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ARTICLE *in* BIOCONJUGATE CHEMISTRY · SEPTEMBER 1991

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Comparison of ELISA with Activity and Ligand-Binding Methods for the Determination of Thymidylate Synthase Concentration[1]

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The determination of enzyme levels in cellular extracts by active site titrations or by catalytic activity measurements is relevant in both science and medicine. However, these techniques assume that enzymes exhibit the same response in crude sample matrices as they do in the purified state. We report here an example of how an enzyme-linked immunosorbent assay (ELISA) was used to determine the true enzyme concentration which was compared to the effective enzyme concentration obtained by ligand binding and catalytic assay methods in a crude bacterial cell extract. Rabbit antibodies specific for *Lactobacillus casei* thymidylate synthase (TS) were used to develop a highly specific and sensitive heterogeneous noncompetitive ELISA assay with a typical detection limit of 1.4 fmol of TS (100 pg) and a dynamic working range of 3 orders of magnitude. The antibodies showed identical responses for TS, its inhibitory binary complex with 5-fluoro-2′-deoxyuridylate, and its inhibitory ternary complex with 5-fluoro-2′-deoxyuridylate and 5,10-methylenetetrahydrofolate in the immunoassay. *L. casei* cell-free extracts were subjected to extraction with CM-Sephadex and the various fractions were analyzed by ELISA, active-site titrations, and catalytic assays which demonstrated that assays which assumed full catalytic or ligand-binding competence underestimated the true enzyme level.

INTRODUCTION

Thymidylate synthase (TS) constitutes the principal mechanism for the biosynthesis of thymidylate (dTMP) in most living cells. Because the supply of dTMP is critical for continued DNA synthesis and cell division, the enzymes of the thymidylate synthesis cycle have often been exploited as targets for chemotherapeutic strategies aimed at growth limitation. Two examples of this approach include the use of fluoropyrimidine-based inhibitors of thymidylate synthase (TS) in cancer chemotherapy (*1*) and the use of pyrimethamine as a specific inhibitor of dihydrofolate reductase in antimalarial treatments (*2*).

Although several effective assay strategies are available for estimating the levels of these enzymes in crude cell extracts, each assay technique is bound by certain limitations. In order to obtain an estimate of enzyme concentration, it must be assumed that all enzyme molecules in the sample exhibit full catalytic or ligand-binding competence. This assumption could prove to be faulty in several cases, such as (*1*) occasions in which specific inhibitors of the enzyme might interfere with ligand binding or catalytic activity; (*2*) circumstances in which an allosteric effector might be up- or down-regulating catalytic or ligand-binding activity; (*3*) cases where the enzyme has unknown catalytic or ligand-binding activity, such as with either naturally occurring mutant enzymes or with site-directed mutants of cloned enzymes; or (*4*) when the enzyme has suffered proteolytic, oxidative, or thermal degradation.

A cogent example of the need to develop a corollary enzyme assay technique is the measurement of thymidylate synthase concentrations in cell-free extracts obtained from cells exposed to fluoropyrimidines, such as 5-fluorouracil and 5-fluorodeoxyuridine. The latter compounds are precursors of 5-fluorodeoxyuridine 5′-monophosphate (FdUMP), a mechanism-based inhibitor, which forms binary and ternary complexes at the active sites of thymidylate synthase. In fact, fluoropyrimidine-treated, drug-resistant cancer cells often exhibit expanded TS pools after prolonged treatment (*1*, *3*). Obviously, since these agents perturb ligand binding and catalysis by interacting with the active site of the enzyme, then activity or ligand-based methods for estimating the concentration of thymidylate synthase in the presence of such antimetabolites will yield underestimates of the enzyme concentration.

In general, the problems associated with conventional ligand-binding or catalytic assays, which actually measure the effective concentration of an enzyme, can be overcome by an assay based on immunological recognition wherein the quantity of enzyme (the true enzyme concentration) is determined with little or no dependence on the status of the active site(s) of the enzyme. We report here the development and characterization of an ELISA for use in determining the true enzyme concentration of *L. casei* thymidylate synthase in purified samples and crude cell extracts.

