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KINETIC MECHANISM OF FORMIMINOTRANSFERASE FROM PORCINE LIVER

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Summary

Formiminotransferase (EC 2.1.2.5) and cyclodeaminase (EC 4.3.1.4) constitute an enzyme complex that catalyses two sequential metabolic reactions. The activity of native formiminotransferase can be measured without interference from cyclodeaminase, and its kinetic mechanism has been investigated. Although initial velocity plots yield families of parallel lines suggesting that the transferase utilizes a ping-pong mechanism, product inhibition and alternate substrate studies with tetrahydropteroic acid clearly show the mechanism to be sequential. Of the possible mechanisms compatible with these observations, several could be ruled out through the effects of various dead-end inhibitors. The data indicate that the transferase mechanism is rapid equilibrium random with formation of a dead-end complex enzyme-tetrahydrofolate-glutamate.

Introduction

Formiminoglutamate : tetrahydrofolate formiminotransferase (EC 2.1.2.5) and formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4) catalyse the following sequential reactions in the pathway of histidine degradation in mammals:

Formiminoglutamate + tetrahydrofolate → formiminotetrahydrofolate + glutamate

Formiminotetrahydrofolate → 5,10-methenyltetrahydrofolate + NH_4^+

The enzymes, which earlier had been shown to copurify [1,2] comprise a molecular complex that has been obtained in crystalline form and shown to be composed of eight apparently identical polypeptide chains of about 64 000 daltons [3]. The formiminotransferase activity was demonstrated to be inde-

pendent of the cyclodeaminase by Tabor and Wyngarden [1] who found conditions to selectively destroy each of the activities.

These two activities are stable mammalian folate-dependent enzyme(s) that can be obtained in reasonable quantities [3] and therefore provide a convenient system to investigate aspects of the mechanism of folate mediated one carbon transfer reactions. The properties of the enzymes are also of interest with respect to their role as an enzyme complex. As a preliminary step to elucidating the functional properties of this complex, the kinetics of the formiminotransferase activity have been investigated by initial velocity and product inhibition studies. Alternative substrates and dead-end inhibitors were used to further test possible kinetic mechanisms.

Materials and Methods

Formiminotransferase-cyclodeaminase was prepared from frozen hog liver according to the method of Drury et al. [3]. Solutions of formiminoglutamate were prepared from the hemibarium salt (Sigma) by addition of 10% excess solid Na_2SO_4 followed by centrifugation to remove the precipitated $BaSO_4$. Formiminoaspartic acid and folic acid were products of Sigma. (±) L-tetrahydrofolic acid was prepared by reduction of a neutral aqueous solution of folic acid at room temperature with 1 atm. of hydrogen over platinum [4], followed by purification on DEAE-cellulose [5] by elution with 0.2 M triethanolamine · HCl, 0.5 M 2-mercaptoethanol, pH 7.0. Similarly, (±)-tetrahydropteroic acid was prepared by reduction of pteroic acid in 0.1 M $NaHCO_3$, and purified on DEAE-cellulose by elution with 0.01 M triethanolamine · HCl, 0.5 M mercaptoethanol, pH 7.2. Concentrations of tetrahydrofolate and tetrahydropteroate were obtained by enzymic assay using excess amounts of formiminotransferase and formiminoglutamate [1] and measuring the concentration of the 5,10-methenyl derivatives using $\epsilon_{350\,nm}$ 24 900 [6]. Only one isomer of tetrahydroflolate is utilized in the enzymic assay [1] and observed values have been doubled to express concentrations as total (±)-tetrahydro derivative.

The assay of the formiminotransferase activity was adapted from previous investigations [1,3] and the conditions optimized. Enzyme activity has been shown to depend on the buffer species used [3] and these observations have been extended to include concentration, pH and temperature of incubation. By varying these parameters optimal conditions were found to be 0.1 M triethanolamine sulfate, pH 7.2, and incubation at 37°C for 2—3 min. Standard assay solutions contain 5 mM formiminoglutamate, 1 mM tetrahydrofolate, 100 mM 2-mercaptoethanol, buffer and enzyme in a total volume of 1 ml. Reactions were stopped by the addition of 1 ml of 0.36 M HCl, followed by heating at 100°C for 55 s to convert formiminotetrahydrofolate to 5,10-methenyltetrahydrofolate.

