Properties of Phosphorylated Thymidylate Synthase

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Abbreviations:AcN, acetonitrile; FdUrd, 5-fluoro-dUrd; FdUMP, 5-fluoro-dUMP; TFA, trifluoroaceticacid; TS, thymidylate synthase; meTHF, N5,10-methylenetetrahydrofolate; KPA, potassium phosphoramidate; MOAC, metal oxide/hydroxide affinity chromatography; m-pTS, hpTS, r-pTS, Tsp-pTS and Ce-pTS, phosphorylated, MOAC-separated from the corresponding nonphosphorylated TS fractions of mouse, human, rat, T. spiralis and C. elegans enzyme recombinant proteins, respectively; m-TS, h-TS, r-TS, Tsp-TS and Ce-TS, non-phosphorylated, MOAC separated from the corresponding phosphorylated TS fractions of mouse, human, rat, T. spiralis and C. elegans enzyme recombinant proteins, respectively.

Abstract

Thymidylate synthase(TS) may undergo phosphorylation endogenously in mammalian cells, and as a recombinant protein expressed in bacterial cells, as indicated by the reaction of purified enzyme protein with Pro-Q®Diamond Phosphoprotein Gel Stain(PGS). With recombinant human, mouse, rat, Trichinella spiralis and Caenorhabditis elegans TSs, expressed in E. coli, the phosphorylated, compared to non-phosphorylated recombinant enzyme forms, showed a decrease in Vmaxapp, bound their cognate mRNA(only rat enzyme studied),and repressed translation of their own and several heterologous mRNAs(human, rat and mouse enzymes studied). However, attempts to determine the modification site(s), whether endogenously expressed in mammalian cells, or recombinant proteins, did not lead to unequivocal results. Comparative ESI-MS/analysis of IEF fractions of TS preparations from parental and FdUrd-resistant mouse leukemiaL1210 cells, differing in sensitivity to inactivation byFdUMP, demonstrated phosphorylation of Ser and Ser in the resistant enzyme only, although PGS staining pointed to modification of both L1210 TS proteins. The TS proteins phosphorylated in bacterial cells were shown by31P NMR to be modified only on histidine residues, like potassium phosphoramidate (KPA)-phosphorylated TS proteins. Nano LC-MS/MS, enabling the use of CID and ETD peptide fragmentation methods, identified several phosphohistidine residues, but certain phosphoserine and phosphorthreonine residues were also implicated. Molecular dynamics studies, based on the mouse TS crystal structure, allowed oneto assess potential of several phosphorylated histidine residues to affect catalytic activity, the effect being phosphorylation site dependent.

Keywords

Mass spectrometry(MS); Molecular dynamics; Nuclear magnetic resonance (NMR); Post-translational modification (PTM); Protein phosphorylation, Thymidylate synthase

1.Introduction

Thymidylate synthase(TS\*; EC 2.1.1.45), akeytarget in chemotherapyofa number of diseases, includingcancer[1], catalyzes the N5,10-methylenetetrahydrofolate (meTHF)-dependent C(5)-methylation of dUMP[2], required for DNA synthesis. Theenzyme protein participates in a nuclear multienzyme complex, associated with the DNA replication machinery, that includes serinehydroxymethyltransferase and dihydrofolate reductase[3]. Particularly interesting are potential non-catalytic activities, e.g.thymidylate synthase(TS)has been proposed to possess an oncogenelike activity[4], and human and bacterial (E. coli) thymidylate synthases (TSs)have been demonstrated to bind their cognate mRNAs within their codingregion.In both cases the enzyme was capable of repressing its own mRNAin vitrotranslation, with both functions consideredto be interrelated[5]. Moreover the human enzyme has been shown both to bind and repress translation of p53 and c-mycmRNA templates, suggestingthat it isa potential translational regulator of cellular gene expression[5].

In view of such arich repertoire of potential activities, of obvious interest is a report on possible endogenous phosphorylation of mammalian TS, demonstrated bydetection oflabelled phosphoserine inthe enzyme isolated from rat hepatoma cells incubated with 32Pi[6].That TSmodification could be physiologically meaningfulasphosphorylation, rather than mutation, was suggested to be a potential cause of differing properties, including sensitivity to inactivation by5fluoro-dUMP (FdUMP)and someanalogues, of TSs from parental and 5-fluoro-dUrd (FdUrd)resistant mouse leukemiaL1210 cells[7].

To learn moreabout molecular aspects of TSphosphorylation, including its sites of localization, and to determineto what extentenzyme properties maybe affected byendogenous orchemicalphosphorylation, studies were undertaken ofthe endogenous enzymefrom parental and FdUrd-resistant mouse leukemiaL1210 cells,calf thymusand C.elegans, and recombinant mouse,rat, human,T. spiralisand C. elegansenzymesexpressed in bacterial cells.Although numerous sites of phosphorylation are found on proteins from human and mouse cells, their functional effects have been less investigated [8].

2. Materialand Methods

2.1 Materials

Pro-Q®Diamond Phosphoprotein Gel Stain and SYPRO®Ruby Protein Gel Stain werefrom Molecular Probes. Inhibitase was from Eppendorf (Hamburg, Germany), restriction endonucleases from Invitrogen, and Rabbit Reticulocyte Lysate System from Promega (Madison, WI).Potassiumphosphoramidate (KPA)was synthesizedaccording to Pirrung et al. [9].

2.2 TS preparation

The endogenous enzyme proteins from parental and FdUrd-resistant mouse leukemiaL1210 cells[7]and calf thymus[10]were purifiedas previously described, but with the use of phosphataseinhibitors throughout[7]. A similar affinitychromatography approach was applied to purifyC. elegans[cf. ref.11]endogenous TS, but using a modified affinity resin, containing Raltitrexed as an affinant. The latter resin, allowing stronger enzyme binding in the presence of phosphataseinhibitors, was synthesized as earlier described[12], using 5 mg of Raltitrexed (Sigma-Aldrich) per ml of CNBr-activated Sepharose. C. elegans[11]and mouse[13]coding regions were cloned into pPIGDM4+stop vector and expressed as HisTag-free proteins in BL21(DE3) or a TS-deficient TX61-(a kind gift from Dr. W. S. Dallas) E. colistrain, respectively. Human[14]and rat[15]TScoding regions were subcloned into pET28a vector and expressed as HisTag-proteins in an E. coliBL21(DE3) strain. T. spiralisTS coding region[16]was subcloned into pQE2 vector and expressed as a HisTag-protein in aJM109 E. colistrain. HisTag-proteins were purified on NiNTA His-Bind resin (Novagen) according to t e manufacturer’s rotocol, and HisTag-freeproteins werepurified as previously described[15]. Phosphatase inhibitors (50 mM NaF, 5 mM Na-pyrophosphate, 0.2 mM EGTA, 0.2 mM EDTA and 2 mM Na3VO4; following the ammonium sulfate precipitation step, NaFand Na-pyrophosphate concentrations were decreased to 10 mM and 2 mM, respectively) werepresent in all purification buffers. TS activity was monitored, and kinetic parameters of the enzymecatalyzed reaction determined, as previouslydescribed[17].

