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PAPER

Tyrosine nitration affects thymidylate synthase properties

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Highly purified preparations of thymidylate synthase, isolated from calf thymus, and L1210 parental and FdUrd-resistant cells, were found to be nitrated, as indicated by a specific reaction with anti-nitro-tyrosine antibodies, suggesting this modification to appear endogenously in normal and tumor tissues. Each human, mouse and *Ceanorhabditis elegans* recombinant TS preparation, incubated *in vitro* in the presence of $NaHCO_3$, $NaNO_2$ and H_2O_2 at pH 7.5, underwent tyrosine nitration, leading to a V_{max}^{app} 2-fold lower following nitration of 1 (with human or *C. elegans* TS) or 2 (with mouse TS) tyrosine residues per monomer. Enzyme interactions with dUMP, meTHF or 5-fluoro-dUMP were not distinctly influenced. Nitration under the same conditions of model tripeptides of a general formula H_2N-Gly-X-Gly-COOH (X = Phe, Tyr, Trp, Lys, Arg, His, Ser, Thr, Cys, Gly), monitored by NMR spectroscopy, showed formation of nitro-species only for H-Gly-Tyr-Gly-OH and H-Gly-Phe-Gly-OH peptides, the chemical shifts for nitrated H-Gly-Tyr-Gly-OH peptide being in a very good agreement with the strongest peak found in ^{15}N-^{1}H HMBC spectrum of nitrated protein. MS analysis of nitrated human and *C. elegans* proteins revealed several thymidylate synthase-derived peptides containing nitro-tyrosine (at positions 33, 65, 135, 213, 230, 258 and 301 in the human enzyme) and oxidized cysteine (human protein Cys^{210}, with catalytically critical Cys^{195} remaining apparently unmodified) residues.

Introduction

Thymidylate synthase (TS; EC 2.1.1.45), a target in chemotherapy of a number of diseases, including cancer,[1] catalyzes the $N^{5,10}$-methylenetetrahydrofolate (meTHF)-assisted C(5)-methylation of dUMP,[2] required for DNA synthesis. It is, consequently, of interest to examine possible post-translational modifications of the enzyme in living cells.

Nitration of protein tyrosine residues is a post-translational modification, potentially affecting the function of a protein. It is associated with more than 50 diseases, including cancer, involving intensified NO biosynthesis.[3] The modification *in vivo* appears to be selective, with not many proteins becoming nitrated and only very few residues being modified in each protein. Moreover, even with good nitration targets, the yield of protein nitro-tyrosine formation is low.[4] Nevertheless, the few known examples show that nitration of one or two tyrosine residues is enough to cause loss or gain of function (for physicochemical consequences of protein tyrosine nitration, *cf*. ref. 4), suggesting a need for studies directed at protein structure-function analysis of specific proteins found to undergo nitration *in vivo*.[5]

As tetranitromethane nitration of sulfhydryl-blocked *Lactobacillus casei* thymidylate synthase protein caused enzyme inactivation,[6] it was of interest to test the possibility of TS tyrosine nitration in animal cells/tissue, and to determine to what extent enzyme properties might be affected by chemical nitration of TS tyrosine. Therefore kinetic and physicochemical (NMR, MS) studies were undertaken of *in vitro* nitrated preparations of human, mouse and *Caenorhabditis elegans* recombinant TSs. In order to enable interpretation of NMR resonances found in nitrated protein spectra, model tripeptides of a general formula H_2N-Gly-X-Gly-COOH (X = Phe, Tyr, Trp, Lys, Arg, His, Ser, Thr, Cys, Gly) were nitrated and analyzed using NMR spectroscopy. Besides, the theoretical calculations of ^{15}N NMR chemical shift for models of the nitrated tripeptides were performed and compared with those found experimentally.

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Results and discussion

Reactivity of purified endogenous TS proteins to anti-tyrosine antibodies

Highly purified TS proteins, isolated from calf thymus, and L1210 parental and FdUrd-resistant cells, were found to be nitrated (Fig. 1), based on a specific reaction with anti-nitrotyrosine antibodies (Sigma-Aldrich, Anti-Nitro-tyrosine, Cat. No. N0409), suggesting the enzyme to undergo this modification endogenously in normal and tumor tissues. The reaction was specific to tyrosine, as the presence of nitro-tyrosine (10 mM) in the buffer containing anti-tyrosine antibodies (Fig. 1B), as well as the reduction of nitro-tyrosine to amino-tyrosine with $Na_2S_2O_4$ (Fig. 1C), prevented anti-tyrosine antibodies binding with the proteins. Of note is that the lack of the signal in panels B and C (Fig. 1) was not caused by an insufficient amount of protein, as demonstrated by the incubation of the same PVDF membrane with anti-TS antibodies (Fig. 1, E–F). It should be worthwhile to add that, according to the manufacturer, the polyclonal antibodies preparation used is fairly specific for protein nitro-tyrosine. While it recognizes nitrated proteins and 3-nitro-L-tyrosine, it does not cross-react with L-tyrosine, *p*-nitro-L-phenylalanine, 3-amino-L-tyrosine, 3-chloro-L-tyrosine and phospho-L-tyrosine BSA conjugates (http://www.sigmaaldrich.com/life-science/cell-biology/antibodies/learning-center/antibody-explorer/spotlights/anti-nitrotyrosine.html).

A comparison of nitration levels observed for the three endogenous enzyme preparations (Fig. 1, lanes 5–7) and that of the *in vitro* nitrated mouse recombinant enzyme determined to contain 0.8 and 1.6 mol/mol of TS monomer (Fig. 1, lanes 2 and 3, respectively), based on the ratio of signal intensities resulting from application of anti-nitro-tyrosine and anti-thymidylate synthase antibodies, indicates the modification level of the endogenous proteins to be much lower and concern presumably only a small fraction of each of those proteins.

