# Catalytically Active Cross-Species Heterodimers of Thymidylate Synthase†

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ABSTRACT: Thymidylate synthase (TS) is a highly conserved homodimeric enzyme with two active sites, each of which contains amino acid residues from both subunits. We show that the conservation at the subunit interface between *Escherichia coli* TS and *Lactobacillus casei* TS is sufficient to permit the formation of a cross-species heterodimer between subunits of *E. coli* TS and *L. casei* TS. Heterodimer formation was monitored by the generation of catalytic activity when combinations of inactive *E. coli* homodimers and inactive *L. casei* homodimers were mixed under conditions of reversible unfolding and dissociation. The inactive *L. casei* mutant enzymes (Lc)C198A, (Lc)C198L, and (Lc)V316Am were tested as Arg donors to the active sites of the inactive *E. coli* mutant enzymes (Ec)R126Q and (Ec)R126E, while the inactive *E. coli* mutant enzymes (Ec)K48Q, (Ec)C146S, (Ec)R166Q, and (Ec)I264Am were tested as Arg donors to the active site of inactive (Lc)R178F. Except for (Lc)V316Am, all of the mutant enzymes tested were able to form catalytically active cross-species heterodimers. (Lc)C198A and (Ec)R126Q were cotransformed on compatible plasmids into a thymine-requiring *E. coli* host, and this combination was able to form sufficient active TS in vivo to support growth. Titration of (Ec)R126Q with (Lc)C198A showed that the cross-species heterodimer formed with the same probability as the intraspecies homodimers in the refolding mixture. The single active site formed by this pair has $k_{cat}$ and $K_m$ values similar to those of an intraspecies heterodimer.

Thymidylate synthase (TS)[1] catalyzes the conversion of dUMP and $CH_2$-$H_4$folate to dTMP and dihydrofolate. TS is a dimer of identical subunits, and the dimeric structure is required for enzyme activity since each of the two active sites contains amino acid residues contributed by the other subunit. For example, Arg 178 and Arg 179 from one subunit of *Lactobacillus casei* TS bind to the phosphate group of the substrate dUMP in the active site of the other subunit (Hardy et al., 1987; Matthews et al., 1990; Montfort et al., 1990). Comparison of amino acid sequences shows that TS is one of the most conserved enzymes known with approximately 18% identical residues among 17 species (Perry et al., 1990). The three dimensional structures of TSs from four different species have been solved by X-ray crystallography, and as expected for enzymes with high sequence similarity, there is a highly conserved structural "core" (Finer-Moore et al., 1990; Perry et al., 1990). The dimer interface of TS is composed primarily of a six-stranded $\beta$-sheet which forms part of the conserved core. If structural conservation of this interface is sufficient between species, it might be possible to form heterodimers between these TSs.

In order to test whether related TSs can form cross-species heterodimers, we have taken advantage of the dimeric nature of the active site. We have previously shown that intraspecies heterodimer formation can be monitored by the acquisition of catalytic activity when certain types of inactive *L. casei* mutant enzymes are mixed *in vitro* under conditions of reversible unfolding and dissociation or *in vivo* by genetic complementation (Perry et al., 1992; Pookanjanatavip et al., 1992). Subunits of inactive Arg 178 and Cys 198 mutant enzymes can combine to form an active heterodimeric enzyme with a single active site which has steady-state parameters similar to those of a single active site in refolded wild-type enzyme (Pookanjanatavip et al., 1992). Similarly, if inactive subunits of *Escherichia coli* and *L. casei* TS could dimerize to form a competent active site (Figure 1), the presence of the cross-species heterodimer could also be monitored by the acquisition of catalytic activity. The inactive pairs of enzymes in this study were selected so that one member of the pair had a mutation at (Lc)Arg 178 [or (Ec)Arg 126]; the other had some other lesion in the active site region which rendered the homodimer inactive but had an intact Arg to donate to the active site of the first subunit (Figure 1). In this paper we report that catalytically active cross-species heterodimers of TS can be generated from pairs of inactive mutant enzymes.

## MATERIALS AND METHODS

*Plasmids and Bacterial Strains.* pMPTS(C198A) (*spectinomycin*$^r$, *choramphenicol*$^r$) (Pookanjanatavip et al., 1992) encodes *L. casei* TS with an Ala substitution at Cys 198. It is a derivative of pDPT2789, which has an incFII origin of replication and is compatible with the other plasmids in this study (Debouck et al., 1987). pSCTS9, a pUC18 derivative containing the synthetic TS gene, was the vector for the *L. casei* TS mutants (Climie et al., 1990). pThyA, a derivative

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[1] Abbreviations: TS, thymidylate synthase; DTT, dithiothreitol; dUMP, 2′-deoxyuridine 5′-monophosphate; dTMP, thymidine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; $CH_2$-$H_4$folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Ec, *Escherichia coli*; Lc, *Lactobacillus casei*; Am, amber.