2.3 TS phosphorylationanalysis with the Phosphoprotein Gel Stain

Assays for TS phosphorylation, and protein detection, were performed using the Multiplexed Proteomics® P os o rotein Gel Stain Kit, according to t e manufacturer’s protocol. Preparations of purified TS from parental and FdUrd-resistant L1210 cells, and calf thymus, as well as isolated recombinant TS proteins, were separated in a Mini-PROTEAN® II. Electrophoresis Cellon either polyacrylamide gel underpreviouslydescribed non-denaturing conditions[12], or on SDS/polyacrylamide gel[18]. Following electrophoresis, protein was fixed in a gel by incubation in 50 mM Tris-HClbuffer pH 7.5, containing 25 % (v/v) 2-propanol. Thegel was subsequentlywashed in distilled water and treated for 90 min 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, the protein again visualized on a 300 nm UV transilluminator, and the image recorded using the 590 .40 nm filter.Pe ermintStick™ phosphoprotein molecular mass standards, containing phosphorylated (ovalbumin, 45 kDa; ßcasein, 23.6 kDa) and non-phosphorylated (ß-galactosidase, 116.25 kDa; bovine serum albumin, 66.2 kDa; avidin, 18 kDa) proteins, provided controls (both positive and negative). TS protein band identity was determined by immunoblotting.

2.4 Separation of TS preparations into phosphorylated and non-phosphorylated fractions

This was done according to Wolschin et al.[19]using metal oxide/hydroxide affinitychromatography(MOAC) on Al(OH)3beads.Theprotein was bound on an Al(OH)3column from 30 mM MES, containing0.2 M aspartate, 0.2 M glutamate, 20 mM imidazole, 20 mM 2mercaptoethanol and 25 % (w/v) CHAPS, pH 6.1.The column was washed with thesame buffer (100-fold bead volume), later with 10 mM HEPES, containing20 mM 2-mercaptoethanol, pH 7.0 (20-fold bead volume), and phosphorylated protein eluted with 100 mM sodium pyrophosphate, containing 20 mM 2-mercaptoethanol, pH 9.0. Both protein fractions, theone not bound by the column,and the one bound and eluted, were concentrated, following dilution, on a small DEAE column, as previously described [12].With each mouse, human rat, T. spiralisand C. elegans enzyme recombinant protein, separationresulted in a pair of fractions: phosphorylated, MOACseparated from thecorresponding non-phosphorylated TS fraction (fractionsof this series arefurther referred to as m-pTS, h-pTS, r-pTS, Tsp-pTS and Ce-pTS, respectivelyand nonphosphorylated, MOAC-separated from the corresponding phosphorylated TS fraction (fractionsofthis series arefurtherreferred to as m-TS, h-TS, r-TS, Tsp-TS and Ce-TS, respectively).

2.5 TS phosphorylationwith potassium phosphoramidate (KPA)

The protein (2-60 mg) was incubated in 50 mM Tris-HCl or phosphate buffer pH 7.5, in the presence of 12-16% KPA, for 24 h at 25-27o C. A constant decrease of enzyme activity was observed with the courseof the reaction, whereas no loss of activity was noted in controls containingKCl in placeof KPA. The reaction product was enriched with the use of MOAC, yielding 10% of protein phosphorylated to an extent similar to that observed with thephosphorylated fraction of recombinant TS protein.

2.6 RNA electrophoreticmobility shift assay

This was performed as previously described[20]. Briefly, before addition of 32P-labelled mRNA (its previous denaturation at 75.C for 5 min, and a subsequent 15 min equilibration at room temperature (22.C), was without effect on thefinal result), TS protein was preincubated, alone orwith addition of RNA, for 15 min at room temperature in the reaction mixture (20 µl), containing, unless otherwise indicated, 10 mM Hepes (pH 7.4), 40 mM KCl, 3 mM MgCl2, 5%(v/v) glycerol, 200 mM 2-mercaptoethanol and 0.1 unit/µl inhibitase. In controls, TS was replaced byanotherprotein, or the incubation mixture contained no protein. After addition of 32P-labelled mRNA, the mixture was incubated for 15 min at 37.C, 12 units of RNase T1 added, and incubation continued at room temperature foranother 20 min, with 150 µg heparin added 10 min after RNase T1. Samples underwent electrophoresis (300 V, 4.C, 2 h 15 min) in a non-denaturing polyacrylamide (4%, w/w, acrylamide/N,N\_-methylenebisacrylamide, 60:1) gel.Before sample loading, gels were run for 30 min at 300 V and 4.C), then dried and visualized byautoradiography. Theautoradiograms were scanned and band densities calculated with use of acomputer program, Scion Image (Scion Corporation, Frederick, MD, U.S.A.).

2.7 In vitro translation assay

Rabbit Reticulocyte Lysate System (Promega) was used according to t e manufacturer’s instructions, but with thefinal volume of the reaction mixture scaled down to 12.6 µl.In brief, each translational reaction mixture, containing 8.8 µl of rabbit reticulocyte lysate, 0.3 µl of 1 mM methionine-free mixture of amino acids, 0.5 µl of Rnasin® Ribonuclease in ibitor (40 units/µl), 0.5µl [35S]-met ionine (10 mCi/ml, 1175 Ci/mmol), 0.5 µl of t e a ro riate mRNA transcri t aqueous solution (1 mole/µl) and 2 µl of rotein in 10.6 mM os ate buffer, containing 20 mM2-mercaptoethanol (in controls the protein solution was replaced with water or 10.6 mM phosphate buffer, containing 20 mM 2-mercaptoethanol), was incubated at 37.C for 30 min. Reaction products were analyzed by SDS/PAGE (12% polyacrylamide; 37.5 : 1 acrylamide: bis-acrylamide ratio)as described byLaemmli[18]. Non-incorporated label was removed by soaking the gel in 50%(v/v) methanol containing 10% (v/v) acetic acid for 30 min, then in 7% (v/v) acetic acid containing 1% (v/v) glycerol for 10 min. After drying for 1 h in the gel dryer, the translation products were identified by autoradiography. Each experiment was repeated at least twice.

2.8 Mass spectrometry analysis

2.8.1 Electrospray ionization tandemmass spectrometry (ESI-MS/MS) analyses Prior to analysis, bands containing TS were cut out from the 12.5% SDS/polyacrylamidegel and subjected to standard “in-gel digestion”[21],during which proteins were reduced, alkylated and digested with trypsin. The resulting peptides wereeluted from thegel with 0.1% TFA. Thepeptide mixture was applied to an 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% AcNin 30 min) inthe presence of 0.05%formic acid at a flow rate of 150 nl/min. The column outlet was coupled directly to a Finningan Nanospray ion source of an LTQ-FT (Thermo) mass spectrometer operating in the regime of data-dependent MS to MS/MS switch. A blank run, ensuring lack of cross contamination from previoussamples, preceded each analysis. The output list of precursor and product ions was compared to theNCBI database with a MASCOT local server (www.matrixscience.com). Spectra of peptides wereanalyzed with the use of the MassLynx v. 3.5 program.

2.8.2 NanoLC-MS/MS analysis

Protein samples (55-65 µg) were transferred to 20 mM TBC buffer (Triethylamine bicarbonate, pH 8.5). To prevent cysteine oxidation, eachsample, following addition of 150 µl of iodoacetamide (IA, 11 mg/ml), was incubated at room temperaturefor 30 min, washed twice with 100 µl TBC buffer to remove IA by spin filtering (3,5 k MWCO), and treated with 1µg (50µl) of trypsin. To achieve full digestion, samples were incubated at 37oC overnight. A digested samplewas either desalted or subjected to a CPP (Calcium Phosphate Precipitation) protocol [22]to enrich phosphopeptides in the mixture. Desaltingwas done byabsorbing peptides on 2-4 µl Oligo 20 R3 reversed Phase Packingresin (Applied Biosystem), washing 4-5 times with 100 µl deionized water, and elution with 45 µl 50% aqueous acetonitrile.

