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Effect of Subunit Dissociation, Denaturation, Aggregation, Coagulation, and Decomposition on Enzyme Inactivation Kinetics: I. First-Order Behavior

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Enzyme inactivation kinetics typically follows what would appear to be simple first-order behavior. However, the inactivation process is known to involve a number of reversible (dissociation and denaturation) as well as irreversible (decomposition, aggregation, and coagulation) reactions. These reactions can combine to form a wide variety of reaction pathways which can potentially demonstrate complex inactivation kinetics. However, it was shown that with appropriate assumptions with regard to the relative magnitudes of the various reaction rates, many complex inactivation pathways can demonstrate apparent first-order behavior. Thus, with this analysis, a more accurate interpretation of the slope of an activity versus time semi-log plot can be obtained.
Key words: enzyme • inactivation • first order • denaturation

INTRODUCTION

Enzyme inactivation experiments are typically analyzed by fitting enzyme activity (A) versus time (t) results to the equation

$$\ln\left(\frac{A}{A_0}\right) = -k_{obsd}t \qquad (1)$$

where A_0 is the enzymatic activity at $t = 0$ and k_{obsd} is the observed inactivation rate constant.[24] If this semi-log plot is linear, it is then usually assumed that enzyme inactivation demonstrates first-order behavior. It is perhaps surprising that enzymes, which are very complex molecules, would exhibit such simple inactivation kinetics. One tentative explanation for this simplicity is that the enzyme contains a single bond or structure whose disruption is sufficient to eliminate activity.[27]

There are indications that enzyme inactivation is generally not as simple a process as the "single-bond" hypothesis might lead us to believe. First, two types of complex non-first-order kinetics have been observed with numerous enzymes: the first is biphasic behavior, where a rapid inactivation is followed by a decelerated decay which eventually approaches an activity plateau; the second is grace period behavior, where an initial stable phase of little activity loss is followed by periods of accelerating and decelerating inactivation. Biphasic behavior has been observed with enzymes such as luciferase[8] and acid phosphatase[14] and grace periods with galactosyl transferase[5] and β-D-fructofuranosidase.[32] The presence of these complex inactivation phenomena would indicate, at least with some enzymes, that the inactivation process involves several reaction steps. In addition, it has been observed that under some experimental conditions, enzyme inactivation is a reversible process (i.e., when the inactivating agent is removed, the enzyme regains its activity); under different conditions, inactivation is irreversible. This is further evidence that several reactions which influence activity can take place simultaneously in solution.

Studies that have previously examined enzyme inactivation phenomena have generally taken two divergent approaches: either specific phenomena such as decomposition[2] or dissociation[13] are examined in detail without attempting to determine how they interrelate with other inactivation mechanisms or general inactivation models are developed that do not define the exact nature of the reactions or intermediates involved.[6,7] Little effort has been directed toward determining how one specific inactivation reaction would influence another to produce complex behaviors. Therefore, the goals of this study were to determine (1) precisely what species could potentially be present during the inactivation of an enzyme and (2) the exact nature (reversible versus irreversible) of the transitions that produce these species. This work will determine what reactions or combinations of reac-

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tions could potentially display first-order inactivation kinetics, thereby providing a clearer idea of the possible phenomenological meanings of the constant k_{obsd} of Eq. (1). In a subsequent publication[25] these same reactions will be used to better understand the underlying phenomena that produce non-first-order biphasic and grace period behavior.

MECHANISMS OF REVERSIBLE INACTIVATION

The majority of work with regard to the disruption of protein structures has focused on the native-to-denatured transition of globular monomeric proteins (Fig. 1a). The native-to-denatured structural transition is generally believed to be a reversible, highly cooperative one-step process.[21] This denaturation can thus be characterized by the following equation:

$$P_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P_1^* \qquad (2)$$

where P_1 and P_1^* are the native and denatured structures of the monomeric protein. The kinetic behavior of such a system has been well documented.[29] If $k_1 \cong k_{-1}$, an initial first-order decay [$k_{obsd} = k_1(1 - k''/k)$, where k and k'' are the proportionality constants relating $[P_1]$ and $[P_1^*]$ to their solution activities, respectively] will eventually level off to an activity plateau [$A_\infty/A_0 = (1 + k''k_1/kk_{-1})/(1 + k_1/k_{-1})$]. On the other hand, if P_1^* is highly favored (i.e., $k_1 \gg k_{-1}$) and has no residual activity, the k_{obsd} of Eq. (1) will then simply be equal to k_1.

