Insertion of an unnatural amino acid into the protein structure: preparation and properties of 3-fluorotyrosine-containing organophosphate hydrolase

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Five Tyr residues present in the native organophosphate hydrolase (OPH) containing a hexahistidine tag at the N-terminus of the protein molecule (His_6-OPH) were replaced by fluorine-containing analogs using a biosynthetic approach. The modified enzyme had an extended pH-optimum of action shifted to acidic pH and an enhanced thermal stability in the alkaline pH region.

Key words: 3-fluorotyrosine, organophosphate hydrolase, unnatural amino acid, paraoxon.

The interest in the preparation of modified proteins containing unnatural amino acids, in particular, fluorinated amino acid analogs, is due, on the one hand, to the possibility of solving a number of fundamental problems related to the studies of the protein structure and structure—property relationships, and, on the other hand, to the probable practical application of these proteins.[1—3]

The replacement of amino acids in proteins by their analogs may give rise to proteins with new properties and, in particular, favorably change the properties of well-known proteins toward their practical use. In particular, the substrate specificity, stability, the pH and temperature optimum of action of the modified enzyme, and folding kinetics can be varied.[4—6] Thus the replacement of the phenylalanine residues in PvuII-endonuclease by 3-fluorophenylalanine (3-F-Phe) results in twice as high specific activity of the modified enzyme as that of the native enzyme, while the introduction of 4-F-Phe reduces it fourfold.[7] X-Ray diffraction analysis of glutathione transferase M-1 in which Tyr residues were replaced by 3-fluorotyrosine (3-F-Tyr) has revealed multiple conformational changes in the structure of the modified enzyme, which changed its spectral and kinetic characteristics.[8]

The replacement of a substantial portion of amino acids in microbial proteins by synthetic analogs[9,10] is a route to the design of novel biotechnological processes and to the preparation of modern products based on previously unknown microbial producers.[11—14] The possibility of biosynthetic preparation of a protein by replacing the natural amino acid by its unnatural analog is based on the lack of absolute specificity of the enzyme aminoacyl-tRNA synthetase[3].

The biosynthesis of a recombinant protein containing an unnatural amino acid implies growing an auxotrophic *E. coli* strain up to the end of the logarithmic phase of growth in a minimum medium containing the required salts, vitamins, and glucose as the only carbon source, a protein synthesis repressor, and a natural amino acid that makes the *E. coli* strain an auxotroph; then the minimum medium with the natural amino acid is replaced by a similar medium containing an unnatural amino acid, glycerol instead of glucose, and an inducer of the target protein synthesis.

Organophosphate hydrolase (OPH or phosphotriesterase (EC 3.1.8.1)) catalyzes hydrolysis of organophosphorus compounds (OPC)[15—20] to give phosphoric or alkylphosphonic acid derivatives, which is accompanied by a decrease in the pH of the reaction medium. Efficient enzyme-catalyzed hydrolysis of OPC requires essential stabilization of the enzyme in the acidic pH region. As a rule, enzyme immobilization makes it more stable.[21,22] This communication describes an attempt to change characteristics of the enzyme containing an N-terminal hexahistidine tag (His_6-OPH)[23,24] by introducing 3-fluorotyrosine into the protein molecule.

Experimental

The following commercial chemicals were used: paraoxon (diethyl *p*-nitrophenyl phosphate), imidazole, CHES (2-(cyclohexylamino)ethanesulfonic acid), HEPES (*N*-(2-hydroxyethyl)-*N*′-(2-sulfoethyl)piperazine), $CoCl_2 \cdot 6H_2O$, $MgSO_4$, thiamine, $CaCl_2$, glycerol, bromophenol blue, Coomassie Brilliant Blue R-250, ampicillin sodium salt, hen egg albumin, sodium dodecyl sulfate (Sigma, USA); tyrosine, acrylamide, *N*,*N*′-methylene-

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bisacrylamide, ethylenediaminetetraacetic acid (EDTA) (Merck, Germany); 3-F-L-Tyr (3-fluoro-4-hydroxy-L-phenylalanine) (Lancaster, UK); isopropyl 1-thio-β-D-galactopyranoside (IPTG), molecular weight markers (14.4, 21.5, 31.0, 45.0, 66.2, 116.0 kDa) for protein electrophoresis (Fermentas, Lithuania); *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA), and ammonium peroxydisulfate (Bio-Rad, USA). Co-IDA-Sepharose (Pharmacia, Sweden), which forms complexes with cobalt ions, was used as the carrier for affinity chromatography. The other chemicals (analytical grade) were purchased from Labtekhnika and Khimmed (Russia).

