# Inhibition of Protein Synthesis by Didemnin B: How EF-1α Mediates Inhibition of Translocation†

Deepika Ahuja, Matthew D. Vera, Bhagyashri V. SirDeshpande, Hiromi Morimoto, Phillip G. Williams, Madeleine M. Joullié, and Peter L. Toogood*,‡

*Willard H. Dow Laboratory, Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1055, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 1904-6323, and The National Tritium Labeling Laboratory, Structural Biology Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720*

*Received September 22, 1999; Revised Manuscript Received January 7, 2000*

ABSTRACT: The antineoplastic cyclic depsipeptide didemnin B (DB) inhibits protein synthesis in cells and in vitro. The stage at which DB inhibits protein synthesis in cells is not known, although dehydrodidemnin B arrests translation at the stage of polypeptide elongation. Inhibition of protein synthesis by DB in vitro also occurs at the elongation stage, and it was shown previously that DB prevents EF-2-dependent translocation in partial reaction models of protein synthesis. This inhibition of translocation displays an absolute requirement for EF-1α; however, the dependence upon EF-1α was previously unexplained. It is shown here that DB binds only weakly to EF-1α/GTP in solution, but binds to ribosome·EF-1α complexes with a dissociation constant $K_d = 4\ \mu M$. Thus, the inhibition of protein synthesis by DB appears to involve an interaction with both EF-1α and ribosomes in which all three components are required. Using diphtheria toxin-mediated ADP-ribosylation to assay for EF-2, it is demonstrated that DB blocks EF-2 binding to pre-translocative ribosome·EF-1α complexes, thus preventing ribosomal translocation. Based on this model for protein synthesis inhibition by DB, and the proposed mechanism of action of fusidic acid, evidence is presented in support of the Grasmuk model for EF-1α function in which this elongation factor does not fully depart the ribosome during polypeptide elongation.

Inhibition of protein biosynthesis has been proposed to account for the cytotoxicity of several potential antitumor agents, including the didemnins (*1*−*5*), and bouvardins (RA-peptides; *6*, *7*). While it is difficult to correlate in vitro data with mechanisms of action in vivo, RA-VII, bouvardin, and didemnin B (DB)[1] (Figure 1) all inhibit protein synthesis in cell-free assays as well as in cells, and mechanisms for these inhibitors have been described (*8*−*10*). One of the more unusual mechanisms is displayed by DB. This naturally occurring cyclic peptide inhibits protein synthesis at the elongation stage by inhibiting EF-2-dependent translocation of mRNA through the ribosome (*8*); however, inhibition is dependent upon the presence of EF-1α, and has been proposed to be mediated through the binding of DB to this factor (*11*).

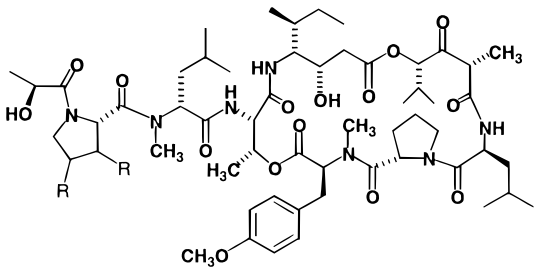


FIGURE 1: Structures of didemnin B (DB) and tritiated didemnin B ($T_2$-DB).

According to the currently accepted model of eukaryotic protein synthesis, EF-1α and EF-2 possess overlapping binding sites on 80S ribosomes and do not bind simultaneously to the same ribosome during polypeptide elongation (*12*). EF-1α delivers the aminoacyl-tRNAs to the ribosome and is subsequently released in a GTP-dependent step prior to the binding of EF-2. Based on this model, it was not immediately obvious how a ligand that binds to EF-1α could inhibit the function of EF-2. To answer this question, we have examined the composition of DB-inhibited ribosomal complexes, with particular emphasis on the presence of specific elongation factors. We have demonstrated that both DB and EF-1α are retained in the inhibited complex, and that didemnin B prevents translocation by inhibiting EF-2 binding. Unexpectedly, while the binding of DB to ribosome·

† This work was supported by NIH Grants R29 CA619178 (P.L.T.) and R01 CA40081 (M.M.J.).

‡ Present address: Parke-Davis Pharmaceutical Research, 2800 Plymouth Rd., Ann Arbor, MI 48105. Tel: (734) 622 1335. FAX: (734) 622 7879. Email: Peter.Toogood@wl.com.

[1] Abbreviations: AcOH, glacial acetic acid; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; DB, didemnin B; DTT, dithiothreitol; EF-1α, eukaryotic elongation factor 1α; EF-2, eukaryotic elongation factor 2; ELISA, enzyme-linked immunosorbant assay; EtOAc, ethyl acetate; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GDPCP, guanylyl-(β,γ-methylene)-diphosphate; HRP, horseradish peroxidase; NMM, 4-methylmorpholine *N*-oxide; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; THF, tetrahydrofuran.

10.1021/bi992202h CCC: $19.00 
Published on Web 03/23/2000

EF-1α complexes could be measured readily, the binding of DB to EF-1α alone was weak. Here, we propose a mechanistic model for the inhibition of protein synthesis by DB and use this inhibitor to probe the association of EF-1α with ribosomes during protein synthesis. In addition, we present new evidence that EF-1α remains associated with ribosomes throughout the elongation cycle.