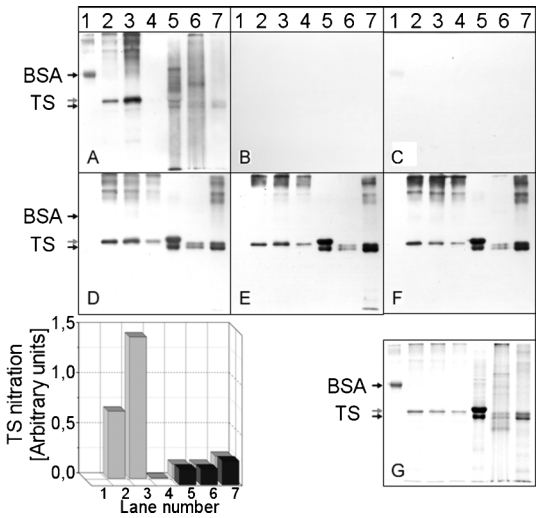

Fig. 1 Nitro-tyrosine detection by specific antibodies in chemically nitrated mammalian recombinant TS and endogenous enzyme preparations purified from tumour and normal tissues and separated by SDS-PAGE (without 2-mercaptoethanol). Proteins were stained with Sypro® Ruby Protein Gel Stain (G) or, following transfer to PVDF membrane, underwent first reaction with anti-nitroY antibodies (A–C), followed by removing of anti-nitroY antibodies and treatment with anti-TS antibodies (D–F). Negative controls included treatment with anti-nitroY antibodies either in the presence of 10 mM nitro-tyrosine (B) or following reduction of nitro-tyrosine to amino-tyrosine with 100 mM $Na_2S_2O_4$ at pH 9,0 (C). Nitrated BSA (positive control; lane 1), mouse recombinant TS nitrated with 8 mM (0.8 mol nitroY/mol TS subunit; lane 2), 12 mM (1.6 mol nitroY/mol TS subunit; lane 3) or 12 mM inactivated (negative control, 0 mol nitroY/mol TS; lane 4) peroxynitrite, and endogenous TS purified from calf thymus (lane 5), and L1210 parental (lane 6) and FdUrd-resistant (lane 7) cells. TS nitration presented in the bar chart was calculated as the ratio of signals deriving from bands of nitrated protein (A) and TS protein (D), with lighter and darker bars corresponding TS bands (marked with lighter and darker arrows in A and B) showing lower and higher mobility, respectively.

Recombinant TS *in vitro* nitration and its effect on enzyme properties

Each human, mouse and *Ceanorhabditis elegans* recombinant TS preparation, incubated *in vitro* at pH 7.5 in the presence of peroxynitrite ($ONOO^-$) producing mixture, containing $NaHCO_3$, $NaNO_2$ and H_2O_2 (1.00 : 1.05 : 1.00), underwent [H_2O_2]-dependent tyrosine nitration (Fig. 1, lanes 2–4, and Fig. 2; *Note*: H_2O_2 concentration reflects concentration of the peroxynitrite producing mixture, its constituents present always in constant proportion). The reaction dependence on H_2O_2 concentration varied in different TS proteins, being similar with the human (Fig. 2) and *C. elegans* proteins (linear progress observed in the range of 10–70 mM H_2O_2), but distinctly different with the mouse protein (linear progress observed in the range of only 5–20 mM H_2O_2, with lower nitration at higher concentrations). While this modification did not distinctly influence the K_m values reflecting enzyme interactions with dUMP and meTHF or the inhibition and inactivation rate constants (not shown) reflecting slow-binding of TS by 5-fluoro-dUMP (*cf.* ref. 7), it affected TS activity, leading to a V_{max}^{app} 2-fold lower following nitration of 1 (with human or *C. elegans* TS) or 2 (with mouse TS) tyrosine residues per monomer (not shown). It should be mentioned that initial experiments, involving TS nitration with synthesized authentic peroxynitric acid, showed the enzyme to undergo an instant inactivation (not shown), presumably due to the catalytic cysteine[2] modification. In accord, application of the peroxynitrite producing mixture, including CO_3^{2-}, known to inhibit sulfhydryl oxidation and enhance nitration of aromatics,[8] allowed to study nitration with the enzyme activity preserved.

NMR analyses of nitrated model compounds

In order to enable interpretation of resonances found in nitrated protein spectra, model compounds, including free amino acids (Phe, Tyr and Trp) and tripeptides of a general formula H_2N-Gly-X-Gly-COOH (X = Phe, Tyr, Trp, Lys, Arg, His, Ser, Thr, Cys, Gly), were nitrated and analyzed using NMR spectroscopy. While the three free amino acids did not allow quantitative analysis due to relatively low solubility of aromatic amino acids, such a study was possible with the use of the tripeptides, serving as simple models of proteins. The ^{15}N chemical shifts resulting from NMR studies, as well as DFT calculations performed for nitrated forms of truncated amino acid moieties (Fig. 3), are presented in Table 1. As the experimental NMR data clearly showed formation of nitro-species only for H-Gly-Tyr-Gly-OH and

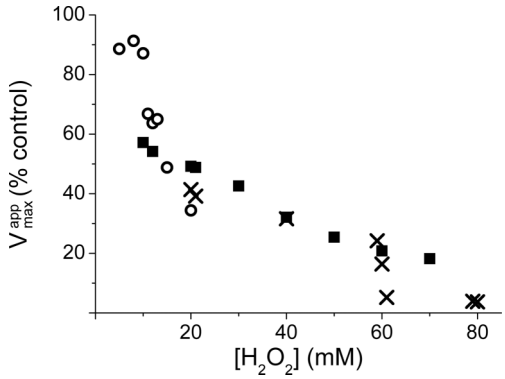

Fig. 2 Chemical nitration of human (squares), mouse (circles) and *C. elegans* (crosses) recombinant TS proteins: dependence of nitrated enzyme catalytic potency, reflected by the V_{max}^{app} value, on H_2O_2 concentration in the reaction mixture (Note: H_2O_2 concentration reflects concentration of the peroxynitrite producing mixture, its constituents present always in constant proportion). V_{max}^{app} value was determined at varying [dUMP] and constant [$N^{5,10}$-methylenetetrahydrofolate].

