Parameters Affecting the Restoration of Activity to Inactive Mutants of Thymidylate Synthase via Subunit Exchange: Further Evidence That Thymidylate Synthase Is a Half-of-the-Sites Activity Enzyme[†]

Ruth L. Saxl,[‡] Li-Ming Changchien,[‡] Larry W. Hardy,[§] and Frank Maley*[,‡]

Wadsworth Center, New York State Department of Health, Empire State Plaza, P.O. Box 509, Albany, New York 12201-0509

Received December 26, 2000; Revised Manuscript Received March 5, 2001

ABSTRACT: In a previous study we demonstrated that *Escherichia coli* thymidylate synthase activity could be restored completely by incubating basically inactive mutants of this enzyme at room temperature with $R_{126}E$, another inactive mutant [Maley, F., Pedersen-Lane, J., and Changchien, L.-M. (1995) *Biochemistry* *34*, 1469−1474]. Since only one of the enzyme's two subunits possessed a functional active site and the restoration of activity could be titrated to be equivalent to that of the wild-type enzyme's specific activity, it was proposed that thymidylate synthase was a half-of-the-sites activity enzyme. We now provide additional support for this thesis by presenting an in-depth analysis of some conditions affecting the restoration of enzyme activity. For this purpose, we employed two mutants with marginal thymidylate synthase activity, $Y_{94}A$ and $R_{126}E$. The parameters that were examined included pH, concentration of protein, temperature, and urea concentration, all of which influenced the rate of activity restoration. It was found, surprisingly, that by maintaining the amount of each protein constant, while increasing the volume of solution, the rate and total activity restored was greatly enhanced. Increasing the pH from 6.0 to 9.0 markedly increased the rate at which the optimal activity was restored, as did increasing the temperature from 4 to 40 °C. A similar effect was obtained when the incubation of the mutants was conducted at 4 °C in the presence of 1.5 M urea, a temperature at which activity is restored extremely slowly. Raising the pH to 9.0 resulted in an almost instantaneous restoration of activity at 4 °C. The manner in which thymidylate synthase activity is restored from the mutants in the presence of varying concentrations of ethanol, ethylene glycol, and glycerol suggests that changes in subunit interaction and enzyme conformation are in part responsible for the observed differences. Most significantly, at solution levels of 10%, ethanol was found to activate, while ethylene glycol inhibited slightly and glycerol was somewhat more inhibitory. At a concentration of 20%, ethanol inhibited rather strikingly, ethylene glycol was slightly more inhibitory than at 10%, and glycerol was strongly inhibitory. Since the net result of these findings is the suggestion that the restoration of thymidylate synthase activity is due to a separation of the mutant dimers into their respective subunits, followed by their recombination to an active heterodimer, evidence for this phenomenon was sought by separating the recombined dimers using nondenaturating polyacrylamide gel electrophoresis. Sequence analysis of the isolated homo- and heterodimers clearly demonstrated that the active enzyme is a product of subunit exchange, one that is very efficient relative to the wild-type enzyme, which did not exchange subunits unless denatured.

Thymidylate synthase (TS)[1] (EC 2.1.1.45), an enzyme first described by Friedkin and Kornberg (*1*), is essential for the survival of most cells due to its critical involvement in the synthesis of DNA. As a consequence it is not surprising that it is one of the most conserved proteins known (*2*).

Since its discovery, the unique mechanism by which TS reduces and transfers a methylene group from CH_2H_4PteGlu to the 5-position of dUMP to yield dTMP has occupied the attention of many laboratories (*2*). As a consequence of X-ray crystallography studies on the substrate/substrate analogue associated structures of *Escherichia coli* TS (*3*−*6*), as well as some intriguing transition state trapping experiments (*7*, *8*), a solution to this problem may be at hand. Still some problems remain to be resolved, particularly those involving the asymmetric manner in which the enzyme's two identical subunits interact with substrate and substrate analogues (*9*), suggesting that only one of the two is catalytically active. This proposal is consistent with numerous studies over a 30 year period that support this thesis (*10*−*18*) and which we first suggested almost 25 years ago (*14*) but for which irrefutable proof could not be presented at that time. More recently, undeniable evidence for this proposal has been

[†] This work was supported in part by Grant CA 44355 from The National Cancer Institute, USPHS/HHS.

* To whom correspondence should be addressed. Telephone: 518-474-4184. Fax: 518-473-2900. E-mail: maley@wadsworth.org.

[‡] New York State Department of Health.

[§] Present address: Arqule Inc., 19 Presidential Way, Woburn, MA 01801.

[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonate; dUMP, 2′-deoxyuridine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; NO_2dUMP, 5-nitro-2′-deoxyuridine 5′-monophosphate; CH_2H_4PteGlu, (*R*,*S*)-5,10-methylenetetrahydrofolate; WT, wild type; TS, thymidylate synthase; DTT, dithiothreitol; PVDF, poly(vinylidene difluoride); TM, triple mutant ($C_{146}W$-$E_{237}R$-$E_{245}R$). Unless specified otherwise, all mutants and WT-TS are considered to be in the dimeric state.

10.1021/bi002925x CCC: $20.00 © 2001 American Chemical Society
Published on Web 04/03/2001

obtained by incubating two basically inactive mutants of *E. coli* TS together, which revealed that not only is one of two subunits active but it is equivalent in activity to that of the wild-type enzyme (*19*). Since, as constituted, only one of the two subunits of the resulting heterodimer can form a single functional active site with a specific activity equivalent to that of WT-TS, it would appear that TS is indeed a half-of-the-sites activity enzyme. Very recent studies based on X-ray analysis of a TS from *Pneumocystis carinii* are in agreement with the half-of-the-sites reactivity mechanism (*20*).

In the present study, we shall present additional evidence for this thesis but will also describe some unusual parameters affecting the interaction of the subunits of this enzyme in solution. It will be shown that while we can describe conditions for separating subunits from inactive mutant homodimers in solution, further study is needed before we can provide a reasonable explanation for how different sets of homodimers dissociate to individual subunits and then reassociate at characteristic rates to form an active heterodimer.