Eluted peptides were brought to dryness on a Speed Vacand dissolved in water prior to MSanalysis. Samples of either non-separated or CPP-enriched peptides were subjected to NanoLC-MS/MS analysis. Samples wereapplied onto an EASY nano-LC system (Thermo Scientific/Proxeon) and preconcentrated on a home-made 3-cm fused silica precolumn (100 .m inner diameter, ReproSil-Pur C18 AQ, 5 µm article size (Dr. Maisc GmB , Germany)). T epeptides were eluted from the precolumn with a two-step linear gradient at 275 nl /min onto an 18cm analytical column (100 .m inner diameter, ReproSil-Pur C18 AQ, 3 .m particle size). Thegradient was as follows: from 100% phaseA (water) to 34% phaseB (90% acetonitrile in water)for10 min, 34%-100% Bfor5 min, and 100% B for 8 min. The pH of the elution mixture was between 5.5 and 6.5. MS analyses were performed using aLTQ-Orbitrap XL mass spectrometer operating in the positive ion mode. Data-dependent analysis was used. Full MS scans from 300 to 1800 m/z were acquired, and the most intensive peptide ions were subjected to further fragmentation by either collision-induced dissociation (CID)alone (Top-6, Multistage activation) orcollision-induced dissociation andelectron-transfer dissociation (CID-ETD)(Top-3). Duplicate detection of a single m/z within 30 s led to dynamic exclusion. The foregoingmethod ofMS sample preparation was optimized, to protect phosphohistidine residues, using model peptides resulting from tryptic (with both phosphorylated and nonphosphorylated fractions, chymotryptic peptides allowed onlyabout 60% sequencecoverage, vs. 80% withtryptic peptides) digests of iodoacetamide-blocked TS preparations, including KPAphosphorylated h-TS, as well as m-TS, m-pTS and KPA-phosphorylated m-TS. Followingdesalting, each peptide mixture was analyzed by ESI-MS/MS (CID), and the number of identified peptides compared. This approach was used to select the desalting method, including pH conditions, and the phosphopeptide enrichment method.AsESI-MS/MS comparativeanalyzes of samples desalted with the use of three different beads, including(i)R2 (POROS 20 R2 Reversed-PhasePacking),(ii) R3 (Oligo 20 R3 Reversed-Phase Packing) and (iii) 1:1 mixture ofR2 and R3 beads, did notshow distinct differences concerningsequence coverage or peptiderecovery, R3 bead was used in further studies.In order toassess the effect of pHduring sample preparation, KPA-phosphorylated and MOAC-enriched h-TS, following tryptic digestion,was either acidified with different concentrations of TFA or left in TBC buffer(pH 8.5), and the resulting peptide samples desaltedon R3 columns. The columns were washed with TFA solutions of different concentrations (pH ~1-5) or deionized water (pH 5.8-6.0), followed byelution with 50 % AcNin a solution used to wash a given column. Whereas in samples treated with 0.1 % TFA (pH ~1)no phosphopeptides wereidentified, in those treated with 0.005 % TFA (pH~3.5) single phosphopeptides, but no phosphohistidine, wererevealed. Phosphopeptides, including phosphohistidine, were observed onlyin samplesremainingat pH 6, 7 or 8.5 and washed with deionized water. Hence, in further steps samples trypsin-digested at pH 8.5 were desalted on R3 columns washed with deionized water, thusallowing phosphohistidine preservation. Analysisofa relatively large sample (7-9 µg) allowed a high level of peptides ionization, resulting in strong signals for particular ions.Three methodsallowing enrichment of phosphopeptides following desalting werecompared: (i) using ProQDiamond Phosphoprotein Enrichment Kit,(ii) Phosphoselect Sigma IMAC Fe-beads, (iii) CPPmethod, based on precipitation of phosphopeptides under alkaline conditions in the presenceofcalcium salts [22].Each method was tested by usingit to enrich peptides resulting from trypticdigestion of a standard phosphoprotein mixture. ESI-MS/MSanalysis showed no phosphopeptides following application of the ProQ Diamond Phosphoprotein Enrichment Kit, phosphopeptides constituting 10 % of all identified peptides following application of Phosphoselect Sigma IMAC Fe-beads and phosphopeptidesconstituting50 % of all identified peptides following application ofthe CPP method.Consideringthenecessity of sample acidification in thecourse of the CPPprocedure, the effectiveness of this method was tested by using it to enrich phosphopeptidesresultingfrom tryptic digestion of ~100 µg of CeTS,followed by 10 h incubation at room temperature with 12 % KPA and desalting(R3 beads washed with deionized water). ESI MS/MSanalysis identified phosphopeptides only in the enriched fraction.

2.9 31P NMR spectroscopy

Spectra wererecorded on a Bruker AvanceII spectrometer operating in the quadrature mode at 500.13 MHz for1H and 202.46 MHz for31P nuclei. All spectra wererecorded at 277 K, both with and without proton decoupling. The sample tubediameter was 5 mm and spectra were recorded at pH 7.5 (for r-pTS, Tsp-pTS, Ce-pTS, r-TS, Tsp-TS, Ce-TS and all chemically phosphorylated TS preparations) or at pH 7.8 (m-pTS, h-pTS, m-TS, h-TS). Eac sam le (740 µl) contained 440 µl of eit er 3.3 µM os orylated TS or 13.4 µM non-phosphorylated TS fraction in 0.2 M Tris-HCl pH 7.5 or 7.8, 20% sacc arose and 20 mM ß-merca toet anol, mixed wit 300 µl D2O. Inorganic phosphate (Pi) was used as aninternal standard, with resonances at 2.14-2.16 ppm (pH 7.8), 2.002.05 ppm (pH 7.5), 1.60-1.70 ppm (at pH 5.0) or 0.0 ppm (at pH 1-1.5). Additionally, the spectrawerereferenced using an external standard (85% H3PO4). All protein samples wereanalyzed by two-dimensional NMR spectroscopy, usinggradient-enhanced 1H-31P heteronuclear multi-bond correlation (HMBC). The HMBC experiments were conducted with optimization for long-rangecouplings, using different 3JPHvalues (1-10Hz).

2.10 31PNMR-monitored amino acid phosphorylation

To a solution of 10 mg of agiven amino acid (histidine, arginine or lysine) in 0.2 M Tris-HCl buffer ( H 7.8, 200 µl) was added otassium os oramidate (at a molar ratio of 7.5:1 KPA to amino acid). After shaking the reaction mixture for 24 h at 277 K, 31P NMR was monitored in a sam le containing 200 µl of t e reaction mixture and 300 µl D2O.

2.11 Immunoblotting

A previously described method was used, with anti-TS polyclonal antibody[11].

2.12 Statistically evaluated results

These 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.Statistical significance of differences was evaluated with the use of Student’st-test, and was accepted at p-value<0.05.

3. Results

3.1 Detection of phosphorylation in purified TS proteins

TS preparations highly purified in the presence of phosphatase inhibitors, includingendogenous enzymefrom L1210 parental and FdUrd-resistant cells, and calf thymus(Fig. 1), as well as mouse, rat, human,Trichinella spiralisand Caenorhabditis elegansrecombinant TSs expressed in bacterial cells(Fig. 1), as analyzed with the Pro-Q®Diamond Phosphoprotein Gel Stain (further referred to as Phosphoprotein Gel Stain) following SDS-PAGE, contain different levels of phosphorylated forms. Although the three preparationsof endogenous TSs were not enriched in phosphorylated forms, in each the level of the modified form was high enough to enableclear identification, with the highest content of phosphorylated fraction present in calf thymus TS(Fig. 1A, lanes 1-3).Interestingly, although protein content and concentration of endogenous TSpurified to aspecific activity of 1.5 µmol/min/mgroteinin the presence of phosphatase inhibitors from C.elegans,were too low to perform extensive electrophoretic studies, itscatalytic properties differed from those of the endogenous enzyme purified in the absence of phosphatase inhibitors, as previously demonstrated for the L1210 parental and FdUrd-resistant cell enzyme forms[7]. Thepresence of phosphataseinhibitors in the purification buffers resulted in aproduct with aKmappfordUMP of 14.5.14%µM (2), hence an order of magnitude higher than that (Kmof 1 µM; [11]) for the reaction catalyzed bytheC. elegansenzyme purified withoutuse of inhibitors.