Relatively stable intermediate structures have been detected in the denaturation of some monomeric proteins.[18] If one intermediate (I) is present, this transition is given by

$$P_1 \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} I \underset{k''_{-1}}{\overset{k''_1}{\rightleftharpoons}} P_1^* \qquad (3)$$

As with the mechanism of Eq. (2), the initial rate of inactivation will appear to be first order with $k_{obsd} = k'_1(1 - k'/k)$, where k' is the proportionality constant relating the concentration of I to its activity. Similarly, at $t = \infty$, a plateau activity of $A_\infty/A_0 = (1 + k'k'_1/kk'_{-1} + k''k'_1k''_1/kk'_{-1}k''_{-1})/(1 + k'_1/k'_{-1} + k'_1k''_1/k'_{-1}k''_{-1})$ will once again be observed. The mechanism of Eq. (3) can also, under certain circumstances, display "disguised" first-order behavior[30] (i.e., a first-order plot is linear). For example, this would be the case if both k'_{-1} and $k''_{-1} \cong 0$ and $k'' = (k'_1 - k''_1)/k'_1$.

Another consequence of the presence of the I species in the denaturation transition of Eq. (3) is that, theoretically, the progression from the initial slope to the final plateau can now be more complex, displaying either sigmoidal grace period behavior or two first-order slopes (biphasic behavior). As a result, intermediates in the native-to-denatured transition have been suggested as the main source of non-first-order behavior.[29] However, denaturation transition intermediates are generally observed only at very low concentrations[3] relative to the other protein species P_1 and P_1^*. Furthermore, the time frame of these native-to-intermediate-to-denatured transitions are normally fast (order of seconds)[18] relative to most protein inactivation experiments (minutes, hours, or days).[29] Thus, it can be assumed that during most inactivation experiments, the native, denatured, and any intermediates present in Eq. (3) are essentially at equilibrium; therefore, the presence of denaturation intermediates should not have a direct effect on the kinetic behavior of an enzyme.[3] However, if other reactions take place that affect some protein forms in Eq. (3) more than others (cf. next section), intermediates could have an indirect impact on enzyme inactivation kinetics.

Most enzymes are oligomeric,[11] consisting of associated monomers. It is known that monomer interactions can have a strong influence on enzymatic activity[11]; quaternary interactions are also known to stabilize monomers.[13] Consequently, oligomer dissociation, which is a

