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Altered mouse leukemia L1210 thymidylate synthase, associated with cell resistance to 5-fluoro-dUrd, is not mutated but rather reflects posttranslational modification

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Thymidylate synthase purified from 5-fluoro-dUrd-resistant mouse leukemia L1210 cells (TSr) was less sensitive to slow-binding inhibition by 5-fluoro-dUMP than the enzyme from the parental cells (TSp), both enzyme forms differing also in sensitivity to several other dUMP analogues, apparent molecular weights of monomer and dimer, and temperature dependence of the catalyzed reaction. Direct sequencing of products obtained from RT-PCR, performed on total RNA isolated from the parental and 5-fluoro-dUrd-resistant cells, proved both nucleotide sequences to be identical to the mouse thymidylate synthase coding sequence published earlier (NCBI protein database access no. NP_067263). This suggests that the altered properties of TSr are caused by a factor different than protein mutation, presumably posttranslational modification. As a possibility of rat thymidylate synthase phosphorylation has been recently demonstrated (Samsonoff *et al.* (1997) *J Biol Chem* 272: 13281), the mouse enzyme amino-acid sequence was analysed, revealing several potential phosphorylation sites. In order to test possible influence of the protein phosphorylation state on enzymatic properties, endogenous TSp and TSr were purified in the presence of inhibitors of phosphatases. Although both enzyme forms were phosphorylated, as shown by electrophoretical separation followed by phosphoprotein detection, the extent of phosphorylation was apparently similar. However, the same two purified enzyme preparations, compared to the corresponding preparations purified in the absence of phosphatase inhibitors, showed certain properties, including sensitivity to the slow-binding inhibition by FdUMP, altered. Thus properties dependence on phosphorylation was indicated.

Keywords: thymidylate synthase, FdUrd resistance, L1210, posttranslational modification, protein phosphorylation

Thymidylate synthase (TS; EC 2.1.1.45) catalyzes the $C_{(5)}$ methylation of 2′-deoxyuridine-5′-monophosphate (dUMP), resulting in the synthesis of 2′-deoxythymidine-5′-monophosphate (dTMP). The donor of both the one-carbon group and reductive equivalents is $N^{5,10}$-methylenetetrahydrofolate (meTHF), transformed in the reaction to dihydrofolate (Carreras & Santi, 1995). As the reaction is the only source of *de novo* synthesis of thymidylate, the enzyme is a prominent target for fluoropyrimidines and antifolates in cancer chemotherapy (Jackman *et al.*, 1985; Douglas, 1987; Danenberg *et al.*, 1999; Napier & Ledermann, 2000; Lehman, 2002).

The anticancer activity of 5-fluoro-dUrd (FdUrd) is based on intracellular conversion of FdUrd to 5-fluoro-dUMP (FdUMP), a potent, slow-binding TS inhibitor. FdUMP forms a ternary complex with the enzyme and meTHF that results in a slowly reversible enzyme inactivation. Clinical effectiveness of antineoplastic drugs is hampered by development of various types of drug resistance. Resistance of tumors to FdUrd was found to be accompanied in most cases by impaired phosphorylation of FdUrd in cells (Kessel & Wodinsky, 1970; Mulkins & Heidelberger, 1982), enhanced phosphatase activity (Fernandes & Cranford, 1985), and increased TS

Abbreviations: FdUrd, 5-fluoro-dUrd; FdUMP, 5-fluoro-dUMP; meTHF, $N^{5,10}$-methylenetetrahydrofolate; PBS, phosphate-buffered saline; TS, thymidylate synthase; TSp, TS from L1210 parental cells; TSr, TS from 5-fluoro-dUrd-resistant cells.

mRNA and protein levels (Berger *et al.* 1985; Cieśla *et al.*, 1995; Aschele *et al.*, 2002; Libra *et al.*, 2004; Ma *et al.*, 2004), with the latter parameter dependent also on cellular TS stability (Kitchens *et al.*, 1999). Several reports appeared on another mechanism of resistance to FdUrd, an alteration of the target enzyme (Heidelberger *et al.*, 1960; Jastreboff *et al.*, 1983; Bapat *et al.*, 1983; Barbour *et al.*, 1992; Hughey *et al.*, 1993; Kawate *et al.*, 2002), four of those reports (Bapat *et al.*, 1983; Barbour *et al.*, 1992; Hughey *et al.*, 1993; Kawate *et al.*, 2002) documenting the role of the enzyme's alteration resulting from mutation(s).