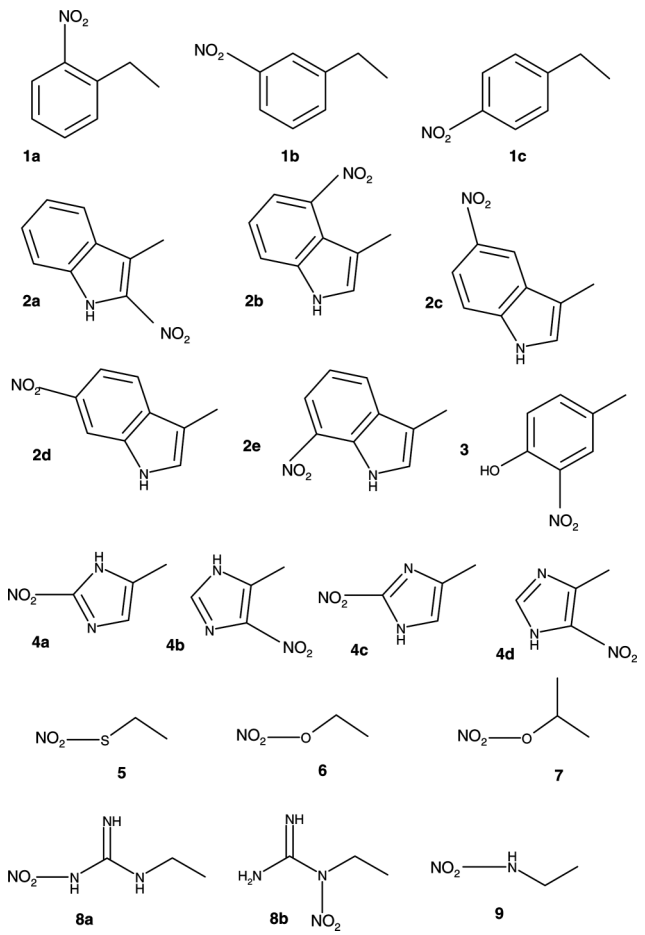

Fig. 3 Models of nitro-aminoacids used for DFT calculations: **1a** – *o*-NO_2Phe, **1b** – *m*-NO_2Phe, **1c** – *p*-NO_2Phe, **2a** – 2-NO_2Trp, **2b** – 4-NO_2Trp, **2c** – 5-NO_2Trp, **2d** – 6-NO_2Trp, **2e** – 7-NO_2Trp, **3** – 3-NO_2Tyr, **4a** – 2-NO_2-His N1-H, **4b** – 5-NO_2-His N1-H, **4c** – 2-NO_2-His N3-H, **4d** – 5-NO_2-His N3-H, **5** – *S*-NO_2Cys, **6** – *O*-NO_2Ser, **7** – *O*-NO_2Thr, **8a** – *N*-ω-NO_2Arg, **8b** – *N*-ε-NO_2Arg, **9** – *N*-NO_2Lys.

H-Gly-Phe-Gly-OH peptides, NMR-DFT comparison of chemical shifts was possible for these two systems. However, calculated ^{15}N chemical shifts, with chemical shift differences calculated as $\Delta\delta = \delta_{exp}{}^{15}N - \delta_{cal}{}^{15}N$ ($\delta_{exp}{}^{15}N$ – experimental chemical shift; $\delta_{cal}{}^{15}N$ – calculated chemical shift) for models of nitrated H-Gly-Tyr-Gly-OH and H-Gly-Phe-Gly-OH peptides (Table 1, column 5) taken into consideration, could be useful for prediction of ^{15}N chemical shifts of similar nitro-systems or even nitro-proteins. Of note is that recently Lehnik and Kirsch analyzed peroxynitrite nitration of L-tyrosine and related compounds with the use of ^{15}N CIDNP method.[9]

Nitration of the tyrosine-containing tripeptide resulted in the formation of 3-nitro-tyrosine moiety, along with only traces of other compounds (Fig. 4). Based on the ^{1}H NMR data, the molar ratio of tripeptide containing nitro-tyrosine to that containing tyrosine was 1 : 4.4. The confirmation of H-Gly-Tyr($^{15}NO_2$)-Gly-OH peptide formation was found in the ^{15}N-^{1}H HMBC spectrum (Fig. 5), clearly showing the heteronuclear coupling between ^{15}N-nitrate group (373.4 ppm) and the adjacent aromatic hydrogen atom at the ring C(2) (7.84 ppm). As indicated in Fig. 5, the ^{15}N resonance of the above mentioned nitro-moiety is a doublet, due to the 3-bond proximity of the hydrogen atom at the ring C(2).

Of note are interesting observations resulting from studies on the nitrated phenylalanine-containing tripeptide. The peaks observed in the ^{15}N-^{1}H HMBC spectrum (not shown) suggest the existence of two compounds, each containing nitro group attached to the aromatic ring. The NMR data, describing the cross peak, showing heteronuclear coupling between $^{15}NO_2$ nitrogen (371.2 ppm) and aromatic hydrogen atom (8.09 ppm), are in a very good agreement with those for 4-nitrotoluene that contains identical 4-nitrobenzyl moiety.[10] Thus the latter pair of chemical shifts points to the formation of H-Gly-Phe(NO_2)-Gly-OH peptide, with the $^{15}NO_2$ group generally connected to electronically and sterically favored *para* position.

The second cross peak (Fig. 5) found in ^{15}N-^{1}H HMBC spectrum (7.88 ppm at H-axis and 374.1 ppm) is in a surprisingly good agreement with our data found for H-Gly-Tyr(NO_2)-Gly-OH (*vide supra*). The NMR data strongly suggest the existence of nitro-tyrosine moiety that must have been formed by nitration of tyrosine moiety. Consequently, the tyrosine moiety must have resulted from phenylalanine moiety hydroxylation, a process described in the literature.[11,12] However, the yield of the overall process is very low, as the molar ratio calculated from ^{1}H NMR spectrum shows the amount of nitro-tyrosine-tripeptide to be almost six-fold lower than that of nitro-phenylalanine-tripeptide. The results obtained by ^{15}N NMR were validated by high resolution mass spectrometry (HRMS). The HRMS spectra revealed the presence of peaks corresponding to H-Gly-Phe($^{15}NO_2$)-Gly-OH (MNa^+ calculated 348.0932, found 348.0932), as well as H-Gly-Tyr($^{15}NO_2$)-Gly-OH (MNa^+ calculated 364.0882, found 364.0888). The intensities of the signals assigned to the nitration products, especially to H-Gly-Tyr(NO_2)-Gly-OH, were much lower than those of H-Gly-Tyr-Gly-OH and H-Gly-Phe($^{15}NO_2$)-Gly-OH.

