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Complete Restoration of Activity to Inactive Mutants of *Escherichia coli* Thymidylate Synthase: Evidence that *E. coli* Thymidylate Synthase is a Half-the-Sites Activity Enzyme†

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ABSTRACT: *Escherichia coli* thymidylate synthase (TS) is a dimeric protein containing identical subunits. When $R_{126}E$, an inactive mutant of this enzyme, was incubated at room temperature with other inactive mutants of *E. coli*, TS enzyme activity gradually reappeared. The rate of activity restoration was dependent on the mutant employed. In the case of $C_{146}W$, an active site mutant, the half-time required for maximal activity restoration was about one hour, which was about 500-fold faster than that obtained with $C_{146}S$. The final specific activity of the mutant mixtures, based on the concentration of $R_{126}E$, was equivalent to that of the wild-type TS (WT-TS). However, when the activities of *E. coli* WT-TS and mutant TS mixtures were compared for their extents of renaturation following denaturation as described for *Lactobacillus casei* TS [Pookanjanatavip, M., Yuthavong, Y., Greene, P. J., & Santi, D. V. (1992) *Biochemistry 31*, 10303−10309], only about one-half of the activity of WT-TS was restored, implying that the denaturation−renaturation procedure was less efficient than allowing the native TS mutant dimers to exchange subunits. If, as proposed, subunit exchange is responsible for the observed restoration of activity to the *E. coli* mutant TS mixtures, it would suggest that only one active site cysteine, that provided by $R_{126}E$ in the dimer $(R_{126}E)-(C_{146}W)$, is sufficient to yield the same k_{cat} as WT-TS, which contains one active site cysteine in each subunit. Other mutant dimers that contain both active site cysteines such as $(R_{126}E)-(Y_{94}A)$ and $(R_{126}E)-(I_{264}Am)$ are also fully active, even though one of the subunits is functionally inactive. Whether TSs in general catalyze their reactions via a half-the-sites activity mechanism remains to be resolved, but the data presented in this paper suggest that *E. coli* TS employs this mechanism.

Not only is thymidylate synthase (TS)[1] (EC 2.1.1.45) one of the most conserved proteins known, its enzyme mechanism is one of the more unique reactions to be described in that the product, dTMP, is formed via a process of reductive methylation involving its substrates, dUMP and CH_2H_4-PteGlu (Pastore & Friedkin, 1962). In the course of this reaction a methylene group and hydride ion are contributed by CH_2H_4PteGlu to the 5-position of dUMP, the latter being activated by a nucleophilic attack at the 6-position of dUMP (Pogolotti & Santi, 1977). The nucleophile was shown subsequently to be a specific cysteinyl residue of the enzyme (Bellisario et al., 1976; Pogolotti et al., 1976) and verified more recently by X-ray crystallographic studies on the

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[1] Abbreviations: dTMP, 2′-deoxythymidine 5′-monophosphate; dUMP, 2′-deoxyuridine 5′-monophosphate; FdUMP, 5-fluoro-dUMP; CH_2H_4-PteGlu, 5,10-methylene tetrahydrofolate; Am, amber; WT, wild type; TS, thymidylate synthase.

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liganded structure of *Escherichia coli* TS (Matthews et al., 1990a,b; Montfort et al., 1990).

The crystal structures of both *Lactobacillus casei* (Hardy et al., 1986) and *E. coli* TS (Matthews et al., 1990 a,b; Montfort et al., 1990) reveal that the enzyme's two identical subunits are held together by a backbone of about 30 amino acid residues with the active site in each subunit widely separated from one another despite the marked change in conformation that occurs on substrate or analogue binding. This being the case, each site should be equally active although a considerable body of evidence exists suggesting either that the two active sites contribute unequally to the enzyme's catalytic efficiency (k_{cat}) or that only one of the two sites is active (Aull et al., 1974; Leary et al., 1975; Galivan et al., 1976a,b; Danenberg & Danenberg, 1979; Beaudette et al., 1980). Paradoxically, other studies suggest that both sites contribute equally to enzyme activity (Plese & Dunlap, 1977), particularly those involving subunit complementation wherein specific inactive mutants of *L. casei* TS were denatured together with urea and then renaturated by dilution, resulting in the restoration of enzyme activity (Perry et al., 1992; Pookanjanatavip et al., 1992; Carreras et al., 1994). In these cases only half the activity of the *L. casei* WT-TS was restored, which would be expected if only one active site was reconstituted in the resulting heterodimer. Similar results were obtained by us in collaboration with Greene et al. (1994) when cross-species complementation studies were conducted with *E. coli* and *L. casei* TS.