The present studies were aimed at explanation of the mechanism of differing properties, including sensitivity to inactivation by FdUMP and its analogues, of TSs from parental and FdUrd-resistant mouse leukemia L1210 cells. Surprisingly, the results indicate that the alteration of the enzyme expressed by the resistant cells does not result from mutation, but rather is due to posttranslational modification(s), with phosphorylation presumably involved.

MATERIALS AND METHODS

Reagents. Multiplexed Proteomics® Phosphoprotein Gel Stain Kit and SYPRO Ruby Protein Gel Stain were from Molecular Probes. Methanol, glacial acetic acid and acetonitrile were from Roth and sodium acetate was from Sigma.

L1210 cells. Mouse leukemia L1210 cells were maintained, harvested, and stored as previously described (Rode *et al.*, 1984). An FdUrd-resistant L1210 cell line, developed as described earlier (Rode *et al.*, 1990), was maintained as for parental L1210 cells, except that the cell-bearing mice were treated with FdUrd (75 mg/kg injected ip as 0.5 ml of PBS solution) on the fourth day following transplantation.

Enzyme assay. The [5-^{3}H]dUMP tritium release assay was performed as previously described (Rode *et al.*, 1984), all measurements being done in triplicate. TS activity unit is defined as μmoles of released tritium per min at 37°C.

Thymidylate synthases. TSs from parental and FdUrd-resistant L1210 cells were purified by means of affinity chromatography on N^{10}-formyl-5,8-dideazafolate as described earlier (Rode *et al.*, 1979). Both enzymes were also purified by essentially the same method but with inhibitors of protein phosphatases included in buffers. All steps were carried out at 4°C. The cell pellets were thawed with 3 vol. of 50 mM phosphate buffer, pH 7.5, containing 0.1 M KCl, 10 mM 2-mercaptoethanol, 50 mM NaF, 5 mM Na-pyrophosphate, 0.2 mM EGTA, 0.2 mM EDTA and 2 mM Na_3VO_4, sonicated and centrifuged for 20 min at 20000 × ***g***. Nucleic acids were precipitated from the supernatant with 2% streptomycin sulfate and spun down as above. The supernatant was fractionated with ammonium sulfate as previously described (Rode *et al.*, 1979), the pellet precipitated with 70% ammonium sulfate was then dissolved in 10 mM phosphate buffer, pH 7.5, containing 0.1% Triton X-100, 20 μM dUMP, 10 mM 2-mercaptoethanol, 50 mM NaF, 5 mM Na-pyrophosphate, 0.2 mM EGTA, 0.2 mM EDTA and 2 mM Na_3VO_4 (buffer A), and passed through the affinity column equilibrated with the same buffer. The column was washed with 5 mM phosphate buffer, pH 7.5, containing 0.1% Triton X-100, 20 μM dUMP, 10 mM 2-mercaptoethanol, 10 mM NaF, 2 mM Na-pyrophosphate, 0.2 mM EGTA, 0.2 mM EDTA and 2 mM Na_3VO_4 (buffer W), and the enzyme eluted with buffer W lacking dUMP into a small DEAE-cellulose column, connected to the affinity column in series. TS was eluted from the DEAE-cellulose column with 0.2 M phosphate buffer, pH 7.5, containing 0.1% Triton X-100, 20% sucrose, 10 mM 2-mercaptoethanol, 10 mM NaF, 2 mM Na pyrophosphate, 0.2 mM EGTA, 0.2 mM EDTA and 2 mM Na_3VO_4. Pooled active fractions were diluted 20-fold with a solution of 0.1% Triton X-100, containing 20 μM dUMP, 10 mM 2-mercaptoethanol, 10 mM NaF, 2 mM Na-pyrophosphate, 0.2 mM EGTA, 0.2 mM EDTA and 2 mM Na_3VO_4, passed through the affinity column equilibrated with buffer A and the purification-concentration procedure was repeated as described above. Active TS fractions eluted from DEAE-cellulose were stored at –20°C.

Kinetic studies. Quantitative analyses of TS interaction with substrate dUMP and its analogues were performed as reported earlier (Dąbrowska *et al.*, 1996). To identify the type of inhibition involved, the effects of an inhibitor on the dependence of the reaction rate on either dUMP or meTHF concentration were examined and analyzed as previously described (Dąbrowska *et al.*, 1996). Quantitative analyses of TS inhibition by dUMP analogues, leading to time-dependent inactivation of the enzyme, were performed as described earlier (Rode *et al.*, 1990).

Electrophoretic analysis. TS preparations purified in the absence of phosphatase inhibitors were analyzed by SDS/polyacrylamide gel electrophoresis according to Weber and Osborn (1969). Samples were prepared as described previously (Rode *et al.*, 1979).