Using these conditions, the purified formiminotransferase had specific activities of 43—45 μmol · min^{-1} · mg^{-1} protein. Kinetic experiments were performed using 0.3—2.0 μg of enzyme and initial velocities (v) are expressed as nmol · min^{-1}.

Results

The kinetics of the formiminotransferase could be examined without interference from the cyclodeaminase because the assay measures all of the formiminotetrahydrofolate product, even if some should be converted to 5,10-methenyl- or 10-formyltetrahydrofolate during the incubation. Under conditions of our transferase assay, the product formiminotetrahydrofolate accumulates and little 5,10-methenyltetrahydrofolate appears (Fig. 1). Although it is unlikely that significant 10-formyltetrahydrofolate would be produced by chemical hydrolysis from such low concentrations of 5,10-methenyltetrahydrofolate present during the short incubation period, we tested for this possibility by acidification of reaction mixtures at various times and followed the appearance of 5,10-methenyltetrahydrofolate at room temperature. The change in absorbance at 350 nm with time was characteristic of the reaction with formiminotetrahydrofolate with no initial rapid changes in absorbance due to 10-formyltetrahydrofolate, which cyclizes about 15 times faster [1]. Thus formiminotetrahydrofolate represents more than 95% of the folate products and is not utilized by the cyclodeaminase under conditions of the assay.

Initial velocity and product inhibition studies with tetrahydrofolic acid and formiminoglutamic acid as substrates

The double reciprocal plots of the initial velocity with tetrahydrofolic acid as variable substrate at different fixed concentrations of formiminogluta-

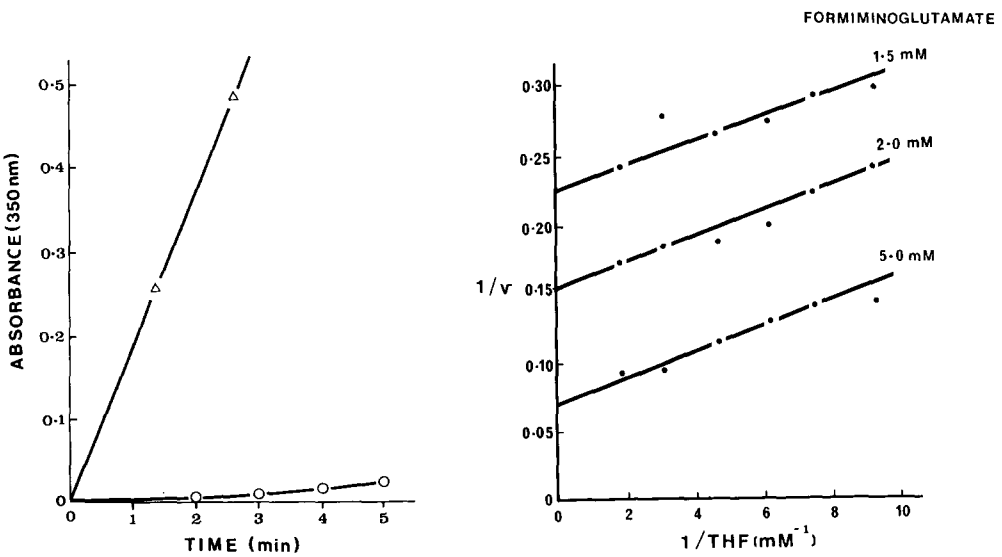


Fig. 1. Time course of the formation of products during the formiminotransferase assay. The appearance of 5,10-methenyltetrahydrofolate at pH 7.2 is given by the circles while the total of formiminotetrahydrofolate and 5,10-methenyltetrahydrofolate is indicated by open triangles.

Fig. 2. Effect of tetrahydrofolate concentration on the initial velocity of the transferase reaction at fixed concentrations of formiminoglutamate.

