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Solvation effects upon the thermodynamic substrate activity; correlation with the kinetics of enzyme catalyzed reactions. II. More complex interactions of alpha-chymotrypsin with dioxane and acetone which are also competitive inhibitors

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Abstract

It is shown that the effects of the addition of various amounts of dioxane and acetone (solvent modifiers) upon the α-chymotrypsin-catalyzed hydrolysis of methylhippurate can be explained in terms of three factors. (A) The effects of the above modifiers on the chemical potential of the substrate. (B) The solvent modifiers dioxane and acetone also act as classical competitive inhibitors. The means of sorting out these contributions is presented. (C) The alterations of the chemical potentials or for free energies of the enzyme, enzyme–substrate complex, and/or other intermediates by the added modifiers appear to cancel out with the substrate used here.

Keywords: Enzyme catalyzed reactions; α-Chymotrypsin; Modifiers; Enzyme kinetics; Enzyme thermodynamics

1. Introduction

As mentioned in the preceding paper in this series, Applewhite et al. [1] were the first to attempt to explain the action of solvent-altering substances from the standpoint of the principles advanced by us in these communications. The enzyme they investigated was α-chymotrypsin in conjunction with methylhippurate as the substrate. The hydrolysis was followed at pH 7.9 by constant titration with alkali at 25°C and standard pressure. Unfortunately, they did not pursue the detailed study of any substance which affected the maximum velocity. Therefore, the only solvent modifiers studied in sufficient depth in this regard were dioxane and acetone. They showed that v_{max} was little affected even in 20% (w/v)

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solutions of these substances. For the reasons mentioned below, they considered their approach to be a failure.

They showed that working at five concentrations between zero and 1.78 *M* dioxane brought about a maximum deviation from the mean of about 3.6% in the maximum velocity. An almost identical result was obtained at five concentrations between zero and 2.79 *M* acetone concentration. Therefore, it is clear that one might expect the results to be interpretable in terms of Case I or some variation of Case I, from the preceding paper, which assumes that the maximum velocity is approximately independent of the modifier concentration (mod). In this case dioxane or acetone is serving as modifier. They expected, as we might, that the entire effect of the added dioxane or acetone upon the substrate thermodynamic activity might account for *all* of the variation of the apparent $\bar{K}$ or K_m; that is to say, after correction for the effect of the modifier on the activity of the substrate, they expected that $\bar{K}$ or K_m would remain absolutely constant within experimental error. To their perplexity, this was not observed. Significant deviation of K_m was observed with dioxane and a much smaller but still demonstrable drift was also seen with acetone. They attempted to compensate for this multiplying the activity coefficients by a factor of 1.2 for acetone and 1.2 times the *square* of the activity for dioxane. A number of other plots were attempted involving the reciprocal of the dielectric constant, the reciprocal of the dielectric constant squared, etc. None of these were found to be satisfactory. Applewhite et al. [1] ended by wondering whether the "empirical relationships we have found are fortuitous or have a rational basis". It is the purpose of this present communication to show that there is indeed a "rational basis" for their findings; it is not necessary to depend on empirical relationships.

The data of Applewhite et al. [1] is entirely explainable by means of the premise that dioxane and acetone do indeed alter the chemical potential or activity of the substrate as discussed in Part I of this series; in addition, it will be seen that both of these substances also act as weak competitive inhibitors.

For this reason, the principles already outlined in the preceding paper must be combined with the process of competitive inhibition. Usually to test for competitive inhibition one plots the reciprocal of the rate as a function of the reciprocal of the substrate concentration in the absence and presence of various concentrations of inhibitor or makes use of some other equivalent plot. Applewhite et al. [1] did not give their information in this form, but rather listed the apparent K_m as a function of dioxane and acetone concentrations.

We may write the rate equation for competitive inhibition in the reciprocal (Lineweaver–Burk) form [2].

$$\frac{1}{v} = \frac{1 + K_i[I]}{k_{cat}\bar{K}[E]_0[S]} + \frac{1}{k_{cat}[E]_0} \qquad (1)$$

where $\bar{K}$ is the apparent *association* constant for enzyme-substrate complex formation, K_i is the corresponding *association* constant for enzyme–inhibitor complex formation, k_{cat} is the first order rate constant for the formation of the product or products from the enzyme–substrate complex, and $[E]_0$ and [S] represent the initial enzyme concentration and substrate concentration, respectively. The concentration of inhibitor is represented by [I].