## MATERIALS AND METHODS

The didemnin B used in this study was provided by the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Fusidic acid and α-sarcin were purchased from Sigma (St. Louis, MO). Rabbit reticulocyte lysates (translation grade) were obtained from Green Hectares (Oregon, WI). Salt-washed ribosomes were obtained from rabbit reticulocyte lysates as described previously (*8*). Elongation factor EF-1α was purified from *S. cerevisiae* according to the literature procedure (*13*). Elongation factors EF-1α and EF-2 from rabbit reticulocytes were provided by Professor William C. Merrick (Case Western Reserve). Rabbit anti-EF-1α was provided by Professor K. Chakraburtty (Wisconsin School of Medicine). Mouse anti-EF-1α was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-EF-2 was a gift from Professor James Bodley (University of Minnesota). [$^{14}$C]-Phenylalanine (497 mCi mmol$^{-1}$), γ-[$^{32}$P]-GTP (30 Ci mmol$^{-1}$), α-[$^{32}$P]-GTP (800 Ci mmol$^{-1}$), [$^{32}$P]-NAD (30 Ci mmol$^{-1}$), and [$^{32}$P]-ATP (3000 Ci mmol$^{-1}$) were obtained from New England Nuclear (Wilmington, DE). γ-[$^{32}$P]-GTP and α-[$^{32}$P]-GTP were diluted prior to use to 440 and 513 dpm pmol$^{-1}$, respectively. Yeast tRNA$^{Phe}$ (Sigma) was charged with [$^{14}$C]-phenylalanine using crude rabbit reticulocyte aminoacyl-tRNA synthetases as described previously (*8*). Scintillation counting was performed using 5 mL of Scintiverse/sample (Fisher Sientific; Pittsburgh) in a Packard 1600 TR scintillation spectrometer. PBS refers to phosphate-buffered saline containing NaCl (200 mM) and potassium phosphate (10 mM), pH 7.6. Poly(U) buffer contains Tris-HCl (20 mM), pH 7.5, $MgCl_2$ (10 mM), KCl (100 mM), and DTT (1 mM). ABTS [2,2′-azino-di-(3-ethyl-benthiazoline-6-sulfonic acid)] and ABTS buffer were puchased from Calbiochem (San Diego, CA). The preparation of $T_2$-DB is described in the Supporting Information.

*Analysis for EF-2 Binding to Inhibited Ribosomes from Cell Lysate*. An active translation system was generated (total volume 100 μL) by incubating supplemented rabbit reticulocyte lysate (40 μL), 2.5 M KOAc (4 μL), 25 mM $Mg(OAc)_2$ (6 μL), and drug solution (5 μL; final concentration 50 μM didemnin B or 3 mM fusidic acid or 3 μM α-sarcin) at 37 °C for 30 min. In control assays, the carrier solvent (EtOH or $H_2O$) was used in place of the drug solution. Each assay mixture was replicated 3 times, and the replicates were pooled at the end of the incubation period. The combined assay mixture (400 μL) was rapidly cooled on ice for 10 min, then layered on buffer A [20 mM Tris/HCl, pH 7.6, 2 mM $Mg(OAc)_2$, 5 mM β-mercaptoethanol; 2 mL] containing 0.75 M sucrose, and centrifuged in an 80 Ti rotor at 32 000 rpm for 3 h. (When 3 mM fusidic acid was used as the drug solution, buffer A also contained 3 mM fusidic acid.) The sucrose-cushioned ribosomes were resuspended in buffer B [0.25 M sucrose, 70 mM KCl, 30 mM HEPES/KOH, pH 7.6, 2 mM $Mg(OAc)_2$, 1 mM DTT; 110 μL], and 4 M KCl was added to a final concentration of 0.5 M. The mixture was stirred on ice for 1.5 h, and then centrifuged in an 80 Ti rotor at 32 000 rpm for 12 h at 4 °C. The salt-wash supernatant obtained at this stage contains 95% of the previously ribosome-bound EF-2. An aliquot (15 μL) of this salt wash was added to a reaction mixture containing buffer C (100 mM KCl, 30 mM HEPES/KOH, pH 7.6, 2 mM magnesium acetate, 1 mM DTT), diphtheria toxin (4 μg), and $^{32}$P-labeled β-nicotinamide adenine dinucleotide ($NAD^+$, 500 pmol) in a total volume of 50 μL. This solution was incubated at 37 °C for 60 min, and then precipitated with an equal volume of acetone overnight at −20 °C. The precipitate was collected by centrifugation and analyzed by SDS−PAGE. The resulting gel was visualized using a phosphorimager.

*EF-2 Binding to Salt-Washed Ribosomes Probed Using [$^{32}$P]-GTP*. Salt-washed ribosomes (15 pmol), with or without rabbit EF-2 (80 pmol), in poly(U) buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) were incubated with α-[$^{32}$P]-GTP at 37 °C for 10 min. This solution was diluted with the same buffer, and filtered through a nitrocellulose filter. The filter was dried, and the amount of bound $^{32}$P-labeled phosphate was measured by scintillation counting. EF-2-dependent guanosine phosphate binding to ribosomes was measured in the presence or absence of 50 μM DB. The binding of γ-[$^{32}$P]-GTP to ribosomes was determined similarly.

*[$^3$H]-DB Binding to the Ribosomes in Rabbit Reticulocyte Lysate*. Reaction mixtures (total volume 200 μL) contained rabbit reticulocyte lysates supplemented with 25 μM hemin and 50 μg mL$^{-1}$ creatine kinase (120 μL), 100 mM KOAc (8 μL), 1.5 mM $Mg(OAc)_2$ (12 μL), and [$^3$H]-DB (660 dpm pmol$^{-1}$). A "blank" assay was prepared without cell lysate. The reaction mixtures were incubated at 37 °C for 30 min, and then cooled rapidly on ice. Next, the reaction mixtures were layered on 1 mL of buffer A containing 0.75 M sucrose, and centrifuged in an 80 Ti rotor at 32 000 rpm for 3 h at 4 °C. The ribosome pellet obtained was resuspended in buffer D (10 mM Tris/HCl, pH 7.6, 1 mM $MgCl_2$; 200 μL), and two aliquots (80 μL each) were removed for scintillation counting.

*DB Binding to Rabbit Reticulocyte Ribosomes Measured by Equilibrium Dialysis*. A microdialyzer (Bel-Art Products, Pequannock, NJ) was set up such that the two half-cells (maximum volume 100 μL) were separated by a dialysis membrane (MW cutoff 10 000 Da). Two reaction mixtures (total volume 75 μL each) were prepared containing $T_2$-DB (specific activity 0.73 Ci/mmol; concentrations ranging from 48 to 66 μM), 18 mM GDPCP (1 μL), glycerol (8 μL), buffer E (3.8 μL; 500 mM Tris/HCl, pH 7.5, 2 M KCl, 20 mM DTT, 200 mM $MgCl_2$), and either 43.4 μM rabbit EF-1α or buffer F (25 mM Tris/HCl, pH 7.5, 100 mM KCl, 1 mM DTT, 10 mM $MgCl_2$, 25% glycerol; 60 μL). The reaction mixtures were incubated at 37 °C for 10 min, and then briefly cooled on ice. One assay mixture (73 μL) was introduced into each half-cell. The dialysis chamber was placed on a platform shaker (100 rpm) at 4 °C for 24 h. Aliquots (10 μL each) were removed from each half-cell for scintillation counting. To control for nonspecific binding, bovine serum albumin (BSA) was substituted for EF-1α in the assay mixture.

