The Origins of Enzyme Catalysis and Reactivity: Further Assessments

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Author’s contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

Alternatives to conventional mechanisms of enzyme catalyzed reactions, although within the ambit of transition state theory, are explored herein. This is driven by reports of a growing number of enzymes forming covalently linked enzyme-substrate intermediates, which clearly deviate from the conventional Michaelis-complex mechanism. It is argued that the formation of the covalent intermediates can be accommodated within the framework of transition state theory and the original Pauling hypothesis. This also obviates the need to invoke intramolecular reactivity to explain enzymic accelerations. Thus, the covalent binding of a substrate distorted towards the transition state, with the binding being fully manifested in the ensuing transition state, would conform to the traditional endergonic pre-equilibrium mechanism. Intriguingly, an alternative exergonic formation of the covalent intermediate would also lead to catalysis: in this case, any of the three steps–covalent binding, turnover or product release–can be rate limiting. Although the exergonic mode has been dismissed previously as leading to a “thermodynamic pit” (Michaelis complex case), this view now needs to be reassessed as it seems inaccurate. Therefore, it remains for the enzyme to stabilize the various transition states via the multifarious mechanisms available to it. The Pauling hypothesis remains vindicated.

Keywords: Covalent linking; enzyme-substrate intermediate; intramolecularity; pauling hypothesis; pre-equilibrium; transition state stabilization.

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1. INTRODUCTION

1.1 General Background

Enzymes are a key piece in the jigsaw puzzle of life and the reductionist exercise that is central to modern science [1-6]. In fact, enzymes are not just catalysts par excellence but also serve as a pivot in the expression of the genetic code, via the multifarious metabolic pathways that regulate and sustain life (enshrined in the one-gene-one-enzyme hypothesis). The importance of understanding the way enzymes perform their appointed duties in defining the tapestry of life, therefore, cannot be overstated!

Indeed, the molecular basis of enzyme catalysis has been a topic of endless fascination over several decades, ever since the isolation and characterization of enzymes were made possible by advances in both chemistry and biology. Not just their sheer catalytic power but also the exquisite selectivity exhibited by enzymes, have intrigued and inspired a generation or two of intrepid explorers dedicated to their study. This has led to the development of experimental and theoretical strategies of great ingenuity, which indeed define a scientific frontier of highest esteem and importance.

Thus, much is now known about enzymes per se, as also their mode of action. Indeed, enzymes can be treated just as other organic catalysts, except that the proteinic enzymes possess far more complicated molecular structures! These are being unraveled by X-ray crystallography in increasing numbers, and serve as the basis for a rigorous understanding of their reaction mechanisms. The kinetics of enzyme catalysis has also played a key role in all aspects of enzymology, with recent conceptual advances paving the way for a deeper understanding of the intricacies of enzyme catalysis [2].

1.2 Current Approaches to Enzyme Reactivity

1.2.1 Conventional enzymology

Current views of enzyme catalysis are based in the relatively rapid formation of a weakly bound enzyme-substrate intermediate (Fig. 1). This results in an endergonic pre-equilibrium, with the enzyme-substrate intermediate turning over to product in an ensuing rate-determining step. This leads in the general case to second order kinetics that is easily accommodated within the framework of transition state theory.

![Gibbs energy (G) profiles for enzyme catalysis. Solid line represents the endergonic formation of the enzyme-substrate intermediate (ES) from enzyme (E) and substrate (S), the dashed lines the exergonic formation of ES ("thermodynamic pit"), P being product; the corresponding reaction schemes are shown as boxed items (solid and dashed lines respectively).](image)
The exergonic formation of the enzyme-substrate intermediate, however, has been firmly rejected previously, as it apparently leads to a “thermodynamic pit” and consequent rate inhibition (Fig. 1). Furthermore, the origins of the phenomenal accelerations exhibited by enzymes have been viewed with awe and fascination that have spawned a variety of hypotheses of varying theoretical rigor. Prime among these was the idea that intramolecular reactivity was an appropriate model for enzymic reactivity [7], although this has now been challenged [8].

Thus, the origins of the catalytic powers of the enzymes most likely reside in the molecular complexity of the enzyme macromolecule itself, as has been argued [2]. In particular, both kinetic and conceptual models for enzyme catalysis need to accord with transition state theory to be credible. The origins of transition state theory and its evolution to the status of the reigning paradigm of chemical reactivity have been discussed [9,10], noting particularly that the theory is essentially based in the Boltzmann equation, the common foundation of both rate and equilibrium.