As an extension of the studies of Greene et al. (1994) we undertook to determine the kinetics of subunit interaction with mutants of *E. coli* TS comparable to those already described for *L. casei* (Pookanjanatavip et al., 1992). Much to our surprise, on just mixing $R_{126}E$ and several inactive *E. coli* TS mutants in the absence of urea denaturation, enzyme activity rapidly appeared. What was even more surprising was that, in contrast to the *L. casei* TS studies, restoration of only *one* of the *two* active sites of *E. coli* TS was sufficient to obtain a k_{cat} comparable to that of the wild-type enzyme. This finding indicates that, unlike *L. casei* TS, the subunits of *E. coli* TS can dissociate reversibly in solution. Somewhat similar findings have been recently described for ornithine decarboxylase (Tobias & Kaban, 1993; Coleman et al., 1994).

MATERIALS AND METHODS

*Preparation of Wild-Type and Mutant Enzymes. ThyA-*TS was prepared using a high expression system which yielded 40−50% of the cellular protein of *E. coli* as TS (L. Changchien, and F. Maley, manuscript in preparation). The enzyme was purified to homogeneity through the first four steps of the procedure described by Maley and Maley (1988). The mutants of TS were prepared from the *thyA* gene by a modification of the procedure of Taylor et al. (1986) using a mutagenesis kit obtained from Amersham. The mutant proteins were purified as above for WT-TS by isolating the major peak from the DE-52 chromatography step. SDS−PAGE was used in these cases to determine which fractions through the peak should be omitted from the enzyme pool.

Measurement of TS Activity. Enzyme activity was measured at 30 °C using essentially the spectrophotometric assay of Wahba and Friedkin (1961), but in the presence of 50 mM $MgCl_2$ due to the high Mg^{2+} requirement of this enzyme (Maley et al., 1979). One unit is defined as the amount of enzyme required to synthesize 1 μmol of dTMP/min under the conditions of the assay. Protein was determined from $\epsilon_{280} = 0.87 \times 10^5\ M^{-1}\ cm^{-1}$, which is equivalent to 1.43 A_{280}/mg of protein. The protein value was obtained by amino acid analysis.

Restoration of Activity to Inactive Mutants. Varying amounts of basically inactive TS mutants (0−2% WT-TS activity) were added to a constant concentration of $R_{126}E$ in 0.5 mL of a solution containing 25 mM potassium phosphate, pH 7.5, and 2−10 mM dithiothreitol. The latter was added to stabilize enzyme activity over extended periods of incubation (15−30 h) at room temperature, although this reducing agent was unnecessary at shorter periods of incubation. Usually 26 μg (0.85 μM) of $R_{126}E$ or $R_{126}Q$ was incubated at room temperature and assayed at various times after the addition of a specific mutant at 5−10 times the concentration of $R_{126}E$.

