Characterization of a Novel Form of Thymidylate Synthase: A Heterodimer Isolated after Specific Chemical Modification of the Immobilized Native Enzyme†

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ABSTRACT: A novel approach, utilizing covalent chromatography and selective chemical modification, is described for application in studying subunit interactions involved in the catalytic and regulatory mechanisms of certain oligomeric proteins. The specific objective was to prepare and characterize a heterodimeric form of thymidylate synthase which would serve as a model for an intermediate stage of the catalytic mechanism in which the active-site cysteine of one subunit would be engaged in covalent catalysis while that of the other subunit would exist in the free sulfhydryl or thiolate anion form. Dimeric *Lactobacillus casei* thymidylate synthase was subjected to covalent chromatography on thiopropyl Sepharose 6B resin under conditions in which a mixed disulfide linkage was formed with the catalytic sulfhydryl group of just one of the two subunits. Specific chemical modification of the remaining essential sulfhydryl group of the immobilized group enzyme with *N*-ethylmaleimide, followed by cleavage and elution with buffer containing 2-mercaptoethanol, yielded the desired soluble heterodimeric form of the enzyme. The specific activity of this unique form of the enzyme (1.55 units/mg) was approximately 60% that of native protein (2.61 units/mg). Gel electrophoretic analysis of the heterodimeric enzyme, incubated in the presence of FdUMP and 5,10-methylenetetrahydrofolate (CH_2H_4folate), resulted in the appearance of a single protein band corresponding to the 1:1:1 enzyme–FdUMP–CH_2H_4folate complex, confirming the new species as dimeric thymidylate synthase containing a single functional active site. The maximum covalent binding ratio, as measured by a trichloroacetic acid precipitation assay, for this inhibitory ternary complex consisting of enzyme, FdUMP, and CH_2H_4 folate was 1.0 (mol FdUMP:mol enzyme), as compared to the typical value of 1.7 observed for native enzyme. Binary and ternary complex binding curves were almost identical in shape to those for native thymidylate synthase; however, the binding ratios were markedly lower for the single-site enzyme form. Comparison of the K_d values obtained by Scatchard analysis showed that the single-site enzyme formed slightly tighter covalent binary and ternary complexes than did native enzyme. Of greater significance was the observation that the k_{cat} value (331 min^{-1}) for single-site enzyme was more than 60% larger than the k_{cat} (203 min^{-1}) of native enzyme, while the K_m(dUMP) values were about the same. These results suggest that the covalent modification of the active-site cysteine in one site produces positive cooperativity in binding and catalysis at the second site.

Much recent attention has been given to thymidylate synthase (EC 2.1.1.45) because the reaction which it catalyzes, the CH_2H_4folate[1]-dependent reductive methylation of dUMP to dTMP, represents the sole *de novo* source of dTMP, which is essential for DNA replication [for reviews, see Danenberg (1977); Lewis and Dunlap (1981); Santi and Danenberg (1984); Cisneros *et al.* (1988)]. The strategic role of this enzyme promoted its use as a target for two classes of anticancer drugs, the fluorinated pyrimidines and the antifolates, and provoked an intense interest in both its mechanistic and its structural properties. The current mechanistic view, which was shaped by studies of mechanism-based inhibitors such as FdUMP, features the sequential transformation of a series of covalent enzyme–nucleotide complexes, including the catalytic covalent ternary complex which is the product of the addition of the N-5 cationic imine form of the cofactor and the thiolate anion of an active-site cysteine residue, respectively, across the C-5,C-6 double bond of dUMP. Cleavage of the folate N-5-methylene group bond in the latter ternary complex leads to formation of the exocyclic methylene form of the covalent nucleotide intermediate which, in turn, is reduced by H_4folate to yield the last enzyme-bound species. This final covalent complex rapidly discharges dTMP concurrent with rehybridization of its C-5,C-6 double bond.

As isolated from most sources, thymidylate synthase is a dimer (MW range 60–75 kDa) of identical subunits, whose primary structure is apparently the most highly conserved of an enzyme studied to date (Perry *et al.*, 1990). Ligand-binding and chemical modification studies indicate the presence of a functional active site on each subunit; some results suggest that the function of the active sites of thymidylate synthase is subject to intersubunit cooperativity effects (Cisneros *et al.*, 1988; Danenberg & Danenberg, 1979). For example, when thymidylate synthase is incubated with saturating levels of FdUMP in the absence of folates, the data suggest the inhibitor is covalently bound at one active site but is noncovalently associated with the active site on the other

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[1] Abbreviations: (±)-N^5,N^{10}-methylenetetrahydrofolate, CH_2H_4folate; 5,5′-dithiobis(2-nitrobenzoic acid), DTNB; 5-fluoro-2′-deoxyuridylate, FdUMP; (±)-7,8-dihydrofolate, H_2folate; (±)-5,6,7,8-tetrahydrofolate, H_4folate; *N*-ethylmaleimide, NEM; polyacrylamide gel electrophoresis, PAGE; 2-thiopyridone, 2-TP; 2,2′-dithiodipyridine, 2-PDS.