*Analysis for EF-1α in DB-Treated Reconstituted Pretranslocative Complexes*. Rabbit reticulocyte ribosomes (400

pmol), [$^{14}$C]-Phe-tRNA (50 pmol), poly(U) (0.2 mg), EF-1α (200 pmol), GTP (1 mM), and DB (50 μM) were incubated in poly(U) buffer at 37 °C for 30 min. A sample (100 μL) of this mixture was removed and diluted in poly(U) buffer (300 μL), and then filtered through a nitrocellulose filter, rinsing with more poly(U) buffer (3 × 1 mL). This filter was dried (60 °C, 10 min) and used to measure the amount of bound [$^{14}$C]-Phe-tRNA by scintillation counting. The remaining assay mixture was layered onto a sucrose cushion (400 μL) containing sucrose (0.25 M) in poly(U) buffer and centrifuged at 48 000 rpm in an 80 Ti rotor for 1.75 h. The clear pellet obtained was rinsed with poly(U) buffer (2 × 1 mL) and then resuspended in the same buffer (250 μL). The amount of ribosomes in this suspension was determined by measuring the absorbance at 260 nm. Samples of the suspension, each containing 10 pmol of ribosomes, were aliquoted into separate wells in a 96-well plate containing PBS (100 μL per well) for analysis of the EF-1α content. The plate was incubated at 30 °C for 90 min with gentle shaking. Excess liquid was removed from the wells, and the plate was blocked by incubating overnight with a solution of PBS buffer containing 3% nonfat dried milk and 0.01% Tween 20 (100 μL per well). The blocking solution was removed, and the wells were rinsed with PBS (2 × 300 μL). To each well was added a solution of anti-EF-1α antibodies in PBS containing 2% nonfat dried milk and 0.1% Tween 20. Following incubation at 30 °C for 90 min, the antibody solution was removed, and the wells were rinsed with PBS (2 × 300 μL). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies in PBS (100 μL) containing 2% nonfat dried milk and 0.1% Tween 20 were added, and the samples were incubated at 30 °C for 90 min. The wells were drained and rinsed as before. A solution of ABTS in ABTS buffer (100 μL, Calbiochem; prepared as described in the manufacturer's instructions) was added, and the mixture was incubated at 37 °C for 30 min. The absorbance of the colored product was measured at 405 nm.

*Analysis for Guanine Nucleotide Binding to Reconstituted Pre-translocative Complexes*. A mixture containing [$^{14}$C]-Phe-tRNA (50 pmol), EF-1α (243 pmol), ribosomes (423 pmol), poly(U) (200 μg), DB (50 μM), and γ-[$^{32}$P]-GTP (100 μM) or α-[$^{32}$P]-GTP (100 μM) in poly(U) buffer (150 μL) was incubated at 37 °C for 30 min. This mixture was overlaid on a 0.25 M sucrose cushion in poly(U) buffer (250 μL) and centrifuged at 48 000 rpm in an 80 Ti rotor for 1.75 h. The pellet obtained was washed with poly(U) buffer (2 × 3 mL) and then resuspended in the same buffer (1.5 mL). The amount of ribosomes was determined by measuring the absorbance at 260 nm. The amount of labeled nucleotide bound to the ribosomes was determined by scintillation counting of an aliquot of this mixture using an energy window of 50−1700 keV. This window counts $^{14}$C disintegrations with approximately 10% efficiency. Given the much lower specific activity of [$^{14}$C]-Phe-tRNA vs [$^{32}$P]-GTP, and the fact that the $^{14}$C-label is present in all of the samples, including the controls, the error in counting $^{32}$P due to the presence of $^{14}$C in these experiments is considered to be negligible.

*Western Analysis for EF-1α Associated with Ribosomes from Rabbit Reticulocyte Lysate*. Reaction mixtures (total volume 50 μL) prepared using supplemented rabbit reticulocyte lysate (20 μL), 2.5 M KOAc (2 μL), 25 mM $Mg(OAc)_2$ (3 μL), drug solution (5 μL; final concentration 50 μM DB or 3 mM fusidic acid), or the carrier solvent (10% aqueous EtOH or $H_2O$) were incubated at 37 °C for 30 min. The assay mixtures were cooled on ice, and then layered on buffer A (250 μL) containing 0.75 M sucrose and centrifuged in a Type 35 rotor at 32 000 rpm for 3 h at 4 °C. The ribosome pellet was briefly rinsed in buffer A, resuspended in 2× Laemmli buffer (80 μL), and then heated at 90 °C for 10 min. Twenty-two microliters of each assay was loaded on a Biorad mini-gel and the gel run under standard operating conditions. After SDS−PAGE, the protein was transferred onto a nitrocellulose membrane by electroblotting. The membrane was soaked in reconstituted 2% nonfat dried milk in PBS buffer overnight at 4 °C to block nonspecific sites. The membrane was washed twice with PBS buffer and then incubated with EF-1α antibody (200 μg/166 μL; Upstate Biotechnology) as a 1:1000 dilution in PBS buffer containing 2% nonfat dried milk and 0.1% Tween 20 for 1.5 h at room temperature with gentle agitation. The excess antibody was washed off with 0.2% nonfat dried milk in PBS. Next, the membrane was incubated with horseradish peroxidase-conjugated IgG goat anti-mouse antibody (1 mg mL$^{-1}$; NEN Life Science, Boston, MA) as a 1:10 000 dilution in PBS buffer containing 2% nonfat dried milk and 0.1% Tween 20 for 1 h at room temperature with gentle agitation. The membrane was washed with PBS buffer, and then incubated with a mixture of equal parts of Luminol/Enhancer solution and peroxide solution (Pierce, Rockford, IL) for at least 5 min. The membrane was placed in protective plastic wrap and exposed to autoradiographic film. The film was developed (Kodak developer/fixer solution) and dried. The relative band intensities were quantified using a densitometer and analyzed using the ImageQuant software.