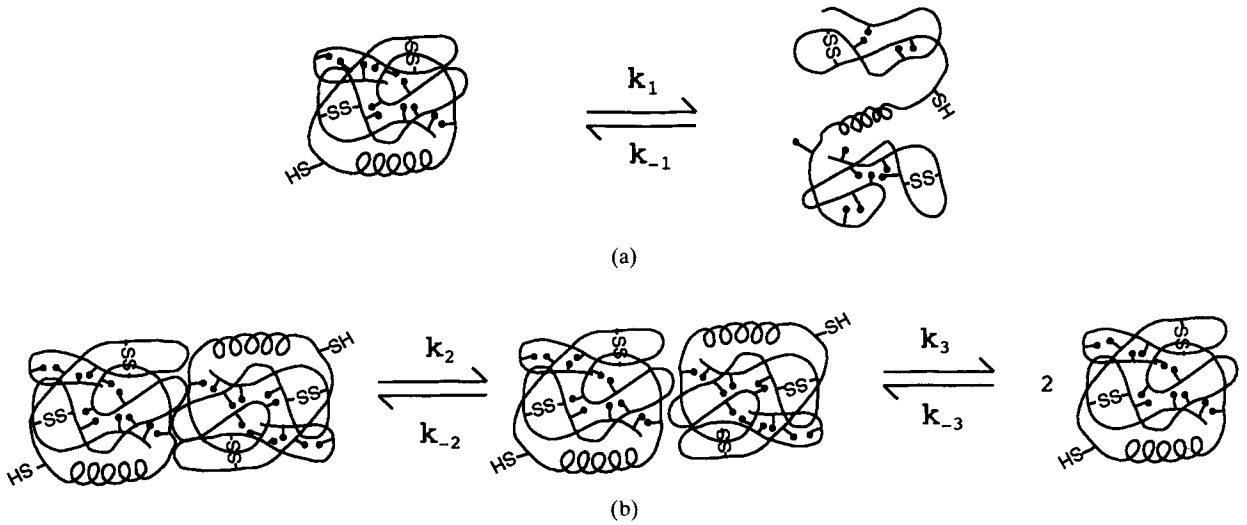

Figure 1. Schematic diagram of reversible inactivation mechanisms of a globular enzyme (●, hydrophobic group; SH, thiol cysteine group; S–S, cystine group): (a) denaturation; (b) dissociation.

relatively slow but in most cases a reversible process,[13] would be expected to also produce intermediate species with differing activities and stabilities. We hypothesize that in most inactivation experiments, it is not the intermediates of the denaturation transition but more likely those produced by oligomeric dissociation that have the greatest influence on enzyme inactivation behavior. For an enzyme with two subunits, it has been hypothesized that the dissociation process (Fig. 1b) would involve two steps[20]:

$$P_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P_1P_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} 2P_1 \qquad (4)$$

where the first stage is an association-dependent structural change and the second is an association/dissociation interaction. The reversible inactivation transition from P_2 to P_1^* would then involve the combination of Eqs. (2) and (4). Once again, like the mechanism of Eq. (3), this system would have a first-order initial rate (in this case, $k_{obsd} = k_2(1 - k^{++}/k^{+})$, where k^{+} and k^{++} are proportionality constants relating $[P_2]$ and $[P_1P_1]$ to the activities of each of their two monomers, respectively) and could potentially display grace period or biphasic inactivation behavior. An analytical solution for the value of the activity plateau cannot be determined for this system because of the presence of the second-order association reaction; it must be calculated by numerical methods.

Enzymes with three or more monomers can conceivably have several oligomer-to-monomer pathways. However, there is usually just one intermediate species and consequently one pathway that will predominate.[12] For example, it has been observed that the hexamer urease can form a stable trimer[10]; therefore, the first dissociation step would most likely be

$$P_6 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} P_3P_3 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} 2P_3 \qquad (5)$$

Since no other dissociation intermediates demonstrate a great deal of stability, it can be assumed that in the next step, the trimer separates directly into three monomers by the following mechanism:

$$P_3 \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} P_1P_1P_1 \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} 3P_1 \qquad (6)$$

For urease, the overall inactivation mechanism would include Eqs. (2), (5), and (6). As with previous examples, the inactivation curve would have an initial first-order slope [$k_{obsd} = k_4(1 - k^{**}/k^{*})$, where k^{*} and k^{**} are proportionality constants relating $[P_6]$ and $[P_3P_3]$ to the activities of each of their six monomers, respectively] but could display a number of kinetic behaviors before reaching an activity plateau. At this point, it should be emphasized that the initial rates of all of the monomer and oligomer inactivation mechanisms illustrated above follow first-order behavior; therefore, if the experimental conditions are such that intermediates are present in significant quantities, and these intermediates or products have residual activity, but the inactivation is halted well before the plateau is reached, then the experimental results could give the false impression that simple first-order kinetics is appropriate.

MECHANISMS OF IRREVERSIBLE INACTIVATION

The dissociation and denaturation processes outlined in the previous section are generally considered to be reversible.[13] Consequently, adjunct processes must be responsible for the irreversibility that is observed in most enzyme inactivation experiments. Most potentially irreversible mechanisms, which occur at higher protein concentrations[4] (>0.01 mg mL^{-1}) involve intermolecular interactions.[22,31] One type of interaction is enzyme aggregation, an irreversible process which could potentially involve either the native oligomer or monomer species (Fig. 2a illustrates the aggregation of the latter). The reaction involving the aggregation of the dimer would take on the form

$$P_2 + P_i' \xrightarrow{k_8'} P_{i+2}' \qquad (7)$$

where P_i' is an aggregated species. The resulting aggregate can be stabilized by several types of molecular interactions. For example, protein thiol (–SH) groups are not reactive in the reducing atmosphere inside the cell. Yet, if these groups are exposed to an oxidizing atmosphere, the formation of disulfide bonds can produce covalently cross-linked high molecular weight species. The production of oligomeric aggregates has been observed experimentally with numerous enzymes. With several enzymes, this aggregation reaction has little effect on enzymatic activity (e.g., urease[10]). However, disulfide bond formation can effectively destroy enzymatic activity, especially if one of the thiol groups is located at or near the active site.[17] Nevertheless, disulfide bond formation can be easily inhibited by the addition of a reducing agent such as