In order to test the presence of phosphate groups on TS molecule, enzyme preparations purified in the presence of phosphatase inhibitors were analyzed by SDS/polyacrylamide gel (12.5%) electrophoresis according to Laemmli (1970).

Analysis of TS phosphorylation. The assays for the TS phosphorylation and protein detection were performed using the Multiplexed Proteomics® Phosphoprotein Gel Stain Kit according to the manufacturer's protocol. Preparations of purified TSs from

parental and FdUrd-resistant L1210 cells were separated on SDS/polyacrylamide gel (Laemmli, 1970), that was subsequently treated with Pro-Q Diamond Phosphoprotein Gel Stain. Phosphorylated species were visualized on a 300 nm UV transilluminator. The gel was subsequently stained with SYPRO Ruby Protein Gel Stain and the protein was again visualized on a 300 nm UV transilluminator. A mixture of the PeppermintStick™ phosphoprotein molecular mass standards, containing phosphorylated (ovalbumin, 45 kDa; β-casein 23.6 kDa) and non-phosphorylated (β-galactosidase 116.25 kDa; bovine serum albumin, 66.2 kDa; avidin 18 kDa) proteins, provided controls (both positive and negative).

Gel filtration. The molecular mass of TS dimer was assessed by Sephadex G-100 filtration. Before the run samples of TSp or TSr were incubated for 15 min at 37°C with 1 μM [6-^{3}H]FdUMP and 1 mM meTHF in 0.2 M Tris/HCl, pH 7.5, containing 0.01 M 2-mercaptoethanol, in a total volume of 1 ml. The column was developed at 4°C under conditions preventing dissociation of the ternary complex TS-[6-^{3}H]FdUMP–meTHF, using 0.05 M Tris/HCl, pH 7.5, buffer containing 0.01 M 2-mercaptoethanol, 0.1 M KCl and 0.1 mM meTHF, and collected fractions were monitored for radioactivity.

RT-PCR and sequencing of TS cDNA. Total RNA was isolated from L1210 cells parental and resistant to FdUrd with TRIZOL reagent (Gibco) according to the manufacturer's protocol. RT-PCR was performed with standard methods (Sambrook *et al*., 1989), using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) and oligo(dT) primer. In *Taq* polymerase-catalyzed reaction the following primers were used: forward primer I (5′-GG**GAATTCATATG**CTGGTGGTTGGCTCCGAG-3′) having sequence identical to the first 21 nucleotides of the coding region plus *Eco*RI and *Nde*I restriction sites (bold), and the reverse primer II (5′-AAA**AAGCTT**TTAAACAGCCATTTCCATTTTAAT-3′) complementary to the last 24 nucleotides of the coding region plus *Hin*dIII restriction site (bold). The resulting TS cDNAs from L1210 parental and FdUrd-resistant cells were purified from agarose gel and sequenced directly (both strands) with Reader DNA sequencing kit (Fermentas) reagents, but according to our own protocol. Primers were labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The matrix (TS cDNA, 0.1 pmol), labelled primer (1 pmol), 10 × polymerase buffer (6 μl), *Taq* polymerase (5 U) and water were mixed in a final volume of 15 μl and 3.5 μl aliquots were added to each of four tubes (A,C,T,G) containing 2 μl of water and 1.5 μl of respective d/ddNTP. The samples were subjected to cycle sequencing with 30 cycles of denaturing (94°C, 1 min), annealing (primer t_m-dependent temperature, 0.5 min) and extension (72°C, 0.5 min), followed by addition of 4 μl of Stop Solution. The samples were separated on polyacrylamide gel and autoradiographed according to standard methods (Sambrook *et al*., 1989).

The *Eco*RI/*Hin*dIII sites were used to clone the TS coding region into pBluescript and the *Nde*I/*Hin*dIII restriction sites were used for subcloning of the TS coding region into an expression vector (Cieśla *et al*., 2002). Restriction, ligation, transformation of *Escherichia coli* XL-1 cells with recombinant plasmids and plasmid purification were performed using standard methods (Sambrook *et al*., 1989). Recombinant plasmids with 1000 bp inserts (pBluescript/TS) were sequenced with the Sanger's method using Sequenase Version 2.0 DNA Sequencing Kit (Amersham).