## RESULTS

*DB Inhibits EF-2 from Binding to the Ribosome Complex.* Ribosome-associated EF-2 was measured by ADP-phosphorylation using [$^{32}$P]-NAD and diphtheria toxin as follows. Ribosomes were isolated from rabbit reticulocyte lysate by centrifugation through a sucrose cushion; then the bound EF-2 was released by treatment of the isolated ribosomes with 0.5 M KCl, and ADP-ribosylated according to the procedure of Nygard and Nilsson (*14*). A single polypeptide with molecular mass ~100 kDa was radiolabeled under these conditions, corresponding to ADP-ribosylated EF-2. Addition of DB to the lysate consistently reduced the amount of EF-2 detected (Figure 2A). In control experiments, the addition of fusidic acid increased the amount of EF-2 bound to ribosomes as expected for this inhibitor, which prevents the release of EF-2·GDP from ribosomes following a single round of translocation (Figure 2B) (*15*−*17*). These results indicate that DB interferes with EF-2 binding to ribosomes in whole lysate. In contrast, DB failed to inhibit EF-2 binding to ribosomes stripped of translation factors by treatment with 0.5 M KCl (*18*), as determined by measuring EF-2-dependent guanosine phosphate binding (Table 1; *19*). Thus, DB inhibits EF-2 binding to ribosomes indirectly.

*DB Binds Weakly to EF-1α in Solution.* Previous results implicate EF-1α as the translation factor responsible for mediating the inhibition of EF-2 binding by DB (*8*). To examine binding of DB to EF-1α, tritiated DB ($T_2$-DB) was prepared synthetically as described in the Supporting Infor-

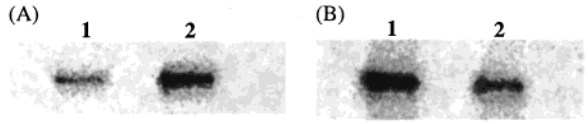


FIGURE 2: Phosphorimaged analysis of [$^{32}$P]-ADP-ribosylated EF-2 following PAGE. (A) A comparison of sample prepared with and without DB. (B) A comparison of sample prepared with and without fusidic acid. Rabbit reticulocyte lysate was incubated with (A) 50 $\mu$M DB (lane 1) or 10% EtOH (control; lane 2) and (B) 3 mM fusidic acid (lane 1) or $H_2O$ (control; lane 2) for 30 min at 37 °C. Following isolation of the ribosome complex by ultracentrifugation, ribosome-bound EF-2 was radiolabeled using [$^{32}$P]-NAD$^+$ and diphtheria toxin. The reaction mixtures were subjected to SDS−PAGE, and then phosphorimaged. The obtained image was analyzed by ImageQuant software. Intensity integrations are as follows: (A) lane 1, 4.9 units; lane 2, 20.7 units; (B) lane 1, 30 units; lane 2, 8.5 units.

Table 1: Effect of DB on EF-2-Dependent GTP Binding to Salt-Washed Ribosomes[a]

| | α-[$^{32}$P]-GTP bound (pmol) | |
|---|---|---|
| | −DB (50 $\mu$M) | +DB (50 $\mu$M) |
| −EF-2 | 3.6 | 6.7 |
| +EF-2 | 7.8 | 9.9 |

[a] Results were obtained as described under Materials and Methods. Data represent the mean of two independent determinations.

mation. Binding then was assessed by equilibrium dialysis. Equal concentrations of $T_2$-DB and GDPCP were placed on either side of a dialysis membrane to achieve rapid ligand equilibration, while a fixed concentration of rabbit EF-1α (kindly provided by Prof. W. C. Merrick) was added only to one half-cell. Following equilibration, aliquots were removed for scintillation counting from each half-cell. A slight increase (10−20%) in radioactive counts in the EF-1α-containing cell was consistently observed at the end of a 24 h incubation period with no further increase in counts even after 48 h, suggesting that an EF-1α·GDPCP·DB complex had formed. The dissociation constant $K_d$ for the complex was determined to be 211.1 ± 83.8 $\mu$M. This relatively high $K_d$ value indicates that there is only a weak association between DB and EF-1α·GDPCP. In control experiments, no binding could be detected between DB and bovine serum albumin.

*Analysis for EF-1α Bound to Pre-translocative Ribosomes.* The weak association between DB and EF-1α in solution could indicate that DB primarily recognizes ribosome-bound EF-1α. Previous mechanistic studies suggest that DB locks ribosomes in a pre-translocative state. To further examine this model, we first attempted to establish whether EF-1α remains bound to ribosomes that have been inhibited by DB. Using anti-EF-1α antibodies in an ELISA format, EF-1α was detected in association with pre-translocative complexes prepared from ribosomes, EF-1α, GTP, poly(U), and Phe-tRNA (control samples contained no EF-1α). The addition of DB to these complexes did not significantly alter the amount of EF-1α detected. This result indicates that EF-1α is a component of the pre-translocative complex and is not displaced by DB (Table 2).

*EF-1α Remains Bound to Ribosomes from DB-Treated Cell Lysate*. The effect of DB on EF-1α binding to ribosomes in cell lysate was further evaluated by quantifying the amount of EF-1α associated with ribosomes in the presence and

Table 2: ELISA Assay[a] for Binding of EF-1α to Reconstituted Pre-translocative Ribosomes

| | absorbance at 405 nm (AU) |
|---|---|
| −DB | 1.12 ± 0.14 |
| +DB | 1.18 ± 0.06 |

[a] Assays were performed as described under Materials and Methods. The background absorbance measured in the absence of EF-1α was 0.64 ± 0.1 absorbance unit.

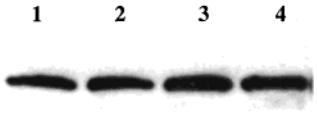


FIGURE 3: Western-blot analysis of ribosome-bound EF-1α following SDS−PAGE. Rabbit reticulocyte lysate was incubated either in the absence (lane 1) or in the presence (lane 2) of 50 $\mu$M DB, or in the absence (lane 3) or presence (lane 4) of 3 mM fusidic acid for 30 min at 37 °C. The ribosome complex was isolated by ultracentrifugation, and then subjected to SDS−PAGE. The presence of EF-1α was detected by Western blot analysis and quantified by densitometry. Intensity integrations, in arbitrary units, are as follows: lane 1, 2213; lane 2, 2401; lane 3, 3449; lane 4, 2863.

absence of DB. Rabbit reticulocyte lysate was incubated at 37 °C for 30 min in the presence or absence of DB; then the ribosome-containing complex was isolated by ultracentrifugation and subjected to SDS−PAGE. The presence of EF-1α in the individual assay mixtures was detected by Western blot analysis and quantified by densitometry. As can be seen in Figure 3, the amount of ribosome-bound EF-1α is the same in both the DB-treated and the DB-free samples. This result confirms the presence of EF-1α in inhibited ribosomal complexes and indicates that DB has no significant effect on the binding of EF-1α to ribosomes. When fusidic acid was used in place of DB in the same assay, no significant difference was observed in the amount of EF-1α bound to ribosomes from fusidic acid-treated and untreated samples (Figure 3).

