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Thiophosphorylation of free amino acids and enzyme protein by thiophosphoramidate ions

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ABSTRACT

In search of an activity-preserving protein thiophosphorylation method, with thymidylate synthase recombinant protein used as a substrate, potassium thiophosphoramidate and diammonium thiophosphoramidate salts in Tris- and ammonium carbonate based buffer solutions were employed, proving to serve as a non-destructive environment. Using potassium phosphoramidate or diammonium thiophosphoramidate, a series of phosphorylated and thiophosphorylated amino acid derivatives was prepared, helping, together with computational (using density functional theory, DFT) estimation of ^{31}P NMR chemical shifts, to assign thiophosphorylated protein NMR resonances and prove the presence of thiophosphorylated lysine, serine and histidine moieties. Methods useful for prediction of ^{31}P NMR chemical shifts of thiophosphorylated amino acid moieties, and thiophosphates in general, are also presented. The preliminary results obtained from trypsin digestion of enzyme shows peak at m/z 1825.805 which is in perfect agreement with the simulated isotopic pattern distributions for monothiophosphate of TVQQQVHLNQDEYK where thiophosphate moiety is attached to histidine (His^{26}) or lysine (Lys^{33}) side-chain.

1. Introduction

Physicochemical properties of phosphates allow them to play a dominating role in the living world [1]. Thiophosphate (or phosphorothioate) analogues, offering, as compared to the phosphate congeners, potential phosphorus-centered chirality and increased stability towards chemical and enzymatic hydrolysis [2–4], are often used in studies on biological activity of phosphorylated compounds [5–8]. The hydrolytic stability is of particular interest with studies on phosphorylation of protein basic amino acids, as due to their acid-labile character phosphoramidates within proteins easily escape detection by analytical methods [9,10]. Moreover, phosphohistidine, representing probably a major protein modification in eukaryotes [9], applied as a hapten has proved too unstable to generate antibodies [11]. In hope that thiophosphoramidates will be useful as more stable substitutes and probes for the biological function of the corresponding phosphoramidates, methods are sought allowing preparation of thiophosphoramidate-modified biomolecules under conditions mild enough to preserve biological function [12,13].

The first thiophosphorylation of peptide was described by Lasker et al. [12]. Surprisingly, despite the successful preparation of thiophosphohistidine by reaction with thiophosphoramidate, the authors were unable to thiophosphorylate histidine-rich peptide. Therefore they developed another method, employing $PSCl_3$ as a phosphorylation reagent.

The present study was done in search of an activity-preserving protein thiophosphorylation, with thymidylate synthase recombinant protein, found to undergo acid-labile phosphorylation [14], used as a substrate. Instead of phosphorus thiochloride that (i) shows high reactivity towards popular buffer ingredients, (ii) produces acidic conditions when used in water solutions and (iii) could act as a cross-linking agent giving products difficult to analyze [12], potassium thiophosphoramidate and diammonium thiophosphoramidate salts in Tris- and ammonium carbonate based buffer solutions were employed, proving to serve as a non-destructive environment. Using potassium phosphoramidate or diammonium thiophosphoramidate, a series of phosphorylated and thiophosphorylated amino acid derivatives was prepared, helping, together with computational (DFT) estimation of ^{31}P

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NMR chemical shifts, to assign thiophosphorylated protein NMR resonances and prove the presence of thiophosphorylated lysine, serine and histidine moieties.

2. Results and discussion

2.1. NMR spectra of phosphorylated and thiophosphorylated amino acids

The ^{31}P NMR results for side-chain P- and TP-derivatives are presented in Table 1.

The most upfield shifted resonances (−5 to −10 ppm) among investigated phosphorylated amino acids belongs to phosphohistidines (P-His). Histidine reaction with phosphoramidates is significantly faster than in case of other amino acids but P–N bond that was created is vulnerable for acidic conditions as was found by Boyer group [15]. Histidine reaction with phosphoramidate produces two isomers 1-phosphohistidine and 3-phosphohistidine, the last one more thermodynamically stable. It should be noted that P-His isomers differ significantly in their hydrolysis rate, with 1-phosphohistidine having a 5 min hydrolytic half-life at pH 2.4 (46 °C), while 3-phosphohistidine has approx. five times longer half-life [13]. ^{31}P NMR spectrum of histidine and phosphoramidate post-reaction mixture contains two major resonances. The resonances at *upfield* region seem to belong to 1- and 3-phosphorylated histidine derivatives, with more *upfield* shifted resonance (−8 to −10 ppm) belonging to 1-phosphohistidine (δ_1-phosphohistidine) and the other one (−6 and −7 ppm) to 3-phosphohistidine (ε_2-phosphohistidine). ^{31}P NMR spectrum contains also two small singlet resonances belonging to 1,3-diphosphohistidine. The assignment of NMR resonances was based on previously published data [13].