Nondenaturing Acrylamide Gel Electrophoresis. To determine the extent of migration of WT-TS and its mutants, as well as mixtures of the latter with $R_{126}E$, aliquots of the protein solutions (about 5 μg in 10 μL) were mixed with an equal volume of a buffer containing 0.16 M Tris-HCl, pH 6.8, 25% glycerol, and 0.08% bromphenol blue. The resulting solution was loaded onto a 4.5% stacking gel, pH 6.8, cross-linked with 2.7% bisacrylamide. The stacking gel was placed over a 10% running gel, pH 8.8, that was cross-linked to the same extent. The size of the gels was 0.7 mm × 16 cm × 20 cm, which were run at 4 °C for 18 h at 75 V in a Bio-Rad Protean II gel apparatus. The running buffer consisted of 0.2 M glycine and 30 mM Tris-HCl, pH 8.3. The acrylamide and bisacrylamide solutions were purchased from National Diagnostics and the gels prepared according to their instructions, but without sodium dodecyl sulfate and mercaptoethanol (Garfin, 1990).

To form ternary complexes of TS that could be detected by autoradiography, 10 μL of 0.34 mM [^{32}P]FdUMP (1.04 × 10^5 cpm/nmol) and 10 μL of 1.6 mM of (RS) CH_2H_4-PteGlu were added to a solution of WT-TS (34 μg) or mutants ($R_{126}E$, 32 μg; $C_{146}W$, 32 μg; $Y_{94}A$, 37 μg) in 0.1 mL of an incubation solution (25 mM potassium phosphate, pH 7.5, and 10 mM DTT). The enzyme mixtures were $R_{126}E$ + $C_{146}W$ and $R_{126}E$ + $Y_{94}A$ at the indicated concentrations. Incubations were maintained at 25 °C for 4 h before ternary complex formation was initiated. The final concentration of the ligands was in excess of the total number of enzyme subunits employed. Electrophoresis samples were prepared as described above. After the completion of the electrophoresis, the gel was stained with Coomassie Blue-R and then destained and dried on a segment of Whatman 3 MM that was slightly larger than the gel. Through the use of [^{32}P]FdUMP, the X-ray film need only be exposed for a few hours at room temperature to obtain a satisfactory autoradiogram.

RESULTS

Restoration of TS Activity to Inactive Mutants. It is seen in Figure 1 that on mixing $R_{126}E$ with either $C_{146}S$ or $C_{146}W$, TS activity gradually appeared in both cases, although the rate of increase is obviously much greater in the latter case. As expected this effect was concentration dependent, with the optimum level of activity being achieved at a ratio of

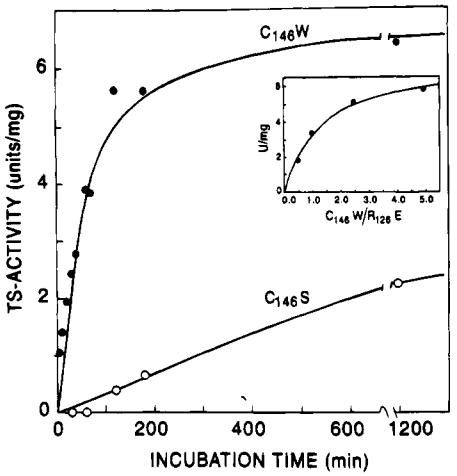

FIGURE 1: Time course of appearance of TS activity on incubating $R_{126}E$ with $C_{146}W$ and $C_{146}S$: U, units of enzyme. $R_{126}E$ (33 μg) was mixed with 250 μg of $C_{146}W$ (640 μg), respectively, in 0.5 mL of 25 mM phosphate buffer, pH 7.5. The times at which TS activity was measured are indicated in the figure. The inset was obtained by incubating 65 μg of $R_{126}E$ with 32, 64, 160, and 320 μg of $C_{146}W$ for 14 h. See Materials and Methods for additional details.