Figure 1. Phosphorylation of TS protein, monitoredbySDS/polyacrylamide gel

electrophoresis. Gels, containing TS preparations from parental (A and B, lane 1) and FdUrdresistant (A and B, lane 2) L1210 cells, calf thymus (A and B, lane 3), and recombinant mouse (Aand B, lanes 4 and 5), human (A and B, lanes 6 and 7), rat (Aand B, lanes 8 and9), Trichinella spiralis(A and B, lanes 10 and 11) and Ceanorhabditis elegans(A and B,lanes 12 and 13),werefirst treated with Pro-Q Diamond Phosphoprotein Gel Stain, to detect phosphorylatedprotein (A) and then with the SYPRO Ruby Protein Gel Stain,to detect total protein (B). Phosphorylated (Aand B, lanes 4, 6, 8, 10 and 12) and non-phosphorylated (A and B, lanes 5, 7, 9, 11 and 13) fractionsof recombinant TS proteins were used, resulting from affinitychromatography (MOAC) separation. The vertical lines separate different gels.

Each endogenous TS preparation(Fig. 1B, lanes 1-3), and thephosphorylated form of each recombinant protein (Fig. 1B, lanes 4, 6, 8 10, 12),separated bySDS electrophoresis into two protein bands, apparently subunits. Interestingly, Fig. 1A shows that,in each case,only one of thesesubunits nderwent phosphorylation(Fig. 1, compare lanes 4 and5, 6 and 7, 8 and 9, 10 and 11, 12 and 13).Thus the modification seems to result in assymetryof the enzyme molecule.

3.2 MS analyses of endogenous TS preparations

Previous unsuccessful attempts to reveal phosphorylated amino-acid residues in L1210 parental and FdUrd-resistant TS preparations[7]suggested a low frequency of phosphorylation ofparticular amino-acid residue in a population of TS molecules. In linewith this, analysis of the sameTS preparations by isoelectric focusing permitted separation ofeach into 8 bands (not shown).MSanalysis of each of these bands revealed several modifications found in both enzyme forms (methylation of Glu, Glu, Glu, Glu, Glu, Glu, Gluand Glu,and acetylation ofMet1).Three amino-acid modifications differentiated the FdUrd-resistant from the parental cell enzyme, including phosphorylation of both Serand Ser(Fig. 2), and methylation of Gluin theresistant (but not parental) cell TS. Whether the presence of these modifications is responsible forthe differing properties of thesetwo TSs [7] remains to be established. Moreover, MS detection ofphosphorylated amino-acids only in resistant cellTS is in clear disagreement with the results of staining with Phosphoprotein Gel Stain (Fig. 1), pointing to phosphorylation of both L1210 TSproteins. HenceMS analysis appeared to be unable to detect part of the enzyme phosphorylation profile. Furthermore, despite high levels of phosphorylation of purified calf thymus TS, attempts to determine the modification sites have hitherto been unsuccessful.

3.3 Properties of MOAC-separated fractions of recombinant TS proteins

The foregoing pointed to the necessity of considerable enrichment of phosphorylated fractions of TS preparations to enable MS analysis. Five recombinant TS preparations (mouse, rat, human, T. spiralis, and C. elegans)were separated into phosphorylated (m-pTS, r-pTS, h-pTS, Tsp-pTS and Ce-pTS, respectively) and non-phosphorylated (m-TS, r-TS, h-TS, Tsp-TS and Ce-TS, respectively) fractions (see2.4). In each case the phosphorylated fraction, reacting with Phosphoprotein Gel Stain (Figs 1 and 3),corresponded to <1% of the total, and showed 3-14-fold lower specific activity than the non-phosphorylated fraction. The latter was found to reflect from 24-fold (human, mouse, rat and C. elegansTSs) to 30-fold (T. spiralisTS) lower Vmaxvalues of thecorresponding phosphorylated forms (Table 1). Each mouse and human phosphorylated TS showed one protein band reacting with Phosphoprotein Gel Stain when analyzed by PAGE under nondenaturing conditions (Fig. 3, lanes 1 and 8 , respectively. Note that lanes3 and 6 show staining of FdUMP phosphate bound to the enzyme protein). However, following SDS-PAGE, each enzyme preparation separated into a pair of bands, with only one band of each pair reacting with Phosphoprotein Gel Stain (Fig. 1, lanes 4 and 6, respectively). Thus it appears that, in each mouseand human recombinant TS, only one enzyme subunit underwent phosphorylation.

Moreover, only the phosphorylated fractions showed ability to repress in vitrotranslation ofTS cognate mRNA (Fig. 4),as well as of several other mRNA species (Fig. 5). With rat recombinant TS (the onlyTS tested) only the phosphorylated form was able to bind the cognate mRNA (Fig. 6).

Figure 2.MS/MS identification ofphosphorylation sites Ser(A) andSer(B) within FdUrdresistant L1210 cells thymidylate synthase peptide 3VVGSELQSDAQQLSAEAPR21.MS/MS spectracorrespond to the peptides phosphorylated on Ser(underlined; A) or Ser(underlined; B). Fragment ions, with mass + 80 Davs. the unmodified,are denoted by“\*”.And ß-eliminated ions, with mass -98 Davs.t e os orylated, are denoted by“.”. Indicated areß-eliminated parent ions ([M+2H]2+.), as well astheb and y cleavage products of the peptides.

Table 1.Catalytic properties of phosphorylated andnon-phosphorylated fractions of human, mouse, rat, T. spiralisandC. elegansrecombinant TS preparations, and KPA-phosphorylatedhuman, mouse andC. elegansrecombinant enzyme.

appapp

FractionVmax Vmax KmfordUMPKmformeFH4vs.varying[dUMP]vs.varying[meFH4]

(µmol/min/mgprotein)(µM)

HumanTS-HisTag

Phosphorylated0.59±0.06(4)\*0.57±0.06(4)\*3.5±0.2(4)42±2.4(4)\*

Non-phosphorylated2.14±0.03(4)2.62±0.11(4)3.6±0.2(4)81±14(4)

KPA-phosphorylated0.11±0.01(2)\*NDa11.8±0.1(2)ND

Mouse TS

Phosphorylated0.59±0.05(5)\*0.65±0.04(5)\*1.9±0.2(5)52±6(6)

Non-phosphorylated1.64±0.05(5)1.84±0.07(5)2.2±0.3(5)50±5(6)

KPA-phosphorylated0.52±0.11(4)\*0.61±0.10(4)\*5.6±0.7(4)43±2(4)

Rat TS-HisTag

Phosphorylated0.51±0.03(3)0.56±0.04(3)\*\*1.6±0.1(3)44±19(3)

Non-phosphorylated2.31±18% (2)1.66±0.08(3)1.6±0.2(3)66±16(3)

T. spiralisTS-HisTagb

Phosphorylated0.020±0.002(3)\*0.016±0.01(3)\*1.9±0.8(3)24±3(3)\*

Non-phosphorylated0.42±0.07(3)0.62±0.05(3)2.6±0.8(3)53±5(3)

C.elegansTS-HisTag

Phosphorylated1,26±11% (2)ND6,91±0,70(4)NDNon-phosphorylated2,06±0,76(3)ND6,07±0,41(4)NDKPA-phosphorylated0,19±0,07(3)\*ND11,02±1,76(3)ND

\*Mean value significantlydifferent (\*, p<0.05)from the corresponding parameter describing the

non-phosphorylated fractionaNot determined

bPrepared as previously described [44]

3.4 31P NMR-monitored phosphorylation of standard amino acids

To identify the phosphorylated amino acid residue(s) responsible for the observed 31P NMR spectra of TS preparations, reactions with KPA ofsome standard amino acids were monitored by31P NMR. The amino acids used included those with side-chains containinggroups (-OH, -SH, NH2and complex arginine or histidine nitrogen-rich fragments) especiallyreactive towards phosphoramidate ion. The resulting spectra (Table 2) were helpful in assigning NMR resonances in the phosphorylated protein spectra.

