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# Heterodimeric Thymidylate Synthases with C-terminal Deletion on One Subunit*

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**We have combined site-directed mutagenesis with the technique of reversible unfolding and subunit dissociation to construct heterodimeric thymidylate synthases that lack the C-terminal valine from only one subunit of the dimer. Removal of this residue either from both subunits of the dimer by mutagenesis (V316Am mutation) or from only one subunit by treatment with carboxypeptidase has been reported to result in an inactive enzyme (Carreras, C. W., Climie, S. C., and Santi, D. V. (1992) *Biochemistry* 31, 6038–6044; Aull, J. L., Loeble, R. B., and Dunlap, R. B. (1974) *J. Biol. Chem.* 249, 1167–1172). Arg-178 is an essential active site residue of thymidylate synthase that is donated from the opposing subunit of the dimer. The R178F-V316Am heterodimer was formed by the unfolding and refolding of a mixture of inactive R178F and V316Am mutants. This enzyme has one intact active site and was found to have half of the activity and the same $K_m$ values as wild-type thymidylate synthase that was unfolded and refolded as a control. We have also formed the V316Am-WT heterodimer and report that this heterodimeric enzyme is also active, has a $k_{cat}$ value that is approximately half of that of the wild-type thymidylate synthase dimer, and binds substrate and cofactor with $K_m$ values similar to those of the wild-type enzyme.**

Thymidylate synthase (TS, EC 2.1.1.45)[1] is a dimeric enzyme that catalyzes the reductive methylation of dUMP by $CH_2H_4$folate to produce dTMP and $H_2$folate. The first indication that the C terminus of TS is involved in catalysis was the report that treatment of *Lactobacillus casei* TS with carboxypeptidase A led to liberation of a single valine from the dimer with concomitant inactivation of the enzyme (Aull *et al.*, 1974). Several subsequent reports also used carboxypeptidase treatment to inactivate TS and abolish folate binding (Galivan *et al.*, 1976, 1977). Their suggestion that the C terminus contributes to the cofactor binding site has since been confirmed crystallographically (Montfort *et al.*, 1990). Comparison of the TS·dUMP crystal structure with the structure of TS complexed with dUMP and the cofactor analog CB3717 has shown that folate binding induces a major conformational change in which the C-terminal residue, Val-316, moves more than 4 Å to anchor a lid that forms over the bound ligands (Finer-Moore *et al.*, 1993; Montfort *et al.*, 1990). The C-terminal carboxylate is especially important and coordinates an extensive hydrogen bond network that links active site residues to groups on both the substrate and the cofactor.

Mutagenesis of the conserved C-terminal valine of *L. casei* TS has shown that most amino acid replacements yield active enzyme; however, deletion of this residue inactivates the enzyme (Carreras *et al.*, 1992; Climie *et al.*, 1992). The defect of the deletion mutant was assigned to events that occur subsequent to binding of substrates on a step of the TS reaction that includes the C-terminal conformational change. The crystal structure of the unliganded V316Am mutant was found to be superimposable onto the structure of the wild-type enzyme (Perry *et al.*, 1993) and did not suggest an explanation for the selectivity of carboxypeptidase for only one subunit of TS or a reason why truncation of one subunit would affect the activity of the remaining subunit.

The observation that a single amino acid truncation of only one subunit with carboxypeptidase caused complete inactivation of the enzyme suggested either that only one of the two active sites observed crystallographically is functional or that modification of the C terminus of one subunit could inactivate the second subunit through a cooperative effect. In this work, we have combined site-directed mutagenesis with the technique of reversible unfolding and subunit dissociation to construct active, heterodimeric enzymes that lack the C-terminal valine from only one subunit of the dimer (Fig. 1).

