BBA 66174

ON KINETIC TREATMENTS OF ENZYME–ANTIENZYME REACTIONS

JOHN C. KHOO AND PERCY J. RUSSELL
Department of Biology, University of California, San Diego, La Jolla, Calif. 92037 (U.S.A.)

(Received April 20th, 1970)

SUMMARY

Antibodies against enzymes usually bind tightly to their antigens, and under such conditions Michaelis–Menten or steady-state kinetics are not applicable. This paper points out some of the misinterpretations which arise when reversible kinetics are applied to tight-binding or essentially irreversible systems.

The antiserum against yeast adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) was used for the purposes of illustration. Several criteria for evidence of a reversible tight-binding system are given. One of the most useful methods is a plot of initial velocities as a function of enzyme concentrations in the presence and absence of antienzyme.

INTRODUCTION

Antienzymes which inhibit enzymatic activity seem potentially as useful as low-molecular-weight, reversible inhibitors in determinations of the relationship of the site of inhibitor addition on the enzyme with respect to the catalytic site. However, antibodies generally have such high affinities for protein antigens that the antibodies usually fall into the classification of tight-binding inhibitors[1,2], in which case Michaelis–Menten or steady-state kinetic treatments of data do not apply. This paper illustrates some of the difficulties that are inherent in the interpretation of double reciprocal plots involving enzyme–antienzyme inhibitions. This paper also points out that inhibition constants for antibodies from double reciprocal plots may not be constant, nor are the inhibition patterns consistent since both are affected by the enzyme concentration. The antienzyme for yeast adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) was used for the purposes of this study.

MATERIALS AND METHODS

Materials

Adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) from bakers' yeast was purified according to CHIU *et al.*[3]. The following reagents were obtained from the Sigma Chemical Co.: pyruvate kinase (Type II), lactic dehydrogenase (Type

III), phosphoenolpyruvate, ATP, 5'-AMP, and 2'-AMP. All nucleotide solutions were standardized spectrophotometrically.

A solution of highly purified yeast adenylate kinase was mixed 1:1 with Freund's complete adjuvant. A single injection of 0.5 ml containing 2 mg of enzyme-adjuvant was given subcutaneously to adult female guinea pigs. Normal serum showed no inhibitory effect on yeast adenylate kinase[4]. The antiserum against yeast adenylate kinase gave a single precipitin band by gel diffusion and immunoelectrophoresis. Antiserum for kinetic studies in Figs. 2 and 3A had 32.5 μg of antibody protein per ml precipitated by 2.2 enzyme units (0.94 μg) of yeast adenylate kinase. Antiserum for kinetic studies in Fig. 3B had 25.2 μg of antibody protein per ml precipitated by 0.94 μg of enzyme.

Methods

The reverse reaction was used exclusively in this paper and is considered as starting with AMP + ATP in the following reaction: 2 ADP = AMP + ATP. The coupling system for the reverse reaction is that of ADAM[5]. 1 ml assay mixture contained 50 μmoles Tris–HCl buffer (pH 8.0); 50 μmoles KCl; 0.1 μmole EDTA; 0.33 μmole phosphoenolpyruvate; 0.0133 μmole NADH; 1 μmole free Mg^{2+}; sufficient amount of pyruvate kinase and lactic dehydrogenase so that the coupling system was not rate-limiting; MgATP and AMP were varied for the needs of the experiment. MgATP was calculated based on stability constant 70 000 (ref. 6). The initial reaction rate at 25° was determined by measuring the decreased absorbancy at 340 mμ with time. One enzyme unit represented the disappearance of 2 μmoles of NADH in 1 min.

The antiserum inhibition experiments were carried out by adding 0.1 ml of antiserum diluted with 0.15 M NaCl to the incubation mixture which contained Tris buffer; KCl; AMP; ATP; EDTA; yeast adenylate kinase and water to the total volume of 0.8 ml. This mixture was incubated at 0° for 16 h to insure that equilibrium was reached. The coupling system, given above, was added to the incubation mixture and then $MgCl_2$ was added to initiate the enzyme assay. For controls, 0.1 ml of diluted normal guinea pig serum was used in place of antiserum.