Figure 3.Phosphorylation of TS protein, monitoredbypolyacrylamide gel electrophoresisunder non-denaturing conditions, and identification of the enzyme based on the gel shift caused by formation of the FdUMP-meTHF-TS ternary complex (cf. [43]).Gelscontainingphosphorylated (Aand B, lanes 1, 4, 5 and 8)and non-phosphorylated (A and B, lanes 2, 3, 6 and 7)fractions of recombinant mouse (A and B, lanes 1-4) and human (A and B, lanes 5-8) TSs, weretreated with Pro-Q Diamond Phosphoprotein Gel Stain, to detect phosphorylatedprotein (A),and then with SYPRO RubyProtein Gel Stain, to detect total protein (B). Priorto electrophoresis, the enzyme was incubated alone (A and B, lanes 1, 2, 7 and 8), or in the presence of FdUMP and meTHF (A and B, lanes 3-6). The signal denoting the presence of phosphate may result from protein phosphorylation (A, lanes 1 and 8), bound FdUMP (containing phosphate group; A, lanes 3 and 6), or both (A, lanes 4 and 5).

Table 2.31P NMR chemical shifts observedfor a reaction mixture containing histidine, arginine, lysine and cysteine, following 24 h reaction with KPA, and for commercially available phosphotyrosine, phosphoserine andphosphothreonine.

Amino acid treated with KPAChemical shifts [ppm]

Histidine-6.52; -6.88; -7.86; -8.93 (at pH 7.5)aArginine6.50 and6.46 (at pH 7.5)

Lysine0.04 (at pH 5.0), 0.24 (at pH 9.5)

Cysteine15.93 (at pH 5.0); 15.97 (at pH 9.5)

Tyrosineb-3.98 (at pH 7.5)

Serineb-0.38 (at pH 7.5); 6.60 (at pH 9.5); 6.79 (at pH 11)

Threonine b-1.40

aFor 3-phosphate of the diphosphate (1,3-diphosphohistidine), 3-phosphohistidine, 1-phosphate ofthe diphosphate (1,3-diphosphohistidine), 1-phosphohistidine respectively, based on the previouslyreported resonance order [9, 23].

bNMR data for commercially available phosphorylated amino acids.

Figure 4. Effect of phosphorylated and non-phosphorylated fractions (upper panels) ofrecombinant human (hHisTagTS, left), rat (rHisTagTS, middle) andmouse(mHisTagTS, right) thymidylate synthases on thein vitrotranslation of their cognatemRNAs.Rabbitreticulocyte lysate was incubated with 40 nM corresponding TS mRNA and the corresponding TS preparation at indicated concentrations. Controls contained, in place of protein, one of the following: (i) water (H2O), (ii) 10.6 mM phosphate buffer, containing 20 mM 2-mercaptoethanol (the buffer associated with each TS preparation studied; B), (iii) ovalbumin (from Sigma)dissolved in water (Ov) and (iv) caseindissolved in water (Cas). Thelower plots show the ependencies oftranslation activity (thedensity of a band reflecting TS protein synthesized in the reaction, expressed as % of the correspondingcontrol B band density) on concentration of phosphorylated (left) or non-phosphorylated (right) human (-¦-), rat (-.-) and mouse (-.-) TS.

Figure 5. Effect of phosphorylated and non-phosphorylated fractionsof recombinant humanthymidylate synthase protein (hHisTagTS) on the in vitrotranslation of heterologous mRNAs encoding human dihydrofolate reductase (hDHFR; A), deoxycitidylatedeaminase (hDCD;B), serine hydroxymethyltransferase (hSHMT; C) and thymidine kinase (hTK; D). Rabbitreticulocyte lysate was incubated with 80 nM corresponding mRNA and the corresponding TS preparation at indicated concentrations. Controls contained,in place of theprotein preparation,either water (lane 1 in panels A-D) or 10.6 mM phosphate buffer, containing 20 mM 2mercaptoethanol (the buffer associated with each TS preparation studied; lane 2 in panels A-D). The right plots present the dependence of translationalactivity (density of a band reflecting TS protein synthesized in the reaction, expressed as % of the corresponding control lane 2 band density) on the concentration of phosphorylated (-¦-) or non-phosphorylated (-.-) TS.

Figure 6. Binding of rat mRNA by ratrecombinant TS (rHisTag-TS), unseparated (lanes 2-5)and its phosphorylated(lanes 6-9) and non-phosphorylated (lanes 10-13) forms, obtained by separation onmetal oxide/hydroxide affinity chromatography(MOAC).32P-labelled rat TSmRNA (0.2 nM, 200 000 c.p.m.) was incubated in the absence of protein (lane 1) and in thepresence of either 1.2 µM (lanes 2, 6 and 10),2.4 µM (lanes 3, 7 and 11),5 µM (lanes 4, 8 and 12), or 10 µM (lanes 5, 9 and 13) appropriate TS preparation (see Material and Methods).

3.5 31PNMR spectra of phosphorylated proteins

All phosphorylated enzymesexhibitedvery similar31P NMR resonance patterns(Table 3; Fig. 7).Apart frominorganic phosphate at ca.2 ppm,therewere two additionalmajor resonances at approx. -7.4 ppm and -9.9 ppm, belonging apparentlyto 3-os o istidine (.2-phosphohistidine) and 1-os o istidine (.1-phosphohistidine), respectively.Assignment of NMR resonances was based on literature data[9, 23],and also on our histidine phosphorylation experiments. The singlet multiplicity and the two-dimensional 31P{1H} heteronuclear correlation (HETCOR) and gradientenhanced 1H-31P heteronuclear single-quantum correlation (HSQC)experiments showed that thereis no measurable coupling between phosphorus and hydrogen atoms withone to four bond distances. The observedresonances also do not correspond to diphosphate species that would show a singlet resonance pattern from 31P-31P coupling,often observable even for long-distancecouplings. Both 1D and 2D experiments stronglysupportidentification of the two resonances asrepresentingphosphohistidine.

Some factors may influencethe 31P NMR results. Bond-angle effects described by Gorenstein[24]show that even a small change of theO-P-O angleof aphosphate moiety results in quitea largechange in chemical shift.It is possible that steric or electronicrepulsion can slightly deform the phosphate of phosphohistidine,aneffectthat should be especially observable forphosphorylated protein samples. This effect could be responsible for the smaller (1-5ppm) shift differences betweenaseemingly identical free phosphorylated amino acid and the samephosphorylated moietyin aprotein.

Table 3.31P NMR chemical shifts for phosphorylated and non-phosphorylated fractions,

separated on Al(OH)3, of human, mouse, rat, T. spiralisandC. eleganspurified recombinant

TS preparations and ofKPA-phosphorylated human, mouse andC. elegansenzyme proteins.