Thymidylate synthase is present in most cells at very low levels, although it is expressed to a greater extent in rapidly dividing cells such as fetal and embryonic tissue, fibroblasts, and certain drug-resistant tumor cell lines (*4*). The extremely low levels of TS present in most cells

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[1] Abbreviations used: TS, thymidylate synthase; dTMP, thymidylate: dUMP, 2′-deoxyuridylate; FdUMP, 5-fluoro-2′-deoxyuridylate; $CH_2H_4PteGlu$, (±)-5,10-methylenetetrahydropteroylglutamic acid; ELISA, enzyme-linked immunosorbent assay; TSAb, rabbit IgG specific for *L. casei* thymidylate synthase; TSAb–HRP, horseradish peroxidase labeled rabbit IgG specific for *L. casei* thymidylate synthase; HRP, horseradish peroxidase; IgG–HRP, horseradish peroxidase labeled goat antibodies specific for rabbit IgG; CFE, cell-free extract; 5-AS, 5-aminosalicylic acid; 2-ME, 2-mercaptoethanol; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; PBS, phosphate-buffered saline consisting of 10 mM KH_2PO_4, and 150 mM NaCl, pH 7.4; PBS-T, PBS containing 0.05% Tween 20; TBS, Tris-buffered saline consisting of 50 mM Tris and 500 mM NaCl, pH 7.4; TBS-T, TBS containing 0.05% Tween 20; TCA, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

1043-1802/91/2902-0327$02.50/0

(estimated to be as low as a few molecules per cell) make sensitivity the critical requirement in designing any assay for this enzyme. The unique features of the heterogeneous noncompetitive enzyme-linked immunosorbent assay (ELISA) yield maximum sensitivity while the highly discriminatory nature of immunorecognition provides an optimal level of selectivity. In the strategy we report here, polyclonal antibodies were isolated from the serum of rabbits immunized with purified thymidylate synthase obtained from methotrexate-resistant *L. casei* and used in the development of an ELISA technique for thymidylate synthase. We chose to employ polyclonal rather than monoclonal antibodies in order to allow the application of this technique to the broadest possible range of analytes, including mutant forms and ligand-bound forms of *L. casei* thymidylate synthase.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals. Goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (IgG–HRP) were obtained from Bio-Rad and used at a 2000-fold dilution in PBS-T. Horseradish peroxidase (HRP), nonspecific rabbit IgG, FdUMP, and 4-chloro-1-napthol were obtained from Sigma Chemical Co. 5-AS was obtained from Sigma and was recrystallized by the method of Ellens and Gielkens (*5*) as described by Tijssen (*6*). Carboxymethyl Sephadex C-50 (CM-Sephadex) and Sephacryl S-300 were obtained from Pharmacia. DEAE–cellulose resin was obtained from Pierce. [6-^{3}H]FdUMP was obtained from Moravek Biochemicals. Racemic tetrahydrofolate was prepared from folic acid by catalytic hydrogenation as described by Hatefi et al. (*7*) and was converted to (±)-5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4PteGlu) by adding a 25-fold molar excess of formaldehyde.

Cell-free extracts (CFE) of methotrexate-resistant *L. casei* (*8*) were prepared from 20 g of cells by sonication at 0–10 °C in 80 mL of CM-20 buffer (50 mM Bis-Tris, 20 mM KCl, 20 mM 2-ME, 1 mM EDTA, pH 6.5) followed by centrifugation for 30 min at 15000*g* at 4 °C to remove cellular debris and insoluble material. *L. casei* MTX^r provides a 200–400-fold increase in TS level over the sensitive organism. Crude TS was prepared from *L. casei* CFE by batch extraction with CM-Sephadex as described by Zapf and Dunlap (*9*). Briefly, 80 mL of CFE was incubated with 20 g of damp CM-Sephadex preequilibrated with CM-20 buffer for 60 min, followed by washing with 500 mL of CM-165 buffer (50 mM Bis-Tris, 165 mM KCl, 20 mM 2-ME, 1 mM EDTA, pH 6.5). Partially purified TS was eluted with 20 mL of CM-500 buffer (50 mM Bis-Tris, 500 mM KCl, 20 mM, 2-ME, pH 6.5).

