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Subunit Complementation of Thymidylate Synthase†

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ABSTRACT: Each of the two active sites of thymidylate synthase contains amino acid residues contributed by the other subunit. For example, Arg-178 of one monomer binds the phosphate group of the substrate dUMP in the active site of the other monomer [Hardy et al. (1987) *Science 235*, 448–455]. Inactive mutants of such residues should combine with subunits of other inactive mutants to form heterodimeric hybrids with one functional active site. In vivo and in vitro approaches were used to test this hypothesis. In vivo complementation was accomplished by cotransforming plasmid mixtures encoding pools of inactive Arg-178 mutants and pools of inactive Cys-198 mutants into a host strain deficient in thymidylate synthase. Individual inactive mutants of Arg-178 were also cotransformed with the C198A mutant. Subunit complementation was detected by selection or screening for transformants which grew in the absence of thymidine, and hence produced active enzyme. Many mutants at each position representing a wide variety of size and charge supported subunit complementation. In vitro complementation was accomplished by reversible dissociation and unfolding of mixtures of purified individual inactive Arg-178 and Cys-198 mutant proteins. With the R178F+C198A heterodimer, the K_m values for dUMP and CH_2H_4folate were similar to those of the wild-type enzyme. By titrating C198A with R178F under unfolding–refolding conditions, we were able to calculate the k_{cat} value for the active heterodimer. The catalytic efficiency of the single wild-type active site of the C198A+R178F heterodimer approaches that of the wild-type enzyme.

Thymidylate synthase (TS)[1] (EC 2.1.1.45) catalyzes the conversion of deoxyuridine monophosphate (dUMP) and 5,-10-methylenetetrahydrofolate (CH_2H_4folate) to thymidine monophosphate (dTMP) and dihydrofolate. Comparison of the amino acid sequences of TS from over 17 sources has revealed that TS is the most conserved enzyme known (Perry et al., 1990). It is a dimer of identical subunits of about 35 kDa each, and structural studies have shown that the dimer interface of each monomer is composed of a five-stranded β-sheet, in which some 29 residues interact with the other monomer (Hardy et al., 1987). Arg-178 and Arg-179 are donated from one monomer to the active site of the other, and these residues interact with the phosphate moiety of the substrate dUMP. Surprisingly, all Arg-179 mutants thus far examined and about half of the Arg-178 mutants were catalytically active (Climie et al., 1990; Michaels et al., 1990). Thus, the Arg-178 and -179 residues per se are not essential, but not all residues at position 178 support catalysis.

We reasoned that an inactive Arg-178 mutant TS, incapable of donating a functional Arg-178 to the other subunit, and a second inactive mutant with Arg at position 178 could combine to form a heterodimer containing one functional active site (Figure 1). In this paper, we show that inactive Arg-178 mutants can complement inactive Cys-198 mutants by forming catalytically active heterodimers. The detection of active heterodimers was facilitated by the use of genetic complementation to select or screen for catalytically active TS in Thy⁻ bacteria.

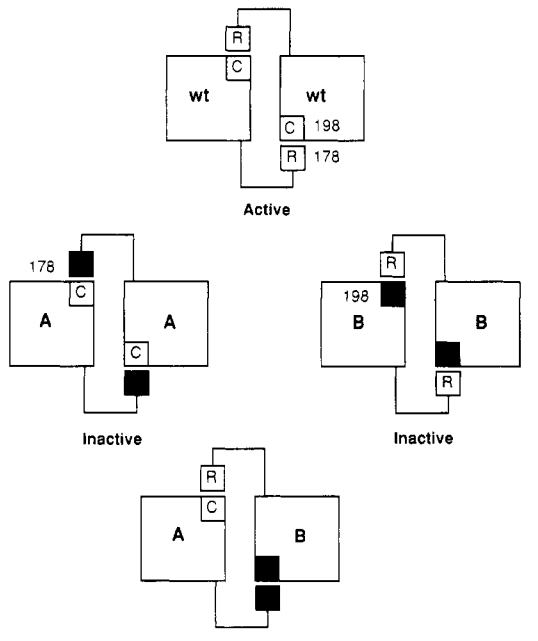

FIGURE 1: Subunit complementation. Inactive R178F and C198A mutants recombine to form a heterodimer with a single intact active site.

MATERIALS AND METHODS

pSCTS9 is a pUC-derived plasmid carrying the synthetic *Lactobacillus casei* TS gene (Climie & Santi, 1990). pDPT2789, a high copy number chloramphenicol-resistant

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[1] Abbreviations: TS, thymidylate synthase; DTT, dithiothreitol; dUMP, deoxyuridine 5′-monophosphate; dTMP, thymidine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; ON, oligonucleotide; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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incFII plasmid derived from pDPT287 (Debouck et al., 1987), was a gift from Dr. A. R. Schatzman (Smith Kline and Beecham Laboratories). *Escherichia coli* strain χ2913RecA (Δ*thyA*572, *recA*56) (Climie et al., 1992) was used for complementation assays and for expression of enzymes. *E. coli* DH5α-competent cells were purchased from Bethesda Research Laboratories. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim. The Qiagen kit for plasmid isolation and purification was purchased from Diagen Inc. The Gene Amp DNA amplification kit was from Perkin Elmer–Cetus. [6-^{3}H]FdUMP (20 Ci/mmol) was from Moravek Biochemicals. DNA sequencing was performed by the dideoxy chain-termination method using the Sequenase kit from U.S. Biochemical Corp. Oligonucleotides were prepared at the UCSF Biomolecular Resource Center and purified as described (Ivanetich et al., 1991). Other materials have been previously described (Climie et al., 1990; Pogolotti et al., 1986) or were obtained commercially.