Table 1: Rate of Activation of Various Inactive TS Mutants by $R_{126}E$[a]

mutant	units/mg	mutant	units/mg
$Y_{94}A$	3.86	$Y_{209}M$	1.40
$E_{58}D$	3.10	$R_{166}Q$	1.38
$C_{50}F$	2.48	$T_{46}MT_{47}V$	0.65
$W_{80}M$	2.43	$K_{48}E$	0.64
$Y_{94}K$	2.39	$C_{146}A$	0.54
$C_{146}W$	2.27	$K_{48}Q$	0.50
$Y_{94}F$	1.59	$C_{146}S$	0.02
$I_{264}Am$	1.42		

[a] $R_{126}E$ (26 μg) was present in 0.5 mL of 25 mM potassium phosphate, pH 7.5, and 4 mM dithiothreitol at room temperature. The individual mutants (160 μg) were added to this solution, and aliquots of each of the binary mixtures were assayed for TS activity after a 30 min incubation.

about 5:1 for $C_{146}W$ to $R_{126}E$ (Figure 1, insert). At this point the TS specific activity was comparable to that of the wild-type dimeric enzyme (average specific activity 7.0). Assuming that subunit exchange is involved in the restoration of TS activity, there would be only one cysteine containing active site in the resulting dimer, suggesting that only one complete active site is necessary to achieve full TS activity. When the extent of reactivation was measured by the urea denaturation–renaturation procedure of Perry et al. (1992) that was used to restore activity to comparable mutant mixtures of *L. casei* TS (Pookanjanatavip et al., 1992), only half the *E. coli* wild-type activity was obtained, which is similar to what these investigators found.

Rate of TS Reactivation. One of the interesting aspects of the reactivation phenomenon is that the rate of reactivation depended on the type of mutant employed. This is suggested in the results presented in Figure 1, where a significant difference in the rates of activity restoration was observed with $C_{146}W$ and $C_{146}S$. Thus it is seen that only about 1 h was required in the former case to restore about 50% of the TS activity, while in the latter instance the increase in activity was about 500-fold slower. Other mutants were found to restore activity even more rapidly than $C_{146}W$ (Table 1). In most cases the activity was eventually restored to that

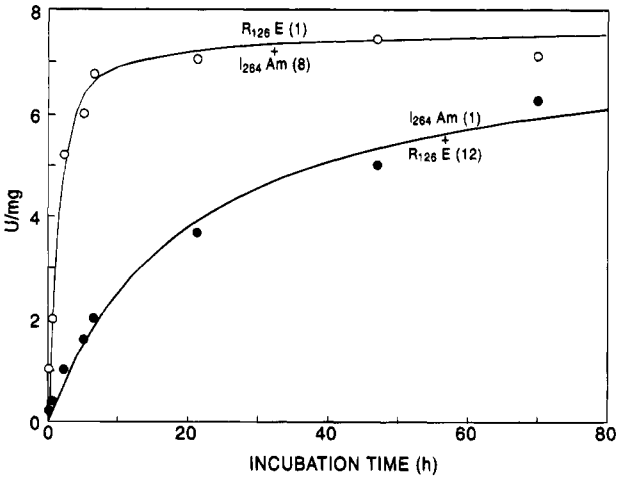

FIGURE 2: Time course of appearance of TS activity on incubating $R_{126}E$ (53 μg) with $I_{264}Am$ (428 μg) (○) and $I_{264}Am$ (43 μg) with $R_{126}E$ (532 μg) (●). The numbers in parentheses in the figure represent relative concentrations of the mutants in the incubation solution. Conditions were similar to those described in the legend to Figure 1 and in Materials and Methods.

expected for WT-TS with the rate being dependent on the nature of the mutant employed and its concentration. This is apparent in Figure 2, which shows that when $I_{264}Am$ was incubated with $R_{126}E$ at a ratio of 1:10 the activity increased at a much slower rate than when the ratio of $I_{264}Am$ to $R_{126}E$ was about 10:1, but in both instances the specific activity approached that of WT-TS. These findings are in contrast to those obtained by Carreras et al. (1994) with the comparable *L. casei* TS mutants. In their study a $(R_{178}F)$–$(V_{316}Am)$ heterodimer prepared by denaturation–renaturation was found to possess half the activity of the *L. casei* WT-TS, a value consistent with the presence of one functionally active subunit in the heterodimer. The same result was obtained with *L. casei* $(R_{178}F)$–$(C_{198}A)$ which also contained only one active site cysteine. It is not clear at this time why their results differ from ours, except for the fact that we used the corresponding *E. coli* TS mutants. Additionally, it should perhaps be re-emphasized that when we employed the denaturation–renaturation procedure of Perry et al. (1992) with the corresponding *E. coli* mutants, only about half the WT-TS activity was restored relative to that obtained under nondenaturing conditions (data not presented).

