acceleration the fewer molecules of protein the organism must produce to obtain a given flux of material through the enzyme-catalyzed pathway.

The synthesis of proteins, including enzymes, is, in fact, a very expensive part of the organismic economy. Atkinson[3] has estimated the cost of synthesis of a millimole of protein of molecular weight 100,000 daltons to be around 36 equivalents of ATP (free energy of hydrolysis *ca.* 263 kcal), even ignoring »overhead and indirect costs such as the cost of synthesis of the messenger RNA and the ribosomes that are required for protein synthesis.« Since protein synthesis is so energy-intensive, the saving in energy from the mutation of an enzyme to a more catalytically powerful structure seems sure to be selectively advantageous to the organism.

However, there are clearly other factors involved which in some cases may be dominant. In addition to possessing high catalytic power, it may be necessary or desirable for the enzyme to be regulated in its activity, and enzymes with appropriate regulatory properties should confer selective advantage. Other features may include a structure with properties appropriate for transport (for example, secretion through membranes), for post- translational modification, and for interaction with other structures such as membranes, polysaccharides or other proteins, *etc.*

Nevertheless, studies of the molecular evolution of enzymes have often concentrated on catalytic power as the pivot of natural selection, since this certainly has been of major importance in the evolution of many enzymes.[4] In this paper, we also will limit our attention to enzymic evolution toward higher catalytic power.

Kinetic Significance, Reactive and Stable Intermediates

The concept of kinetic significance,[5,6] which makes the question of what determines the rate of reaction a quantitative and definite matter, is accordingly useful in the discussion of the catalytic power of enzymes. Our discussion will be couched in terms of the simplest possible model, for the irreversible steady-state reaction of a one-substrate enzyme. Let us consider such an enzyme E that catalyzes a stepwise reaction of substrate A in which A is converted to the intermediate M and then to product P (Chart 1).

Chart 1 shows the two enzyme-kinetic parameters k_{cat} and k_{cat}/K_m and their definitions in terms of the so-called microscopic parameters, the rate constants. As the table shows, each reciprocal observed rate constant is a simple sum of effective rate constants for individual state-to-state (reaction state to transition state) conversions. Each of these terms is thus a property of two states: the reactant state and the transition state. The magnitude of the term is fixed by the difference in Gibbs free energies of the transition state and reactant state, and is independent of the properties of all other states, including the intervening ones.

CHART 1

Summary of Enzyme Kinetics for a Simple Stepwise Mechanism

Enzyme-catalyzed reaction mechanism with microscopic parameters:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EM \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EP \xrightarrow{k_7} E + P$$

The kinetic law for the reaction (v/E_o, the velocity per unit concentration of enzyme, the turnover rate; A the substrate concentration):

$$\frac{E_o}{v} = \frac{K_m}{k_{cat}} \frac{1}{[A]} + \frac{1}{k_{cat}}$$

The two observable kinetic parameters and their contributions from the microscopic parameters:

$$\frac{K_m}{k_{cat}} = \frac{1}{k_1} + \frac{k_2}{k_1 k_3} + \frac{k_2 k_4}{k_1 k_3 k_5} + \frac{k_2 k_4 k_6}{k_1 k_3 k_5 k_7}$$

$$\frac{1}{k_{cat}} = \frac{1}{k_3} + \frac{k_4}{k_3 k_5} + \frac{k_4 k_6}{k_3 k_5 k_7} + \frac{1}{k_5} + \frac{k_6}{k_5 k_7} + \frac{1}{k_7}$$

When the reactant concentration [A] is low, the relevant kinetic parameter is k_{cat}/K_m. Note from Chart 1 that the only reactant state for which the Gibbs free energy contributes to the magnitude of k_{cat}/K_m is the state E + A. Thus none of the intermediates EA, EM or EP has any kinetic significance – none of their properties can have any effect on the kinetics.

