Time-Dependent Inhibition of Protein Farnesyltransferase by a Benzodiazepine Peptide Mimetic

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ABSTRACT: Protein farnesyltransferase (FTase) and protein geranylgeranyltransferase-I (GGTase-I) catalyze the prenylation of proteins with a carboxy-terminal tetrapeptide sequence called a CaaX box, where C refers to cysteine, "a" refers to an aliphatic residue, and X typically refers to methionine, serine, or glutamine (FTase), or to leucine (GGTase-I). Marsters and co-workers [(1994) *Bioorg. Med. Chem. 2*, 949−957] developed inhibitors of FTase with cysteine and methionine attached to an inner hydrophobic benzodiazepine scaffold. We found that the most potent of these compounds (BZA-2B) resulted in the time-dependent inhibition of FTase. The K_i of BZA-2B for FTase, which is the dissociation constant of the initial complex, was 79 ± 13 nM, and the K_i*, which is the overall dissociation of inhibitor for all enzyme forms, was 0.91 ± 0.12 nM. The first-order rate constant for the conversion of the initial complex to the final complex was 1.4 ± 0.2 min^{-1}, and that for the reverse process was 0.016 ± 0.002 min^{-1}. The latter rate constant corresponds to a half-life of the final complex of 45 min. Our experiments favor the notion that the inhibitor binds to the FTase−farnesyl diphosphate complex which then undergoes an isomerization to form a tighter FTase*−farnesyl diphosphate−BZA2-B complex. Diazepam, a compound with a benzodiazepine nucleus but lacking amino acid extensions, was a weak (K_i = 870 μM) but not time-dependent inhibitor of FTase. Cys-Val-Phe-Met and Cys-4-aminobenzoyl-Met were instantaneous and not time-dependent inhibitors of FTase. Furthermore, BZA-4B, with a leucine specificity determinant, was a classical competitive inhibitor of GGTase-I and not a time-dependent inhibitor.

The joining of the 15-carbon farnesyl group and the 20-carbon geranylgeranyl group to protein-cysteines is catalyzed by protein farnesyltransferase (FTase)[1] and protein geranylgeranyltransferase-I (GGTase-I), respectively (*1*). Substrates for FTase include Ras, lamins A and B, and the α-subunit of transducin, the predominant retinal signal transduction protein. Substrates for GGTase-I include a variety of G-proteins such as Rap1A, Rac1, and the γ-subunit of heterotrimeric ($\alpha\beta\gamma$) G-proteins such as G_s, G_i, and G_o (*1*, *2*). Substrates for the prenyltransfases require the attachment of these lipid groups to become functional, and these posttranslational protein modifications are pivotal.

FTase and GGTase-I are heterodimers consisting of an α- and a β-subunit; the α-subunits are identical, and the β-subunits are homologous to the α-subunits and to each other (*3*). The β-subunit of FTase functions in the Zn^{2+}-dependent binding of the peptide or protein substrate and in the binding of the prenyl donor (*4*, *5*). X-ray diffraction studies of FTase by Park et al. (*6*) show that zinc occurs at a junction between a hydrophilic surface groove near the subunit interface (peptide binding site) and a deep lipophilic cleft in the β-subunit lined with aromatic residues (farnesyl diphosphate binding site). The peptide binding site involves residues on both the α- and β-subunits, and the farnesyl diphosphate binding site is a cleft in the β-subunit. Furfine et al. (*7*) studied steady-state and pre-steady-state kinetics of recombinant rat FTase expressed in insect cells in culture. They reported that farnesyl diphosphate binds first (although the peptide can bind first, it is not on a catalytic pathway), and the enzyme−farnesyl diphosphate complex reacts with the peptide to give the product.

Both FTase and GGTase-I can recognize short peptides containing appropriate CaaX box sequences as substrates. Stoichiometries of these modifications are shown by the chemical equations:

$$\text{farnesyl}-PP_i + HS-\text{acceptor}_f \rightarrow \text{farnesyl}-S-\text{acceptor}_f + PP_i$$

$$\text{geranylgeranyl}-PP_i + HS-\text{acceptor}_{gg} \rightarrow \text{geranylgeranyl}-S-\text{acceptor}_{gg} + PP_i$$

The isoprenoid groups become linked to polypeptidic cysteines through thioether (C−S−C) bonds. Following prenylation, the terminal three residues (aaX) are removed by proteolysis, and the carboxyl group of the terminal cysteine is methyl-esterified (*1*).