Bacterial culture, $CaCl_2$/heat shock transformation, and routine DNA manipulations were performed as described (Sambrook et al., 1989). Protein concentration was determined by the method of Read and Northcote (1981) using BSA as a standard. The concentration of purified TS was determined by measuring A_{278} ($\epsilon = 1.07 \times 10^5$ M^{-1} cm^{-1}) (Santi et al., 1974). SDS–PAGE was performed as described (Laemmli, 1970). Plasmid copy number was measured as described (Taylor & Brose, 1988).

Complementation Assays. Individual colonies were streaked on duplicate minimal agar plates containing ampicillin and chloramphenicol containing or lacking 50 μg/mL thymine. Transformants producing catalytically active TS were identified by the ability of Thy$^-$ χ2913RecA cells harboring both plasmids to grow on minimal agar lacking thymine.

Inactive Arg-178 and Cys-198 Mutant Plasmid DNA Pools. Mixtures of mutant plasmids were prepared by casette mutagenesis of the synthetic *L. casei* TS gene using derivatives of pSCTS9 (Climie & Santi, 1990; Climie et al., 1990). pSCTS9(ST-*Nco*I/*Sna*BI) is a derivative of pSCTS9 with a noncoding sequence which contains unique *Not*I and *Sph*I sites between the *Nco*I and *Sna*BI sites (Climie & Santi, 1990). A synthetic double-stranded oligonucleotide which restored the TS coding sequence between the *Nco*I and *Sna*BI sites and replaced the Cys-198 codon with NN(G+C) (where N = GATC) was ligated into gel-purified *Nco*I–*Sna*I-digested vector, and the ligated mixture was used to transform *E. coli* DHFα. Approximately 1.4×10^4 colonies were pooled in 30 mL of LB medium and incubated at 37 °C for 30 min. Pooled plasmid DNA was isolated by alkaline lysis, purified using a Qiagen column, digested with *Not*I to reduce or eliminate the background of parent vector (Wells et al., 1986), and used to transform *E. coli* χ2913RecA.

The Arg-178 total mutant pool was prepared in pSCTS9-(*Sma*I). This vector is a Y176G mutant of pSCTS9 which contains a *Sma*I site at nucleotide 523 and a unique *Sal*I site spanning the Arg-178 codon (D. V. Santi, unpublished experiments). A synthetic double-stranded oligonucleotide which restores Y176 and contains NN[G+C] at codon 178 was ligated into gel-purified *Sma*I–*Bsu*36I-digested vector, and the ligated mixture was used to transform *E. coli* DH5α. Approximately 7×10^3 transformants were pooled in 30 mL of LB medium and incubated at 37 °C for 30 min. Pooled plasmid DNA was isolated by alkaline lysis, purified using a Qiagen column, digested exhaustively with *Sal*I to reduce the background of parent vector, and used to transform *E. coli* χ2913RecA.

Inactive pools of Arg-178 and Cys-198 plasmids were prepared from the total pools of transformants in χ2913RecA; 150 Arg-178 mutants and 200 Cys-198 mutants were transferred to duplicate minimal agar plates containing or lacking thymine to determine which transformants contained active enzyme. Colonies which grew on plates lacking thymine were removed from the corresponding plates containing thymine and discarded, and the remaining colonies (76/150 Arg-178 mutants; 186/200 Cys-198 mutants) were suspended in 3 mL of LB and grown for 3 h. Plasmid DNA from the Arg-178 and Cys-198 pools of inactive mutant colonies was isolated by alkaline lysis and purified using Qiagen columns.

A mixture of 0.4 μg of DNA from the inactive Arg-178 and Cys-198 pools was used to transform *E. coli* χ2913RecA. The individual inactive pools were also used individually to transform *E. coli* χ2913RecA. Transformation cultures were plated in duplicate on minimal agar containing 50 μg/mL ampicillin containing or lacking 50 μg/mL thymine and incubated at 37 °C overnight. Colonies which grew in the absence of thymine were isolated, plasmid DNA was prepared, and the DNA sequence of the Arg-178–Cys-198 region of the TS gene was determined. These DNA samples were also used to retransform *E. coli* χ2913RecA. Transformed cells from the secondary transformation were plated in duplicate on minimal medium containing or lacking thymine. Plasmid DNA was isolated from Thy$^-$ and Thy$^+$ colonies derived from the secondary transformation, and the DNA sequence of the Arg-178–Cys-198 region of the TS gene was again determined.