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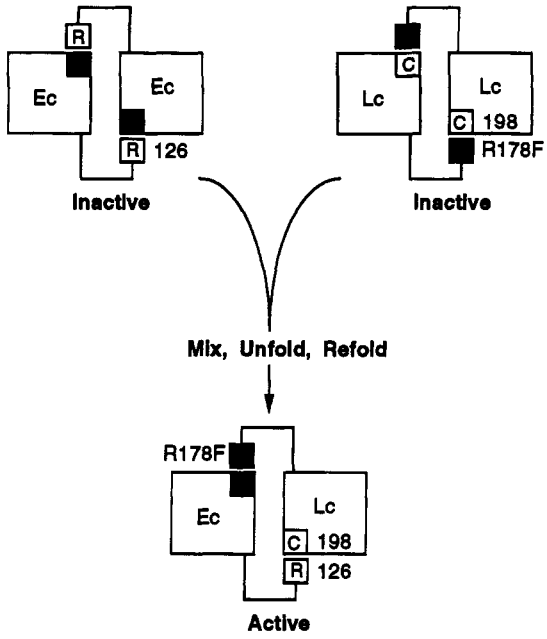


FIGURE 1: Cross-species subunit complementation. An inactive *E. coli* mutant TS which has an intact R126 is depicted acting as an Arg donor with an inactive *L. casei* TS which is inactive because of a mutation at R178 but has an otherwise intact active site. In cross species heterodimer formation, these two mutant enzymes would recombine to form a heterodimeric enzyme with a single intact active site sharing *E. coli* and *L. casei* contributions and a doubly defective active site.

of Bluescript (Stratagene), expresses *E. coli* TS at a high level (F. Maley, in preparation) and was used to prepare *E. coli* TS mutants.

*E. coli* strain χ2913RecA (Climie et al., 1992), which has a partial deletion of the *thy*A gene, was used for expression of *L. casei* enzymes. *E. coli* strain XAC25 [F⁻Δ(*gpt-lac*), *thy*A, *kan*$^r$, *gyr, rpo, arg, ara*] (Kim et al., 1992) was obtained from Jeffrey Miller, UCLA. This strain has the entire *thy*A gene deleted and was used in complementation assays.

*DNA Manipulations.* Bacterial culture, $CaCl_2$/heat shock transformation, and routine DNA manipulations were performed as described (Sambrook et al., 1989). The rapid transformation method of Chung and Miller (1988) was also used to obtain cotransformants.

*Preparation of TS Mutants. L. casei* TS mutants have been described previously (Climie et al., 1990; Climie & Santi, 1990; Pookanjanatavip et al., 1992). *E. coli* TS mutants were prepared by mismatch oligonucleotide mutagenesis of pThyA with a phosphorothioate/restriction selection enrichment step (Taylor et al., 1985) using the Amersham kit version 2.1.

*In Vivo Complementation.* pMPTS(C198A) was cotransformed with pThyA-TS[R126Q] into XAC25. pMPTS-(C198A) was also cotransformed with pUC18, while pThyA-TS[R126Q] was cotransformed with pDPT2789 so that all strains carried the same antibiotic resistance markers. Several single colonies from each transformation were purified by streaking on LB agar containing 50 μg/mL thymine, 50 μg/mL ampicillin, and 10 μg/mL chloramphenicol. Purified chloramphenicol/ampicillin-resistant colonies were tested for complementation by streaking on duplicate minimal agar plates [M-9 plus casamino acids (Sambrook et al., 1989)] containing 50 μg/mL ampicillin and 10 μg/mL chloramphenicol and containing or lacking 50 μg/mL thymine. Colonies producing catalytically active TS were identified by growth of Thy⁻ XAC25 cells on minimal agar lacking thymine.

*Protein Purification. L. casei* mutant enzymes were purified to homogeneity as judged by SDS–PAGE using successive automated chromatography steps on phosphocellulose (Whatman P11) and hydroxylapatite (Bio-Gel HTP, Bio-Rad) as described (Kealey & Santi, 1992). *E. coli* mutant enzymes were purified by DE52 chromatography as described (Maley & Maley, 1988). The pThyA expression system yields 30–50% of cell protein as TS, and with this expression level the single DE52 chromatography step yields homogeneous enzyme (F. Maley, in preparation).

*Unfolding/Refolding Reaction.* Purified enzymes were mixed in freshly made unfolding buffer [7–8 M urea (enzyme grade from Gibco BRL), 20 mM $KH_2PO_4$, 0.1 mM EDTA, 1 mM DTT, 0.4–0.45 M KCl, pH 7.0]. Unfolding mixtures were kept on ice for 1–3 h, then diluted 10-fold with 20 mM $KH_2PO_4$, 0.1 mM EDTA, 1 mM DTT and, 0.5 M KCl, pH 7.0, and kept on ice for 2–3 h. Enzyme activity is maximum during this time period. Up to 100 μL of each refolding reaction was assayed for TS activity in a total reaction volume of 1 mL. These conditions of unfolding/refolding and assay were optimized for *L. casei* TS (Perry et al., 1992).