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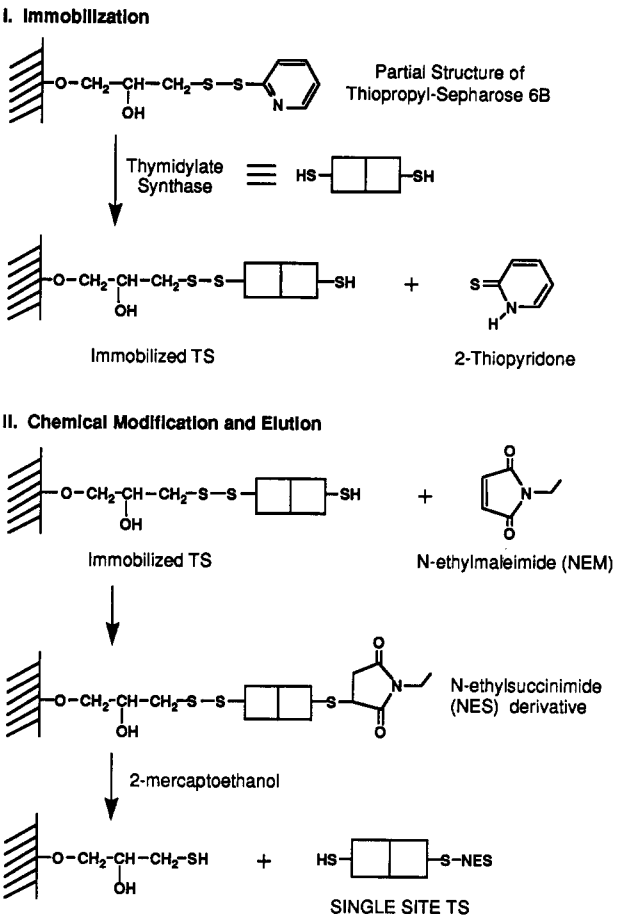
subunit (Galivan *et al.*, 1976; Lewis *et al.*, 1980), thus producing nonequivalent, asymmetrically oriented subunits. The addition of CH_2H_4folate to this system results in the formation of stable covalent inhibitory ternary complexes at both active sites, which presumably places the two subunits in a symmetrical relationship. In fact, the binding of folates, including CH_2H_4folate, at one of the active sites has been shown to produce large conformational changes (Lewis *et al.*, 1981; Moore *et al.*, 1986; Matthews *et al.*, 1990; Lockshin & Danenberg 1980; Pogolotti *et al.*, 1986; Danenberg & Danenberg, 1979; Kamb *et al.*, 1992a,b), whose consequences include lowering the K_d for nucleotide binding, thus enhancing the extent of covalently bound FdUMP or dUMP at both active sites. It was postulated that folate binding at one site is communicated to the other site and that the conformational change following ligand binding may be responsible for the asymmetric behavior (Finer-Moore *et al.*, 1990). The crystal structure determined by Kamb *et al.* (1992a) has illustrated the structural basis for recognition of polyglutamyl folates by thymidylate synthase, suggesting that cofactor binding, independent of dUMP interaction, produces a major conformational change in the protein (Kamb *et al.*, 1992b). Finally, X-ray crystallographic analyses of the apoenzyme and selected ternary complexes reveal that the intersubunit boundary is populated by some 29 residue interactions between the individual monomers. Two residues from each subunit, Arg178′ and Arg179′, participate in the operation of the active site on the other subunit (Hardy *et al.*, 1987; Matthews *et al.*, 1990). Collectively, these findings are thought to reflect various aspects of the operation of an intersubunit communication system in the thymidylate synthase dimer.

The results of gel filtration, fluorescence spectroscopy, and ultracentrifugation studies by Reinsch *et al.* (1979) showed that the unfolding of *Lactobacillus casei* thymidylate synthase at 4 °C by increasing concentrations of denaturants was accompanied by a significant dissociation of dimer to monomers only at or above 4 M guanidine hydrochloride or 6 M urea. The fact that the dimeric protein does not dissociate to monomeric species under native conditions formed the basis for two different approaches leading to the generation of interesting heterodimeric species of the enzyme which have been employed to probe the nature and basis of subunit communication. First, and perhaps the most dramatic illustration of subunit communication in this enzyme, was the approach based on the finding that the carboxypeptidase A-dependent removal of the C-terminal residue from just one of the two identical subunits corresponds to the loss of dTMP synthesis activity at both enzymic active sites (Loeble & Dunlap, 1972; Aull *et al.*, 1974a). Even more surprisingly, the subtly modified heterodimer resulting from limited treatment of thymidylate synthase with carboxypeptidase A continues to catalyze the formation and decay of covalent binary and ternary complexes, but these events are restricted to just one of the two subunits of the enzyme (Cisneros *et al.*, 1993). Clearly, this approach provides both further evidence supporting the notion of subunit communication and a unique heterodimer in which to study the phenomenon. Second, an approach termed subunit complementation employs both *in vivo* (Climie *et al.*, 1990) and *in vitro* (Perry *et al.*, 1992) systems to generate heterodimers of thymidylate synthase (Pookanjanatavip *et al.*, 1992). For example, inactive R178F and C198A mutants combine to form a heterodimeric species with a single intact active site which is capable of covalent inhibitory ternary complex formation (restricted to that site) and exhibits a k_{cat} per active site close to that of the wild-type

Scheme I: Production of Dimeric Thymidylate Synthase Containing One Functional Active Site[a]



[a] (I) Immobilization of native enzyme on thiopropyl Sepharose 6B; (II) chemical modification of the immobilized enzyme with NEM followed by elution of the heterodimer with 50 mM 2-mercaptoethanol.

enzyme (Pookanjanatavip *et al.*, 1992).

Since covalent catalysis involving the active-site cysteine residue, Cys198, plays such a central role in the mechanism of action of this enzyme, we chose to design a heterodimeric form of thymidylate synthase which would permit us to evaluate the possible role of these crucial residues in subunit communication. In particular, we sought a thymidylate synthase heterodimer which would mimic a plausible intermediate stage of the reaction in which the active-site cysteine of one subunit would be engaged in covalent catalysis while that of the other active site would be in the free sulfhydryl or thiolate anion form. Since formidable technical problems prohibit the trapping and isolation of such a catalytic intermediate (or its inhibitory ternary complex counterpart) or the solution-phase selective chemical modification of the catalytic sulfhydryl group at just one of the two active sites, we devised a novel approach to this problem utilizing an adaptation of enzyme immobilization.

We have recently characterized the covalent chromatography of *L. casei* thymidylate synthase on thiopropyl Sepharose 6B resin (Bradshaw & Dunlap, 1993) and have described experimental conditions which yield immobilized enzyme attached through a mixed disulfide linkage specifically and exclusively through just one of the two catalytic sulfhydryl groups (Scheme I). The immobilized enzyme, which itself constitutes a heterodimeric species, exhibited both catalytic activity and inhibitory ternary complex formation, though at much reduced levels when compared to the native enzyme (Bradshaw & Dunlap, 1992). We now report that a desired

model form of thymidylate synthase in an intermediate stage of catalysis, henceforth referred to as single-site thymidylate synthase, can be generated by the specific, irreversible chemical modification of the lone essential active-site cysteine remaining on the immobilized enzyme and its subsequent cleavage and elution from the resin (Scheme I). In an initial characterization, the biochemical and kinetic properties of this novel, heterodimeric form of thymidylate synthase were determined and compared directly with those obtained for native enzyme.