Evidence That Activity Restoration Is Associated with Dimer Dissociation. As expected the rate of activity restoration was temperature dependent; 37 °C gave a much faster rate but was associated with enzyme instability while incubation at 4 °C yielded an extremely slow rate of enzyme appearance. Room temperature (25 °C) gave the best results as the enzyme was stable for days under these conditions. Another finding that suggested that dimer dissociation and reassociation was the basis for the restoration of activity was the observation that by diluting the mutant enzymes 10-fold an even faster rate of activity recovery could be obtained (data not presented). In addition the inclusion of 1–2 M urea in the reaction enhanced the rate of appearance of enzyme activity by 2–3-fold. By contrast, increasing $MgCl_2$ levels inhibited the appearance of activity (Table 2), which could be explained as due to the impairment of dissociation or reassociation of the enzyme subunits by Mg^{2+}.

Analysis of the Mutant Interactions by Nondenaturing Gel Electrophoresis. In an effort to find some physical property

Table 2: Effect of Increasing Mg^{2+} Concentration on Restoration of TS Activity

Mg^{2+} (mM)	% initial activity[a]	Mg^{2+} (mM)	% initial activity[a]
0	100	40	54
20	74	100	26

[a] The reaction solution contained $R_{126}E$ and $C_{146}W$ at concentrations similar to that in Table 1, but with $MgCl_2$ present at the indicated concentrations. TS was assayed 3 h after the start of the incubation at room temperature and was found to be 4.39 μmol/(min·mg) based on $R_{126}E$ in the absence of $MgCl_2$.

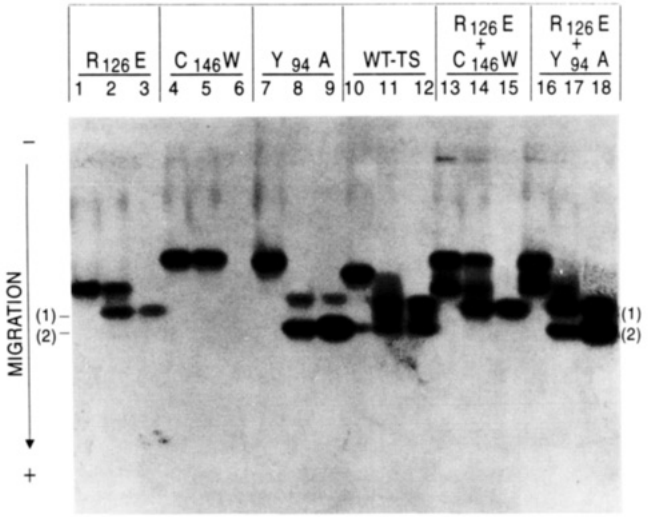

FIGURE 3: Nondenaturing gel electrophoresis of WT-TS and mutant mixtures. The first two lanes for each protein or protein mixture (lanes 1, 2, 4, 5 etc.) represent the migration of proteins as revealed by Coomassie Blue staining. The middle band in each grouping (lanes 2, 5, 8 etc.) is a result of adding [^{32}P]FdUMP and CH_2H_4-PteGlu to the various proteins and their mixtures. The third lane in each grouping represents the location of each ternary complex as revealed by autoradiography. The designation (1) indicates the effect that a single FdUMP-ternary complex has on the migration of the dimer and (2) the effect that two FdUMP−ternary complexes/dimer has on its rate of migration. Details for the formation of the ternary complexes and the acrylamide gel system employed are described in Materials and Methods.