MATERIALS AND METHODS

Preparation of Wild-Type and TS Mutant Enzymes. TS was prepared using a high-expression system, which produced the enzyme to the extent of 50−60% of the cellular protein of *E. coli* (*21*). The enzyme was purified to homogeneity in just three steps: sonication, ammonium sulfate precipitation, and purification through a DE-52 anion-exchange column as described (*21*). The mutants of TS were prepared from the thyA gene with the appropriate oligonucleotides using the Quick-Change kit from Stratagene (La Jolla, CA). The mutant proteins were purified as above for WT-TS by isolating the major peak from the DE-52 chromatography step. Where necessary, a phenyl-Sepharose purification step was included (*22*) after the DE-52 column. SDS−PAGE was used to determine which fractions should be pooled. The enzyme pools were concentrated in an Amicon ultrafiltration device (W. R. Grace & Co., Beverly, MA) with a 10 kDa cutoff filter. Proteins were stored as ammonium sulfate pellets at −70 °C. Prior to use, the proteins were dissolved in a small volume of a solution containing 50 mM potassium phosphate, pH 7.5, 10% ethylene glycol, and 1 mM DTT against which it was thoroughly dialyzed. The dialyzed solutions were usually stored at −20 °C.

Construction and Expression of the Triple Mutant ($C_{146}W$-$E_{237}R$-$E_{245}R$). The double mutant of thyA ($E_{237}R$-$E_{245}R$) (*23*) was cloned into the *Hin*dIII site of pKS^+ (Stratagene, LaJolla, CA) in a manner similar to that described by us for the WT-TS (*21*) to develop a high expression vector. To introduce the $C_{146}W$ mutation into the double mutant, a pKS^+ construct containing the $C_{146}W$−thyA mutant was cleaved with restriction enzymes *Cla*I and *Mlu*I to yield a 762 bp fragment containing the amino-terminal two-thirds of thyA including the codon for C_{146} and a 3355 bp backbone fragment containing $E_{237}R$ and $E_{245}R$. The latter fragment was separated from the smaller by agarose gel electrophoresis and extracted with phenol. The purified backbone fragment was ligated to a 762 bp *Cla*I−*Mlu*I fragment prepared from a similar pKS^+ plasmid containing the $C_{146}W$ thyA gene mutation. Competent *E. coli* (*recA*) were transformed with the ligated DNA, and the DNA from the resulting ampicillin-resistant bacterial clones was analyzed by restriction enzyme analysis to confirm the correct DNA structure for plasmids carried by the bacteria. The DNA for the entire plasmid-borne mutated thyA gene was sequenced in its entirety to confirm the gene and the desired mutants. The overexpression of the encoded gene product was accomplished by transforming *E. coli* X2913 (RecA) with the above pKS^+ plasmid followed by growth in TBYE (tryptone broth yeast extract) containing 50 μg of ampicillin/mL and 10 μg/mL tetracycline, induction with isopropyl β-D-galactopyranoside, and purification as described (*22*). Although this variant of *E. coli* used to express the enzyme differed from that described earlier (*22*), the expression of the TM was still about 50% of the cellular protein.

Measurement of Thymidylate Synthase Activity. Thymidylate synthase activity was measured at 30 °C using a kinetic spectrophotometric assay (*24*) in the presence of 50 mM $MgCl_2$ due to the high Mg^{2+} requirement of this enzyme (*25*). Kinetic assays were performed using a Model 7400 Beckman spectrophotometer equipped with a Peltier temperature controller and an enzyme mechanism software package. CH_2H_4PteGlu was obtained from Schircks Labs (Jona, Switzerland). One unit is defined as the amount of enzyme required to synthesize 1 μmol of dTMP/min at 30 °C under the conditions of the assay. The protein concentration of TS was calculated from the *thyA*−TS amino acid sequence (*26*) using the method of Gill and von Hippel (*27*), which provided a molar extinction coefficient of 0.591×10^5 M^{-1} cm^{-1} at 280 nm. This value is equivalent to 1.71 A_{280}/mg of protein.

Restoration of Activity to Inactive Mutants. Inactive TS mutants ($Y_{94}A$, $C_{146}S$, $C_{146}W$, or $C_{146}W$-$E_{237}R$-$E_{245}R$), specific activities of 0−0.04 unit/mg, which ranged from 0 to 0.4% of the WT-TS activity, were added to a constant concentration of $R_{126}E$. Each reaction was carried out in a volume of 0.5−1.0 mL containing either 25 mM potassium phosphate, pH 7.5, or 25 mM CHES, pH 9.0, 0.02% sodium azide, and 10 mM DTT and incubated for varying periods of time at the specified temperature. DTT was added to stabilize enzyme activity over extended periods of incubation (15−30 h) at 25 °C, although this reducing agent was unnecessary for shorter periods. Usually, 25 μg in 0.5 mL volume (0.41 μM) of $R_{126}E$ was incubated at 25 °C and assayed by removing aliquots (10−50 μL) at the indicated times after the addition of a specific mutant at 10 times the concentration of $R_{126}E$ (250 μg, 4.1 μM).

Nondenaturing Polyacrylamide Gel Electrophoresis. A shift in the migration of the TS homo- and heterodimers on nondenaturing polyacrylamide gel electrophoresis was used to verify the dissociation and exchange of the subunits in the dimer of TS. The following protein solutions were prepared: (1−4) $R_{126}E$ (62 μg); (5−8) WT-TS (160 μg); (9−12) $R_{126}E$ (62 μg) + $C_{146}W$ (172 μg); and (13−16) $R_{126}E$ (62 μg) + TM (171.2 μg). Each solution was incubated at room temperature (25 °C) overnight in approximately 0.4 mL of 25 mM potassium phosphate, pH 7.5, and 5 mM DTT. To form complexes, various ligands were added to the protein solutions corresponding to the respective lanes in Figure 10: lanes 1, 5, 9, and 13, none; lanes 2, 6, 10, and 14, 2 μL of 15 mM FdUMP and 112 μL of 90 mM CH_2H_4PteGlu;

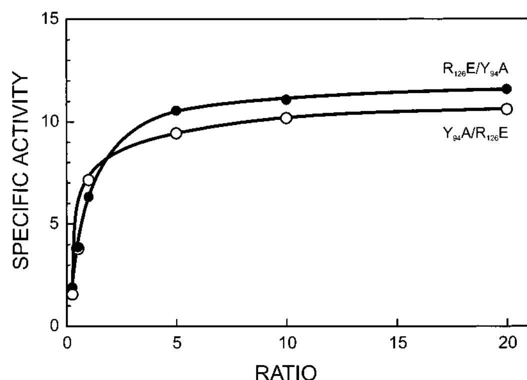

FIGURE 1: Effect of increasing ratio of $Y_{94}A$ to $R_{126}E$ (○) and $R_{126}E$ to $Y_{94}A$ (●) on the restoration of *E. coli* TS activity. The time of incubation at room temperature was 6 h. The first four points represent ratios of 0.25, 0.5, 1.0, and 5.0. See Materials and Methods for the quantities of mutant proteins employed and assay procedures.