*Enzyme Assays and Protein Measurements.* TS activity was determined spectrophotometrically by monitoring the conversion of $CH_2$-$H_4$folate to 7,8-dihydrofolate (Wahba & Friedkin, 1961) with the changes described by Pogolotti et al. (1986). Reaction mixtures (1.0 mL) contained 50 mM TES [*N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid], pH 7.4, 25 mM $MgCl_2$, 6.5 mM HCHO, 75 mM 2-mercaptoethanol, 150 μM $CH_2$-$H_4$folate, 125 μM dUMP, and 0.02–2.6 μM enzyme. Reactions were carried out at 25 or 30 °C as noted in the text or figure legends. One unit of enzyme activity is the amount of enzyme that produces 1 μmol of dTMP $min^{-1}$.

Protein concentration was determined by the method of Read and Northcote (1981) using BSA as a standard. Concentrations of purified *L. casei* and *E. coli* TS were determined using $\epsilon_{278} = 1.26 \times 10^5$ $M^{-1}$ $cm^{-1}$ for *L. casei* TS[2] and $\epsilon_{278} = 0.84 \times 10^5$ $M^{-1}$ $cm^{-1}$ for *E. coli* TS.

*TS–FdUMP–$CH_2$-$H_4$folate Ternary Complexes.* [$^{32}$P]FdUMP was synthesized from FUdR (Aldrich Chemical Co.) and [γ-$^{32}$P]ATP (>3000 Ci/mmol) (Du Pont NEN) using partially purified *E. coli* thymidine kinase (F. Maley, unpublished experiments). Purified [$^{32}$P]FdUMP was diluted with unlabeled FdUMP, and 10 μL containing 2.3 nmol ($6 \times 10^5$ cpm) was added to 1 mL of renaturation solution containing a maximum of 0.8 nmol of heterodimer. $CH_2$-$H_4$folate was added to a final concentration of 160 μM, and the reaction was allowed to proceed for 1 h at room temperature. The reaction mixture was concentrated to approximately 300 μL in a Centricon-30 (Amicon) apparatus with two 1-mL additions of 25 mM Tris-HCl (pH 7.5). Fifty-microliter aliquots of each reaction were analyzed by 12.5% SDS–PAGE (Laemmli, 1970). The gels were stained with Coomassie blue, dried, and autoradiographed.

## RESULTS

*In Vitro Subunit Complementation. L. casei* mutant enzymes (Lc)R178F, (Lc)C198A, (Lc)C198L, and (Lc)-V316Am and *E. coli* mutant enzymes (Ec)K48Q, (Ec)R126Q, (Ec)R126E, (Ec)C146S, (Ec)R166Q, and (Ec)I264Am were purified to homogeneity as judged by SDS–PAGE. Struc-

[2] The $\epsilon_{278}$ of *L. casei* TS has been corrected to $1.26 \times 10^5$ $M^{-1}$ $cm^{-1}$ using the spectrophotometric method of Edelhoch (1967); it is based on the number of tryptophan (7) and tyrosine (14) residues as determined from the protein sequence. The previous value of $1.07 \times 10^5$ $M^{-1}$ $cm^{-1}$ (Santi et al., 1974) had been calculated using the number of tryptophan (6) and tyrosine (11) residues determined by amino acid analysis.

Table I: Equivalent Residues[a]

| *E. coli* | *L. casei* |
|---|---|
| K48 | K50 |
| R126 | R178 |
| C146 | C198 |
| R166 | R218 |
| I264 | V316 |

[a] Equivalence is based on both sequence alignment and crystallographic analysis.

Table II: *In Vitro* Complementation by Intraspecies or Interspecies Heterodimer Formation[a]

| | acceptor TS | | |
|---|---|---|---|
| donor TS | (Ec)R126Q | (Ec)R126E | (Lc)R178F |
| (Ec)I264Am | + | nt | +[b] |
| (Ec)R166Q | + | nt | +[b] |
| (Ec)C146S | + | + | +[b] |
| (Ec)K48Q | + | nt | +[b] |
| (Lc)V316Am | – | nt | + |
| (Lc)C198A | + | + | + |
| (Lc)C198L | + | nt | + |

[a] Homodimers do not catalyze dTMP synthesis at a level detectable by the spectrophotometric assay (less than ~0.001 unit/mg). Activities measured for all of the active heterodimers are at least 0.05 unit/mg. nt = not tested. [b] Unstable during the enzyme reaction.