The classical theory of enzyme action was based in the Michaelis-Menten equation and the Pauling hypothesis of transition state stabilization. Although the Michaelis-Menten equation did not contravene transition state theory per se, the equation led to a thermodynamic conundrum at high substrate levels (cf. the “one-way enzymes” idea). Hence, the equation stands discredited although the Pauling hypothesis remains as leitmotif [2,11].

1.2.2 Covalent catalysis

The original Pauling theory of enzyme action was based on the stabilization of the rate-determining transition state [2,11]. All the same, recent years have witnessed attempts to circumvent the Pauling hypothesis [6,12], apparently on the grounds that it fails to account for cases of rate accelerations > 10^{11} M^{-1}. Although these claims were purportedly supported by the observation that a large number of these enzymes form covalent enzyme-substrate intermediates, it is unclear how this militates against the Pauling hypothesis. Indeed, that covalent enzyme-substrate intermediates are formed is an enlightening revelation, although it falls short of providing a comprehensive mechanistic rationale for abandoning the Pauling hypothesis.

In fact, the covalent catalysis proposal apparently draws a fine line between circumventing the Pauling hypothesis and abandoning transition state theory in toto! The crux of the covalent catalysis proposal is the idea that there is a change of mechanism, with the enzymatic reaction being routed through lower barriers. However, how are these lower barriers attained by the enzyme (if not by transition state stabilization)? If it is only by a change in mechanism, then smaller non-enzymatic catalysts should also be equally effective!

Whilst scholarly analysis indeed establishes that covalent catalysis is the norm for the most efficient enzymes, this also leads to intriguing questions as to the origins of the accelerations. In fact, much of the quantitative conclusions that assume $k_{\text{cat}}$ and $K_M$ values [6] are now invalidated because of the collapse of the Michaelis-Menten equation [2,11]. This particularly concerns the correlations of rate enhancements and association constants, which now appear dubious. In fact, the role of covalent intermediates was an early concern [12] that continues apace to the present [13-20], hence demanding a thorough conceptual analysis.

Furthermore, it is noteworthy that the formation of covalent enzyme-substrate intermediates does not necessarily justify the idea that intramolecular reactivity is the basis of enzyme catalysis. As has been argued at length [8], intramolecular reactivity is based in a raised ground state Gibbs energy content, arising from both enthalpy and entropy effects. Indeed, a unimolecular reaction (intramolecular case) cannot serve as a model for a bimolecular reaction (enzyme case), as the ground states are vastly different in the two cases. Also, although multifunctional catalysis is indeed an important contributor to enzyme catalysis, and can be replicated in intramolecular models, the overall enzyme-substrate reaction remains bimolecular, hence subject to the Pauling hypothesis.

It is the purpose of this paper to examine the formation of covalent enzyme-substrate intermediates and its mechanistic consequences. The formation of these intermediates prior to the rate-determining step is indeed intriguing, as it apparently begs the question of how their formation is itself catalyzed! In fact, deeper mechanistic analysis of this phenomenon leads to a fundamental reappraisal of the basis of enzyme catalysis, clearly with far-reaching consequences in the broad area of chemical biology.
2. DISCUSSION

2.1 General Mechanistic Background to Enzyme Catalysis

The Pauling hypothesis of transition state stabilization is generally considered to imply weak binding of the substrate at the enzyme active site, followed by strong binding of the rate determining transition state [1,2]. Thus, the substrate is "lured" to the hydrophobic active site pocket, which is not only complementary to the transition state in shape but also brings to bear a variety of weak interactions that cumulatively stabilize the transition state. Weak non-covalent interactions acting in concert at the ephemeral transition state are thus the key to enzyme accelerations, as these interactions are "switched off" once the product ground state is reached in the reaction energy profile. This ensures the rapid release of the free enzyme that can participate in further catalytic cycles.

Thus, the subtle balance between weak ground state binding (whether of substrate or product) and strong transition state binding, ensures not only a desired level of rate enhancement but also the rapid release of free enzyme. The formation of a strongly bound enzyme-substrate intermediate, however, can in principle lead to the opposite consequence, as indicated in the "thermodynamic pit" idea. Apparently, therefore, the Pauling hypothesis is predicated on an endergonic pre-equilibrium formation of the enzyme-substrate intermediate, with the exergonic counterpart representing the "thermodynamic pit" (cf. Fig. 1).

