Enzyme Inhibition in Open Systems
SUPERIORITY OF UNCOMPETITIVE AGENTS

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Investigations of the open system behavior of reversible dead-end inhibitors were carried out by means of computer simulations and experimental studies. The results from both approaches indicate that substrate-competitive inhibition may often be an inappropriate basis for design of potential therapeutic agents. The use of uncompetitive (also called anticompetitive) inhibitors in this role is likely to be far more effective. Chemical analogs of pathogen-specific enzymatic reaction products rather than analogs of substrates provide a promising basis for the systematic design of such uncompetitive inhibitors.

A substantial fraction of the literature on rational drug design continues to be based on searches for substrate-competitive antimetabolites. On first consideration, this approach has appeared to be a sensible one, especially where inhibition of a pathogen-specific enzyme was the goal. However, considered in the kinetic context of open systems (constant input of substrates and removal of products), such a strategy appears virtually certain to yield disappointing results. In vivo, as in theoretical open systems, the tendency of substrates to accumulate at blockage points cannot safely be ignored. Cornish-Bowden (1) has documented the striking difference between the effects of competitive and uncompetitive inhibitors on substrate accumulation in a theoretical metabolic pathway, and such considerations indicate that an approach based on uncompetitive (or anticompetitive) behavior would be far superior. The present report explores the relationships relevant to this distinction, both in explicit mathematical modeling studies and in experimental work with a simple enzymic model system. It further seeks to identify a more promising basis for the systematic design of inhibitory agents that can be expected to be effective in vivo.

EXPERIMENTAL PROCEDURES

The numerical integration program of Franco and Canela (2) was used to model time courses of product formation and substrate accumulation in a simple bisubstrate enzyme system that was open and overall irreversible. The theoretical behavior of this system was then compared with the experimental behavior of an actual enzyme system that is understood to approximate the same formal mechanism. The effects of various types of inhibitors on both the theoretical and experimental open systems were evaluated. Progress curves for inhibition are presented as plots of the percentage of inhibition versus time, based on point-by-point comparisons of the instantaneous velocities of product formation in inhibited systems with those for the same times in the same systems at zero inhibitor concentration.

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Recrystallized bovine liver rhodanese (thiosulfate:cyanide sulfur-transferase, EC 2.8.1.1) was prepared by the method of Horowitz (3) and assayed by following the formation of inorganic thiocyanate (4). Sodium 2-aminoethanethiosulphonate was synthesized (5) and characterized (6) as reported previously. In the uninhibited rhodanese assay system with an organic thiocyanate as the sulfur donor substrate and cyanide anion as the acceptor substrate, the only enzymic species present at kinetically significant steady-state concentrations are free rhodanese and the sulfur-substituted (persulfide) enzyme (5). The formation of thiocyanate from rhodanese persulfide and cyanide anion is essentially irreversible. Neither of the products of the rhodanese-catalyzed reaction (hypotaurine and thiocyanate) serves as a product inhibitor (5, 7). The experimental data collected in the present study establish that the dead-end inhibitors used here (iodide and acetate anions) occur in rapid equilibrium with their enzymic complexes.

The categories of formal kinetic mechanisms considered here are those first developed exhaustively by Wong and Hanes (8) and by Cleland (9). Rate equations for initial steady-state velocity in the absence and the presence of reversible inhibitors were derived by the procedures of King and Altman (10) and Cha (11). All experimental steady-state velocities used in this work were shown to be constant with time and linear with enzyme concentration over the ranges used. Steady-state initial velocity saturation curves are presented in the traditional linear double reciprocal form, but all statistical evaluation of these results was carried out by nonlinear least squares analysis without inversion or other transformation of the data.

RESULTS

The Open System Analog—To document the behavior of inhibitors in open systems, a very simple formal mechanism that corresponds to a known enzymatic reaction was selected. The scheme in Fig. 1 was considered under the circumstances that donor substrate A is supplied at a constant rate, reaction 1 is effectively irreversible (either because of a large equilibrium constant in the indicated direction or because product X is maintained at concentrations near zero by rapid X removal in a coupled reaction), acceptor substrate B is buffered so as to be present at effectively constant concentration, reaction 2 is chemically irreversible, and product Y simply accumulates.