to distinguish between the various inactive mutants and their active heterodimeric products, we tried various chromatographic separations without success. However, when we resorted to nondenaturing acrylamide gel electrophoresis, we were able to obtain distinct separations between some of the mutant dimers. The nature of these separations is shown in Figure 3, where it is seen that $R_{126}E$ migrates faster than $C_{146}W$ (lane 1 vs lane 4). Lane 7 reveals that $Y_{94}A$, a mutant that is probably inactive because it cannot affect the release of a hydride ion from the 5-position of the uracil ring of dUMP (Matthews et al., 1990), migrates similar to $C_{146}W$. It is not clear why WT-TS and its mutants migrate somewhat differently on nondenaturing gels, except perhaps that their conformations may be somewhat altered from one another. Slightly different isoelectric points do not appear to be responsible for the mobility differences, as isoelectric focusing studies on WT and mutant TSs did not reveal any significant changes in migration between them. It was hoped that only the active TS heterodimers would form a ternary complex with [^{32}P]FdUMP and CH_2H_4PteGlu, as opposed to the inactive mutants, so that the number of functionally active TS sites of heterodimer could be established by the extent of migration of the radioactive ternary complexes. This thesis is based on the finding of Aull et al. (1974) that each of the active subunits forms a ternary complex with FdUMP and CH_2H_4PteGlu, and the enzyme dimer containing one FdUMP migrates slower than that containing two FdUMP. However, as will be shown below, basically inactive mutants can still form ternary complexes. The second lane in each of the proteins in Figure 3 presents the results of ternary complex formation as visualized by protein staining alone while the third lane reveals only those bands that have formed a radioactive ternary complex with [^{32}P]FdUMP.

It is evident from lanes 2, 3, 5, and 6 that while $R_{126}E$ forms a weak radioactive band with [^{32}P]FdUMP, $C_{146}W$ does not interact with this compound at all in the presence of CH_2H_4PteGlu. The radioactive band associated with $R_{126}E$ coincides with the binding of a single FdUMP/dimer, which increases the migration of this complex (lanes 2 and 3) relative to $R_{126}E$ alone (lane 1). When those species that contain 1 and 2 FdUMP/dimer are formed such as in the case of WT-TS, two bands become evident (lanes 11 and 12). As shown in lanes 13−16, the incubation of $R_{126}E$ and $C_{146}W$ together yielded bands corresponding to each mutant protein (lane 13, also see lanes 1 and 4), but in the presence of [^{32}P]FdUMP and CH_2H_4PteGlu an additional faster moving band appears. The latter is most probably due to the presence of a ternary complex containing one FdUMP/dimer (lanes 14 and 15). This result is consistent with the thesis that a single active site is sufficient to provide the same activity seen with WT-TS, except that the latter is capable of forming two ternary complexes, one containing 1 FdUMP/dimer and another 2 FdUMPs/dimer (lanes 11 and 12). If sufficient FdUMP was present, only the band corresponding to 2 FdUMPs/dimer would be present as shown for the case of the inactive mutant $Y_{94}A$ (lanes 8 and 9). The findings with $R_{126}E$ and $C_{146}W$ are also supported by the results obtained with $R_{126}E$ and $Y_{94}A$. The latter mutant, as indicated, is capable of binding 2 FdUMPs/dimer but when incubated with $R_{126}E$ a new band results which is evident both on Coomassie Blue staining (lane 17) and radioactivity (lane 18). The fact that full activity results from this combination, which is associated with the binding of a single FdUMP/dimer, again emphasizes the point that the presence of a single functionally active site in the ($R_{126}E$)−($Y_{94}A$) heterodimer is sufficient to yield a specific activity equivalent to that of WT-TS.