The NMR data for nitrated H-Gly-Trp-Gly-OH peptide demonstrated only traces of nitrated forms as judged from ^{15}N (369.0 ppm) and ^{1}H (8.07 ppm) chemical shifts (not shown). The strongest cross peak in ^{15}N-^{1}H HMBC spectrum showed coupling between positions 400.7 ppm ^{15}N and 1.66 ppm ^{1}H of the spectrum but the chemical shift of the ^{1}H resonance (singlet resonance) can

Table 1 Experimental and calculated ^{15}N NMR chemical shifts of nitrated side chains of selected amino acid moieties

Residue	δ_{exp} ^{15}N (experimental; rel. to NH_3(lq)) [ppm][a]	δ_{exp} ^{1}H (experimental; H adjacent to N) [ppm]	δ_{cal} ^{15}N (calculated; rel. to NH_3(lq)) [ppm][b]	$\Delta\delta$[c] [ppm]	Nitro-product to substrate ratio[g]
o-NO_2Phe			382.8	—	—
m-NO_2Phe			389.7	—	—
p-NO_2Phe			389.7	–18.5	1 : 28.8
	371.2; 374.1[d]	8.09; 7.88[d]			1 : 156.3[d]
2-NO_2Trp	394.1[e]	2.78[e]	400.3	—	—
4-NO_2Trp	360.0[e]	1.70[e]	381.9	—	—
5-NO_2Trp	400.7[e]	1.66[e]	388.2	—	—
6-NO_2Trp			388.9	—	—
7-NO_2Trp			386.7	—	—
		7.84 (s, H-2)			
3-NO_2Tyr	373.4	7.37 (dd, H-6)	389.3	–15.9	1 : 4.4
		6.93 (d, H-5)			
2-NO_2-His N_1-H	—	—	408.0	—	—
5-NO_2-His N_1-H			396.6		
2-NO_2-His N_3-H			408.0		
5-NO_2-His N_3-H			403.6		
S-NO_2Cys		8.38 (s)[f]	370.0	—	—
O-NO_2Ser	—	8.39 (s)[f]	418.2	—	—
O-NO_2Thr	—	—	419.8	—	—
N-ω-NO_2Arg	—	—	429.1	—	
N-ε-NO_2Arg	—	—	416.9	—	
N-NO_2Lys	—	8.39 (s)[f]	407.7	—	

[a] Chemical shifts for nitrated tripeptides. [b] Calculated chemical shifts for truncated nitrated amino acids (Fig. 3). [c] $\Delta\delta = \delta_{exp}$ ^{15}N – δ_{cal} ^{15}N. [d] NO_2-Tyr (see Results and discussion). [e] See Results and discussion. [f] Side chain oxidation product. [g] Calculated from ^{1}H NMR spectrum.

not be associated with aromatic nitro-forms. It appears that the above mentioned singlet resonance reflects a tryptophan ring-cleavage product that contains an aliphatic region with ^{15}N atom.[12] Similar results, with only traces of nitro-form noticed (not shown), were obtained when post reaction mixture containing H-Gly-His-Gly-OH peptide was monitored. Moreover, the ^{15}N-^{1}H HMBC spectrum of the nitrated H-Gly-His-Gly-OH sample did not show any noticeable peaks corresponding to the ^{15}N and ^{1}H shifts of nitro-histidine.

The NMR spectra of nitrated serine and lysine peptides showed only traces of new forms. The ^{1}H NMR spectra of H-Gly-Cys-Gly-OH, H-Gly-Ser-Gly-OH and H-Gly-Lys-Gly-OH peptides contained small resonances at 8.4 ppm, suggesting oxidation of small percentage of peptides side chain heteroatoms to produce aldehydes. The ^{1}H spectra of nitrated threonine-containing peptide also showed traces of *O*-nitro-threonine moiety (CH–O–NO_2), with its ^{1}H resonance at 5.12 ppm and ^{15}N resonance at 535.5 ppm (not shown).

^{1}H NMR spectrum of nitrated H-Gly-Cys-Gly-OH showed almost all peptide used to be converted to various products. The literature suggests that the reaction of cysteine moiety with peroxynitrite may produce various amounts of disulfide (RSSR), sulfenic acid (RSOH), sulfinic acid (RSO_2H), sulfonic acid (RSO_3H), nitrosocysteine (RSNO), nitro-cysteine ($RSNO_2$) and various radicals.[12] Although the existence of RSNO and $RSNO_2$, containing ^{15}N-nitrogen atom originating from peroxynitrite, should be easy to confirm in 1D and 2D NMR spectra, it was not apparent. Consequently, the new products found in the ^{1}H NMR spectrum appear to be mainly disulfide, as well as sulfenic acid, sulfinic acid and sulfonic acid. The mass spectrometric analysis confirmed the presence of sulphonic acid in the sample.

NMR analyses of nitrated TS

The nitration of protein conducted under acidic conditions (pH 2–5) resulted in the precipitation of protein and consequently very low concentration of soluble ^{15}N-nitro-forms that could be analyzed by NMR method. The best results were achieved at pD of 7.2–7.4; when in NMR tube no presence of visible solid residues was apparent during the reaction and measurements. The chemical shifts for the strongest peak found in ^{15}N-^{1}H HMBC spectrum (7.67 ppm on the H-axis and 374.9 ppm on the ^{15}N axis) of nitrated protein (not shown) were in a very good agreement with our data for nitrated H-Gly-Tyr-Gly-OH peptide (*vide supra*). Due to relatively low concentrations of protein and very low yields of nitration process, it was impossible to confirm identity of other nitrated moieties.

MS analyses of nitrated TS

To localize the *in vitro* modifications of TS, nitrated human and *C. elegans* proteins underwent proteolytic digestion and the resulting peptides were analyzed by ESI. For each of the two proteins several TS-derived peptides were found to contain nitro-tyrosine (Table 2) and oxidized cysteine (Table 3) residues. It should be mentioned that although sequences of several peptides listed in Table 2 contained, besides tyrosine, also histidine residues, a possibility of their nitration appears negligible. The nitration of aromatic compounds is performed by the electrophilic aromatic substitution mechanism. Susceptibility of tyrosine side chain for electrophilic substitution is very high, in particular in its phenolate form at pH > 7. In contrast to this, the electron density of the imidazole ring of histidine is lower. Consequently, the electrophilic substitution of histidine residue is much slower and requires