This simple open system analog requires only the assignment of constant values to \(k_{in}, k_{1-2}, k_{2-1}, \) and \(k_{1-2}[B]\) to generate time courses for \([A], [E], [E']\), and \([Y]\). Qualitatively, such a system is expected to establish steady-state concentrations of \(E, E',\) and \(A\) such that \(Y\) will be generated at the same rate that \(A\) is supplied. When there is a perturbation of any sort, these steady-state concentrations will tend to adjust in predictable ways that minimize the effect on overall flux and, if possible, will stabilize at new steady-state values.

Simulations—Computer simulations of product accumulation in such a system when various types of dead-end inhibitors are included in the reaction mixture show that the most striking behavior is generated by the competitively inhibited system, which automatically adjusts over time to produce a velocity closely approaching that in the uninhibited system. Fig. 2 illustrates this recovery in terms of the percentage of inhibition as a function of time. In marked contrast to the competitive case, the simulation for an uncompetitively (anticompetitively)
inhibited system shows that following a brief partial recovery phase, this system persists indefinitely in a state of diminished flux. Behavior in a noncompetitive or "mixed" system is, as expected, intermediate between the competitive and uncompetitive cases.

The cause of the behavior documented in Fig. 2 is easily understood in terms of the effects of substrate accumulation in inhibited open systems. Fig. 3 shows the constant, low steady-state level of [A] in the uninhibited system and the nearly constant but elevated level ultimately achieved in the presence of an inhibitor competitive with A. Also illustrated is the fact that accumulation of A is predicted to proceed indefinitely in the uncompetitive system, where increased substrate concentration can have only a very limited "corrective" effect on overall flux.

Experimental Time Courses—The formal mechanism given in Fig. 4 is a steady-state representation of the sulfur transfer reaction catalyzed by bovine liver mitochondrial rhodanese with an organic thiosulfonate and cyanide anion as the substrates. As indicated, kinetic studies have established the occurrence of no kinetically significant transient complexes in this system. That is, the secondary double reciprocal plots of primary intercepts intersect the origin (5). The overall reaction is essentially irreversible (12), and the product sulfinate is not an effective product inhibitor (5). Inhibition by high levels of thiocyanate results from formation of a dead-end complex with the sulfur-substituted enzyme rather than from reversal of the second reaction (7).

An experimental reaction corresponding to Fig. 4 can be run as an open system (eg. Fig. 1) to be limited ultimately by accumulation of thiocyanate to dead-end inhibitory levels, simply by providing for continuous input of the substrates. For cyanide anion, an essentially constant level is easily arranged by supplying a high total cyanide concentration ([HCN] + [CN⁻]) and buffering the reaction mixture at a pH value well below the cyanide pK of 9.1.

Evaluation of reaction velocity as a function of time in this system then involves the measurement of successive instantaneous velocities in a series of reaction mixtures set up to reflect the progression in concentrations as time passes. All samples receive the same quantity of fully active enzyme and of total cyanide, leaving the concentration of donor substrate and the instantaneous reaction velocity as the only significant variables. The former is calculated as the initial substrate concentration plus the product of the experimental time t and the constant rate of substrate input adopted for the experiment minus the concentration of substrate that has been converted to product SCN⁻ at times that are less than or equal to t. Reaction velocity is measured experimentally as the rate of SCN⁻ production during brief incubations at the calculated substrate concentrations. In this way, by alternately carrying out the measurement of average instantaneous velocity during short incubation periods and then the calculation of the donor concentration for use in the next incremental period, it is possible in principle to construct experiment-based progress
curves for product concentration, donor substrate concentration, and percentage inhibition that are fully analogous to those generated in the computer simulation studies reported in Figs. 2 and 3. All that is required for comparison of inhibition in this experimental open system with expectations from simple theory is a set of rhodanese inhibitors that genuinely represent the classic competitive, noncompetitive, and uncompetitive (anticompetitive) types.

Inhibitors—Steady-state kinetic data were obtained for rhodanese-catalyzed reactions of 2-aminoethane thiosulfonate with cyanide in the presence of acetate as the inhibitor in pH 6.1 MES buffer at 25 °C. In all experiments carried out under these conditions, the slopes of the double reciprocal plots were increased linearly by the presence of acetate, whereas the intercepts were not significantly altered. The value for acetate as a competitive inhibitor was 37 ± 2 mM.