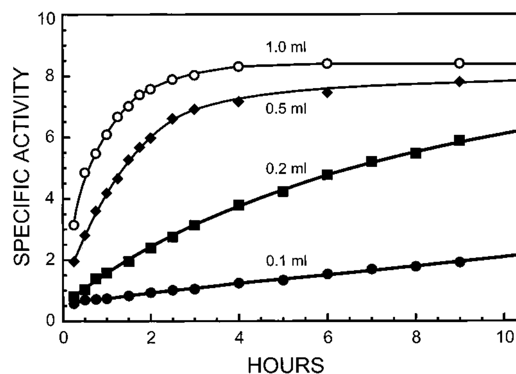

FIGURE 2: Effect on TS activity of maintaining a constant protein concentration of each mutant in the indicated volume of solution. $R_{126}E$ (26.6 μg) was mixed with $Y_{94}A$ (242 μg) in the indicated volume of 25 mM potassium phosphate, pH 7.5.

lanes 3, 7, 11, and 15, 1 μL of 12 mM NO_2dUMP; lanes 4, 8, 12, and 16, 1 μL of NO_2dUMP and 112 μL of 90 mM CH_2H_4PteGlu. In addition, 25 μL of 1 M $MgCl_2$ was added to each solution. The final concentration of the ligands was in excess of the total number of enzyme subunits employed. These aliquots of protein and ligands in 0.5 mL volume were incubated for an additional 6 h at room temperature. Electrophoresis samples were prepared by mixing 15 μL of each protein solution with an equal volume of loading buffer (0.16 M Tris-HCl, pH 6.8, 25% sucrose, 0.08% bromphenol blue). The resulting solutions were pipetted onto a 0.7 mm × 16 cm × 20 cm native gel comprised of a 4.5% stacking gel, pH 6.8, over a 10% running gel, pH 8.8, both of which were cross-linked with 2.7% bisacrylamide (*28*). The gels were run at 4 °C for 20 h at 75 V in a Bio-Rad Protean II gel apparatus using a running buffer of 0.2 M glycine and 30 mM Tris-HCl, pH 8.3. After the completion of the electrophoresis, the gel was stained with Coomassie blue R, then destained with a solution of 20% methanol in 30% acetic acid, and dried.

Sequence Analysis of TS Amino Ends. To sequence the amino ends of WT-TS and its mutants, the nondenaturing gel above was transblotted to a PVDF membrane (Bio-Rad, Hercules, CA), and the membrane was lightly stained with 0.01% Coomassie blue. The transferred proteins were excised and sequenced with a model 477A protein/peptide sequencer from Applied Biosystems (Foster City, CA) using their Blott cartridge.

RESULTS

As indicated earlier (*19*), the rate at which TS activity is restored on mixing $R_{126}E$ with various inactive mutants of TS is a function of the second mutant. Thus, the most rapid reappearance of activity occurred with $Y_{94}A$, and the slowest was with $C_{146}S$. In this study we present an in-depth analysis of parameters affecting the restoration of activity.

Effect of the Ratio of $Y_{94}A$ to $R_{126}E$ and Vice Versa on TS Activity. As described earlier (*4, 29*), each subunit of TS contributes a side chain from its residue 126 to the active site of the other subunit. Therefore, increasing the amounts of $Y_{94}A$ added to a constant level of $R_{126}E$ should eventually reach a plateau in TS specific activity. This plateau is achieved at about a ratio of 10−20/1 of $Y_{94}A$ to $R_{126}E$ (Figure 1). It is of interest to note that in going from a ratio of 1/1 to 20/1 the specific activity increases by only 40%, which suggests rather tight binding between the heterodimer subunits. Similar results were obtained when the level of $Y_{94}A$ was maintained at a constant concentration and $R_{126}E$ was varied in amount. It should be emphasized that, in both cases, the specific activity was the same as that of the wild-type enzyme despite the fact that the resulting heterodimers had only one active site

Effect of Dilution on the Rate of TS Activity Restoration. One of the more unusual effects observed is described in Figure 2 where dilution of a mixture of $Y_{94}A$ and $R_{126}E$ (each maintained at a constant quantity and at a ratio of 10:1) from 0.1 to 1.0 mL results in a sharp increase in the rate of appearance of TS. Even more surprising was the finding that when this mutant mixture was diluted even further to 5.0, 10, and 20 mL (data not shown), there was no reduction in the optimal rate of enzyme restoration nor in its optimal specific activity which was found to occur at 1.0 mL. This effect would appear to be opposite to that expected if the mass action principle was in effect and again emphasizes the complexity of this process. One explanation would be that at high protein concentrations the mutants ($Y_{94}A$ and $R_{126}E$) do not dissociate to subunits as well as at lower concentrations, which is necessary for active heterodimer formation.

Thus, the greater the dilution the more subunits available for reassociation to form active enzyme, which as indicated below appears to be more stable than the inactive mutant homodimers.

Effect of Urea on the Restoration of TS Activity. When the mutant proteins $R_{126}E + Y_{94}A$ are exposed to 1 M urea, it is clearly seen that TS activity is restored much more rapidly than in its absence (Figure 3). This response is in sharp contrast to what occurs when the concentration of urea is increased to 2 M, where there is an immediate increase in activity followed by a rapid fall to about zero in about 10 h. One could propose in this instance that low concentrations of urea (1.0−1.5 M) enhance the separation of enzyme subunits from the mutants but favor their reassociation to the more stable active heterodimeric state of the enzyme, (R_{126}-$Y_{94}A$) ($R_{126}E$-Y_{94}). The same cannot be said apparently for these proteins in 2 M urea where an almost immediate reassociation of the mutant subunits occurs followed by a time-dependent decrease in activity, which could result from the perturbation of subunit or heterodimer structure or both

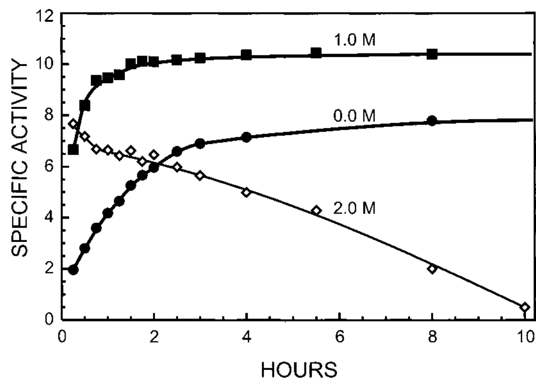

FIGURE 3: Effect of urea concentration on the rate of appearance of TS activity. $R_{126}E$ (27.2 μg) was incubated with $Y_{94}A$ (242 μg) in 0.5 mL of 25 mM potassium phosphate containing urea at the indicated concentrations. Aliquots were taken from the 0.5 mL reaction for assay of TS activity at the times presented by the different symbols: 0 M urea (●); 1.0 M urea (■); 2.0 M urea (◇).