The relevance of this feature to the molecular evolution of enzymes is that, for physiological regimes in which this kinetic parameter dominates, it is quite irrelevant

TABLE I

Kinetic terms corresponding to state-to-state conversions

Reactant states	Transition states			
	step 1,2	step 3,4	step 5,6	step 7
E + A	$(k_1)^{-1}$	$(k_1k_3/k_2)^{-1}$	$(k_1k_3k_5/k_2k_4)^{-1}$	$(k_1k_3k_5k_7/k_2k_4k_6)^{-1}$
EA	–	$(k_3)^{-1}$	$(k_3k_5/k_4)^{-1}$	$(k_3k_5k_7/k_4k_6)^{-1}$
EM	–	–	$(k_5)^{-1}$	$(k_5k_7/k_6)^{-1}$
EP	–	–	–	$(k_7)^{-1}$

what changes in the free energy of EA, EM, EP or any other state occur. Only the stabilization of the transition states can increase the rate (*catalytic fundamentalism*).[6,7]

Usually physiological concentrations of enzyme substrates are high enough for the parameter k_{cat} to influence the rate also.[2-4] There the »enzyme-bound« states including the enzyme-bound intermediate state EM may be accessible to molecular evolution. Thus it is in respect to k_{cat} that we must seek any evolutionary significance of the effects of mutations in enzyme structure on the Gibbs free energy of the state EM.

Overall Rate and the Kinetic Significance of EM

Chart 2 outlines the main points in the kinetic significance of EM, which we take to be the fraction of total enzyme in the steady state which is present as EM. If this fraction is zero, then the properties of EM can have no effect on the rate. If this fraction is unity, then EM is the only reactant state for which the properties will affect the rate. Between these limits, the importance of the free energy of EM for the rate varies accordingly, and thus the attribution of the term, *kinetic significance.*

Chart 2 first confirms from the general expression that the kinetic significance of EM indeed becomes negligible at negligible A concentration. It then considers the rate as a function of the properties of EM if all other free energies are held constant. It emerges that the rate will be maximized if the off-rate or rate of exit from the state EM is maximized. As is also shown, the kinetic significance of EM then becomes negligible: it becomes analogous to a reactive intermediate in an organic reaction. This is a further manifestation of catalytic fundamentalism: the stabilization of intermediate states is anti-catalytic, and their destabilization to the point of kinetic insignificance is catalytically optimum.

The next question brings us to the issue of concertedness. Once the kinetic significance of EM is negligible, is there a catalytic advantage in completely removing EM as a stable state – rendering the conversion of EA to EP a concerted reaction?

The Catalytic Advantage of Concertedness

The points at issue are summarized in Chart 3. There it is shown that, when the formation and decomposition of a reactive (*i.e.* unstable and therefore kinetically insignificant) intermediate are roughly equally rate-limiting for a reaction, the reaction will proceed at a rate only about one-half the rate that would be observed if the reaction were concerted, passing through a single transition state of free energy equal to the free energies of the transition states for formation and decomposition of the reactive intermediate. There are several ways of explaining why this is so. Perhaps the simplest is that, when the intermediate provides a »stopping point«, no matter how unstable, between the two transition states, half the molecules which have passed the first transition state will return back over it to the reactant state.

Thus an enzyme can in principle, under these circumstances, realize a catalytic advantage of a factor of two in rate by rendering a stepwise reaction concerted. Note that this will also apply to the reaction at low [A] with rate constant k_{cat}/K_m if the formation and decomposition of a reactive intermediate are equally rate-limiting here also (see Chart 1 for the relevant equation).

Is there a reasonable circumstance in which the relatively modest factor of two in enzymic acceleration could be regarded as an effective response to evolutionary pressure? If the transition states flanking the reactive intermediate were fully rate-limiting

CHART 2

Kinetic Significance of Intermediate EM

Let the kinetic significance of EM be the fraction $[EM]/E_o$:

$$\frac{[EM]}{E_o} = \frac{\dfrac{k_1[A]k_3}{(k_2 + k_3)}}{\dfrac{k_1[A]k_3}{k_2 + k_3}\left(\dfrac{k_5}{k_3}\dfrac{(k_3 + k_7)}{(k_6 + k_7)} + \dfrac{k_4}{k_3} + 1\right) + \dfrac{k_5k_7}{k_6 + k_7} + \dfrac{k_2k_4}{k_3 + k_4}}$$

Note that when [A] becomes very small, the kinetic significance of EM becomes very small.