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[1] Abbreviations: ABA, aminobenzoate; BZA-2B, Cys-(*N*-methyl)-3-amino-1-carboxymethyl-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-Met; BZA-4B, Cys-(*N*-methyl)-3-amino-1-carboxymethyl-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-Leu; B581, *N*-{2(*S*)-[2(*R*)-amino-3-mercaptoamino]-3(*S*)-methylbutyl}-Phe-Met; FTase, protein farnesyltransferase; FTI-249, Cys-4-aminobenzoate-Met; GGTase, protein geranylgeranyltransferase; L-739,750, 2(*S*)-[[2(*S*)-[[2(*S*)-[(2(*R*)-amino-3-mercaptopropyl)amino]-3-methylpentyl]oxy]-1-oxo-3-phenylpropyl]amino]-4-(methylsulfonyl)-butanoic acid.

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Mutants of *ras* occur in 20−30% of all human cancer cells (*8*). Conversion of the protein-cysteine acceptor site to protein-serine in H-Ras prevents prenylation, precludes membrane attachment, and abolishes the malignant transforming ability of oncogenic H-*ras* (*9*). Thus, inhibition of Ras prenylation represents an important strategy for the treatment of cancer (*10*). Compounds based upon the structure of peptide substrates have been used to design inhibitors for FTase (*11*−*19*).

Several enzymes that serve as therapeutic drug targets are subject to time-dependent inhibition by substrate analogues. Time-dependent, or slow-binding, enzyme inhibitors, in contrast to classical competitive inhibitors, require seconds-to-minutes to exert their inhibitory effect. In contrast, classical competitive inhibitors bind instantaneously (millisecond time scale) to the active sites of enzymes to exert their inhibitory effect. The theoretical advantage of slow-binding inhibitors is not that they take seconds-to-minutes to inhibit their target enzyme. Rather, after inhibition occurs, it takes minutes-to-hours for the inhibitor to spontaneously dissociate from the enzyme, and this reversal cannot be accelerated by substrate (*20*). With classical competitive inhibitors, an increase in substrate concentration due to decreased metabolism can overcome inhibition. Examples of slow-binding inhibitors and their target enzymes include methotrexate and dihydrofolate reductase (*21*−*24*), enalopril and angiotensin converting enzyme (*25*−*27*), allopurinol and xanthine dehydrogenase (*28*), lovastatin and HMG-CoA reductase (*29*), finestride and steroid 5 α-reductase (*30*), and indomethacin and prostaglandin H synthases 1 and 2 (*31*−*34*). Slow-binding inhibitors of enzymes dissociate from their target slowly, and this can be therapeutically advantageous.

Marsters and co-workers (*35*) developed benzodiazepine peptidomimetic inhibitors of FTase that inhibit H-Ras transformation of cells in culture (*12*). We found that the most potent of these compounds, called BZA-2B, results in the time-dependent inhibition of FTase. Our experiments favor the notion that the inhibitor binds to the enzyme−farnesyl diphosphate complex which then undergoes an isomerization to form a tighter enzyme−farnesyl diphosphate−BZA2-B complex. The half-time for the dissociation of the complex is on the order of 45 min.

MATERIALS AND METHODS

Materials. The peptidomimetic benzodiazepines were the generous gift of Dr. James C. Marsters, Jr. (Genentech, South San Francisco, CA). Recombinant rat FTase (*36*) and GGTase-I (*37*), which were expressed in Sf9 insect cells using baculovirus expression systems in culture, were the generous gift of Drs. Patrick W. Casey and John Moomaw (Duke University, Durham, NC). B581 (*11*) and Cys-4-ABA-Met (*14*) were obtained from Bachem (Torrance, CA). The sources of the other materials were noted previously (*36*, *37*).

Enzyme Assays. Progress curves were determined by adding enzyme to the reaction mixture containing farnesyl diphosphate, peptide (Lys-Lys-Ser-Ser-Cys-Val-Ile-Met or Lys-Arg-Lys-Cys-Val-Leu-Ser), and the specified concentration of inhibitor. In the absence of inhibitor, time courses were linear for 40 min, and less than 5% of substrate depletion occurred during the experiment. The final reaction mixture (600 μL) contained 50 mM Tris-HCl (pH 7.5), 20 μM $ZnCl_2$, 100 mM KCl, 1 mM dithiothreitol, 0.33% octylmethylglucoside, acceptor peptide, 400 nM [^{3}H]farnesyl diphosphate (≈15 000 dpm/pmol), and recombinant rat FTase. To measure the amount of product formed, portions (25 μL) were removed at intervals and applied to strips of phosphocellulose paper prior to emersion in phosphoric acid/ethanol (*38*). The conditions for the GGTase assay were previously described (*39*).

