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Phosphorylation of thymidylate synthase from various sources by human protein kinase CK2 and its catalytic subunits

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ABSTRACT

Thymidylate synthase (TS) was found to be a substrate for both catalytic subunits of human CK2, with phosphorylation by CK2α and CK2α′ characterized by similar K_m values, 4.6 μM and 4.2 μM, respectively, but different efficiencies, the apparent turnover number with CK2α being 10-fold higher. With both catalytic subunits, phosphorylation of human TS, like calmodulin and BID, was strongly inhibited in the presence of the regulatory subunit CK2β, the holoenzyme being activated by polylysine. Phosphorylation of recombinant human, rat, mouse and *Trichinella spiralis* TSs proteins was compared, with the human enzyme being apparently a much better substrate than the others. Following hydrolysis and TLC, phosphoserine was detected in human and rat, and phosphotyrosine in *T. spiralis*, TS, used as substrates for CK2α. MALDI-TOF MS analysis led to identification of phosphorylated Ser^{124} in human TS, within a sequence $LGFS^{124}TREEGD$, atypical for a CK2 substrate recognition site. The phosphorylation site is located in a region considered important for the catalytic mechanism or regulation of human TS, corresponding to the loop 107–128. Following phosphorylation by CK2α, resulting in incorporation of 0.4 mol of phosphate per mol of dimeric TS, human TS exhibits unaltered K_m values for dUMP and $N^{5,10}$-methylenetetrahydrofolate, but a 50% lower turnover number, pointing to a strong influence of Ser^{124} phosphorylation on its catalytic efficiency.

1. Introduction

Thymidylate synthase (TS; EC 2.1.1.45) catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) by $N^{5,10}$-methylenetetrahydrofolate (meTHF) to generate thymidylate (dTMP) and dihydrofolate. The reaction provides the sole intracellular *de novo* source of dTMP [1], making the enzyme essential for regulating the balanced supply of DNA precursors required for DNA replication [2,3], and is consequently also a key target for chemotherapy.

In addition to its catalytic function, TS is an RNA-binding protein. The human and bacterial (*E. coli*) enzymes have been demonstrated to bind their cognate mRNAs within their coding regions, and each is capable of repressing its own mRNA in *in vitro* translation, with both functions considered to be connected [4]. This characteristic of TS may be of more general importance, since the human enzyme has been shown to bind and repress translation of p53 and *c-myc* mRNA templates, suggesting it is a potential translational regulator of cellular gene expression [5]. Thus TS may also be involved in several unknown non-catalytic functions, e.g. it has been postulated to possess an oncogene-like activity [6].

Phosphorylation of TS was shown to occur in rat hepatoma H35 [7] and mouse leukaemia L1210 [8] cells, with staurosporine-inhibited modification of a serine indicated in the former study. Recombinant rat TS was also found to be phosphorylated *in vitro* by calmodulin kinase and protein kinase C [7], but no data on phosphorylation site(s), or effects of phosphorylation on enzyme properties, were reported.

Bearing in mind that CK2 is known to phosphorylate more than 300 proteins [9], we herein report on the possible substrate properties of recombinant human, rat, mouse and *Trichinella spiralis* TS for human CK2 and its catalytic subunits, and the effects of phosphorylation of human TS on its properties.

Protein kinase CK2 is a ubiquitous and evolutionary conserved enzyme. It exists as a heterotetrameric complex, consisting of a regulatory β subunit (26 kDa) dimer and two catalytic, α (42 kDa)

Abbreviations: TS, thymidylate synthase; meTHF, $N^{5,10}$-methylenetetrahydrofolate.

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or α′ (38 kDa), subunits with holoenzyme stoichiometries of $\alpha_2\beta_2$, $\alpha'_2\beta_2$ or $\alpha\alpha'\beta_2$ [10]. Genetic studies in *Saccharomyces cerevisiae* [11] and in mice [12] demonstrated that CK2 is essential for cell viability, cell cycle progression, cell polarity, ion homeostasis, and other functions. Analysis of available bioinformatic resources shows that CK2 targets are largely involved in various aspects of global gene expression [9]. CK2 phosphorylation sites appear to be specified by multiple acidic residues located mainly downstream of a phosphorylatable amino acid residue, Ser being preferred over Thr and Tyr [13]. The minimum consensus sequence is S/TXXD/E [13,14], and the most crucial function is played by the amino acid residue at position $n + 3$ [14,15]. The third C-terminal amino acid from the phosphorylatable residue (Glu or Asp) can be replaced by phosphoserine or phosphotyrosine, but not phosphothreonine [14]. Otherwise a negative role is exerted by basic residues at any position close to the phosphoacceptor residue, as well as by a proline residue at the position $n + 1$. Analysis of the frequency of negatively charged amino acid residues (Glu or Asp) present in CK2 phosphorylation sites showed a single acidic determinant to be present only in very rare cases, and then invariably at the crucial position $n + 3$ [9,14].

2. Materials and methods

2.1. Plasmids, enzymes and reagents

All reagents were of molecular biology grade. [γ-^{32}P]ATP (spec. act. 4500 TBq/mmol) was from Hartmann Analytic GmbH. The N-terminal 6-His-tagged CK2α (MW 46,090 Da; specific activity ~1980 μmol/min/g protein, determined with the peptide substrate RRRDDDSDDD) was obtained from Millipore (formerly Upstate). The reconstituted human recombinant CK2 holoenzyme ($\alpha_2\beta_2$) was obtained from Biaffin GmbH & Cp KG, and its specific activity, determined with the synthetic peptide substrate RRRDDDSDDD (see below), was ~1200 μmol/min/g protein. Polylysine (Poly-L-lysine; average chain length # 4) was from Sigma.