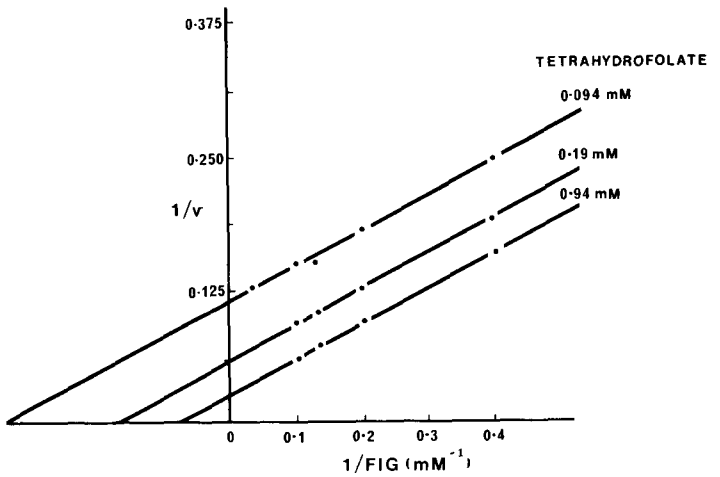


Fig. 3. Effect of formiminoglutamate concentration on the initial velocity of the transferase reaction at different fixed concentrations of tetrahydrofolate.

mate yield a series of lines that do not appear to intersect to the left of the vertical axis (Fig. 2). When formiminoglutamic acid is the variable substrate and tetrahydrofolic acid is used at different fixed concentrations, again the lines do not intersect to the left of the vertical axis and the plots appear parallel (Fig. 3). Secondary replots of intercepts versus the reciprocal of the concentration of the fixed substrate were linear in both cases. The Michaelis constants for formiminoglutamic acid and tetrahydrofolic acid as estimated from Lineweaver-Burke plots where the fixed substrate was at the concentration described in Materials and Methods, are $1.2 \cdot 10^{-2}$ and $1 \cdot 10^{-4}$ M respectively, and agree with the results of Tabor and Wyngarden [1]. The turnover number for formiminotransferase is calculated to be 26 000 per min per mol or 3250 per min per chain, assuming eight identical subunits per molecule [3].

The initial velocity data suggest that the formiminotransferase utilizes a ping-pong mechanism [7]. The only likely possibility for such a mechanism from a chemical stand point, would involve binding of formiminoglutamic acid, formation of a formimino-enzyme with the release of glutamate followed by reaction of the formimino-enzyme intermediate with tetrahydrofolate to produce the last product, formiminotetrahydrofolate. Product inhibition studies were used to attempt to test the ping-pong mechanism, but were limited in that only glutamate could be used because the other product formiminotetrahydrofolic acid, is a substrate for the associated cyclodeaminase activity. Glutamate was found to be non-competitive against tetrahydrofolic acid when formiminoglutamic acid was held constant (Fig. 4) and competitive against formiminoglutamic acid when tetrahydrofolic acid was held constant (Fig. 5). All secondary replots of slopes and intercepts were linear. These results indicate that glutamate binds to the same enzyme form as does formiminoglutamic acid and to a different enzyme form than tetrahydrofolic acid, which is inconsistent with the ping-pong mechanism suggested by the initial velocity studies. It is possible that the mechanism may appear ping-pong but in reality be sequential