turally equivalent residues between *E. coli* and *L. casei* TSs are listed in Table I. The *L. casei* mutant enzymes (Lc)-C198A, (Lc)C198L, and (Lc)V316Am were tested as Arg 178 donors to the (Ec)R126 mutants. The *E. coli* mutant enzymes (Ec)C146S, (Ec)K48A, (Ec)R166Q, and (Ec)-I264Am were tested as Arg 126 donors with (Lc)R178F. Purified enzymes were mixed under conditions of reversible dissociation and unfolding as described in Materials and Methods and assayed for the generation of TS activity. (Lc)-C198A and (Lc)C198L mutant enzymes were able to complement both (Ec)R126Q and (Ec)R126E mutant enzymes, while (Lc)V316Am did not show cross-species complementation. In the reverse combinations, (Ec)K48A, (Ec)C146S, (Ec)R166Q, and (Ec)I264Am all complemented (Lc)R178F. These latter cross-species heterodimers, in which (Lc)R178F acts as the Arg acceptor, had lower specific activities than the heterodimers in which (Ec)R126Q or (Ec)-R126E acts as the Arg acceptor, and in addition they appear to be less stable during the TS reaction. That is, the rate of reaction is initially linear but falls off markedly after approximately 4-min reaction at 30 °C; the reaction rates of heterodimers containing (Ec)R126Q as the Arg acceptor remain linear until substrate becomes limiting. Intraspecies pairs tested as controls complemented in every case. Complementation results are summarized in Table II. No activity was recovered when (Ec)R126Q and (Lc)C198A were mixed and incubated on ice for up to 4 h in the absence of urea or in the presence of 0.8 M urea, the concentration present in refolding buffer. Thus the recovery of activity was dependent on reversible denaturation-induced dissociation.

*FdUMP Binding.* When TS is incubated with FdUMP in the presence of $CH_2$-$H_4$folate, a covalent ternary complex is formed which is stable during SDS–PAGE (Santi & McHenry, 1972). We incubated unfolded–refolded TSs with [$^{32}$P]-FdUMP plus $CH_2$-$H_4$folate and analyzed the reaction products by SDS–PAGE followed by autoradiography (Figure 2). Under the conditions used here, the inactive *E. coli* or *L. casei* mutant enzymes did not form significant amounts of covalent ternary complex. In contrast, the cross species heterodimer (Ec)C146S + (Lc)R178F and the intraspecies heterodimers

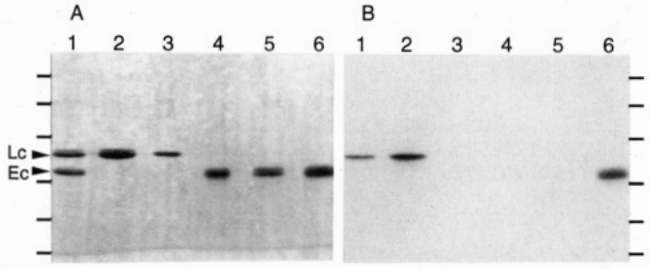


FIGURE 2: Enzyme–FdUMP–$CH_2$-$H_4$folate complexes with intraspecies and cross-species heterodimers. Reactions were carried out with [$^{32}$P]FdUMP as described in Materials and Methods. (Panel A) Coomassie blue stained gel. (Panel B) Autoradiogram of the gel shown in panel A. Lanes: (1) (Lc)R178F + (Ec)C146S; (2) (Lc)-R178F + (Lc)C198A; (3) (Lc)C198A; (4) (Ec)R126Q (5) (Ec)-C146S; (6) (Ec)R126Q + (Ec)C146S. The positions of *L. casei* and *E. coli* TSs are indicated at the left of panel A. The positions of standards at the following molecular weights ($\times 10^3$) are also indicated at the edges of the panels: 97.4, 66.2, 42.7, 31.0, 21.5, and 14.4.

(Lc)C198A + (Lc)R178F and (Ec)C146S + (Ec)R126Q each showed high incorporation of [$^{32}$P]FdUMP. The (Lc)C198A + (Ec)R126Q heterodimer also showed high incorporation of [$^{32}$P]FdUMP (data not shown). The subunit molecular masses of *E. coli* and *L. casei* TSs differ by approximately 5 kDa so they are easily distinguishable on SDS–PAGE. In the heterodimers, [$^{32}$P]FdUMP label comigrates with the subunit that contains the intact cysteine nucleophile, as would be predicted.

*In Vivo Complementation.* We also monitored cross-species heterodimer formation in *E. coli* cells by genetic complementation. pMPTS(C198A) and pThyA-TS[R126Q] were cotransformed into the Thy$^-$ host strain XAC25 (Kim et al., 1992). XAC25 was used for complementation since the entire ThyA gene has been deleted, and therefore complementation could not be achieved by recombination between the host and the ThyA plasmid. In order to obtain control strains with the same antibiotic resistance markers as controls, pMPTS-(C198A) was cotransformed with pUC18 to confer ampicillin resistance, and pThyA-TS[R126Q] was cotransformed with pDPT2789, the vector used in the construction of pMPTS-(C198A), to confer chloramphenicol resistance. All of these cotransformants were selected and restruck on rich medium (LB plus thymine) containing both ampicillin and chloramphenicol. Purified single colonies were then tested for their ability to grow on minimal medium lacking thymine. XAC25 containing the plasmid encoding (Lc)C198A plus the plasmid encoding (Ec)R126Q was able to grow in the absence of thymine, indicating the presence of an active cross-species heterodimeric TS in this strain. Neither pMPTS(C198A) nor pThyA-TS[R126Q] was able individually to confer the ability to grow in medium lacking thymine.