2.2. Thymidylate synthase overexpression and purification

The enzyme preparations (recombinant human, rat, mouse and *T. spiralis* TSs) were overexpressed and highly purified in the presence of phosphatase inhibitors in the purification buffers. Mouse HisTag-free TS (mTS) was overexpressed and purified as previously described [16]. Rat HisTag-TS (rTS) was overexpressed in *Escherichia coli* BL21(DE3) cells transformed with the pET-28a(+)::*TS* vector (Novagen, Madison, WI) containing the TS protein coding region [8] as the NdeI/BamHI fragment. Human HisTag-TS (hTS) was expressed in *E. coli* BL21(DE3) strain as previously described [17], using the plasmid constructed by subcloning of the human gene excised with the use of NdeI/Hind III from the pET17xb/hTS(LVAG) plasmid [18] into pET-28a(+) vector (Novagen, Madison, WI). *T. spiralis* TS coding region [19] was subcloned as SphI/HindIII fragment into pQE2 vector and overexpressed as HisTag-containing protein in JM109 *E. coli* strain. All HisTag-containing TSs were purified on Ni–NTA HisBind Resin (Novagen) according to the manufacturer protocol. Removal of phosphorylated TS from the purified enzyme preparation was done according to [20], using metal oxide/hydroxide affinity chromatography on $Al(OH)_3$ beads.

2.3. Kinase overexpression, purification and assay of activity

Human CK2 α and α′, and the regulatory β subunits, were expressed as fusion proteins with N-terminal GST, a set of the corresponding plasmids, pGEX-3x::*CK2α*, pGEX-3x::*CK2α′* and pGEX-3x::*CK2β* [21], a generous gift from Prof. D. Litchfield, University of Western Ontario. The plasmids were used to transform *E. coli* BL21 *trxB* DE3 cells (applied for further protein overexpression) which were cultured in LB medium containing ampicillin and kanamycin at final concentrations of 100 and 10 μg/ml, respectively. Overexpression was induced by IPTG addition to a final concentration of 0.1 mM just after the OD_{600} attained a value of 0.5. After 24 h incubation at 30 °C, cells were harvested, rinsed with PBS and stored at −70 °C.

Following overexpression of GST-CK2α/α′ or GST-CK2β, bacterial cells were resuspended in lysis buffer containing 50 mM Tris–HCl, pH 7.5, 300 mM NaCl, 0.5 mM PMSF and 6 mM β-ME, and sonicated. The resulting suspension was centrifuged 15 min at 15,000 rpm (Sorvall, SS-34), and a sample of the supernatant loaded on a 1-ml column of glutathione-agarose beads. Unbound protein was washed out with TBS buffer containing 1% Triton X-100, and the appropriate GST-CK2 subunit was eluted (elution buffer containing 50 mM Tris–HCl, pH 9.4, and 10 mM reduced glutathione). Eluted proteins were collected as 0.5-ml fractions and analyzed on 11.25% SDS/PAGE. Fractions containing pure GST-CK2 subunit were combined, dialyzed against TBS buffer containing 50% glycerol, and stored at −20 °C.

Activity of CK2 holoenzyme and its catalytic subunits was determined as the rate of incorporation of phosphate from [γ-^{32}P]ATP into the RRRDDDSDDD peptide substrate under conditions described below for TS phosphorylation.

Specific activities of the CK2 catalytic subunit preparations, determined with the RRRDDDSDDD peptide substrate (see below), were ~12,500 μmol/min/g protein for GST-CK2α (MW 72,160 Da) and ~1005 μmol/min/g protein for GST-CK2α′ (MW 68,400 Da). Factor Xa protease was used for site-specific removal of the GST tag from CK2β protein (according to Sigma procedure).

2.4. Thymidylate synthase phosphorylation

Phosphorylation reactions were conducted at 37 °C for 5–80 min in 50 μl samples, each containing human recombinant CK2α, CK2α′ or CK2 holoenzyme (amounts given in experimental descriptions), appropriate concentration of thymidylate synthase (see experimental descriptions); 20 μM [γ-^{32}P]ATP (specific radioactivity 300–1000 cpm/pmol); 15 mM Mg^{2+}; 20 mM Tris–HCl pH 7.5 and 6 mM 2-mercaptoethanol.

Phosphorylation was terminated by addition of SDS/PAGE sample buffer and proteins resolved by electrophoresis, followed by Coomassie Brilliant Blue staining. Dried gels were exposed to Kodak X-Omat film. ^{32}P-labeled bands of TS were excised from the gel and radioactivity determined in a scintillation counter by Cerenkov counting.

To determine phosphate incorporation into protein (when measuring apparent K_m and V_{max} values), the reaction was stopped by addition of 100 μl of ice-cold TCA. Samples were filtered through GF/C filters and incorporated radioactivity counted in a scintillation counter.

2.5. Phosphoamino acids analysis

Following CK2α-catalyzed phosphorylation, the reaction mixture, containing ^{32}P-phosphorylated human, rat or parasite TS protein (10 μg), was subjected to SDS/PAGE in 10% gel, and transferred onto a PVDF membrane. The thymidylate synthase band, stained with Ponceau Red solution, was excised from the membrane and the protein transferred to a hermetic glass vial containing 150 μl 6 N HCl. Protein hydrolysis was conducted at 110 °C for 1 h. After HCl evaporation, the sample was taken up in 5 μl distilled water and loaded onto a cellulose plate (20 cm × 20 cm, Merck). Two-dimensional high voltage electrophoresis was performed, using formic-acetic buffer pH 1.9 and 1.5 kV in the first, and pyridine

buffer pH 3.5 and 1.6 kV in the second, dimension. Phosphoserine, phosphothreonine and phosphotyrosine, used as standards, were visualized by ninhydrine staining (0.1% solution in acetone-acetic acid). Positions of ^{32}P-labeled amino acids were detected by exposure of the dried plates to Kodak X-Omat film.