When [A] becomes very large, the kinetic significance of EM becomes:

$$\frac{[EM]}{E_o} = \frac{1}{\left(\dfrac{k_5}{k_3}\dfrac{(k_3 + k_7)}{(k_6 + k_7)} + \dfrac{k_4}{k_3} + 1\right)}$$

This can be thought of as a ratio involving k_{on}, the rate constant for entry into the state EM, and k_{off}, the rate constant for exit from EM:

$$\frac{[EM]}{E_o} = \frac{k_{on}}{k_{on} + k_{off}}$$

where

$$\frac{1}{k_{on}} = \frac{1}{k_3} + \frac{1}{k_3k_5/k_4} + \frac{1}{k_3k_5k_7/k_4k_6} + \frac{1}{k_7}$$

and, as Chart 1 shows, is just the exit rate from all other forms of the enzyme present at very high [A], and where

$$\frac{1}{k_{off}} = \frac{1}{k_5} + \frac{1}{k_5k_7/k_6}$$

and, as Chart 1 shows, is indeed the exit rate from EM.

The overall specific rate v/E_o is then given by

$$\frac{v}{E_o} = k_{off}\frac{[EM]}{E_o} = \frac{k_{on}k_{off}}{k_{on} + k_{off}}$$

If it is noted that only k_{off} will be affected by a change in the Gibbs free energy of EM, then evolutionary changes in rate as a result of alteration in the EM free energy will maximize the rate when $k_{off} \gg k_{on}$, *i.e.*, when the kinetic significance of EM becomes negligible.

CHART 3

The Kinetic Advantage of Concertedness

Consider a circumstance (Chart 2) where entrance to EM completely determines the rate, with rate constant given by

$$\frac{1}{k_{on}} = \frac{1}{k_3} + \frac{1}{k_3k_5/k_4} + \frac{1}{k_3k_5k_7/k_4k_6} + \frac{1}{k_7}$$

Note that the only terms related to EM are those for passage through the transition state preceding EM and for passage through the transition state succeeding EM:

$$\frac{1}{k_3} + \frac{1}{k_3k_5/k_4} = \frac{1}{k_3}\left(\frac{k_4+k_5}{k_5}\right)$$

Recall that EM is already of relatively high free energy so that the two rate constants leading out of EM, k_4 and k_5, are both quite large. If either is much larger than the other, then the kinetic term above collapses to the rate constant for a single state-to-state conversion. This is the equivalent of a concerted reaction, since one of the barriers separating EM from adjacent stable states will effectively have vanished. On the other hand, if the transition-state free energies are similar, the two rate constants will be similar and

$$\frac{1}{k_3}\left(\frac{k_4 + k_5}{k_5}\right) \approx \frac{2}{k_3}$$

Thus a kinetically insignificant, reactive intermediate flanked by barriers of roughly equal height, confers as much as a two- fold retardation of the rate. If the EA is the only kinetically significant species, the effect is two-fold; as EP becomes more kinetically significant, the effect diminishes to no effect at all.

Conclusion: a two-fold catalytic advantage may be accrued by conversion of a stepwise reaction through a kinetically insignificant intermediate into a concerted reaction.

under physiological circumstances, then the realization of this full factor of two would be possible. This would result in the saving to the organism of one-half the cost of protein production for the step catalyzed by the enzyme in question. In consideration of the energy-economical argument of Atkinson, cited above, this would appear to constitute a substantial advantage for survival under many conditions.

Thus it seems likely that there have been circumstances in which an evolutionary advantage could have been seized through a molecular-evolutionary conversion of a stepwise into a concerted reaction. What strategies are open to molecular evolution in effecting such a conversion?

Transition-Sate Stabilization and Concertedness

We take up the question of evolutionary increases in enzyme catalytic power in the circumstance where the rate is limited by two barriers of roughly equal height, those for the transition states leading into and out of the reactive intermediate EM, itself of no kinetic significance. The evolutionary drive toward greater catalytic power will tend to reduce the Gibbs free energies of these two transition states. The following argument shows that, in general, they will tend to be reduced in free energy about equally. If the intermediate EM is very unstable, and thus very close in energy to the two transition states, the structures of the two transition states will tend to be about the same (*Bema Hapothle*).[8] Then the effects of an enzyme mutation on the free energy of interaction of the two transition states with the enzyme will tend to be the same. Furthermore, the structure of the intermediate EM should also approximate the structure of the transition state, and mutations should affect its free energy equally with the free energies of the preceding and succeeding transition states.