Preparation of Immunoreagents. Homogenous thymidylate synthase (TS) was obtained from amethopterin resistant *L. casei* according to the method of Lyon et al. (*8*). The purity and quality of the enzyme was verified by a single protein band under native gel electrophoresis which exhibited a specific activity of 2.5–3.0 units/mg (*8*) and a ternary complex binding ratio of 1.7 (*9*). TS was prepared for immunization by centrifugal column chromatography (*10*) to exchange the buffer from thiol-free activation buffer (50 mM Tris, 50 mM KCl, pH 6.8). The latter enzyme solution was then passed through a 0.45-μm sterile filter and mixed 1:1 with Freund's Complete Adjuvant, and three New Zealand white rabbits were immunized subcutaneously with the resulting emulsion (100 μg of TS/rabbit). Rabbits were boosted twice at 3-week intervals with 190 μg of TS in Freund's Incomplete Adjuvant, and whole blood samples were collected by arterial puncture of the dorsal ear artery. Anti-TS IgG was isolated from rabbit serum by caprylic acid precipitation and DEAE–cellulose chromatography (*11*). The purity of the pooled rabbit IgG fractions was determined by native and denaturing PAGE, and the immunochemical specificity was demonstrated by using the IgG pool as a probe of cell lysates of *L. casei* MTX^r.

TSAb–HRP was prepared as described by Tijssen (*5*) by activating 2.5 mg of HRP (Sigma) in 0.25 mL of 0.1 M Na_2CO_3, pH 9.2, and 0.25 mL of 10 mM $NaIO_4$ at room temperature for 2 h. TSAb (6 mg) in 1.0 mL of 0.1 M Na_2CO_3, pH 9.2, was then added to the activated HRP solution. Dry Sephadex G-25 resin (Pharmacia, 85 mg) was then added to increase the effective protein concentration (by absorbing water) and to consume excess periodate. The mixture was incubated for 3 h at room temperature and the Schiff's base adduct was reduced by incubating for 60 min at 4 °C with 0.25 mg of $NaBH_4$. The TSAb–HRP conjugate was purified by chromatography on a 1 × 100 cm Sephacryl S-300 column equilibrated in PBS (10 mM KH_2PO_4, 150 mM NaCl, pH 7.4).

Enzyme Assays. TS catalytic activity was determined spectrophotometrically by the method described by Lyon et al. (*8*). Various amounts of purified *L. casei* TS (23, 46, or 92 pmol of TS, specific activity 2.0 units/mg) were added to 100-μL aliquots of freshly prepared *L. casei* CFE and incubated for 30 min at room temperature. These samples were then added to quartz cuvettes and diluted with a substrate-containing mixture, the reaction was monitored spectrophotometrically for 30 s at 25 °C, and the increase in absorbance at 340 nm due to the production of dihydrofolate was recorded. The final assay mixture contained 0.1 mM CH_2H_4PteGlu, 0.1 mM dUMP, and 0.1 M Tris, pH 7.3, in a total volume of 1.01 mL. These samples were compared to similar 100-μL aliquots of CFE to which only activation buffer was added and to samples of purified TS to which only cell-break buffer and no CFE were added.

Ligand-binding competence was measured by the trichloroacetic acid precipitation assay (TCA) described by Cisneros (*12*) and Moore et al. (*13*). In the TCA assay, purified or crude samples containing 0.08–0.25 nmol of TS were incubated with 0.5 μmol of CH_2H_4PteGlu and 1 nmol of [6-^{3}H]FdUMP at 0.76 Ci/mmol in 0.5 mL of buffer containing 0.2 M sodium acetate, 0.1 M 2-ME, and 0.1 mg of BSA/mL, pH 5.8, for 60 min at 37 °C. The reactions were quenched by adding TCA to a final concentration of 10% and the protein precipitates were collected by centrifugation for 3 min at 16000*g*. The pellets were washed three times with 1.5-mL portions of 10% TCA, solubilized in three 125-μL aliquots of 0.2 M NaOH in 50% ethanol, and the radioactivity of the covalently attached ligand was quantitated in 10 mL of Beckman HP scintillation cocktail with 75 μL of glacial acetic acid with a Beckman LS 7500 liquid scintillation counter.