2.6. Mass spectrometric analyses

Two protein bands (about 4 μg each) of phosphorylated and non-phosphorylated TS protein (control sample) were excised from the Coomassie blue-stained SDS/PAGE gel and digested *in situ* with porcine trypsin as elsewhere described [22]. After digestion and extraction, a small aliquot of the peptide mixture was analyzed by MALDI–TOF MS with the use of a Bruker Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), following the manufacturer's recommendation. To enhance detectability of phosphorylated peptides, we used the matrix 2,5-dihydroxybenzoic acid, spiked with 1% phosphoric acid. The two spectra were manually scrutinized for differences due to addition of 80 Da in the phosphorylated sample. In order to position the phosphate group in the detected peptide, the digest was sulfonated for improved amino acid sequencing using post-source decay [23].

2.7. Kinetics

Kinetic parameters of the TS-catalyzed reaction were determined as earlier reported [24], based on non-linear regression [25]. Statistically evaluated results are presented as means ± SEM, followed by the number of experiments (N) in parentheses.

2.8. Protein content

This was determined using the Bradford reagent (Sigma) according to the manufacturer, with bovine serum albumin as a standard.

3. Results and discussion

According to the consensus sequence S/TXXD/E [14], and results of (i) analyses of the four TS sequences with the use of bioinformatic tools, including ELM [26] (http://elm.eu.org), NetPhosK 1.0 [27] (http://www.cbs.dtu.dk/services/NetPhosK/), ScanProsite [28] (http://www.expasy.ch/tools/scanprosite/) and GPS 2.1 [29] and (ii) accessibility of amino acid residues at the protein surface, based on either available crystallographic structures of human (PDB 1I00) [30], rat (PDB 2TSR) [31] and mouse (PDB 3IHI) TS, or the ternary structure of *T. spiralis* TS modelled assuming similarity to the foregoing structures (in view of the high sequence homology), each TS polypeptide harbors several putative CK2 phosphorylation sites. The sequence of the human enzyme contains a serine residue at position 124, and a threonine residue at position 170, as possible targets for CK2-mediated phosphorylation. Rat, similar to mouse, TS sequence reveals the presence of one serine residue at position 10 and two threonine residues at positions 164 and 281. The *T. spiralis* enzyme contains serine residues at positions 12 and 14, and a threonine residue at position 13, which fulfil criteria for recognition by CK2.

Phosphorylation of all four TS proteins was catalyzed by CK2α (Fig. 1) and CK2α′ (results not shown), with the human enzyme being by far the best substrate, as reflected by initial rates of phosphorylation, assessed as ^{32}P incorporation following 5 min incubation (Fig. 1). Notably, dUMP (up to 200 μM) did not interfere with the reaction (tested with CK2α′). Assuming one amino acid residue phosphorylated per TS dimer, the number of phosphate groups incorporated per protein molecule was found to be ~0.4 for human, ~0.2 for rat, ~0.16 for *T. spiralis*, and only 0.08 for the mouse enzyme, suggesting in all cases incorporation of a maximum of one phosphate residue per TS dimer. Of note is the significantly lower level of mouse, relative to rat, TS phosphorylation (Fig. 1), notwithstanding the high sequence identity (*cf.* [17]) of the two (only eight residues different out of 307 per subunit). However, the rat, but not the mouse, TS protein contained the HisTag. Similar apparent partial availability of phosphorylation sites on a CK2 substrate has been previously reported, e.g. with the protein BID [42], which was phosphorylated to the extent of only 0.3 mol of ATP per mol with CK2α, or 0.05 mol of ATP per mol with the CK2 holoenzyme. This may be related to the different state of dissociation and/or conformational availability of the target/neighboring amino acid residues, resulting from various combinations of posttranslational modifications of substrate molecules.

Two-dimensional electrophoresis indicated serines to undergo phosphorylation in the human and rat, but a tyrosine residue in the parasite, enzyme (Fig. 2).

Although CK2 is classified as a protein Ser/Thr kinase, it also phosphorylates tyrosine residues in *S. cerevisiae* [32,33] and in mammalian cells [10,33,34]. However, an investigation of the kinetic parameters of phosphorylation by CK2 of synthetic pep-

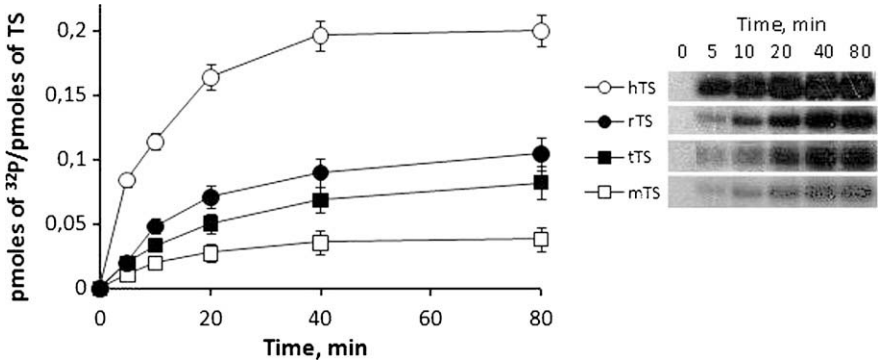

Fig. 1. Time-dependent phosphorylation by CK2α of human (hTS), rat (rTS), mouse (mTS) and *Trichinella spiralis* (tTS) thymidylate synthase. Reactions were conducted at 37 °C for 80 min in 50 μl samples containing 107.5 nmol/min of human recombinant GST-CK2α (MW 72,150 Da), 2 μg (dimer final concentration ~0.5 μM; monomer mol. wt. 37, 677 for human HisTag-TS, 36,704 Da for rat HisTag-TS, 34,958 Da for mouse TS and 37,188 Da for *T. spiralis* HisTag-TS) of appropriate thymidylate synthase protein, 20 μM [γ-^{32}P]ATP (specific activity 300–1000 cpm/pmol), 15 mM Mg^{2+}, 20 mM Tris–HCl pH 7.5 and 6 mM 2-mercaptoethanol. The reaction was terminated by addition of 10 μl SDS/PAGE sample buffer, samples resolved on SDS/PAGE, and incorporated ^{32}P-radioactivity determined by Cerenkov counting and autoradiography. Each point is the average of three independent experiments, with standard deviations indicated. Note: Results were unchanged when the experiment, following phosphorylation of human, rat and T. spiralis TS, was done under the same conditions, but with 150 μM [γ-^{32}P]ATP in the reaction mixture.