The analogical reaction, carried out with thiophosphoramidate instead of phosphoramidate, led to histidine thiophosphates that are more hydrolytically stable than the corresponding phosphates, thus having significantly longer half-life [15]. The ^{31}P NMR spectrum of thiophosphorylated histidine contained resonances belonging to more thermodynamically stable 3-thiophosphohistidine isomer (3-TP-His; at 34.2 ppm), less stable 1-thiophosphohistidine (at 34.5 ppm) and two much smaller resonances belonging most likely to the 1,3-dithiophosphate species (at 33.7 and 34.4 ppm).

All phospho and thiophospho derivatives of cysteine, serine and lysine showed their resonances in the form of triplets, as an effect of the three-bond proximity of two magnetically equivalent hydrogen atoms. The coupling constants $^3J_{P-H}$ for those systems are in the 6–9 Hz range, in agreement with published data concerning similar compounds [16,17].

The most significant distinction between NMR properties of phosphate and thiophosphate compounds concerns large differences between chemical shifts. While the ^{31}P NMR resonances of P-His are in the −7 to −10 ppm range, those of TP-His are in the 33–35 ppm region. The magnitude of those differences may reflect a distinctly weaker electronic withdrawing effect of sulfur, as compared with oxygen which causes strong electronic deshielding of phosphorus. This *downfield* shifting effect is observable for all phosphorylated and thiophosphorylated amino acid pairs, and is especially visible for thiophosphocysteine, its two sulfur atoms connected to phosphorus and ^{31}P resonance shifted *downfield* to 54.2 ppm.

It should be noted that the averaged differences between ^{31}P NMR resonances of different pairs of P- and TP-amino acids are comparable, ranging from 43.2 ppm difference with 1-P-/1-TP-His, 43.4 ppm with P-/TP-Lys and 44.1 ppm with P-/TP-Ser to 41.0 ppm with 3-P-/3-TP-His and 38.3 ppm difference with P-/TP-Cys. Thus knowing the mean difference value, roughly 42 ppm, may be useful in prediction of ^{31}P NMR shifts of various thiophosphates when chemical shifts of the corresponding phosphates are known.

2.2. DFT calculations of ^{31}P shieldings of TP-amino acids

With an accurate and reliable estimation of chemical shifts for ^{31}P NMR method being still a long-term challenge, little is known in this respect about thiophosphates and their derivatives. While estimation of phosphorus chemical shift $\delta_{P,}$ based on comparison of electronic shielding of particular atoms, might provide very useful information regarding NMR properties of thiophosphates, it has so far poorly described.

Phosphorus electronic shielding calculations of side-chain thiophosphate derivatives of selected amino acids are presented in Table 2. The TP-amino acid models used in these calculations were divided into three groups based on molecular charge of model compound. All of the shielding values of TP-amino acids were converted into chemical shift values using analogically obtained

Table 1
^{31}P NMR chemical shifts of phosphorylated and thiophosphorylated amino acids.

P- or TP-amino acid	^{31}P NMR chemical shift [ppm] and/multiplicity	Averaged P-/TP-amino acid shift difference [ppm]
P-His[a]	−5 to −10/s	–
P-Arg	5–6/s	–
P-Lys	0–0.2/tr	–
P-Cys	15.9/tr	–
P-Ser	−0.3/tr	–
P-Tyr	−3.8–0.0/s	–
P-Thr	−1.4/m	–
TP-His[a]	32.5–34.5/s	43.2 (1-P-His)[e], 41.0 (3-P-His)[e]
TP-Arg	–	–
TP-Lys	43.4/tr	43.4[f]
TP-Cys	54.2/tr	38.3
TP-Ser	43.8/[b]	44.1
$PSCl_3$[c]	54.6/s, 49.7[d]; 28.6[d]; 15.7[d]; −0.1[d]/s	–

[a] Histidine phosphorylation product contain 3-P-His, 1-P-His and diphosphate (with phosphate groups both at 1- and 3-positions) with relative integrals being 13.9:1:0.5 respectively. Also the thiophosphorylation product contain 3-TP-His, 1-TP-His and analogical dithiophosphate with relative integrals being 8.5:1:0.14.
[b] Two doublets.
[c] Common trace contaminant of thiophosphoramidates.
[d] Phosphorus thiochloride hydrolysis products.
[e] Calculated using $\delta_{1\text{-P-His}} = -8.7$ ppm and $\delta_{3\text{-P-His}} = -6.8$ ppm.
[f] $\delta_{P\text{-Lys}}$ of 0.0 ppm was used for calculations (pH 7.5–7.8); higher values up to 0.2 ppm were observed at different pH values.

Table 2
Phosphorus isotropic shielding constants (σ_P, ppm) of side-chain thiophosphorylated amino acids estimated with the B3LYP/aug-cc-pVTZ GIAO calculations. δ_P stands for ^{31}P chemical shift in relation to H_3PO_4, $H_2PO_4^-$ or HPO_4^{2-}.