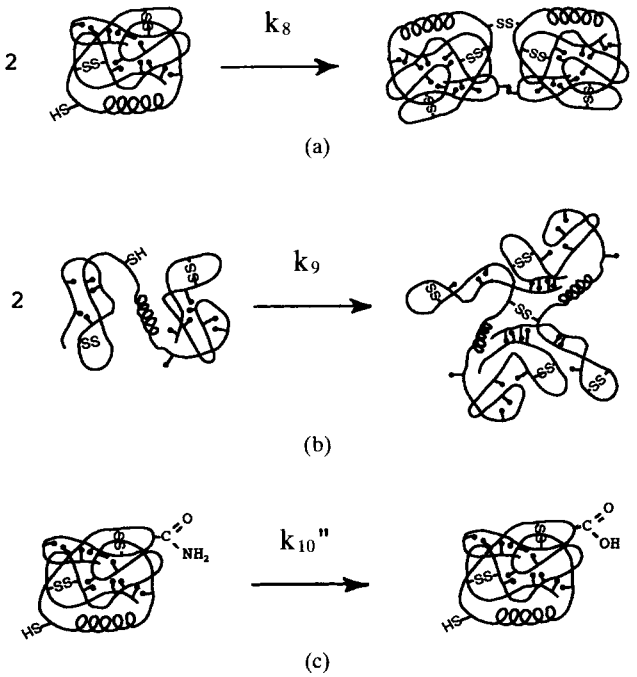

Figure 2. Schematic diagram of irreversible inactivation mechanisms of a globular enzyme (●, hydrophobic group; SH, thiol cysteine group; S–S, cystine group; $CONH_2$, asparagine group): (a) aggregation; (b) coagulation; (c) decomposition.

dithiothreitol,[9] so this reaction is normally not a factor in most inactivation experiments.

It has been suggested that high temperatures could have the opposite effect of actually destroying cystines and forming irreversibly inactive "scrambled" structures.[2] However, this reaction appears to occur, at least with the enzyme lysozyme, only at pH > 8. Hence, it is also doubtful whether the formation of scrambled structures will be a significant irreversible inactivation mechanism at the neutral pHs that are typically used during enzyme inactivation experiments.

Intermolecular interactions can also occur via the formation of hydrogen and ionic bonds.[19] With enzymes, it is in fact these two types of noncovalent bonding that stabilize subunit interactions.[19] However, the specific pairing of interacting groups within the oligomeric structure makes it less likely, with dilute protein solutions under normal aqueous conditions, that these types of interactions will lead to high molecular weight enzyme aggregate formation.[13] On the other hand, the dissociation of an oligomeric enzyme can expose hydrophobic amino acid groups (bold circles in Figs. 1 and 2). These hydrophobic patches can subsequently interact irreversibly in an aqueous milieu to form inactive aggregates. For a dimeric enzyme, this reaction would take on the form

$$P_1 + P_i' \xrightarrow{k_8} P_{i+1}' \tag{8}$$

As an example, Contaxis and Reithel[10] have shown that the active hexamer of urease can dissociate into two trimeric species which aggregate to form an inactive hexamer.

The conversion of the monomer to a denatured structure (Fig. 1a) can expose additional hydrophobic side chains that were previously buried in the center of the protein globule. The interaction of these exposed side chains can also lead to intermolecular associations (Fig. 2b). This type of interaction will be referred to as coagulation since this term usually denotes an aggregation that can be reversed only with the greatest amount of difficulty.[22] By making the simplifying assumption that denatured monomers are added to a coagulate P_i^* one at a time, this process can be characterized by

$$P_1^* + P_i^* \xrightarrow{k_9} P_{i+1}^* \tag{9}$$

It should also be emphasized that the reactions of Eqs. (8) and (9) can only be considered irreversible under normal experimental conditions; both of these reactions can theoretically be reversed with the use of high concentrations of chaotropic compounds such as guanidine hydrochloride.[2]