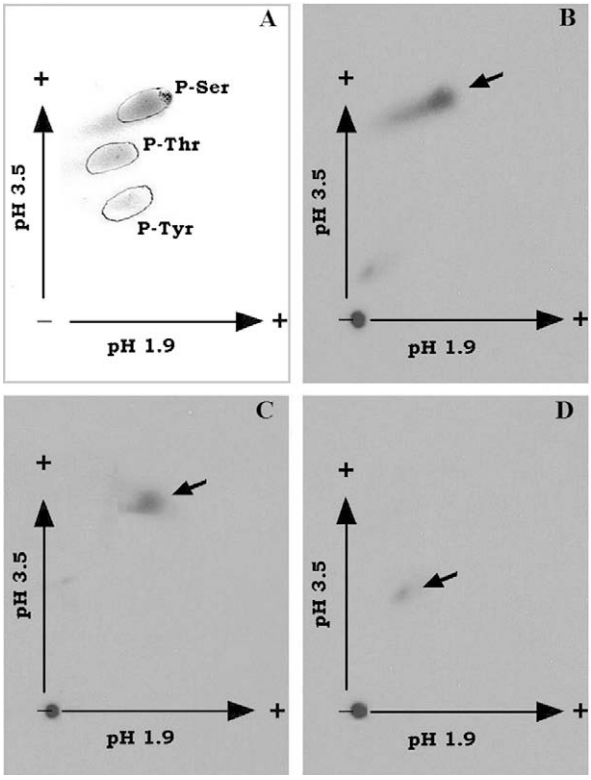

Fig. 2. Two-dimensional electrophoresis of amino acids phosphorylated by CK2α in human, rat and *Trichinella spiralis* thymidylate synthase. Following CK2α-catalyzed phosphorylation, the reaction mixture, containing ^{32}P-phosphorylated human (B), rat (C) or *T. spiralis* (D) TS protein (10 μg), was subjected to SDS/PAGE in 10% gel, transferred onto a PVDF membrane and hydrolysed in 6 N HCl. Amino acids obtained after hydrolysis were separated in two dimensions. Standard phosphoamino acids were visualized by ninhydrine staining (A) and positions of ^{32}P-labeled amino acids determined by autoradiography (B, C and D).

tides containing Ser, Thr and Tyr residues indicated serine-containing peptides to be better substrates than those containing tyrosine [33].

The human TS phosphorylation site was identified by MALDI–TOF MS peptide analysis. Peptides resulting from trypsin digestion of phosphorylated and non-phosphorylated TS were compared by peptide mass fingerprinting, using a Bruker Ultraflex TOF/TOF mass spectrometer. A weak, but significant, signal at MH+ 1337.58 was unique in the phosphorylated sample (Fig. 3A), This mass is a result of phosphorylation (addition of 80 Da) of the tryptic peptide DFLDSLGFSTR. To determine which of the two serine residues was modified, the sample was sulfonated (adding 136 Da to each arginine-containing peptide) and the peptide of mass 1473.58 was selected for sequence analysis (Fig. 3B). Phosphorylation was shown to occur at Ser^{124} in the region D^{116}FLDSLGFpSTREEGDLGP-VY^{135} of human TS.

However, although the human enzyme sequence contains a CK2 consensus site at Ser^{124} (underlined), the presence of a basic arginine residue at position $n+2$ (Fig. 4), representing a negative determinant of phosphorylation [14], should be noted. At the same time, the human CK2α-phosphorylated peptide sequence contains positive acidic determinants at positions $n+3$, $n+4$ and $n+6$. With regard to the arginine residue present in the sequence at position $n+2$, and its apparent lack of expected negative influence on phosphorylation, the corresponding human (PDB 1I00) 3D structure [30] shows the Arg side-chain to be displaced from the protein surface and buried (Fig. 5). The possibility of human thymidylate synthase Ser^{124} phosphorylation by CK2 was previously suggested (Bell et al. Proc. Am. Assoc. Cancer Res. 45, Abstr. # 1026, 2004; Proc. Am. Assoc. Cancer Res. 46, Abstr. # 4152, 2005) but with no direct evidence reported.

Identification of phosphotyrosine in the CK2α-phosphorylated parasite thymidylate synthase, allows speculation as to the most probable site of the modification, as analysis of potential tyrosine phosphorylation sites, considering the sequence requirements [34], points to only two residues, Tyr^{118} and Tyr^{152}. Of the two, the most probable site should be Tyr^{118}, corresponding to human Ser^{124}, despite the presence of asparagine, instead of the required aspartate [34], immediately C-terminal to the phosphorylatable residue (Fig. 4). In favor of Tyr^{118} as the site of phosphorylation, the amino acid sequence of *T. spiralis* TS contains positive acidic determinants at positions $n+3$, $n+4$ and $n+6$. Of note also is the high level of homology of this fragment of *T. spiralis* TS to the CK2 protein fragment being the most probable target of autophosphorylation at a tyrosine residue [34]. Moreover, based on the ternary structure of *T. spiralis* TS, modelled assuming similarity to the 3D structures of human (PDB 1I00) [30], rat (PDB 2TSR) [21] and mouse (PDB 3IHI) enzymes, it is highly probable that Glu^{110}, Asp^{113}, Glu^{121} and Glu^{122} are close to the putative site of phosphorylation, and Tyr^{120} assumes a position homologous to that of Arg^{126} in human TS (Fig. 5), i.e. with the side-chain removed from the protein surface and buried, thus unavailable for modification. Besides, Tyr^{118} is located in the middle of a loop, well exposed, and thus accessible to modification. By contrast, Tyr^{152} is probably located at the beginning of a loop, following an α-helix, with its movement limited, and having three basic residues (Lys^{160}, Arg^{163} and Arg^{170}) in close vicinity.