EXPERIMENTAL PROCEDURES

Materials

Thiopropyl Sepharose 6B, activated thiol Sepharose 4B, 2-PDS, NEM, DTNB, FdUMP, dUMP, and G-25 fine Sephadex beads were obtained from Sigma Chemical Co. (St. Louis, MO). [6-^{3}H]FdUMP (20 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Racemic H_4folate was synthesized by catalytic hydrogenation of folic acid according to the method of Hatefi *et al.* (1960) and converted to CH_2H_4folate by addition of a 20-fold molar excess of formaldehyde.

Methods

Enzyme. Thymidylate synthase was purified from amethopterin-resistant *L. casei* according to the method described by Lyon *et al.* (1975) and stored by dialysis at 4 °C in fresh 100 mM Tris buffer (pH 7.3) containing 20 mM KCl, 1 mM EDTA, and 50 mM 2-mercaptoethanol. The enzyme was routinely concentrated by vacuum dialysis (Micro ProDiCon; Beaverton, OR) in 100 mM potassium phosphate buffer (pH 6.5) containing 20 mM KCl, 1 mM EDTA, and 50 mM 2-mercaptoethanol.

Protein Determination and Activity Assay. The concentration of thymidylate synthase was determined in a Hewlett-Packard 8450A spectrophotometer using a molar absorptivity of 105 000 cm^{-1} M^{-1} for the protein absorbance at 278 nm (Lyon *et al.*, 1975). Enzyme activity was determined spectrophotometrically by following the increase in absorbance at 340 nm due to conversion of CH_2H_4folate to H_2folate, according to the procedure of Dunlap *et al.* (1971). One unit of activity is defined as 1 micromole of 7,8-dihydrofolate formed per minute at 25 °C. Immobilized thymidylate synthase activity was measured according to procedures developed and described by Bradshaw and Dunlap (1992).

Polyacrylamide Gel Electrophoresis. Nondenaturing gel electrophoresis was routinely performed on native and NEM-modified thymidylate synthase, in the presence and absence of FdUMP and CH_2H_4folate, essentially as described by Aull *et al.* (1974b). The gels (0.15 × 16 cm), composed of 8.0% w/v acrylamide, were run at 4–6 °C using a constant current of 20 mA per gel. A Protean II (Bio-Rad, Hercules, CA) gel electrophoresis apparatus was used. The gels were then stained for protein with a 0.1% solution of Coomassie Brillant Blue R-250 stain (40% methanol, 10% acetic acid).

Production of Single-Site Thymidylate Synthase. The procedure for production of dimeric thymidylate synthase containing one functional active site (single site) is presented in Scheme I. Thiopropyl Sepharose 6B gel was equilibrated by washing with 20–30 volumes of 100 mM potassium phosphate buffer (pH 6.5) containing 20 mM KCl and 1 mM EDTA (Buffer A). Prior to enzyme immobilization, thymidylate synthase was separated from exogenous thiol by the centrifugal column chromatography method of Bradshaw *et al.* (1988). In a typical experiment, approximately 1 mL of dethiolated enzyme (15.8 mg; specific activity, 2.29 units/mg) was loaded onto a 1-mL thiopropyl Sepharose 6B column as previously described (Bradshaw & Dunlap, 1993). After the coupling reaction was allowed to proceed for 6 h at 4 °C, the column was washed with 10 mL of Buffer A to remove the released 2-TP and any unbound protein. Approximately 2.4 mg of enzyme was recovered in this step. The column was then washed with 10 mL of Buffer A including 5 mM L-cysteine to remove the remaining 2-thiopyridyl groups, followed by 10 mL of Buffer A alone to reequilibrate the resin for the modification reaction.

The *in situ* modification of immobilized thymidylate synthase with NEM was based on methods described for soluble enzyme which showed that only the two catalytic sulfhydryl groups were chemically modified (Plese & Dunlap, 1977). Irreversible covalent modification of the free active-site sulfhydryl group of the resin-bound enzyme was accomplished by washing the column with 10 mL of Buffer A containing 5 mM NEM. Halfway through this step, the column flow was stopped and the NEM allowed to react with the enzyme for 2 h. The column was then washed with 20 mL of 100 mM Tris buffer (pH 8.0) containing 20 mM KCl and 1 mM EDTA (Buffer B) to ensure both the removal of all unreacted NEM and the equilibration of the resin to pH 8.0. The modified enzyme was then eluted in 30 mL of Buffer B containing 50 mM 2-mercaptoethanol, and 1-mL fractions were collected. Three separate modified enzyme pools were constructed on the basis of their order of elution (Pool 1: fractions 1–10; Pool 2: fractions 11–20; and Pool 3: fractions 21–30). Before analysis, these were dialyzed separately in Buffer A containing 50 mM 2-mercaptoethanol.

Kinetic Analysis. Kinetic parameters were determined at 25 °C by Lineweaver–Burk analysis (Lineweaver & Burk, 1934). A 2000-fold molar excess of CH_2H_4folate over enzyme was included in the assay mixtures for native and single-site enzyme; a 200-fold excess was used with immobilized enzyme. Each native enzyme reaction mixture (1.01 mL) contained 0.11 μM native thymidylate synthase, 220 μM CH_2H_4folate and 0.52–77.3 mM dUMP. The single-site enzyme reaction mixtures (1.01 mL) included 0.06 μM single-site thymidylate synthase, 120 μM CH_2H_4folate, and 0.5–50.9 mM dUMP. Before kinetic analysis of immobilized thymidylate synthase, dethiolated enzyme (139 nmol; specific activity, 2.76 units/mg) was covalently coupled to a 1-mL sample of thiopropyl Sepharose 6B. After the sample was washed to remove unbound protein and 2-TP, the 1-mL resin–enzyme suspension was then diluted to 5 mL with Buffer A. Each immobilized thymidylate synthase reaction mixture (3.0 mL) contained 210 μM CH_2H_4folate and 3.4–168.3 μM dUMP. The assay was initiated by introducing 100 μL of the immobilized enzyme (2.78 nmol). All assays were conducted a minimum of three times, with the mean values obtained used for further calculations. The Lineweaver–Burk diagrams were determined by linear regression analysis.