The biosynthesis of the fluorine-substituted analog of His_6-OPH (F-Tyr-His_6-OPH) was carried out using tyrosine-auxotrophic *E. coli* B-2935 cells (VKM). The plasmid pTES-His-OPH encoding the synthesis of His_6-OPH was used for their transformation.[15]

The *E. coli* B-2935 cells containing the plasmid pTES-His-OPH were incubated at 37 °C in the M9 medium containing (per L) glucose (4 g), thiamine (1 mmol), $MgSO_4$ (1 mmol), $CaCl_2$ (0.1 mmol), L-tyrosine (25 μmol) and ampicillin (100 mg).

The 16-h inoculate was introduced into a flask containing the nutrient medium M9 of the same composition (100 mL). The cells were grown to an optical density of 0.8 (540 nm) and centrifuged under sterile conditions (a Beckman J2-21 centrifuge (USA), 6000 g, 20 min, 4 °C). The cells were re-suspended in M9 with ampicillin (100 μg mL^{-1}) containing (per L) glycerol (20 g), thiamine (1 mmol), $MgSO_4$ (1 mmol), $CaCl_2$ (0.1 mmol), and 3-F-Tyr (25 μmol), and the enzyme synthesis was induced by adding IPTG up to a final concentration of 0.25 mmol L^{-1}. Simultaneously, $CoCl_2$ was introduced up to a concentration of 10^{-4} mol L^{-1}. The cells were incubated at 37 °C with continuous stirring (180 rpm) in a thermostated Adolf Kuhner AG shaker (Switzerland) for 20 h. The biomass was sedimented by centrifugation (a Beckman J2-21 centrifuge, 6000 g, 20 min).

F-Tyr-His_6-OPH was isolated and purified on Co-IDA-Sepharose. The cell biomass was re-suspended in a phosphate buffer, pH 8.0 (50 mmol L^{-1}), containing NaCl (300 mmol L^{-1}) up to a concentration of 0.2 g mL^{-1}. Then the cells were destroyed using an ultrasonic disintegrator (44 kHz, 6×45 s). The cell debris were separated by centrifugation (15000 g, 30 min), and an equal volume of a Co-IDA-Sepharose suspension pre-equilibrated in a phosphate buffer (50 mmol L^{-1}) was added to the supernatant. The resulting suspension was applied onto a chromatographic column and eluted with a phosphate buffer, pH 8.0 (50 mmol L^{-1}) containing NaCl (300 mmol L^{-1}) and imidazole (10 mmol L^{-1}) at a flow rate of 0.5 mL min^{-1} to the eluate optical density of 0.01 (280 nm). The enzyme was eluted with an imidazole solution (250 mmol L^{-1}). Imidazole was removed from the collected fractions by dialysis against a phosphate buffer with pH 8.0 (50 mmol L^{-1}), the fractions were analyzed by electrophoresis under denaturing conditions in a 10% polyacrylamide gel using a Miniprotean II Bio-Rad cells (USA) according to the manufacturer´s manuals to the instruments and then stained with Coomassie R-250. The protein concentration and the enzymatic activity were determined in the fractions.

The fact and the degree of Tyr replacement by 3-F-Tyr in the protein were determined by MALDI TOF mass spectrometry of the trypsine hydrolyzate of the purified protein sample.

The protein concentration was determined by spectrophotometry[25] using a Bio-Rad reagent (USA).

Processing of the experimental data gave the average values and the standard deviations. All experiments were performed in triplicate.

The enzymatic activity with respect to paraoxon was determined by spectrophotometry (an Agilent 8453-UV spectrophotometer, Germany) based on the accumulation of the hydrolysis product, 4-nitrophenolate anion, at 25 °C (405 nm). The catalytic reaction was initiated by adding a solution of His_6-OPH or F-Tyr-His_6-OPH into the cell with the buffer and the substrate up to a concentration of 10^{-8}—10^{-7} mol L^{-1}.

The amount of the enzyme needed to hydrolyze 1 μmol of the substrate in 1 min at 25 °C and pH 10.5 was taken to be a unit enzymatic activity.