PCR Amplification of the TS Gene from pSCTS9. Reaction mixtures (100 μL) contained 5 pmol of each of the two oligonucleotide primers, ON1 and ON2, 1 ng (0.43 fmol) of pSCTS9 DNA, all four dNTPs at 200 μM each, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 10% DMSO, and 5 units of *Thermus aquaticus* DNA polymerase. ON1 (5′-AGCAAGCTTCTAGAGGC-CTCCGACTGAAAGCGGGCA-3′) contains sites for *Hin*dIII (bases 4–9), *Xba*I (bases 9–14), and *Stu*I (bases 14–19) followed by 17 bases complementary to the sequence immediately upstream of the lacZ promoter of pUC18 (nucleotide positions 2074–2080, 2082–2091 of pUC18; nucleotide 2081 was inadvertently omitted), the parent of pSCTS9. ON2 (5′-AGTGCCAAGCTTTTAAACAG-3′) is a 20-base oligonucleotide which starts at position 944 of the TS coding sequence and extends 6 bases past the *Hin*dIII site of pSCTS9. It contains 19 complementary bases; an extra T was inadvertently inserted after the coding sequence, and this extra base is present in the final plasmid. PCR was performed using a Cetus/Perkin-Elmer DNA thermocycler. PCR parameters were 35 thermal cycles consisting of 1-min denaturation at 94 °C, 2-min annealing at 37 °C, and 3-min polymerization at 72 °C. After the last cycle, the polymerization step was extended by 10 min to complete all strands. After analysis of the PCR product on a 1.5% agarose gel, the product was purified by phenol/chloroform extraction, precipitated by ethanol, dissolved in TE buffer, and digested with 30 units of *Hin*dIII. The 1.1-kb band was purified by 1.5% low-melting agarose gel electrophoresis.

Cotransformation of Compatible Plasmids. Heterodimers derived from the compatible pUC- and pDPT2789-derived plasmids were prepared by two methods. In one method, pMPTS(C198A) DNA and DNA from individual pSCTS9R178 mutant plasmids were mixed and used to cotransform *E. coli* χ2913RecA. In the other method, *E.*

coli χ2913RecA cells containing pMPTS(C198A) were transformed with individual pSCTS9R178 mutants. In both cases, transformants were plated on LB agar containing 50 μg/mL thymine, 50 μg/mL ampicillin, and 10 μg/mL chloramphenicol to select for both vectors. Chloramphenicol/ampicillin-resistant colonies were isolated and tested for complementation in χ2913RecA.

Protein Purification. Wild-type, R178F, C198R, C198E, C198L, C198S, and C198A TS's were purified to homogeneity as judged by SDS–PAGE using successive chromatography steps on phosphocellulose (Whatman P11) and hydroxylapatite (Biogel HTP, Bio-Rad) as described by Kealey and Santi (1992). TS-containing fractions eluting from hydroxylapatite were pooled, concentrated, and equilibrated with 20 mM KH_2PO_4 (pH 6.8) and 0.5 mM EDTA using an Amicon ultrafiltration device equipped with a YM-10 membrane. Strains containing the appropriate plasmids were grown in LB medium containing 50 μg/mL thymine and 50 μg/mL ampicillin. One-liter cultures yielded 30–60 mg of pure enzyme. Enzymes were stored at −80 °C.

In Vitro Complementation of Subunits. Purified TS R178F was mixed with each of the purified Cys-198 mutant proteins in unfolding buffer. Except in the titration experiments where the amount of protein varied, the unfolding reactions contained 150 μg/mL each protein in 7–8 M urea, 20 mM KH_2PO_4, 0.1 mM EDTA, 1 mM DTT, and 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. One hundred microliters of each refolding reaction was assayed for TS activity in a total reaction volume of 1 mL. Individual mutant and wild-type TS proteins were treated identically to serve as controls.

Enzyme Assays and Protein Measurements. TS activity was determined spectrophotometrically by monitoring the conversion of CH_2H_4folate to 7,8-dihydrofolate (Wahba & Friedkin, 1962) 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, 340 μM (6-*R*,*S*)-CH_2H_4 folate, 125 μM dUMP, and 0.5–1.0 μg of enzyme. Reactions were carried out at 25 °C. One unit of enzyme activity is the amount of enzyme that produces 1 μmole of dTMP min^{-1}.

TS–FdUMP–CH_2H_4folate covalent complexes were formed by incubating 1.0–2.5 μM enzyme with 0.3–0.5 μM [6-^{3}H]-FdUMP (20 Ci/mmol) and 140 μM CH_2H_4folate at 25 °C for 30 min in the standard assay buffer and analyzed by 10% SDS–PAGE (Laemmli, 1970). The gels were stained with Coomassie Blue, and fluorography was carried out as described (Chamberlain, 1979).

RESULTS

In Vivo Subunit Complementation: Cotransformation of pSCTS9-Derived Plasmid Mixtures. In this strategy, we attempted to generate all possible heterodimers by cotransformation of plasmid mixtures of inactive Arg-178 and Cys-198 mutants into χ2913RecA cells and to identify active heterodimers by genetic complementation.