Protein Concentration. Protein concentrations in crude extracts were determined by the Bio-Rad protein assay kit and confirmed by the BCA protein assay kit (Pierce). Concentrations of purified TS were calculated from absorbance values at 278 nm and a molar absorptivity of 1.10×10^5 M^{-1} cm^{-1} (*14*).

Western Blotting. Proteins from native and denaturing (SDS) polyacrylamide gels were electrophoretically transferred to nitrocellulose membranes in a Bio-Rad Trans Blot apparatus. Membranes were then soaked for 60 min in TBS-T (50 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.4) with 3% gelatin added to block excess protein binding sites on the membranes. The membranes were then incubated for 3 h in 100 mL of TBS-T with 4.0

μg of TSAb/mL on a tilting table. The membranes were then washed three times with 250 mL of TBS-T, incubated for 3 h in a 2000-fold dilution of Bio-Rad goat anti-rabbit IgG-HRP stock in TBS-T, then washed three times with TBS-T as before and once with TBS. Immunoreactive bands were revealed by adding 50 mL of TBS with 0.008% H_2O_2 and 10 mL of 6 mg of 4-chloro-1-napthol in 100% methanol.

Optimization of ELISA. To ensure that both the primary and secondary antibody preparations would always be in excess over the amount of TS in the sample and in order to drive the reaction forming the immune complexes as far toward completion as possible (to enhance sensitivity), we first endeavored to determine the maximal TSAb loading capacity of the microtiter plates. This was accomplished by adding various amounts of TSAb in PBS (0.02–20.0 μg of TSAb/mL) to the wells of a fresh Immulon II 96-well microtiter plate (Dynatech), incubating for 4 h at room temperature, and washing away the unbound TSAb with a Bio-Rad Model 1550 microplate washer adjusted to deliver three wash cycles of 300 μL of PBS-T (10 mM, KH_2PO_4, 150 mM NaCl, 0.05% Tween-20, pH 7.4) with 8-s soaks before aspiration of the spent wash liquid. The amount of TSAb necessary to saturate protein binding sites in the wells was then determined by adding 200-μL aliquots of Bio-Rad HRP-labeled goat anti-rabbit IgG antibodies (IgG–HRP) diluted 2000-fold with PBS-T and allowing the immune complexes to form for 4 h at room temperature. The excess IgG–HRP was then washed away with the plate washer as described above and the immune complexes were detected colorimetrically at 490 nm with a Dynatech MR 650 microtiter plate reader by adding HRP substrate cocktail (10 mM potassium phosphate, 150 mM NaCl, 1.0 mg of 5-AS/mL, 0.005% H_2O_2, pH 6.0) and incubating for 10 min at room temperature.

Optimization of the second antibody concentration was performed similarly by first adding 200 μL of TSAb in PBS per well of a fresh microtiter plate at twice the concentration of TSAb found to yield a maximal response in the previously mentioned experiment. The plate was maintained at room temperature for 4 h, then stored overnight at 4 °C, and the excess TSAb was washed away with the microplate washer as described above. The TS binding sites were saturated by adding 200 μL of 0.1 mg of purified *L. casei* TS/mL of PBS-T per well and incubating for 4 h at room temperature. The excess TS was washed away as described above, various amounts of TSAb–HRP in PBS-T was added to each well (0.01–10 μg/mL) and allowed to react for 4 h at room temperature. The excess TSAb–HRP second antibody was washed away as described above and the extent of immune complex formation was determined by adding 200 μL of HRP substrate cocktail per well and recording the absorbances at 490 nm with the microtiter plate reader.