To determine the kinetic parameters associated with slow-binding inhibition of FTase, progress curves with 20 or more data points, typically at 0.5 min intervals, were obtained at several inhibitor concentrations and a fixed concentration of peptide acceptor substrate. The concentration of FTase (90−450 pM) was at least 20-fold less than that of the inhibitor so that depletion of inhibitor by the enzyme was less than 5%. The pseudo-first-order decrease in velocity to the steady state occurs under these substrate and inhibitor concentrations (*40*, *41*).

Kinetic Analysis. The kinetic analysis of time-dependent inhibition of FTase by BZA-2B was performed by the procedure described by Morrison and Walsh (*20*). This algorithm was derived from the work of Cha (*40*, *42*, *43*), Strickland et al. (*44*), and Williams and Morrison (*45*), Williams et al. (*22*), and Morrison (*41*). When the reaction was initiated by the addition of the enzyme to a mixture of substrates and inhibitor, we assumed that the difference between the pre-steady-state rate and the steady-state rate decreased exponentially with an observed first-order rate constant, k_{obs}, as shown in eq 1:

$$v - v_s = (v_0 - v_s) \exp(-k_{obs}t) \quad (1)$$

where v is the pre-steady-state rate at time t, v_0 is the initial pre-steady-state rate at t equal to zero, and v_s is the steady-state rate. This equation can be integrated to yield

$$P = v_s t + (v_0 - v_s)[1 - \exp(-k_{obs}t)]/k_{obs} \quad (2)$$

where P is the concentration of product (*20*). The initial velocity (v_0), final steady-state velocity (v_s), and k_{obs} values were determined for each progress curve by nonlinear regression fitted to eq 2 using the Marquardt−Levenberg algorithm (*46*) incorporated in SigmaPlot software (Jandel Scientific, Corte Madera, CA). In general, the asymptotic standard errors of the parameters for each fit were within 25% of the calculated value.

Using the values for v_0 and the corresponding inhibitor concentrations, we calculated K_i using the equation:

$$v_0 = \frac{VA}{K_a\left(1 + \frac{I}{K_i}\right) + A} \quad (3)$$

We calculated K_i* from a similar equation substituting v_s for v_0 and K_i* for K_i. k_4 was calculated according to eq 4 using the values of k_{obs} at several concentrations of inhibitor. k_3 was then calculated according to eq 5 in a similar fashion using the value of k_4 obtained from eq 4 (*20*):

$$k_{obs} = k_4\left[\frac{1 + \frac{I}{K_i^*\left(1 + \frac{A}{K_a}\right)}}{1 + \frac{I}{K_i\left(1 + \frac{A}{K_a}\right)}}\right] \quad (4)$$

$$k_{obs} = k_4 + k_3\left[\frac{\frac{I}{K_i}}{1 + \left(\frac{A}{K_a}\right) + \left(\frac{I}{K_i}\right)}\right] \quad (5)$$

The values of the rate and dissociation constants in this paper are the means and standard errors of at least three independent experiments.

RESULTS

General Characteristics of FTase Inhibition by Peptides and Peptidomimetics. We performed steady-state kinetic experiments to determine the apparent K_i of FTase for BZA-2B, a benzodiazepine peptidomimetic, and we obtained values of 6.5 nM with a 15 min incubation period and 3.4 nM with a 30 min incubation. This result prompted us to determine the rate of product formation, and we found that the rate was concave-downward in the presence of BZA-2B (Figure 1). Nonlinear rates of product formation and apparent K_i values that decrease as a function of incubation time suggested that BZA-2B was a slow-binding inhibitor of FTase. Progress curves initiated by adding enzyme to substrates in the presence of slow-binding inhibitors are concave-down (*20*). With classical inhibitors, in contrast, reaction rates are linear over the period during which there is no substrate depletion.

In contrast to BZA-2B, the rate of product formation was linear in the presence of other inhibitors (Figure 2) including Cys-Val-Phe-Met (*47*, *48*), B581 (*11*), Cys-4-ABA-Met, or FTI-249 (*14*), and diazepam, an anxiolytic drug with the benzodiazepine scaffold of BZA-2B (Figure 3). We found that each of these compounds was a linear competitive inhibitor with respect to the acceptor peptide substrate, Lys-Arg-Lys-Cys-Val-Leu-Ser. Additional experiments were performed to more fully understand the slow-binding inhibition process.