Individual mixtures of plasmids encoding all amino acid substitutions at positions 178 and 198 were prepared by inserting mixed-oligonucleotide cassettes into derivatives of the TS synthetic gene (Climie et al., 1990). Large pools of mutant plasmids (14 000 and 7000 for C198X and R178X, respectively) were collected so that all 20 amino acids would be represented. Each of these two plasmid pools was used to transform *E. coli* χ2913RecA. Randomly selected colonies (200 from the C198 mixture; 150 from the R178 mixture) were tested for complementation. Of these, 76 Arg-178 mutants (51%) and 186 Cys-198 mutants (93%) showed no growth in the absence of exogenous thymine. These inactive colonies were combined, and plasmid DNA was isolated.

Competent *E. coli* χ2913RecA cells were transformed with each of the DNA pools and with a mixture of the pools, and transformants were plated on minimal medium containing or lacking thymine. Transformation of *E. coli* χ2913RecA with the individual or combined pools gave about 10^4 transformants/μg of DNA in medium containing thymine. The individual inactive mutant pools gave no transformants in medium lacking thymine; however, a mixture of both pools gave about 100 complementing transformants/μg of DNA, or about 1% of the transformants obtained under nonselective conditions.

Eleven of the complementing colonies from the mixed transformation were picked and grown in minimal medium lacking thymine. Plasmid DNA was isolated, and the mutations responsible for the Thy$^+$ phenotype were identified by DNA sequence analysis. In all cases, mixtures of bases were found at positions representing codons 178 and 198 of the TS gene. At codon 178, bases corresponding to Arg were always present in the mixture, and at codon 198, bases corresponding to Cys were always present. Assuming that in each case one of the plasmids responsible for complementation contained CGT (the Arg codon of the parent) at 178 and a mutant at 198, and the other contained TGT (the Cys codon of the parent) at 198 and a mutant at 178, we deduced the plasmid components of the primary transformants. In 10 of the 11 colonies examined, a single mutant sequence could be identified for at least 1 position: Phe, Leu, Ile, Val, and Cys were found at position 178; Ser, Leu, Gly, Arg, Thr, and Asp were found at position 198. In five of the colonies, a single mutant could be identified at both positions (Table IC).

Of the 11 characterized colonies from the primary transformation, 4 were chosen for further study. Plasmid DNA

Table I: Complementation and TS Activity of Homodimers and Heterodimers Produced by Cotransformation

plasmids	origin of replication	genetic complementation	TS activity (units/mg of protein in crude extract)
(A) Single Plasmids			
pSCTS9(WT)	ColE1	+	0.2–0.3
pMPTS(WT)	incFII	+	0.03–0.07
pSCTS9R178X[a]	ColE1	−	<0.001
pMPTSC198A	incFII	−	<0.001
(B) Cotransformation of Compatible Plamids[b]			
C198A+R178D	ColE1/incFII	+	0.020
C198A+R178E	ColE1/incFII	+	0.016
C198A+R178F	ColE1/incFII	+	0.068
C198A+R178I	ColE1/incFII	+	0.001
C198A+R178L	ColE1/incFII	+	0.001
C198A+R178P	ColE1/incFII	+	0.022
C198A+R178Am	ColE1/incFII	−	<0.001
(C) Cotransformation Selected by Complementation by pSCTS9-Derived Mixtures[c]			
C198S+R178F	ColE1/ColE1	+	0.010
C198L+R178I	ColE1/ColE1	+	0.020
C198G+R178L	ColE1/ColE1	+	nd[d]
C198D+R178V	ColE1/ColE1	+	nd
C198S+R178C	ColE1/ColE1	+	nd

[a] X designates inactive Arg-178 mutants: Asp, Glu, Phe, Ile, Leu, Pro, Amber. [b] C198A indicates pMPTSC198A; R178F, I, etc. indicate pSCTS9 carrying the designated mutation. [c] All plasmids are mixtures of pSCTS9 with indicated pairs of mutations identified by DNA sequence analysis. [d] nd, not determined.

was retransformed into *E. coli* χ2913RecA, and ampicillin-resistant colonies were selected both in the presence and in the absence of thymine. These secondary transformations gave primarily Thy⁻ segregants; however, a small number of complementing colonies was again obtained. DNA sequence analysis was carried out on plasmid DNA isolated from both Thy⁺ and Thy⁻ secondary transformants. With one exception, only a single sequence was detectable at the codons representing positions 178 and 198 in the Thy⁻ secondary transformants. In contrast, the Thy⁺ transformants retained a mixture of sequences at these codons. As an example of this analysis, one of the primary transformants and also the Thy⁺ secondary transformants derived from it showed (C+T)(G+T)(T+C) at codon 178 [encoding Arg (CGT) and Phe (TTC)] and (T)(C+G)(T+C) at codon 198 [encoding Cys (TGT) and Ser (TCC)]. Plasmid DNA isolated from individual Thy⁻ segregants each showed a single sequence containing either TTC (Phe) at 178 and TGT (Cys) at 198, or CGT (Arg) at 178 and TCC (Ser) at 198, indicating that a complementing pair formed a heterodimer of R178F+C198S.