ELISA. New Dynatech Immulon II 96-well microtiter plates were coated with TSAb solutions (typically 4.0 μg of TSAb/mL) in PBS for 4 h at room temperature and stored at 4 °C until use within 1 week. The coated plates were then washed with PBS-T with the Bio-Rad microplate washer as described above and either purified *L. casei* TS standards (0.05 ng–100 μg TS/well), TS-containing crude cytoplasmic extracts, or PBS-T blanks were then added and incubated for 4 h at room temperature before washing with PBS-T as described above. TSAb–HRP was then added (200 μL of 2.0 μg/mL solution) in PBS-T and incubated for 4 h at room temperature. The TSAb::: TS:::TSAb–HRP sandwiches were then revealed after washing of the plate as described above by adding 200 μL of HRP substrate cocktail and measuring the absorbance values at 490 nm with the microplate reader.

RESULTS AND DISCUSSION

Characterization of Immunoreactants. TS levels in cell-free extracts from *L. casei* MTX^r were determined by the TCA assay. Aliquots from these samples containing ca. 2.0 μg of TS were incubated either with activation buffer (50 mM Tris, 50 mM KCl, 20 mM 2-ME, pH 7.3) or with activation buffer containing a 10-fold molar excess of FdUMP and a 500-fold molar excess of CH_2H_4PteGlu for 60 min at 37 °C. These samples and purified *L. casei* TS standards were separated by native 8.0% PAGE and subjected to Western blotting. Similar samples and purified TS standards were denatured and separated by denaturing 9.0% PAGE and also subjected to Western blotting. The results of Western blot analyses conducted on both pure TS and cell-free extracts, in the presence or absence of ligands, and in the presence or absence of denaturants failed to show any bands other than those that could be attributed to TS or its complexes. Under native conditions, *L. casei* TS is a dimer of identical subunits, yielding a single band on native PAGE (with an apparent MW of 73 kDa), which is converted into two additional faster moving bands upon incubation with FdUMP and CH_2H_5folate (formation of covalent inhibitory ternary complexes). SDS denaturation of native enzyme or its inhibitory ternary complexes yields a single band (with an apparent MW of 35 kDa). In no case was evidence obtained that indicated the existence of any "structurally similar impurity in addition to TS" in the samples which we studied. These blots demonstrated that TSAb exclusively recognized bands which could be attributed to TS or TS–ligand complexes with no noticeable cross reactivity with other bands in the gel (data not shown).

Characterization of ELISA. The maximum capacity for loading TSAb on Immulon II plates was found to be ca. 1 μg of TSAb/mL of PBS (Figure 1), so all subsequent plates were coated with 4.0 μg of TSAb/mL of PBS. Similarly, the maximum ELISA response was observed at TSAb–HRP working concentrations of 2.0 μg of TSAb–HRP/mL of PBS-T. Subsequent plates were processed with a TSAb–HRP working concentration of 4.0 μg/mL.

Various amounts (0.01–100 ng) of purified *L. casei* TS were diluted with PBS-T to 200 μL/well in a microplate that had been coated with TSAb and washed with PBS-T. After incubation for 4 h at room temperature, the plates were washed with PBS-T, TSAb–HRP was added, and the plates were incubated as described above. The plates were washed again, and HRP substrate cocktail was added. The ELISA response was judged to be linear over 3 orders of magnitude when expressed as A_{490} vs log (ng of TS/well) (Figure 2) with typical detection limits around 100–200 pg of TS (1.4–2.8 fmol).

Recognition of Denatured TS and Native TS-Ligand Complexes. Denatured *L. casei* TS samples were prepared by incubating purified TS samples in a boiling-water bath for 10 min in the presence of 1% SDS. The buffer was exchanged by five cycles of concentration and dilution with PBS-T in a Centricon-10 device, and the concentration of the denatured protein was quantitated by UV absorbance at 278 nm and by the BCA protein assay kit (Pierce). The denatured TS sample was then compared to native *L. casei* TS standards by using the ELISA protocol described above (Figure 3). As shown in Figure 3, the ELISA assay was nearly 100-fold less sensitive