Characterization of Time-Dependent Inhibition. There are two general mechanisms that describe time-dependent inhibition (*20*). Mechanism A is represented by the following equation where E represents the enzyme and I, the inhibitor:

$$E + I \rightleftharpoons EI \quad (6)$$

For mechanism B, binding of the inhibitor I (BZA-2B) involves the rapid formation of an initial collision complex (EI) that undergoes a slow isomerization. Binding of inhibitor thus occurs in the following sequence:

$$E + I \rightleftharpoons EI \rightleftharpoons EI^* \quad (7)$$

where the forward and reverse rate constants for the first process (formation of EI) are k_1 and k_2, respectively, and for the second process (formation of EI*) are k_3 and k_4, respectively. The overall dissociation constant (K_i*) is

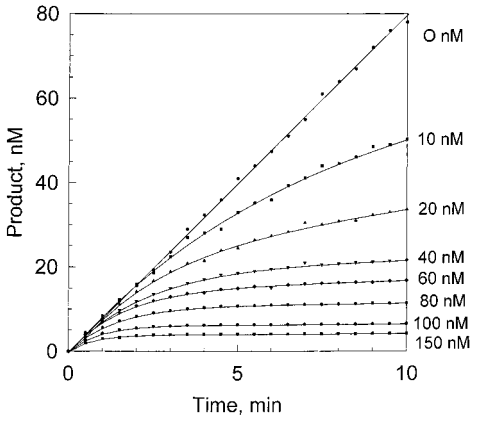

FIGURE 1: Progress curves of product formation illustrating time-dependent inhibition of FTase by BZA-2B. The solid lines are the computer-generated curves predicted by fitting the data to eq 2. Reactions contained 90 pM FTase (8.4 pg/mL), 100 μM Lys-Arg-Lys-Cys-Val-Leu-Ser acceptor peptide, 400 nM farnesyl diphosphate, the specified concentration of BZA-2B, and the other components given under Materials and Methods. The averages of duplicate determinations are given for a representative experiment. Similar results were obtained in eight other experiments.

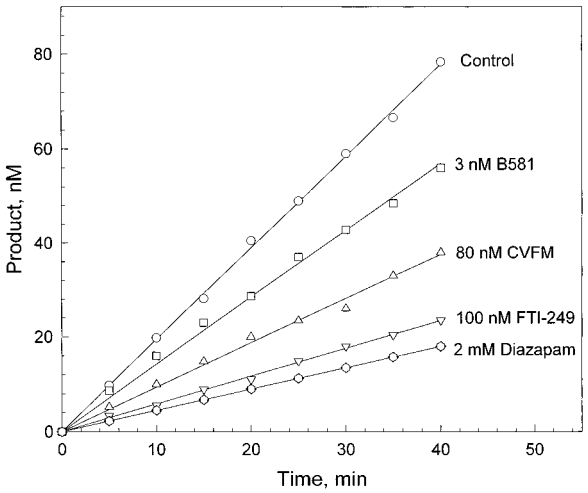

FIGURE 2: Progress curves of product formation in the presence of classical inhibitors of FTase. The reaction was performed as described in Figure 1 with the specified concentrations of the designated compounds. The data represent the mean of three independent experiments.

$$K_i^* = [E][I]/[EI + EI^*] = K_i k_4/(k_3 + k_4) \quad (8)$$

where $K_i = k_2/k_1$. In mechanism A, the equilibrium between E, I, and EI is reached slowly. In mechanism B, the equilibrium between E, I, and the initial EI complex is attained rapidly, but this EI complex subsequently isomerizes to a tighter EI* complex (*41*).

We performed experiments with BZA-2B concentrations ranging from 2 to 600 nM (Figure 1), and we determined v_0, v_s, and k_{obs} by fitting the data to eq 2. The computer analysis yielded values of k_{obs} that reached a plateau at increasing concentrations of BZA-2B (Figure 4), and this result indicated that we were most likely dealing with a situation that resembles mechanism B, the two-step slow-binding inhibition pattern.