Over the course of this study, DNA from 15 Thy⁺ colonies and 20 Thy⁻ segregants was sequenced. In the Thy⁺ colonies, we always observed mixed sequences at the Arg-178 and Cys-198 codons, rather than a single sequence at these positions. In the Thy⁻ segregants, we never observed a plasmid with mutations at both positions; plasmids always had either Arg at 178 and a mutation at 198, or Cys at 198 and a mutation at 178. These results indicate that complementing colonies contain two or more plasmids which individually are unable to complement the TS deficiency of χ2913RecA, but when present together produce sufficient TS activity to support growth.

In Vivo Subunit Complementation: Cotransformation of Compatible Plasmids. (A) Preparation of Plasmids. We have also generated heterodimers in vivo utilizing two separate compatible plasmids. Many mutant TS derivatives of the ColEI plasmid, pSCTS9, were available from previous studies (Climie et al., 1990), but we wanted to construct a mutagenesis vector based on the incFII plasmid pDPT2789. The strategy used was to clone the entire TS coding sequence of pSCTS9 along with its promoter and ribosome binding site into pDPT2789. Mutant TS sequences could then be directly transferred from pSCTS9 to the incFII vector by fragment exchange.

The approach used for insertion of the synthetic TS gene (WT) into pDPT2789 to obtain pMPTS(WT) is shown in Figure 2. First, a *Hin*dIII fragment containing the sequence of pSCTS9 starting from slightly upstream of the lacZ promoter and extending through the synthetic gene was prepared by PCR. After amplification, the *Hin*dIII-digested PCR product was cloned into the *Hin*dIII site of pUC18 to obtain pUCTS(WT). The 1.1-kb *Hin*dIII insert from pUCTS-(WT) was cloned into the *Hin*dIII site of pDPT2789 to obtain pDPTS(WT) which contained two *Hin*dIII sites. To create a mutagenesis vector with a single *Hin*dIII cloning site, the *Hin*dIII site at the 5′ end of the TS gene was removed. In one orientation of the *Hin*dIII fragment, the *Hin*dIII site at the 5′ end of the gene was located on a 300 bp *Stu*I fragment which also contained a unique *Xba*I site. The final step in the construction of pMPTS(WT) was the removal of the 300 bp *Stu*I fragment. This was accomplished by digesting pDPTS-(WT) with *Stu*I, religating, digesting with *Xba*I to destroy remaining pDPTS(WT), and transforming into χ2913RecA. The authenticity of the product, pMPTS(WT), was verified by its resistance to *Xba*I digestion, by linearization following

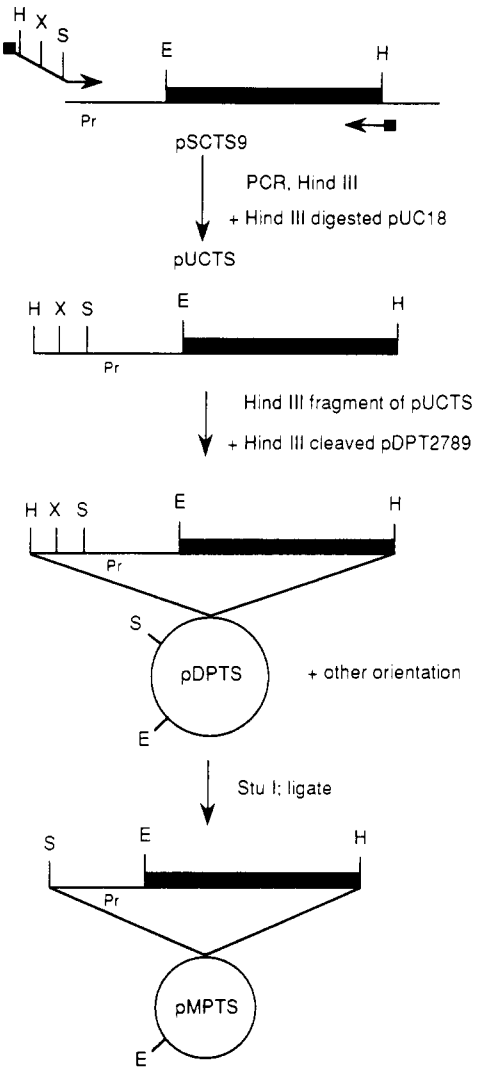

FIGURE 2: Construction of pMPTS(WT). Arrows depict PCR primers. Abbreviations used are H = *Hin*dIII, X = *Xba*I, S = *Stu*I, E = *Eco*RI, and Pr = LacZ promoter. The closed bar represents the TS coding sequence and ribosome binding site from pSCTS9.

*Hin*dIII or *Stu*I digestion, and by complementation of the Thy⁻ phenotype of χ2913RecA. DNA sequence analysis of the TS gene revealed a single base change from T to C at position 583 in the PCR-derived gene; however, this change did not alter the predicted amino acid sequence.

The *Bam*HI/*Hin*dIII fragment from the TS gene of pSCTS9(C198A) was cloned into the *Bam*HI/*Hin*dIII sites of pMPTS(WT) to give pMPTS(C198A) which did not complement χ2913RecA.