Human TS was used as a substrate to determine kinetic parameters for both catalytic subunits of human CK2 (Table 1). While the K_m^{app} values for CK2α and CK2α′ did not differ significantly, the catalytic efficiency of CK2α was almost an order of magnitude higher than that of CK2α′, in accord with the similar 11-fold different catalytic efficiencies of the subunits with the RRRDDDSDDD peptide substrate (see Section 2).

It is of interest that the phosphorylation site is located within a region corresponding to the human thymidylate synthase loop 107–128, which is considered important for its catalytic mechanism or its regulation (but not directly in dUMP binding; *cf.* the lack of dUMP interference with phosphorylation), as it has been found to assume a well-defined conformation only in association with the "active" conformation, one of the two conformations differing by positioning of the loop 181–197, hitherto described only for the human enzyme, and assumed to be characteristic for primates [35]. Whether the latter may be related to the higher substrate activity of human TS relative to the other enzyme forms, remains to be established.

The possible function of phosphorylation (*cf.* [36]) on human TS catalytic activity is of obvious interest. Comparison of the properties of the two (phosphorylated and non-phosphorylated) TS samples separated from the respective reaction mixtures showed the phosphorylated enzyme-catalyzed reaction to be described by a significantly lower apparent V_{max} value, determined with either dUMP or meTHF as the variable substrate, pointing to a two-fold lower turnover number, with similar K_m^{app} values describing interactions of both enzyme forms with each substrate (Table 2). However, it should be noted that the number of phosphate groups incorporated per human TS dimer was ~0.4 (see above; Fig. 1), suggesting that the observed decrease of turnover number is a consequence of modification of less than half the TS molecules, pointing to the possibility that phosphorylation eliminates or very strongly diminishes catalytic activity of the enzyme molecule. In fact, the latter, together with the previously reported assumption that only one TS subunit undergoes phosphorylation (*cf.* Frączyk et al., Acta Biochim. Pol. 54 (S3): 37–38, 2007), seems to be the only plausible explanation of the results (modification of 20% molecules on both subunits would hardly agree with a two-fold lower catalytic activity). Consequently, the unaltered K_m values, determined