*DB Binds to Ribosome·EF-1α Complexes*. The extent of didemnin binding to ribosome·EF-1α complexes was analyzed by titration of translation grade rabbit reticulocyte lysate with $T_2$-DB, followed by isolation of the ribosomes by centrifugation. The amount of ribosome-associated $T_2$-DB was determined by scintillation counting, and the data were analyzed using a Scatchard plot as shown in Figure 4. From this analysis, the dissociation constant for DB binding to the ribosome·EF-1α complex is $K_d$ = 4.1 $\mu$M, and the number of binding sites for DB on the ribosome·EF-1α complex is 1.7. This analysis suggests that the stoichiometry of DB binding to ribosomes is approximately 2:1. To examine whether DB could bind to ribosomes alone, we quantified the amount of $T_2$-DB bound to salt-washed ribosomes (i.e., ribosomes stripped of all initiation and elongation factors by treatment with 0.5 M KCl). The extent of $T_2$-DB binding to these salt-washed ribosomes was 10−20% of the binding observed between $T_2$-DB and the complete translation complex. These results show that DB binds to ribosomes with micromolar affinity in a translation factor dependent manner.

*DB Does Not Inhibit EF-1α-Dependent GTP Hydrolysis*. To analyze the guanosine phosphate bound to the pre-translocative ribosomal complex, rabbit reticulocyte ribosomes were incubated with EF-1α, Phe-tRNA$^{Phe}$, poly(U),

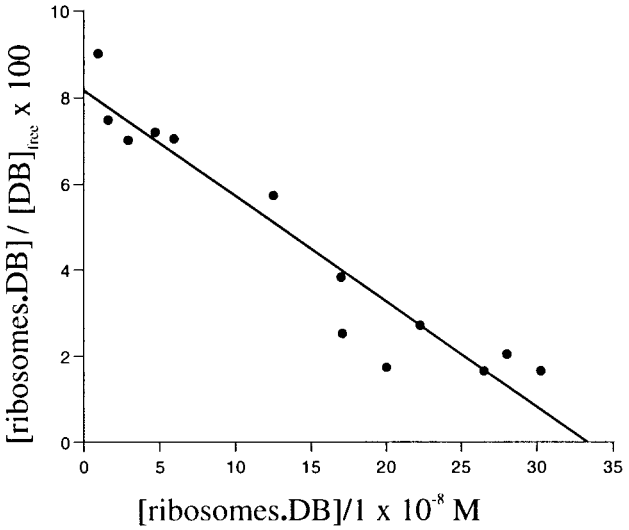


FIGURE 4: Scatchard plot of $T_2$-DB binding to ribosome·EF-1α complexes. The dissociation constant for DB binding was obtained from the slope of this plot, and the stoichiometry of binding is indicated by the *x*-intercept.

and either α-[$^{32}$P]-GTP or γ-[$^{32}$P]-GTP, with or without DB (50 μM). The pre-translocative complex formed from these components was isolated from free protein and nucleotides by centrifugation through a sucrose cushion, rinsed, and analyzed for GTP by nitrocellulose filter binding and scintillation counting. The amount of ribosomes isolated by this procedure was determined by measuring the absorbance at 260 nm of a sample of the ribosomal pellet. In the absence of DB, the ratio of $^{32}$P-labeled guanosine phosphate to ribosomes was approximately 2:1 when using α-[$^{32}$P]-GTP and 1:1 when using γ-[$^{32}$P]-GTP, suggesting that one of the GTP molecules is cleaved to GDP prior to isolation of the complex (Table 3). The same results were obtained in the presence of DB, indicating that DB does not affect EF-1α-dependent guanosine phosphate binding, or GTP hydrolysis on the ribosome.

Table 3: Binding of Labeled Guanosine Phosphates to Pre-translocative Ribosomes[a]

| DB (μM) | ribosomes (pmol) | guanosine phosphate (pmol) | guanosine:ribosomes ratio |
|---|---|---|---|
| | | α-[$^{32}$P]-GTP | |
| 0 | 58 | 131 | 2.0 |
| 50 | 45 | 137 | 3.0 |
| 0 | 45 | 101 | 2.2 |
| 50 | 39 | 107 | 2.7 |
| | | γ-[$^{32}$P]-GTP | |
| 0 | 118 | 179 | 1.5 |
| 50 | 112 | 189 | 1.7 |
| 0 | 61 | 56 | 0.9 |
| 50 | 55 | 50 | 0.9 |

[a] Experiments were performed as described under Materials and Methods.

## DISCUSSION

The cyclic depsipeptide DB possesses several interesting biological activities (*5*, *21*). It is immunosuppressive and inhibits T-cell blastogenesis at picomolar concentrations (*22*); it prevents cell proliferation at nanomolar concentrations (*23*); and it is cytotoxic at nanomolar to micromolar concentrations against a wide range of human tumor cell types (*24*). The observation that DB inhibits protein synthesis in cells led to the suggestion that this activity might represent the primary mechanism of cytotoxicity for this agent (*2*−*4*). Didemnins inhibit protein synthesis in cell-free assays indicating that they can interfere directly with the translational machinery (*8*, *25*). Comparison of the inhibitory potency of various didemnin analogues (Ahuja et al., unpublished results), and the identification of EF-1α as a didemnin binding protein (*11*), indicates that the inhibition of protein synthesis by didemnins occurs as the result of a specific recognition event rather than through nonspecific interference with the ribosome. However, several results indicate that the immunosuppressive and the antiproliferative properties of didemnins in cells are mediated through a different mechanism. For example, DB inhibits T-cell blastogenesis at concentrations approximately 3 orders of magnitude lower than required to inhibit general protein synthesis in the same cells (*22*). It is active in the mixed-lymphocyte reaction at concentrations below which it is cytotoxic toward lymphocytic cells (*21*). In addition, the DB analogue nordidemnin inhibits *myo*-inositol uptake into $WRK_1$ cells at concentrations that do not significantly affect the expression of vasopressin receptors (*26*). We have shown recently that inhibition of protein synthesis is insufficient to induce apoptosis in DB-sensitive cell lines (*27*). A report of didemnin binding to protein palmitoyl thioesterase may lead to the delineation of other mechanisms that could explain these effects of didemnins, but the significance of this protein to didemnin function is presently unclear (*28*, *29*).