When the reciprocal of the velocity is plotted against $1/[S]$ a straight line will results with the intercept on the x-axis representing the apparent $\bar{K}$. It is easy to show from eq. (1) that

$$\bar{K}_{app} = \frac{\bar{K}}{1 + K_i[I]}$$

or

$$\frac{1}{\bar{K}_{app}} = K_{m\,app} = \frac{1}{\bar{K}} + \frac{K_i[I]}{\bar{K}} \qquad (2)$$

Where $\bar{K}$ is that term in the absence of inhibitor. Equation (2) indicates that a plot of $K_{m\,app}$ against [I] would be expected to produce a straight line. The intercept on the $K_{m\,app}$ axis is equal to the reciprocal of the true $\bar{K}$ which is equal to the true K_m. The negative intercept on the [I] axis may be shown to be numerically equal to the

reciprocal of K_i, the *association* constant for the formation of the enzyme inhibitor complex.

2. Results and discussion

Equation (2) is a suitable place to start the analysis of the data of Applewhite et al. [1]. Figures 1 and 2 show their results for the modifiers dioxane and acetone, respectively. The very considerable curvatures observed confirm the comments of those authors (unconfirmed by graphical results) that despite the fact that v_{max} is unchanged, dioxane and acetone are not competitive inhibitors in the ordinary sense. Particular note should be made of point A on the [I] axis of each plot which is an extension of what would appear to be the limiting slope obtained at low values of [I]. The significance of these points will be discussed a little later.

The next step is to test the internal consistency of their results insofar as the solubility studies on methylhippurate are concerned. Although they did not realize it at the time, much later work of Bennett and Canady [3] indicates that one would expect that a plot of the logarithm of the solubility of substrate (methylhippurate) vs. concentration of dioxane or acetone modifiers would be linear over a considerable concentration range of the modifier. The slope of this line is approximately numerically equal to the McMillan–Mayer second virial coefficient [4] but of opposite sign. Figures 3 and 4 show the logarithmic plots of this kind for dioxane- and acetone–water solutions, respectively. The fine linearity observed in these plots, as well as in others derived from their data, is a testament to their experimental skills. Dioxane is a somewhat better solubilizer than acetone on a molar basis.

If the simple Case I from the discussion in Part I of this series pertains to these systems involving dioxane and acetone, it would be expected that a plot of ln $\bar{K}$(or ln K_m) vs. concentration of modifier would be linear and approxi-

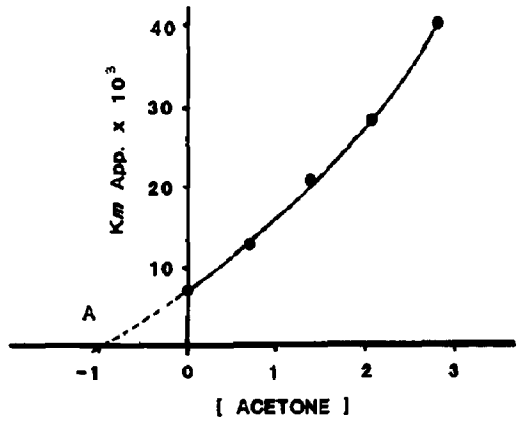

Fig. 2. Plot of $K_{m\,app}$, the apparent Michaelis constant, vs. acetone concentration for the same reaction as in Fig. 1.

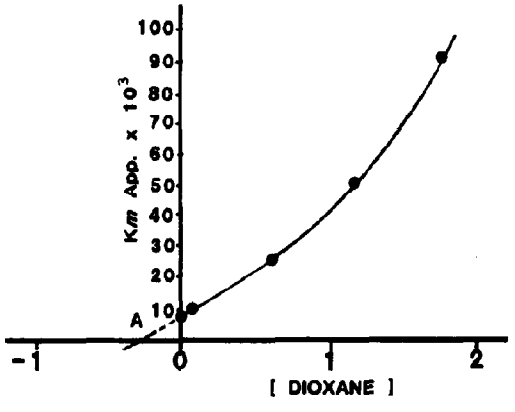

Fig. 1. Plot of $K_{m\,app}$, the apparent Michaelis constant, associated with the α-chymotrypsin catalyzed hydrolysis of methylhippurate vs. dioxane concentration. The curvature indicates that we are not dealing with simple competitive inhibition.

