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The effect of Arg209 to Lys mutation in mouse thymidylate synthase✪

Joanna Cieśla[1], Barbara Gołos[1], Elżbieta Wałajtys-Rode[2], Elżbieta Jagielska[1], Andrzej Płucienniczak[3] and Wojciech Rode[1]✉

[1]*Nencki Institute of Experimental Biology, Warszawa, Poland;* [2]*Institute of Biology and Environmental Protection, Rzeszów University, Rzeszów, Poland; and* [3]*Institute of Biotechnology and Antibiotics, Warszawa, Poland*

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Mouse thymidylate synthase R209K (a mutation corresponding to R218K in *Lactobacillus casei*), overexpressed in thymidylate synthase-deficient *Escherichia coli* strain, was poorly soluble and with only feeble enzyme activity. The mutated protein, incubated with FdUMP and $N^{5,10}$-methylenetetrahydrofolate, did not form a complex stable under conditions of SDS/polyacrylamide gel electrophoresis. The reaction catalyzed by the R209K enzyme (studied in a crude extract), compared to that catalyzed by purified wild-type recombinant mouse thymidylate synthase, showed the K_m value for dUMP 571-fold higher and V_{max} value over 50-fold (assuming that the mutated enzyme constituted 20% of total crude extract protein) lower. Thus the ratios $k_{cat,\ R209K}/k_{cat,\ 'wild'}$ and $(k_{cat,\ R209K}/K_{m,\ R209K}^{dUMP})/(k_{cat,\ 'wild'}/K_{m,\ 'wild'}^{dUMP})$ were 0.019 and 0.000032, respectively, documenting that mouse thymidylate synthase R209, similar to the corresponding *L. casei* R218, is essential for both dUMP binding and enzyme reaction.

Thymidylate synthase (EC 2.1.1.45) catalyzes the C(5) methylation of 2′-deoxyuridylate (dUMP) in a concerted transfer and reduction of the one-carbon group (at the aldehyde oxidation level) of $N^{5,10}$-methylenetetrahydrofolate, with concomitant production of thymidylate (dTMP) and dihydrofolate (Carreras & Santi, 1995; Rode & Leś, 1996). As the sole *de novo* source of thymidylate synthesis, it is an attractive target in chemotherapy (Ealick & Armstrong, 1993).

The enzyme's active center requires the phosphate moiety of dUMP to be dianionic for optimum binding (Gołos *et al.*, 2001). Crystallographic studies of thymidylate synthase-bound dUMP have shown the dianionic phos-

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✉Corresponding author: Wojciech Rode at the Nencki Institute of Experimental Biology, L. Pasteura 3, 02-093 Warszawa, Poland; fax: (48 22) 822 5342; e-mail: rode@nencki.gov.pl

phate to be ligated by a quartet of highly conserved arginines (Arg23, Arg178′, Arg179′ and Arg218 in the *Lactobacillus casei* enzyme) and one serine (Ser219). This pattern appears to be common for the bacterial (Fines-Moore *et al.*, 1993; Stroud & Finer-Moore, 1993; Fauman *et al.*, 1994; reviewed in Carreras & Santi, 1995), fungal (Anderson *et al.*, 2000) and mammalian (Sotelo-Mundo *et al.*, 1999) synthases. The arginines appear to bind cooperatively to the phosphate oxygens, as their energetic contributions indicate ideal complementation of the charge and geometry of the phosphate moiety (Morse *et al.*, 2000).

Kawase *et al.* (2000) replaced each of the four arginine residues in the *L. casei* enzyme by mutagenesis, proving their differing contributions to binding and catalysis. Interestingly, only one of the four phosphate-binding arginine residues (Arg218) appeared to be indispensable for catalysis (Kawase *et al.*, 2000) and its guanidinium group was suggested to lower, *via* ion-pairing, the pK_a of the catalytic thiol of Cys198, enhancing its nucleophilicity (Hardy *et al.*, 1987). So far no data on the effects of the corresponding replacements in the enzyme of higher organisms have been reported.