*Kinetic Characterization of (Lc)C198A + (Ec)R126Q Heterodimeric TS.* The recovery of active heterodimer was measured as a function of protein concentration. Equimolar mixtures of (Lc)C198A + (Ec)R126Q were mixed under unfolding conditions (over the range of 4.0–16 $\mu$M each homodimer), diluted into refolding buffer, and assayed for TS activity. TS activity increased linearly with protein concentration (data not shown). We determined $K_m$ values for dUMP and $CH_2$-$H_4$folate for the (Lc)C198A + (Ec)-R126Q heterodimer. The cross-species heterodimer had $K_m$ values of 7.2 $\mu$M for dUMP and 6.4 $\mu$M for $CH_2$-$H_4$folate, similar to those of the component wild-type enzymes and the intraspecies heterodimers [see below and Pookanjanatavip et al. (1992)] (Table III).

Table III: Steady-State Kinetic Parameters of Heterodimers

| | $K_m$(dUMP) ($\mu$M) | $K_m$($CH_2$-$H_4$folate) ($\mu$M) | $k_{cat}$/active site ($s^{-1}$) |
|---|---|---|---|
| (Lc)C198A + (Lc)R178F | 3.6[a] | 16[a] | 1.1[b] |
| (Ec)C146S + (Ec)R126Q | 3.8 | 7.1 | 1.2[c] |
| (Lc)C198A + (Ec)R126Q | 7.2 | 6.4 | 1.2[c] |

[a] Data from Pookanjanatavip et al. (1992); activity measurements at 25 °C. [b] Activity measurements at 25 °C. [c] Activity measurements at 30 °C.

To assess the concentration of the heterodimer in the cross-species mixtures so that we could estimate a $k_{cat}$, we carried out a titration as described previously for intraspecies heterodimers (Pookanjanatavip et al., 1992). A constant concentration of (Ec)R126Q was mixed with increasing concentrations of (Lc)C198A, and the initial rate of enzyme activity was plotted as a function of (Lc)C198A concentration. The dependence of initial rate on the concentration of (Lc)-C198A fits the model described in Pookanjanatavip et al. (1992) which assumes random reassociation of all monomers (Figure 3A). The excellent fit of the experimental data to the theoretical curve ($R = 0.99$) is consistent with a model where the (Lc)C198A + (Ec)R126Q heterodimer forms with the same probability as the (Lc)C198A and (Ec)R126Q homodimers. This result makes it possible to calculate the concentration of heterodimer in any refolding mixture. In the experiment shown in Figure 3A, the calculated $k_{cat}$ was 0.37 $s^{-1}$ at 25 °C; however, in some experiments we have observed a $k_{cat}$ of up to 1.2 $s^{-1}$ at 30 °C (see Table III).

*Kinetic Characterization of (Ec)C146S + (Ec)R126Q.* We characterized an intraspecies heterodimer of (Ec)TS for comparison to the cross-species heterodimer. The intraspecies *L. casei* heterodimer has been characterized previously (Pookanjanatavip et al., 1992).

The (Ec)R126Q + (Ec)C146S heterodimer was formed, and the $K_m$ values for dUMP and $CH_2$-$H_4$folate were determined to be 3.8 and 7.1 $\mu$M, respectively. The concentration of heterodimeric (Ec)TS in refolding mixtures was determined by titrating a constant concentration of (Ec)-R126Q with increasing concentrations of (Ec)C146S (Figure 3B) as described for the intraspecies heterodimer (Pookanjanatavip et al., 1992) and the cross-species heterodimer (above). The *E. coli* mixture of (Ec)R126Q and (Ec)C146S showed random reassociation of monomers in the refolding mixture. Using the calculated concentration of heterodimer, we obtained a $k_{cat}$ of 1.2 $s^{-1}$ for (Ec)R126Q + (Ec)C146S. The catalytic constants of intraspecies and interspecies heterodimers are compared in Table III.

## DISCUSSION

We sought to determine whether dimer formation in TS could tolerate differences in the subunit interface which would result from an *E. coli–L. casei* TS heterodimer. The strategy was to combine inactive mutant enzymes from one species with inactive mutant enzymes from the other species; pairs of enzymes were chosen so that if a cross-species heterodimer formed, it could potentially have one catalytically functional active site and one catalytically incompetent active site (see Figure 1). We found that subunits of *E. coli* TS and *L. casei* TS can associate to form catalytically active heterodimers. Heterodimer formation does not occur under native conditions but readily occurs when urea-denatured subunits are refolded to the native state. In the experiments reported here, several inactive mutant enzymes which had intact (Ec)Arg 126 or (Lc)Arg 178 were tested as Arg donors to the active sites of (Lc)R178F, (Ec)R126Q, or (Ec)R126E. Of the Arg donors tested, (Lc)Cys 198 and (Ec)Cys 146 mutant enzymes are inactive because they do not possess the essential active site nucleophile; (Lc)V316Am and (Ec)I264Am have lost the C-terminal residue which is required for an essential conformational change that accompanies formation of the ternary complex (Carreras et al., 1992; Matthews et al., 1990; Montfort et al., 1990); (Lc)Lys 50 [or (Ec)Lys 48] is involved in binding the folate cofactor (Finer-Moore et al., 1990; Maley et al., 1982); and (Lc)Arg 218 [or (Ec)Arg 166] binds the phosphate of dUMP and also hydrogen bonds to other residues within the active site (Finer-Moore et al., 1990; Montfort et al., 1990). All of the mutant enzymes used as Arg donors were inactive as homodimers but formed catalytically active dimers with an inactive Arg mutant from the same species. Most importantly, with the exception of (Lc)V316Am, these mutant enzymes also formed catalytically active *E. coli–L. casei* hybrid heterodimers.