Steady-state data for iodide as a rhodanese inhibitor under the same conditions that yielded competitive inhibition for acetate gave significant increases in both the slope and the intercept of the double reciprocal plots. If these effects are to be attributed to irreversible dead-end inhibition, this behavior corresponds to either classical noncompetitive or mixed inhibition. In this case, the fact that the inhibitor-induced increases of the slope and the intercept are by the same factor, with the consequent invariance of the value (3.8 ± 0.5 mM), indicates classical noncompetitive behavior. However, data in this pattern might conceivably also be generated by an agent that simply caused irreversible inactivation of part of the enzyme (13). This possible alternative explanation for the present observations was tested by an experiment in which donor substrate saturation curves were determined for several enzyme concentrations at fixed inhibitor concentration (Fig. 5). It is easily shown from simple steady-state theory that in these circumstances, secondary plots of both reciprocal slope and reciprocal intercept (from the primary double reciprocal plots) against enzyme concentration will be linear through zero for the reversible noncompetitive case but would appear parabolic for the case of irreversible partial inactivation. In the present work, both secondary plots were linear (correlation coefficients > 0.99) and passed within standard error limits of the origin. These results confirm the assignment of classical, reversible noncompetitive inhibition of rhodanese by iodide at pH 6.1 with an organic thiosulfonate as the sulfur donor substrate. The value is 0.64 ± 0.14 mM.

Steady-state data for iodide as a rhodanese inhibitor under different conditions (pH 5.0 acetate buffer at 25 °C with inorganic thiosulfate as the sulfur donor) were also obtained. As noted previously (7), rhodanese assays show marked hysteresis during the first minute under these conditions, but this effect is easily avoided simply by delaying the observation of steady-state velocity until the second minute of assay. Accordingly, the velocity values used in the present analysis were those for the second minute of incubation (v = [SCN]2 min − [SCN]1 min) and are true steady-state values directly proportional to total enzyme concentration. The intercepts of the double reciprocal plots were strongly affected by the presence of iodide; the slopes were not significantly altered. The value of iodide as an uncompetitive inhibitor with respect to thiosulfate in this system was 2.0 ± 0.1 mM.

Experimental Open System Time Courses in the Presence of Inhibitors—When open system experiments of the type described in the foregoing sections were carried out in the presence of dead-end inhibitors, the results varied according to the type of inhibitor tested. The data displayed in Fig. 6 show that the rate of product accumulation, initially diminished by the presence of acetate at pH 6.1 as a competitive inhibitor, completely recovered during the course of a 10-min experiment. The further data in Fig. 6, obtained with iodide at pH 5 as an uncompetitive inhibitor, show that the open rhodanese system was permanently inhibited under these conditions. When the iodide was used at pH 6.1 as a classical noncompetitive inhibitor, partial recovery was seen. The time courses of the percentage of inhibition in the foregoing experiments are displayed in Fig. 7. Although this conversion of the data appears to magnify the experimental errors, these plots clearly support the conclu-

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1 The abbreviation used is: MES, 2-(N-morpholino)ethanesulfonic acid.
The causes of the observed behavior are addressed by the plots given in Fig. 8, which show the extent of donor substrate accumulation in the presence of competitive and uncompetitive inhibitors. Clearly, the recovery from inhibition in the competitive case was associated with substrate accumulation to a new steady-state level. In the uncompetitive case, substrate accumulation proceeded at a constant rate throughout the entire experiment, but no recovery ensued. The noncompetitive case, as expected, was intermediate between the competitive and uncompetitive extremes, showing a partial recovery associated with a rate of substrate accumulation that diminished during the experimental period.

**DISCUSSION**

The immediate objective of this study is a documentation of inhibitor effects on the kinetic behavior of a very simple experimental catalytic open system as compared with the behavior implied in principle by the corresponding formal mechanism. In particular, attention is focused on the contrast between competitive inhibitors, which are expected to exhibit only transient effects in open systems, and uncompetitive (anticompetitive) inhibitors, which are expected to yield permanent inhibition. Finally, these considerations direct attention to the question of inhibitor design for the purpose of predicting structures of compounds most likely to be effective in particular open systems.

Cornish-Bowden (1) has compared the "catastrophic" response of a theoretical metabolic pathway in the presence of an uncompetitive inhibitor with the much milder response expected for an otherwise comparable competitive inhibitor. Documentation of the differential effect was in terms of the accumulated concentration of a metabolic intermediate as a function of the inhibitor concentration. That work provided valuable insight into the causes of the rarity of uncompetitive inhibition in metabolic systems but was less helpful in providing guidance for therapeutic inhibitor design, asserting only that "...this will certainly be much more difficult." The present work proposes to ease this difficulty for particular cases in the context of pathogen-specific enzymes.