An unexpected byproduct of our recent experiment, aimed at overexpression of mouse thymidylate synthase and involving PCR-assisted replacement of certain bases in the coding sequence, was the enzyme containing an R209K mutation (R218K in *L. casei*), resulting probably from a Taq polymerase error. The present paper presents the properties of the recombinant mutated and wild-type mouse thymidylate synthases.

MATERIALS AND METHODS

pBluescript SK$^-$ vector and *Escherichia coli* XL-1 Blue strain were from Stratagene, TX61$^-$, a derivative of the *E. coli* BL21(DE3) strain with the endogenous thymidylate synthase gene inactivated by transposon-mediated insertion, was a gift from Dr. W.S. Dallas (Glaxo Wellcome, U.K.).

Maintenance of L1210 leukemia cells and RNA isolation. The cells were maintained as previously described (Rode *et al.*, 1984). Transplantations were done once every 7 days by intraperitoneal inoculation of 10^4 cells in 0.5 ml of ascites fluid diluted with 0.9% NaCl. The cells were harvested 7 days after inoculation by centrifuging the ascites fluid at 300 × ***g*** for 10 min. The pellet was washed twice with 0.9% NaCl and immediately used for total RNA isolation with TRIzol reagent (Gibco) according to the manufacturer's protocol.

RT-PCR and cDNA cloning. General methods for DNA manipulations were as described (Sambrook *et al.,* 1989). Reverse transcription was performed on total RNA with Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) and oligo(dT) primer with an additional round of reverse transcription reaction – after the first round the sample was denatured for 3 min at 95°C, cooled on ice and, after addition of reverse transcriptase (50 units), incubated for 1 h at 42°C. In the PCR procedure the following primers were used: forward (primer I) 5′-GGGAATTCATATGCTGGTGGTTGGCTCCGAG-3′, complementing the 21 nucleotide region upstream of the start codon, and the reverse (primer II) 5′-AAAAAGCTTTTAAACAGCCATTTCCATTTTAAT-3′, complementing the last 24 bases of the coding region. *Eco*RI and *Nde*I (in primer I) or *Hin*dIII (in primer II) restriction sites were included to permit cloning of the amplified fragment. This fragment, being thymidylate synthase coding region, was ligated into the *Eco*RI/*Hin*dIII sites of the pBluescript SK$^-$ vector and the ligation mixture was used to transform *E. coli* XL-1 cells. One-thousand bp inserts in the isolated recombinant plasmids (pBluescript/TS) were sequenced using USB Sequenase Version 2 DNA sequencing kit. The coding region was restricted from pBluescript as an *Nde*I/*Hin*dIII fragment and ligated into the

pPIGDM4+stop vector, either straight away or after prior PCR with primers III (5′-GGGAATTCATATGCT**T**GT**T**GTTGG**T**TCCGG-3′) and II (in order to substitute T for G or C in the 2nd, 3rd and 5th codons). The pPIGDM4+stop expression vector is a derivative of the pPIGDM1 plasmid (Mikiewicz *et al.*, 1997), containing ampicillin resistance gene (inserted into the putative *rom* gene), T7 phage promoter, transcription terminator and rare arginine tRNA genes ($tRNA^{Arg(AGG/AGA}$). DH5αF′ cells were transformed, recombinant plasmid isolated and the construction was confirmed. A pPIGDM4+stop/TS plasmid pool was used to transform the thymidylate synthase-deficient *E. coli* strain TX61$^-$.