FdUMP Binding Assays. The maximum covalent binding ratio of the nucleotide inhibitor, [6-^{3}H]FdUMP, to soluble native and single-site thymidylate synthase was determined by a trichloroacetic acid precipitation procedure developed by Moore *et al.* (1984). The binary complex reaction mixtures (0.5 mL) contained from 0.10 to 0.25 nmol of thymidylate synthase in 100 mM phosphate buffer (pH 6.8), including 20 mM KCl and a 20-fold molar excess of [6-^{3}H]FdUMP (approximately 0.1 Ci/mmol). The maximum covalent ternary complex binding ratio was determined by adding a 500-fold molar excess of CH_2H_4folate into the above mixture,

maintaining the 0.5-mL volume. The binding ratios were calculated by dividing the amount of [6-^{3}H]FdUMP bound to the precipitate by the amount of protein in the reaction mixture.

In experiments involving titration of native and single-site enzyme with the nucleotide, binary binding curves were constructed by addition of increasing amounts of [6-^{3}H]-FdUMP (from 0.25 to 100 times the amount of enzyme) to 0.10 nmol thymidylate synthase in 100 mM potassium acetate buffer (pH 5.8) containing 50 mM 2-mercaptoethanol. For ternary complex titrations, CH_2H_4folate at a constant 1000-fold molar excess was added to the above binary reaction mixtures, maintaining the same reaction volume (0.5 mL). Prior to this study, both native and single-site enzymes were dialyzed in 100 mM Tris buffer (pH 7.5) containing 20 mM KCl, 1 mM EDTA, and 50 mM 2-mercaptoethanol. The binding ratios and dissociation constants (K_d) for the binary and ternary complexes formed with native or single-site thymidylate synthase were determined by Scatchard analysis. Data from duplicate assays were weighted (weight factor = 1/range) for nonlinear regression analysis according to the methods described by Connors (1987).

RESULTS AND DISCUSSION

Specificity of the Immobilization Reaction and Production of Single-Site Thymidylate Synthase. A requirement for the production of single-site thymidylate synthase was that native enzyme attach to the thiopropyl Sepharose 6B resin through only a single catalytic sulfhydryl group (Cys198). Coupling through a nonessential cysteine would permit NEM-modification of both catalytic sulfhydryl groups. However, we have shown previously that thymidylate synthase is bound to the resin specifically and exclusively through an active-site cysteine (Bradshaw & Dunlap, 1992). In our previous studies of the covalent chromatography of thymidylate synthase, we showed that the initial relatively rapid coupling of the enzyme to the resin depicted in Scheme I was followed by a slower reaction of the catalytic sulfhydryl group on the second subunit with the resin (Bradshaw & Dunlap, 1993). Since this phenomenon, termed multiple-site attachment, would, of course, preclude the NEM modification step and interfere with the isolation of single-site thymidylate synthase, the immobilization reaction time was limited to 6 h at 4 °C. After elution of the NEM-treated immobilized enzyme, three separate modified enzyme pools were constructed (see Experimental Procedures). The pools were then characterized to identify which pool(s) contained only the single-site thymidylate synthase species.

Initial Characterization of Immobilized Thymidylate Synthase Eluted after NEM Treatment: Catalytic and FdUMP-Binding Activities. After exhaustive dialysis of the three eluted, NEM-modified enzyme pools, the amount of protein in each was determined and assayed for catalytic and ligand-binding activity. The three modified enzyme pools (Pools 1–3) contained 5.1, 2.3, and 1.3 mg of protein, respectively (Table I). Each enzyme pool exhibited catalytic and ligand-binding competence. Interestingly, the specific activity of the modified enzyme pools increased with elution volume, with Pool 1 having the lowest specific activity of 1.55 units/mg. Modified enzyme Pools 2 and 3 exhibited specific activities of 2.05 and 2.13 units/mg, respectively. The enzyme in Pool 1 displayed a maximum covalent binding ratio for FdUMP in the inhibitory ternary complex of 1.0 (mol FdUMP: mol enzyme), the exact value predicted for single-site enzyme. The FdUMP binding ratios for the other two pools were both approximately 1.2 (mol FdUMP: mol enzyme), which suggested that a small fraction of the enzyme in these pools was not modified by NEM, probably due to multiple-site attachment of the protein to the matrix.

Table I: Characterization of the NEM-Modified Enzyme Pools Generated in the Production of Single-Site Thymidylate Synthase

modified enzyme pool	protein (mg)	specific activity (units/mg)[a]	FdUMP binding ratio[b]
1	5.1	1.55	1.0
2	2.3	2.05	1.2
3	1.3	2.13	1.2

[a] The specific activity is expressed in units of micromoles of H_2folate produced per minute per milligram of protein at 25 °C. [b] The maximum covalent binding ratio for FdUMP in the inhibitory ternary complex (mol FdUMP:mol enzyme) was determined as described in the Materials and Methods section.

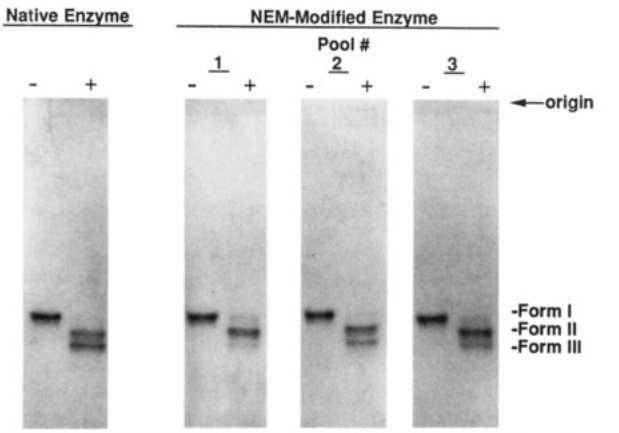


FIGURE 1: Effect of NEM modification on gel electrophoretic patterns of thymidylate synthase (TS). NEM-modified enzyme eluting from the thiopropyl Sepharose 6B column was collected in 1-mL fractions. Three separate pools were constructed (see Experimental Procedures) and dialyzed extensively in Buffer B containing 50 mM 2-mercaptoethanol. Following this step, the protein (5 μg) was subjected to electrophoresis, under nondenaturing conditions, either in the presence (+) or absence (–) of ligands FdUMP and CH_2H_4folate. A sample of native, unmodified TS was analyzed in identical fashion.