Neutral TP-amino acid	TP-Arg	TP-Cys	3-TP-His	TP-Lys	TP-Ser	TP-Thr
σ_P [ppm]	248.11	189.71	244.01	234.51	232.93	232.49
δ_P [ppm] rel. to H_3PO_4	62.62	121.02	66.72	76.22	77.80	78.24
δ_P [ppm] rel. to $H_2PO_4^-$	56.07	114.47	60.17	69.67	71.25	71.69
δ_P [ppm] rel. to HPO_4^{2-}	38.46	96.86	42.56	52.06	53.64	54.08
TP-amino acid monoanion						
σ_P [ppm]	255.65	223.81	257.59	255.88	254.84	245.13
δ_P [ppm] rel. to H_3PO_4	55.08	86.92	53.14	54.85	55.89	65.60
δ_P [ppm] rel. to $H_2PO_4^-$	48.53	80.37	46.59	48.30	49.34	59.04
δ_P [ppm] rel. to HPO_4^{2-}	30.92	62.76	28.98	30.69	31.73	41.44
TP-amino acid dianion						
σ_P [ppm]	276.55	216.67	255.33	266.40	256.36	244.65
δ_P [ppm] rel. to H_3PO_4	34.17	94.06	55.40	44.33	54.37	66.08
δ_P [ppm] rel. to $H_2PO_4^-$	27.63	87.51	48.85	37.78	47.82	59.53
δ_P [ppm] rel. to HPO_4^{2-}	10.02	69.90	31.24	20.17	30.21	41.92
TP-Cys 'thiol' form (P–SH) neutral		*TP-Cys 'thiol' form (P–SH) monoanion*				
σ_P [ppm]	261.38	σ_P [ppm]		275.16		
δ_P [ppm] rel. to H_3PO_4	49.35	δ_P [ppm] rel. to H_3PO_4		35.57		
δ_P [ppm] rel. to $H_2PO_4^-$	42.80	δ_P [ppm] rel. to $H_2PO_4^-$		29.02		
δ_P [ppm] rel. to HPO_4^{2-}	25.19	δ_P [ppm] rel. to HPO_4^2		11.41		

values of reference compounds – neutral H_3PO_4, monoanionic $H_2PO_4^-$ and HPO_4^{2-} dianion using the following equation:

$$\delta_P = \sigma_{ref} - \sigma_x \quad (\text{Ref. } [15])$$

where δ_P is phosphorus NMR chemical shift, σ_{ref} – isotropic phosphorus shielding of reference compound and σ_x – isotropic phosphorus shielding of analyzed compound.

It should be noted that precise estimation of phosphorus NMR chemical shift is still an unsolved and very difficult problem because of (i) unreliable estimation of concentration of neutral, mono- and dianionic forms in solution, (ii) complexity of interactions between analyzed compounds and buffer ingredients and also (iii) calculations of shieldings of those mentioned forms being still inaccurate. In our calculations the best match of obtained shift values with experimental ones was achieved with neutral TP-amino acid models (Table 2, Fig. 1). The experimental values of ^{31}P NMR chemical shifts for 3-TP-His (32.5 ppm), TP-Lys (43.4 ppm) and TP-Ser (43.8 ppm) could be correlated with neutral TP-amino acid models (related to H_3PO_4) by subtracting an additional factor of 33.7 ppm value. This subtraction yields the following values of correlated chemical shifts: 33.0 ppm (TP-His, −0.4 ppm difference), 42.5 ppm (TP-Lys, 0.88 ppm difference) and 44.1 ppm (TP-Ser, −0.30 ppm difference). These values are in a good agreement with experimental ones that showed ^{31}P NMR shifts of phosphates and thiophosphates to have their resonances within the range of almost 150 ppm. The computational prediction of ^{31}P chemical shift for TP-Arg and TP-Thr was also made (Table 2), suggesting chemical shifts of 28.9 ppm for TP-Arg and 44.5 ppm for TP-Thr.

Of interest is that the chemical shift values of TP-Cys phosphorus (see Table 2), as compared with those for TP-His, TP-Ser and TP-Lys, are much higher, although calculated in an identical manner. Furthermore, this value (87.3 ppm) differs considerably from experimental one (54.2 ppm). The explanation of this phenomenon was described previously by our research group [18]. The calculation results strongly suggest that TP-cysteine is existing in equilibrium of 'thione' tautomeric form with P=S formal double bond along with 'thiol' form with P=O formal double bond and ionized or protonated sulfur P–S^-/P–SH. Those 'thiol' tautomeric forms are having their ^{31}P NMR resonances *upfield* shifted by a factor of 20–30 ppm [18]. In order to estimate the chemical shift of 'thiol' form, we have conducted another set of calculations and those results are presented in Table 2 (bottom part). The calculated phosphorus chemical shift of neutral form of 'thiol' TP-Cys (in relation to H_3PO_4, with 33.7 ppm correlation factor) is approx. 16.7 ppm