Current thinking is thus based on the idea that weak, non-covalent interactions—primarily van der Waals but also hydrogen bonding— are the key to enzyme catalysis, as these can be easily switched on (at the transition state) or off (at the ground state of substrate or product). The formation of covalent enzyme-substrate intermediates in a large number of cases, therefore, represents a major departure from conventional norms and patterns. However, as argued below, these cases represent extensions rather than departures from established conceptual frameworks and conventions, in fact, essentially of degree rather than of kind.

\[ V = \frac{v_{\text{cat}}}{K_{\text{M}}} [E][S] \]  
(1)

The kinetics of enzyme catalyzed reactions are best described by a straightforward second-order rate equation (Eq. 1: \( V \) is rate, \( k_{\text{cat}} \) turnover number, \( K_{\text{M}} \) the Michaelis constant, \( E \) and \( S \) concentrations of enzyme and substrate respectively) [2]. Note that the earlier Michaelis-Menten equation now stands discredited [2,11], although its kinetic symbols have been retained in Eq. 1. The familiar "saturation kinetics" feature, in fact, indicates the gradual inhibition of the enzyme by the weak binding of a second molecule of substrate at high substrate concentration, apparently preventing the release of product.

2.2 Covalent Enzyme-substrate Intermediates: A New Normal?

2.2.1 General considerations

The observation that a large number of enzymes have evolved to form covalent enzyme-substrate intermediates, and that these enzymes are among the most efficient known, implies that the covalent binding brings extraordinary features particularly conducive to enhanced reactivity. What, indeed, may these be?

In attempting to answer this onerous question within the ambit of transition state theory, two broad mechanistic possibilities may be envisaged. In short, these conform to the formation of the covalent intermediate in a prior step that is either endergonic or exergonic, which are discussed sequentially below. It should also be noted that, in forming the enzyme-substrate covalent bond, an existing covalent bond is replaced by another: examples would be esterification (O-H by O-CO-) or addition to an unsaturated moiety (a \( \pi \) bond is replaced by a stronger \( \sigma \) bond), etc.

2.2.2 Endergonic formation of the covalent intermediate

This case is closely similar to the conventional mechanism of enzyme catalysis, except that the initial formation of a weak (Michaelis) intermediate is immediately followed by the formation of the covalent intermediate \( [(ES)_{\text{COV}}] \), in an overall endergonic equilibrium step (Fig. 2, the reaction scheme is shown in the box).

However, there are two problems with this approach: firstly, explaining how the covalent bonding is itself catalyzed, and secondly, explaining how the formation of \( (ES)_{\text{COV}} \) remains endergonic.
Indeed, if this mode of reaction is to be viable, the formation of the covalent intermediate itself needs to be catalyzed by the enzyme via the mechanisms available to it. This thus assumes that the formation of the covalent intermediate is much faster (easier to catalyze) than the subsequent rate determining step. The latter, apparently, needs the assistance of the covalent bond in some manner (vide infra).

Also, the formation of the covalent intermediate can be endergonic if it involves the distorted substrate. For instance, esterification could normally be exergonic, but steric and other distortions in the active site cavity could render it endergonic.

Furthermore, the strain involved in these distortions could be relieved upon reaching the transition state structure: at this stage, the formation of the enzyme-substrate covalent bond becomes effectively exergonic, resulting in a corresponding stabilization of the transition state! (This can be understood by comparing the uncatalyzed transition state with the covalently bonded one, noting that the substrate is now essentially strain free; cf. Fig. 2, dashed profile, \(\Delta G_{\text{cat}}\) being the difference in Gibbs energy of activation.)

Thus, if the strain-free covalent intermediate is more stable than the unbound substrate by (say) 10 kcaals mol\(^{-1}\), the transition state would be stabilized by about the same amount. This would be in addition to the other weak interactions between enzyme and substrate, so would be substantial. The release of the product from the enzyme, again, must be catalyzed by the enzyme in a manner analogous to the formation of the covalent enzyme-substrate intermediate.

This mode of reaction clearly depends on a delicate balance of ground and transition state effects, and particularly on the relatively easy covalent binding of the distorted substrate at the enzyme active site (and final release of product). The heavy catalytic machinery is apparently reserved for stabilizing the rate determining transition state, towards which the covalent bonding contributes substantially, as seen above. Also, this mode of reaction is feasible if the substrate molecule is relatively flexible and can be distorted in a manner conducive to enhanced reactivity (as noted above).
2.2.3 Exergonic formation of the covalent intermediate

2.2.3.1 General considerations: three distinct cases

The relative strength of the covalent bond implies that the formation of the covalent enzyme-substrate intermediate could well be exergonic, although the implications for catalysis need careful examination. Indeed, previously the (analogous) exergonic formation of a Michaelis complex was dismissed as leading to a “thermodynamic pit” (cf. Fig. 1), hence inhibition rather than acceleration. However, it now appears these assumptions need to be reassessed.