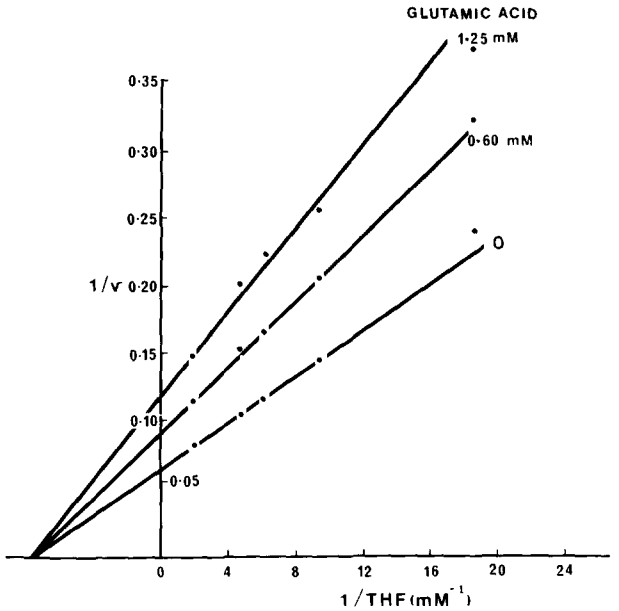


Fig. 4. Product inhibition of formiminotransferase by glutamic acid with tetrahydrofolate (THF) as variable substrate and formiiminoglutamate held constant at $5 \cdot 10^{-3}$ M.

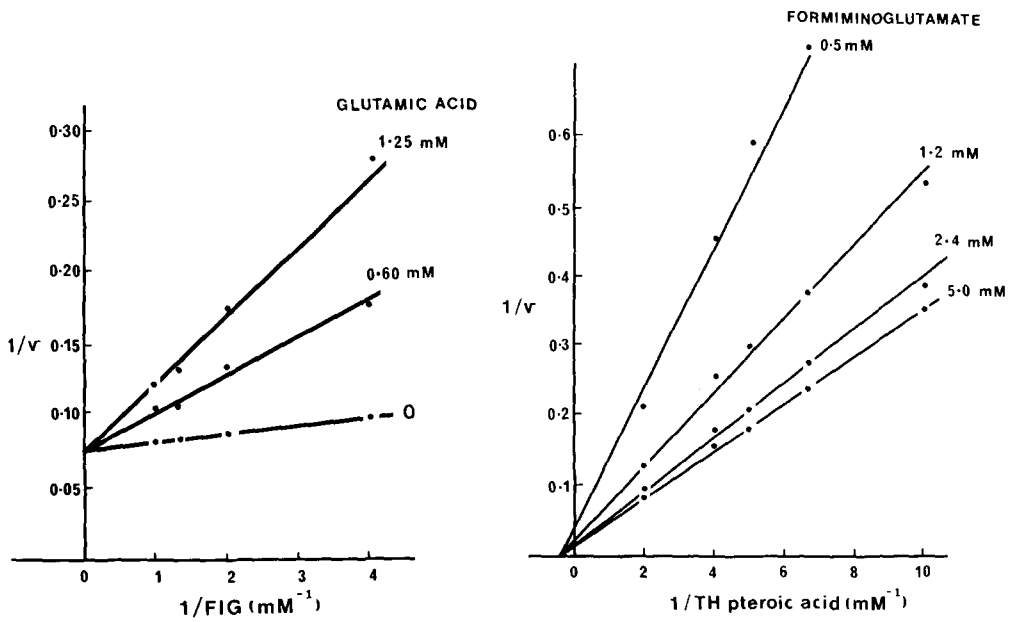


Fig. 5. Product inhibition of formiminotransferase by glutamic acid with formiminoglutamate (FIG) as variable substrate and tetrahydrofolate held constant at $1 \cdot 10^{-4}$ M.

Fig. 6. Effect of concentration of the alternate substrate, tetrahydropteroic acid, on the initial velocity of the transferase reaction at different fixed concentrations of formiminoglutamate.

with the reciprocal initial velocity plots actually intersecting far below the abscissa.

The general initial rate equation [7] for a two substrate sequential mechanism is given by:

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (1)$$

In a ping-pong mechanism, the equation is the same except for the term $K_{ia}K_b$ which is equal to zero. We can then suspect that the transferase mechanism could be sequential with a very low numerical value for this term. Thus an increase in the $K_{ia}K_b$ term might be obtained using alternate substrates and could produce an intersecting pattern in initial velocity studies, confirming a sequential mechanism. Tetrahydropteroic acid was used and found to be a good alternative substrate for tetrahydrofolic acid.