TS mRNA level assay. mRNA was isolated from total RNA from L1210 parental and FdUrd-resistant cells with the use of PolyATtract mRNA Isolation System (Promega) according to the manufacturer's protocol. Various amounts of both mRNA preparations were separated on agarose/formaldehyde gel, transferred to nylon membrane, hybridized with ^{33}P-labelled TS probe (entire coding region) and subjected to autoradiography. Then the TS probe was stripped, the membrane rehybridized with ^{33}P-labelled β-actin probe (entire coding region) and autoradiography performed again. The densities of TSp and TSr mRNA bands in relation to those of actin mRNA were compared with the use of the computer program Scion Image (Scion Corporation, Frederick, MD, USA).

Mass spectrometry analysis. Prior to analysis, bands containing TS were cut out from a 12.5% SDS/polyacrylamide gel and subjected to standard "in-gel digestion" procedure during which proteins were reduced, alkylated and digested with trypsin. The resulting peptides were eluted from the gel with 0.1% TFA. Peptide mixture was applied to RP-18 precolumn (LC Packings), using water containing 0.1% TFA as a mobile phase, and then transferred to a nano-HPLC RP-18 column (LC Packings, 75 μm i.d.) and developed with an acetonitrile gradient (0–50% AcN in 30 min) in the presence of 0.05% formic acid at a flow rate of 150 nl/min. Column outlet was directly coupled to a Finningan Nanospray ion source of an LTQ-FT (Thermo) mass spectrometer working in the regime of data dependent MS to MS/MS switch. A blank run, ensuring lack of cross contamination from previous samples, preceded each analysis. The output list of precursor and product ions was compared to the NCBI database with a MASCOT local server (www.matrixscience.com). Spectra of peptides were analyzed with the use of the MassLynx v. 3.5 program.

Statistically evaluated results. These are presented as means ± S.E.M. or means ± percentage difference between the mean and either two results, followed by the number of experiments (N) in parentheses.

RESULTS AND DISCUSSION

The FdUrd-resistant L1210 cells were developed from L1210 cells grown intraperitoneally in mice (Rode *et al*., 1984). Compared to the parental cells, the resistant ones were found to express TS less sensitive to the slow-binding inhibition by FdUMP. This concerned both the enzyme activity studied in crude cell extracts (not shown) and highly purified TS preparations (Table 1). The purified preparations of the enzyme from the parental (TSp) and resistant (TSr) L1210 cells showed also different sensitivities to time-dependent (slow-binding) inhibition by other dUMP analogues (Table 1; discussed in Rode *et al*., 1990; Dzik *et al*., 1993) and classical competitive inhibition (reflected by the intersection at the ordinate of the Lineweaver-Burk plot; not shown) by the same analogues, as well as by the reaction product dTMP (Table 2). Interestingly, while the basic kinetic properties (specific activity, molecular activity and the K_m values describing interaction with the substrates) of both preparations appeared the same (Table 3; cf. Jastreboff *et al*., 1983; Bapat *et al*., 1983), the enzyme monomers separated with the use of SDS/polyacrylamide electrophoresis in a continuous buffer system (Weber & Osborn, 1969), as well as dimers analyzed by Sephadex gel filtration, showed small but reproducible differences of apparent molecular mass. Notably, while the molecular mass of the monomer was apparently lower for the resistant than parental cell enzyme, that of the dimer was apparently lower for

Table 1. Parameters for inactivation by FdUMP, N^4-OH-dCMP, N^4-OH-5-FdUMP, 2-thio-FdUMP and 4-thio-FdUMP of thymidylate synthases from L1210 cells parental (TSp) and FdUrd-resistant (TSr) cells.

The plots of log(remaining activity) *vs* time were usually biphasic (cf. Rode *et al*., 1990), suggesting different interactions of each inhibitor with the two binding sites on a TS molecule. Consequently, inhibition constants and inactivation rate constants were calculated with the use of apparent inactivation rate constants during the initial (0.0–1.5 min) and later (4–10 min) periods of preincubation with a given inhibitor at various concentrations. The corresponding inhibition constants and inactivation rate constants were then K_i' and k_2' and K_i'' and k_2'', respectively.