The rates of enzymatic reactions (v) were calculated from the initial linear sections of the kinetic curves ($v_0 = \tan\alpha$). The highest rate of the enzymatic reaction and the Michaelis constant (K_m) were determined both using the Lineweaver—Burk double reciprocal coordinates ($1/v$—$1/[S]$) and from the full curve reaction rate *vs.* substrate concentration ([S]). The kinetic constants for His_6-OPH and F-Tyr-His_6-OPH were found using a carbonate buffer, pH 10.5 (50 mmol L^{-1}).

The protein isoelectric points were determined by analytical isoelectrofocusing on a Model 111 Cell instrument (Bio-Rad, USA) according to the manufacturer´s manual.

The pH dependence of the catalytic activity of enzymes was studied using buffers (50 mmol L^{-1}) with overlapping pH values: HEPES (pH 7.5—8.5), CHES (pH 8.5—10.0), and the phosphate—carbonate buffer (pH 9.5—12.0).

The optimum temperatures for the action of the native and modified enzymes were determined by maintaining the cell with the buffer and the substrate for 10 min at a temperature from 25 to 70 °C. The reaction was initiated by adding an aliquot (10 μL) of a solution of His_6-OPH or F-Tyr-His_6-OPH.

The thermal inactivation kinetics of His_6-OPH and F-Tyr-His_6-OPH were studied by incubating enzyme samples in a phosphate—carbonate buffer (pH 8.5, 50 mmol L^{-1}) for 15 min at a specified temperature in the range of 20—85 °C; after that, the sample was cooled in ice and the residual enzymatic activity was determined.

Results and Discussion

Preparation of F-Tyr-His_6-OPH. The replacement of Tyr by its fluorinated analog (3-F-Tyr) was carried out by performing the biosynthesis of His_6-OPH in auxotrophic *E. coli* B-2935 cells (VKM) devoid of the metabolic ways of tyrosine biosynthesis, thus compelling the cell to use the unnatural amino acid for the protein biosynthesis. Incubation for 20 h gave 4.6 g of cell biomass from 1 L of the medium. Metal chelating chromatography gave 1.2 mg of a high-purity F-Tyr-His_6-OPH sample from 1 g of cells with an average specific activity of 160 units per mg of the protein. The electrophoregram reflecting the level of His_6-OPH synthesis in a medium with an unnatural analog and the homogeneity of isolated and purified F-Tyr-His_6-OPH is shown in Fig. 1. The yield of the enzyme was 78.2% based on the initial amount of

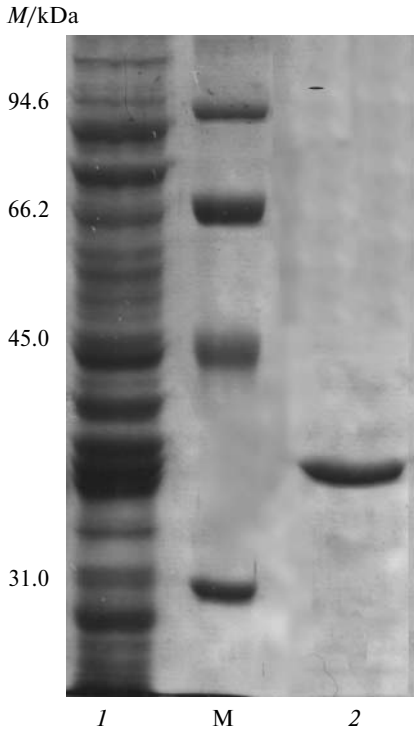

Fig. 1. SDS electrophoresis in 10% polyacrylamide gel of the cell homogenate (*1*) and purified F-Tyr-His_6-OPH (*2*) (M are protein molecular weight markers).