Induction of thymidylate synthase synthesis. Overnight cultures of untransformed TX61$^-$ cells or TX61$^-$ cells transformed with the TS/pPIGDM4+stop plasmid wild-type or mutated were used for inoculation of superbroth medium (Pedersen-Lane *et al.*, 1997) with 30 μg/ml kanamycin, 50 μg/ml ampicilin and 50 μg/ml thymidine. Cells were shaken at 270 r.p.m. at 37°C until the culture attained an A_{600} of 0.6. IPTG (1 mM) was then added and shaking was continued for another 4 h, at which time the cells were centrifuged at 10 000 × ***g*** for 15 min at 4°C. The cell pellets were frozen at –20°C until use.

Cell extracts. The cell pellets were suspended in lysis buffer containing 50 mM Tris/HCl, pH 7.5, 0.1% Triton X-100, 0.1 mM EDTA and 10 mM 2-mercaptoethanol and sonicated. The resulting homogenate was centrifuged for 20 min at 20 000 × ***g*** at 4°C and the pellet discarded. The supernatant, further referred to as crude extract, was used for thymidylate synthase activity, FdUMP binding and protein assays.

Recombinant mouse thymidylate synthase purification. A previously described method (Cieśla *et al.*, 1995) was modified as follows. Purification was done at 2–4°C and all buffers contained 20 mM 2-mercaptoethanol. Cell pellet, corresponding to 1 l of culture, was suspended in 100 ml of 50 mM sodium/potassium phosphate buffer, pH 7.5, containing 0.1 M KCl, immersed in an ice-ethanol bath and sonicated (Branson Sonifier 250) for ten pulses of 60 s each, separated with 15 s pauses. After removal of cell debris by centrifugation for 20 min at 20 000 × ***g***, 2% streptomycin sulfate was added to the supernatant, the suspension stirred for 20 min and the precipitated nucleic acid removed by 20 min centrifugation at 20 000 × ***g***. Solid ammonium sulfate was added to the stirred supernatant to 30% saturation and, after additional 20 min stirring, the resulting mixture centrifuged for 20 min at 20 000 × ***g***. The ammonium sulfate content of the supernatant was raised to 80% saturation and, after stirring for 20 min, the resulting precipitate was spun down. It was dissolved in 10 mM sodium/potassium phosphate buffer, pH 7.5, dialyzed overnight against two 2-liter changes of the same buffer and loaded onto a DE-52 column (2.5 × 7 cm) equilibrated with the above buffer. The column was washed with 150 ml of the same buffer, followed by 250 ml of 25 mM sodium/potassium phosphate buffer pH 7.5, and the enzyme eluted with 50 mM sodium/potassium phosphate buffer, pH 7.5. Fractions containing thymidylate synthase activity were pooled and precipitated with solid ammonium sulfate at 80% saturation. Following centrifugation the pellet was dissolved in 10 mM sodium/potassium phosphate buffer, pH 7.5, containing 1 M ammonium sulfate, and loaded onto a phenyl-Sepharose column (1.5 × 7 cm) equilibrated in the same buffer. The column was washed with 200 ml of the same buffer and the enzyme eluted with 10 mM sodium/potassium phosphate buffer, pH 7.5, containing 0.8 M ammonium sulfate. The most active and purest fractions (assessed electrophoretically) were pooled and concentrated in an Amicon Column Eluate Concentrator apparatus.

Thymidylate synthase. This was determined by monitoring the release of tritium from [5-^{3}H]dUMP (Rode *et al.*, 1984). A unit of activity was defined as the amount of enzyme

catalyzing formation of 1 μmole of dTMP (or release of 1 μequivalent of $^3H^+$) per minute at 37°C. Protein was determined as described by Spector (1978) with bovine serum albumin as a standard.

Kinetic studies. Quantitative analyses of thymidylate synthase interaction with dUMP were performed as reported earlier (Jastreboff *et al.*, 1983). Results are presented as means ± S.E.M., or means ± % difference between the mean and each of two results, followed by the number of experiments (n) in parentheses.