As a result, in many mutations, the free energies of the two transition states and the intermediate EM will move together. The stepwise character of the reaction will be preserved. The rate through the enzymic pathway will, at all such points in biological history, remain two-fold smaller than could have been the case if the reaction had become concerted. The organism would continue to suffer twice as large a burden of protein synthesis for this enzyme. Can molecular evolution evade this blockade?

Linear Free-Energy Relationships

We will argue that it is probable that the relative Gibbs free energies of the transition state P preceding the intermediate M, the intermediate M and the transition state S succeeding the intermediate M will obey linear free-energy relationships (we omit the designation E for the enzyme at this point because we wish to consider the effects of mutation in E – changes from E to E′ to E″, *etc.* – on the free energies of the enzyme-bound states). This requires a defense because we have recently argued that, in the general case, linear free-energy relationships for »on-enzyme« reactions need not and probably do not govern their rates and equilibria.[9] This is contrary to one of the mathematical assumptions underlying the well-known Albery-Knowles model of enzyme »evolution to perfection«.[4] Our study of a stochastic model for molecular evolution of catalytic power[9] showed this assumption to be unnecessary to account for the observed results, and thus the original Albery-Knowles concept to be broader in its validity than may have been believed.

Contrary to the general case, we consider the linear free-energy relationships presented in Chart 4 to be likely in the present case. When the structural resemblance between a highly reactive (*i.e.*, kinetically insignificant) intermediate and the two flanking transition states are as close as is argued here, then even if the mutations and thus the changes in enzyme-ligand interaction energies are entirely random in character, the free energies of the three consecutive, nearly isergonic states in the reaction sequence should trace each other at least at the level of similar free-energy relationships.

We argue in Chart 4 that a change from stepwise to concerted on-enzyme reaction can occur, within certain limitations of the nature of stabilizing enzyme-ligand interactions, by means of the development of a reaction subject to *enforced concertedness*, *i.e.*, a reaction in which the structure corresponding to the intermediate in a stepwise

CHART 4

Limitations Imposed by the Bema Hapothle

Let the Gibbs free energies of the preceding transition state P, the reactive intermediate M and the succeeding transition state S be governed by linear free-energy relationships:

$$G(\mathrm{P}) = p\Gamma + \pi$$
$$G(\mathrm{M}) = m\Gamma + \mu$$
$$G(\mathrm{S}) = s\Gamma + \sigma$$

where Γ is a parameter describing the effect of a mutation in the enzyme structure. Forward direction in time (evolution) will be defined to correspond to an overall tendency of $\delta\Gamma$ to be negative so that a positive coefficient of the $\delta\Gamma$ will correspond to an overall tendency toward evolutionary stabilization of the state.

The mutation-induced or evolutionary change in the free energy of the intermediate relative to each of the transition states will be given by:

$$\delta G(\mathrm{M}) - \delta G(\mathrm{P}) = [\mathrm{m} - p]\,\delta\Gamma + [\mu - \pi]$$
$$\delta G(\mathrm{M}) - \delta G(\mathrm{S}) = [\mathrm{m} - s]\,\delta\Gamma + [\mu - \sigma]$$

The evolutionary selection for faster rate will require that transition states be stabilized: thus $p > 0$ and $s > 0$.

If evolution to concertedness is to occur by development of enforced concertedness, the disappearance of a barrier before or after, or both before and after the reactive intermediate, so that the reactive intermediate is transformed into a transition state, then it is required that:

$$m < p \quad \text{and/or} \quad m < s.$$

This will ensure an overall greater stabilization of either P or S than M, so that one or the other barrier or both will disappear.

Thus the nature of mutations leading to concertedness must be such that transition-state stabilization exceeds intermediate stabilization, even in this context of strong structural resemblance between the intermediate and flanking transition states.

mechanism comes to suffer no barrier to decomposition in one or the other or both directions (toward reactants or toward products).[10]

As is argued in Chart 4, this requires that the linear free-energy slope m for intermediate stabilization be smaller than for the preceding and/or succeeding transition state in order for enforced concertedness to be reached in the course of an overall evolutionary stabilization of high-energy points on the energy surface. This in turn can offer suggestions about what kinds of transition-state stabilization will and will not tend to convert stepwise to concerted reactions.