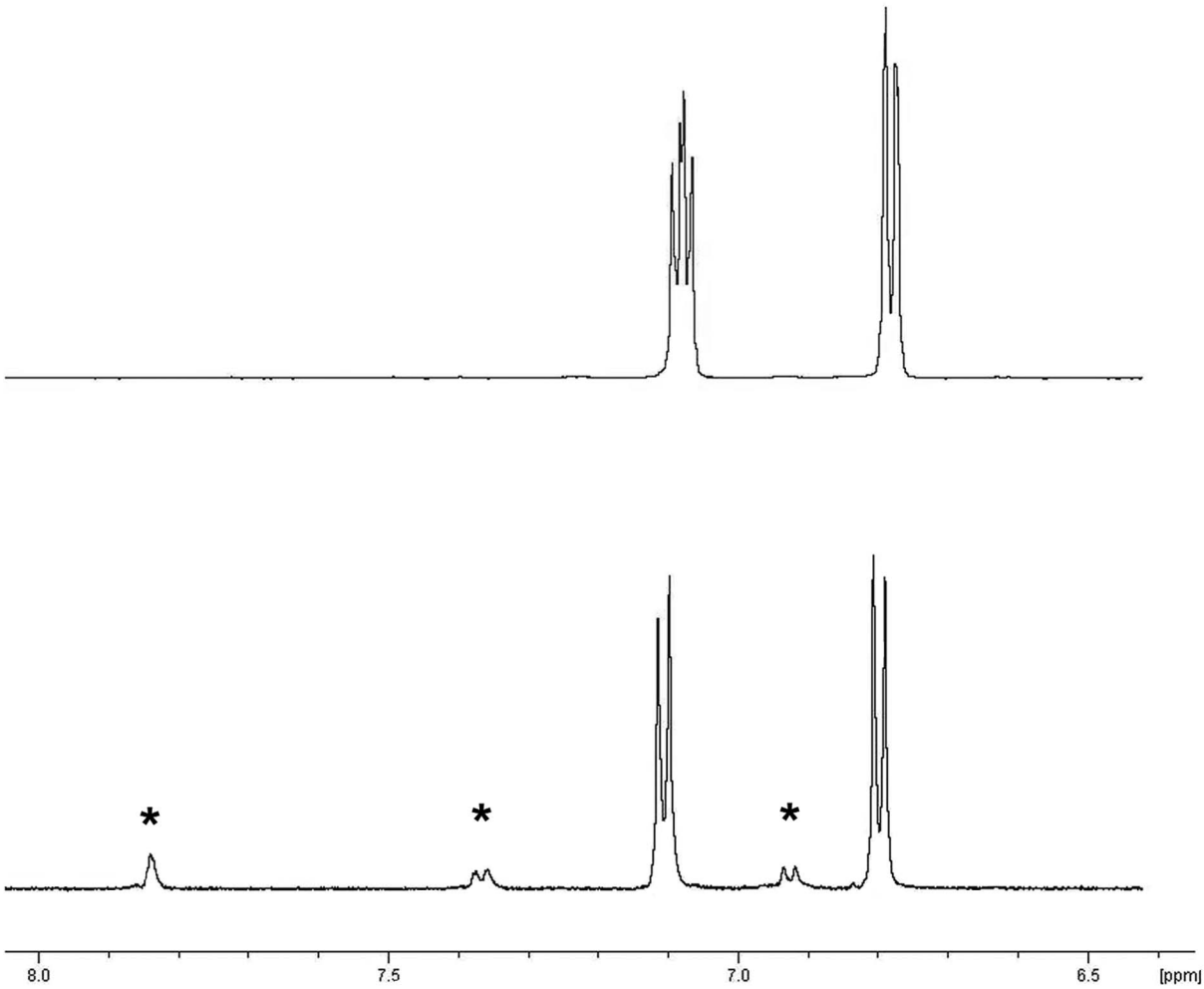

Fig. 4 Aromatic regions of H-Gly-Tyr-Gly-OH peptide (top) and post-reaction mixture of peptide ^{15}N-nitration (bottom). Asterisks indicate aromatic hydrogens of ^{15}N-nitro-tyrosine moiety.

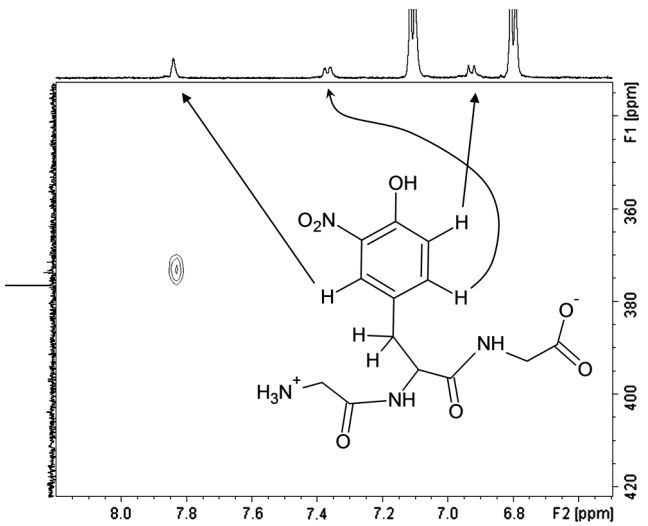

Fig. 5 The fragment of ^{15}N-^{1}H HMBC spectrum of the nitrated H-Gly–Tyr-Gly-OH peptide showing ^{15}N-H(Tyr) coupling. The largest peak on ^{15}N axis belongs to NO^{3-} anion.

higher pH than that of tyrosine. Therefore it appears hardly possible that in the presence of very reactive phenolate side chain the imidazole ring of histidine would be modified. In accord, ESI-MS analysis revealed only one nitration site in the 255–261 fragment of human thymidylate synthase, containing one tyrosine and two histidine residues (Fig. 6). Moreover, low susceptibility to nitration of histidine *vs.* tyrosine was shown also by the results of NMR analyses of nitrated model compounds (*vide supra*).

While the non-nitrated counterparts of several nitrated peptides could be identified, in most cases their proportion was probably too low to allow unambiguous recognition.

Following nitration at pH 7.5 with 20 mM H_2O_2, tyrosine modification to nitro-tyrosine was found at positions 33, 65, 135, 213, 230, 258 and 301 in human TS (Fig. 6 and 7), and 34, 66, 137, 148 and 232 (homologous to human 33, 65, 135, 146 and 230, respectively) in the *C. elegans* protein. Interestingly, when 50 mM H_2O_2 was applied with *C. elegans* TS, a different nitration profile, including positions 66, 137, 148, 204, 215 and 260 (homologous to human 65, 135, 146, 202, 213 and 258, respectively), was apparent. Of note is a tendency for homologous