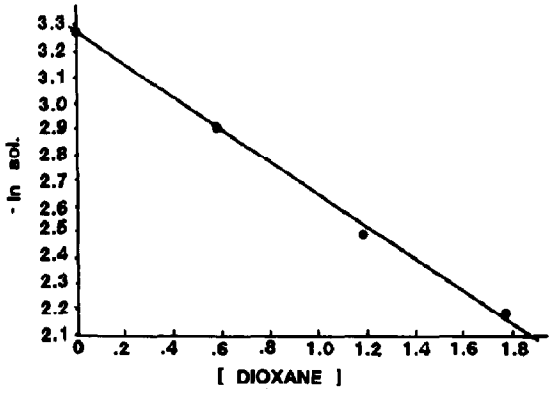

Fig. 3. Plot of the natural logarithm of the solubility of the substrate methylhippurate in various dioxane–water mixtures. The slope was determined by the method of least squares.

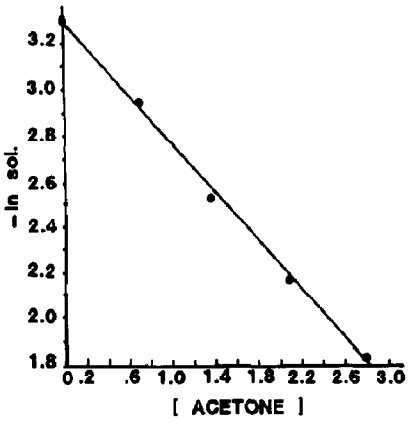

Fig. 4. Plot of the natural logarithm of the solubility of the substrate methylhippurate in various acetone–water mixtures. The slope was determined by the method of least squares.

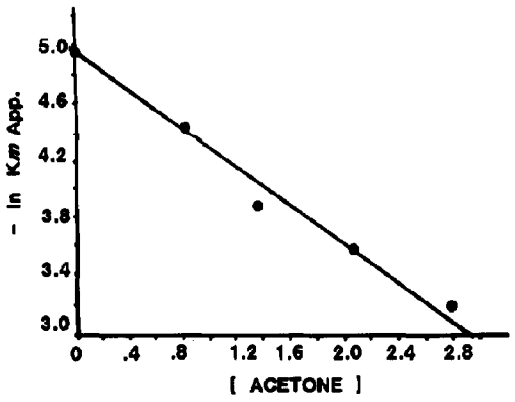

Fig. 6. Plot of the natural logarithm of K_{mapp} vs. acetone concentration. The slope was determined by the method of least squares. The reasonable agreement with the slope obtained in Fig. 4 indicates that we are dealing with an approximation of Case I of the previous paper in this series.

mately numerically equal in slope to that of the plot of ln solubility vs. concentration of modifier. Reference to Fig. 3 and 4 (ln solubility) and Figs. 5 and 6 (ln K_{mapp}) shows that there is no agreement whatsoever as far as the slopes are concerned in the case of dioxane; indeed there is pronounced curvature in the plot involving ln K_{mapp} vs. modifier concentration. Also the dependence of ln K_{mapp} upon dioxane concentration is much greater than that which would be anticipated from just the effect of dioxane on the thermodynamic activity of the substrate (Fig. 3). On the other hand, in the case of acetone, the agreement between effects of ln(solubility) and ln K_{mapp} is reasonably good, but not excellent. The numerical value for the slope for the solubility experiment is 0.53 while that for the K_m values is 0.6. The discrepancy is quite small but is apparently real; there may be a little curvature in Fig. 6, but not much. If dioxane and acetone are both competitive inhibitors, the greater deviation observed with dioxane would suggest that perhaps dioxane is the more potent inhibitor of the two.