TS Fraction separated

on Al(OH)3Chemical shifts [ppm]

Human [37]h-pTS2.16 (s, Pi); -7.36 (s); -9.91 (s)

h-TS-KPA2.30 (s, Pi); -6.47 (s); -7.34 (s); -7.70 (s); -8.15(s); 8.56

(s); -9.19(s); -9.53 (s); -12.11(s)

h-TS2.15 (s, Pi)

Mousem-pTS2.14 (s, Pi); -7.39 (s); -9.87 (s)

m-TS-KPA2.20 (s, Pi); -7.62 (s); -8.02 (s); -8.52 (s); -9.10 (s); 9.39

(s); -12.00 (s)

m-TS2.15 (s, Pi,)

Ratr-pTS2.02 (s, Pi, HW 2,6Hz); -7.85 (s); -9.91 (s)

r-TS2.07(s, Pi)

T. spiralisTsp-pTS2.01 (s, Pi, HW 2,7Hz); -7.80 (s); -9.50 (m); -9.90 (s)

Tsp-TS2.00 (s, Pi)

C. elegansCe-pTS2.20 (s, Pi); -7.59 (s); -9.35 (s)

Ce-TS-KPA 2.30 (s, Pi); -7.40 (s); -9.16 (s); -9.50 (s)

Ce-TS2.34 (s, Pi)

Figure 7. 31P NMR spectrum of purified mouserecombinant TS.Upper panel: enriched

phosphorylated fraction (m-pTS;cf. [44]).Lower panel: corresponding spectrum of the nonphosphorylated

TS fraction (m-TS).

Figure 8. Influenceof acidic conditions on the31P NMR spectrum of recombinant rat TSphosphorylated fraction.The enzyme solution (pH adjusted to 3.0 with 1 M HCl)was incubated at 4°C, and the 31P NMR spectrarecorded at the same temperature. Theupfieldshifted resonances, in the vicinity of-10 ppm,correspond to phosphohistidine, hydrolysis of which results in theobserved increase of inorganic phosphate concentration (.=0 ppm).

3.6 31PNMR-monitored acid hydrolysis of phosphorylated TS preparations

The half-life values at pH 2.4 are 5 and 25 min for 1-phosphohistidine and 3phosphohistidine, respectively[9]. Seekinga further test of the nature of TS phosphorylation, thepH ofa phosphorylated rat enzyme (r-pTS)sample (at 278 K)was adjusted to 3.0 with 1M HCl and the 31P NMR spectrum recordedat the start,andafter 1 hour. The startingspectrum contained resonances with integral values (vsinorganic phosphate) of 1.0, 14.3 and 17.3. After 1 hourat pH 3.0, the integral values for inorganic phosphate, 3-phosphohistidine and 1-phosphohistidinewere1.0, 3.28 and 1.01,respectively(Fig. 8). Results forhydrolysisofthemouse enzyme (m-pTS)werevirtuallythe same.Note that the 3-phosphohistidine moiety hydrolyzed approximatelyfour times slowerthan the 1-phosphorylated, ingood agreement with literature data for hydrolysis of phosphorylated histidines at slightly more acidicconditions[9].

3.7 31P NMR spectraand properties ofKPA-phosphorylated TS proteins

In search ofa source of larger quantities (.50 mg)of histidine-phosphorylated TS protein, required forcrystallographic studies, non-phosphorylated human, mouse and C. elegansTS preparations were phosphorylated with KPA,and enriched with the use of MOAC. NMRanalysisof the chemically phosphorylated enzyme showed a series of peaks assigned to 1-phosphohistidine, 3-phosphohistidine and 1,3-diphosphohistidine, and no other phosphorylated residues (Table3).This clearly affected enzyme properties. The Vmaxofthe reaction catalyzed by the in vitro phosphorylated m-TS-KPA and Ce-TS-KPA vs.unmodifiedTS was significantly decreased. Parallel control incubations showednoloss of enzymeactivity (cf.Materialand Methods), and the Kmwas increased almost 2-fold (Table1).

3.8 31PNMRstudies ofphosphorylationsites inrecombinant TSs, phosphorylatedeitherendogenously in bacterial cells,or with the use of KPA The31PNMR spectraofthecorrespondingTSpreparations (Table3) contain,in each case,theresonanceat 2.1-2.3ppmofinorganic phosphate, and 2-6 upfieldshifted peaks ofavisible singletmultiplicityin theca.-7 to -10 ppm region.Notably, most31PNMR spectracontain onlytwomajorpeaks in this region,accompanied bya few peaks ofconsiderablysmallerarea. Theassignment ofthe singletresonances,based on thosepreviouslydescribed[25], stronglysuggeststhatpeaksin thenegativespectrum regionreflect N-phosphorylated histidines.Thereareno visible peaks thatmightsuggest the presenceofa phosphate moietyattached covalentlyto otheramino-acids (Fig.7). Moreover, analyses of time-dependent changes of the 31PNMR spectrum followingacidification (1h at pH 3) confirmed the presence of phosphorus in a phosphoramidate (acid-labile) bond(Fig. 8).

Possiblepresencein thesesamples ofanother phospho-amino acid, escaping detection, wasassessed byasimple calculation ofpeak areaand intensity. Takingthe inorganicphosphateresonanceareaas 1.0, the total areaofphosphohistidine species ranges from 4.9 to 93.7.Assumingthat allthe enzymehistidineside-chains weremonophosphorylated, theresonancearea,resulting from the presenceofanothersinglyphosphorylated amino acid (forexampleserine), should benot less than 0.08 (0.71/9 histidineresidues foran experiment with the highest levelofinorganicphosphate)to 10.40 (experiment with lowest inorganic phosphate concentration). Moreover, the fewer the phosphorylated histidineresidues in the protein, the larger the areaofsinglephosphorylated species. Consideringthe inorganic phosphate resonanceareaas 1.0, itis ratherunlikelyforaphophoserineresonanceofdd ortr-multiplicity, foundalways in the 6 to -1 ppm range, to becompletelynon-visible,furthersupported bythe signal-to-noise ratios ofhighest pHis resonances being in the 33 to 970 range.

3.9 MS studies of phosphorylation sites in recombinant TSs, phosphorylated eitherendogenously in bacterial cells,or with the use of KPA Surprisingly, whereas31P NMR spectroscopyclearlydemonstrated the presence of phosphorylated residues in the enriched fractions of in vivophosphorylated enzyme (Table3), ESIMS/MS analyses did not reveal the presence of phosphorylated residues in any of these. The results were negative even whenIEF fractionsofthe m-pTS preparation wereanalyzed, although this approach was at least partiallysuccessful with FdUrd-resistant L1210 cellTS (see3.2). However, the results indicating histidine phosphorylation pointed to the possibility of missing thesemodifications in MS analyses[26, 27],especiallyconsideringthe acidic conditions used for elution from the gel,and further separationof digested peptides (2.8.1; cf.[28]). HencefurtherattemptsinvolvedESI-MS/MSanalysis, in a search forhistidine-containingpeptides,separated from therecombinant mouse TS preparation trypsin digest using TiO2beads (Phos-Trap, PerkinElmer).This led to enrichment andMS/MSdetection of a singlehistidine-containingpeptide KVETIDDFKVEDFQIEGYNPHPTIK.Assumingthat enrichment resultedfrom phosphohistidine binding by the beads,the resultspoint to His298asthe most probable phosphorylation site, althoughthe mass spectra did not show direct evidenceof the presenceofpHis. Apparently,whileneutral and basic pH applied during the digestion and enrichment steps allowed preservation ofthe pHis298residue, the subsequent MS/MS measurement,involvingacidicpH,probablyled to loss of the phosphate group.Consideringthepossibility of another cause of thepeptide's enrichment, the presenceofsixacidic amino acid residues should not be responsible, in view of their low affinity towards TiO2[29].