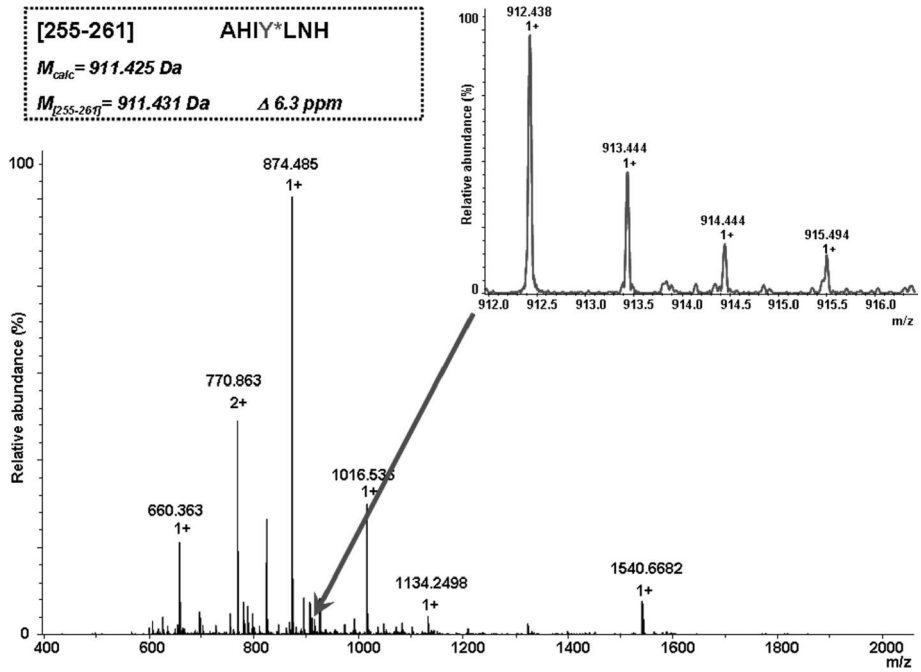

Fig. 6 The ESI-MS spectrum of the chymotryptic products of human TS. The expanded part of spectrum corresponds to the fragment 255–261 containing the nitrated tyrosine at position 258. The monoisotopic molecular mass of the presented fragment was compared with one simulated basing on the molecular formula.

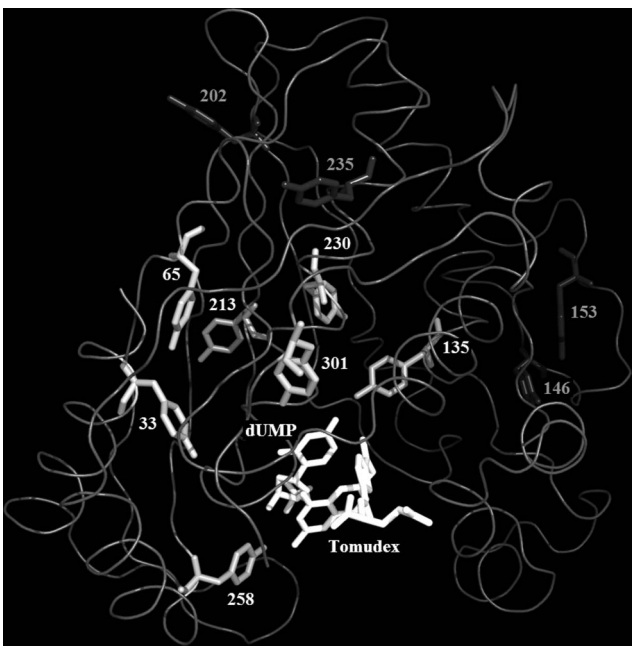

Fig. 7 Ribbon representation of monomer A from the crystal structure of human thymidylate synthase with bound dUMP and Tomudex (PDB code: 1I00). Tyrosine residues are shown as sticks, labelled with sequence numbers and marked either lighter or darker depending on whether a residue has been found to be nitrated or not, respectively. dUMP and Tomudex are shown as sticks and marked light.

tyrosine residues in both TS sequences to undergo nitration (Table 2). Considering localization of nitrated tyrosine residues in human TS, those at positions 135, 230 and 258, being closest to the active center (Fig. 7), were of particular interest, in view of the influence of nitration on the catalytic potency (Fig. 2). It should be mentioned that at the conditions used to nitrate the human enzyme (pH 7.5, 20 mM H_2O_2) the only cysteine residue to undergo modification was Cys^{210}, with catalytically critical Cys^{195} remaining apparently unmodified. In *C. elegans*, the homologous catalytic cysteine residue TS (Cys^{197}) underwent modification only at 50 mM H_2O_2, in accord with the dependence presented in Fig. 2, and cysteine residues at positions 67, 151 and 243, oxidized at 20 mM H_2O_2, correspond to human TS homologous amino acid residues different from cysteine (Ser^{66}, Met^{149} and Thr^{241}, respectively).

In order to asses a potential of nitration of different tyrosine residues to affect TS catalytic potency, a parallel molecular modeling study (using the molecular dynamics method, followed by post-processing of the resulting trajectories) has been performed. The simulations were based on the crystal structure of the ternary complex of human thymidylate synthase with dUMP and Tomudex (PDB accession code 1I00), with the Tomudex molecule replaced by the molecule of tetrahydrofolate (close analogue of methylenetetrahydrofolate), according to the previously described superimposition.[13] Initial results indicated nitration in human TS of either of the four residues, Tyr^{33}, Tyr^{135}, Tyr^{213} and Tyr^{258}, to differently influence the binding alignment between the substrate, dUMP, and cofactor, THF, in the enzyme active site. The impact ranges from (i) a strong misalignment that is likely to significantly reduce the catalytic activity of TS (nitration on Tyr^{135}) to (ii) moderate deviations from the native alignment (nitrations on Tyr^{33} and Tyr^{258}), and (iii) the lack of deviation from the native alignment (nitration on Tyr^{213}) that suggests preservation of TS native catalytic activity.[14] Considering the apparent strong influence of Tyr^{135} nitration on the substrates alignment, of interest is that mutation (Y94F) of the corresponding residue

Table 2 Mass spectrometric determination of tyrosine residues modification of $-NO_2$ type in human and *C. elegans* recombinant thymidylate synthase proteins nitrated *in vitro* at pH 7.5. Purified recombinant protein, following nitration, was analyzed by ESI-MS after proteolytic digestion. The peptides listed encompass those resulting from digestion by trypsin, as well as chymotrypsin