Enzyme	K_i' (nM)	K_i'' (nM)	k_2' (min^{-1})	k_2'' (min^{-1})
		FdUMP		
TSp[a]	1.8 ± 0.4 (6)	20 ± 5 (4)	0.17 ± 0.02 (6)	0.12 ± 0.04 (5)
TSr[a]	12.2 ± 1.4 (6)	14 ± 3 (4)	0.25 ± 0.04 (6)	0.06 ± 0.02 (4)
TSp*	15.3 ± 3.8 (3)	6.5 ± 2.2 (3)	0.22 ± 0.06 (3)	0.10 ± 0.04 (3)
TSr*	47.5 ± 10.7 (3)[c]		0.59 ± 0.27 (3)	
		N^4-OH-dCMP		
TSp[a]	63 ± 9 (4)	226 ± 35 (5)	0.17 ± 0.05 (4)	0.02 ± 0.00 (5)
TSr[a]	184 ± 61 (6)	1460 ± 330 (5)	0.20 ± 0.04 (6)	0.09 ± 0.02 (5)
		N^4-OH-5-FdUMP		
TSp[a]	73 ± 13 (4)	71 ± 6 (7)	0.24 ± 0.04 (4)	0.07 ± 0.01 (7)
TSr[a]	93 ± 18 (4)	56 ± 9 (7)	0.24 ± 0.03 (4)	0.06 ± 0.00 (7)
		2-Thio-FdUMP		
TSp[b]	41 ± 9 (3)	46 ± 25 (3)	0.12 ± 0.02 (3)	0.02 ± 0.01 (3)
TSr[b]	297 ± 93 (3)	93 ± 31 (3)	0.40 ± 0.04 (3)	0.04 ± 0.01 (3)
		4-Thio-FdUMP		
TSp[b]	102 ± 36 (3)	202 ± 36 (3)	0.21 ± 0.04 (3)	0.05 ± 0.00 (3)
TSr[b]	14 ± 4 (5)	34 ± 9 (5)	0.11 ± 0.03 (5)	0.03 ± 0.01 (5)

[a]Rode *et al*. (1990); [b]Dzik *et al*. (1993); [c]The inactivation rate did not change during preincubation of the enzyme with inhibitor; TSp* and TSr*, enzyme purified in the presence of protein phosphatase inhibitors. Results are presented as means ± S.E.M., followed by the number of separate experiments in parentheses.

Table 2. Apparent K_i values for inhibition of thymidylate synthase from parental (TSp) and FdUrd-resistant (TSr) L1210 cells by FdUMP, N^4-OH-dCMP, N^4-OH-5-FdUMP, 2-thio-FdUMP, 4-thio-FdUMP and dTMP[a].

Inhibitor	TSp		TSr	
	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m
FdUMP	0.02	0.01	0.03	0.02
N^4-OH-dCMP	1.70	0.68	9.30	5.47
N^4-OH-5-FdUMP	0.80	0.32	1.40	0.82
2-Thio-FdUMP	0.07	0.03	0.14	0.08
4-Thio-FdUMP	0.57	0.23	0.44	0.26
dTMP	11.90	4.76	51.50	30.29

[a]Each analogue was added to the reaction mixture simultaneously with the substrate and cofactor.

the parental than resistant cell enzyme (Table 3 and Fig. 1), suggesting that the observed differences may reflect different capacity of the monomers to bind SDS or different conformation of dimers, rather than distinctly different molecular mass. The two enzyme preparations differed also in the dependences of TS activity on temperature. Although both Arrhenius plots, reflecting those dependences in the 24–42°C temperature range, were biphasic (Fig 2; cf. Rode *et al.*, 1986), the resistant cell enzyme showed a lower activation energy, compared to the parental one, in the temperature range 37–42°C (6.36 ± 0.14 Kcal/mol and 8.25 ± 0.23 Kcal/mol for the resistant and parental cell enzyme, respectively; N=3), but not at 24–37°C (12.01 ± 0.27 Kcal/mol and 12.46 ± 0.26 Kcal/mol with the resistant and parental cell enzyme, respectively; N=3). On the other hand, the two enzyme preparations did not appear to differ in the mechanism of substrate binding and product release, with the sequential reaction mechanism suggested by the kinetics of interaction with the substrates reflected by 1/v *vs* 1/[dUMP] or 1/[meTHF] plots intersecting to the left of the ordinate (not shown) and ordered substrate addition, dUMP prior to meTHF, indicated by the successful application of affinity chromatography (cf. Rode *et al.*, 1979).

It should be mentioned that TS mRNA levels, estimated by Northern blot analysis (Fig. 3), as well as TS protein levels, judged based on the enzyme specific activity determined in cell extracts (0.30 ± 0.04 unit/g and 0.42 ± 0.09 unit/g with the parental and resitant cells, respectively; N=11) and molecular activity of purified preparations (Table 3), were similar for both parental and resistant cells.