Using the values for v_0 and the corresponding inhibitor concentrations, we calculated K_i using eq 3. For these experiments, we used three concentrations (100, 200, and

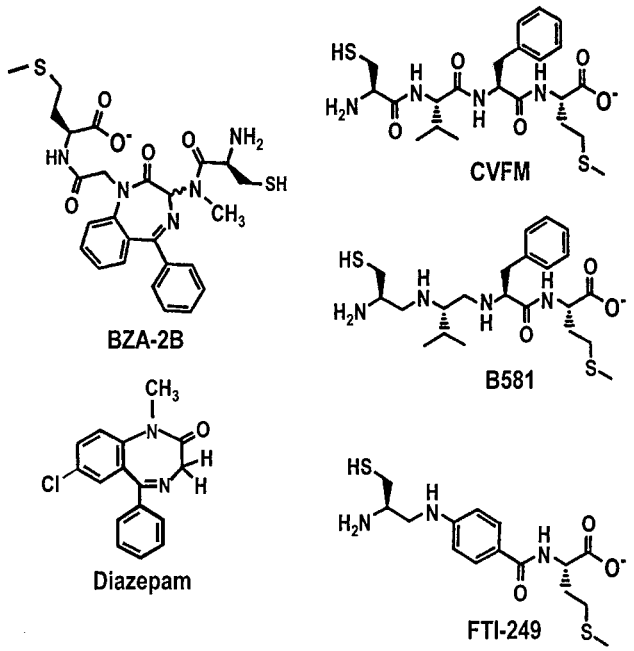

FIGURE 3: Structures of FTase inhibitors.

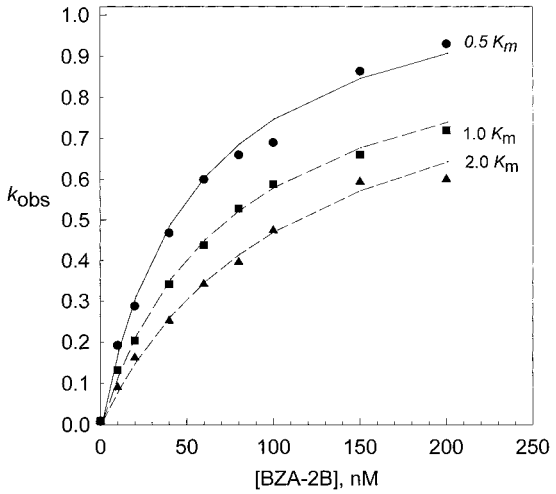

FIGURE 4: k_{obs} values as a function of BZA-2B concentrations at three concentrations (100, 200, and 400 μM) of acceptor peptide substrate (Lys-Arg-Lys-Cys-Val-Leu-Ser). k_{obs} was calculated by nonlinear regression of progress curves as described under Materials and Methods. The data are from one representative experiment. Similar data were obtained in two other experiments. The concentration of FTase was 90 pM (8.4 ng/mL).

400 μM) of Lys-Arg-Lys-Cys-Val-Leu-Ser with a Michaelis constant, or K_m, of 200 μM. Higher peptide concentrations were not used because they were inhibitory. We obtained a K_i value of 79 ± 13 nM, which is much higher than the low nanomolar concentration usually associated with BZA-2B (*12*). We calculated K_i* from eq 3 and obtained a value K_i* value of 0.91 ± 0.12 nM.

From the values of K_i, K_i*, and k_{obs}, we calculated the value for k_4, which represents the rate of conversion of the EI* complex to EI, using eq 4. k_4 represents the rate of conversion of the EI* complex to EI, and we obtained a value of 0.016 ± 0.002 per minute. The time required for half of the EI* complex to isomerize to EI is given by 0.693/k_4, and the half-time of this reaction is about 45 min. k_3 was then calculated according to eq 5. k_3 represents the rate of conversion of EI to EI*, and we obtained a value of 1.4 ± 0.19 per minute. The half-time for the isomerization to form the EI* complex (0.693/1.4) is therefore 0.5 min. These data are summarized in Table 2. BZA-2B exhibited product formation rates that were concave-downward with peptide acceptors (Lys-Arg-Lys-Cys-Val-Leu-Ser and Lys-Lys-Ser-Ser-Cys-Val-Ile-Met) and with recombinant H-Ras protein (not shown). At a given concentration of BZA-2B, the k_{obs} values decreased as the concentration of peptide substrate increased, a pattern confirming that the inhibition was competitive with respect to the peptide (*42*).