The combination of Eqs. (8) and (9) with, for example, Eqs. (2) and (4) will most likely lead to complex inactivation behavior.[25] However, under conditions where other irreversible inactivation reactions are insignificant and the reversible reactions of Eqs. (2) and (4) reach equilibrium very rapidly and favor the native structure, Eq. (8) or (9) can be the dominant inactivation mechanism. The formation of small amounts of P_1 or P_1^* could go unnoticed at the beginning of the experiment since this could easily happen within a few minutes and produce a relatively minor decrease in activity. Nonetheless, small amounts of the native or denatured monomers could then aggregate or coagulate, producing inactive species. The dissociation and denaturation equilibria then will shift to replace the reacted monomers, leading to more inactivation via aggregation or coagulation.

If aggregation is the most important irreversible reaction (i.e., $k_8 \gg k_9$), initially, when aggregate concentrations are low compared to the monomer, the controlling inactivation reaction could be approximately characterized by

$$\frac{d[P_1]}{dt} = -2k_8[P_1]^2 \tag{10}$$

Using a dimeric enzyme as an example, if the dimer, native, and denatured species are all approximately at equilibrium ($K_1 = [P_1^*]/[P_1]$, $K_2 = [P_1P_1]/[P_2]$, and $K_3 = [P_1]^2/[P_1P_1]$) and P_2 is the only active species [i.e., $(A/A_0) = 2[P_2]/[P]_T$, where $[P]_T$ is the total number of monomer species in all forms in solution], then it can be shown that

$$\frac{d(A/A_0)}{dt} = -k_8(8K_2K_3[P]_T)^{1/2}\left(\frac{A}{A_0}\right)^{3/2} \tag{11}$$

The integrated form of this equation is the following:

$$\left(\frac{A}{A_0}\right)^{-1/2} = 1 + \frac{k_8}{2}(8K_2K_3[P]_T)^{1/2}t \tag{12}$$

Taking the natural logarithm of both sides of Eq. (12) and using a truncated series expansion simplification [i.e., $\ln(1 + x) \cong x$], Eq. (12) becomes

$$\ln\left(\frac{A}{A_0}\right) \cong -k_8(8K_2K_3[P]_T)^{1/2}t \tag{13}$$

Therefore, k_{obsd} will approximately equal $k_8(8K_2K_3 \times [P]_T)^{1/2}$. If coagulation [Eq. (9)] dominates over aggregation, a similar analysis will show that $k_{obsd} \cong k_9K_1 \times (8K_2K_3[P]_T)^{1/2}$. In both cases, even though the underlying inactivation reactions are second order, a first-order plot can still be approximately linear.

Ahern and Klibanov[2] have recently suggested that intermolecular interactions are not the only source of inactivation. For example, with the enzyme lysozyme at neutral pH, they showed that exposure to very high temperatures (i.e., 100°C) can lead to first-order irreversible amino acid decomposition reactions such as the deamidation of asparagine. Other experiments with model peptides have shown that the stability of protein asparaginyl residues can vary by a factor of up to 30, depending on the catalytic ability and precise location of adjacent amino acids.[23,26] Therefore, each species resulting from the reversible reactions described in the previous section could potentially have its own rate of decomposition (Fig. 2c illustrates one possible deamination reaction for the native monomer P_1).

Accordingly, the mechanism for a simple monomer following a combined denaturation/decomposition inactivation would be

$$\begin{array}{ccc} P_1 & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & P_1^* \\ \Big\downarrow k_{10} & & \Big\downarrow k_{11} \\ P_1'' & \underset{k_{-12}}{\overset{k_{12}}{\rightleftharpoons}} & P_1^{*\prime\prime} \end{array} \qquad (14)$$