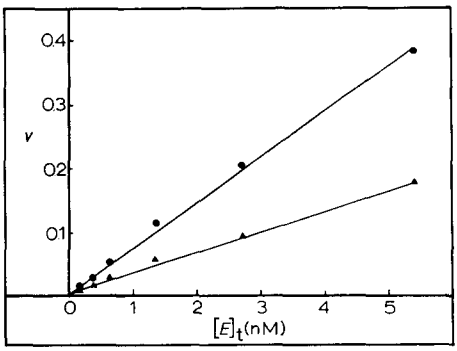
RESULTS

Reversible inhibition

Fig. 1 shows the plots of the initial reaction rates of yeast adenylate kinase as a function of enzyme concentrations, in the presence and absence of the reversible inhibitor, 2'-AMP. An apparent K_i value of 0.26 mM was calculated by using Eqn. 3 of MORRISON[2]. The required kinetic constants were previously determined[7]. The data from inhibited reaction rates plotted as a straight line and passed through zero. This is one of the criteria of reversibility.

Antiserum inhibition

Fig. 2 shows plots of the initial reaction rates of yeast adenylate kinase as a function of enzyme concentrations, in the presence and absence of guinea pig antiserum against the enzyme. The presence of the inhibiting antiserum did not result in a linear relationship, but yielded a curve, concave upward from the origin and approaching an asymptote. This pattern is typical of tight-binding inhibitors[1,2] and has been observed



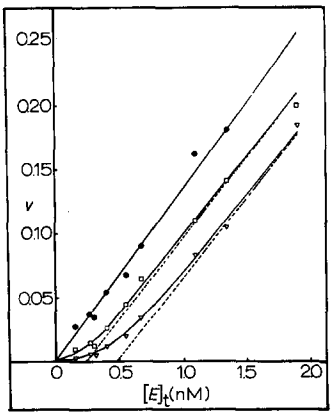


Fig. 1. Plots of initial reaction rate as a function of enzyme concentration in the absence and presence of a reversible inhibitor, 2′-AMP. The MgATP and 5′-AMP concentrations were both 0.83 mM. The concentrations of 2′-AMP were 0 mM (●—●) and 2.5 mM (▲—▲).

Fig. 2. Plots of initial velocity as a function of total enzyme concentration in the absence and presence of antiserum. The dilutions of antiserum were 1:2000 (□—□) and 1:1000 (▽—▽). The control was run with normal serum, 1:1000 (●—●). The concentrations of AMP and MgATP were both 0.83 mM. The assay system is as described in *Methods*.

for other enzyme–antienzyme systems[8–10]. Patterns typical of irreversible inhibitors do not pass through the origin.

For the sake of argument, if one assumes that one molecule of antibody inhibits one molecule of enzyme, then the point of intersection with the abscissa by the asymptote is equivalent to the concentration of inhibiting antibody. Final concentrations of 0.235 and 0.475 nM were obtained from Fig. 2 for final antiserum dilutions of 1:2000 and 1:1000, respectively. Based on the determination of antibody concentrations and previously determined kinetic constants[7], the apparent K_i value can be calculated from the Eqn. 11 of MORRISON[2] for tight-binding inhibition:

$$v^2 + k\mathrm{AB}\left[\frac{K_i}{K_b\mathrm{A}} + \frac{I_t - E_t}{D}\right]v - \frac{k^2 K_i E_t \mathrm{AB}^2}{K_b D} = 0$$

where D is the denominator of the rate equation for a sequential mechanism[11]; I_t is the inhibitor concentration; and E_t is the enzyme concentration. The apparent K_i value calculated for the antiserum was 0.005 nM for the conditions in Fig. 2.