Table 1: Apparent K_i Values for Selected Inhibitors of FTase and GGTase[a]

compound	K_i for FTase (μM)	K_i for GGTase (μM)
B581	0.0063 ± 0.0009	3.8 ± 0.2
BZA-2B	0.0034 ± 0.000[b]	0.19 ± 0.02
BZA-4B	0.0068 ± 0.001[b]	0.26 ± 0.02
Cys-4-ABA-Met	0.059 ± 0.008	0.169 ± 0.063
Cys-Val-Phe-Leu	5.1 ± 0.7	0.89 ± 0.05
Cys-Val-Phe-Met	0.11 ± 0.01	35 ± 4
diazepam	874 ± 93	2000 ± 190

[a] The K_i values were determined at several concentrations of Lys-Arg-Lys-Cys-Val-Leu-Ser (FTase) or Lys-Lys-Ser-Ser-Cys-Ala-Ile-Leu (GGTase) as previously described (*39*). The values represent the means ± SEM of at least three independent experiments. Date were obtained using a 15 min incubation, unless noted otherwise. [b] Data obtained using a 30 min incubation.

Table 2: Kinetic Constants for Time-Dependent Inhibition of FTase by BZA-2B[a]

constant	experimental value	defining equation
K_i	79 ± 13 nM	3
K_i*	0.91 ± 0.12 nM	8
k_4	0.016 ± 0.002 min	4
k_3	1.4 ± 0.2 min	5

[a] The values were determined from progress curves determined as described in Figure 1. The values represent the means ± SEM determined from nine progress curves.

Preincubation of FTase, Substrates, and BZA-2B. Steady-state and stopped-flow kinetic studies indicate that the FTase reaction is ordered with farnesyl diphosphate as the leading substrate and the acceptor peptide or protein as the following substrate (*7, 49*). The peptide or protein can bind to FTase, but this is not on the main catalytic pathway. To determine whether BZA-2B leads to time-dependent inhibition by binding to free enzyme or the enzyme−farnesyl diphosphate complex, we preincubated FTase with BZA-2B alone, with BZA-2B and farnesyl diphosphate, or with BZA-2B with acceptor peptide for 15 min at 25 °C. (This temperature was chosen because the enzyme was inactivated when it was incubated at 37 °C for 15 min in the absence of farnesyl diphosphate.) The rate of product formation was then measured with the standard assay at 37 °C. We found that preincubation of the enzyme and inhibitor with farnesyl diphosphate resulted in a linear time course of product formation (Figure 5). Preincubation of enzyme with BZA-2B in the absence of farnesyl diphosphate resulted in a concave-downward time course. During the preincubation of enzyme and farnesyl diphosphate, enzyme−substrate complex formation readily occurs (*7*). We surmise that BZA-2B binds to the complex and results in the formation of the EI* complex during the 15 min preincubation. When the

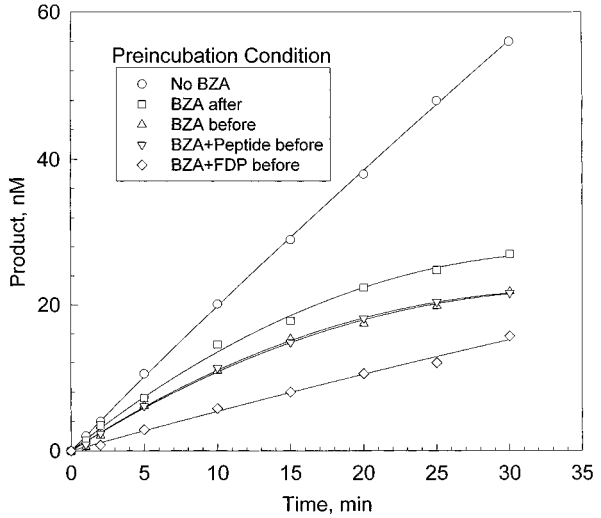

FIGURE 5: Preincubation of FTase with BZA-2B. FTase (13.5 pM) was incubated in the assay buffer for 15 min at 25 °C (200 μL) alone (□), with 9 nM BZA-2B (△), with 9 nM BZA-2B and 150 μM Lys-Arg-Lys-Cys-Val-Leu-Ser (▽), or with 9 nM BZA-2B and 600 nM farnesyl diphosphate (◇). The time course of product formation at 25 °C was measured after 100 μL of buffer containing missing inhibitor and substrates was added. The concentrations of the various components after dilution were as follows: FTase, 9.0 pM; BZA-2B, 6.0 nM; farnesyl diphosphate, 400 nM; acceptor peptide, 150 μM. Portions (25 μL) of sample were withdrawn at the indicated times and quenched with phosphoric acid/ethanol as previously described (*38*). (○) No BZA-2B during the preincubation or assay. The data are from a representative experiment. Similar results were obtained in three other experiments.

acceptor peptide is added during the 15 min assay, the time course of product formation reflects the steady-state velocity (v_s) as near-equilibrium conditions of enzyme, substrate, and inhibitor prevail, and the time course of product formation is essentially linear. In contrast, preincubation of enzyme with BZA-2B without farnesyl diphosphate does not lead to formation of the EI* complex. These results suggest that BZA-2B interacts with the FTase−farnesyl diphosphate complex and thereby produces time-dependent inhibition.