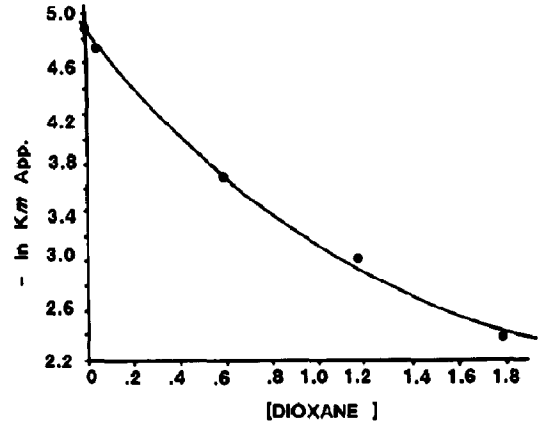

Fig. 5. Plot of the natural logarithm of K_{mapp} vs. dioxane concentration. The curvature and much greater dependence of ln K_{mapp} upon dioxane concentration indicates that the simple cases discussed in Part I of this series do not apply. The slope was determined by the method of least squares, ignoring the curvature.

Our premise is that both dioxane and acetone may be weak competitive inhibitors, so weak in fact that in order to produce a measurable amount of inhibition by the law of mass action, the concentration of that inhibitor must be raised to such a high level that its effect on the thermodynamic activity or effective concentration of the substrate is comparable in magnitude to the inhibition. The result is even more inhibition of the enzyme catalyzed reaction than was due to the competitive effect alone. If this is the case, we should, upon correction of the $1/\bar{K}$ or K_m values by

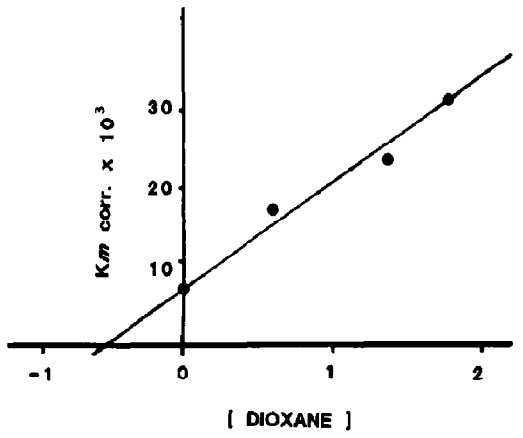

Fig. 7. Plot of K_{mcorr}, the K_m value corrected for the effect of dioxane on the thermodynamic activity of the substrate vs dioxane concentration. The result indicates that dioxane is also behaving as a classical competitive inhibitor.

introducing the activity coefficients previously mentioned in Part I be able to plot the *corrected* values for K_m against the "inhibitor" concentration (dioxane and acetone) and obtain linear results indicating that classical competitive inhibition is indeed taking place, this process having been masked by the substrate solubilizing effect of the inhibitors.

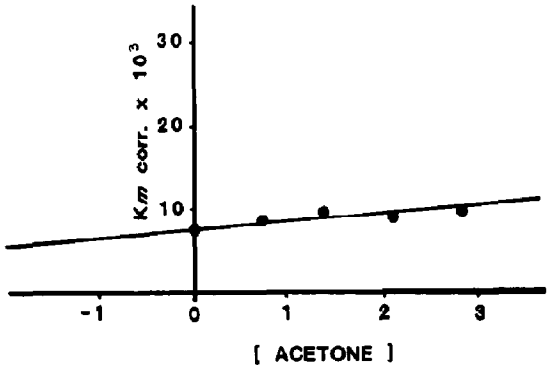

Fig. 8. Plot of K_{mcorr}, the apparent K_m value corrected for the effect of acetone on the thermodynamic activity of the substrate vs. acetone concentration. The result indicates that acetone is also behaving as a competitive inhibitor, but is extremely weak in this regard. By far the greatest effect is that exerted upon the activity of the substrate.

Figures 7 and 8, the corrected plots, show that this is indeed the case for both dioxane- and acetone water systems. It is also clear that dioxane is a much more powerful competitive inhibitor than is acetone. In either case, those lines which exhibited pronounced curvature in Figs. 1 and 2 are linear within experimental tolerances (Figs. 7 and 8).