## EXPERIMENTAL PROCEDURES

*Materials*—Plasmids that express mutant *L. casei* TSs in the Thy⁻ *Escherichia coli* strain χ2913 have been described (Climie *et al.*, 1990). Between 50 and 90 mg each of wild-type and V316Am, R178F, and C198A mutant *L. casei* TSs were purified from each liter of plasmid-containing χ2913 *E. coli* using an automated method and stored at −80 °C in 10 mM $KPO_4$, pH 7.0, 1 mM EDTA until use (Kealey and Santi, 1992). The enzyme preparations were >95% homogeneous as judged by SDS-polyacrylamide gel electrophoresis. Ultrapure urea was obtained from BDH Chemicals Ltd. (Pool, England). dUMP was obtained from Sigma, as was (6*R*,6*S*)-$H_4$folate, which was condensed with formaldehyde to produce (6*R*,6*S*)-$CH_2H_4$folate (Bruice and Santi, 1982). All concentrations of $CH_2H_4$folate given refer to the concentration of (6*R*)-$CH_2H_4$folate competent for the TS reaction. All other materials were obtained from commercial sources and used without purification.

*Assay of TS Activity*—TS activity was monitored spectrophotometrically at 340 nm using a Perkin-Elmer λ15 or Hewlett-Packard 8452A spectrophotometer as previously described (Pogolotti *et al.*, 1986). Assays were performed at 25 °C in the standard assay buffer, which contained 50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 75 mM β-mercaptoethanol. The concentration of wild-type TS was determined spectrophotometrically using $\epsilon_{278}$ = 125,600 $M^{-1}$ $cm^{-1}$; we assume that the V316Am, C198A, and R178F mutations have an insignificant effect on this value. Specific activities were determined

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[1] The abbreviations used are: TS, thymidylate synthase; V316Am TS, mutant *L. casei* TS lacking the C-terminal valine from both subunits; WT, wild-type; $CH_2H_4$folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; $H_2$folate, 7,8-dihydrofolate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

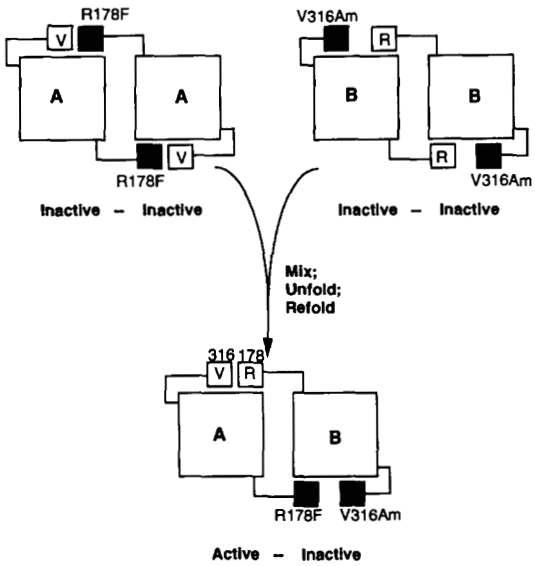


FIG. 1. **Subunit complementation in thymidylate synthase.** Inactive V316Am and R178F enzymes recombine to create a heterodimeric enzyme with one functional active site.