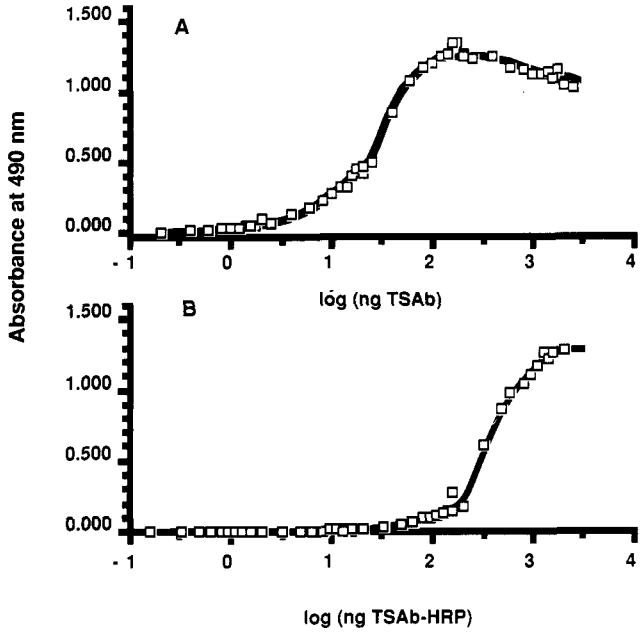

Figure 1. Optimization of conditions for ELISA. The optimum antibody concentrations for ELISA were established by determining the concentration thresholds necessary for maximal response. (A) 200-μL aliquots containing various amounts of TSAb (0.02–20.0 μg/mL TSAb) in PBS were added to individual wells of a 96-well microtiter plate, incubated for 4 h, and washed with PBS-T. Saturating amounts of IgG–HRP specific for rabbit IgG were added, incubated for 4 h, washed with PBS-T, and incubated for 10 min at room temperature with HRP substrate cocktail, and the absorbance at 490 nm was recorded. (B) 200-μL aliquots of 4.0 TSAb/mL PBS were added to the wells of a microtiter plate, allowed to incubate for 4 h, and washed with PBS-T, 200 μL of 0.1 mg of TS/mL of PBS-T was added, and the mixture was incubated for 4 h and washed with PBS-T. TSAb–HRP (0.01–10.0 μg/mL, 200-μL aliquots in PBS-T) was added, incubated for 4 h, and washed with PBS-T, HRP substrate cocktail was added, and the absorbance at 490 nm was recorded.

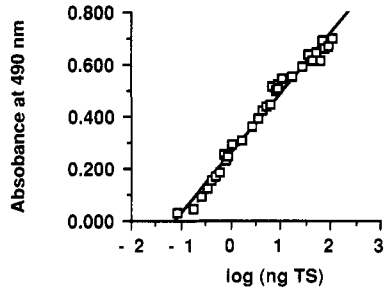

Figure 2. Sensitivity and dynamic working range of ELISA. The dynamic working range of ELISA was determined by adding various amounts of purified TS (0.10–100 ng TS/mL) in PBS-T to a microtiter plate previously coated with TSAb and washed with PBS-T, incubating for 4 h, washing with PBS-T, and developing with TSAb–HRP.

to denatured enzyme than to native TS, even though the concentration of the antibody used in both trials was the same. These results might be attributed to either the destruction or alteration of certain epitopes present in the tertiary structure of the native protein because of unfolding of the polypeptide chains or the blocking or shielding of the epitopes from the antibody by the denaturant.

Binary and ternary complexes of TS were formed by incubating purified TS (1.4–55 pmol) with 10 μM FdUMP alone or with 10 μM FdUMP and 27 μM (±)-CH_2H_4PteGlu, respectively, in the wells of a 96-well microplate. Ligand-free controls were prepared similarly to purified TS standards in 200 μL of PBS-T. The plate was incubated

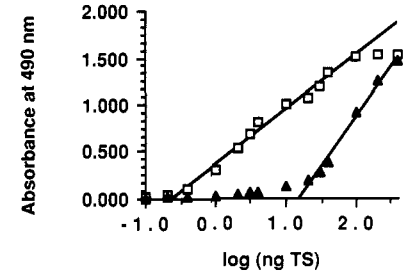

Figure 3. Recognition of denatured thymidylate synthase by ELISA. Purified TS was denatured with 1.0% SDS, the buffer was exchanged for PBS-T, and the ELISA response of the denatured sample (closed triangles) was compared to native TS (open squares).