Although inhibition of protein synthesis may not explain all of the biological properties of DB, we saw several reasons to study this activity in more detail. First, the mechanism of inhibition of protein synthesis by DB is unusual, making this molecule a useful probe for studying the details of protein synthesis in eukaryotes. Second, the concentrations of didemnins required to inhibit protein biosynthesis in vitro are similar to the cytotoxic concentrations ($LC_{50}$) of DB determined in screens against 60 human tumor cell lines.[2] If the toxicity associated with the clinical use of didemnins is related to the ability of these compounds to inhibit protein synthesis, then an understanding of how DB inhibits protein synthesis may facilitate the design of less toxic didemnins for therapeutic application. Third, the inhibition of translation by DB is mediated by EF-1α, and this protein has been linked to metastatic potential (*30*, *31*) and increased susceptibility to transformation (*32*, *33*); the expression of a closely related protein is associated with a human hepatic carcinoma (*34*). Together, these observations suggest that EF-1α may be an appropriate target for novel antitumor or antimetastatic agents. With these considerations in mind, we have examined the composition of the inhibited ribosomal complex resulting from treatment of rabbit reticulocyte lysate with DB, and we have determined in more detail the mechanism by which DB prevents ribosomal translocation.

We envisaged two possibilities for the way that DB inhibits translocation: inhibition of EF-2 function or inhibition of EF-2 binding. Although only the latter possibility is consistent with the generally accepted model for eukaryotic elongation, some workers have provided evidence that this

[2] $LC_{50}$ values for DB were determined by the Drug Synthesis and Chemistry Branch of the National Cancer Institute.

model may not be entirely correct (see below). In probes of EF-2 binding to ribosomes in reticulocyte lysate, DB consistently reduced the amount of EF-2 to background levels similar to those obtained using α-sarcin[3] (Figure 2; *35, 36*). Thus, DB appears generally to inhibit translocation by preventing EF-2 binding.

We have shown previously that ribosomal translocation is not prevented by DB in the absence of EF-1α, suggesting that EF-2 binding to ribosomes may not be inhibited directly (*8*). Consistent with this hypothesis, DB does not inhibit EF-2-dependent GTP binding to ribosomes stripped of translation factors using 0.5 M KCl (Table 1). Thus, the inhibition of EF-2 binding by DB appears to be an indirect result of interactions between didemnin B and a translation factor, most likely EF-1α. Further support for the formation of a complex between DB and EF-1α is provided by the demonstration that *N*-Ac-DA binds to EF-1α (*11*). However, using a variety of different approaches, including co-immunoprecipitation and gel-filtration, we initially were unable to detect any significant binding of DB to EF-1α either in the presence or in the absence of GTP. After much experimentation to identify appropriate conditions, we eventually were able to detect weak binding using microdialysis. From these experiments, we determined a $K_d \sim 0.2$ mM for DB binding to EF-1α/GDPCP. In control experiments, no binding was detected to bovine serum albumin. This result is in contrast to the observation of Crews and co-workers, who estimated a $K_d = 15$ μM for *N*-acetyl-DA binding to EF-1α/GTP by gel-filtration, and is higher than may be expected based on the $IC_{50}$ for inhibition of protein synthesis by DB.

In contrast, DB binding to ribosomes isolated directly from cell lysate occurs with $K_d = 4$ μM (Figure 4). This value is similar to the $IC_{50}$ for inhibition of protein synthesis in vitro ($4.0 \pm 0.5$ μM)[4] and to the $K_d$ determined for *N*-Ac-DA binding to EF-1α in solution (15 μM). The presence of EF-1α in association with these ribosomes was verified by Western analysis (Figure 3), and this result was supported by data from reconstituted pre-translocative complexes (Table 2). We did not observe significant binding of DB to salt-washed ribosomes that are no longer associated with translation factors, supporting the notion that DB binding is elongation factor dependent. The stoichiometry of DB binding to ribosomes was calculated to be approximately 2:1, which might explain the steep concentration dependence exhibited by didemnins in assays of protein synthesis inhibition (*8*). We can speculate either that both DB molecules bind to a single EF-1α molecule or that while one DB molecule binds to EF-1α the other molecule binds somewhere else on the ribosome·EF-1α complex. Alternatively, the 2:1 stoichiometry of binding could indicate the presence of two molecules of EF-1α on the ribosome, each bound to a single DB molecule. Janssen and co-workers have postulated that two EF-1α chains can associate with a single ribosome (*37*). Moreover, other studies have indicated that EF-Tu, the prokaryotic equivalent of EF-1α, can form a pentameric complex containing 2 GTP molecules and 2 aminoacyl-tRNAs (*38, 39*).

According to the generally accepted model of eukaryotic protein synthesis (*12, 40, 41*), EF-1α functions similarly to its prokaryotic analogue EF-Tu. A ternary complex is formed between EF-1α, GTP, and aminoacyl-tRNA, which delivers the aminoacyl-tRNA to the ribosomal A-site. Once the aminoacyl-tRNA is bound to the ribosome, EF-1α hydrolyzes a molecule of GTP to GDP and is released from the ribosome as an EF-1α·GDP complex. The GDP must be replaced by GTP in the cytosol before the factor can bind and deliver another aminoacyl-tRNA molecule. EF-2 is thought not to bind to ribosomes when EF-1α is present at the ribosomal A-site. Several pieces of evidence have been presented that are consistent with the hypothesis that EF-1α and EF-2 possess overlapping binding sites on the ribosome (*14, 42*−*45*).