The exergonic formation of a covalent intermediate implies that it is essentially strain free. For this mode to be viable in the overall catalytic scheme, the stabilization accruing from the formation of the covalent intermediate needs to be carried over to the ensuing transition states. This is critical for avoiding the thermodynamic pit problem. This exergonic mode appears more general as it does not depend on the flexibility of the substrate (relative to the above endergonic mode).

The advantage of covalently linking enzyme and substrate to catalytic activity is not generally clear. However, it is possible that the “anchoring” of the substrate and the consequent increase in the “residence time” at the active site, allows the enzyme to undergo conformational changes—perhaps induced by the covalent linking itself—that would facilitate the reaction.

In fact, three distinct cases may be considered, depending on whether the substrate binding step [formation of (ES)_{COV}, the turnover step [conversion of (ES)_{COV} to product P] or the product release step is rate determining, as discussed below. The overall reaction also needs to be strongly exergonic if a build-up of the enzyme-bound intermediates is to be avoided. Furthermore, the desired level of catalytic activity would be determined by the needs and demands of metabolic regulation and control, and the following analysis needs to be viewed in this light.

The kinetic forms of these three modes would be closely similar, being overall second order involving enzyme and substrate (cf. Eqs. 2-4 below, derived by standard procedures [2, 10]). This implies that the three modes cannot be distinguished easily by kinetic means alone.

2.2.3.2 Substrate binding is rate determining

This case is represented by the energy profile in Fig. 3. (For the reaction scheme, cf. Fig. 2, ES being omitted for convenience.) This implies that the enzyme has evolved to speed up all the other steps by stabilizing their transition states, with the first covalent binding step possibly lagging behind. (However, the first step too may have reached evolutionary perfection.)

In this mode, practically all the enzyme is converted to the covalent intermediate, essentially irreversibly. This implies that a maximal turnover rate is attained relative to the enzyme concentration employed (although this is irrelevant to the overall rate as the first step is rate limiting.) Also, the stability of the covalent bond formed needs to be fully carried over to the ensuing transition states, if a thermodynamic pit is to be avoided.

The rate equation for this mode is straightforward as shown in Eq. 2 (k_{ex} is the overall rate constant by the exergonic mechanism, other symbols cf. Eq. 1):

\[ V = k_{ex}(E)(S) \]  

(2)

2.2.3.3 The turnover step is rate determining

This case is represented by the energy profile in Fig. 4. (For the reaction scheme, cf. Fig. 2, ES being omitted for convenience.) Once again, as long as the stability of the enzyme-substrate covalent bond is fully carried over to the turnover transition state, a thermodynamic pit can be avoided. This case also indicates that the transition state of the uncatalyzed reaction is relatively high in energy, so its stabilization by the enzyme is particularly demanding.

Interestingly, enhanced stabilization of the covalent enzyme-substrate intermediate, which is also fully carried over to the turnover transition state, would now result in enhanced reactivity (as turnover is rate limiting). Evolutionary efforts would thus be directed toward these ends.
less than maximal relative to the enzyme concentration employed. This case, therefore, appears the least efficient in terms of catalytic power, possibly because stabilizing the turnover transition state is particularly demanding (as noted above).

Fig. 3. Gibbs energy (G) profile for the exergonic formation of a covalent enzyme-substrate intermediate [(ES)\textsubscript{COV}] that is also rate-determining; (ES) is not shown for convenience (letter symbols have the usual meaning, cf. Fig. 1)

Fig. 4. Gibbs energy (G) profile for the exergonic formation of a covalent enzyme-substrate intermediate [(ES)\textsubscript{COV}], the ensuing turnover step being rate determining; ES is not shown for convenience (letter symbols have the usual meaning, cf. Fig. 1)
The rate equation for this mode involves the pre-equilibrium constant \((K_{eq})\) and the turnover rate constant \((k_{TO})\), but still remains overall second order (Eq. 3, other symbols cf. Eq.1).

\[
V = k_{eq}(E)(S) = k_{TO}K_{eq}(E)(S)
\]

\(K_{eq} = [[ES]_{cov}]/(E)(S)\)

Interestingly, also, these cases would lead to a muted temperature coefficient of the overall rate constant, as the rate determining step is linked to an endergonic equilibrium between the intermediate and the starting reactants. This equilibrium shifts towards the reactants with increasing temperature, decreasing the concentration of the reactive intermediate, thus creating the illusion of a lower activation energy.