Initial velocity studies with tetrahydropteroic acid and formiminoglutamic acid as substrates

The double reciprocal plots of the initial velocities with tetrahydropteroic acid as variable substrate at different concentrations of formiminoglutamic acid yields a series of lines intersecting to the left of the vertical axis (Fig. 6). The K_m value for tetrahydropteroic acid is $2.5 \cdot 10^{-3}$ M. Similarly the double reciprocal plots with formiminoglutamic acid as variable substrate at different concentrations of tetrahydropteroic acid (Fig. 7) also intersect at a common point to the left of the vertical axis. With tetrahydropteroic acid as substrate the K_m value of formiminoglutamic acid is $2 \cdot 10^{-3}$ M.

These intersecting plots rule out a ping-pong mechanism and are consistent with a sequential mechanism. The product inhibition studies limit the kinetic possibilities to three different sequential mechanisms: ordered, Theo-

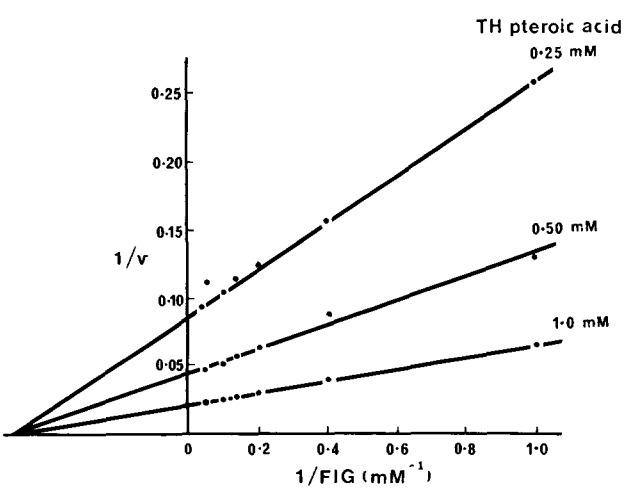


Fig. 7. Effects of formiminoglutamate (FIG) concentration on the initial velocity at different fixed concentrations of the alternate substrate, tetrahydropteroic acid.

TABLE I

INHIBITORS OF FORMIMINOTRANSFERASE

Inhibitor	K_i (M)	Type of inhibition	
		Formiminoglutamic acid	Tetrahydrofolic acid
Tris	$4 \cdot 10^{-3}$	C	NC
Imidazole	$6 \cdot 10^{-3}$	C	UC
Formiminoaspartate	$1 \cdot 10^{-2}$	C	NC
Maleate	$3 \cdot 10^{-2}$	C	NC
Citrate	$6 \cdot 10^{-2}$	C	NC
Glutamate	$9 \cdot 10^{-4}$	C	NC
Folic acid	$1 \cdot 10^{-4}$	NC	C

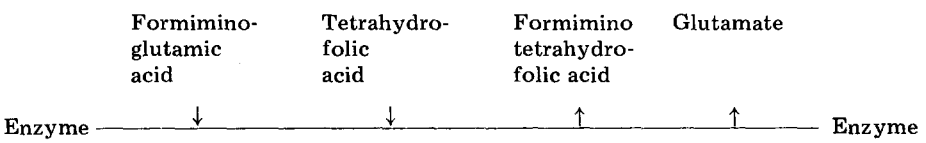
C, competitive; NC, non-competitive; UC, uncompetitive.

rell-Chance, and rapid equilibrium random with a dead-end complex enzyme-tetrahydrofolate-glutamate.

Dead-end inhibitors

Initial velocity and product inhibition studies alone often cannot rule out possible kinetic mechanisms. The use of dead-end inhibitors can assist in this regard, and become particularly important with enzymes such as the transferase where the reverse reaction cannot be assayed accurately. A number of inhibitors have been used to attempt to eliminate some of the possible sequential mechanisms, and their effects on the transferase activity are listed in Table I. Formiminoaspartate, which is not a substrate, as well as Tris, maleate and citrate were found to be competitive against formiminoglutamic acid and non-competitive against tetrahydrofolic acid; imidazole, while also competitive against formiminoglutamic acid is uncompetitive against tetrahydrofolic acid. Folic acid is competitive against tetrahydrofolic acid and non-competitive against formiminoglutamic acid as shown in Figs 8 and 9.