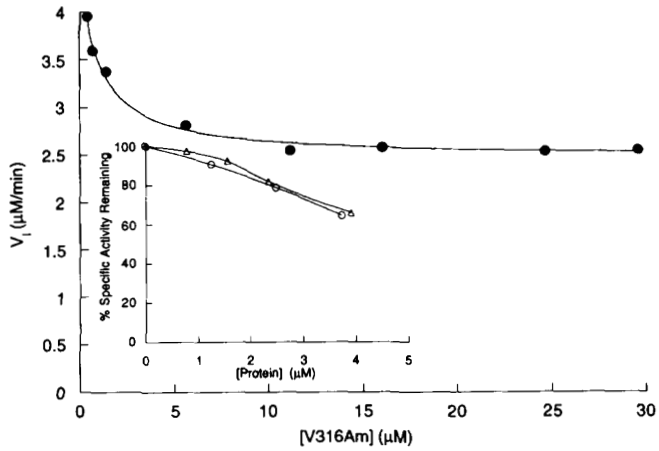


FIG. 2. **Formation of the V316Am-WT heterodimer.** *Main plot*, activity of 0.05 μM wild-type TS dimer refolded with increasing amounts of V316Am TS. The *x* axis refers to the concentration of V316Am TS in the refolding reaction. *Inset*, effect of protein concentration on the unfolding/refolding of wild-type TS. In this experiment 0.28 μM wild-type TS was refolded in the presence of increasing concentrations of either V316Am TS (△) or additional wild-type TS (○).

in 1-ml reaction mixtures that contained the standard assay buffer, 125 μM dUMP, and 170 μM $CH_2H_4$folate. One unit of TS is the amount of enzyme that will produce 1 μmol of product/min in a 1-ml reaction mixture. Using a highly sensitive radioactive assay, V316Am TS was shown to be inactive ($<4 \times 10^{-8}$ units/mg) (Carreras *et al.*, 1992). The R178F and C198A mutants had specific activities less than $10^{-3}$ units/mg as assessed spectrophotometrically. Wild-type TS used in these studies had a specific activity between 3.5 and 4.5 units/mg.

*Formation of R178F-V316Am and V316Am-WT Heterodimers*—Heterodimeric thymidylate synthases were prepared by the reversible dissociation of mixtures of mutant or wild-type enzymes as previously described (Perry *et al.*, 1992; Pookanjanatavip *et al.*, 1992). Unfolding was accomplished by mixing the enzymes with 3 volumes of unfolding buffer containing 20 mM $KH_2PO_4$, pH 7.0, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 M KCl, and 9 M urea and incubating at 4 °C for 2 h. Refolding was achieved by 10-fold dilution of the unfolding reaction into refolding buffer (unfolding buffer without urea) and incubating at 4 °C for 4 h. R178F-V316Am heterodimers were formed in refolding reactions that contained 0.3 μM V316Am TS and 0–1.3 μM R178F TS. Similarly, V316Am-WT heterodimers were formed in refolding reactions that contained 0.05 μM wild-type TS and 0–30 μM V316Am TS. TS assays were performed on 380-μl aliquots from the refolding reactions, and specific activities were determined by dividing initial rates by the monomer concentration of the constant enzyme. Urea and KCl were present in TS assays at 250 and 185 mM, respectively, and have been previously shown not to affect TS activity at these concentrations (Perry *et al.*, 1992). Controls were included in which (*a*) 0–4 μM wild-type TS was refolded, (*b*) urea was omitted from unfolding reactions, or (*c*) the well characterized R178F-C198A heterodimer was formed (Pookanjanatavip *et al.*, 1992).

*Steady-state Kinetics of Heterodimers*—Heterodimeric R178F-V316Am TS used in steady-state kinetic experiments was formed as described above from refolding mixtures containing 0.65 μM V316Am TS and 0.75 μM R178F TS. Heterodimeric V316Am-WT TS was formed from refolding mixtures containing 3 μM V316Am TS and 0.09 μM wild-type TS. TS assays used for the determination of $K_m$ values included 65–220 nM heterodimeric TS in the standard assay buffer. The $K_m$ for dUMP was determined by varying dUMP concentration from 0 to 250 μM in the presence of 170 μM $CH_2H_4$folate. $K_m$ values for $CH_2H_4$folate were determined by variation of the $CH_2H_4$folate concentration from 0 to 220 μM in the presence of 125 μM dUMP. Urea and KCl were present in assay mixtures at 200 and 150 mM, respectively. Kinetic constants were determined from a nonlinear least square fit to the Michaelis-Menten equation using the program Kaleidagraph (Abelbeck Software) run on a Macintosh II computer.