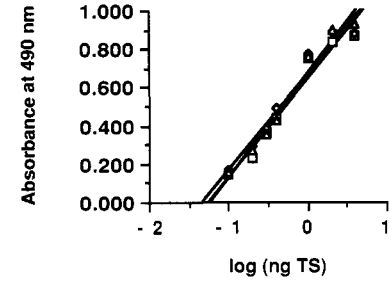

Figure 4. Recognition of thymidylate synthase ligand complexes by ELISA. Purified TS was incubated with excess FdUMP (open triangles) or FdUMP plus CH_2H_4PteGlu (open diamonds) in a microtiter plate and the ELISA response was compared to ligand-free TS (open squares).

Table I. Thymidylate Synthase from Cell-Free Extract

sample	quantitation of thymidylate synthase: by TCA,[a,d] μM	by ELISA,[d] μM	ELISA result/ TCA result
CFE	1.2	2.0	1.7
CM-Sephadex[b] (unretained)	0.020	0.068	3.4
CM-Sephadex[c] (retained)	0.74	0.87	1.2

[a] Quantitation of TS by TCA assay of the inhibitory ternary complex assumed an FdUMP:TS binding stoichiometry of 1.7, found to be the typical binding ratio by Moore et al. (*13*). [b] CFE fraction which passed directly through CM-Sephadex. [c] CFE fraction which was retained by CM-Sephadex and eluted with CM-500 buffer. [d] These concentration values were derived from primary data for binding ratio determination accurate to within 5%.

for 4 h at room temperature and processed as described above. The ELISA responses from all three data sets were almost indistinguishable, indicating that TSAb would detect either ligand-bound or uncomplexed forms of TS with nearly identical responses (Figure 4), thus validating the utility of an assay based on the polyclonal antibodies.

Quantitation of TS Levels in *L. casei* Cytoplasmic Extracts. Samples of freshly prepared CFE were subjected to ELISA and the TCA ligand-binding assay. The ligand-binding assay measured the presence of only 1.2 μM TS in *L. casei* CFE while ELISA predicted 2.0 μM TS in that fraction (Table I). Among the possible explanations for the difference in the ligand binding and ELISA results are the following: (1) inactivation of TS by proteolysis, (2) protein denaturation or oxidation of the active-site sulfhydryl groups resulting in inactivation, and (3) the presence of an endogenous inhibitor of TS. First, the fact that Western blots (see above) exhibited only those bands known to be native TS or TS–ligand complexes effectively dismisses the possibility that TS in the CFE has undergone proteolytic degradation. Second, if partial denaturation or sulfhydryl group oxidation of TS had occurred during

Table II. Thymidylate Synthase Catalytic Activity in Cell-Free Extract

sample	TS added, pmol	volume CFE, μL	thymidylate synthase activity × 10^{-3}, units observed[a]	expected	difference
purified TS	23.1	0	3.7		
	46.2	0	6.6		
	92.4	0	13.0		
cell-free extract	0	100	7.9		
cell-free extract	23.1	100	9.4	11.7	2.3
with TS spike	46.2	100	12.3	14.5	2.7
	92.4	100	18.9	20.6	1.7

[a] Averages of two catalytic activity measurements with a spectrophotometric method (*8*). Pooled standard deviation was ±0.0005 units. A unit was defined as the amount of enzyme capable of catalyzing the production of 1 μmol of dihydrofolate per min at 25 °C.

cell storage, sonication, or centrifugation, then spiking the CFE with known amounts of purified TS should result in observed activities of the mixtures which are the simple sums of the activities of the individual components. When such an experiment was performed employing the spectrophotometric assay to measure TS activities, the results (Table II) indicated that the observed activities of the mixtures were uniformly lower (an average of 0.022 units/mL of CFE) than the expected sum of the activities of the two components.