In view oftheunequivocal determination ofphosphorylated residue sites, NanoLC-MS/MSwas additionally used, allowingtwo methodsofpeptide fragmentation induction: CID (collisioninduced dissociation)and ETD(electron transfer dissociation).Enriched phosphoproteinforms of recombinant TSs were studied that underwent phosphorylation in vivo(i.e. when overproducedin bacterial cells) orin vitro(chemically).While the results showed identified phosphorylated histidine residues in in vivophosphorylated mouse TS (His33) and chemicallyphosphorylated mouse(His, potentiallyHis), human (His, Hisand His) and C. elegans(His, Hisand His258) recombinant TS proteins, phosphorylationof several hydroxyamino acid residues was also indicated(Table4; Fig. 9).Several oftheidentified serine residueshadpreviouslybeen reported to undergo phosphorylation,with mouse TS Serand Serfound phosphorylatedendogenously in FdUrd-resistant L1210 cells (seeabove) and human TS Ser124found to be a substrate for CK2 kinase[30]. Interestingly, mouse TS Ser, homologous to human Ser, was found to undergo phosphorylatation both in vivo, in the recombinant protein expressed in bacterial cells, andin vitro (Table 4; Fig. 9).And C.elegansTS Thr, also homologous to human Ser, underwent phosphorylationin vitro(Table4).

Table 4. Mass spectrometric(NanoLC-MS/MS) determination of phosphorylated amino acidresidues in selected purified TS proteins, including the MOAC-enriched phosphorylatedfraction of mouse recombinant enzyme, andtheKPA-phosphorylated fractions (MOACenriched) of recombinant mouse, human andC. elegansTS proteins.

Sequence of modified peptideModification SiteFragmentation method

Phosphorylated fraction (MOAC-enriched) of mouse recombinant TS protein (m-pTS)

QVEpHILRCGFKKEDRHis(HisCID

DFLDSLGFpSARSer(Ser)CID

KPA-phosphorylated fraction (MOAC-enriched)of mouse recombinant TS protein (m-TS-KPA)

DFLDSLGFpSARSer(Ser)CID

DFLDSLGFpSARQEGDLGPVYGFQWRSer(Ser)CID

MLVVGSELQpSDAQQLSAEAPRSerCID

MLVVGSELQSDAQQLpSAEAPRSer

KVETIDDFKVEDFQIEGYNPpHPpTIKHis(His) CID

KVETIDDFKVEDFQIEGYNPpHPpTIKThr(Thr)

VEDFQIEGYNPpHPTIKHis(His)CID

pHFGAEYKDMDpSDYpSGQGVDQLQKSerorSerorHisCID

KEDRpTGTGTLSVFGMQARThrCID andETD

DLPLMALPPCpHALCQFYVVNGELSCQLYQRHisCID

KPA-phosphorylated fraction (MOAC-enriched)of human recombinant TS protein (h-TS-KPA)

DAEPRPPpHGELQYLGQIQHILRHisCID

DAEPRPPpHGELQYLGQIQpHILRHisandHisCID

DAEPRPPpHGELQYLGQIQpHILRcGVRKDDRHisandHisCID

DFLDSLGFpSTREEGDLGPVYGFQWRSerCID

DFLDSLGFpSTRSerCID

AEDFQIEGYNPpHPpTIKHisorThrCID

KPA-phosphorylated fraction (MOAC-enriched)ofC. elegansrecombinant TS protein (Ce-TSKPA)

ENIIADAPSDVVKTVQQQVpHLNQDEYKHisCID

TVQQQVpHLNQDEYKHisCID

YVDCpHTDYSGQGVDQLAEVIRHisCID

VCGLKPGTLVpHTLGDApHVYSNHVDALKHisorHisCID

VCGLKPGTLVpHpTLGDAHVYSNHVDALKHisorThrCID andETD

AFLDNLGFpTpSpREEGDLGPVYGFQWRThr(Ser)orSerorCID

Arg128

aHomologous human TS site, if also modified, is in parentheses

Figure9. MS/MS (CID)identification of phosphorylationofHis298ofKPA-phosphorylated mouserecombinant TS (A), Serand HisofKPA-phosphorylated human recombinant TS(B andC, respectively), His252of KPA-phosphorylatedC. elegansrecombinant TS (D). Fragment ions, with mass + 80 Davs.those unmodified, are denoted by ”\*”. Thecleavage products of peptides aredenotedbyb and y.

3.10 Molecular dynamicsassessment of potential effect of phosphorylation of selectedhistidine residues To assess the potential ofphosphorylation of different histidine residues on the catalyticactivityof TS, molecular dynamics (MD) studies werecarried out (for MD simulation protocol see[31, 32]). Candidates for phosphorylation were selected,based on initial results (some of them not finally confirmed)of MS analysis of different protein samples. Both the N1-and N3phosphorylated variants of certain histidine residues wereselected for simulations,since theyareindistinguishable byMS,and NMR analysis showedthat both variants were present in phosphorylated samples. Simulations started from the crystal structure of mouse TS (mTS) in a ternarycomplex(PDBID: 4EB4)with the substrate, dUMP, and the quinazoline antifolate inhibitor, Tomudex, the latter molecule mimicking binding of the TS natural cofactor, N(5,10)methylenetetrahydrofolate. Initial results, based ona monomeric structure,indicated phosphorylation in mTS of eachof the four histidines, His, His, His, and His(Fig. 10), to differently influence the binding equilibrium between dUMP and Tomudex. The observed effects can be divided in threegroups:(i) well-preserved binding of ligands, dUMP and Tomudex, resulting in parallel, or close to parallel, alignment between the dUMP pyrimidine and the Tomudexquinazoline rings, suggestingalack of, or very weak, influence of phosphorylation on TS catalyticactivity (His135 and His298 phosphorylated at either the N1 orN3 positions); (ii) binding of ligands strongly affected in one form and preserved in the other form of phosphorylated protein (His255 phosphorylated at N1 or N3, respectively), pointing to a phosphorylation site-dependent effect;(iii)binding affected in both forms of phosphorylated protein (His233 phosphorylated at either N1 or N3), resulting ina perturbed alignment between the dUMP pyrimidine and Tomudexquinazoline rings,as well as (in the N3-phosphorylated form) an elongated distance between both rings, suggesting considerable reduction of TS catalyticactivity. Interestingly, while two ofthethree distant(from the ligand binding site)modifications (His135 and His298) show no apparent effect on binding of ligands, the third one (His233, located ina long .-helix (residues 217-235)), exerts a strongeffect. The reasons forthis,as well asthe mechanism behind the effect of (approximately2.5-foldless distant) phosphorylation at His 255,arecurrently under study.