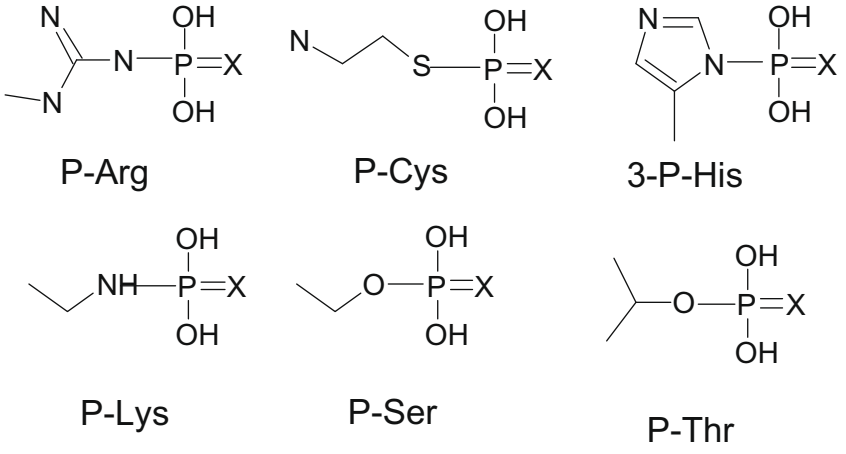

Fig. 1. Structures of the models of phosphorylated and thiophosphorylated amino acids. The symbol *X* denotes O or S atom.

being very similar to previously described thiophosphate 'thiol' forms [18]. The almost equimolar equilibrium of 'thione' (87.3 ppm) and 'thiol' (16.7 ppm) forms should give observed value of 54.2 ppm.

2.3. Thiophosphorylation of Caenorhabditis elegans thymidylate synthase

Fig. 2 presents thiophosphate 'fingerprint' region of ^{31}P NMR spectrum. Spectrum B at Fig. 2 presents results of the thiophosphorylation of Tris–HCl buffer, identical to one used for modification of thymidylate synthase. The thiophosphorylated Tris (fragment B at Fig. 2) presents two multiplets at approx. 42.3 and 43.9 ppm that are also observable in thiophosphorylated protein NMR spectrum (spectrum C at Fig. 2). The resonances of thiophosphorylated Tris at 42.3, 43.9 ppm (J_{P-H} = 7.6 Hz and 6.6 Hz respectively) are triplets as a result of heteronuclear coupling of thiophosphate phosphorus with two magnetically equivalent hydrogen atoms from neighboring methylene group [16]. Spectrum A in Fig. 2 presents the above described fingerprint region of thiophosphorylated protein with H-decoupling applied, showing clearly that thiophosphorylated Tris resonances are singlets, thus proving the three-bond P–H couplings.

The thiophosphorylated thymidylate synthase NMR spectrum is presented in Fig. 2, part C. Besides the thiophosphorylated Tris resonances, two triplets are present at approx. 43.2 and 43.3 ppm (coupling constants J_{P-H} = 6.5 and 5.6 Hz respectively) and two doublets at 43.8 ppm (J_{P-H} = 5.7 and 8.5 Hz). Coupling constants mentioned above are in the range typical for three-bond P–H coupling [19]. All of the resonances from thiophosphorylated protein are singlets with H-decoupling applied (Fig. 2, spectrum A), proving the three-bond proximity of thiophosphate phosphorus to the methylene hydrogen atoms. The resonance at approx. 43.8 ppm apparently belongs to the thiophosphorylated serine side-chain hydroxyl. The ^{31}P resonance of newly formed TP-Ser is in the form of two doublets, resulting from the heteronuclear coupling with phosphorus of two magnetically nonequivalent (shift difference 0.04 ppm) hydrogen atoms from TP-serine moiety methylene group. Those two magnetically nonequivalent hydrogen atoms have very similar coupling constants $^{3}J_{P-H}$ = 5.65 and 5.67 Hz. TP-Ser thesis is strongly supported by an almost identical chemical shift of TP-serine obtained by direct thiophosphorylation of serine (see Table 1) and ^{31}P–^{1}H gradient enhanced HMBC spectra pointing to 3.6 ppm as ^{1}H chemical shift of phosphorus-coupled hydrogen atoms.

Two triplet resonances at approx. 43.3 and 43.4 ppm correspond apparently to TP-Lys as thiophosphorylation of free amino acid lysine gave a very similar result (Table 1). The ^{31}P NMR spectrum of TP-Lys that was measured at identical pH as thiophosphorylated thymidylate synthase sample, contains a triplet resonance at 43.4 ppm. Also, the ^{31}P–^{1}H HMBC spectra confirm that phosphorus-coupling hydrogen atoms have ^{1}H NMR shifts of approx. 3.1 ppm, corresponding to ε-CH_2 group of lysine moiety. Another interesting observation that stems from the 2D spectrum is that the phosphorus atoms of both triplet resonances in TP-lysine region (43.3–43.4 ppm) are coupled to exactly the same ^{1}H chemical shift (3.1 ppm), suggesting those two triplets to reflect two forms of protein TP-lysine moiety resulting probably from a slow, at NMR-scale, protonation/deuteration of lysine ε-nitrogen atom caused by bulky thiophosphate substituent.