## RESULTS AND DISCUSSION

Studies of the effect of carboxypeptidase treatment on TS provided the initial indication of the importance of the C terminus in TS catalysis and showed that truncation of a single amino acid of one subunit is sufficient to inactivate the TS dimer (Aull *et al.*, 1974). V316Am TS, a C-terminal deletion mutant that lacks the C-terminal valine from both subunits of the dimer, is also completely inactive (Carreras *et al.*, 1992). The crystal structures of wild-type TS and V316Am TS (Hardy *et al.*, 1987; Montfort *et al.*, 1990; Perry *et al.*, 1993) show that the C terminus of the enzyme has no direct interaction with the dimer interface and that deletion of the C-terminal residue does not affect the overall structure of the enzyme or the local structure of the C terminus. None of these studies explained the selectivity of carboxypeptidase for only one subunit of the dimer or why its removal would cause the inactivation of both subunits of the dimer (Carreras *et al.*, 1992; Perry *et al.*, 1993). Recently, it was demonstrated that dimeric thymidylate synthase could be reversibly unfolded and dissociated with only a small loss of activity (Perry *et al.*, 1992). It was shown that refolding two appropriate, inactive TS mutants can create heterodimeric enzymes that contain one functional active site (Pookanjanatavip *et al.*, 1992; Greene *et al.*, 1993). In the present work, the approach has been used to create heterodimeric enzymes that lack the C-terminal valine on one subunit of the TS dimer (Fig. 1).

We attempted to form the V316Am-WT heterodimer by refolding the WT subunit in the presence of increasing amounts of the inactive V316Am, expecting one of several results. First, if the V316Am-WT heterodimer is completely inactive, excess V316Am would abolish the activity. Second, if each subunit of the wild-type dimer is partially or fully active, whether paired with a V316Am subunit or with another wild-type subunit, then the specific activity of wild-type TS would decrease or not change when it is converted to the V316Am-WT heterodimer. Third, if only one subunit of the wild-type dimer is active, refolding with an excess of V316Am TS would effectively double the concentration of active subunits, and up to a 2-fold increase in activity could occur.

When increasing concentrations of inactive V316Am TS were refolded with a constant amount of wild-type TS, the activity first decreased to about 65% of the initial value and then remained constant (Fig. 2). Kinetic parameters of the putative heterodimer were similar to the R178F-C198A heterodimer previously reported and the R178F-V316Am heterodimer reported here (Table I). One explanation for the behavior ob-

TABLE I
*Steady-state kinetic constants of heterodimeric thymidylate synthases*

| Enzyme | dUMP $K_m$[a] | $CH_2H_4$folate $K_m$ | $k_{cat}$ | $k_{cat}$/active site[b] |
|---|---|---|---|---|
| | μM | μM | $s^{-1}$ | $s^{-1}$ |
| WT TS[c] | 9 ± 1 | 17.5 ± 4 | 2.0 ± 0.2 | 1.0 ± 0.2 |
| V316Am-WT TS | 10.5 ± 2 | 10.5 ± 1 | 1.0 ± 0.2 | 1.0 ± 0.2 |
| R178F-C198A TS | 3.5 ± 2 | 7 ± 1 | 1.0 ± 0.1 | 1.0 ± 0.1 |
| R178F-V316Am TS | 6.5 ± 1 | 14 ± 4 | 1.0 ± 0.1 | 1.0 ± 0.1 |

[a] Values given include standard errors from nonlinear least square fit of the Michaelis-Menten equation.
[b] Heterodimers that lack essential active site residues on one subunit have only one active site/dimer.
[c] Unfolded and refolded as a control.