On binding to the ribosome·EF-1α complex, DB could prevent EF-2 binding by inhibiting EF-1α release from the ribosomal A-site. The observed stimulation in Phe-tRNA$^{Phe}$ binding to ribosomes as a function of DB would be consistent with this hypothesis (*8*). Since GTP hydrolysis is required for EF-1α release from the ribosomal A-site, one way in which DB could prevent EF-1α release is by inhibiting GTP hydrolysis. Therefore, the effect of DB upon EF-1α-mediated GTP hydrolysis was examined by analyzing the nature of the guanosine phosphate bound to ribosomes in an EF-1α-dependent fashion in the presence and absence of DB. In two independent experiments using either α-[$^{32}$P]-GTP or γ-$^{32}$P-labeled GTP, the total amount of guanosine phosphate bound to ribosomes was found to be independent of DB. Moreover, the ratio of GTP to GDP was unaltered by the addition of DB (Table 3). This observation suggests that DB does not inhibit EF-1α-dependent GTP hydrolysis. Independently, Crews and co-workers showed that *N*-acetyl-DA stimulates GTP hydrolysis by EF-1α in vitro in the absence of aminoacyl-tRNA or ribosomes. Also, we have shown previously that peptide bond formation can still occur in the presence of DB (*8*). Since peptide bond formation does not occur without prior GTP hydrolysis (*46*), it seems unlikely that DB is an inhibitor of EF-1α GTPase activity.

We sought to probe more directly the effect of DB upon EF-1α binding to ribosomes by quantifying the amount of EF-1α associated with the ribosome in the presence and absence of the inhibitor. According to the accepted model for polypeptide elongation, EF-1α and EF-2 possess overlapping binding sites on the ribosome and bind in a mutually exclusive manner. Therefore, we expected the amount of EF-1α bound to ribosomes to increase in the presence of DB since the amount of EF-2 decreased; however, as stated above, we found instead that the amount of ribosome-bound EF-1α was the same for both DB-treated and DB-free samples. To examine this result further, we used fusidic acid in place of DB in the same assay. Again, according to the standard model, the retention of EF-2 on ribosomes by fusidic acid should prevent EF-1α from associating with these ribosomes; however, addition of fusidic acid did not alter the amount of ribosome-bound EF-1α. *This result, together*

[3] α-Sarcin prevents both EF-1α and EF-2 binding to ribosomes (*51*). However, at concentrations sufficient to completely inhibit protein synthesis, α-sarcin failed to reduce to zero the amount of EF-2 associated with ribosomes. This result suggests that a subpopulation of the ribosomes in the cell lysate are not participating in protein synthesis and therefore are not susceptible to inhibition by either didemnin B or α-sarcin. These ribosomes may exist in an EF-2-bound terminated state as proposed by Davydova and co-workers (*52*).

[4] This $K_d$ value is slightly lower and more accurate than the previously published value as a result of repeated determinations over a 5 year period.

*with the known mechanism of fusidic acid, is not compatible with the classical model proposed for the association of EF-1α with the ribosome; however, it is consistent with the model proposed by Grasmuk and co-workers* (*47*, *48*). According to the Grasmuk model, EF-1α is not released from the ribosome following aminoacyl-tRNA binding, but remains bound throughout the elongation cycle, although not necessarily at the ribosomal A-site. This view has been supported by Crechet and Parmegianni (*49*) but is generally disregarded in reviews of this area (*12*). Retention of EF-1α on the ribosome, throughout polypeptide elongation, would explain the inability of both DB and fusidic acid to alter the amount of ribosome-bound EF-1α.

Based on the totality of our data, we propose the following model for the inhibition of protein synthesis by DB. DB binds to ribosome·EF-1α complexes in a manner that does not inhibit the EF-1α-dependent or ribosome-catalyzed reactions, but that does inhibit EF-2 binding. We submit two possible mechanisms by which DB may block EF-2 from associating with the inhibited ribosome: (1) **Competitive**: DB binds to EF-1α such that either DB or EF-1α physically occupies the binding site on the ribosome where EF-2 normally binds; i.e., DB/EF-1α competes with EF-2 for the same binding site. (2) **Uncompetitive**: DB binds to the ribosome·EF-1α complex and locks the complex in a conformation that disfavors EF-2 binding. A similar mechanism has been proposed for ricin (*50*). Either mechanism is consistent with the observation that increasing the amount of EF-2 can overcome DB inhibition (*8*).

In conclusion, we have found that DB inhibits ribosomal translocation by indirectly preventing EF-2 from binding to ribosomes. The present work is consistent with the hypothesis that EF-1α is the principal site of action for inhibition of protein synthesis by DB, but indicates that the part played by EF-1α is more complex than initially appreciated. Two mechanisms have been proposed for how DB may prevent EF-2 binding. Both of these mechanisms invoke the Grasmuk model for the role of EF-1α during polypeptide elongation, a model that is supported by our experiments. Contrary to accepted dogma, EF-1α appears not to cycle on and off the ribosome during polypeptide elongation.

## SUPPORTING INFORMATION AVAILABLE

Experimentals for the preparation of $T_2$-DB and proton and tritium NMR spectra for $T_2$-DB (7 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