where P_1'' and $P_1^{*\prime\prime}$ are the decomposed native and denatured species, respectively. This kinetic mechanism can be greatly simplified if it is assumed that the denaturation reactions are much more rapid than the decompositions (i.e., k_1, k_{-1}, k_{12}, and $k_{-12} \gg k_{10}$ and k_{11}). Then the denatured species are essentially at equilibrium ($K_1 = [P_1^*]/[P_1]$ and $K_{12} = (P_1^{*\prime\prime}]/[P_1'']$). Under these circumstances, this mechanism will display first-order behavior with $k_{obsd} = [k_{10}(1 - k^{\dagger}/k) + k_{11}K_1 \times ((k'' - k^{\dagger\dagger})/k)]$, where $k^{\dagger}$ and $k^{\dagger\dagger}$ are proportionality constants relating $[P_1'']$ and $[P_1^{*\prime\prime}]$ to their activities, respectively. With dimeric proteins, the overall inactivation mechanism would involve a combination of Eqs. (14) and (15):

$$\begin{array}{ccccc} P_2 & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & P_1P_1 & \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} & 2P_1 \\ \Big\downarrow k_{13} & & \Big\downarrow k_{14} & & \\ P_2'' & \underset{k_{-15}}{\overset{k_{15}}{\rightleftharpoons}} & P_1P_1'' & \underset{k_{-16}}{\overset{k_{16}}{\rightleftharpoons}} & P_1 + P_1'' \end{array} \qquad (15)$$

Once again, if it is supposed that the dissociation and denaturation reactions are at equilibrium and dimer inactivation reactions dominate (i.e., k_{13} and $k_{14} \gg k_{10}$ and k_{11}), first-order behavior will again be observed [$k_{obsd} = (1/k^{+})((k^{\ddagger\ddagger} - k^{++})k_{14}K_2 + (k^{\ddagger} - k^{+})k_{13})$, where $k^{\ddagger}$ and $k^{\ddagger\ddagger}$ are proportionality constants relating $[P_2'']$ and $[P_1P_1'']$ to their activities, respectively].

In contrast, if the dissociation, denaturation, and decomposition reactions occur at more or less the same rates, complex non-first-order kinetics would result. From the sparse literature on deamidation of asparaginyl residues, it is impossible to predict a priori whether this is the case for most enzymes under moderate experimental conditions. The half-life of deamidation with lysozyme at pH 6 and 100°C is approximately 1.4 h, but this enzyme is known to be very susceptible to deamination reactions.[2] Model peptides containing asparaginyl residues at 37°C in a pH 7.4 phosphate buffer have half-lives ranging from 12 of 20 days.[26] If these results could be extrapolated to proteins, this reaction would most likely be extremely slow as compared to the dissociation and denaturation reactions [viz., the simplifying assumptions made for Eqs. (14) and (15) are most likely valid].

DISCUSSION AND CONCLUSIONS

A general model for enzyme inactivation, if it were to simultaneously include all possible inactivation reactions (e.g., decomposition, dissociation, denaturation, aggregation, and coagulation) as well as all inactivation species would be much too complex to be of any practical use. Fortunately, in the vast majority of cases, only one ot two reactions will dominate at a particular set of experimental conditions. A few logical assumptions can lead to relatively simple mechanisms with rudimentary kinetic behaviors (i.e., real or apparent first-order behavior). Nevertheless, it is obvious from the above analysis that the slope of a first-order plot can have a wide variety of phenomenological meanings.

The above analysis can also be used to better understand the meaning of the various steps of some of the semi-empirical inactivaton models that currently exist in the literature. For example, the standard model used by Gianfreda et al.[14] and Sadana[28] among others,

$$P \longrightarrow I \longrightarrow D \qquad (16)$$

could result from several mechanism scenarios. If the enzyme is oligomeric, the first step could be related to dissociation and the second step to denaturation. In very dilute solutions, subunit reassociation (a higher order reaction) will be extremely slow, giving the impression that the first dissociation step is irreversible. With monomeric enzymes, the two steps could be denaturation along with decomposition or coagulation. Henley and Sadana, in fact, have suggested a modification of the mechanism of Eq. (16) so that the first step is reversible.[16] Gianfreda et al. have modified Eq. (16) in a similar fashion by adding several I species, all of which are at equilibrium and react in parallel to form inactive species.[15] This could approximately represent a dissociation followed by decomposition, aggregation, or coagulation. Parallel mechanisms, such as those developed by Agarwal[1] or Henley and Sadana[16] for systems of isoenzymes, also show great similarities to the dissociation/decomposition mechanism of Eqs. (14) and (15). Thus, it would appear that our analysis is not only compatible with models currently found in the literature but also provides greater insight into their meaning.

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