However, we required two stringent criteria to establish that single-site thymidylate synthase was produced. In addition to determining the maximum covalent FdUMP binding ratios in the inhibitory ternary complex, we also carried out nondenaturing polyacrylamide gel electrophoresis of NEM-modified enzyme incubated in the presence and absence of ligands, FdUMP and CH_2H_4folate (see below).

Polyacrylamide Gel Electrophoresis Studies of the NEM-Modified Enzyme Pools. Though the possibility of disproportionation of the thymidylate synthase dimer under native conditions was considered to be remote in light of results published by Reinsch *et al.* (1979), Aull *et al.* (1974a), Perry *et al.* (1992), and Pookanjanatavip *et al.* (1992), we sought to design an experiment whose results could discriminate between an enzyme dimer with a single functional active site and possible dimers produced by the dissociation/reassociation of the protein eluted from the thiopropyl Sepharose 6B resin. We chose to employ native polyacrylamide gel electrophoresis for this study since the migration of the native dimeric enzyme (designated Form I) is distinctly altered by prior incubation with FdUMP and CH_2H_4folate and results in its replacement by two faster migrating bands designated as Form II and Form III (see Figure 1) (Aull *et al.*, 1974b). In previous work, this laboratory demonstrated that Forms II and III are inhibitory ternary complexes which can be fractionated from each other and from Form I by either carboxymethyl Sephadex

chromatography (Aull *et al.*, 1974c) or native gel electrophoresis (Aull *et al.*, 1974b). By employing radiolabeled ligands, we further showed that the stoichiometry of the FdUMP:CH_2H_4folate:enzyme dimer was 1:1:1 for Form II and 2:2:1 for Form III (Aull *et al.*, 1974c). Native thymidylate synthase, which has been purified to homogeneity as a protein (Figure 1, native enzyme, "–" lane) is heterogeneous with respect to its extent of ternary complex formation (Figure 1, native enzyme, "+" lane).

Thus, if authentic single-site enzyme was produced by the procedure described earlier, its incubation with FdUMP and CH_2H_4folate should lead to the formation of a ternary complex with a 1:1:1 stoichiometry which should migrate at the same rate as Form II on native gels. However, if the species produced were to undergo disproportionation and reassociation to form enzyme dimers with no functional active sites *and* enzyme dimers with two fully active subunits, then their incubation with FdUMP and CH_2H_4folate followed by electrophoresis on native gels should yield protein bands migrating at the same rates as Form I and Form III, respectively.

The differences in the FdUMP-binding stoichiometries of the above NEM-modified enzyme pools are illustrated and corroborated by the gel electrophoretic patterns in Figure 1, where protein bands for both native thymidylate synthase and NEM-modified enzyme are shown. In Figure 1, the lanes for native enzyme (specific activity, 2.61; FdUMP binding ratio, 1.6) incubated in the presence (+) and absence (–) of ligands contain the bands typical for pure thymidylate synthase. A different protein band pattern appears in the remaining lanes. As expected, the NEM-modified enzyme from Pool 1, subjected to electrophoresis in the absence of ligands, migrates to the same position as that for native enzyme. However, when the ligands are included, only the single band corresponding to Form II (or the 1:1:1 complex) appears. Taken together with the FdUMP binding ratio of 1.0 for this species, these data represented direct physical and chemical evidence that Pool 1 consisted almost exclusively of dimeric enzyme containing only one functional active site.

As the FdUMP-binding stoichiometries for enzyme Pools 2 and 3 had suggested, the electrophoretic evidence confirmed that incomplete NEM-modification had occurred with these enzyme forms. Although the bands for enzyme samples subjected to electrophoresis in the absence of ligands comigrated with single-site thymidylate synthase, a band for Form III also appeared in the two lanes for enzyme preincubated with FdUMP and CH_2H_4folate. This result established that in a small percentage of immobilized enzyme, neither of the two active-site sulfhydryl groups was accessible for covalent modification by NEM. These enzyme pools did not represent single-site thymidylate synthase and thus were not subjected to further characterization.

As demonstrated by these results, our procedure has allowed for isolation of a soluble heterodimeric form of thymidylate synthase, in which one active site remains functional while the other is irreversibly blocked by NEM. Upon isolation, the specific activity of the modified enzyme (1.55 units/mg) was always significantly less than that of pure dimeric thymidylate synthase (2.61 units/mg), whereas the FdUMP binding ratio for the inhibitory ternary complex was near the expected value of 1.0. In contrast, enzyme subjected to exactly the same covalent chromatography procedure, except for omitting the NEM-modification step, did not exhibit any loss in either catalytic activity or ligand-binding ability (Bradshaw & Dunlap, 1993).

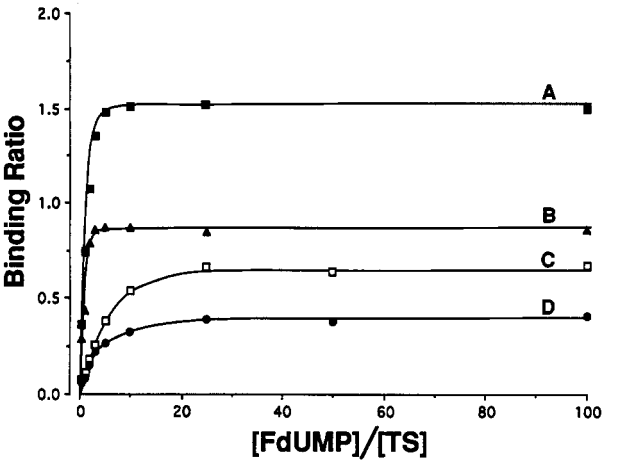


FIGURE 2: FdUMP binding curves for native and single-site thymidylate synthase (TS). FdUMP binding curves were obtained by incubating increasing amounts of [6-^{3}H]FdUMP with 0.1 nmol of enzyme (native or single-site) in the presence or absence of a 1000-fold molar excess of (±)-CH_2H_4folate. Binding ratios were calculated by dividing the amount of FdUMP bound to the precipitate by the amount of protein in the reaction mixture. Binding curves are shown for: (A) ternary complex of native enzyme, (B) ternary complex of single-site enzyme, (C) binary complex of native enzyme, and (D) binary complex of single-site enzyme.