The dimer interface of TS is formed by contact between the surfaces of a six-stranded $\beta$-sheet from each monomer plus several residues from other parts of the monomer (Hardy et al., 1987; Matthews et al., 1989, 1990; Montfort et al., 1990; Perry et al., 1990) (Table IV). Perry and co-workers have made a detailed comparison of the positions of atoms between (Ec)TS and (Lc)TS and found that the positions of $\alpha$-carbons of the $\beta$-sheet are virtually superimposable (Perry et al., 1990). The overall sequence conservation of the $\beta$-sheet is approximately 80%; 19 of 24 residues whose side chains are in close contact across the interface are identical (Table IV). The regions of contact which are in loops between secondary structural elements show greater differences in the $\alpha$-carbon positions (Perry et al., 1990), and the sequence conservation is also less; 5 of 11 residues in the loops vary, and all of the differences are nonconservative. Of the five variant residues in the $\beta$-sheet, one of the changes is the conservative difference of Ile (Lc252)–Val (Ec200). A more striking difference is the covariant pair of Thr-Leu/Ala-Phe (Ec148–149) (Lc200–201) which lies on the axis of 2-fold symmetry of the dimer (Perry et al., 1990). Attempting to pair the larger Phe with Thr might cause steric problems; however, inspection of the sequences of several species reveals that these positions are tolerant to substitutions, with a side chain as large as Ile paired with Phe (*Saccharomyces cerevisiae*) and one as small as Ala paired with Leu (human and mouse). Table V summarizes the nonidentical residues between *L. casei* and *E. coli* TSs and lists contacts at the dimer interface. Seven of the ten nonidentical residues interact with residues which are also variant residues. One notable feature of the varying residues is that all occur either at the edges of the $\beta$-strands or in loops between secondary structural elements (see Table IV). Another important feature of the TS subunit interface is the high percentage of hydrophilic and charged residues, many of which form hydrogen bonds across the subunit interface (Matthews et al., 1990; Perry et al., 1992) [see the kinemage appendix in Perry et al. (1992)]. All of the residues which clearly form hydrogen bonds across the interface are conserved.

In intraspecies subunit complementation by TS, the concentration of heterodimers is determined by random reassociation of the monomers in the refolding mixture (Pookanjanatavip et al., 1992). We wanted to determine whether the differences at the subunit interface would result in preferential association of the homodimers in refolding mixtures containing *E. coli* and *L. casei* TSs. The titration experiment shown in Figure 3A indicates that the probability of (Lc)C198A +

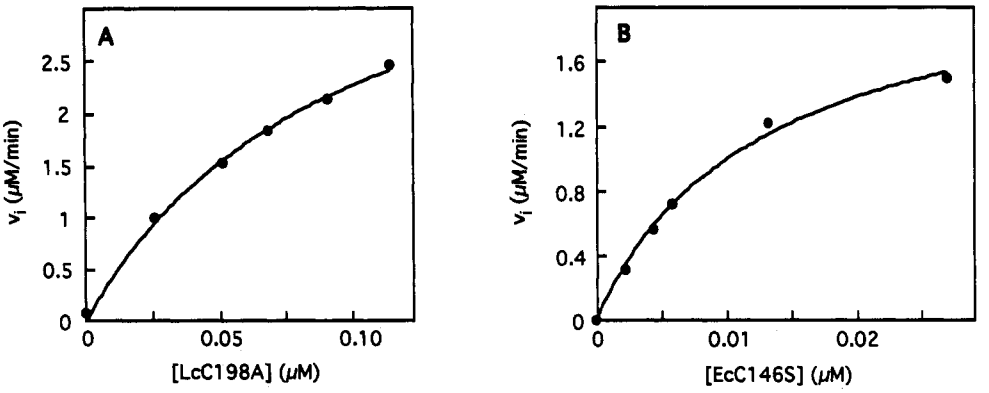


FIGURE 3: (Panel A) Initial velocity of dTMP synthesis by the reassociated Ec/Lc heterodimer plotted as a function of (Lc)C198A concentration with a constant concentration of (Ec)R126Q (0.10 μM). (Lc)C198A was combined with (Ec)R126Q under reversible unfolding/refolding conditions as described (Materials and Methods). Initial velocity is proportional to the concentration of the heterodimer; the solid line is the best fit of the data to the equation $v_i = V_{max}[(Lc)C198A]/([(Lc)C198A] + [(Ec)R126Q])$ which describes the behavior of the system when monomers reassociate randomly (i.e., heterodimer probability equals homodimer probability) (Pookanjanatavip et al., 1992). (Panel B) Initial velocity of dTMP synthesis by the reassociated Ec/Ec heterodimer plotted as a function of (Ec)C146S concentration with a constant concentration of (Ec)R126Q (0.015 μM). The homodimers were combined under reversible unfolding/refolding conditions as described (Materials and Methods). The solid line is the best fit to the equation specified in the legend to panel A.