2.2.3.4 Product release is rate determining

This case is represented by the energy profile in Fig. 5. (For the reaction scheme, cf. Fig 2, ES being omitted for convenience; EP is the enzyme-product intermediate, also omitted in previous profiles for convenience.)

Clearly, the enzyme has succeeded in stabilizing all the previous transition states, with the last step remaining rate limiting. Again, avoiding the thermodynamic pit implies that the stabilization provided by the covalent linking of enzyme and substrate is carried over to the last transition state too. It is also likely that the product release step is per se rapid, so need not be perfected further.

The rate equation for this mode involves the two pre-equilibrium constants but remains overall second order (Eq. 4):

\[
V = k_{eq}(E)(S) = k_{TO}K_{eq}K'_{eq} (E)(S)
\]

\(K_{eq} = [(ES)_{cov}]/(E)(S); \ K'_{eq} = (EP)/(ES)_{cov}\)

2.2.3.5 The exergonic modes in sum

The exergonic modes represent a new mechanistic addition to the palette of enzyme kinetics. Clearly, it is possible to circumvent the thermodynamic pit problem as long as the ground state binding is fully carried over to the ensuing transition state. In fact, even if the covalent binding is only partly carried over to the ensuing transition state, the thermodynamic pit can be avoided if the enzyme employs additional stabilization modes for lowering the energy of the transition state.

**Fig. 5.** Gibbs energy \((G)\) profile for the exergonic formation of a covalent enzyme-substrate intermediate \([ES]_{cov}\), product release being rate determining; ES is not shown for convenience, EP is the enzyme-product intermediate (for letter symbols, cf. Fig. 1)
Furthermore, the thermodynamic pit problem is a very relative one, as the imputed loss in reactivity depends on the standard for comparison! Thus, if the standard is the uncatalyzed reaction, the thermodynamic pit can be irrelevant as seen in the above discussion. This would be true even if the covalent binding stability is not fully carried over to the transition state, as some rate enhancement would still accrue.

However, if the standard is an ideal reaction in which the ground state stabilization is fully carried over to the ensuing transition state, the thermodynamic pit would be manifested in cases in which the said stabilization is not fully carried over to the transition state. (Thus, the comparison standard is a perfected enzyme.)

In fact, even a partial carry-over of the ground state covalent binding to the transition state can be supplemented by additional binding modes at the transition state, which may themselves be aided by the covalent “anchoring” of the substrate (vide supra). It is also important that the overall reaction be substantially exergonic in order to avoid a build-up of the various enzyme-substrate intermediates at the putative thermodynamic pit, with a consequent decrease in the free enzyme concentration.

Finally, it certainly bears mention that the exergonic formation of an enzyme-substrate intermediate can be viable even in the general case (without covalent binding), as long as the binding is carried over to the transition state! However, it would appear that the exergonic binding mode would be more likely in the covalent binding case because of the relative strength of the covalent bond.

3. CONCLUSION

The formation of a covalent enzyme-substrate intermediate can indeed be accommodated within the framework of transition state theory, although certain mechanistic details need careful consideration. Prime among these is the manner in which the formation of the covalent bond is itself catalyzed, although this may indicate that the covalent bond is part of the “heavy catalytic machinery” reserved for the key turnover step. (However, additional binding modes at the transition state would supplement the effect of the covalent binding itself.)

Both endergonic and exergonic formation of the covalent intermediate need to be considered, and can apparently lead to overall catalysis, although with interesting differences in terms of reactivity and substrate characteristics. The endergonic mode is apparently suitable for flexible substrates that can be bound in a distorted form, with the resulting strain being released at the transition state thus manifesting the binding energy in full. The exergonic mode is apparently more general, although there is a risk of encountering a thermodynamic pit leading to the inhibition of the reaction. However, this can be avoided if the strength of the covalent linking can be fully carried over to the ensuing transition state.

In the exergonic binding mode, the “anchoring” of the substrate at the active site is possibly critical to stabilizing the turnover transition state: this perhaps increases the residence time which allows the enzyme-substrate intermediate to undergo conformational changes to reach the most reactive state.

Thus, the covalent binding of a substrate to the enzyme represents a new addition to the palette of enzymology, offering hitherto unsuspected opportunities for mechanistic exploration. However, fears that the covalent binding indicates the abandonment of transition state theory appear unfounded, as this mode can be viewed within the normal theoretical framework of chemical reactivity.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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