Enzyme-Ligand Interactions Favoring Concertedness

Among the stabilizing interactions that clearly favor concertedness are specific transition-state stabilization features that lead to no stabilization of stable states. Preeminent here is general acid-base catalysis. One of the strangest, and still ill-understood, features of the powerful hydrogen bridging in general acid-base catalysis is that it is highly specific for transition states and occurs only rarely in stable states.[11] Such interactions will tend to lower transition-state free energies while leaving the intermediate »high and dry« and thus a transition state itself.

Interactions that stabilize positive charge on carbocation intermediates (for example by the electrostatic effects of nearby glutamate or aspartate residues) are unsuitable and will lead away from, not toward concertedness. This is because the cation will tend to have a relatively high and concentrated charge relative to the transition states (where bonds are forming to the cationic center and dispersing the charge). Thus its linear free-energy slope (*m* in Chart 4) will be larger rather than smaller than the slopes *p* and *s* for the two transition states.

By this kind of reasoning, familiar to physical organic chemists and a part of the heritage created by Professor Dionis E. Sunko and the generations of mechanisms chemists he led, trained and inspired, it should be possible to construct a set of guideposts to examine enzymic reactions with a view to determining the degree to which evolutionary enforced concertedness has played a role in the biological history of catalysis.

Acknowledgment: – This work was supported by the National Institutes of Health (USA) and the University of Kansas General Research Fund.

REFERENCES

1. A few recent examples: H. Vančik, K. Percač and D. E. Sunko, *J. Chem. Soc., Chem. Commun.* (1991) 807; P. Buzek, P. v. R. Schleyer, S. Sieber, W. Koch, J. W. de M. Carneiro, H. Vančik, and D. E. Sunko, *J. Chem. Soc., Chem. Commun.* (1991) 671; H. Vančik, K. Percač, and D. E. Sunko, *J. Amer. Chem. Soc.* **112** (1990) 7418; W. Koch, B. Liu, D. J. DeFrees, D. E. Sunko, and H. Vančik, *Angew. Chem.* **102** (1990) 198; B. Juršić, M. Ladika, and D. E. Sunko, *Gazz. Chim. Ital.* **118** (1988) 613; H. Vančik and D. E. Sunko, *J. Amer. Chem. Soc.* **111** (1989) 3742.
2. S. A. Benner, *Chem. Rev.* **89** (1988) 789.
3. D. E. Atkinson, *Cellular Energy Metabolism and Its Regulation,* Academic Press, New York, 1977, pp. 75-79.
4. J. J. Burbaum, R. T. Raines, W. J. Albery, and J. R. Knowles, *Biochemistry* **28** (1989) 9293 and preceding papers from this group.
5. W. J. Ray, *Biochemistry* **22** (1983) 4625; R. L. Stein, *J. Org. Chem.* **46** (1981) 3328; R. M. Noyes, *Prog. Reac. Kin.* **2** (1964) 339.
6. R. L. Schowen in R. D. Gandour and R. L. Schowen, eds., *Transition States of Biochemical Processes*, Plenum Press, New York, 1978, p. 77.
7. J. Kraut, *Science* **242** (1988) 533.
8. W. P. Jencks, *Chem. Rev.* **85** (1985) 511; the specific concept is the Hammond postulate (G. S. Hammond, *J. Amer. Chem. Soc.* **77** (1955) 334).
9. D. W. Huhta, T. Heckenthaler, F. J. Alvarez, J. Ermer, G. Hübner, A. Schellenberger, and R. L. Schowen, *Acta Chem. Scand.*, in press.
10. For full references and many examples, see: A, Williams, *Adv. Phys. Org. Chem.* **27** (1992) 1; I. Lee, *Adv. Phys. Org. Chem.* **27** (1992) 57; C. F. Bernasconi, *Adv. Phys. Org. Chem.* **27** (1992) 119.
11. C. G. Swain, D. A. Kuhn, and R. L. Schowen, *J. Amer. Chem. Soc.* **87** (1965) 1553.

SAŽETAK

Reaktivni intermedijari i problem usklađenosti katalitičkog djelovanja enzima

Richard L. Schowen i K. Barbara Schowen

Polazeći od osnovnih načela fizikalne organske kemije diskutira se o uvjetima pod kojima bi se neka enzimski katalizirana višestepena reakcija mogla preusmjeriti tako da teče usklađenim mehanizmom.