Comparison of Experimental and Theoretical Results—The product accumulation time courses from experimental studies were compared with the computer simulations of the open system analog shown in Fig. 1. In both cases, product accumulation to an new steady-state level acts by mass action to overcome the inhibition. In contrast, the accumulation of donor substrate, which also occurs in the uncompetitive open systems, is neither self-limiting nor corrective in its effect on the velocity of product accumulation. In this case, even raising the donor substrate concentration to infinite levels clearly would not overcome the inhibition in the open system. These results carry the implication that uncompetitive therapeutic agents will be far superior to competitive ones in vivo.

It is to be noted that the observed differences in inhibitor behavior in the open and closed systems reported here represent only the limit cases. Obviously, not all intermediates are free to accumulate to high concentrations in vivo. In particular, substrates at metabolic branch points and those subject to other forms of biochemical regulation may fail to do so. Such circumstances can account for the success of some substrate-competitive antimetabolites, but they do not operate counter to the success of uncompetitive inhibitors. In general, the contrasts in behavior documented in the present work should be observable in the great majority of situations in which the intermediate that tends to accumulate in the presence of an inhibitor has no access to a rapid alternative pathway.

Validity of the Model Systems—It appears that the foregoing conclusions must be generally valid if both the formal mechanism in Fig. 1 is an adequate minimal representation of an open system and also the rhodanese-catalyzed reactions studied experimentally here accurately represent the assigned classical dead-end inhibitory modes. Open systems in general are
characterized by constant input of reactants and constant removal of products. A reasonable analog for use in modeling kinetic behavior over limited concentration ranges was obtained by maintaining the steady reactant input feature while substituting irreversibility of product discharge for the product removal feature. Application of this approach requires the understanding that product accumulation must not be allowed to exceed limits imposed by the possibility of product side reactions with other components of the system. Taken together, these considerations have led to the very simple bisubstrate open system analog given in Fig. 1, which also incorporates the simplifying restriction that the input rate of second substrate is such as to maintain its concentration level essentially constant throughout the observation time.

The well established transfer of sulfane sulfur to cyanide, catalyzed by rhodanese, provides a reaction system capable of satisfying the foregoing requirements. As indicated in Fig. 4, with an organic thiosulfonate as sulfur donor substrate, this enzyme alternates between free and sulfur-substituted (persulfide) forms, with no significant accumulation of either possible binary enzyme-substrate complex (5). Both halves of this simple two-reaction catalytic cycle are essentially irreversible under appropriate conditions, as indicated by the failure of either product to serve as a characteristic product inhibitor. At sufficient levels under some conditions, thiocyanate does inhibit the overall reaction, but it has been established that this effect (uncompetitive with respect to the sulfur donor at pH 5.0) is caused by reversible dead-end complex formation with the sulfur-substituted enzyme, not by reversal of the second reaction (7).

Strictly competitive inhibition with respect to the (anionic) sulfur donor substrates of rhodanese is exhibited by many anions (14, 15); acetate was used as the competitive inhibitor in the present work (Fig. 6). This inhibition is understood to result from the formation of a dead-end complex with the divalent cationic site for donor substrate binding to the free enzyme (16, 17).

Because thiocyanate is known to be a dead-end inhibitor of rhodanese (7) and thiocyanate is a pseudohalide (18), iodide was also tested at various pH values as a potential rhodanese inhibitor. In pH 6.1 MES buffer, iodide proved to be a classical noncompetitive inhibitor capable of forming dead-end complexes equally well with free rhodanese and the sulfur-substituted enzyme. It was established experimentally in this work that the observed noncompetitive behavior was not caused by partial irreversible inactivation of the enzyme by iodide (Fig. 5).

Because uncompetitive inhibition of rhodanese by thiocyana-
tate had previously been observed with inorganic thiosulfate as the sulfur donor substrate in pH 5.0 acetate buffer (7), iodide was also tested in the pH 5.0 system and also found to be an uncompetitive inhibitor. Under these conditions, iodide continues to have access to the sulfur-substituted enzyme but cannot compete with the high level of acetate anion (in the buffer) for dead-end complex formation with the unoccupied active site of the free enzyme.

Clearly, the rhodanese reaction in pH 6.1 MES buffer with acetate and with iodide can provide legitimate examples of classical competitive and noncompetitive behavior, respectively. Moreover, the use of the rhodanese reaction with iodide in pH 5.0 acetate buffer can provide a legitimate example of classical uncompetitive behavior, all in the context of a simple model open system in which input of the donor substrate is maintained at a constant rate, and the reaction mixture is buffered to maintain a practically constant concentration of the acceptor substrate.