Sequence of the modified peptide	Modification site
Human TS protein nitrated at 20 mM H_2O_2	
A^{228}S**Y**ALL^{233}	Tyr^{230}
N^{205}SELSCQL**Y**QRSG^{217}	Tyr^{213}
F^{117}LDSLGFSTREEGDLGPV**Y**GFQW^{139}	Tyr^{135}
A^{255}HI**Y**LNH^{261}	Tyr^{258}
A^{22}EPRPPHGELQ**Y**LGQIQHILRCGV^{45}	Tyr^{33}
T^{251}LGDAHI**Y**^{258}	Tyr^{258}
S^{209}CQL**Y**QRSGDMGLGVPF^{225}	Tyr^{213}
K^{292}AEDFQIEG**Y**NPHPTIKM^{309}	Tyr^{301}
G^{60}MQAR**Y**SLRD^{69}	Tyr^{65}
C. elegans *TS protein nitrated at 20 mM $H_2O_2^-$*	
K^{33}**Y**LKQVE^{39}	Tyr^{34} (Tyr^{33})[a]
L^{133}GPV**Y**^{137}GFQW^{141}	Tyr^{137} (Tyr^{135})
F^{139}QWRHFGAK**Y**VDCHTD**Y**^{155}	Tyr^{148}; Tyr^{155}
G^{134}PV**Y**GFQWRHF^{144}	Tyr^{137} (Tyr^{135})
M^{221}GLGVPFNLAS**Y**GL^{234}	Tyr^{232} (Tyr^{230})
E^{274}P**Y**AFPK^{280}	Tyr^{276}
G^{61}MQSK**Y**CLRNG^{71}	Tyr^{66} (Tyr^{65})
C. elegans *TS protein nitrated at 50 mM H_2O_2*	
G^{61}MQSK**Y**CLRNG^{71}	Tyr^{66} (Tyr^{65})
Q^{40}ILREGTRRDDRTGTGTISIFGMQSK**Y**CLRNGTIPLLTTKRV^{81}	Tyr^{66} (Tyr^{65})
G^{190}QMVLPPCHTMCQF**Y**^{204}	Tyr^{204}
T^{126}SREEGDLGPV**Y**GFQW^{141}	Tyr^{137} (Tyr^{135})
A^{146}K**Y**VDCHTD^{154}	Tyr^{148}
Y^{204}VDNGELSCQL**Y**QRSGDMG^{222}	Tyr^{204}; Tyr^{215} (Tyr^{213})
G^{61}MQSK**Y**CLRNGTIPLLTTKRV^{81}	Tyr^{66} (Tyr^{65})
T^{56}ISIFGMQSK**Y**CL^{68}	Tyr^{66} (Tyr^{65})
T^{126}SREEGDLGPV**Y**GFQW^{141}	Tyr^{137} (Tyr^{135})
V^{251}HTLGDAHV**Y**^{260}	Tyr^{260} (Tyr^{258})

[a] Homologous human TS site, if also modified, is presented in parentheses.

in *E. coli* TS caused an apparent weakening of dUMP binding and associated enhancement of dUMP release, resulting in both substrates (dUMP and meTHF) interacting in a random binding sequence.[15]

In view of the recently presented concept of hydrogen bond bridges playing an important role in the reaction of protein tyrosine nitration,[16] it was of interest to extend our molecular modeling studies to analyze how the results of nitration of tyrosine residues in TS protein conformed to that concept. The results were unequivocal. While the distance between the nitrated tyrosine hydroxyl and the closest acidic/basic amino acid side chain heavy atom matches always satisfactorily the distance required for the nitrating species to form hydrogen bond bridge connecting the tyrosine and corresponding charged amino acid,[16] the corresponding distances measured to heteroatoms of the dissociable groups of the same amino acids are noticeably different from those suggested to be optimal,[16] and in accord, orientations between those dissociable groups and the nitrated tyrosine hydroxyls appear to be in most cases incorrect for forming geometrically reasonable hydrogen bond bridges. However, the latter statement should be taken with caution, as our molecular modeling was performed for the systems that had already undergone tyrosine nitration, hardly allowing evaluation of the intermediate conformational states occurring during nitration reactions.

Conclusions

The present study suggests that thymidylate synthase protein, expressed endogenously in normal and tumour (calf thymus and L1210 cells) tissues undergoes nitration *in vivo*. The modification may influence properties of the enzyme, as chemical reaction with peroxynitrite ($ONOO^-$) produced *in situ* at pH 7.5 of human, mouse and *C. elegans* recombinant TS proteins, resulting primarily in nitration of tyrosine residues, as confirmed by NMR and MS, distinctly lowers the catalytic potency reflected by the V_{max}^{app} value.

Experimental

Materials

Tripeptides of general formula H-Gly-X-Gly-OH where X = Phe, Tyr, Trp, Lys, Arg, His, Ser, Thr, Cys, Gly were purchased from Lipopharm (Poland).

Thymidylate synthase preparation

The endogenous enzyme proteins from parental and FdUrd-resistant mouse leukemia L1210 cells,[17] and calf thymus,[18] were purified as previously described. *Ceanorhabditis elegans*[19] and mouse[20] TS coding regions were cloned into pPIGDM4+stop vector and expressed as HisTag-free proteins in BL21(DE3)

Table 3 Mass spectrometric determination of cysteine residues modification of –O type in human and *C. elegans* recombinant thymidylate synthase proteins nitrated *in vitro* at pH 7.5. Purified recombinant protein, following nitration, was analyzed by ESI-MS after proteolytic digestion. The peptides listed encompass those resulting from digestion by trypsin, as well as chymotrypsin[a]

Sequence of the modified peptide	Modification site
Human TS protein nitrated at 20 mM H_2O_2	
N^{205}SELS**C**QLYQRSG^{217}	Cys^{210}
C. elegans *TS protein nitrated at 20 mM H_2O_2*	
G^{61}MQSKY**C**LRNG^{71}	Cys^{67}
F^{144}GAKYVD**C**HTDYSG^{157}	Cys^{151}
Q^{140}WRHFGAKYVD**C**H^{152}	Cys^{151}
R^{142}HFGAKYVD**C**HTDYSGQGVDQL^{163}	Cys^{151}
M^{238}IAKV**C**GLKPGTLVH^{252}	Cys^{243}
C. elegans *TS protein nitrated at 50 mM H_2O_2*	
G^{61}MQSKY**C**LRNG^{71}	Cys^{67}
Q^{40}ILREGTRRDDRTGTGTISIFGMQSKY**C**LRNGTIPLLTTKRV^{81}	Cys^{67}
G^{190}QMVLPPCHTM**C**QFY^{204}	Cys^{201}
A^{146}KYVDCHTD^{154}	Cys^{151}
K^{171}EQPDSRRIIMSAWNPSDLGQMVLPPCHTM**C**QFYVDNGE^{209}	Cys^{197}; Cys^{201}
G^{190}QMVLPP**C**HTM**C**QFYVDNGELS**C**QL^{214}	Cys^{197}; Cys^{201}; Cys^{212} (Cys^{210})

[a] Homologous human TS site, if also modified, is presented in parentheses.