The results of our studies on free amino acid phosphorylation and thiophosphorylation pointed to histidine being the fastest reacting amino acid in the phosphorylation and thiophosphorylation reactions (not shown). Histidine thiophosphorylation reactions were performed with the free amino acid, as well as *C. elegans* recombinant thymidylate synthase protein (see Figs. 3 and 4, Table 1).

The free amino acid histidine thiophosphorylation reaction was carried out in ammonium carbonate buffer (pH 7.4). Series of resonances were found at 32.5–34.5 ppm region that is very near to that from an older work of Pirrung group showing approx 35 ppm chemical shift of TP-His [13]. However, that spectrum was obtained in deuterium oxide and no exact pH or pD value were presented; it should be noted that ^{31}P NMR chemical shifts of ions significantly depend on pH (or pD). The ^{31}P NMR spectrum of thiophosphorylated thymidylate synthase also presents two singlet resonances at 32.5 and 32.7 ppm laying in the TP-His region (Fig. 3). The more *downfield* shifted resonance has relative integral of 1.0 with another *upfield* shifted one of 1.7. The assignment of those two resonances was made by analysis of free histidine thiophosphorylation NMR results and limited literature data [13,15]. It seems that more *upfield* shifted resonance belongs to hydrolytically more stable 3-thiophosphohistidine (3-TP-His) and another one *downfield* shifted resonance from less hydrolytically stable 1-thiophosphohistidine (1-TP-His).

The exceptionally interesting part of the spectrum of thiophosphorylated thymidylate synthase is presented in Fig. 4. There are two singlet resonances at −6.2 and −6.3 ppm laying in region characteristic for phosphohistidines (P-His). Our free amino acid hisitidine phosphorylation results confirm that those two singlet resonances almost certainly belong to the 1-P-His and 3-P-His being most likely a hydrolysis product of 1-TP-His and 3-TP-His respectively. Relative integrals measured for those two resonances

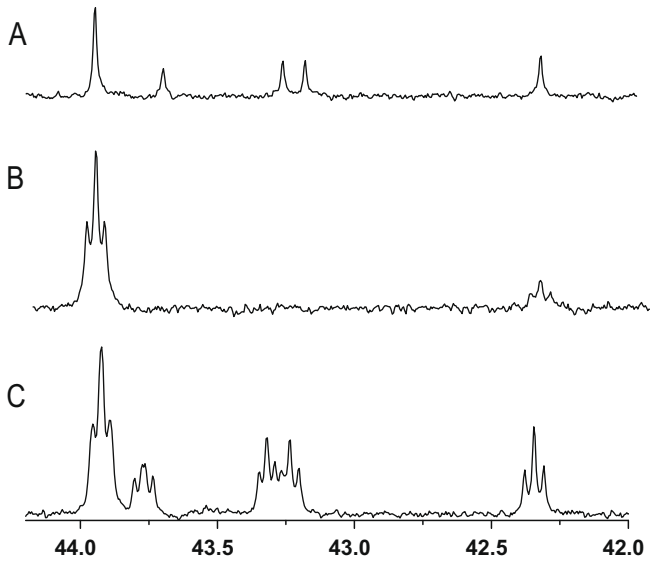

Fig. 2. Thiophosphate fingerprint region of ^{31}P NMR spectrum of thymidylate synthase thiophosphorylation product with ^{31}P–^{1}H decoupling (A) and without H-decoupling (C). Spectrum B shows thiophosphorylation product of Tris–HCl buffer.

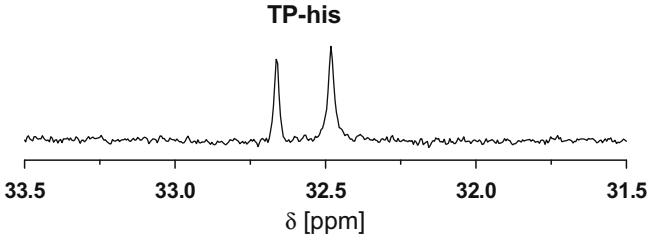

Fig. 3. Thiophosphohistidine resonances of ^{31}P NMR spectrum of thiophosphorylated thymidylate synthase.

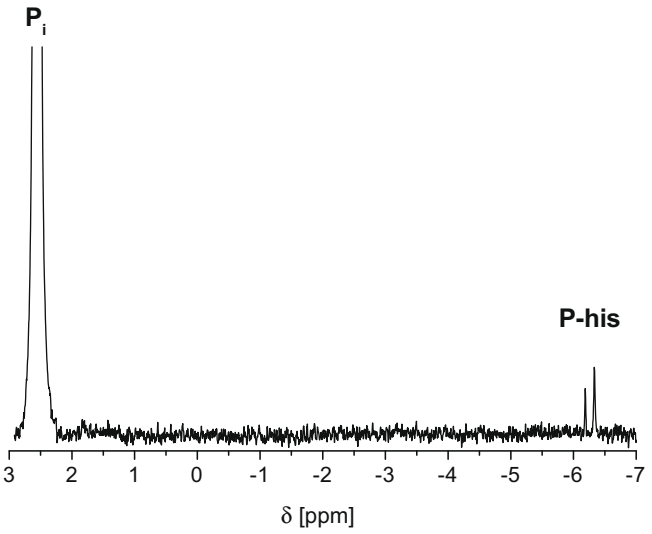

Fig. 4. Phosphohistidine resonances of ^{31}P NMR spectrum of thiophosphorylated thymidylate synthase. P_i = inorganic phosphate.

are 1:2.4 for 1-P-His and 3-P-His respectively. Thiophosphate may hydrolyze to phosphate in aqueous solutions, as evidenced by the time-dependent growth of the inorganic phosphate resonance at approx 2.2–2.4 ppm (see Fig. 4).