Table II: Comparison of FdUMP Binding Stoichiometries and K_d Values for Native and Single-Site Thymidylate Synthase (TS)

	binary complex		ternary complex	
enzyme form	B.R.[a]	K_d[a] (M)	B.R.[a]	K_d^{app} [a,b]
native TS	0.8	1.5×10^{-6}	1.6	9.4×10^{-8}
single-site TS	0.4	0.6×10^{-6}	0.9	7.3×10^{-8}

[a] K_d values and maximum binding ratios (B.R.) were calculated by weighted fit to the Scatchard equation: $B = [L](B_{max}/[L] + K_d$; B = binding. [b] The dissociation constants determined for the ternary complexes of both native and single-site enzyme are apparent values.

Comparison of FdUMP Binding and Dissociation Constants for Single-Site and Native Thymidylate Synthase. To examine the ligand-binding characteristics of single-site thymidylate synthase, we performed FdUMP titrations for both the covalent binary and inhibitory ternary complexes formed with this species. The results of these studies were then compared directly to those obtained with native enzyme. The binary and ternary complex binding curves (Figure 2) for the native enzyme were almost identical in shape to those for the single-site enzyme and represent evidence that the same ligand-binding phenomena may occur with both enzyme forms (i.e., that with respect to binding, one active-site behaves similarly to the other).

The FdUMP binding stoichiometries and dissociation constants (K_d), both determined by Scatchard analysis, are shown in Table II. In this experiment, the binding ratios for FdUMP in the inhibitory ternary complex were 1.6 and 0.9 (mol FdUMP:mol enzyme) for the native and single-site species, respectively. The former value approximated those previously reported in the literature (Santi & Danenberg, 1984; Cisneros *et al.*, 1988), whereas the latter agreed well with our own data on single-site enzyme. Significantly, the binding ratio for FdUMP in the binary complex formed with single-site thymidylate synthase (0.4) was one-half that determined for the native enzyme (0.8). Comparison of the K_d values for the covalent FdUMP bindary and inhibitory ternary complexes showed that the single-site enzyme bound the nucleotide inhibitor more tightly than did the native enzyme with two available active sites. Thus, the covalent modification

of the active-site cysteine by NEM at one site apparently results in a subtle repositioning of residues in the second active site, which thereby strengthens the covalent binding interaction between FdUMP and the single-site enzyme.

The ability of certain folates, especially CH_2H_4folate, to enhance the covalent binding of nucleotides (i.e., dUMP and FdUMP) in native thymidylate synthase ternary complexes has been evoked as evidence for asymmetrical subunit interactions in the protein (see introduction). Our results demonstrated that this feature is also operable in the single-site enzyme form. Therefore, the enhanced covalent binding of nucleotide in the presence of folate is probably not solely due to the opening of a second active site through conformational changes upon ligand binding at the first site, unless the covalent modification by NEM is serving the same function as the covalent binding of FdUMP and CH_2H_4folate. Results from X-ray crystallographic studies of *Escherichia coli* thymidylate synthase have suggested that folate enhancement results from base-stacking interactions between the pterin of the cofactor and the pyrimidine ring of the nucleotide (Finer-Moore *et al.*, 1990). The results obtained with single-site thymidylate synthase are consistent with this hypothesis.

Furthermore, the observation that the binary binding ratio decreased from 0.8 (mol FdUMP:mol enzyme) for the native enzyme to 0.4 for the single-site enzyme indicated that both subunits in the native enzyme were capable of covalent nucleotide binding in the binary complex and corroborates the results of the FdUMP titration experiments. This decrease in the binding ratio of the binary complex for single-site enzyme (as compared to the native form) is not consistent with the hypothesis of Danenberg and Danenberg (1979), which states that one active site must be filled with covalently bound FdUMP and CH_2H_4folate before the other site becomes accessible for ligand binding. Furthermore, the crystal structure of native *L. casei* thymidylate synthase indicates that both active sites are accessible to FdUMP binding before the addition of folates (Hardy *et al.*, 1987).

Immobilized thymidylate synthase is analogous to the soluble single-site species, the only difference being attachment to the solid support versus chemical modification. The maximum inhibitory ternary complex binding ratio for immobilized enzyme has been measured to be 0.32 (mol FdUMP:mol enzyme), about half the predicted value of 0.6–0.7 (Bradshaw & Dunlap, 1992). Most probably this value was less than that obtained with soluble single-site enzyme because immobilization of thymidylate synthase to the solid support resulted in either an obstruction of the one free active site to ligands or a restriction in the flexibility of localized enzyme structure such that the binding reaction was impeded. However, the fact that immobilized enzyme, which has only one functional active site, is still active and able to bind FdUMP and CH_2H_4folate covalently (Bradshaw & Dunlap, 1992) suggests that both subunits have some capacity to operate independently of one another.

Comparison of the Kinetic Properties of Native, Single-Site, and Immobilized Thymidylate Synthase. The final step in these initial characterization studies of single-site thymidylate synthase was a comparison of the kinetic properties (e.g., K_m and k_{cat}) of this species with those obtained for both native and immobilized enzyme. For a valid comparison, the same experimental conditions, except for substrate and cofactor concentrations, were used. Accordingly, exogenous thiols used in dialysis buffers were removed from native and single-site enzymes before the experiment, and thiol-free cofactor was used in all assay mixtures.

Table III: Comparison of Kinetic Parameters for Native, Single-Site, and Immobilized Thymidylate Synthase (TS)

enzyme form	K_m (μM)[a]	k_{cat} (min^{-1})[b]	k_{cat}/K_m
native TS	5.2	203	39
single-site TS	5.8	331	57
immobilized TS[c]	22.6	13	0.6

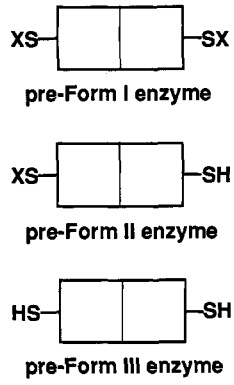
[a] Michaelis constant for the substrate, dUMP. [b] k_{cat} values (turnover numbers) were calculated by dividing the number of micromoles of H_2folate produced per minute by the concentration of available active sites. [c] Kinetic parameters for immobilized enzyme (35) are more appropriately referred to as apparent values (e.g., K_m^{app}, k_{cat}^{app}).