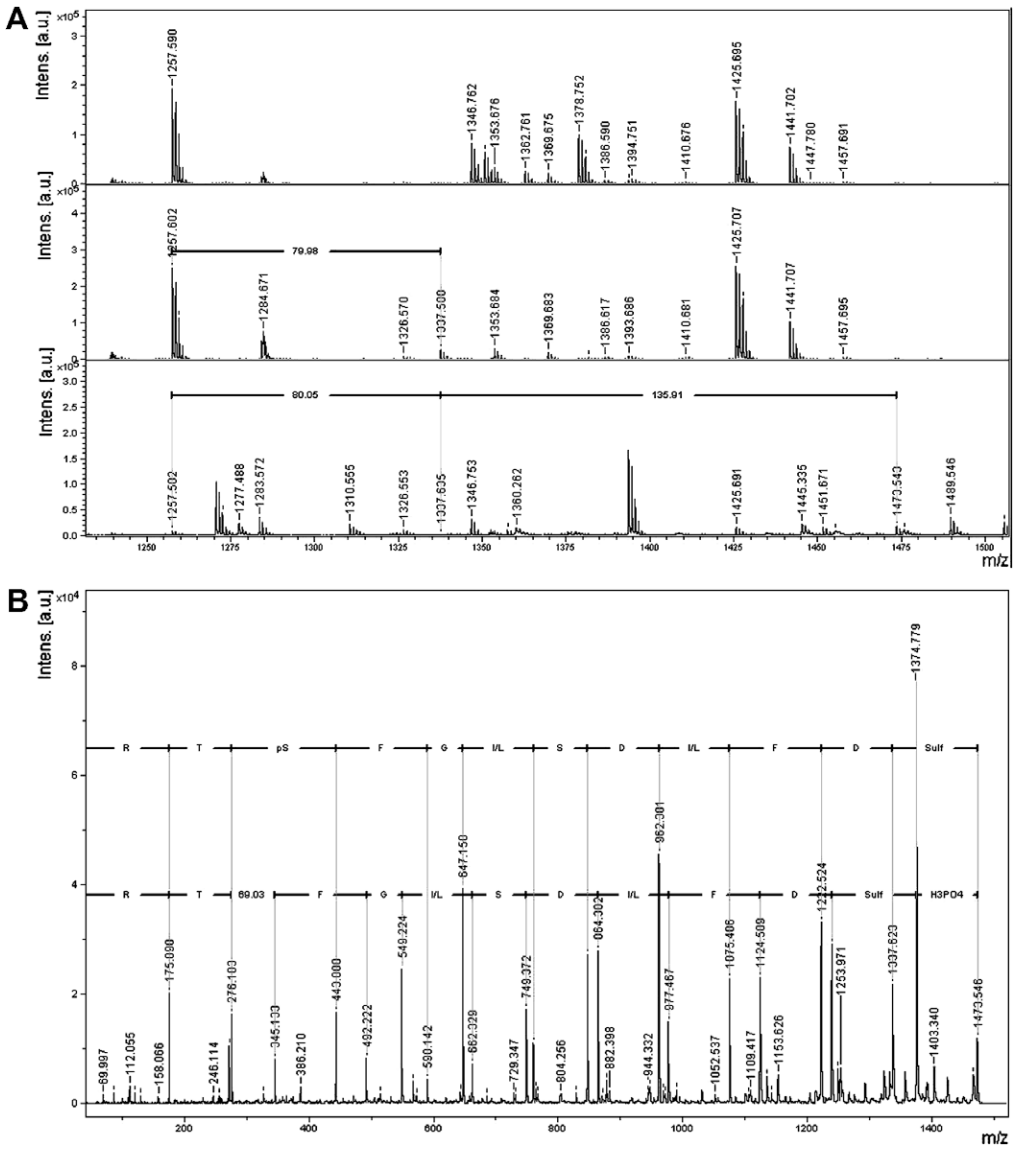

Fig. 3. MALDI mass spectrometry of tryptic digests of human TS. (A) Enlarged region of three spectra: top panel, non-phosphorylated human TS; middle panel, phosphorylated TS; bottom panel, phosphorylated and sulfonated TS. (B) Post-source decay of the peptide of mass 1473.55. The peptide sequence is demonstrated in two versions by complete y-ion series, one with the common loss of phosphoric acid, leaving 69 Da for pSer, and one with an intact pSer (167 Da).

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hTS       DFLDSLGF S124 TREEGDLGPVY
rTS/mTS   DFLDSLGF S118 ARQEGDLGPVY
tTS       EFLDSRGL Y118 NYEEGDLGPVY
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Fig. 4. Multiple alignment of the discussed TS sequence parts.

by monitoring the enzyme activity, appear to characterize active, unmodified enzyme molecules, rather than those phosphorylated, whose activity seems to be very low or zero. In accord with the latter hypothesis, results of molecular modeling studies have indicated human TS Ser^{124} phosphorylation to result in strong attenuation of catalytic potency, resulting from: (i) incorrect binding alignment between the pyrimidine ring of the substrate, dUMP, and the pterine ring of the cofactor, in the active site, and (ii) changes in behavior of collective motions in the phosphorylated enzyme, pointing to hindrance in formation of the closed ternary complex (Jarmuła A, Frączyk T, Cieplak P, Rode W, in preparation).

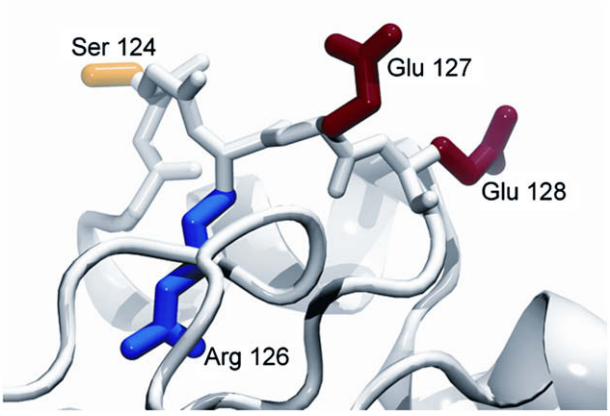

Fig. 5. Conformation of a human thymidylate synthase molecular fragment, encompassing the phosphorylatable Ser 124 and adjacent residues, based on the enzyme crystal structure (PDB code 1I00) [30], with use of VMD [52] software.

Table 1
Kinetic parameters of CK2α- and CK2α′-catalyzed human thymidylate synthase phosphorylation.

Parameter determined with TS dimer as variable substrate	CK2 catalytic subunit	
	CK2α	CK2α′
K_m^{app} (μM)	4.58 ± 0.03	4.20 ± 0.05
Turnover number[a] (mol/min/mol)	2.70 ± 0.06	0.30 ± 0.01

Increasing concentrations (0, 0.19, 0.38, 0.76, 1.52, 2.66, 5.32, 10.62 and 21.26 μM) of human TS (dimer, MW 75,354 Da) were phosphorylated with GST-CK2α (0.1 μg; 27.7 nM; total activity 1.25 nmol/min; MW 72,150 Da) or GST-CK2α′ (1.24 μg; 0.36 μM; total activity 1.25 nmol/min; MW 68,400 Da) in the presence of 20 μM [γ-^{32}P]ATP, 20 mM Tris–HCl pH 7.5, 15 mM $MgCl_2$ and 6 mM 2-mercaptoethanol at 37 °C for 10 min. Reaction was stopped by addition of 100 μl of ice-cold TCA. Samples were passed through GF/C filters and incorporated radioactivity counted in a scintillation counter. Kinetic parameters were determined using non-linear regression [25].

[a] Calculations were based on the V_{max}^{app} values (3.69 ± 0.09 pmol/min and 5.37 ± 0.20 pmol/min for CK2 α and CK2α′, respectively).

Table 2
Influence of phosphorylation by human recombinant (GST-tagged) CK2 α′ on catalytic properties of human recombinant thymidylate synthase.

Parameters of reaction catalyzed by TS isolated from the phosphorylation reaction mixture/variable substrate	Composition of phosphorylation reaction mixture		
	Complete	CK2α′ omitted	ATP omitted
K_m^{app} (μM)/dUMP	3.2 ± 0.5 (3)[a]	2.8 ± 0.3 (3)[a]	2.2 ± 0.1 (3)[a]
K_m^{app} (μM)/meTHF	42 ± 2 (4)[b]	38 ± 5 (4)[b]	43 ± 3 (4)[b]
Turnover number[c] (mol/min/mol TS)/dUMP	5.7 ± 0.5 (3)[d]	10.7 ± 1.3 (3)[d]	10.9 ± 0.6 (3)[d]
Turnover number[c] (mol/min/mol TS)/meTHF	6.7 ± 0.7 (4)[e]	12.1 ± 1.6 (4)[e]	12.4 ± 1.1 (4)[e]

Thymidylate synthase (100 μg) was phosphorylated with CK2α′ (3 μg, GST-tagged) at 30 °C for 60 min, in the presence of 20 μM ATP, 20 mM Tris–HCl pH 7.5, 15 mM $MgCl_2$ and 6 mM 2-mercaptoethanol in the complete reaction mixture, and with either CK2α′ or ATP omitted (controls). Following the reaction, thymidylate synthase was isolated with the use of glutathione-agarose beads, dialyzed against 50 mM Tris–HCl pH 7.5 containing 6 mM 2-mercaptoethanol and 20% sucrose, and tested for catalytic activity (see Section 2). With dUMP or meTHF as variable substrates, the reaction mixture contained 0.63 mM meTHF or 50 μM dUMP, respectively. Kinetic parameters were determined using non-linear regression [25]. Statistically evaluated results are presented as means ± SEM, the number of experiments (N) in parentheses.