The enzyme thiophosphorylation was carried out also in ammonium carbonate buffer, pH 7.4, with the use of diammonium thiophosphoramidate. The results were similar to those obtained with Tris buffer but for (i) much lower total amount of thiophosphohistidines, (ii) higher 3-TP-His/1-TP-His molar ratio and (iii) much smaller corresponding resonances from TP-Ser and TP-Lys.

The mass spectrometry was also used to propose the location of the thiophosphate moiety in the thymidylate synthase molecule. The preliminary results obtained from trypsin digestion of enzyme shows peak at *m*/*z* 1825.805 The experimental isotopic patterns of the positively charged ion are in perfect agreement with the simulated isotopic pattern distributions for monothiophosphate of TVQQQVHLNQDEYK where thiophosphate moiety is attached to histidine (His^{26}) or lysine (Lys^{33}) side-chain (Fig. 1 in supplementary material).

Potential influence of the thiophosphorylation on thymidylate synthase activity was tested with recombinant human and *C. elegans* preparations of the enzyme. Each preparation underwent thiophosphorylation with diammonium TPA in Tris–HCl buffer, pH 7.5, at 4 °C under conditions described in Material and methods, with the enzyme activity determined in samples of the reaction mixture taken just after addition of diammonium TPA, and at several time points during 24 h period. Separate samples were incubated under the same conditions, but without diammonium TPA added, in order to test the enzyme stability. The results indicated the enzyme activity to be lowered, following 24 h reaction, to ~50% of the control value due to the thiophosphorylation process alone, pointing to a possibility that the modification influences the enzyme-catalyzed reaction parameters, as reported for phosphorylation [14].

3. Conclusions

The thiophosphorylation reactions of recombinant thymidylate synthase protein were performed using potassium thiophosphoramidate and diammonium thiophosphoramidate salts in Tris- and ammonium carbonate based buffer solutions at non-destructive environment. The NMR spectra of obtained modified proteins were assigned, based on NMR spectra of thiophosphorylated amino acid samples and the computational (DFT) estimation of ^{31}P NMR chemical shifts, indicating the presence of thiophosphorylated lysine, serine and histidine moieties. Several methods presented here should be useful for prediction of ^{31}P NMR chemical shifts of thiophosphorylated amino acid moieties and even thiophosphates in general.

4. Experimental

4.1. Materials and methods

Cellulose dialysis tubing, DE52-Cellulose, O-phosphotyrosine and O-phosphothreonine were purchased from Sigma–Aldrich. Diammonium- and potassium phosphoramidates (diammonium TPA and KTPA respectively) were prepared according to Pirrung et al. (2000).

4.2. NMR analysis

All NMR spectra were obtained with Bruker Avance spectrometer operating in the quadrature mode at 500.13 MHz for ^{1}H and 202.46 MHz for ^{31}P nuclei. The residual peaks of deuterated solvents were used as internal standards in ^{1}H NMR method. ^{31}P NMR spectra were recorded at 277 K both with and without proton decoupling. The internal standard used in ^{31}P NMR was inorganic phosphate (P_i) having its resonance at 2.15 ppm (at pH 7.8), 2.05 ppm (pH 7.5), 1.65 ppm (at pH 5.0) and 0.0 ppm (at pH 1.5). All samples were analyzed using gradient enhanced ^{1}H–^{31}P Heteronuclear Multiple Bond Correlation (HMBC) experiments. The HMBC experiments were optimized for long range couplings by using different $^{3}J_{P-H}$ values (1–20 Hz). The ^{1}H NMR spectra were obtained with the use of HDO suppression method. All buffer solutions used for NMR spectroscopy were based on deuterium oxide of 100%D purity (Armar Chemicals AG).

4.3. Mass spectrometry

The mass spectrum was obtained on a Bruker MicrOTOF-Q spectrometer (Bruker Daltonik, Bremen, Germany), equipped with Apollo II electrospray ionization source with ion funnel. The mass spectrometer was operated in the positive ion mode. The instrumental parameters were as follows: scan range *m*/*z* 200–2200, dry gas–nitrogen, temperature 180 °C, ion source voltage 4500 V, reflector voltage 1300 V, detector voltage 1920 V. The instrument was calibrated externally with the Tunemix™ mixture (Bruker Daltonik, Germany) in quadratic regression mode. The sample (0.1 M Tris–HCl buffer of pH 7.5 containing approx. 50 μg of protein and traces of thiophosphoramidate and thiophosphate salts) was diluted twice with methanol and infused at a flow rate of 3 μl/min. The mass spectrum was deconvoluted using the maximum entropy method.