In view of the foregoing, the altered TS present in FdUrd-resistant L1210 cells was assumed to be mutated (cf. Barbour *et al.*, 1992). To verify this hypothesis, RT-PCR was performed on total RNA isolated from parental and FdUrd-resistant cells (see Materials and Methods for details). Surprisingly, the sequences of the PCR products (TS cDNAs), determined directly (without cloning), perfectly matched the mouse TS coding sequence (Deng *et al.*, 1986; NCBI protein database access number NP_067263), pointing to identical amino-acid sequences of TSp and TSr. Interestingly, when cDNA sequencing was done after cloning into pBluescript vector of cDNA originating from either parental or FdUrd-resistant

Table 3. Properties of highly purified thymidylate synthase preparations from parental (TSp) and FdUrd-resistant (TSr) L1210 cells

Enzyme	Specific activity (unit/mg)	Apparent monomer mol. wt (kDa)	Apparent dimer mol. wt (kDa)	Molecular activity[a] (min^{-1})	K_m (μM) dUMP	K_m (μM) meTHF
TSp	0.26	34.0 ± 0.0 (3)	74.2 ± 0.0% (2)	18	2.5 ± 0.3 (3)	21.2 ± 1.5 (3)
TSr	0.35	31.0 ± 0.0 (3)	84.2 ± 6.5 (3)	22	1.7 ± 0.3 (3)	27.6 ± 2.1 (3)
TSp*	4.4 ± 31% (2)	35.2±0.5(3)[b] 33.4±0.2(3)[c]	ND	310	2.9 (1)	ND
TSr*	8.9 ± 69% (2)	35.3±0.4(3)[b] 33.3±0.2(3)[c]	ND	628	2.5 (1)	ND

[a]Based on specific activity and dimer molecular mass; [b]Slower band; [c]Faster band; TSp* and TSr*, thymidylate synthases purified from parental and FdUrd-resistant L1210 cells in the presence of inhibitors of protein phosphatases.

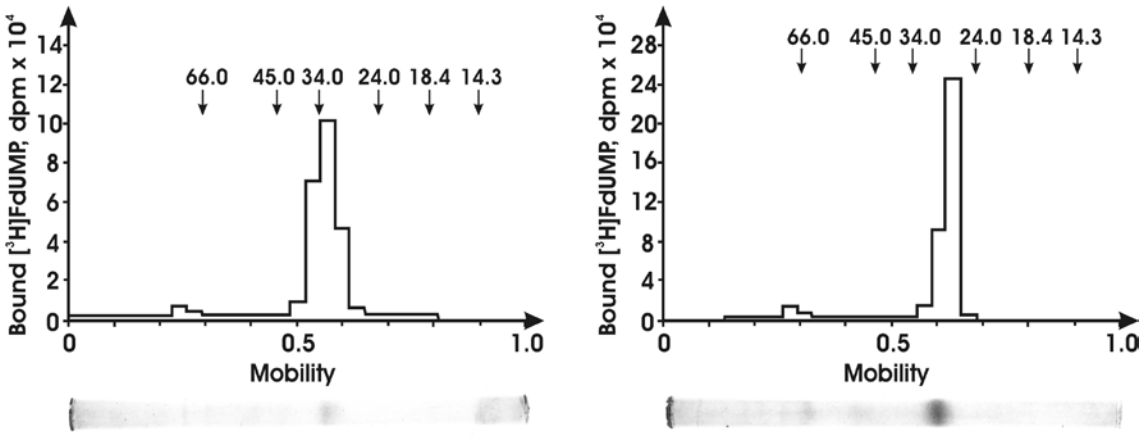

Figure 1. SDS/polyacrylamide gel electrophoresis in the continuous buffer system according to Weber and Osborn (1969).
TSp (left) and TSr (right) were pretreated with [6-^{3}H]FdUMP and meTHF prior electrophoresis. Arrows indicate molecular mass standards in kDa.

cells, several clones with single, double or triple mutations were always found, beside those encoding normal mouse TS sequence (not shown), suggesting certain genetic variation within both parental and FdUrd-resistant L1210 cell populations. However, results of the sequencing of the PCR products, indicating non-mutated mouse TS, point to low frequencies of those mutations.

In view of the foregoing, the only apparent explanation of the differing properties of the parental and FdUrd-resistant L1210 cell TSs should assume differing posttranslational modification(s). However, very little is known about the relation between the enzyme molecule modification and its properties. Although a possibility has been demonstrated of rat TS phosphorylation (Samsonoff *et al.*, 1997), no data show the influence of this modification on the enzyme properties. The only other information in this respect, published by the same laboratory, concerns modification, by N-acetylation, of the NH_2 terminal methionine in the rat endogenous enzyme. The modification was speculated to be a possible cause of a lower, by several-fold, specific activity of electrophoretically homogeneous enzyme preparation, relative to a similarly pure recombinant enzyme, of corresponding amino-acid sequence, with a free NH_2 terminal methionine (Cieśla *et al.*, 1995).