Using these inhibitors, we can begin to eliminate some of the possible mechanisms. An ordered BiBi mechanism is consistent with the initial velocity studies but product inhibition by glutamate indicates that the order of addition of substrates would have to be as indicated, because glutamate and formiminoglutamic acid must bind to the same enzyme form.



Inhibition by folic acid rules out this mechanism, since it binds to the same enzyme form as tetrahydrofolic acid forming a dead-end complex, and would be expected to exhibit uncompetitive inhibition against formiminoglutamic acid, rather than the non-competitive inhibition that is obtained (Fig. 9).

Two possible Theorell-Chance mechanisms must be considered, with

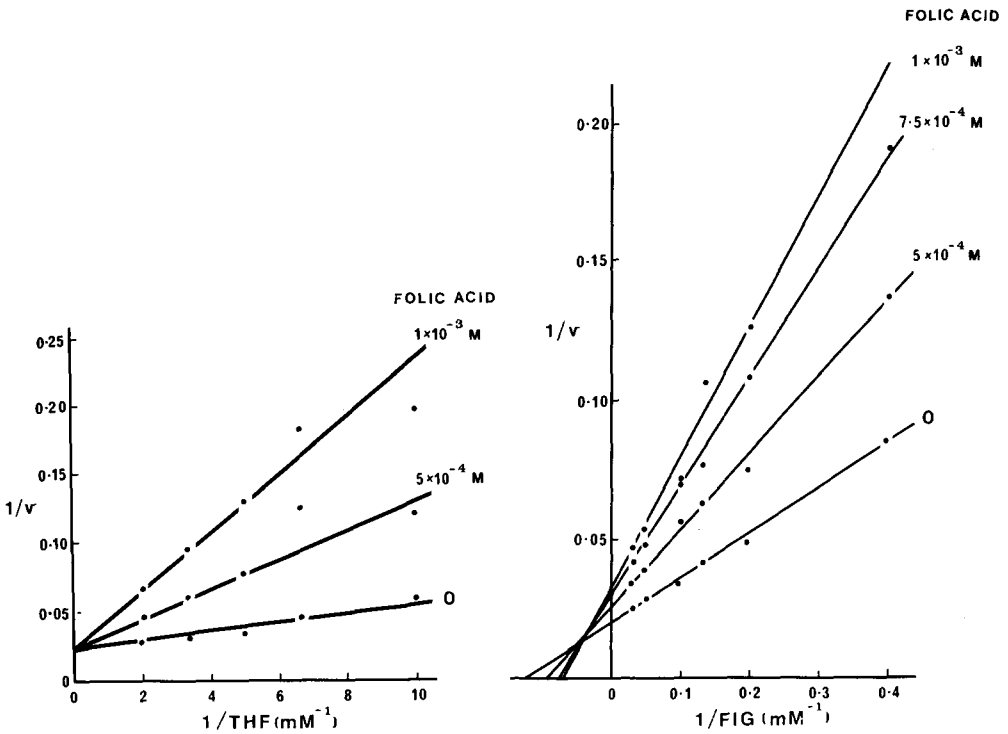


Fig. 8. Effect of folic acid as inhibitor of the transferase with tetrahydrofolate (THF) as variable substrate and formiminoglutamate held constant at $5 \cdot 10^{-3}$ M.

Fig. 9. Effect of folic acid on the transferase reaction with formiminoglutamate (FIG) as variable substrate and tetrahydrofolate at a concentration of $5 \cdot 10^{-4}$ M.

either tetrahydrofolic acid or formiminoglutamic acid being the first substrate to bind. Both of these can be ruled out on the basis that dead-end competitive inhibitors of the second substrate, such as formiminoaspartate for formiminoglutamic acid and folic acid for tetrahydrofolic acid, should then be uncompetitive against the first substrate [7], rather than non-competitive as observed. The inhibition by imidazole is different from the other competitive inhibitors of formiminoglutamic acid, but appears to have more complex effects because replots of slopes and intercepts are parabolic and suggest that this compound binds to more than one enzyme form.