Electrophoretic analysis. In FdUMP binding experiments a crude extract of recombinant thymidylate synthase ('wild' or mutated) was incubated at 37°C for 30 min with 2.5 mM $N^{5,10}$-methylenetetrahydrofolate and 1 μM [^{3}H]FdUMP (18 Ci/mmol). Polyacrylamide (12%) gel electrophoresis in the presence of sodium dodecyl sulfate (Bollag *et al.*, 1991) was employed to test enzyme homogeneity and to determine molecular mass of its monomer. To identify thymidylate synthase on the gel, based on the enzyme's ability to form a ternary complex with 5-fluoro-dUMP and $N^{5,10}$-methylenetetrahydrofolate, a sample of the crude extract of the recombinant enzyme ('wild' or mutated) for electrophoresis was prepared as described elsewhere (Rode *et al.*, 1979).

Western blotting. Standard method was applied (Bollag *et al.*, 1991) with an anti-rat thymidylate synthase monoclonal antibody that recognized both the recombinant and L1210 mouse thymidylate synthases (Gołos *et al.*, 2002) and goat anti-mouse IgG (H+L)-HRP conjugate (BioRad) as a second antibody.

RESULTS

RT-PCR was performed on total RNA isolated from mouse leukemia L1210 cells, with the use of primers designed based on the known mouse thymidylate gene sequence (Deng *et al.,* 1986), and the resulting entire thymidylate synthase coding region was cloned into the pBluescript vector. A direct attempt to express the mouse enzyme in *E. coli* by subcloning of the *Nde*I/*Hin*dIII fragment, restricted from pBluescript/TS, into the pPIGDM4+stop expression vector was unsuccessful. In order to optimize the coding region sequence, following the approach of Pedersen-Lane *et al.* (1997), substitutions were made of G or C in the 2nd, 3rd and 5th codons with T (without changing the encoded amino-acid sequence). Unexpectedly, the procedure resulted in two products: a plasmid with the expected sequence and another one having an additional substitution of A for middle G in the AGG codon encoding Arg209 (amino-acid numbers starting from the first Met in the mouse enzyme), apparently introduced incidentally by Taq polymerase during the subcloning procedure. The latter substitution resulted in Lys209 present in the expressed protein. Both mouse thymidylate synthase forms ('wild' and R209K) were successfully overexpressed in the thymidylate synthase-deficient *E. coli* TX61$^-$ cells, constituting about 20% of total protein (Fig. 1A).

Crude extracts of bacteria expressing the R209K enzyme showed a specific activity of 0.016 ± 0.009 munit/mg protein (n = 4), almost 4000-fold lower than that found with bacteria expressing 'wild' mouse thymidylate synthase (61.8 ± 28.8 munit/mg protein; n = 3). The enzyme activity was undetectable in extracts of untransformed TX61$^-$ cells.

Anti-rat thymidylate synthase monoclonal antibodies, recognizing also mouse recombinant enzyme (Gołos *et al.*, 2002), used in immunoblot analysis following electrophoretic separation under denaturing conditions, allowed us to identify the overexpressed protein, present in extracts of bacteria harboring the mutated plasmid, as thymidylate synthase (Fig. 2, cf. Fig. 1A). Interestingly, with both the 'wild' and mutated enzymes the antibodies showed heterogeneity of the enzyme subunits.

While [^{3}H]FdUMP, binding in the presence of $N^{5,10}$-methylenetetrahydrofolate to 'wild'