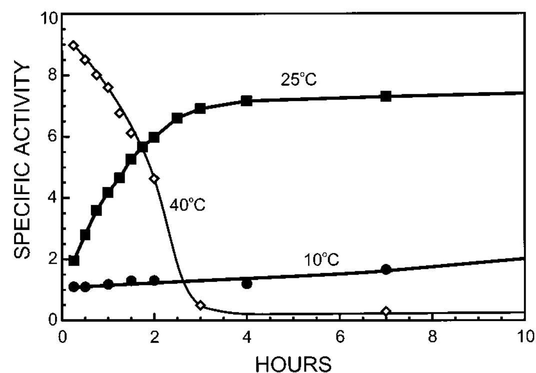

FIGURE 4: Effect of temperature on the rate of appearance of TS activity. The mutants were mixed in 0.5 mL of 25 mM phosphate buffer, pH 7.5, at the concentrations indicated in the legend to Figure 3 and incubated at the designated temperatures. Aliquots were taken for assay at the times indicated by the symbols: (●) 10 °C; (■) 25 °C (◇) 40 °C.

by this concentration of urea with time. Adding to the complexity of this problem is that the response of each mutant to different concentrations of urea may differ, which can also be influenced by temperature. The effect of these two factors together could have a dramatic effect on the restoration of activity as discussed below.

Effect of Temperature on the Restoration of Activity. Compared to 25 °C, the restoration of activity at pH 7.5 and 10 °C using the combination of $(R_{126}E)_2$ and $(Y_{94}A)_2$ is extremely slow, while that at 40 °C is optimal within the mixing period. However, in contrast to the results obtained at 10 and 25 °C, the TS activity at 40 °C falls rapidly with time and at about 2.5 h the enzyme is practically inactive (Figure 4). This result suggests that the enzyme denatures with time at this temperature, since dropping the temperature to 25 °C did not restore TS activity (data not shown). As shown in this figure, the activity only slowly increases with time at 10 °C and even more slowly at 4 °C (data not shown). However, it does so more rapidly at 4 and 10 °C in the presence of 1.5 M urea (Figure 5), and at 25 °C maximal activity is obtained within 1 h, relative to 4 h in the absence of urea (Figure 4). Comparable results were obtained with $R_{126}E$ and other inactive mutants as well, particularly $C_{146}S$. As shown previously (*19*), the latter mutant when mixed with $R_{126}E$ was the slowest of several tested in restoring TS activity, but in the presence of 1.5 M urea its activity was

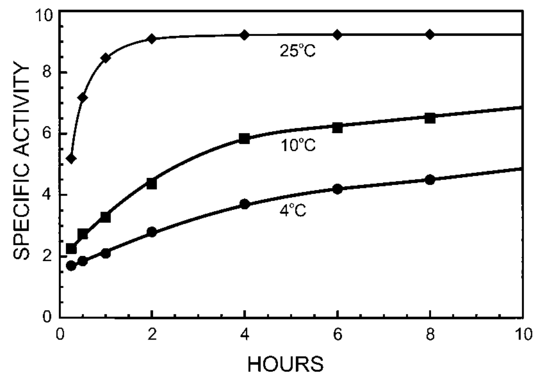

FIGURE 5: Effect of urea on the rate of appearance of TS activity at various temperatures. The mutants ($R_{126}E$ and $Y_{94}A$) were mixed in 1.5 M urea at the concentrations presented in the legend to Figure 3 and incubated at the indicated temperatures, with aliquots taken for assay at the times represented by the various symbols.

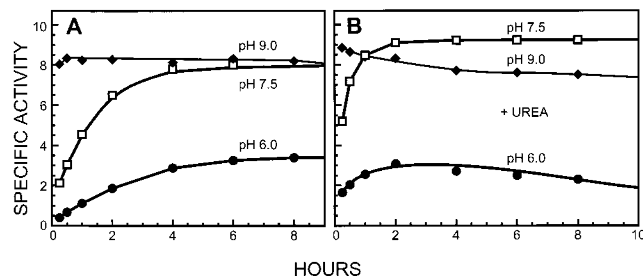

FIGURE 6: Effect of temperature, pH, and urea on the rate of appearance of TS activity. Each sample containing the same concentrations of $R_{126}E$ and $Y_{94}A$ as presented in the legend to Figure 3 was incubated at 25 °C at the indicated pH in the absence (A) and presence of 1.5 M urea (B). Aliquots were removed for assay at the times represented by the symbols.

restored at a rate similar to that obtained on mixing $R_{126}E$ with $Y_{94}A$ (data not shown).

Effect of pH on the Restoration of TS Activity. Another anomaly encountered in the restoration of TS activity on mixing the inactive mutants $R_{126}E$ and $Y_{94}A$ is the dramatic effect that pH has on this process. As shown in Figure 6A activity is only slowly restored at pH 6.0 but is much more rapid at pH 7.5. At pH 9.0 the rate of restoration of activity is so fast that it cannot be measured under the conditions of the assay. On the basis of the results in Figure 3, the presence of 1.5 M urea should enhance this effect, and does so at pH 7.5 and 9.0, but surprisingly had little effect on the restoration of activity at pH 6.0 (Figure 6B).

To emphasize the remarkable effect that pH has on the restoration of TS activity, it is shown in Figure 7 that while activity is only restored slowly at 10 °C, and pH 7.5, optimal activity is obtained relatively rapidly at this temperature when the pH is raised to 9.0. This result contrasts with that at 4 °C where even pH 9.0 has little effect on the rate of activity restoration with $Y_{94}A$ and $R_{126}E$ (Figure 8), but when 1.5 M urea is included in the reaction, it is seen that optimal activity occurs within 1 h.

Effect of Ethanol, Ethylene Glycol, and Glycerol on the Restoration of TS Activity. In an attempt to demonstrate that subunit separation and recombination are involved in restoring TS activity solutions of three related alcohols with differing hydrophobicities, dielectric constants and viscosities were examined. As shown in Figure 9 the presence of glycerol had a dramatic effect on impairing the increase in

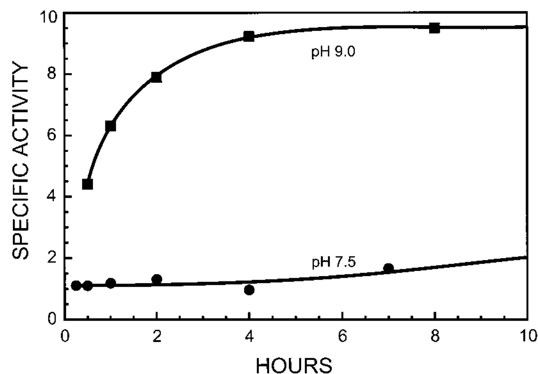

FIGURE 7: Effect of pH on the rate of TS appearance at 10 °C. $R_{126}E$ and $Y_{94}A$ were mixed at the concentrations indicated in the legend to Figure 3 in 25 mM potassium phosphate, pH 7.5, and 25 mM CHES, pH 9.0. Aliquots were removed for assay at the times indicated by the symbols.