DISCUSSION

There is little doubt that regardless of the source from which TS has been isolated the enzyme is a dimer of identical subunits. This appears true also for the bifunctional DHFR-TS present in protozoan parasites (Ivanetich & Santi, 1990; Knighton et al., 1994). The crystal structures of *L. casei* TS (Hardy et al., 1987) and *E. coli* TS (Montfort et al., 1990) reveal the enzyme subunits to be held together at a dimer interface involving 29 and 30 amino acids, respectively, most of which are hydrophobic although some hydrogen bonds are involved. The significance of Arg-126 is that although it is close to the interface and may play a role in maintaining the integrity of the dimer structure, its major contribution appears to be that of being donated from one monomer to the other so as to complete the dUMP binding site of each monomer (Montfort et al., 1990). From what we have described in this communication, the restoration of enzyme activity to basically inactive mutants of TS appears to require

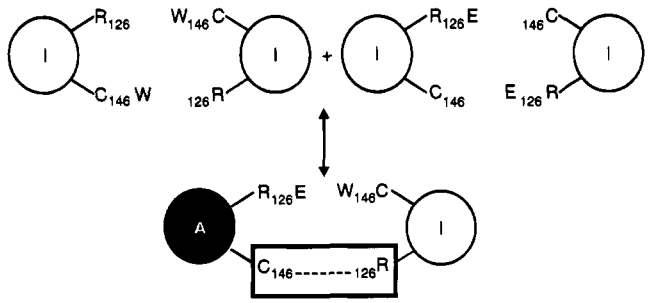

FIGURE 4: Diagrammatic presentation of the proposed active dimer complex resulting from the interaction of $R_{126}E$ and $C_{146}W$. R_{126} from a subunit of the mutant $C_{146}W$ is donated to the active site of a subunit of mutant $R_{126}E$ to restore the wild-type composition of this subunit (boxed region). E_{126} from a subunit of the mutant $R_{126}E$ is in turn donated to the active site region of the inactive $C_{146}W$ subunit. (A) Active subunit; (I) inactive subunit.

that the subunits separate and then recombine in such a manner as to yield one functionally active subunit/dimer as shown in Figure 4. As shown in this figure, the basically inactive TS mutants of $R_{126}E$ and $C_{146}W$ exchange subunits to form a heterodimer in which a subunit from the latter donates Arg-126 to the active site of the $R_{126}E$ subunit, while this subunit in turn donates its mutated Glu-126 to the $C_{146}W$ subunit. The presence of both a Glu at residue 126 and a Trp at residue 146 provides the heterodimer with one completely inactive subunit designated by I, a subunit which was inactive to begin with due to the absence of Cys-146. By contrast, the donation of Arg-126 from the $C_{146}W$ subunit to the $R_{126}E$ subunit, which contains an unmodified active site Cys at residue 146, restores activity to the heterodimer equivalent to that of the wild-type enzyme even though only one of the two subunits is active (A). Although other explanations are certainly possible, the subunit dissociation–reassociation thesis appears to be the most plausible explanation so far for the restoration of activity. How the subunits separate then recombine is yet to be established. It is of interest to note that attempts to restore activity to comparable mutant mixtures of *L. casei* TS ($R_{178}F + C_{198}A$) and T_4 phage-TS ($R_{136}E + C_{156}S$) were unsuccessful. In a similar vein, mixing comparable mutants from different species of TS (*L. casei* and *E. coli* and *E. coli* and T_4) did not restore activity (data not presented). However, activity could be obtained in these cases by denaturation–renaturation as already described (Greene et al., 1993). That dissociation of oligomers can occur without denaturation has been amply documented for allosteric enzymes, but in these cases specific ligands were involved in promoting this effect (Traut, 1994). Attempts to enhance the rate of reactivation of *E. coli* TS mutants through the use of TS substrates were not successful. The only requirement noted so far for the restoration of activity is to simply mix $R_{126}E$ with any other inactive mutant. While it can be claimed that the substrates in the assay cuvette are sufficient to promote subunit exchange, this would not explain how incubation of the mutant mixtures at 37 °C in the absence of ligands greatly enhances the reactivation or how incubation at 4 °C diminishes their activation rate. Nor would it explain why a 10-fold dilution of $R_{126}E + C_{146}W$ results in an enhancement of the rate of activation, that is unless all of the above conditions affect the extent of dissociation. Additional suggestive evidence is presented in Table 2, where it is shown that increasing Mg^{2+} concentration inhibits the extent of activation. This response is not unusual since high salt can act as a deterrent to both dissociation and reassociation.