We may now fully appreciate the almost incredible errors caused by ignoring the solvation effects of added inhibitors.

In this regard, the reader's attention is directed to Figs. 1 and 2 especially to those points labeled A on each plot. Point A is the intersection on the [I]-axis of a line representing the apparent limiting slope of each plot. Conventional wisdom would intuitively suggest that in order to study such substances as dioxane and acetone as competitive inhibitors, one should work at low concentrations of inhibitor (limiting concentrations) in order to minimize the effects of those modifiers on the character of the solvent, leaving their competitive character dominant. For example, we might choose to work at dioxane concentrations from zero to 0.5 M or less. Perhaps working at acetone concentrations from zero to 0.6 M would be suitable. Then, hopefully, the points designated A in Figs. 2 and 3 would indeed approximate a reasonable estimate of K_i, the relevant inhibitor constant. Figures 7 and 8 reveal the fact that such a procedure can, contrary to intuitive thinking, lead to enormous errors in the estimation of K_i. For example, point A in Fig. 1 indicates that the apparent competitive association inhibitor constant for dioxane would be about 4 M^{-1}. The least squares analysis of Fig. 7, the *corrected* plot produces a value of 1.6 M^{-1}, a very large error indeed. An even more dramatic situation is seen with acetone. A rough estimate of K_i by our intuitive method (Fig. 2) would be about 1.0 M^{-1}. The best least squares value calculated from the corrected Fig. 8 is about 0.07 M^{-1}, an error of *more* than an order of magnitude. This is convincing evidence that intuitive efforts which do not take the thermodynamic activity of the substrate into account may be highly unreliable.

We have still not attempted to correct for the

variation of the activity of the modifier (mod) itself with concentration. If has often been conventional to neglect this effect in solution chemistry studies [17] where mod is a nonelectrolyte. It would be especially important not to do so if mod were an ionic species, especially at low ionic strengths. This latter effect of an ionic modifier (potassium chloride) upon enzyme–substrate complex formation in the case of α-chymotrypsin with the substrate methylhippurate at acid pH has been considered in some detail by this laboratory [5–8].

Another interesting fact emerges from the available experimental data for α-chymotrypsin with the substrates discussed here. Addition of methanol, dioxane and acetone appears to alter the activities or free energies of the free enzyme and the Michaelis–Menten complex in the same direction and to about the same extent, apparently cancelling out, thus leaving the effect on the substrate the dominant one. Some effect almost certainly *must* be exerted upon the state of these proteins if for no other reason than that the number of particles in the system is changed when a modifier is substituted for some water molecules. In addition, Timasheff [16] has astutely pointed out that the effects of modifiers of the kind considered here do indeed exert even more effect on protein structure than measurements involving viscosity, circular dichroism, etc., might indicate to the casual observer. We can not argue that there is *no* effect of mod on the proteins involved; cancellation of one type or another is a virtual certainty with α-chymotrypsin. This may also be true for at least some cytochrome P-450 enzymes, an example of which has already been mentioned [9] in the first paper of this series. The results also infer that pseudoequilibrium is reasonably likely for the substrates methylhydrocinnamate and methylhippurate with α-chymotrypsin. It is difficult to imagine deacylation (k_3) as the rate governing step in the hydrolysis of methylhippurate when v_{max} deviates from the mean by about three and a half percent over a range of modifier content which decreases the concentration of water by about 20%. Unless there is a very high affinity water binding site, significant decreases in v_{max} would be expected.

The principles enunciated here throw serious doubt on many previous experiments performed in the presence of such modifiers; this includes some work from our own laboratory [10,11]. From the earliest kinetic studies by Brown [12,13], O'Sullivan and Thompson [14] and Henri [15], the effects described have, for some reason, been virtually ignored for more than a hundred years. They certainly can not be ignored. New research can be done in which interactions of added modifiers with substrates and inhibitors as well as traditional interactions with enzyme and enzyme–substrate complex are taken into account. The present work shows it is by no means impossible to do this. Some older experiments may be reinterpreted in terms of principles suggested here.

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