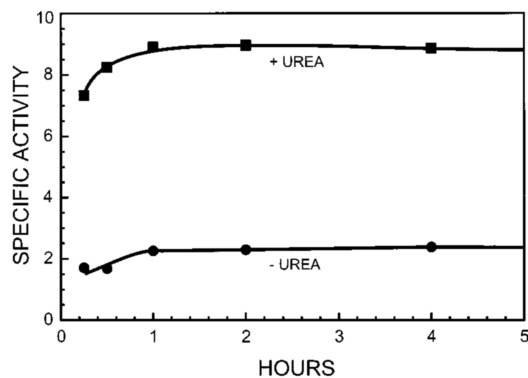

FIGURE 8: Effect of urea on the rate of appearance of TS activity at 4 °C. $R_{126}E$ and $Y_{94}A$ at the same concentrations as in the legend to Figure 3 were incubated in the presence and absence of 1.5 M urea. Aliquots were taken for assay at the times indicated by the symbols.

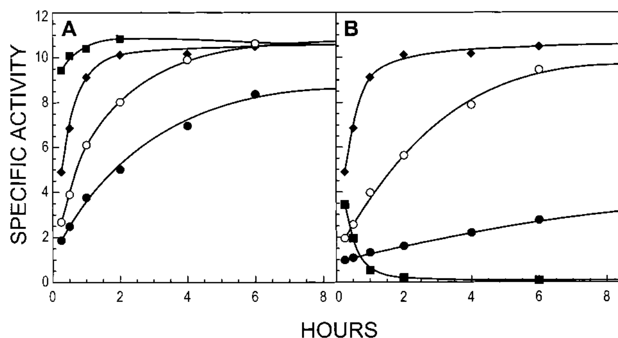

FIGURE 9: Effect of increasing concentrations of ethanol (■), ethylene glycol (○), and glycerol (●) on the rate of appearance of TS activity. $R_{126}E$ and $Y_{94}A$ at the same concentrations as in the legend to Figure 3 were incubated at room temperature with (A) 10% of above additions and (B) 20% of the indicated compounds. Aliquots were taken for assay at the times indicated by the symbols. The increase in TS activity due to the mixing of $R_{126}E$ and $Y_{94}A$ in the absence of any additions is shown by (◆).

TS activity, particularly at 20% (panel B). Ethylene glycol was less inhibitory than glycerol at both 10% (panel A) and 20% (panel B), but ethanol had contrasting effects in that it promoted an activating effect at 10% and a marked inhibition at 20%. While viscosity might play a role in influencing the rate of TS activity appearance, particularly in the case of glycerol, it is more likely that other factors including the water structure associated with the enzyme play a greater role, as will be discussed below.

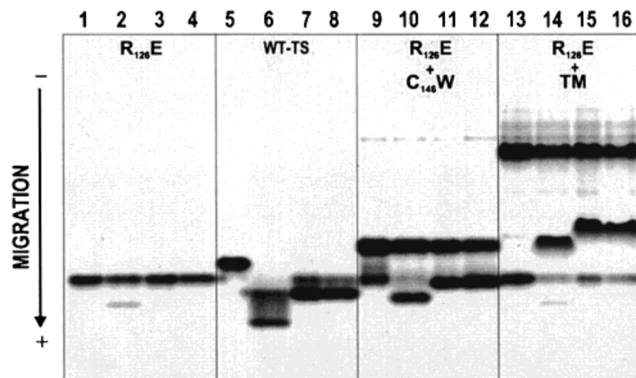

FIGURE 10: Nondenaturing polyacrylamide gel electrophoresis of WT-TS and TS mutants. In addition to the various proteins employed as presented in the panels, various additions were made as follows: lanes 1, 5, 9, and 13, none; lanes 2, 6, 10, and 14, FdUMP and CH_2H_4PteGlu; lanes 3, 7, 11, and 15, 5-NO_2dUMP; lanes 4, 8, 12, and 16, 5-NO_2dUMP and CH_2H_4PteGlu. The incubation conditions, concentrations of ligands, and the electrophoresis procedure used are described in Materials and Methods.

Evidence for Subunit Exchange as the Mechanism for TS Activity Restoration. In an effort to demonstrate conclusively that subunit exchange between homodimers was associated with the reappearance of TS activity, it was necessary to isolate the proposed reconstituted heterodimer and to verify that each subunit was derived from a different homodimer. As shown earlier (19), nondenaturing PAGE can be used as a means of separating intact dimers since mutant and WT-TS electrophorese to distinct positions on the gel. Thus, as revealed in lanes 1, 5, 9, and 13 of Figure 10, each protein ($R_{126}E$, WT-TS, $C_{146}W$, and TM) migrates to a different position in the gel despite the fact that their masses are about the same, indicating that charge and possibly conformation play an important role in protein migration. To take advantage of this property and to effect an even greater separation between $R_{126}E$ and $C_{146}W$, the latter mutant was altered at positions E_{237} and E_{245} to arginines. This modification increases the isoelectric point of *E. coli* TS, thus altering its elution from an anion-exchange column (*23*). As anticipated, the resulting TM migrates much more slowly than unmodified $C_{146}W$ (see lane 9 vs lane 13). In addition, tight complexes with ligands also affect the migration of proteins, which is seen in lane 6 where a tight ternary complex of WT-TS with FdUMP and CH_2H_4PteGlu yields two faster migrating bands than WT-TS alone (lane 5). The faster of the two most likely represents the dimer with each subunit containing a bound FdUMP and CH_2H_4PteGlu, while the slower moving band contains but one of the two subunits bound to the ligands. These results are in contrast to those reported earlier (*30*) where saturation of *Lactobacillus casei* TS with FdUMP and CH_2H_4PteGlu yielded only a single band corresponding to the dimer−ternary complex. We could not, however, reproduce this study with either *E. coli* or *L. casei* TS since two bands were obtained despite saturation of these proteins with the above ligands, results that are consistent with equilibrium dialysis studies demonstrating that each subunit of TS had a different affinity for its ligands and saturation of the dimer was rarely, if ever, observed (*9*).