F-Tyr-His_6-OPH synthesized by the cells. The presence of 3-F-Tyr in the medium inhibited the cell growth. The overall yield of F-Tyr-His_6-OPH was more than 10 times lower than the yield of His_6-OPH synthesized by *E. coli* SG cells on a full LB medium.[24]

In the analysis of the fact and completeness of the replacement of Tyr residues in the protein by 3-F-Tyr, the His_6-OPH sample isolated from the biomass grown in a tyrosine-containing medium was used as the reference sample containing no replacements. Theoretically, treatment of F-Tyr-His_6-OPH with trypsine was expected to

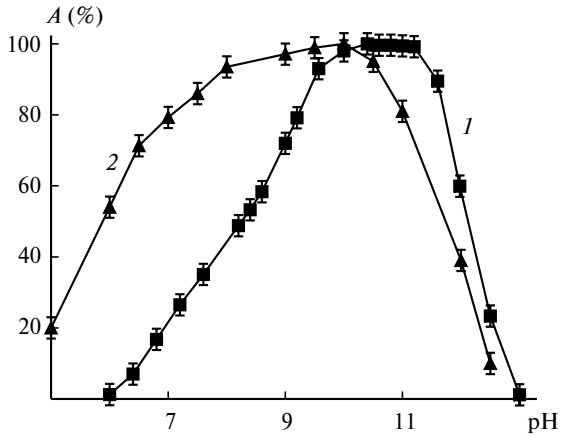

Fig. 2. Enzymatic activities (*A*) of His_6-OPH (*1*) and F-Tyr-His_6-OPH (*2*) *vs.* pH.

give five peptide fragments containing a Tyr residue. The results of processing of the mass spectra are summarized in Table 1, from which it follows that the Tyr292, Tyr239, and Tyr248 residues have been completely replaced by the fluorinated analog, while the Tyr156 residue has been replaced by 90%. From the bulk of individual peptide fragments, it is unclear whether the Tyr309 residue has been replaced by the fluorinated analog, as the mass spectrum contains no peak for the peptide fragment with the required residue. This is due to the drawbacks of the method, caused by different efficiency of trypsinolysis. A comparison of the molecular masses of the F-Tyr-His_6-OPH and His_6-OPH proteins, which were equal to 37691.1 and 37602.91 amu, respectively, has shown that the replacement of Tyr309 by 3-F-Tyr occurred by 98%.

Properties of F-Tyr-His_6-OPH. The replacement of Tyr by 3-F-Tyr extends substantially the pH range of action of the fluorine-substituted enzyme compared with the unsubstituted one (Fig. 2). Indeed, the fluorinated enzyme exhibits stable catalytic activity in the pH range of 5.5—12.0, whereas the original enzyme is

Table 1. Results of the mass spectrometric analysis of the trypsine hydrolyzate of F-Tyr-His_6-OPH

Tyrosine residue in the His_6-OPH molecule	Peptide containing a tyrosine residue	*m/z* for the peak of the Tyr-containing peptide (calculated)	*m/z* for the peak of the 3-F-Tyr-containing peptide (found)	Intensity for the peak of the Tyr-containing peptide[a]	Intensity for the peak of the 3-F-Tyr-containing peptide[a]
Tyr292	ALIDQG<u>Y</u>MK	1038.5	1056.5	—	0.073
Tyr156	EIQ<u>Y</u>GIEDTGIR	1393.6	1411.6	0.066	0.600
Tyr239	VCIGHSDDTDDLS<u>Y</u>LTALAAR	2307.0	2324.8	—	0.175
Tyr248	G<u>Y</u>LIGLDHIPHSAIGLEDNASASALLGIR	2973.5	2991.1	—	0.128
Tyr309	QILVSNDWLFGFSS<u>Y</u>VTNIMDVMDR	2950.4	[2968.4]	—	[b]

[a] Relative ionic current.
[b] See the text.

active in the pH range of 7.0—12.0. The rate of the F-Tyr-His_6-OPH-catalyzed reactions in the extreme points of this pH range is 35—50% of the maximum value.

The pH dependences of the enzymatic activities of F-Tyr-His_6-OPH and His_6-OPH vary in the alkaline region according to a common pattern manifested as a sharp decrease in the catalytic activity at pH > 11.5. The pH optimum of action of both enzymes is rather broad, from 7.5 to 10.5 for F-Tyr-His_6-OPH and from 9.5 to 11.5 for His_6-OPH. The optimum pH range of F-Tyr-His_6-OPH is shifted to an acidic region by 2.0—2.5 pH units with respect to the pH optimum of the unsubstituted enzyme.