The only mechanism that is consistent with initial velocity studies, as well as the effects of both product and dead-end inhibitors is rapid equilibrium random with a dead-end complex enzyme-tetrahydrofolic acid-glutamate.

Discussion

Formiminotransferase and cyclodeaminase form a molecular complex wherein it appears likely that both activities are contained in a single polypeptide chain [3]. In our efforts to more fully understand the functional aspects of this complex and the relationship between the two activities, the kinetic

properties of the transferase have been examined. Transferase activity is independent of the cyclodeaminase [1] and we have further demonstrated that this is the case with native enzyme under our assay conditions. The kinetics have been used in a qualitative manner to eliminate all but one possible mechanism.

Initial velocity studies using tetrahydrofolate and formiminoglutamate as substrates of the transferase reaction indicated a possible ping-pong mechanism with a formimino-enzyme intermediate. We could not demonstrate exchange between [^{14}C]glutamate and formiminoglutamate when these were incubated with the enzyme, and, in addition, kinetic data using product inhibition and an alternate substrate effectively ruled out a ping-pong mechanism. The initial velocity equation for a ping-pong mechanism is the same as for sequential reactions, except that a constant term is equal to zero. While sequential mechanisms might have a finite value for the $K_{ia}K_b$ term, it has been suggested that in some reactions this product could be so small as to have a negligible effect on the initial velocity plots. This situation has been demonstrated using alternate substrates with brain hexokinase [8], phosphofructokinase [9] and D-amino acid oxidase [10]. Formiminoaspartate is inactive, but tetrahydropteroic acid was found to be a good alternate substrate, and was used to demonstrate that the initial velocity plots actually intersect. The apparent K_m for tetrahydropteroic acid is 10 times larger than that for tetrahydrofolic acid, but since both substrates were (±)-mixtures, this comparison is not quantitatively valid because we do not have information on the possible inhibition by the unnatural isomer in each case [11,12].

The kinetic mechanism of the transferase is clearly sequential, and the possible mechanisms were selected on the basis of product inhibition, with glutamate competitive against formiminoglutamic acid and non-competitive against tetrahydrofolate. Ordered mechanisms such as ordered BiBi and Theorell-Chance were eliminated by means of dead-end inhibitors competitive with either tetrahydrofolate or formiminoglutamic acid. Several inhibitors are competitive against formiminoglutamic acid, but imidazole is unusual in that it appears uncompetitive against tetrahydrofolic acid. Secondary replots of slopes and intercepts with imidazole gave parabolic plots indicating that this compound could be binding to different enzyme forms. Because of the apparent complex nature of the inhibition by imidazole, we have relied on the other inhibitors, which have a greater resemblance to tetrahydrofolic acid, in assigning a kinetic mechanism.

The mechanism that fits the data is rapid equilibrium random with a dead-end complex. Regular rapid equilibrium random would predict that glutamate would be competitive against both substrates, and thus the dead-end complex (enzyme-tetrahydrofolic acid-glutamate) must exist to explain the product inhibition. Such a complex is quite reasonable because the structural differences between the substrates and products are minimal. Also in agreement with this assignment are the inhibition patterns by folate, Tris, maleate, citrate and formiminoaspartate.

The rapid equilibrium random type of mechanism dictates that each substrate has an independent binding to a part of the active site of the enzyme, and that either substrate can add first. Preliminary results with chemical modi-

fication by diethylpyrocarbonate, a selective reagent for histidine residues [13], indicates that the rate of inactivation of the transferase is reduced about 6-fold in the presence of 10^{-3} M folic acid (Beaudet, R. and MacKenzie, R., unpublished). This result suggests that tetrahydrofolate binding does not require formiminoglutamate, and provides further evidence to rule out the ordered mechanisms which require formiminoglutamate as the first substrate. Binding studies will be required to confirm that each substrate can bind to the enzyme in the absence of the other, and thus further test the kinetic mechanism.

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