Figure10. Tube representation of the monomer structure of His135-phosphorylated mTS with bound dUMP and Tomudex.Coloringaccording to secondary structureelements (.-helix: purple; 3(10)- elix: blue; ß-sheet:yellow; turn: cyan; coil: white). Histidine residues examined forphosphorylation effect are shown as white sticks and labelled with sequence numbers. Both dUMPand Tomudex are shown as orange sticks. The distances between the centroids of the histidineimidazole and dUMP pyrimidine rings are: 24.4 A for His135–dUMP, 27.8 A for His233–dUMP,

11.1 A for His255–dUMP,and 23.1 A for His298–dUMP.

Considering the above results, pointingto an apparent lack of influence of mTS His298 phosphorylation on ligand binding, itis worth notinginitial results ofparallel MD simulations based onthe crystal structure 1I00 [33] of human TS-dUMP-Tomudexcomplex, with the antifolatereplaced bythe tetrahydrofolate (THF) molecule [32]. When the enzyme dimeric structurewas considered in simulations, an interesting effect was noted on the binding behavior of ligands byphosphorylation at His 304, homologous to His 298 in mTSand also found to undergo phosphorylation (Table 4 and Results).When only a single active site of the dimeric enzyme is occupied by dUMP andTHF, t e ligands are always “in lace”, i.e. aligned parallel to each other, regardless of which single subunit, or both subunits, are actually phosphorylated. However, when both subunits are occupied by the ligands, thealignment between the dUMPpyrimidine ring and thepterine ofTHFdepends on whetherphosphorylation occurs on a single or both subunits. With His 304 in subunit A as the solephosphorylation site,the alignment is substantially disturbed in both subunits. On the other hand,with both His 304 A and His 304 B phosphorylated, the alignment partially improves, being parallel in subunit B (although with the dUMP pyrimidine and THFpterine slightly displacedfrom each other),but disturbed in subunit A, where the pyrimidine and pterine position themselves perpendicular to each other, precluding possibility of a bond-formingreaction. Thus the latterresults show,albeit unexpectedly,that phosphorylation at His 304 residue(s)of one/bothhTS subunit(s) mayhave an overall negativeeffect on ligand binding, causingpredictablereduction,or even elimination,ofthe catalyticactivity,provided the active sites in both subunits are simultaneously occupied by ligands. Control simulations on dimeric structures of mTS complexes arein progress.

4. Discussion

The present results indicate that TS may undergo phosphorylation endogenously in mammalian cells, as previously reported [6,7]. Theonly indications of a potential physiological roleof this modification are(i)influence on catalytic properties (Kmappfor dUMP) of endogenous C. elegansTS(see3.1)and(ii) affectingdiffering properties, including molecular activity, as wellassensitivity to inactivation by 5-fluoro-dUMP (FdUMP) and some analogues, of TSsfrom parental and 5-fluoro-dUrd (FdUrd)-resistant mouse leukemiaL1210 cells[7]. It should be noted that in the studies of theC. elegansenzyme, as well as those ofboth L1210 TS forms, a similar approachwas used, based on comparison of the properties of the enzyme preparations purified without,orin the presence of,phosphatase inhibitors. Sincethe enzyme was found to undergo phosphorylation also as a recombinant protein overexpressed in bacterial cells, thisallowed a direct comparison of phosphorylated vs. nonphosphorylated forms (Table 1 andResults)and document the influence of the modification’s not only on catalytic, but also on non-catalytic propertiesof TS.Considering the latter, the modification was found critical forinteraction ofphosphorylated rat recombinant TS with its own mRNA, demonstrated by the appearance of a radiolabelled, RNase T1-resistant and electrophoreticallyseparable complexafter incubation of labelled mRNA with the enzyme under conditions described by Chu et al. [20]. Of note is that the phenomenon to be shownrequired a protein/mRNA molar concentration ratio in the rangeof103(Fig.6), indicating that onlya small fraction (~0.1%) of TS was involved in binding. One possibilityis that arare monomer of the TS dimer is involved,assuggested byVoeller et al. [34],to be the only TS form capable of mRNA binding.Whilerat pTS is capable of both binding and inhibitingin vitrotranslation of its own mRNA, the same enzymepreparation used in the two tests revealed that translation was affected at a considerably lower protein/mRNA molecular concentration ratio (at a ratio in the range 4-5 the rate of translation was decreased to 50 % of thecontrol; Fig. 4) than was binding (~1000). This low protein/mRNA ratio (in the range 2-10), diminishing the rate of translation by 50 %, was seen also with hTS and mTS mRNAs (Fig. 4), as well with mRNAs of several other enzymes (Fig. 5).The lattersuggested either the presence in the reticulocyte preparation of afactor strengthening the mRNA–protein interaction or a mechanism whereby TS impaired translation differentlyrelative to mRNA binding (e.g. interaction of the enzyme with ribosome; Note: not only RNA-protein, but also protein-protein binding may be influenced by phosphorylation [35]).

Of interest is asymmetry of TS homodimer caused by phosphorylation, almost exclusively of only one of the subunits (Fig. 1). It should be noted that,although TS is a homodimer with two equivalent active sites composed of residues from both subunits, it shows half-the-siteactivity, with associated negative cooperativity [36-38]. Would the latter phenomenon belinked with phosphorylation?

The enriched phosphorylated fractions of recombinant TS proteins, albeit representing in each case only . 1% of t e total urified TS rotein, rovided enoug rotein (with each TSabout 1 mg)to attempt 31P NMRqualitativeidentification ofphosphorylated amino acid residue(s). An unexpected finding of phosphorus in a phosphoramidate (acid-labile) bond, pointing to modification of histidine residue(s), raises thequestion as to whethera similar modification may be present in the endogenous mammalian TS proteins studied. Such a possibilitymayexplainwhyESI-MS/MSstudies showed onlylimited success with the two L1210 TS forms,and lack of success with the calfthymus enzyme. Especially that even with highly enriched phosphorylated fractions of recombinant TSs onlyapplication ofNanoLC-MS/MS analysis with carefullyselected conditions of samplepreparation (2.8.2) allowed to determine certain phosphohistidine positions(Table 4; Fig. 9).

The present MS results are in obvious disagreement with the31P NMR results thatshow no apparent phosphoserine in the TS preparations studied (Table 3), andargueagainst the ability of KPA to phosphorylate serine (Table 2), serine-containing peptide (Gly-Ser-Gly; not shown) or C. elegansrecombinant TS protein at pH 7.5 (not shown). Especially thatthe possibility of frequent modification of phosphohistidine-containing samples during preparation for the NanoLC-MS/MSexperiments may probablybe neglected, in view of the careful selection ofconditions protectingthis modification (cf.2.8.2). Would it then be feasible to expect modification of samples in the course of MS measurements.Recently Schmidt et al. [39] reported that application of collisional activation in analysis of arginine-phosphorylated peptides mayresult in false localizations, as wellas in rearrangement of phosphorylation onto serine and glutamicacid residues. Possibility of transfer of the phosphate moietyofaphosphohistidine residue to an acceptor aspartate residue to occur as an experimental artefact in the course of a short LC-MS analysis has also been reported byGonzalez-Sanchez et al. [40]. The same group reported intramolecular gas-phase phosphate transfer (under conditions of collision-induced dissociation) from one phosphopeptide, containing pHis or pLys, to another, resulting in a doubly phosphorylated peptide product ion[41]. Of interest in this respectis the crystal structure of chemically phosphorylated C. elegansrecombinant TS in a complex with dUMP. Although this structure, in light of the 31P NMR spectrum (Table 3), was expected to contain phosphorylated histidine residue(s),and showed no indication of serine phosphorylation, the only exceptional electron densityregion, pointingtocovalent bonding with an amino acid residue, and conforming to a phosphate group,was at Ser127,pointing to possible transphosphorylation, either intermolecular or intramolecular, between histidine and serine residues[43].

Although MS results did not allow tolink phosphorylation effects with modification of particular histidine residue(s), at least mouse TS His, homologous to human enzyme His , is strongly indicated as being phosphorylated in theprotein preparations studied. Considering how distant from the active center is this residue, it was not unexpected that molecular dynamicsstudies performed on a mouse monomeric structure suggested lack of a phosphorylation effect. However similar studies performed on the human TS dimer revealed a strong effect under certain conditions, pointing to the need of further careful molecular modelingofpotential cooperative interactions.

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Highlights

Thymidylate synthase may undergo phosphorylation endogenouslyin mammalian cells.

Recombinantenzyme,expressed in bacterial cells, was found also phosphorylated.

Properties ofphosphorylatedand non-phosphorylatedrecombinant enzyme forms differ.

The modification of the recombinant enzyme was shown by31P NMR to concern histidine.