(B) Expression of Heterodimeric TS. It has been shown previously (Climie et al., 1990) and here that individually neither C198A nor any of the Arg-178 mutants used in this analysis complement *E. coli* χ2913RecA (Table IA). In the pSCTS9 vector, SDS–PAGE of crude extracts of the R178 mutant homodimers showed intense bands (5–20% of soluble protein) at the mobility of TS, except for R178Am in which TS could not be detected. *E. coli* χ2913RecA was cotransformed with pMPTS(C198A) plus the inactive R178 mutants derived from pSCTS9: R178E(GAG), R178I(ATC), R178L-(TTG), R178P(CCG), R178D(GAC), R178F(TTC), and R178Am. Cotransformants were selected on plates containing ampicillin and chloramphenicol and tested for their ability to grow on medium lacking thymine. All of the cotransformants except for C198A+R178Am complemented the Thy⁻ phe-

notype of χ2913RecA (Table IB). Thus, the cotransformants produce sufficient TS activity to support the growth of χ2913RecA, apparently through the formation of heterodimers of the two resident mutant TS's.

To verify that the complementation tests reflected TS activity, cell extracts of the single transformants and cotransformants were assayed for TS activity. pSCTS9, the parent of the R178 mutants, expressed 3–10-fold higher levels of TS than does the pDPT-based pMPTS(WT), the parent of the C198A mutant. None of the cell extracts containing individual mutants had TS activity detectable by the spectrophotometric assay (<0.001 unit/mg). The presence of TS activity in extracts containing mixed mutants reflected the complementation results. That is, extracts from cells containing C198A+R178Am which did not complement Thy⁻ *E. coli* also did not show TS activity, while extracts from the complementing pairs gave measurable TS activity (Table IB).

The expression of TS activity in pMPTS(WT)/χ2913RecA and in the complementing cotransformants was lower than we had expected (Table IA,B). Therefore, we measured the plasmid copy number of pMPTS(WT), pSCTS9, and pDPT2789. The copy numbers of these three plasmids are 60, 200, and 360, respectively. pDPT2789 has a copy number equivalent to or greater than pSCTS9; however, the derivative pMPTS(WT) has a reduced copy number. The reduced gene dosage could account, in part, for the low expression.

In Vitro Subunit Complementation. Conditions have been described for unfolding and dissociating TS in urea and refolding and reassociating the enzyme to a catalytically active form (Perry et al., 1992). Purified C198 mutant enzymes were mixed with purified R178F under the described conditions to test in vitro complementation. Equivalent amounts of C198S and R178F were diluted into unfolding buffer, incubated on ice, and then diluted 10-fold into folding buffer. This equimolar refolded mixture acquired 0.5 unit of TS activity/mg of total protein. Each of the other purified C198 mutant enzymes (C198A, C198E, C198R, and C198L) was also mixed with R178F under conditions of reversible unfolding and dissociation, and each pair yielded a specific activity of approximately 0.5 unit/mg. Similarly treated wild-type enzyme yields a specific activity of 2.0–2.4 units/mg, representing recovery of approximately 70% of the starting activity [our data and Perry et al. (1992)]. A mixture of native mutant proteins incubated overnight at 4 °C in assay buffer in the absence of urea did not acquire catalytic activity. Thus, the subunits of TS do not exchange to form active heterodimers under native conditions.

Characterization of Heterodimeric TS. We have determined the concentration and k_{cat} of the heterodimer in refolding mixtures of C198A and R178F. We have assumed complete dissociation of the homodimers and random distribution of the monomers during reassociation. If the monomers associate to form heterodimers with the same probability that they reassociate to form homodimers, the concentration of the heterodimer in the refolding reaction can be calculated by eq 1, where AB is the heterodimer and AA and BB are the homodimers.

$$[AB] = 2[AA][BB]/([AA] + [BB]) \quad (1)$$

At constant [BB] and infinite [AA], all B is in the form of AB, and the concentration of the heterodimer is 2[BB]. Since the initial rate of the enzyme reaction (v_i) is proportional to the concentration of heterodimer, V_{max} at 2[BB] can be obtained by eq 2 or the double-reciprocal form (eq 3). A constant amount of C198A plus varying amounts of R178F (0.2–2.3-fold relative to C198A) were mixed in unfolding buffer, refolded by dilution, and assayed. A nonlinear least-squares fit of eq 2 to the data (v_i vs [R178F]) yields a k_{cat} of 1.2 s^{-1} for the heterodimer wihch has single wild-type active site (Figure 3). The validity of eq 1 for the calculation of heterodimer concentration is supported by the good fit of this titration data to the theoretical curve for eq 2. After unfolding/refolding, wild-type TS with two active sites has a specific activity of ~2.0–2.4 units/mg and a calculated k_{cat} of 2.3–2.8 s^{-1}.

$$v_i = V_{max}[AA]/([AA] + [BB]) \quad (2)$$

$$V_{max}/v_i = 1 + [BB]/[AA] \quad (3)$$

We determined the K_m values for dUMP and CH_2H_4folate and the k_{cat} for the R178F+C198A heterodimer formed from an equimolar mixture of mutant enzymes. The heterodimer showed K_m values of 3.6 μM for dUMP and 16 μM for CH_2H_4folate, values which are essentially identical to those of the wild-type enzyme. Using eq 1 to calculate the concentration of the heterodimer, a k_{cat} of 1.0 s^{-1} was calculated in these experiments. Assuming one active site for the heterodimer and two for the wild-type enzyme, this k_{cat} is similar to that of wild-type TS which has been unfolded and refolded by the same method (Table II).