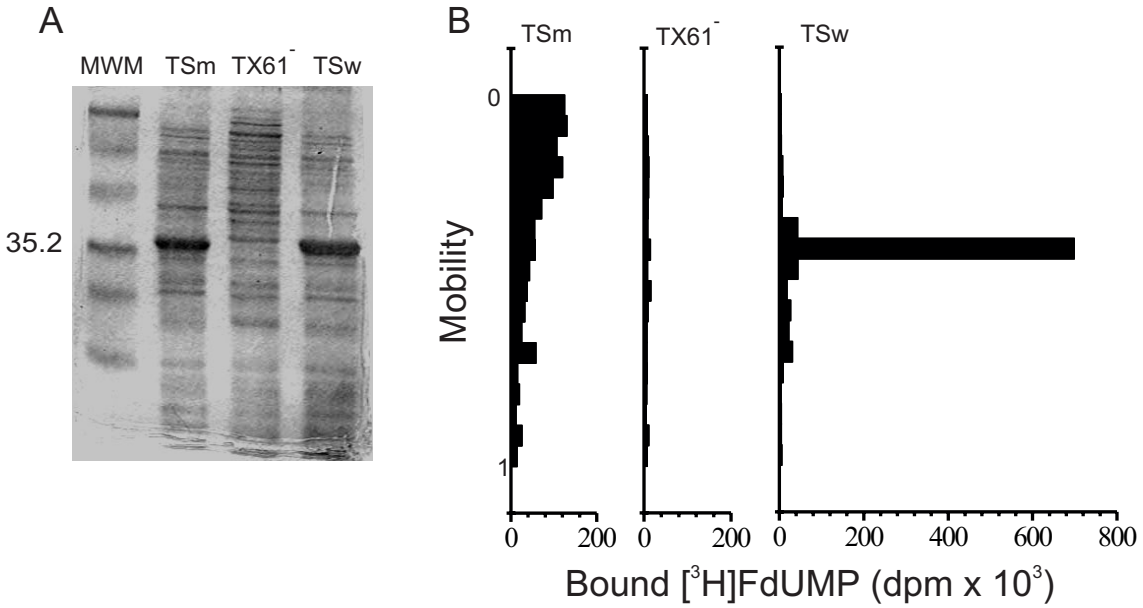

Figure 1. SDS/PAGE of crude extracts from IPTG-induced TX61⁻ cells untransformed (TX61⁻), overexpressing mutant R209K TS (TSm) or 'wild' TS (Tsw).

Samples were treated for 30 min at 37°C with [^{3}H]FdUMP and $N^{5,10}$-methylenetetrahydrofolate before electrophoresis (see Materials and Methods) and loaded onto gel in duplicates. Part of the gel, containing four lanes (MWM, TSm, TX61⁻, and TSw), was stained for protein (A) and the remaining part, containing three lanes (TSm, TX61⁻, and TSw), was sliced and assayed for bound label (B). MWM, molecular mass markers (in kDa).

thymidylate synthase, remained bound under the denaturing condition of SDS polyacrylamide electrophoresis, it failed to form so stable a complex with the R209K enzyme (Fig. 1B).

Table 1 compares the K_m and V_{max} values describing the reaction catalyzed by the crude extract R209K enzyme, and purified 'wild' recombinant, L1210 cell and mouse thymus thymidylate synthases. The affinity of the R209K enzyme for dUMP, reflected by the K_m value, was 571-fold lower than that of the 'wild' recombinant thymidylate synthase, the latter showing 2.6–3.1-fold higher affinity than the two enzyme forms purified from the natural sources. The R209K enzyme was assessed to constitute about 20% of total crude extract protein. Assuming the latter, the V_{max} value, corresponding to 1 mg of pure R209K thymidylate synthase, would amount to 0.024 unit/mg protein and would be over 50-fold lower than that estimated for 'wild' recombinant thymidylate synthase ($k_{cat,\ R209K}/k_{cat,\ 'wild'}$ = 0.019; the k_{cat}/K_m^{dUMP} ratio over 30 000 higher for the 'wild' then R209K enzyme). In accord, the resulting low catalytic potency of the mouse R209K thymidylate synthase was not sufficient to support growth