Besides discounting the second possibility, these results are consistent with the third possibility, namely, that an endogenous inhibitor is present in the CFE. Should this be the case, then the subsequent separation of the enzyme from the putative inhibitor should lead to an increase in the total number of enzyme units as measured by the spectrophotometric assay. Interestingly, Zapf and Dunlap (*9*) found that batchwise extraction of the CFE with CM-Sephadex, which is a facile step in the purification of TS, resulted in the recovery of 114% of the TS activity originally present in the CFE. Accordingly, if CM-Sephadex separates an endogenous inhibitor from TS, then the quantitation of the TS in the fraction retained by CM-Sephadex using the ligand-binding assay should give a result which is similar to that from ELISA. In fact, as shown in Table I, the ratio of the ELISA result to the ligand binding result is 1.2, which suggests that the true concentration of thymidylate synthase protein (ELISA) is now quite similar to the effective enzyme concentration as reflected by a measure of the concentration of enzyme active sites (TCA assay). In our experience, both assay methods can routinely be performed, yielding results with less than 5% error.

Since these results suggest that the CM-Sephadex step resulted in the separation of the endogenous inhibitor from the bulk of the TS present in CFE, then it is reasonable to expect that the inhibitor might be present in another fraction from the CM-Sephadex extraction. When TS levels were examined in the fraction which was not retained by CM-Sephadex extraction (CM-20 wash), ELISA predicted 3.4 times as much TS as the TCA assay. These results suggest that the small but easily measurable level of TS present in the fraction which passed directly through CM-Sephadex must be inhibited to a greater extent than the TS molecules present in the fraction which was retained by CM-Sephadex. These results are especially interesting in light of the fact that Friedkin et al. (*15*) described a naturally occurring TS inhibitor in *E. coli* which they identified as N^5-formyltetrahydropteroyl oligoglutamate with a K_i value of 1.3 μM and speculated that it may play some role in the regulation of TS activity.

The foregoing results aptly demonstrate a fundamental problem in the determination of enzyme concentrations in biological samples. In this regard, a distinction must be made between the true enzyme concentration and its effective concentration. The true enzyme concentration is defined as the total number of protein molecules representing the particular enzyme per unit volume and can be readily measured by ELISA, while the effective concentration of enzyme is the number obtained by the use of methods which measure the extent of catalysis or ligand binding by the enzyme per unit volume under the prevailing conditions. Ideally, the true enzyme concentration should equal the effective enzyme concentration. However, as discussed in the Introduction, many situations are encountered in which the true enzyme concentration is not the same as the effective enzyme concentration. In this report, we described a situation where the effective concentration of TS in the bacterial cell extract is substantially lower than its true concentration, probably because of the presence of an endogenous inhibitor. Unfortunately, many investigators simply assume that enzyme concentrations measured by methods such as catalysis and ligand binding, which depend on the competence of the active site of an enzyme, are the true concentrations. Such an assumption can result in misleading conclusions. For example, consider the folllowing problem which is relevant to cancer chemotherapy. Treatment of colorectal tumor cells with 5-fluorouracil is often accompanied by at least some of the following events (for review, see ref 16): (1) the conversion of 5-fluorouracil to FdUMP, (2) its interaction with TS and CH_2H_4PteGlu to form a reversible inhibitory ternary complex, (3) an increase in the intracellular dUMP concentration, and (4) an increase in mRNA coding for the biosynthesis of TS. If one or more of the latter conditions pertain, the use of a ligand-binding or catalytic method will yield an effective TS concentration which is substantially different from the true concentration of the enzyme and could result in the administration of an inappropriate dose of the drug. The solution to this problem is to employ a combination of methods to estimate enzyme levels, at least one of which should be an ELISA to measure the true enzyme concentration. We are now in the process of extending the methodology described in these studies to measure true and effective TS concentrations in a variety of mammalian cell lines.

ACKNOWLEDGMENT

Support for this work was provided by the National Institutes of Health Grant CA 15645 from the National Cancer Institute. The purchase of the equipment necessary to perform ELISA was supported in part by NIH BRSG S07RR07160 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

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