[a] Values corresponding to the complete and control reaction mixtures did not differ significantly ($p > 0.1$).

[b] Values corresponding to the complete and control reaction mixtures did not differ significantly ($p > 0.5$).

[c] Calculations were based on the V_{max}^{app} value.

[d] Values corresponding to the complete reaction mixture were significantly lower than those determined with omission of CK2α′ ($p < 0.05$) or ATP ($p < 0.002$).

[e] Values corresponding to the complete reaction mixture were significantly lower than those determined with omission of CK2α′ ($p < 0.02$) or ATP ($p < 0.005$).

The regulatory CK2β subunit plays an important role in assembly of the CK2 tetrameric complex, as well as enhancing its stability [10,12,15]. Furthermore, in many cases the β-subunit is responsible for modulation of the catalytic activity and substrate selectivity of CK2 α/α′. Therefore phosphorylation of the human enzyme by human CK2 catalytic subunits α and α′ was compared with that of the tetrameric holoenzyme. Whereas the TS was phosphorylated by both subunits (Fig. 6, lanes 2 and 4), only traces of phosphorylation were apparent with the CK2 holoenzyme (Fig. 6, lane 1). To confirm the inhibitory effect of CK2β on CK2 activity, *in vitro* reconstitution of the $\alpha_2\beta_2$ and $\alpha'_2\beta_2$ isoforms of the holoenzyme was performed and their activities, with human TS as substrate, compared (Fig. 6, lanes 3 and 5). With both tetrameric forms of CK2, an inhibitory effect of the regulatory β-subunit on phosphorylation is apparent.

The regulatory β-subunit was previously shown to enhance the stability of the catalytic subunits and to modulate their substrate selectivity [37], including stimulation of CK2 activity towards certain substrates, such as topoisomerase II [38] and p53 [39]. An effect similar to that demonstrated here for thymidylate synthase was observed with calmodulin as a CK2 substrate, with the regulatory β-subunit almost completely inhibiting its phosphorylation. This inhibitory effect could be overcome by polybasic peptides, e.g. polylysine, but not polyamines [40], with an acidic stretch from residues 55–64, shown to be responsible for both the inhibitory effect and polylysine stimulation [40], and acidic residues at positions 55 and 57 suggested to play a general down-regulatory role [41]. Recently BID, a BH3-domain-containing proapoptotic member of the BCL-2 family, was found to be another CK2 substrate behaving similarly to calmodulin [42]. Considering human TS to be another CK2 substrate of this type, we examined the possible influence of polylysine on the inhibitory effect of CK2β on CK2α′ activity, with an *in vitro* reconstituted $\alpha'_2\beta_2$ isoform of the holoenzyme with human TS as substrate. Polylysine was, in fact, found to activate TS phosphorylation by CK2 $\alpha'_2\beta_2$ holoenzyme form to a level even higher than observed with the catalytic subunit alone (Fig. 7). The effect of polylysine was concentration-dependent, with distinct activation observed already at 20 μM polylysine (not shown). Polylysine concentration required to reactivate the holoenzyme was distinctly higher than those previously used [31–33], probably due to the fact that our polylysine preparation was of shorter chain length. Since the results showed a similar effect of reactivation with $\alpha_2\beta_2$ holoenzyme (not shown), this problem requires further study.

In view of the foregoing, possibility of thymidylate synthase to undergo CK2-catalyzed phosphorylation in a cell would apparently depend on availability of the free catalytic subunits. Although CK2

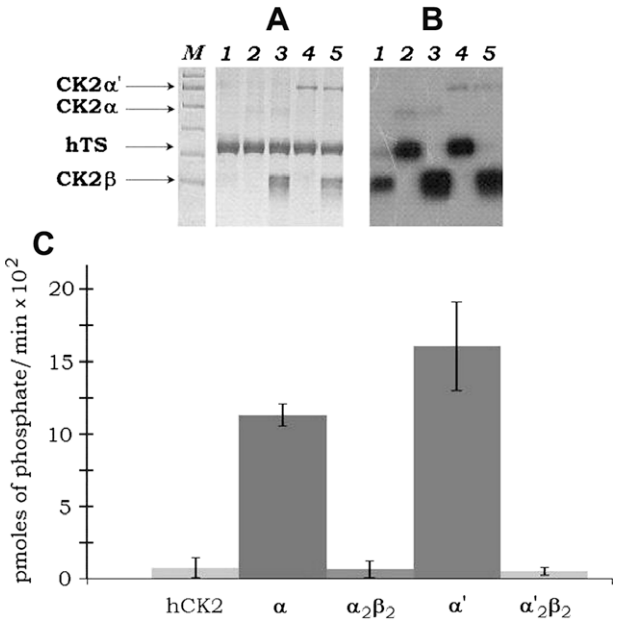

Fig. 6. Influence of the regulatory CK2β subunit on thymidylate synthase phosphorylation. Human TS (2.8 μg; monomer MW 37,677 Da) was incubated with 0.65 nmol/min of CK2 holoenzyme (1), 0.65 nmol/min of recombinant His_6-CK2α (MW 46,090 Da; lanes 2 and 3) and 0.65 nmol/min of GST-CK2α′ (MW 68,400 Da; lanes 4 and 5) in the standard incubation mixture (see Section 2), in the absence (lanes 2 and 4) or presence lanes 3 and 5) of 1.5 μg of the regulatory β subunit (tag-free; MW 24,925 Da). The reaction was conducted at 37 °C for 10 min, and terminated by addition of 15 μl of SDS/PAGE sample buffer. Phosphorylated proteins were resolved in 12% SDS/PAGE (A), with components of Fermentas PageRuler Prestained Protein Ladder (#SM0671), corresponding to molecular weight standards (top to bottom) 100, 70, 55, 40, 35 and 25 kDa (lane M). The level of phosphate incorporation was determined by autoradiography (B) and by Cerenkov counting of ^{32}P-radioactivity incorporated into TS protein (C). Positions of recombinant proteins used in the experiment are denoted by arrows. Each point is the average of three independent experiments, with standard deviations indicated.