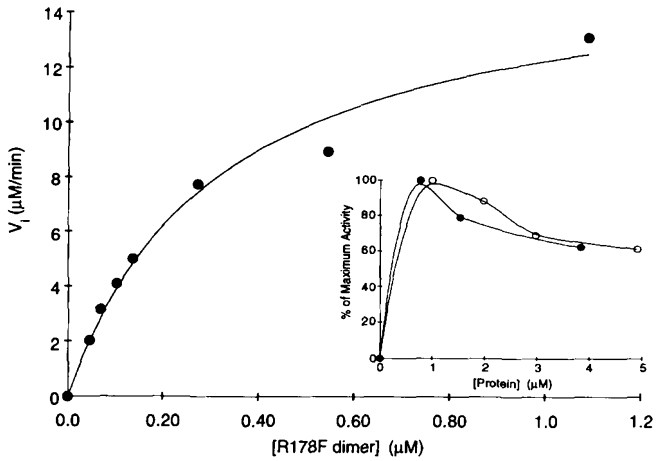


FIG. 3. **Formation of active heterodimers from inactive TS mutants.** *Main plot*, formation of the R178F-V316Am heterodimer. V316Am dimer (0.32 μM) was refolded in the presence of increasing concentrations of R178F dimer. The *x* axis refers to the concentration of R178F TS in the refolding reaction. *Points* are experimental, and the *line* is a best fit ($r > 0.99$) to equation 1 of Pookanjanatavip *et al.* (1992). *Inset*, formation of R178F-C198A and R178F-V316Am heterodimers in refolding reactions with high protein concentrations. Refolding reactions contained 0.34 μM R178F dimer and increasing amounts of either V316Am (●) or C198A TS (○).

served in Fig. 2 is that the V316Am-WT heterodimer is about 65% as active as the WT. However, we also observed a similar decrease of activity upon the refolding of high concentrations (>1 μM) of wild-type TS (Fig. 2, *inset*) or other heterodimers (Fig. 3, *inset*), so we believe the initial decrease in activity is due to a decreased recovery of activity from refolding reactions that contain high protein concentrations. Similar observations have been made on the dependence of protein concentration on the renaturation of inclusion bodies (Kohno *et al.*, 1990). Thus, it seems that the V316Am-WT heterodimer is indeed catalytically active. The only other explanation we can conceive of is that, regardless of the concentration of V316Am TS, only WT homodimers are formed in the refolding experiment. This seems highly unlikely in view of the facile formation of other heterodimers (Pookanjanatavip *et al.*, 1992; Greene *et al.*, 1993) and the noninvolvement of Val-316 in the dimer interface (Perry *et al.*, 1993).

Nevertheless, to unequivocally demonstrate the catalytic activity of a heterodimer containing a single V316Am subunit, we sought to create TS activity by forming heterodimers from two completely inactive mutants (one possessing a C-terminal deletion); any TS activity observed in this experiment could only be derived from the heterodimer. The inactive V316Am TS was folded in the presence of increasing concentrations of the inactive R178F mutant. In wild-type TS, Arg-178 is donated from one subunit to the active site of the second subunit to assist in binding the phosphate moiety of the substrate dUMP. In the R178F-V316Am heterodimer, the R178F mutation is part of the same active site as the V316Am mutation and prevents dUMP from binding to this active site. The second active site of the heterodimer has no mutations and contains the wild-type residues necessary for catalysis of dTMP formation (Fig. 1).

When inactive V316Am TS is refolded with inactive R178F TS, the resulting R178F-V316Am heterodimer catalyzes dTMP formation at a rate that is about 2-fold lower than wild-type TS (Fig. 3). Since neither of the two mutants is active on its own, the ability of inactive R178F monomers to complement inactive V316Am monomers unequivocally demonstrates that a TS heterodimer with a C-terminal deletion on one subunit can catalyze dTMP formation. In addition, the observation that the $k_{cat}$ value of the R178F-V316Am heterodimer was approximately 50% of the $k_{cat}$ value obtained with refolded wild-type TS suggests that both subunits of the wild-type dimer contribute equally to the catalytic activity of the enzyme.

Our observation of active heterodimers with a C-terminal deletion on one subunit is not in accordance with previous studies of carboxypeptidase-inactivated TS (Aull *et al.*, 1974; Cisneros *et al.*, 1993), but we do not know the reason for this discrepancy.

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