The protein (0.2 mg) digestion was performed in the 0.2 M Tris–HCl buffer (pH 7.5) by addition of trypsin (10 μg) the reaction mixture was incubated at 24° C overnight. The solution was desalted on C18 Sep-pak Plus Cartridges (Waters, Ireland). Washed with water (10 ml) and eluted with acetonitrile (0.7 ml). The solution was concentrated *in vacuo* and introduced to mass spectrometer.

Analysis of the obtained spectra was performed with the Bruker Daltonics Compass DataAnalysis v.4 software The mass accuracy together with the isotopic pattern (using SmartFormula) enabled an unambiguous confirmation of the elemental composition of the obtained tryptic peptides.

4.4. Calculations

The theoretical calculations have been performed with the density functional B3LYP/aug-cc-pVTZ method. To save computational time, the structures of phosphorylated and thiophosphorylated amino acids were truncated by removing some groups of atoms remote from the phosphorylated regions (Fig. 1). The optimal geometries were obtained and confirmed with positive harmonic frequencies, then NMR shieldings for models of phosphorylated and thiophosphorylated amino acids in their neutral, monoanionic and dianionic forms were calculated. All the calculations were performed with the Gaussian G03 (rev. C.02) suite of programs [20].

4.5. Thiophosphorylation of amino acids (L-histidine, L-arginine, L-lysine, L-cysteine, L-serine, L-proline and L-tyrosine)

Ammonium carbonate buffer solution of amino acid (0.2 mmol of amino acid in 500 μl of buffer at pH 7.4) was reacted with diammonium thiophosphoramidate (1:3 amino acid to diammonium TPA molar ratios). Reaction mixture was shaken for 72 h at 277–278 K temperature. NMR sample was prepared by mixing 400 μl of post-reaction mixture with 300 μl of deuterium oxide. NMR results are presented in Table 1.

4.6. Phosphorylation of amino acids (L-histidine, L-arginine, L-lysine, L-cysteine, L-serine, L-proline, L-tyrosine and L-threonine)

Water solution of amino acid (0.2 mmol of amino acid in 500 μl of water) was reacted with potassium phosphoramidate (1–10 amino acid to KPA molar ratios). Reaction mixture was shaken for 48 h at 277–278 K temperature. NMR sample was prepared by mixing 100 μl of post-reaction mixture with 200 μl of deuterium oxide and 400 μl of Tris–HCl buffer (pH 7.5). NMR results are presented in Table 1.

4.7. Thymidylate synthase purified preparations and assay of activity

Human recombinant enzyme was expressed in thymidylate synthase-deficient *E. coli* TX61$^-$ strain (a kind gift from Dr. W.S. Dallas) as previously described [21], with the use of the plasmid construct pET17xb/hTS(LVAG) described by Pedersen-Lane et al., [22] and purified using the methods described for the rat enzyme [23]. *C. elegans* recombinant thymidylate synthase was expressed and purified as previously described [24]. The enzyme activity spectrophotometric assay [25] and protein content determination [26] were done by earlier described methods.

4.8. Thiophosphorylation of thymidylate synthase with potassium thiophosphoramidate (KTPA)

The reaction was started by introducing potassium thiophosphoramidate (42.0 mg) into 290 μl of the protein (1.064 mg) in 0.2 M Tris–HCl, pH 7.5. The reaction mixture was then shaken for 24 h at 277 K and analyzed using NMR spectrometer (300 μl of D_2O was added).

4.9. Thiophosphorylation of thymidylate synthase with diammonium thiophosphoramidate (diammonium TPA)

The reaction mixture was obtained by mixing 290 μl of the protein (1.065 mg) solution in 0.2 M Tris–HCl, pH 7.5, with diammonium thiophosphoramidate (41.0 mg). The reaction mixture was then shaken for 24 h at 277 K. The NMR sample contained obtained post-reaction mixture and 300 μl of deuterium oxide.

4.10. Thiophosphorylation of thymidylate synthase with diammonium TPA with buffer exchange

To the protein buffer solution (1 ml of 0.2 M Tris–HCl, pH 7.5 containing 2.1 mg of protein) diammonium TPA (23.0 mg) was added and shaken for 7 h at 277 K. Dialysis of the post-reaction mixture was then conducted by introducing obtained mixture into dialysis bag and dialyzed four times in ammonium carbonate buffer (4×300 ml of 0.1 M ammonium carbonate buffer, pH 7.4). NMR sample contained 0.5 ml of dialysis product and 0.3 ml of deuterium oxide.

4.11. Thiophosphorylation of thymidylate synthase with diammonium TPA in ammonium carbonate buffer

Diammonium TPA (13.4 mg) was introduced into protein solution (840 μg of *C. elegans* thymidylate synthase in 0.1 M ammonium carbonate buffer pH 7.4).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2009.11.002.