Wild-type TS forms a covalent complex with FdUMP and CH_2H_4folate which is stable to SDS–PAGE. Some mutant TS's which are not able to catalyze the synthesis of dTMP do form covalent complexes with FdUMP plus CH_2H_4folate (Carreras et al., 1992). C198A and R178F homodimers were incubated separately with [^{3}H]FdUMP and CH_2H_4folate and analyzed by SDS–PAGE. Neither the C198A nor the R178F homodimer formed detectable covalent complexes with [^{3}H]-FdUMP and CH_2H_4folate. In contrast, the unfolded–refolded mixture forms a radioactive complex which is readily detectable on SDS–PAGE (Figure 4).

DISCUSSION

Several workers have reported in vivo and in vitro systems in which two or more inactive mutants of multisubunit proteins form active hybrid proteins (Distefano et al., 1990; Larimer et al., 1987, 1990; Scrutton et al., 1989; Wente & Schachman, 1987). We have used similar approaches for designing and detecting heterodimers of TS by the restoration of catalytic activity resulting from a combination of subunits from two inactive homodimers. An inactive homodimer with an aberrant residue which is donated by the opposite subunit was used as the "acceptor" subunit. The other homodimer (the "donor" subunit) could be inactive for any reason, but must contain an active residue corresponding to that which is defective in the acceptor subunit. Inactive Arg-178 mutants were chosen as potential acceptor subunits since Arg-178 forms part of the active site of the opposing monomer (Hardy et al., 1987). As the inactive donor subunit, we used mutants of Cys-198, the essential catalytic nucleophile of TS. Combination of inactive Cys-198 and Arg-178 mutant subunits should form a heterodimer with one functional active site containing intact Cys-198 and Arg-178 residues and one incapacitated active site which contains both mutations (Figure 1).

Two in vivo approaches are described for identifying mutations which support subunit complementation of TS. In the first method, a TS-deficient host is cotransformed with plasmid DNA mixtures which encode pools of inactive Arg-178 and pools of inactive Cys-198 mutant enzymes. Colonies containing active TS are selected by complementation of a

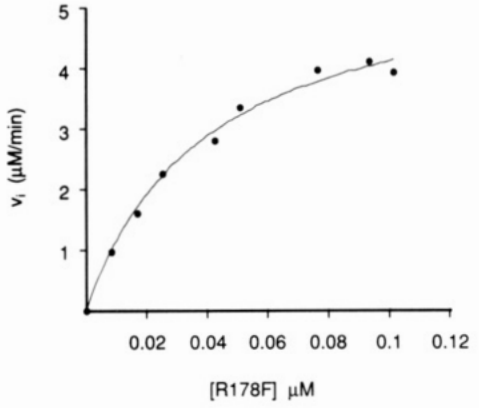

FIGURE 3: Determination of the V_{max} for the C198A+R178F heterodimer. Increasing amounts of unfolded R178F homodimer were combined with 0.04 μM C198A homodimer and refolded (Materials and Methods), and the initial velocities were determined. The solid line is the best fit of the data to eq 2.

Table II: Kinetic Parameters of the Heterodimer and Wild-Type TS

	K_m(dUMP) (μM)	K_m(THF) (μM)	k_{cat} (s^{-1})	k_{cat}/active site[b] (s^{-1})
wild-type TS	4.3	11	2.3–2.8[a]	1.2–1.4
C198A+R178F TS	3.6	16	1.0–1.2	1.0–1.2

[a] Calculated from the specific activity of 2.0–2.4 units/mg measured after unfolding and refolding wild-type TS with a specific activity of 3.2 units/mg. [b] Assumes one active site per heterodimer and two for wild-type TS.

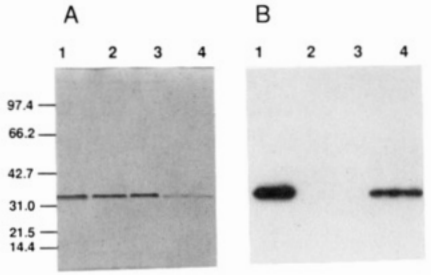

FIGURE 4: Enzyme–FdUMP–CH_2H_4folate complexes with TS homodimers and an active heterodimer. Reactions were carried out with [6-^{3}H]FdUMP as described under Materials and Methods. (Panel A) Coomassie blue stained SDS–PAGE. (Panel B) Autoradiogram of the gel shown in panel A. Lane 1, wild-type TS; (2) C198A; (3) R178F; (4) C198A+R178F heterodimer.