As an independent assessment of the magnitude of K_i*, we preincubated FTase, farnesyl diphosphate, and several concentrations of BZA-2B (0.5, 1.0, 2.0, 3.0, and 4.0 nM) for 15 min (25 °C) to allow for the formation of the EI* complex. We then measured the rate of product formation, or v_s, in solutions containing the same concentration of BZA-2B (37 °C). Using eq 3, we obtained a value of 0.74 ± 0.21 nM, and this value is in satisfactory agreement with K_i* determined from the algorithm described above.

Studies with GGTase and Benzodiazepine Compounds. FTase and GGTase-I are similar enzymes made up of identical α-subunits and homologous β-subunits (*3*). James et al. (*12*) prepared a benzodiazepine derivative (BZA-4B) containing leucine as specificity determinant for GGTase-I. To determine whether BZA-4B was a time-dependent inhibitor of GGTase-I, we measured the rate of product formation. We found that the reaction was linear in the presence of BZA-4B (leucine derivative) and BZA-2B (methionine derivative) as shown in Figure 6. We found that BZA-4B was also a time-dependent inhibitor of FTase (not shown).

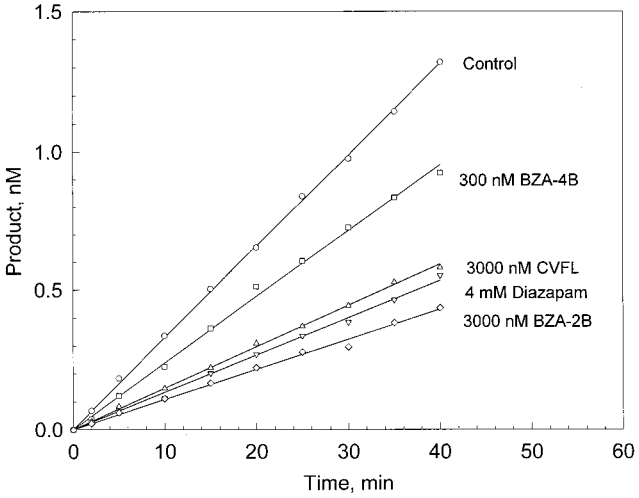

FIGURE 6: Time course of product formation for GGTase-I. The reaction was initiated by the addition of GGTase-I (450 pM, or 42 pg/mL, final concentration). The reaction conditions are described under Materials and Methods. The data are from one representative experiment. Similar results were obtained in three other experiments.

DISCUSSION

Tetrapeptides containing the Cys-A_1-A_2-Met motif can act as substrates or inhibitors of FTase; a free *N*-terminal thiol and a free *C*-terminal carboxylate promote binding to enzyme. Similar requirements are observed for substrates or inhibitors of GGTase-I with a preference for leucine at the *C*-terminus. Because of the requirement for cysteine and methionine for FTase, initial studies focused on modifying the central hydrophobic residues to produce protease-resistant drugs (*50*). Based on the requirement of zinc for the binding of peptide substrates by FTase, Marsters et al. (*35*) hypothesized that the *N*-terminal cysteine side chain and the *C*-terminal carboxylate are coordinated to zinc. Based upon the Cys-X-X-Cys tetrapeptide motif found in aspartate transcarbamoylase (*51*) and other zinc-containing proteins, Marsters et al. (*35*) proposed that peptides bind to FTase with a turn-like backbone conformation. Marsters et al. (*35*) chose BZA as a turn mimic which would enforce the required geometry of the zinc-liganded thiol and carboxylate groups and would provide the hydrophobic groups occurring in substrates.