or thymidylate synthase-deficient TX61$^-$ (a kind gift from Dr W. S. Dallas) *E. coli* strain, respectively. Human TS coding regions[21] were subcloned into pET28a vector and expressed as HisTag-containing proteins in *E. coli* BL21(DE3) strain. HisTag containing proteins were purified on NiNTA His-Bind resin (Novagen) according to manufacturer protocol, and HisTag-free proteins were purified as previously described.[22] Phosphatase inhibitors (50 mM NaF, 5 mM Na-pyrophosphate, 0.2 mM EGTA, 0.2 mM EDTA and 2 mM Na_3VO_4) were present in the purification buffers. Each purified TS preparation was separated from phosphorylated fraction according to Wolschin *et al.*,[23] using metal oxide/hydroxide affinity chromatography on $Al(OH)_3$ beads. The enzyme activity was measured and kinetic parameters of the enzyme-catalyzed reaction were determined as previously described.[24]

Thymidylate synthase tyrosine *in vitro* nitration

The reaction was performed at 4 °C in the presence of 20 μM dUMP (stabilization in the absence of 2-mercaptoethanol) in a reaction mixture containing 200 mM Na/K phosphate buffer pH 7.5, equimolar concentration of $NaHCO_3$ and H_2O_2 (5–70 mM), $NaNO_2$ at concentration by 5% exceeding the latter (5.25–73.5 mM) and the enzyme (5 μM dimer). To start the reaction, H_2O_2 was added, the sample mixed 30 s and next incubated 5 min. While nitro-tyrosine content was determined spectrophotometrically,[25] to the remaining reaction mixture 2-mercaptoethanol (20 mM) was added, followed by either protein precipitation with 10% (w/v) TCA or sample dilution (≥300-fold) with 50 mM Na/K phosphate buffer pH 7.5, containing 0.1% Triton X-100 and 10 mM 2-mercaptoethanol. The diluted preparation preserved TS activity for at least 2 h, allowing enzyme properties to be studied. To the control reaction mixture TS was added after mixing and incubating the remaining components, in order to inactivate the produced peroxynitrite.

Immunoblotting

Previously described method was used,[13] with anti-tyrosine antibody from Sigma–Aldrich (Cat. No. NO409) and anti-TS antibody.[19]

Peptide nitration

Peptides of the general formula H-Gly-X-Gly (where X = Phe, Thr, Trp, Lys, Cys, His, Ser, Arg, Gly) were nitrated in deuterium oxide system using sealed 5 mm NMR tubes. Each tripeptide (8 μmol in 0.1 ml of deuterium oxide) was mixed with solutions containing (i) 76 μmol H_2O_2 in 0.1 ml of D_2O, (ii) 80 μmol sodium ^{15}N-nitrite in 0.1 ml of D_2O and (iii) sodium bicarbonate (80 μmol in 0.1 ml D_2O), followed by addition of 3.3 μl of conc. sulfuric acid in 0.1 ml of D_2O and additional D_2O to the final volume of 0.6 ml (resulting pD of 1.9).

NMR analyses

All NMR spectra were obtained with Bruker Avance spectrometer operating in the quadrature mode at 500.13 MHz for ^{1}H and 50.69 MHz for ^{15}N nuclei. The residual peaks of deuterated solvents were used as internal standards in ^{1}H NMR method. ^{15}N NMR spectra were recorded at 277 K both with and without proton decoupling. All ^{15}N chemical shifts presented in this work are related to liquid ammonia (0.0 ppm). The internal standard used in ^{15}N NMR was sodium nitrite (609.6 ppm rel. to liquid NH_3) and sodium nitrate (376.5 ppm rel. to liquid NH_3). All samples were analyzed using the gradient-enhanced ^{1}H-^{15}N Heteronuclear Multiple Bond Correlation (HMBC) approach. The ^{1}H NMR spectra were obtained with the use of the HDO suppression method. All buffer solutions used for NMR spectroscopy were based on deuterium oxide of '100%D' purity (Armar Chemicals AG, Germany).

Calculations

The theoretical calculations have been performed with the density functional B3LYP/aug-cc-pVTZ method. To save computational time, the structures of amino acids were truncated by removing some groups of atoms remote from the nitrated regions (Fig. 3). Where it was possible, the calculations for full nitro-tripeptide (GCG-NO2) were performed for comparison with the truncated model. The optimal geometries were obtained and confirmed with positive harmonic frequencies, then NMR shielding values for models of nitroamino-derivatives of acids were calculated. All the calculations were performed with the Gaussian G03 (rev. C.02) suite of programs.

Mass spectrometry analysis

Enzymatic hydrolysis. A sample of modified protein (1 mg) was dissolved in water (100 µl). The obtained solution (10 µl) was diluted with 0.03 M NH_4HCO_3 (50 µl). After adding the 0.1% water solution of the proteolytic enzyme (5 µl; trypsin or chymotrypsin) the mixture was incubated at r.t. for 12 h. The products of the hydrolysis were absorbed on OMIX C4 100 µl pipette tips (Varian). The tip was washed 10 times with water (100 µl), than the peptides formed by proteolysis of modified protein were eluted with 60% water solution of acetonitrile (100 µl).

Mass spectrometry. The enzymatic digest was analyzed on high resolution ESI-FT mass spectrometer (Apex-Ultra Qe 7T; Bruker Daltonics; Germany) equipped with an electrospray ionization (ESI) source. The instrument was operated both in the positive and negative ion mode and calibrated with the Tunemix mixture (Bruker Daltonics). The mass accuracy was better than 5 ppm. The instrumental parameters were as follows: scan range, 300–2500 m/z; drying gas, nitrogen; temperature of drying gas, 200 °C; potential between spray needle and orifice, set at 4.5 kV; source accumulation time, 0.5 s; and ion accumulation time, 0.5 s. The analyzed solution was infused directly to the ion source at a flow rate 3 µl min^{-1}. Each spectrum is an average of more than 100 individual scans.

Data analysis. The analysis of spectra was performed using the SNAP algorithm (Data Analysis, Bruker). The generated mass list, including m/z ratio, monoisotopic mass and z, was further analyzed using Excel spreadsheet basing on the following assumptions: (i) The cleavage sites for trypsin: [K,R] and for chymotrypsin [Y,W,F,L,M,H,N,G,I,V,E,D]; (ii) accepted chemical modifications nitration of tyrosine and oxidation of the methionine and cysteine; (iii) Accepted error max. 10 ppm; (iv) Peptide length max. 20 amino acid residues; (v) Only protonated peptides were accepted, the metal adducts (Na, K, Ca) were neglected.

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