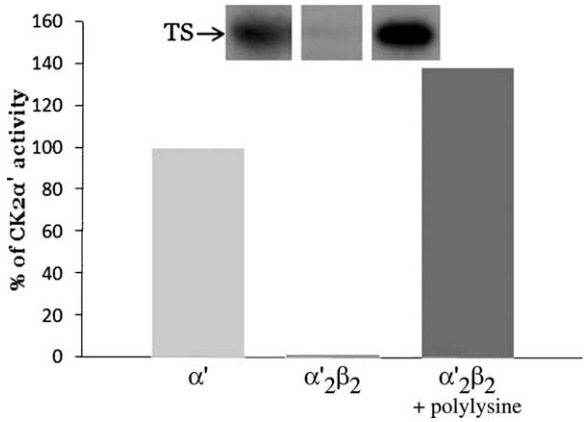

Fig. 7. Polylysine overcomes the inhibitory effect of CK2β subunit on thymidylate synthase phosphorylation. Human TS (2.8 μg; monomer MW 37,677 Da) was incubated in 50 μl medium, containing 3.02 nmol/min (3 μg) of GST-CK2α′ (MW 68,400 Da; all lanes), 100 μM [γ-^{32}P]ATP (specific radioactivity 300–1000 cpm/pmol), 15 mM Mg^{2+}, 20 mM Tris–HCl pH 7.5 and 6 mM 2-mercaptoethanol, in the absence (lane α′) or presence lanes $\alpha'_2\beta_2$ and $\alpha'_2\beta_2$ + polylysine) of 3 μg of the regulatory β subunit (tag-free; MW 24,925 Da), and in the absence (lanes α′ and $\alpha'_2\beta_2$) or presence (lane $\alpha'_2\beta_2$ + polylysine) of 200 μM polylysine (average chain length # 4). The reaction was conducted at 37 °C for 20 min, and terminated by addition of 15 μl of SDS/PAGE sample buffer. Phosphorylated proteins were resolved in 12% SDS/PAGE. The level of phosphate incorporation was determined by autoradiography (upper panel) and by Cerenkov counting of ^{32}P-radioactivity incorporated into TS protein.

has traditionally been classified as a stable tetrameric complex of two catalytic and two regulatory subunits [9,10,15], unbalanced expression of catalytic and regulatory CK2 subunits has been observed in a variety of normal and tumor tissues. In particular, evidence exists supporting CK2 dysregulation in all cancers so far examined [10,43], an example being SAGE analysis of a number of metastatic tumors that showed elevated expression of CK2α′ [44]. It appears that the presence of elevated levels of CK2 catalytic subunits correlates with the pathological state of the cancer, and may serve as an unfavorable prognostic marker [43,45–48]. In contrast to the foregoing, a recent study [49] presented evidence arguing against the existence of free subunits in CHO cells. Therefore, the possibility of activation of TS phosphorylation by CK2 holoenzyme (Fig. 7) is of interest, considering its potential role *in vivo*. It should also be noted that TS [50] and CK2 [10] appear to be colocalized in a cell, both in the cytoplasm and the nucleus, with CK2 showing a higher nuclear/cytoplasmic CK2 ratio in tumor, relative to normal, cells [10,43,46].

Although, in view of the foregoing, TS is phosphorylated by CK2 catalytic subunits *in vitro*, human TS, compared to other enzyme forms studied, is a much better substrate for the human kinase, and the modification of human TS Ser^{124} markedly affects the kinetics of the enzyme, further studies are needed, as with the majority of other known protein phosphorylations (*cf.* [36]), to provide unequivocal evidence of a possible functional role of phosphorylated TS. It should be added that recombinant human, mouse, rat and *T. spiralis* TSs, expressed in *E. coli*, may also undergo phosphorylation on histidine residues, the modification strongly influencing catalytic and non-catalytic properties of the enzyme. L1210 and calf thymus endogenous TS proteins also seem to undergo acid-labile phosphorylation [51].

4. Conclusions

Recombinant human thymidylate synthase is a substrate for both human CK2 catalytic subunits, CK2α and CK2α′, characterized by similar K_m values, 4.6 μM and 4.2 μM, respectively, but different efficiencies, the apparent turnover number for CK2α being 10-fold higher. With both catalytic subunits, phosphorylation of human TS, like the previously reported calmodulin [40], and BID [42], is strongly inhibited in the presence of the regulatory subunit CK2β, the holoenzyme being activated by polylysine. Although recombinant rat, mouse and *T. spiralis* TSs proteins are also phosphorylated by human CK2α and CK2α′, the human enzyme is apparently a much better substrate than the others. Phosphoserine was identified in human and rat, and phosphotyrosine in *T. spiralis*, TS, used as substrates for CK2α. The human enzyme phosphorylation site, identified at Ser^{124} by MALDI-TOF MS analysis, is located in the loop 107–128, considered important for its catalytic mechanism or regulation. In accord, incorporation of 0.4 mol of phosphate per mol of CK2α-phosphorylated human TS resulted in unaltered K_m values for dUMP and meTHF, but a 50% lower turnover number, pointing to a strong influence of Ser^{124} phosphorylation on the enzyme catalytic efficiency.

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