BZA-2B, which was a time-dependent inhibitor of FTase, has a hydrophobic benzodiazepine scaffold from which cysteine and methionine residues emanate to produce an overall configuration that resembles a peptide turn. Peptide mimetics that are flexible or extended are also potent inhibitors of FTase, but not of the slow-binding variety. Cys-Val-Phe-Met and B581 are flexible and lack a rigid turn conformation. Cys-4-ABA-Met is rigid, but it assumes an extended and not turn conformation (*16*). Based upon X-ray crystallographic studies, Terry et al. (*52*) reported that the CaaX portion of peptide substrates and an inhibitory Cys-Ile-Phe-Met analogue (Merck L-739,750) bind to FTase in an extended and not a turn-like conformation. Moreover, the thiol group of the substrates or inhibitor binds to zinc. In contrast, the respective carboxylates form a hydrogen bond with asparagine 167 located on the α-chain, and they do not bind with zinc.

Terry et al. (*52*) have shed light on the mechanism of inhibition by peptide mimetics. The *N*-terminus of L-739,-

750 binds differently than the analogous residues of the peptide substrates, and this alternative binding disallows the reaction of farnesyl diphosphate and the thiol group of the inhibitor. In our case, BZA-2B likely binds to FTase−farnesyl diphosphate in conformations differing from those of peptide substrates such that the EI and EI* complexes are catalytically inactive. Moreover, the EI* complex assumes a conformation that is long-lived with a half-time of dissociation of 45 min. Owing to the unusual property of time-dependent inhibition of FTase, the conformation of bound BZA-2B most likely differs from the conformations of bound peptide substrates or instantaneous peptide inhibitors. We suggest that the ability of BZA-2B to inhibit FTase in a time-dependent fashion is related in part to its peptide turn conformation. Whether BZA-2B can form a bidentate complex with the zinc atom of FTase (*35*) and whether such complex formation is important in yielding a time-dependent inhibitor remain to be established.

The hydrophobic interactions of peptide substrates and inhibitors with FTase are important (*35*, *50*), and it is likely that the hydrophobic benzodiazepine ring played a role in generating a compound that produced time-dependent inhibition. The benzodiazepine ring, however, was not sufficient to produce time-dependent inhibition as indicated by the inability of diazepam, which contains the ring, to produce this phenomenon.

Cys-4-ABA-Met and B581, like BZA-2B, have K_i values that are in the low nanomolar range. Time-dependent inhibition thus did not result from high affinity per se. The concentrations of BZA-2B that we used in our studies (2−600 nM) were higher than the enzyme concentrations so that the inhibitor was not depleted, and it was not necessary to take inhibitor depletion into account (*42*, *45*). The main finding of this paper is that the rate of product formation was concave-downward in the presence of BZA-2B. The data support the notion that the time-dependent inhibition represents a two-step process with the formation of an EI complex with a K_i value for BZA-2B of about 79 nM. The EI complex underwent an isomerization to form a tighter EI* complex. The overall dissociation constant for BZA-2B with all of the enzyme forms (K_i*) was 0.94 nM. This value does not differ from the IC_{50} value reported by James et al. (*12*), whereas one might expect that the K_i*, the overall binding constant of a time-dependent inhibitor, would be less. The first-order rate constant for the formation of the EI* complex was 1.4/min, corresponding to a half-time of 30 s. James et al. (*12*) used a 30 min incubation for their IC_{50} determination, and this allowed for the majority of the enzyme to be converted to the EI* form, and this may account for the similar values.

Preincubation of FTase with farnesyl diphosphate and BZA-2B for 15 min minimized or eliminated the concave-down time course of product formation, whereas preincubation of FTase with BZA-2B alone did not linearize the time course of product formation. This result suggests that time-dependent inhibition by BZA-2B involved the enzyme−farnesyl diphosphate complex and not the free enzyme. This resembles the time-dependent inhibition of dihydrofolate reductase by methotrexate where the inhibited complex is enzyme−NADPH−methotrexate (*21*).

Our first clue concerning the nature of inhibition of FTase by BZA-2B was the time-dependent decrease in the apparent K_i value as determined by traditional steady-state kinetic methods. Until this observation was made, we assumed that the time course of product formation in the presence of BZA-2B was linear. Time-dependent inhibition appeared to follow a two-step process. This is based upon the variation of the initial velocity (v_0) and the hyperbolic dependence of k_{obs} on the concentration of inhibitor. The importance of time-dependent inhibition is related to the kinetics of the overall process where the half-life of the inhibited enzyme is on the order of tens of minutes. In our case, the half-life for the conversion of EI* to EI was about 45 min. In the therapeutic situation, one cannot overcome inhibition by increasing the substrate concentration. The enzyme, once inhibited, remains inhibited for a relatively long time. In the case of captopril and enalopril, time-dependent inhibitors of angiotensin-converting enzyme, the better therapeutic drug is that with the longer half-life (*26*).

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