References

[1] F.H. Westheimer, Science 235 (1987) 1173–1178.
[2] Z. Zhao, Biochem. Biophys. Res. Commun. 218 (1996) 480–484.
[3] L.A. Woźniak, A. Okruszek, Chem. Soc. Rev. 32 (2003) 158–169.
[4] J.J. Allen, S.E. Lazerwith, K.M. Shokat, J. Am. Chem. Soc. 127 (15) (2005) 5288–5289.
[5] F. Eckstein, Annu. Rev. Biochem. 54 (1985) 367–402.
[6] F. Eckstein, G. Gish, Trends Biochem. Sci. 14 (1989) 97–100.
[7] O. Heidenreich, W. Pieken, F. Eckstein, FASEB J. 7 (1993) 90–96.
[8] A. Mescalchin, A. Detzer, M. Wecke, M. Overhoff, W. Wünsche, G. Sczakiel, Expert Opin. Biol. Ther. 7 (2007) 1531–1538.
[9] H.R. Matthews, Pharmacol. Ther. 67 (1995) 323–350.
[10] S. Klumpp, J. Krieglstein, Biochim. Biophys. Acta 1754 (2005) 291–295.
[11] X.L. Zu, P.G. Besant, P.V. Attwood, Compr. Anal. Chem. 52 (2009) 315–352.
[12] M. Lasker, C.D. Bui, P.G. Besant, K. Sugawara, P. Thai, G. Medzihradszky, C.W. Turck, Protein Sci. 8 (10) (1999) 2177–2185.
[13] M.C. Pirrung, K.D. James, V.S. Rana, J. Org. Chem. 65 (2000) 8448–8453.
[14] T. Frączyk, T. Ruman, D. Rut, E. Dąbrowska-Maś, J. Cieśla, Z. Zieliński, K. Sieczka, J. Sikora, E. Wałajtys-Rode, D. Shugar, W. Rode, Anticancer Res. 28 (2008) 3462–3463.
[15] D.E. Hultquist, R.W. Moyer, P.D. Boyer, Biochemistry 5 (1966) 322–331.
[16] A.A. Isab, M.S. Hussain, M.N. Akhtar, M.I.M. Wazeer, A.R. Al-Arfaj, Polyhedron 18 (1999) 1401.
[17] J. Jankowska, A. Sobkowska, J. Cieślak, M. Sobkowski, A. Kraszewski, J. Skawiński, D. Shugar, J. Org. Chem. 63 (1998) 8150.
[18] T. Ruman, K. Długopolska, A. Jurkiewicz, D. Kramarz, T. Frączyk, A. Leś, W. Rode, The synthesis, reactivity and NMR investigation on^{15} N-thiophosphoramidates, Lett. Org. Chem. 6 (8) (2009).
[19] M.J. Gallagher, I.D. Jenkins, Topics in Stereochemistry, vol. 3, John Wiley & Sons, Inc., New York, 1968.
[20] M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, J.A. Montgomery Jr., T. Vreven, K.N. Kudin, J.C. Burant, J.M. Millam, S.S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G.A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J.E. Knox, H.P. Hratchian, J.B. Cross, C. Adamo, J. Jaramillo, R. Gomperts, R.E. Stratmann, O. Yazyev, A.J. Austin, R. Cammi, C. Pomelli, J.W. Ochterski, P.Y. Ayala, K. Morokuma, G.A. Voth, P. Salvador, J.J. Dannenberg, V.G. Zakrzewski, S. Dapprich, A.D. Daniels, M.C. Strain, O. Farkas, D.K. Malick, A.D. Rabuck, K. Raghavachari, J.B. Foresman, J.V. Ortiz, Q. Cui, A.G. Baboul, S. Clifford, J. Cioslowski, B.B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R.L. Martin, D.J. Fox, T. Keith, M.A. Al-Laham, C.Y. Peng, A. Nanayakkara, M. Challacombe, P.M.W. Gill, B. Johnson, W. Chen, M.W. Wong, C. Gonzalez, J.A. Pople, Gaussian 03, Revision C.02, Gaussian, Inc., Wallingford, CT, 2008.

[21] J. Cieśla, B. Gołos, E. Wałajtys-Rode, E. Jagielska, A. Płucienniczak, W. Rode, Acta Biochim. Pol. 49 (2002) 651–658.
[22] J. Pedersen-Lane, G.F. Maley, E. Chu, F. Maley, Protein Exp. Purif. 10 (1997) 256–262.
[23] J. Cieśla, K.X. Weiner, R.S. Weiner, J.T. Reston, G.F. Maley, F. Maley, Biochim. Biophys. Acta 1261 (1995) 233–242.
[24] P. Wińska, B. Gołos, J. Cieśla, Z. Zieliński, T. Frączyk, E. Wałajtys-Rode, W. Rode, Parasitology 131 (2005) 247–254.
[25] A.J. Wahba, M. Friedkin, J. Biol. Chem. 236 (1961) PC11–PC12.
[26] T. Spector, Anal. Biochem. 86 (1978) 142–146.