Similarly 5-NO_2dUMP, a tight active site binding nucleotide (*31*, *32*) of *E. coli* TS, presented comparable findings in that WT-TS when saturated with this compound was barely evident, replaced by two faster migrating bands, most

Table 1: Sequence Analysis of the Upper and Middle Protein Bands Following Nondenaturing PAGE[a]

cycle	amino acid	
	upper band	middle band
1	Met (342)[b]	Met (128)
2	Lys (328)	Lys, Leu (58) (87)
3	Gln (274)	Gln (104)
4	Tyr (387)	Tyr (96)

[a] The protein bands were transblotted onto a PVDF membrane from the nondenaturing PAGE gels, and each of the Coomassie Blue stained bands was excised and sequenced. [b] The figures in parentheses represent amino acid recovery in picomoles.

likely due to one NO_2dUMP/dimer and two NO_2dUMP/dimer (data not shown). The presence of CH_2H_4PteGlu did not appear to alter the binding or migration of NO_2dUMP-bound TS nor its mutants (lanes 8, 12, and 16). $R_{126}E$ as shown does not bind either nucleotide analogue (FdUMP or NO_2-dUMP) well, although a small degree of single subunit binding to FdUMP when CH_2H_4PteGlu is present is evident in lane 2. In the case where a single functional active site is present, resulting from mixing $R_{126}E$ with $C_{146}W$, a single band corresponding to a dimer ($R_{126}E$-$C_{146}W$) saturated at a single site with FdUMP and CH_2H_4PteGlu is noted (compare lanes 6 and 10). A similar result should be obtained in the mixture of $R_{126}E + C_{146}W$ and NO_2dUMP and does indeed occur (lane 11), but the separation of the NO_2dUMP-containing heterodimer from unliganded heterodimer (cf. lanes 9 and 11, lower band) was insufficient to enable the individual proteins to be isolated. However, in the case of TM and $R_{126}E$ a distinct separate band with either FdUMP + CH_2H_4PteGlu (lane 14) or NO_2dUMP is obtained (lanes 15 and 16). Since the TM is in excess of $R_{126}E$, it is not surprising that the band corresponding to $R_{126}E$ is greatly reduced in lanes 14−16.

To establish that these intermediate bands or heterodimers contain one subunit from each of the homodimers, residue 2 of $R_{126}E$ was mutated from a lysine to a leucine, and the experiment presented in Figure 10 was repeated on a larger scale in an attempt to isolate the middle band in lane 14. This band should consist of the heterodimer (K_2L-$R_{126}E$) ($C_{146}W$-$E_{237}R$-$E_{245}R$) liganded to one NO_2dUMP, which would clearly establish that the individual subunits from the homodimers, $(K_2L\text{-}R_{126}E)_2$ and $(C_{146}W\text{-}E_{237}R\text{-}E_{245}R)_2$, had separated and reassociated in solution. After the gels were transblotted to a PVDF membrane, the stained protein bands were excised and sequenced. It is clear from Table 1 that the middle band contains both lysine and leucine in equivalent amounts, thus verifying that the composition of the active species is a heterodimer.

DISCUSSION

It was first reported by Wente and Schachman (33) that in some cases an individual subunit of an oligomeric enzyme may be inactive, but when recombined with the other subunits, its activity can be restored. As pointed out by them, the restoration process can result from the interacting subunits yielding a conformation conducive to enzyme activity or from a sharing of amino acids from adjacent polypeptides to complement each others active sites. Since the presentation of this proposal, which was mainly to explain the restoration of activity for mutants of aspartate transcarbamylase, several other enzymes have been shown to possess similar properties such as ornithine decarboxylase (*34*, *35*), orotate phosphoribosyltransferase (*36*), arginosuccinate lyase (*37*), adenyl succinate synthetase (*38*), and diacylglycerol kinase (*39*), as well as TS (*19*), the subject of this paper. Undoubtedly, other instances of this phenomenon will be found to occur. This unusual property of active site sharing of amino acids from adjacent polypeptide chains is also associated with another unusual property, that of half-of-the-sites reactivity, particularly in the case of TS as described in this paper and previously (*19*). How this process is effected, particularly in the case of a protein that is composed of two identical subunits, is not clear at present, but the purpose of the second apparently inactive subunit may be to provide both structural and conformational support for the active subunit.

In the case of TS, this situation may be even more complex, since on comparing the liganded TS crystal structures of *P. carinii* and *E. coli,* substrate binding to the second subunit of the *P. carinii* TS is impaired (*20*), which is not apparently the case for *E. coli* TS (*3*, *4*). These differences in substrate or substrate analogue binding are even more evident in equilibrium dialysis studies, where the ligands are not present in saturating quantities as they usually are in preparing crystals for X-ray diffraction studies. In the case of *L. casei* TS differences in the binding to each subunit were clearly evident (*9*) and were supported subsequently by binding studies to *E. coli* TS in the absence and presence of the folate analogue BW1843U89 (*40*). Similar findings were also obtained with human TS (*16*). Thus, whatever differences there are between the two subunits of *E. coli* TS, they may be more apparent in solution than as part of a crystal, particularly in the presence of ligands.

Nature of the Forces Involved in Subunit Exchange. E. coli TS appears to be unlike other proteins that exchange subunits in that attempts to inactivate the WT-TS with the double mutant $R_{126}E$-$C_{146}W$ was unsuccessful unless both were first denatured together in 8 M urea and then renatured as described earlier (*29*). However, very recent studies indicate that complete denaturation was not necessary to promote WT-TS subunit exchange as 2 M urea was sufficient to enable the complete inactivation of WT-TS by $R_{126}E$-$C_{146}W$ but at some expense to the stability of WT-TS, as about 50% of the latter was inactivated also. Thus, from this and other studies it appears that the subunits of WT-TS and those of the active heterodimer are held together more tightly than the inactive mutant homodimers. How a single amino acid change can affect the structure of the TS so dramatically as to enhance the capacity of the subunits to dissociate remains to be determined. The fact that each mutant appears to dissociate at a different rate will require a creative explanation indeed or at least will require creative enough experiments to explain the nature of the exchange process and the forces promoting it. In this vein, recent studies by us (R. Saxl and F. Maley, unpublished) have revealed that single mutations in TS ($R_{126}E$, $C_{146}W$, $Y_{94}A$, etc.) render these proteins structurally less compact than WT-TS when examined for their susceptibility to trypsin digestion. This property is consistent with the apparent ability of the mutant subunits to dissociate and to recombine, as evidenced by the restitution of active enzyme when $R_{126}E$ is mixed with various inactive mutants.