Freshly isolated single-site thymidylate synthase was subjected to kinetic analysis as described below. Lineweaver–Burk analysis indicated that the K_m(dUMP) value for the single-site species was 5.8 μM. The calculated k_{cat} was 331 min^{-1}. A competitive inhibition pattern, with respect to FdUMP, was observed with the single-site enzyme (K_i = 0.11 μM). This same pattern of inhibition has been obtained with native thymidylate synthase (Danenberg & Lockshin, 1981). The kinetic properties of native thymidylate synthase were evaluated next. Lineweaver–Burk analysis revealed that the native enzyme displayed a K_m(dUMP) of 5.2 μM. The value of k_{cat} was determined to be 203 min^{-1}. The kinetic parameters for thymidylate synthase immobilized on thiopropyl Sepharose 6B have been determined and reported previously (Bradshaw & Dunlap, 1992). Those studies indicated that the apparent values of K_m and k_{cat} were 22.6 μM (dUMP) and 13 min^{-1}, respectively. A comparison of these kinetic parameters is presented in Table III.

Interestingly, both single-site enzyme and native thymidylate synthase had similar affinities (K_m) for the substrate, dUMP. The k_{cat} values (turnover numbers) were defined as "catalytic activity units" and calculated by dividing the number of micromoles of H_2folate produced per minute by the number of active sites (i.e., two for native and one for single-site enzyme). After such normalization, the k_{cat} value for the single-site enzyme was more than 60% larger than that determined for native enzyme. Furthermore, the specificity constant (k_{cat}/K_m) for the single-site enzyme was nearly 50% larger than that for the native enzyme. Both of these results suggest that modification of the active-site sulfhydryl group at one site substantially enhances catalysis at the open active site and lends further support for the concept of regulation of enzyme activity by a mechanism involving subunit–subunit communication.

Data obtained previously with immobilized thymidylate synthase (Bradshaw & Dunlap, 1992) support this hypothesis. As shown in Table III, the kinetic parameters of this resin-bound form of single-site enzyme are very different from those determined for either the native or the soluble single-site species. The restricted flexibility of the immobilized protein along with partitioning effects encountered through problems in ligand diffusion clearly contributed to these differences. Inspection of the nature of these discrepancies suggests, however, that the covalent modification of one active site (in this case via immobilization) has altered the functional properties of the other. Specifically, whereas the K_m value (22.6 μM) for the immobilized species is approximately 4-fold greater than that seen with native thymidylate synthase, there was a 16-fold change in the k_{cat} value of the coupled protein. Thus, as was the case with the soluble single-site enzyme, the rate of catalysis is affected much more than the extent of ligand binding.

Chart I: Binding Forms for Native Thymidylate Synthase[a]



[a] Shown are the proposed binding species of homogeneous enzyme. Electrophoretic nomenclature is used to describe the three binding forms (Forms, I, II, and III). In this representation, XS• indicates a nonreactive catalytic sulfhydryl group.

CONCLUSIONS

Native thymidylate synthase, homogeneous as a protein, is nevertheless heterogeneous with respect to ligand-binding sites, such that upon incubation with FdUMP and cofactor, two new and distinct electrophoretic bands appear (Forms II and III) (Zapf & Dunlap, 1989). These differences in the ability of the enzyme to bind FdUMP and cofactor have been evoked as evidence for an asymmetrical arrangement between the subunits of the protein. Pre-Form III enzyme has two active-site sulfhydryl groups available for binding substrate and cofactor, while one of the catalytic sulfhydryl groups is incapable of covalently binding FdUMP in pre-Form II enzyme (Chart I). This nonfunctional thiol may exist as some type of mixed disulfide derivative or higher oxidation state. Alternatively, and more suggestive of subunit asymmetry, this sulfhydryl group may be in its required reduced ("SH") form but held in some unreactive conformation (Bradshaw & Dunlap, 1993).

Immobilization of thymidylate synthase on thiopropyl Sepharose 6B, followed by the NEM modification step, allowed for isolation of an enzyme pool homogeneous with respect to binding sites (i.e., dimeric thymidylate synthase containing one functional active site; Figure 1). There is no stringent requirement for the level of activity exhibited by the enzyme which is to be used to prepare single-site thymidylate synthase, as the resin naturally discriminates between pre-Form III, pre-Form II, and inactive enzyme (Bradshaw & Dunlap, 1993). The advantage in using enzyme of the highest quality is that the yield of single-site enzyme is maximized. This single-site species is believed to be one of the first examples of a dimeric enzyme consisting of identical subunits, each containing an active site, being modified such that only one of the catalytic sites remains functional. Single-site enzyme is apparently as stable as native thymidylate synthase; we observed only slight decreases in specific activity and binding ratios during a 2-week period of storage at 4 °C in Tris buffer (pH 7.5) containing 50 mM 2-mercaptoethanol (data not shown). Of importance, this general methodology should facilitate other studies addressing issues of active-site symmetry not only in thymidylate synthase but also in other oligomeric proteins.