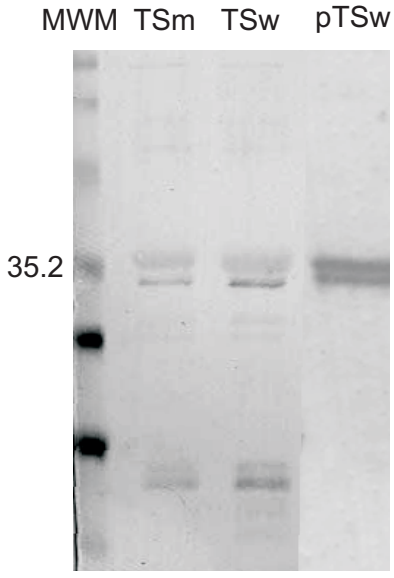

Figure 2. Western blots of crude extracts from TX61⁻ cells overexpressing mutated (TSm) or 'wild' (TSw) mouse thymidylate synthases and of purified 'wild' TS (pTSw).

Anti-rat TS monoclonal antibody was used. MWM, molecular mass markers (in kDa).

of *E. coli* TX61⁻ cells in the Luria-Bertani medium in the absence of exogenous thymidine (not shown).

DISCUSSION

Construction of a system capable of overexpressing mouse thymidylate synthase in *E. coli* required optimization of the coding sequence. In accord, the poor expression of the enzyme obtained with the original coding region of the mouse gene, was improved by 17-fold (up to 5–10% of total cell protein) when the second codon was changed from CUG to CUU (Zhang *et al.*, 1989). Also Pedersen-Lane *et al.* (1997) substituted G or C in the 2nd, 3rd and 5th codons with T (without changing the encoded amino-acid sequence) in human thymidylate synthase gene and obtained thymidylate synthase expression in *E. coli* to the extent of 25–30% of total protein. The latter approach, followed in the present studies, allowed successful expression of mouse thymidylate synthase in *E. coli*.

The equivalent of mouse R209 in *L. casei* thymidylate synthase is R218, suggested, in addition to direct contributions to dUMP phosphate binding, to coordinate the active site in the structure necessary to bind the nucleotide (Kawase *et al.*, 2000). In accord with the results concerning mutagenetic replacement of Arg218 in the *L. casei* enzyme (Kawase *et al.*, 2000), the corresponding Arg209 in the mouse enzyme appears to be of great importance for catalysis and nucleotide binding (Table 1, Fig. 1b). In fact, the consequence of the R209K mutation of the mouse enzyme, reflected by a decrease of the affinity for the substrate, appears to be even deeper than that described for the corresponding mutation (R218K) of the bacterial enzyme (Kawase *et al.*, 2000).

The heterogeneity of enzyme subunits indicated by immunoblot analysis of both the 'wild' and mutated enzymes present in crude extracts, as well as of the purified 'wild' enzyme (Fig. 2), points to a possibility that the subunits underwent different posttranslational modifications (cf. Gołos *et al.*, 2002). The same is suggested by the K_m and V_{max} values, reflecting higher affinity for dUMP and catalytic potency of highly purified 'wild' mouse recombinant enzyme, as compared with several highly purified thymidylate synthases originating from natural sources, including normal mouse thymus (Table 1). The sequence of the enzyme from the latter source may certainly be expected to be the same as the sequence of the recombinant thymidylate synthase. It should be mentioned that recombinant rat and human thymidylate synthases were also found to show catalytic potencies higher than those of the corresponding enzyme species (with each enzyme pair, either rat or human, characterized by the same

Table 1. Kinetic parameters of the reaction catalyzed by the mutant R209K (monomer m. mass 34 928.26 Da) and 'wild' (monomer m. mass 34 956.27 Da) mouse thymidylate synthases, and earlier described preparations of the enzyme isolated from mouse tumour cells and normal tissue

Thymidylate synthase	K_m^{app} for dUMP (μM)	V_{max} (unit/mg protein)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\ \mu M^{-1}$)
Mouse R209K[a]	559.5 ± 6.6%(2)	0.0047 ± 14.6% (2)	0.0055	0.0000097
Mouse 'wild' recombinant[b]	0.98 ± 0.14 (3)	1.26 ± 0.43 (3)	1.47	1.5
Mouse leukemia L1210[b]	2.6[c]; 3.15[d]		≥ 0.52[c, d]	
Mouse Ehrlich carcinoma[b]	2.0[e]		≥ 0.52[f]	
Mouse thymus[b]	4.2[g]		≥ 0.13[g]	