Table IV: Amino Acids Whose Side Chains Are Involved in Intersubunit Contacts[a]

| β-sheet | | | other structural elements | | |
|---|---|---|---|---|---|
| *L. casei* | *E. coli*[b] | β-strand | *L. casei* | *E. coli*[b] | structure |
| His 18 | Thr 16 | –i | Pro 21 | Asn 19 | loop between β-strand –i and i |
| Lys 20 | 18 | | Asp 22 | 20 | |
| Ser 30 | 28 | i | | | |
| Phe 32 | 30 | | | | |
| Gln 35 | 33 | | Trp 153 | 101 | loop immediately after helix G |
| Arg 37 | 35 | | Thr 155 | 103 | |
| Ile 181 | 129 | v | Ser 156 | Pro 104 | |
| Ser 183 | 131 | | | | |
| Thr 200 | Ala 148 | iv | Tyr 176 | Asp 124 | loop between helix H and β-strand v |
| Leu 201 | Phe 149 | | Arg 178 | 126 | |
| Gln 203 | 151 | | | | |
| Tyr 205 | 153 | | | | |
| Val 206 | 154 | | Trp 185 | 133 | loop between β-strand v and helix I |
| Asn 207 | Ala 155 | | Pro 187 | Val 135 | |
| Ser 212 | 160 | iii | Glu 188 | Gly 136 | |
| Gln 214 | 162 | | | | |
| Tyr 216 | 164 | | Asp 208 | 156 | turn between β-strand iv and β-strand iii |
| Arg 218 | 166 | | | | |
| Ser 219 | 167 | | | | |
| Ile 252 | Val 200 | ii | | | |
| Thr 254 | 202 | | | | |
| Asp 257 | 205 | | | | |
| His 259 | 207 | | | | |
| Tyr 261 | 209 | | | | |

[a] This list was compiled from a computer-generated list of symmetry-related atoms from opposite subunits within a distance of 4 Å in the unliganded *L. casei* TS dimer (supplied by Janet Finer-Moore). Published descriptions of the TS dimer interface were also consulted (Hardy et al., 1987; Matthews et al., 1989, 1990; Montfort et al., 1990). The list in the table is not comprehensive:backbone interactions and water-mediated pairs are not included. [b] The residue name is given only when it differs from *L. casei* TS.

(Ec)R126Q dimer formation is equivalent to the probability of homodimer formation. However, an equal probability of dimerization does not mean that interspecies heterodimer stability is unaffected by the differences at the subunit interface. More likely, the composition of dimers in the refolded mixture is under kinetic control, and once dimers are formed, they do not dissociate in nondenaturing conditions. In support of this, mixtures of homodimers that readily form heterodimers upon unfolding/refolding do not do so under nondenaturing conditions. Random reassociation of cross-species heterodimers has also been reported for triosephosphate isomerase in cases where the amino acids at the interface are 100% conserved (Sun et al., 1992). In more distantly related pairs, the homodimers reassociate preferentially.

In order to determine the competency of the active site formed in a cross-species heterodimer, kinetic parameters were measured for the (Lc)C198A + (Ec)R126Q pair. The $k_{cat}$ and $K_m$ values for dUMP and $CH_2$-$H_4$folate are essentially identical to the (Ec)TS and (Lc)TS wild-type values and to the values for the intraspecies pairs. The similar kinetic constants indicate that, in the best case, the cross-species active site is catalytically very similar to the single species active site. However, cross-species dimers are more sensitive to the nature of the mutation than intraspecies dimers. Thus, while the combinations (Lc)C198A or (Lc)C198L mixed with (Ec)-R126Q or (Ec)R126E yield heterodimers with enzyme activity nearly equivalent to that of the intraspecies heterodimers, when (Ec)C146S serves as the Arg donor to (Lc)R178F, the

Table V: Nonidentical Interface Residues and Dimer Contacts

| nonidentical residue[a] | interacts with *L. casei* residue[a] |
|---|---|
| His 18 (Thr 16) | Asn 207 (Ala 155) |
| Pro 21 (Asn 19) | Tyr 176 (Asp 124) |
| Ser 156 (Pro 104) | Ser 156 (Pro 104), Thr 155 (Thr 103) |
| Tyr 176 (Asp 124) | Pro 21 (Asn 19) |
| Pro 187 (Val 135) | Trp 153 (Trp 101) |
| Glu 188 (Gly 136) | Thr 155 (Thr 103) |
| Thr 200 (Ala 148) | Leu 201 (Phe 149) |
| Leu 201 (Phe 149) | Thr 200 (Ala 148) |
| Asn 207 (Ala 155) | His 18 (Thr 16) |
| Ile 252 (Val 200) | Phe 32 (Phe 30) |