Thy$^-$ host, and the mutations present in the active pairs are identified by DNA sequencing. In the present work, *E. coli* χ2913RecA was cotransformed with a mixture of all 19 inactive Cys-198 mutants and a mixture of about 10 inactive Arg-178 mutants, giving 190 potential heterodimers. From a limited sampling of the cotransformants which complemented Thy$^-$ hosts, we identified five Arg-178 mutants and six Cys-198 mutants which could participate in active heterodimer formation. Five pairs of mutants which yielded active TS were directly identified. In this experiment, we used pUC-derived plasmids with origins of replication in the same incompatibility group, and the selection for dTMP production was sufficient for cohabitation of the host; however, when thymine was included in the medium, cotransformation was not stably maintained. An advantage of this method is that individual mutants at each position need not be isolated or identified until active heterodimers are found. Further, in the present case, the mutant mixtures needed were available from a previous study (Climie et al., 1990). However, this approach suffers from requiring a significant effort in the identification of mutants by DNA sequencing. Regardless, this method would have great value as an initial screen in systems where the diversity of possible active heterodimers is so high or the ability to predict active pairs is so low that it precludes prior isolation of individual mutants.

The second in vivo approach was a more directed one, analogous to those reported in other systems [see Distefano et al. (1990) and Larimer et al. (1990)]. Here, two inactive homodimers encoded by plasmids of different incompatibility groups with different antibiotic resistance were cotransformed into a Thy$^-$ host. Cohabitation of host cells by both plasmids was selected by acquisition of resistance to both antibiotics, and formation of catalytically active heterodimers was tested by growth in the absence of thymine. For the donor subunit, we used the inactive C198A mutant TS cloned into pDPT2789. As the acceptor, we used six Arg-178 mutants which were inactive as homodimers and expressed well in *E. coli* hosts. All of these R178 mutant–C198A heterodimers complemented Thy$^-$ *E. coli* and showed activity in crude extracts.

Subunit complementation was permissive of most substitutions at the two positions tested. Of the six different mutations at position 178 which were tested (Asp, Glu, Phe, Ile, Leu, and Pro), all formed active heterodimers with C198A; Val and Cys at position 178 were also detected in the Thy$^+$ cotransformants derived from the mixture transformation (see above). In addition to C198A, mutants containing Ser, Leu, Gly, Arg, Thr, and Asp at position 198 were detected in the Thy$^+$ cotransformants derived from the mixture transformation. The amino acids identified at both positions in Thy$^+$ cotransformants show a wide range of size, charge, and hydrophobicity. Thus, we think that most, if not all, inactive mutants at positions 178 and 198 which are expressed and appropriately folded may be suitable components for subunit complementation.

The range of TS activity observed in crude extracts from complementing pairs varied by 60-fold. Although some of this variation may be due to differential activities of the heterodimers, the differential expression levels of the R178 mutants (5–20% of total cellular protein) and the differential copy number of the two vectors complicate evaluation of the specific activity of individual heterodimers produced in vivo. Therefore, we turned our efforts toward in vitro mixing of the inactive subunits for evaluation of the heterodimeric active site.

Using conditions developed for refolding TS (Perry et al., 1992), we have formed active heterodimers in vitro by refolding and reassociation of a mixture of two unfolded homodimers. We tested four different C198 mutant enzymes (C198R, C198E, C198S, and C198A) with side chains of varying size and charge. In each case, active heterodimers were formed with R178F, and each heterodimer exhibited a similar specific activity. These results confirm that subunit complementation can accommodate diverse substitutions, and in the pairs tested, the efficiency of the single wild-type active site is not affected by the size and charge of the substitutions in the mutant active site. Using eq 1, we calculated the concentration of heterodimer in a known mixture of homodimers, and thus we were able to derive k_{cat} values for the heterodimer. If both subunits of the wild-type enzyme are assumed to be equally active in the production of dTMP, the catalytic activity of the single wild-type active site in the refolded R178F+C198A heterodimer (k_{cat} = 1.0–1.2 s^{-1}) is 83–86% that of each active site of the wild-type enzyme (k_{cat} = 1.2–1.4 s^{-1} per site). The K_m values of dUMP and CH_2H_4folate for the R178F+C198A heterodimer are equivalent to those of wild-type TS. Thus,

the steady-state kinetic parameters of the heterodimer active site are similar to those of the wild-type enzyme. Each of the C198 mutant enzymes tested had similar specific activity; thus, each of these substitutions yields a single active site which is equivalent to the active sites in the wild-type enzyme.

Neither C198A nor R178F homodimers form a covalent complex with FdUMP and CH_2H_4folate, but the C198A+ R178F heterodimer forms a complex which can readily be detected after SDS–PAGE. This experiment clearly shows the inadequacy of the active sites of the homodimers and the integrity of the active site of the reconstituted heterodimer.

Because heterodimer formation in TS can be simply monitored by acquisition of catalytic activity by two inactive homodimers, it is an excellent system for studies of folding or dimer interface interactions. Indeed, this system provides unambiguous proof for a monomer to dimer dissociation and reassociation in folding studies of TS (Perry et al., 1992). The inactive Arg-178 mutants could also be used to complement a donor subunit which is inactive for any reason, providing it contains an Arg-178 residue to donote to the other subunit, and folds to create a suitable dimer interface. It should therefore be possible to study structural elements and modifications in one subunit which preclude activity in the corresponding homodimer. Further, subunit complementation provides access to heterodimers with a single active site, which are potentially useful in studies of subunit cooperativity. Finally, the method could be used to detect subunit chimeras formed from TS subunits of different species.

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