Although nothing else is known about the possible influence of post-translation modification on TS properties, several reports should be mentioned on considerable differences between certain properties of different enzyme forms of the same specific origin (Rode & Leś, 1996; Table 1), sometimes also documented to have the same amino-acid sequence. In this context, our present and previous (Rode *et al.*, 1986; 1987; Cieśla *et al.*, 2002; Dąbrowska *et al.*, 1996; 2004) studies (performed with the use of the same methods) showed differing catalytic efficiencies, and interactions with dUMP and FdUMP, of several mouse TS preparations (the enzyme forms from the L1210 parental and 5-FdUrd-resistant cells and the wild-type recombinant enzyme do not differ in amino-acid sequence; most probably the same is true for the enzyme from mouse thymus and Ehrlich ascites cells) and of two *Trichinella spiralis* TS preparations (the enzyme from muscle larvae also does not differ in amino-acid sequence from the recombinant form). It should also be mentioned that each of the following pairs of enzyme forms, mouse L1210 and the corresponding recombinant wild-type mouse enzyme, and muscle larva *T. spiralis* and the corresponding recombinant TS, showed differing interactions with monoclonal antibodies generated against recombinant rat TS. While some of those

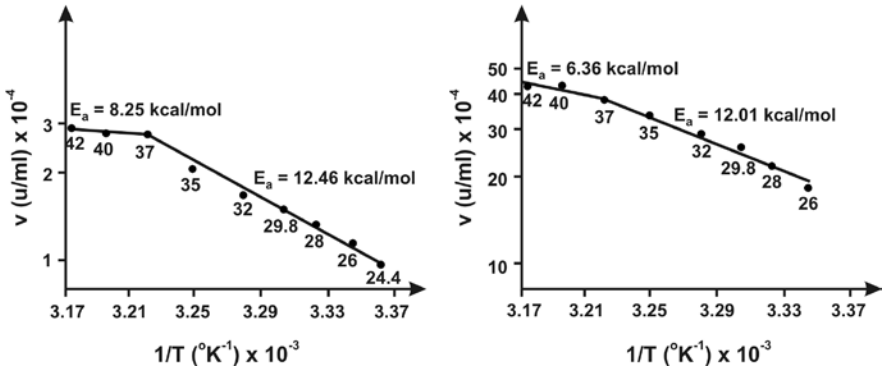

Figure 2. The dependence on temperature of the velocity of the reaction catalyzed by thymidylate synthase from parental (left) and FdUrd resistant (right) L1210 cells, presented as Arrhenius plots.

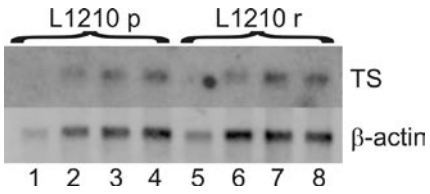

Figure 3. Thymidylate synthase mRNA level in parental (L1210p, lanes 1–4) and FdUrd-resistant (L1210r; lanes 5–8) L1210 cells related to β-actin mRNA.
mRNA samples of 0.38 μg (lanes 1 and 5), 0.76 μg (lanes 2 and 6), 1.14 μg (lanes 3 and 7) or 1.52 μg (lanes 4 and 8), were separated on agarose/formaldehyde gel and subjected to Northern assay. The membrane was first probed with ^{33}P-labelled TS probe and then with ^{33}P-labelled β-actin probe. Four samples containing different amounts of RNA were used for each kind of cells, in order to be able to control the range of linear dependence of the signal on RNA content.

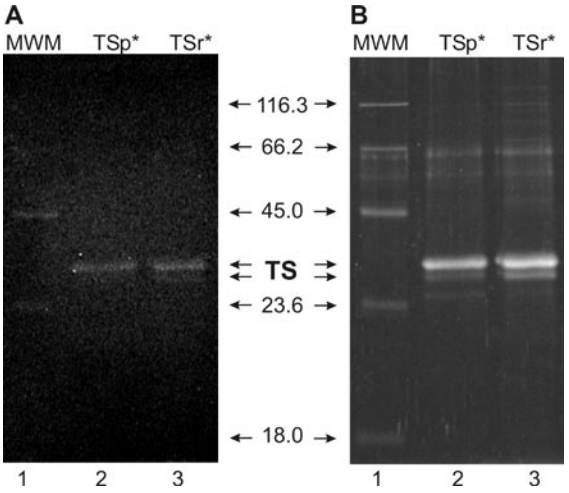

Figure 4. SDS/polyacrylamide gel (12.5%) electrophoresis (Laemmli, 1970) of thymidylate synthase from parental (TSp*; A, lane 2 and B, lane 2) and FdUrd-resistant (TSr*; A, lane 3 and B, lane 3) cells purified in the presence of inhibitors of protein phosphatases.
The gel was stained with Pro-Q® Diamond Phosphoprotein Gel Stain to visualize phosphorylated species (A) and subsequently with SYPRO® Ruby Gel Stain to detect protein (B). The PeppermintStick™ phosphoprotein molecular mass markers (MWM; A, lane 1 and B, lane 1) provide a mixture of phosphorylated and non-phosphorylated proteins (see Materials and Methods for details).