[a] The list of residues and the interactions indicated are based on the unliganded *L. casei* dimer. Corresponding *E. coli* TS residues are listed in parentheses.

specific activity and apparent stability of this heterodimer are reduced. This is in contrast to *L. casei* TS intraspecies heterodimer formation where Arg 178 and Cys 198 substitutions representing a wide range of size and charge combine to form active heterodimers which, in every pair assayed, had enzyme activity nearly comparable to that expected for a single wild-type active site. Furthermore, at least one cross-species pair was inactive [(Lc)V316Am + (Ec)R126Q]. The reduced level or absence of activity in these less active combinations could result from lowered efficiency of the mixed active site or in preferential reassociation of the homodimers.

The comparable catalytic activities of the best cross-species heterodimer and the intraspecies heterodimer indicate that the structural conservation between *E. coli* and *L. casei* is sufficient for strong dimerization between these two different TSs. We have also attempted to form cross-species heterodimers of *E. coli* TS or *L. casei* TS with phage T4 TS. Although T4 TS is able to form active intraspecies heterodimers, we have been unable to detect complementation between T4 TS and either *E. coli* TS or *L. casei* TS (F. Maley, unpublished data). T4 TS is more distantly related, with the sequence conservation of the side chains making contact at the subunit interface reduced to approximately 60% in the $\beta$-sheet [compared to 80% between (Ec)TS and (Lc)TS] and less than 50% in the other structural elements [compared to 60% between (Ec)TS and (Lc)TS]. The differences provide a framework for a mutagenic analysis of the subunit interface directed at defining requirements for subunit recognition.

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## REFERENCES

Carreras, C. W., Climie, S. C., & Santi, D. V. (1992) *Biochemistry 31*, 6038–6044.

Chung, C. T., & Miller, R. H. (1988) *Nucleic Acids Res. 16*, 3580.

Climie, S. C., & Santi, D. V. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 633–637.

Climie, S. C., Ruiz-Perez, L., Gonzalez-Pacanowska, D., Prapunwattana, P., Cho, S. W., Stroud, R. M., & Santi, D. V. (1990) *J. Biol. Chem. 265*, 18776–18779.

Climie, S. C., Carreras, C. W., & Santi, D. V. (1992) *Biochemistry 31*, 6032–6038.

Debouck, C., Gorniak, J. G., Strickler, J. E., Meek, T. D., Metacalf, B. W., & Rosenberg, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8903–8906.

Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.

Finer-Moore, J. S., Montfort, W. R., & Stroud, R. M. (1990) *Biochemistry 29*, 6977–6986.

Hardy, L. W., Finer-Moore, J. S., Montfort, W. R., Jones, M. O., Santi, D. V., & Stroud, R. M. (1987) *Science 235*, 448–455.

Kealey, J. T., & Santi, D. V. (1992) *Protein Expression Purif. 3*, 380–385.

Kim, C. W., Michaels, M. L., & Miller, J. H. (1992) *Proteins: Struct., Funct., Genet. 13*, 352–363.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Maley, G. F., & Maley, F. (1988) *J. Biol. Chem. 263*, 7620–7627.

Maley, G. F., Maley, F., & Baugh, C. M. (1982) *Arch. Biochem. Biophys. 216*, 551–558.

Matthews, D. A., Appelt, K., & Oatley, S. J. (1989) *J. Mol. Biol. 205*, 449–454.

Matthews, D. A., Appelt, K., Oatley, S. J., & Xuong, N. H. (1990) *J. Mol. Biol. 214*, 923–936.

Montfort, W. R., Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Maley, G. F., Hardy, L., Maley, F., & Stroud, R. M. (1990) *Biochemistry 29*, 6964–6977.

Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Montfort, W. R., Maley, G. M., Maley, F., & Stroud, R. M. (1990) *Proteins: Struct., Funct., Genet. 8*, 315–333.

Perry, K. M., Pookanjanatavip, M., Zhao, J., Santi, D. V., & Stroud, R. M. (1992) *Protein Sci. 1*, 796–800.

Pogolotti, A. L., Danenberg, P. V., & Santi, D. V. (1986) *J. Med. Chem. 29*, 478–482.

Pookanjanatavip, M., Yuthavong, Y., Greene, P. J., & Santi, D. V. (1992) *Biochemistry 31*, 10303–10309.

Read, S. M., & Northcote, D. H. (1981) *Anal. Biochem. 116*, 53–64.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Santi, D. V., & McHenry, C. S. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1855–1857.

Santi, D. V., McHenry, C. S., & Perriard, E. R. (1974) *Biochemistry 13*, 467–470.

Sun, A.-Q., Umit Yuksel, K., & Gracy, R. W. (1992) *J. Biol. Chem. 267*, 20168–20174.

Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8765–8785.

Wahba, A. J., & Friedkin, M. (1961) *J. Biol. Chem. 236*, 11–12.