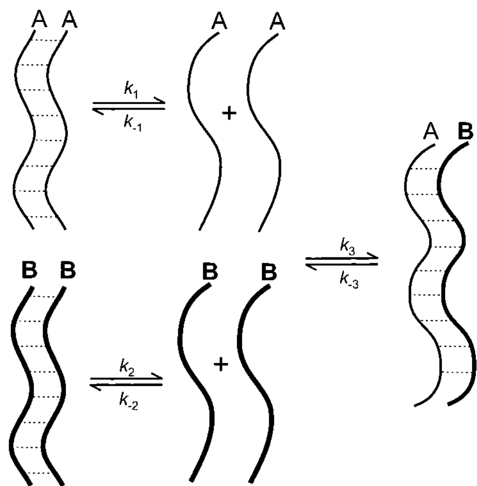

FIGURE 11: Proposed intermediates involved in the restoration of TS activity from the inactive mutant dimers, AA and **BB**. In this case AA represents $R_{126}E$ and **BB** represents $Y_{94}A$, although many other mutants could substitute for the latter. As depicted, AA dissociates to 2A at a rate of k_1, while **BB** dissociates to 2**B** at a rate of k_2, while the active TS heterodimer forms at rate equivalent to k_3; as discussed, numerous factors can affect the rate of A**B** formation.

These studies on interacting protein subunits raise a number of pertinent questions regarding how the homodimer subunits of *E. coli* TS, which are held together at their interface by 14 hydrophobic, 11 polar, and 5 charged side chains including 6 water molecules, of which three are completely buried in the interface (*4*), manage to dissociate, particularly if the mutations do not cross the boundaries of the subunit interface as in the case of $Y_{94}A$ and $C_{146}W$. Since dissociation of the mutant dimers occurs much more readily than WT-TS, it is apparent that the rate of active heterodimer formation is dependent on the quantity of mutant subunits in solution, depicted in Figure 11 as A ($R_{126}E$) and **B** ($Y_{94}A$). Any factor that promotes dissociation of AA and **BB**, without greatly perturbing the structure of the subunits, should increase the rate (k_3) of heterdimer formation. It is assumed that the equilibrium constants of dissociation of AA (k_1/k_{-1}) and **BB** (k_2/k_{-2}) greatly favor the presence of dimer. However, dilution, as shown in Figure 2, must shift the equilibrium toward a greater accumulation of individual subunits, which combine at a rate equivalent to k_3 to yield the active heterodimer (A**B**). The fact that the rate of A**B** formation increases on continued dilution of a fixed amount of the dimers A and **B** suggests that A**B** is fairly stable once formed and that k_3/k_{-3} is in favor of the formation of the heterodimer. Alternatively, it can be proposed that the individual subunits of A and **B** possess multiple conformations, some of which react more readily than others to form the active heterodimer. That this may be the case is described for those experiments (Figure 3) where urea can activate at low concentrations (1 M) and then inhibit at higher concentrations following activation (2 M). Similarly, the rate of restoration of activity is dramatically pH dependent (Figure 6), in that pH 9 > pH 7.5 > pH 6.0. In both instances conditions supporting subunit dissociation can be proposed (1 M urea and pH 9.0), but then unfavorable conditions could lead to perturbations in subunit structure. The latter is probably encountered in the case of 2 M urea (Figure 3) where a rapid dissociation to subunits occurs initially, followed by a slower denaturation or conversion of subunit structures to forms that do not favor A**B** formation. In fact, it would appear that the equilibrium constant (k_3/k_{-3}) is shifted back to A and **B**.

In the case of Figure 4, which demonstrates the temperature dependence of the reaction, the subunit interaction leading to active heterodimer appears to be endergonic in nature since the reaction improves with temperature until at 40 °C other factors come into play. One of these could be monomer (Figure 11) instability at the higher temperature leading to a time-dependent loss in activity, similar to that seen in the case of 2 M urea (Figure 3).

Effect of Altering the Solvent Environment on the Restoration of Activity. Since subunit interaction is undoubtedly responsible for the restoration of activity, it was believed that by increasing the viscosity of the solution environment that the reaction could be slowed. While this response appeared to be the case with increasing amounts of glycerol, the explanation became more complex when the effects of ethanol and ethylene glycol were examined (Figure 9). As indicated in this figure, ethanol (Figure 9A) has a decisively different effect at 10% than at 20% (Figure 9B). In the former case ethanol activates the appearance of TS activity above that obtained in buffer alone, while in the latter it markedly impairs this effect. These two diametrically opposite effects can be explained by ethanol's lower dielectric constant relative to ethylene glycol and glycerol (*41*) and an even lower relative free energy pairwise interaction coefficient (*42*), which is indicative of ethanol's greater capacity for interactions with hydrophobic regions within the homodimer that would lead to subunit dissociation in 10% ethanol solutions. The net result is similar to that found for urea activation (Figure 3) and activation at pH 9.0 (Figure 9). At 20% ethanol, the disruption of water due to the low dielectric constant of this solution relative to 20% ethylene glycol and glycerol becomes a factor in promoting protein aggregation and the inhibition seen in Figure 9B. As a result of ethylene glycol's pairwise interaction coefficient and dielectric constant falling between that of ethanol and glycerol, a response is obtained that falls somewhere between the other solvents (Figure 9, panels A and B). In the case of 20% glycerol (Figure 9B) it is probable that glycerol's stabilizing effect on proteins, which favors their native folded state (*43*) and limits dimer dissociation, results in the apparent inhibitory response to glycerol noted in Figure 9B, although some effect of viscosity on impairing diffusion cannot be ruled out.

CONCLUSIONS

Although our knowledge of protein folding mechanisms has improved markedly in recent times (*44*), there is still much to be learned, as evidenced by the studies presented in this paper. Most of the studies on potential intermediates involved in protein folding are those based on refolding of proteins following complete denaturation. In contrast, the studies described on the restoration of TS activity following the intermixing of specific inactive mutants under nondenaturing conditions suggest that tightly folded dimers can separate and unfold somewhat and then find their appropriate partners to restore both structure and function to TS. Comparable cases have been described for several other inactive mutants that share amino acids in their active sites

(*34*−*39*), indicating that the results described for TS are not unique to this protein. Still to be determined are what enables the monomeric subunits to separate from their parent dimers, the extent to which they separate, the degree to which they unfold, and their rates of refolding to their original inactive homodimers and active heterodimeric state. As indicated recently, these rates can be of the order of micro- to nano-seconds (*45*), depending on the conditions employed, and as shown in the present TS studies can also be extremely rapid, particularly at pH 9.0. The nature of the various forces involved in influencing subunit interaction is still to be resolved, but as implied recently (*46*), subunit interaction is affected by binding free energy that is not evenly distributed across the dimer interface but by hot spots of binding energy, which are composed mainly of tryptophan, tyrosine, and arginine. Shielding of these residues from bulk solvent is proposed to decrease the effective dissociation constants of the dimers. This being the case, it will be of great interest to determine how mutations, particularly those not within the dimer interface, influence the extent of dimer dissociation.