What is even more intriguing is the effect that a single amino acid change can have on the rate of restoration of TS activity. Thus, as shown in Table 1, there is a wide disparity in the rates of appearance of TS activity on mixing various mutants with $R_{126}E$. From the data in this table, $Y_{94}A$ was the most effective in promoting the appearance of activity, $I_{264}Am$ was intermediate, and $C_{146}S$ was the poorest of the mutants tested. These data suggest that the location of a specific mutation in the TS molecule may affect the conformation at the dimer interface in such a manner as to increase or decrease the rate of subunit dissociation. How or if this occurs remains to be determined, but it is an intriguing possibility that should be investigated further. That mutant forms of TS may have slightly different conformations is suggested from their rates of migration in nondenaturing gels (Figure 3). Assuming that WT-TS migrates as a dimer (Figure 3), it would have to be assumed that $R_{126}E$, $Y_{94}A$, and $C_{146}W$ migrate similarly as dimers. Yet it is obvious that $C_{146}W$ (lane 4) and $Y_{94}A$ (lane 7) migrate more slowly that $R_{126}E$ (lane 1) and WT-TS (lane 10). Charge differences do not appear to be involved since these mutants when subjected to isoelectric focusing do not show any significant differences in mobility (data not presented). If it is assumed that complex formation with FdUMP and CH_2H_4PteGlu yields a faster migrating species depending on whether one or two FdUMPs are bound/dimer (lane 12) (Aull et al., 1974), it can be assumed that the faster moving labeled band in lane 15 is the result of a single FdUMP being bound/dimer of $(R_{126}E)-(C_{146}W)$. This band was not seen with $C_{146}W$ (lanes 5 and 6) and only weakly in $R_{126}E$ (lanes 2 and 3), which suggests that the strong radioactive band in lane 15 is in all likelihood due to the enhanced binding of FdUMP to the active subunit of the mixed heterodimer $(R_{126}E)-(C_{146}W)$. An apparent paradox results on comparing ternary complex formation by $R_{126}E$ (lane 3) and $Y_{94}A$ (lane 6) where it is seen that while the former mutant (lane 3) binds FdUMP rather weakly, the latter binds two FdUMPs/dimer rather strongly. This effect appears to be due to the fact that the R_{126} is involved in nucleotide binding while Y_{94} is probably involved in the release of a hydride ion from the 5-position of dUMP and is not involved in the binding of dUMP or CH_2H_4PteGlu. However, when $Y_{94}A$ forms a mixed heterodimer with $R_{126}E$, a new strong radioactive band appears which migrates to the position expected for a single bound FdUMP/dimer (lane 18). This band is proposed to be due to the enzymically active dimer $(R_{126}E)-(Y_{94}A)$ which is not unlike that obtained on mixing $R_{126}E$ and $C_{146}W$ (lane 15). Similarly, when $I_{264}Am$ was incubated with $R_{126}E$, the active dimer $(R_{126}E)-(I_{264}Am)$ was obtained (Figure 2), a result which contrasts with that reported by Aull et al. (1974) who showed that removal of a single valine from the carboxyl end of the *L. casei*–TS dimer resulted in a complete loss in TS activity. Why the other intact subunit is not active is unclear since analogous experiments performed by Carreras et al. (1994) using *L.casei* $R_{178}F$ and $V_{316}Am$ yielded the dimer $(R_{178}F)-(V_{316}Am)$ with a specific activity half that of the *L. casei* WT-TS, a reasonable finding if it is assumed that both subunits are active in the unmutated enzyme. However, these results contrast with those reported for the comparable *E. coli*–TS mutant heterodimer of $(R_{126}E)-(I_{264}Am)$, which yielded a specific activity comparable to

that of WT-TS (Figure 2). One possible explanation for these perplexing findings is that heterodimers behave differently than homodimers as catalytic units due to small differences in conformation at their respective active sites.

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