One consequence of a tight-binding inhibitor is that an initial velocity *versus* substrate concentration plot yields a hyperbola which is not rectangular when inhibitor is present. Therefore, double reciprocal plots yield non-linear plots, but this is not always apparent. Fig. 3A shows double reciprocal plots with MgATP as the varied substrate and antiserum against yeast adenylate kinase as the inhibitor. At lower substrate concentrations, plots appear linear, but at higher substrate concentrations a downward curvature toward the ordinate is evident. When AMP was used as the varied substrate, a marked substrate inhibition obtained[7] at pH values below 8 in the absence of antiserum. As shown in Fig. 3B, antiserum decreased the upward concavity.

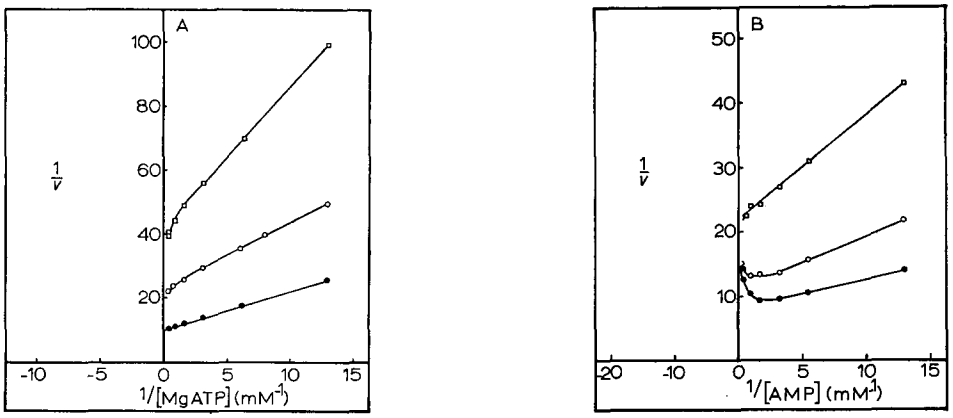


Fig. 3. A. Double reciprocal plots with MgATP as the varied substrate in the absence and presence of antiserum. The concentration of 5′-AMP was fixed at 0.83 mM. The dilutions of antiserum used were 1:2000 (○—○) and 1:1000 (□—□). The control was run with normal serum, 1:1000 (●—●). The assay conditions are described in *Methods*. B. Double reciprocal plots at pH 7.0 with 5′-AMP as the varied substrate in the absence and presence of antiserum. MgATP concentration was fixed at 0.83 mM. The amounts of antiserum used were 1:400 (○—○) and 1:200 (□—□). The control was run with normal serum, 1:200 (●—●).

Replots of intercepts

As shown in Figs. 4A and 4B, the replots of the intercepts of Figs. 3A and 3B *versus* the antiserum concentrations are non-linear, concave upward. Similar non-linearity of replots can be shown for the intercepts extrapolated from the linear portions of the theoretical plots of tight-binding inhibitors of MORRISON[2] and other enzyme–antienzyme systems[12,13].

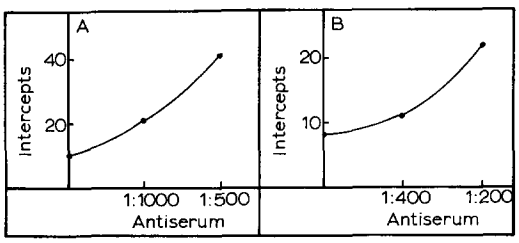


Fig. 4. A. The secondary plot of vertical, extrapolated, intercepts of Fig. 3A against the dilutions of antiserum. B. The secondary plot of vertical, extrapolated, intercepts of Fig. 3B against the dilutions of antiserum.

DISCUSSION

ACKERMANN AND POTTER[1] showed that the use of percent inhibition of enzymic activity is in danger of giving misleading results when the inhibitor is tightly bound or irreversible. The same investigators showed that when the inhibition constants are small compared to the K_m of the substrate, then the inhibitor should be considered as tightly bound. MORRISON[2] has recently developed equations for tight-binding inhibitors. Antibodies generally fall into the category of tight-binding inhibitors. Therefore, it is necessary to demonstrate the reversibility of an enzyme–antienzyme system

before applying kinetic treatments derived for reversible systems. If the enzyme–antienzyme system is not freely reversible, then a calculated K_i value has no meaning, nor do concepts such as competitive inhibition and non-competitive inhibition[12].