[a]Crude extract; [b]preparation apparently homogeneous; [c]Zieliński *et al.*, 1989; [d]Gołos *et al.*, 2001; [e]Jastreboff *et al.*, 1983; [f]Jastreboff *et al.*, 1982; [g]Rode *et al.*, 1986.

sequence) isolated from natural sources, and with the rat enzyme the difference was correlated with the presence of an N-acetylated methionine at the NH_2 end (Cieśla *et al.*, 1995).

REFERENCES

Anderson AC, Perry KM, Freymann DM, Stroud RM. (2000) The crystal structure of thymidylate synthase from *Pneumocystis carinii* reveals a fungal insert important for drug design. *J Mol Biol.*; **297:** 645–57.

Bollag DM, Edelstein SJ. (1991) *Protein methods.* Wiley-Liss, New York, Chichester, Brisbane, Toronto, Singapore.

Carreras CW, Santi DV. (1995) The catalytic mechanism and structure of thymidylate synthase. *Annu Rev Biochem.*; **64:** 721–62.

Cieśla J, Weiner KX, Weiner RS, Reston JT, Maley GF, Maley F. (1995) Isolation and expression of rat thymidylate synthase cDNA: phylogenetic comparison with human and mouse thymidylate synthases. *Biochim Biophys Acta.*; **1261:** 233–42.

Deng T, Li D, Jenh C-H, Johnson LF. (1986) Structure of the gene for mouse thymidylate synthase. Locations of introns and multiple transcriptional start sites. *J Biol Chem.*; **261:** 16000–5.

Ealick SE, Armstrong SR. (1993) Pharmacologically relevant proteins. *Curr Opin Struct Biol.*; **3**: 861–7.

Fauman EB, Rutenber EE, Maley GF, Maley F, Stroud RM. (1994) Water-mediated substrate/product discrimination: the product complex of thymidylate synthase at 1.83 Å. *Biochemistry.*; **33**: 1502–11.

Finer-Moore JS, Fauman EB, Foster PC, Perry KM, Santi DV, Stroud RM. (1993) Refined structures of substrate-bound and phosphate-bound thymidylate synthase from *Lactobacillus casei*. *J Mol Biol.*; **232:** 1101–16.

Gołos B, Dzik JM, Kazimierczuk Z, Cieśla J, Zieliński Z, Jankowska J, Kraszewski A, Stawiński J, Rode W, Shugar D. (2001) Interaction of thymidylate synthase with the 5′-thiophosphates, 5′-dithiophosphates, 5′-*H*-phosphonates and 5′-*S*-thiosulfates of 2′-deoxyuridine, thymidine and 5-fluoro-2′-deoxyuridine. *Biol Chem.*; **382:** 1439–45.

Gołos B, Wałajtys-Rode E, Porębska A, Cieśla J, Dąbrowska M, Zieliński Z, Rode W. (2002) Thymidylate synthase heterogeneity assessed by monoclonal antibodies. In *Chemistry and biology of pteridines and folates 2001*. Milstien S, Kapatos G, Levine RA, Shane B. eds, pp 519–23. Kluwer Academic Publishers, Boston, Dordrecht, London.

Hardy LW, Finer-Moore JS, Montfort WR, Jones MO, Santi DV, Stroud RM. (1987) Atomic structure of thymidylate synthase: target for rational drug design. *Science.*; **235**: 448–55.

Jastreboff M, Kędzierska B, Rode W. (1982) Properties of thymidylate synthetase from Ehrlich ascites carcinoma cells. Effect of Mg2+ and MgATP2-. *Biochem Pharmacol.*; **31:** 217–23.