antibodies reacted with the enzyme from *T. spiralis*, but not with the corresponding recombinant form, the same antibodies reacted with the recombinant wild-type mouse, but not with the L1210, enzyme (Gołos *et al.*, 2002).

In order to test the capacity of mouse TS to undergo phosphorylation, its amino-acid sequence was analysed with three bioinformatics tools (NetPhos 2.0 Server [http://www.cbs.dtu.dk/services/NetPhos/], PPsearch [http://www.ebi.ac.uk/ppsearch/] and ScanProsite [http://www.expasy.org/tools/scanprosite/]) to reveal nine potential phosphorylation sites: S60, S96, S118, T69, T70, T161, T245, T281 and T300. NetPhos 2.0 Server and ScanProsite (but not PPSearch) pointed also to Y147 as a potential phosphorylation site. Although the structure of the mouse TS protein is not known, the homology between mouse, rat and human TSs is ≥90% (Perrymann *et al.*, 1986; Cieśla *et al.*, 1995). Therefore we used the RasMol program for visualization of the known three-dimensional structures of rat and human TS proteins to find out which of those potential phosphorylation sites are present on the protein surface. The results showed that five amino-acid residues corresponding to S60, S96, S118, T161, T300 of mouse TS are exposed, being potentially available for protein kinases.

We also tested directly the phosphorylation state of the two types of endogeneous TSs. In order to prevent potential dephosphorylation, the enzyme was purified from both parental and FdUrd-resistant L1210 cells in the presence of inhibitors of phosphatases (see Materials and Methods), with the resulting enzyme preparations referred to as TSp* and TSr*, respectively. When subjected to SDS/polyacrylamide gel electrophoresis in a discontinuous buffer system (see Materials and Methods), each of the two preparations showed two closely located bands of different protein content in the region of the expected TS molecular mass (Fig. 4B). The two bands resulting from separation of each of the two TS preparations were cut out from the gel individually and analyzed by mass spectrometry, the results identifying both the upper and the lower band of TSp* and TSr* as thymidylate synthase. Notably, the analysis of the spectra indicated also that the enzyme present in each of the four bands analyzed has its amino-terminal methionine N-acetylated (cf. Cieśla *et al.*, 1995).

Analysis of TSp* and TSr*, following their separation by polyacrylamide gel electrophoresis, with the use of a phosphoprotein detection system revealed each TS band to be phosphorylated (Fig. 4A), apparently to a similar extent, as indicated by the phosphoprotein to protein signal ratio (not shown).

While the presence of phosphatase inhibitors during enzyme purification did not influence the K_m values for dUMP with either enzyme (Table 3), the slow-binding inhibition of TSp* and TSr* by FdUMP, reflected by time-dependent enzyme inactivation, was weaker than that of the respective enzymes purified in the absence of the inhibitors (Table 1). Interestingly, compared to TSp*, TSr* was still some three-fold less sensitive to FdUMP inactivation. Moreover, unlike with TSp*, TSr* inactivation rate did not change during preincubation with FdUMP, pointing to similar interactions of both its active sites with the inhibitor (cf. Rode *et al.*, 1986; 1987; 1990). Of note is that both TSp* and TSr* showed much

higher molecular activities compared to TSp and TSr (Table 3), the difference being clearly too large to be accounted for by the higher degree of purification of TSp* and TSr* than TSp and TSr preparations. The above quantitative and qualitative differences between the enzyme preparations purified in the presence and in the absence of protein phosphatases inhibitors suggest that phosphorylation may influence TS inactivation by FdUMP, although for a precise description of this influence, and its dependence on the site(s) and/or extent of modification of the enzyme molecule, further studies are needed.

Thus, although the present results do not make it possible to identify precisely the cause of the different properties of TSs from mouse leukemia L1210 parental and FdUrd-resistant cells, they suggest it to be connected with a posttranslational modification, pointing to phosphorylation as presumably involved.

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