It is not the authors' intention to be hypercritical, but the literature does contain studies of enzyme–antienzyme systems which do use reversible kinetic treatments in the absence of evidence for reversibility[13,14]. In the absence of such evidence, conclusions based on these measures are in doubt. A plot of initial velocities *versus* enzyme concentrations will indicate whether the antibody is a reversible inhibitor, tight-binding inhibitor, or an irreversible inhibitor[1,2]. The distinctive patterns of reversible and tight-binding inhibitors with respect to initial velocities and enzyme concentrations were shown in Figs. 1 and 2, respectively. Such patterns hold regardless of the mechanisms of inhibition[2].

A double reciprocal plot of initial velocities *versus* substrate concentrations in the presence of a tight-binding inhibitor, such as an antienzyme, can be misleading (see Figs. 3A and B). The plots may appear linear, particularly when high substrate concentrations are not studied. In Fig. 3A, the downward curvature towards the ordinate at high substrate concentrations obtains in the presence of a tight-binding inhibitor because the true steady-state, initial velocity is described by an equation which contains both v and v^2 terms[2]. In Fig. 3B, the double reciprocal plot with AMP as the varied substrate showed marked substrate inhibition in the absence of antiserum. Antiserum appeared to overcome substrate inhibition, but the effect on the double reciprocal plot was most likely the result of opposing concavities, *i.e.*, the upward concavity due to substrate inhibition and the downward concavity due to tight-binding inhibition by antibody.

This paper has pointed out fallacies inherent in treatments of enzyme inhibitions by antienzymes as a reversible, steady-state system in the absence of evidence for reversibility. In a freely reversible system, the degree of inhibition is independent of enzyme concentration. The degree of inhibition is not affected by the sequence of addition of inhibitor and substrate; and is also independent of the preincubation period with inhibitor. The inhibitor concentrations are in great excess compared to enzyme concentrations; and K_i values are generally within one or two orders of magnitude of K_m values[1]. These tests can be applied to any enzyme–antienzyme system.

REFERENCES

1 W. W. ACKERMANN AND V. R. POTTER, *Proc. Soc. Exptl. Biol. Med.*, 72 (1949) 1.
2 J. F. MORRISON, *Biochim. Biophys. Acta*, 185 (1969) 269.
3 C. S. CHIU, S. SU AND P. J. RUSSELL, *Biochim. Biophys. Acta*, 132 (1967) 361.
4 S. MINATOGAWA AND P. J. RUSSELL, *Biochem. Biophys. Res. Commun.*, 31 (1968) 48.
5 H. ADAM, in H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Verlag Chemie–Academic Press, New York and London, 1965, p. 539.
6 J. F. MORRISON, W. J. SULLIVAN AND A. G. OGSTON, *Biochim. Biophys. Acta*, 52 (1961) 82.
7 J. C. KHOO AND P. J. RUSSELL, *J. Biol. Chem.*, 245 (1970) 4163.
8 P. A. MARKS AND E. A. TSU TSUI, *Ann. N.Y. Acad. Sci.*, 103 (1963) 902.
9 P. FEIGELSON AND O. GREEGARD, *Ann. N.Y. Acad. Sci.*, 103 (1963) 1075.
10 A. S. PIETRO, *Ann. N.Y. Acad. Sci.*, 103 (1963) 1093.
11 W. W. CLELAND, *Biochim. Biophys. Acta*, 67 (1963) 104.
12 B. CINADER, *Proc. 2nd Meeting Fed. European Biochem. Socs.*, 1 (1965) 85.
13 L. A. FAHIEN, H. G. STEINMAN AND R. MCCANN, *J. Biol. Chem.*, 241 (1966) 4700.
14 E. SHAPIRA AND R. ARNON, *Biochemistry*, 6 (1967) 3951.