The goal of these studies with single-site thymidylate synthase was the initial characterization of this unique enzymic species. Whether as a result of steric influences or the alteration of a hydrogen-bonding network or molecular packing, the covalent modification of the catalytically essential side chain in one site results in single-site enzyme which exhibits enhanced dTMP synthesis activity and a moderate increase in the tightness of FdUMP binding when compared to native, two-active-site enzyme. While the results do not fully describe the phenomenon of subunit communication in thymidylate synthase, they certainly suggest that the catalytic sulfhydryl groups and their chemical status play a major role in the communication network. Since covalent catalysis via the attachment of the active-site cysteine at carbon-6 of dUMP is involved in each step in the reaction pathway (exclusion of the initial noncovalent binding of dUMP and the final discharge of noncovalently bound dTMP), the simplest model of a dimeric thymidylate synthase with one covalently occupied active site and one available active site, and thus of the enzyme in an intermediate stage of catalysis, is the single-site enzyme described in this study. This heterodimeric form of thymidylate synthase should, as such, provide a valuable model for further exploration of the unique properties of the enzyme. Taken together, the results suggest that the covalent association, whether by FdUMP or NEM modification, of the active-site cysteine in one site produces positive cooperativity in binding and catalysis at the second site. The relative proximity of the two active sites at the interface of the two identical subunits also makes this contention plausible. The delicate interplay of the two active sites is further illustrated by carboxypeptidase A studies (Loeble & Dunlap, 1972; Aull *et al.*, 1974a; Cisneros *et al.*, 1993), which demonstrate that the removal of the carboxyl-terminal valine from one subunit results in a form of negative cooperativity expressed as the complete loss of dTMP synthesis activity but the retention of ternary complex formation at one active site. Why does the single-site dimer form produced in this study exhibit positive cooperativity while the heterodimer resulting from carboxypeptidase A treatment evidences negative cooperativity? The answer most certainly lies in the fact that NEM modification and carboxypeptidase A treatment occur at different subsites of the active site, each of which is involved in distinct aspects or stages of the reaction. It is not clear, for example, that the carboxypeptidase-dependent inactivation of the enzyme, which results from the removal of just one of the two C-terminal valine residues of the enzyme dimer, should produce cooperativity effects which are necessarily coupled to those observed when the active-site cysteine on one subunit is selectively modified. These two active-site components play widely different roles in the enzyme and do not have direct interaction. What is clear is that the cooperativity effects resulting from NEM modification and from carboxypeptidase A treatment represent two different modes of subunit communication.

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REFERENCES

Aull, J. L., Loeble, R. B., & Dunlap, R. B. (1974a) *J. Biol. Chem. 249*, 1167–1172.

Aull, J. L., Lyon, J. A., & Dunlap, R. B. (1974b) *Microchem. J. 19*, 210–218.

Aull, J. L., Lyon, J. A., & Dunlap, R. B. (1974c) *Arch. Biochem. Biophys. 165*, 805–808.

Bradshaw, T. P., & Dunlap, R. B. (1992) *Oncol. Res. 4*, 249–254.

Bradshaw, T. P., & Dunlap, R. B. (1993) *Biochim. Biophys. Acta 1163*, 165–175.
Bradshaw, T. P., Ahmed, F., & Dunlap, R. B. (1988) *J. Biochem. Biophys. Methods 17*, 159–165.
Cisneros, R. J., Silks, L. A., & Dunlap, R. B. (1988) *Drugs Future 13*, 859–881.
Cisneros, R. J., Zapf, J. W., & Dunlap, R. B. (1993) *J. Biol. Chem. 249*, 1167–1172.
Climie, S., Ruiz-Perez, L., Gonzalez-Pacanowska, D., Prapanuwattana, P., Cho, S. W., Stroud, R., and Santi, D. V. (1990) *J. Biol. Chem. 265*, 18776–18779.
Connors, K. A. (1987) in *Binding Constants*, Chapter 3, pp 103–138, John Wiley and Sons, New York.
Danenberg, P. V. (1977) *Biochim. Biophys. Acta 473*, 73–92.
Danenberg, K. D., & Danenberg, P. V. (1979) *J. Biol. Chem. 254*, 4345–4348.
Danenberg, P. V., & Lockshin, A. (1981) *Pharmacol. Ther. 13*, 69–90.
Dunlap, R. B., Harding, N. G. L., & Huennekens, F. M. (1971) *Biochemistry 10*, 88–97.
Finer-Moore, J. S., Montfort, W. R., & Stroud, R. M. (1990) *Biochemistry 29*, 6977–6986.
Galivan, J. H., Maley, G. F., & Maley, F. (1976) *Biochemistry 15*, 356–362.
Hardy, L. W., Finer-Moore, J. S., Montfort, W. R., Jones, M. O., Santi, D. V., & Stroud, R. M. (1987) *Science 235*, 448–455.
Hatefi, Y., Talbert, P. J., Osborn, M. J., & Huennekens, F. M. (1960) *Biochem. Prep. 7*, 89–92.
Kamb, A., Finer-Moore, J. S., Calvert, A. H., & Stroud, R. M. (1992a) *Biochemistry 31*, 9883–9890.
Kamb, A., Finer-Moore, J. S., and Stroud, R. M. (1992b) *Biochemistry 31*, 12876–12884.
Lewis, C. A., & Dunlap, R. B. (1981) in *Topics in Molecular Pharmacology* (Burgen, A. S. V., & Roberts, G. C. K., Eds.) pp 169–219, Elsevier, Amsterdam.
Lewis, C. A., Ellis, P. D., & Dunlap, R. B. (1980) *Biochemistry 19*, 116–123.
Lewis, C. A., Ellis, P. D., & Dunlap, R. B. (1981) *Biochemistry 20*, 2275–2285.
Lineweaver, H., & Burk, D. (1934) *J. Am. Chem. Soc. 56*, 658–666.
Lockshin, A., & Danenberg, P. V. (1980) *Biochemistry 19*, 4244–4251.
Loeble, R. B., & Dunlap, R. B. (1972) *Biochem. Biophys. Res. Commun. 49*, 1671–1677.
Lyon, J. A., Pollard, A. L., Loeble, R. B., & Dunlap, R. B. (1975) *Cancer Biochem. Biophys. 1*, 121–128.
Matthews, D. A., Appelt, K., Oatley, S. J., & Xuong, Ng. H. (1990) *J. Mol. Biol. 214*, 923–936.
Moore, M. A., Ahmed, F., & Dunlap, R. B. (1984) *Biochem. Biophys. Res. Commun. 124*, 37–43.
Moore, M. A., Ahmed, F. & Dunlap, R. B. (1986) *J. Biol. Chem. 261*, 12745–12749.
Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Montfort, W. R., Maley, G. M., & 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.
Plese, P. C., & Dunlap, R. B. (1977) *J. Biol. Chem. 252*, 6139–6144.
Pogolotti, A. L., Jr., 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.
Reinsch, J. W., Smith, L. L., and Dunlap, R. B. (1979) *Cancer Biochem. Biophys. 3*, 57–64.
Santi, D. V., & Danenberg, P. V. (1984) in *Folates and Pterins* (Blakely, R. L., & Benkovic, S. J., Eds.) Vol. 1, Chapter 9, Wiley, New York.
Zapf, J. W., & Dunlap, R. B. (1989) *Adv. Enzyme Regul. 29*, 189–207.