Jastreboff MM, Kędzierska B, Rode W. (1983) Altered thymidylate synthetase in 5-fluorodeoxyuridine-resistant Ehrlich ascites carcinoma cells. *Biochem Pharmacol.*; **32:** 2259–67.

Kawase S, Cho S-W, Rozelle J, Stroud RM, Finer-Moore J, Santi DV. (2000) Replacement set mutagenesis of the four phosphate-binding arginine residues of thymidylate synthase. *Protein Engin.*; **13**: 557–63.

Mikiewicz D, Wróbel B, Węgrzyn G, Płucienniczak A. (1997) Isolation and characterization of a ColE1-like plasmid from *Enterobacter agglomerans* with a novel variant of rom gene. *Plasmid.*; **38:** 210–9.

Morse RJ, Kawase S, Santi DV, Finer-Moore J, Stroud RM. (2000) Energetic contributions of four arginines to phosphate-binding in thymidylate synthase are more than additive and depend on optimization of “effective charge balance”. *Biochemistry.*; **39**: 1011–20.

Pedersen-Lane J, Maley GF, Chu E, Maley F. (1997) High-level expression of human

thymidylate synthase. *Protein Expr Purif.*; **10**: 256–62.

Rode W, Leś A. (1996) Molecular mechanism of thymidylate synthase-catalyzed reaction and interaction of the enzyme with 2- and/or 4-substituted analogues of dUMP and 5-fluoro-dUMP. *Acta Biochim Polon.*; **43:** 133–42.

Rode W, Scanlon KJ, Hynes J, Bertino JR. (1979) Purification of mammalian tumor (L1210) thymidylate synthetase by affinity chromatography on stable biospecific adsorbent. Stabilization of the enzyme with neutral detergents. *J Biol Chem.*; **254:** 11538–43.

Rode W, Kulikowski T, Kędzierska B, Jastreboff M, Shugar D. (1984) Inhibition of mammalian thymidylate synthetase by 5-alkylated 2′-deoxyuridine 5′-phosphates. *Biochem Pharmacol.*; **33:** 2699–705.

Rode W, Cieśla J, Zieliński Z, Kędzierska B. (1986) Purification and properties of mouse thymus thymidylate synthase. Comparison of the enzyme from mammalian normal and tumour tissues. *Int J Biochem.*; **18:** 361–8.

Sambrook J, Fritsch EF, Maniatis T. (1989) *Molecular cloning. A laboratory manual.* 2nd edn. Cold Spring Harbor Laboratory Press.

Sotelo-Mundo RR, Cieśla J, Dzik JM, Rode W, Maley F, Maley GF, Hardy LW, Montfort WR. (1999) Crystal structures of rat thymidylate synthase inhibited by Tomudex, a potent anticancer drug. *Biochemistry.*; **38:** 1087–94.

Spector T. (1978) Refinement of the Coomasie Blue method of protein quantitation. *Anal Biochem.*; **86:** 142–6.

Stroud RM, Finer-Moore JS. (1993) Stereochemistry of a multistep/bipartite methyl transfer reaction: thymidylate synthase. *FASEB J.*; **7:** 671–7.

Zhang HC, Cisneros RJ, Dunlap RB, Johnson LF. (1989) Efficient synthesis of mouse thymidylate synthase in *Escherichia coli*. *Gene.*; **84**: 487–91.

Zieliński Z, Dzik JM, Rode W, Kulikowski T, Bretner M, Kierdaszuk B, Shugar D. (1990) Interaction of N^4-hydroxy-5-FdCMP with L1210 thymidylate synthase differing in sensitivity towards 5-FdUMP inhibition. In *Proc Ninth International Symposium on Pteridines and Folic Acids Derivatives Chemical, Biological and Clinical Aspects.* Zurich, Switzerland, September 3–8, 1989. Curtius H-Ch, Ghisla S, Blau N. eds, pp 817–20. Walter de Gruyter, Berlin, New York.