Systematic Design of Inhibitors for Use in Open Catalytic Systems—The foregoing considerations lead to the conclusion that substrate-competitive inhibitors cannot be expected to (and do not) provide effective long term inhibition in simple open systems. In contrast, inhibitors uncompetitive with respect to open system substrates can be expected to (and do) yield effective long term inhibition. This situation might seem to be particularly unfortunate because the rational design of competitive inhibitors, as substrate analog structures, is relatively straightforward. It remains to be considered here whether there might also be some general principle for guiding the systematic design of uncompetitive inhibitors.

These considerations are here developed in the context of a bisubstrate enzyme, but the general conclusions are not limited to this case. The enzyme is presumed to be involved in the formation of a specific intermediate in the reproduction or replication of a pathogen. Effective specific inhibition of such an enzyme in vivo would very likely be of therapeutic value. Because most systems in vivo behave as open systems, as noted above, competitive inhibitors are unlikely to be effective, but uncompetitive inhibitors are much more likely to succeed.

In general, a bisubstrate enzyme might function by any of several varieties of formal mechanism: a substituted enzyme mechanism, a rapid equilibrium ordered ternary complex mechanism (in either order), a steady-state-ordered ternary complex mechanism (in either order), or a random mechanism (8, 9). Each of these forms can be explored in principle for the identity of possible inhibitors uncompetitive with the leading substrate.2

In substituted enzyme mechanisms, the leading substrate forms a noncovalent complex with the enzyme, and a reaction occurs to generate a covalent enzyme intermediate and discharge the first product. The substituted enzyme then reacts with a second substrate to release the final product. In this mechanism, uncompetitive inhibitors are compounds that form dead-end complexes with the substituted enzyme. That is, they occupy binding groups that are uncovered when the first product is discharged from the noncovalent complex. Obviously, analogs of the first product will be prime candidates for uncompetitive inhibition in this type of mechanism. Product analogs also have some further advantages as inhibitors. To whatever extent the target enzyme uses intrinsic binding energy to “pay for” catalysis (19, 20), product analogs as inhibitors would be relieved of such a burden and might therefore be expected to achieve tighter binding than would otherwise comparable substrate analogs. Moreover, product analogs can be smaller and simpler to synthesize than full substrate analogs.

In ternary complex mechanisms that have an obligatory order of substrate binding, no matter which substrate is the leading substrate, binding analogs of the first product released are uncompetitive inhibitors with respect to the substrate protein. This assertion is true both for steady-state-ordered mechanisms including the Theorell-Chance mechanism and for rapid equilibrium ordered mechanisms. It will be noted that for all of the formal mechanisms in these classes, it is not obvious a priori which product might be the first released. Clearly, if the formal mechanism has not previously been elucidated, analogs of both products should be tested (in simple initial velocity studies) for activity as uncompetitive inhibitors.

Finally, random order mechanisms require consideration. On the face of it, the only purely uncompetitive inhibitors in bisubstrate reactions that have a random order release of products would be compounds that form dead-end complexes with

2 An exactly analogous treatment, here omitted for the sake of brevity, is applicable to the case of the second substrate.
the ternary complex of the enzyme with both its substrates, and this possibility can hardly be taken seriously in the context of systematic inhibitor design. However, there is an additional possibility hidden in the guise of noncompetitive or mixed inhibition.

In general, such random mechanisms provide two enzyme forms to which any particular product analog might bind: the free enzyme and the binary complex with the other product. Reaction with the former will result in purely competitive behavior, but that with the latter will result in behavior uncompetitive with respect to the substrates. These two actions add up to noncompetitive or mixed inhibition overall. In an open system, the competitive part of the action will be lost as the concentration of substrate builds up, but the uncompetitive component will persist.

It should be noted that the group of bisubstrate formal mechanisms considered here constitutes the entire set of common forms, and no new principles become involved when the set is extended to include trisubstrate reactions. That is, the results may be generalized in the form of a simple, rational strategy: design and if possible synthesize chemical analogs of all the products of a pathogen-specific enzyme-catalyzed reaction. Then test these product analogs as inhibitors of the reaction in a simple closed steady-state system. Those that yield noncompetitive inhibition or noncompetitive inhibition with a major uncompetitive component are prime candidates for testing as potential therapeutic agents in vivo. To the extent that biochemical pathways in vivo approximate open systems, this approach through product analogs should prove to be far superior to the common alternative that involves synthesis and testing of competitive substrate analogs.

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