ACKNOWLEDGMENT

We thank Joan Petersen-Lane for able assistance with some of the experiments reported in the manuscript and Lynda M. Jury for patience and competence in typing it. We also thank Dr. Chang-Hwei Chen for helpful comments.

REFERENCES

1. Friedkin, M., and Kornberg, A. (1957) *The Chemical Basics of Heredity* (McElroy, W. D., and Glass, B., Eds.) pp 609−614, Johns Hopkins Press, Baltimore, MD.
2. Carreras, C. W., and Santi, D. V. (1995) *Annu. Rev. Biochem. 64*, 721−762.
3. Matthews, D. A., Villafranca, J. E., Janson, C. A., Smith, W. W., Welsh, K., and Freer, S. (1990) *J. Mol. Biol. 214*, 937−948.
4. Montfort, W. R., Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Maley, G. F., Hardy, L., Maley, F., and Stroud, R. M. (1990) *Biochemistry 29*, 6964−6977.
5. Fauman, E. B., Rutenber, E. E., Maley, G. F., Maley, F., and Stroud, R. M. (1994) *Biochemistry 33*, 1502−1511.
6. Sage, C. R., Michelitsch, M. D., Stout, T. J., Bierman, D., Nissen, R., Finer-Moore, J. S., and Stroud, R. M. (1998) *Biochemistry 37*, 13893−13901.
7. Barrett, J. E., Maltby, D. A., Santi, D. V., and Schultz, P. G. (1998) *J. Am. Chem. Soc. 120*, 449−450.
8. Variath, P., Liu, Y., Lee, T. T., Stroud, R. M., and Santi, D. V. (2000) *Biochemistry 39*, 2429−2435.
9. Galivan, J. H., Maley, G. F., and Maley, F. (1976) *Biochemistry 15*, 356−362.
10. Dunlap, R. B., Harding, N. G. L., and Huennekens, F. M. (1971) *Biochemistry 10*, 88−97.
11. Danenberg, P. V., Langenbach, R. J., and Heidelberg, C. (1974) *Biochemistry 13*, 926−933.
12. Aull, J. L., Loeble, R. B., and Dunlap, R. B. (1974) *J. Biol. Chem. 249*, 1167−1172.
13. Leary, R. D., Beaudette, N., and Kisliuk, R. L. (1975) *J. Biol. Chem. 250*, 4864−4868.
14. Galivan, J., Noonan, J., and Maley, F. (1977) *Arch. Biochem. Biophys. 184*, 336−345.
15. Danenberg, K., and Danenberg, P. (1979) *J. Biol. Chem. 254*, 4345−4348.
16. Dev, I. K., Dallas, W. S., Ferone, R., Hanlon, M., McKee, D. D., and Yates, B. B. (1994) *J. Biol. Chem. 269*, 1873−1882.
17. Chen, C.-H., Davis, R. A., and Maley, F. (1996) *Biochemistry 35*, 8786−8793.
18. Spencer, T., Villafranca, J., and Appleman, J. (1997) *Biochemistry 36*, 4212−4222.
19. Maley, F., Pedersen-Lane, J., and Changchien, L. (1995) *Biochemistry 34*, 1469−1474.
20. Anderson, A. C., O'Neil, R. H., DeLano, W. L., and Stroud, R. M. (1999) *Biochemistry 38*, 13829−13836.
21. Changchien, L.-M., Garibian, A., Frasca, V., Lobo, A., Maley, G. F., and Maley, F. (2000) *Protein Expression Purif. 19*, 265−270.
22. Maley, G. F., and Maley, F. (1988) *J. Biol. Chem. 263*, 7620−7627.
23. Hardy, L. W., Pacitti, D. F., and Nalivaika, E. (1994) *Structure 2*, 833−838.
24. Wahba, A. J., and Friedkin, M. (1961) *J. Biol. Chem. 236*, 11−12.
25. Maley, G. F., Maley, F., and Baugh, C. M. (1979) *J. Biol. Chem. 254*, 7485−7487.
26. Belfort, M., Maley, G. F., and Maley, F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1858−1861.
27. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.
28. Garfin, D. E. (1990) *Methods Enzymol. 182*, 426−441.
29. Greene, P. J., Maley, F., Pedersen-Lane, J., and Santi, D. V. (1993) *Biochemistry 32*, 10283−10288.
30. Aull, J. L., Lyon, J. A., and Dunlap, R. B. (1974) *Microchem. J. 19*, 210−218.
31. Mertes, M. P., Chang, C. T.-C., De Clerq, E., Huang, G.-F., and Torrence, P. F. (1978) *Biochem. Biophys. Res. Commun. 84*, 1054−1059.
32. Wataya, Y., Matsuda, A., and Santi, D. V. (1980) *J. Biol. Chem. 255*, 5538−5544.
33. Wente, S. R., and Schachman, H. K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 31−35.
34. Tobias, K. E., and Kahana, C. (1993) *Biochemistry 32*, 5842−5847.
35. Coleman, C. S., Stanley, B. A., Visanath, R., and Pegg, A. E. (1994) *J. Biol. Chem. 269*, 3155−3158.
36. Ozturk, D. H., Dorfman, R. H., Scapin, G., Sacchettini, J. C., and Grubmeyer, D. (1995) *Biochemistry 34*, 10764−10770.
37. Turner, M. A., Simpson, A., McInnes, R. R., and Howell, P. L. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 9063−9068.
38. Kang, C., Kim, S., and Fromm, H. J. (1995) *J. Biol. Chem. 271*, 29722−29728.
39. Lau, F. W., Chen, X., and Bowie, J. V. (1999) *Biochemistry 38*, 5521−5527.
40. Weichsel, A., Montfort, W. R., Ciesla, J., and Maley, F. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3493−3497.
41. Okamoto, B. Y., Wood, R. H., and Thompson (1978) *J. Chem. Soc., Faraday Trans. 74*, 1990−2007.
42. Franks, F., and Smith, H. T. (1968) *Trans. Faraday Soc. 64*, 2962−2972.
43. Gekko, K., and Timasheff, S. N. (1981) *Biochemistry 20*, 4667−4676.
44. Chamberlin, A. K., and Marqusee, S. (2000) *Adv. Protein Chem. 53*, 283−328.
45. Mayor, U., Johnson, C. M., Daggett, V., and Fersht, A. R. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 13518−13522.
46. Bogan, A. A., and Thorn, K. S. (1998) *J. Mol. Biol. 280*, 1−9.

BI002925X