## REFERENCES


1. Rinehart, K. L., Jr., Gloer, J. B., Hughes, R. G., Jr., Renis, H. E., McGovren, J. P., Swynenberg, E. B., Stringfellow, D. A., Kuentzel, S. L., and Li, L. H. (1981) *Science 212*, 933−935.
2. Rinehart, K. L., Jr., Gloer, J. B., Wilson, G. R., Hughes, R. G., Jr., Li, L. H., Renis, H. E., and McGovren, J. P. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 87−90.
3. Li, L. H., Timmins, L. G., Wallace, T. L., Krueger, W. C., Prairie, M. D., and Im, W. B. (1984) *Cancer Lett. 23*, 279−288.
4. Crampton, S. L., Adams, E. G., Kuentzel, S. L., Li, L. H., Badiner, G., and Bhuyan, B. K. (1984) *Cancer Res. 44*, 1796−1801.
5. Li, W.-R., and Joullie, M. M. (1992) *Stud. Nat. Prod. Chem. 10*, 241−302.
6. Itokawa, H., and Takeya, K. (1993) *Heterocycles 35*, 1467−1501.
7. Itokawa, H. (1991) *Top. Pharm. Sci.*, 607−630.
8. SirDeshpande, B. V., and Toogood, P. L. (1995) *Biochemistry 34*, 9177−9184.
9. SirDeshpande, B. V., and Toogood, P. L. (1995) *Bioorg. Chem. 23*, 460−470.
10. Zalacain, M., Zaera, E., Vazquez, D., and Jimenez, A. (1982) *FEBS Lett. 51*, 869−900.
11. Crews, C. M., Collins, J. L., Lane, W. S., Snapper, M. L., and Schreiber, S. L. (1994) *J. Biol. Chem. 269*, 15411−15414.
12. Merrick, W. C., and Hershey, J. W. B. (1996) in *Translational Control* (Hershey, J. W. B., Mathews, M. B., and Sonnenberg, N., Eds.) pp 31−70, Cold Sping Harbor Press, Cold Spring Harbor, NY.
13. Thiele, D., Cotrelle, P., Iborra, F., Buhler, J.-M., Sentenac, A., and Fromageot, P. (1986) *J. Biol. Chem. 260*, 3084−3089.
14. Nygard, O., and Nilsson, L. (1984) *Eur. J. Biochem. 145*, 345−350.
15. Bodley, J. W., Lin, L., Salas, M. L., and Tao, M. (1970) *FEBS Lett. 11*, 153−156.
16. Bodley, J. W., Zieve, F. J., and Lin, L. (1970) *J. Biol. Chem. 45*, 5662−5667.
17. Bodley, J. W., Zieve, F. J., Lin, L., and Zieve, S. T. (1970) *J. Biol. Chem. 21*, 5656−5661.
18. Merrick, W. C. (1979) *Methods Enzymol. 60*, 108−123.
19. Bermek, E., and Matthaei, H. (1971) *J. Biol. Chem. 10*, 4906−4912.
20. Davydova, E. K., Sitikov, A. S., and Ovchinnikov, L. P. (1984) *FEBS Lett. 176*, 401−405.
21. Sakai, R., Rinehart, K. L., Kishore, V., Kundu, B., Faircloth, G., Gloer, J. B., Carney, J. R., Namikoshi, M., Sun, F., Hughes, R. G., Jr., Gravalos, D. G., de Quesada, T. G., Wilson, G. R., and Heid, R. M. (1996) *J. Med. Chem. 39*, 2819−2834.
22. Montgomery, D. W., and Zukoski, C. F. (1985) *Transplantation 40*, 49−56.
23. Teunissen, M. B. M., Pistoor, F. H. M., Rongen, H. A. H., Kapsenberg, M. L., and Bos, J. D. (1992) *Transplantation 53*, 875−881.
24. Jiang, T. L., Liu, R. H., and Salmon, S. E. (1983) *Cancer Chemother. Pharmacol. 11*, 1−4.
25. Legrue, S. J., Sheu, T.-L., Carson, D. D., Laidlaw, J. L., and Sanduja, S. K. (1988) *Lymphokine Res. 7*, 21−29.
26. Dominice, C., Dufour, M.-N., Patino, N., Manzoni, P., Jouin, P., and Guillon, G. (1994) *J. Pharmacol. Exp. Ther. 271*, 107−117.
27. Beidler, D. R., Ahuja, D., Wicha, M. S., and Toogood, P. L. (1999) *Biochem. Pharmacol. 58*, 1067−1074.
28. Crews, C. M., Lane, W. S., and Schreiber, S. L. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4316−4319.
29. Meng, L., Sin, N., and Crews, C. M. (1998) *Biochemistry 37*, 10488−10492.
30. Pencil, S. D., Toh, Y., and Nicolson, G. L. (1993) *Breast Cancer Res. Treat. 25*, 165−174.
31. Taniguchi, S., Miyamoto, S., Sadano, H., and Kobayashi, H. (1991) *Nucleic Acids Res. 19*, 6949.
32. Sonenberg, N. (1993) *Curr. Opin. Cell Biol. 5*, 955−960.
33. Tatsuka, M., Mitsui, H., Wada, M., Nagata, A., Nojima, H., and Okayama, H. (1992) *Nature 359*, 333−336.
34. Koch, I., Hofschneider, P. H., Lottspeich, F., Eckerskorn, C., and Koshy, R. (1990), 839−843.
35. Brigo, Rambelli, F., Zamboni, M., Montanaro, L., Simonetta, J., and Sperti, H. (1989) *Biochem. J. 157*, 723−727.
36. Ahuja, D. (1999) Ph.D. Thesis, University of Michigan.
37. Janssen, G. M. C., Van Damme, H. T. F., Kriek, J., Amons, R., and Moller, W. (1994) *J. Biol. Chem. 269*, 31410−31417.
38. Bilgin, N., and Ehrenberg, M. (1995) *Biochemistry 34*, 715−719.
39. Rodnina, M., and Wintermeyer, W. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1945−1949.
40. Negrutskii, B. S., and El'skaya, A. V. (1998) *Prog. Nucleic Acid Res. Mol. Biol. 60*, 47−78.

41. Merrick, W. C. (1992) *Microbiol. Rev. 56*, 291−315.
42. Baliga, B. S., Schechtman, M. G., and Munro, H. N. (1973) *Biochem. Biophys. Res. Commun. 51*, 406−413.
43. Grasmuk, H., Nolan, R. D., and Drews, J. (1974) *Eur. J. Biochem. 48*, 485−493.
44. Nombela, C., and Ochoa, S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3556−3560.
45. Richter, D. (1973) *J. Biol. Chem. 218*, 2853−2857.
46. Moldave, K. (1985) *Annu. Rev. Biochem. 54*, 1109−1149.
47. Grasmuk, H., Nolan, R. D., and Drews, J. (1976) *Eur. J. Biochem. 71*, 271−279.
48. Grasmuk, H., Nolan, R. D., and Drews, J. (1977) *Eur. J. Biochem. 79*, 93−102.
49. Crechet, J.-B., and Parmeggiani, A. (1986b) *Eur. J. Biochem. 161*, 655−660.
50. Nilsson, L., and Nygard, O. (1986) *Eur. J. Biochem. 161*, 111−117.
51. Brigo, Rambelli, F., Zamboni, M., Montanaro, L., Simonetta, J., and Sperti, H. (1989) *Biochem. J. 157*, 723−727.
52. Davydova, E. K., Malanin, N. L., and Ovchinnikov, L. P. (1993) *Eur. J. Biochem. 215*, 291−296.

BI992202H