The pK_a values for the hydroxyl groups of 3-F-Tyr and Tyr are known to be 8.54 and 10.46, respectively (see Refs 26, 27). Taking the logarithm of the k_{cat}/K_m *vs.* pH dependences for the modified and native enzymes showed that the $pK_{a,1}$ values are 6.8 and 9.8 in the case of F-Tyr-His_6-OPH and His_6-OPH, respectively, whereas $pK_{a,2}$ for both enzymes proved to be equal (11.4). These data correlate with the change in the isoelectric point (pI) of the protein upon replacement of the hydrogen atom in all Tyr residues by the highly electronegative fluorine. For F-Tyr-His_6-OPH, pI = 7.6, whereas for His_6-OPH, pI = 8.5.[24]

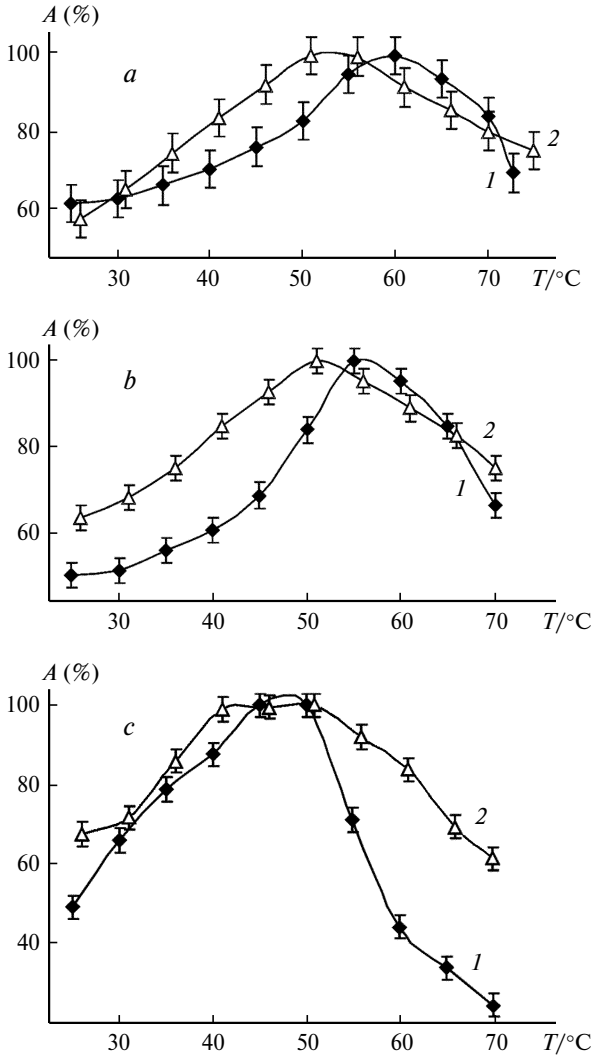

Fig. 3. Enzymatic activities (*A*) of His_6-OPH (*1*) and F-Tyr-His_6-OPH (*2*) *vs.* temperature at different pH: 8.0 (*a*), 9.0 (*b*), and 10.5 (*c*).

The temperature optimum of the F-Tyr-His_6-OPH-catalyzed hydrolysis of OPC virtually does not depend on pH, being equal to 45—50 °C (Fig. 3, *a*—*c*). The temperature optimum of action of His_6-OPH at pH 8.0 lies in the 57—62 °C range, while that at pH 9.0 is at lower temperatures (53—58 °C).

An essential difference is found at pH 10.5: the temperature optimum for F-Tyr-His_6-OPH is 40—55 °C (see Fig. 3, *c*), while that for His_6-OPH is 47—52 °C. At temperatures above 50 °C, the enzymatic activity of His_6-OPH sharply drops, while the activity of F-Tyr-His_6-OPH decreases more smoothly. At 70 °C, the modified enzyme retains ~60% of the activity, while the native one, only 23%.

Even more pronounced distinctions were revealed in the thermal stability of the two enzymes (Fig. 4). The thermal stability was estimated from the residual activity of the enzyme after 15-min incubation at a particular temperature in the phosphate—carbonate buffer (pH 8.5). The replacement of Tyr by 3-F-Tyr resulted in an enhancement of thermal stability of the enzyme. Indeed, F-Tyr-His_6-OPH was barely inactivated up to 60 °C; on further increase in temperature, the residual activity sharply decreased, and at 70 °C, it was 20%. Full inactivation of the enzyme took place at 85 °C. The residual activity of His_6-OPH at 50 °C was 60% of the initial level. Further increase in temperature led to a substantial de-

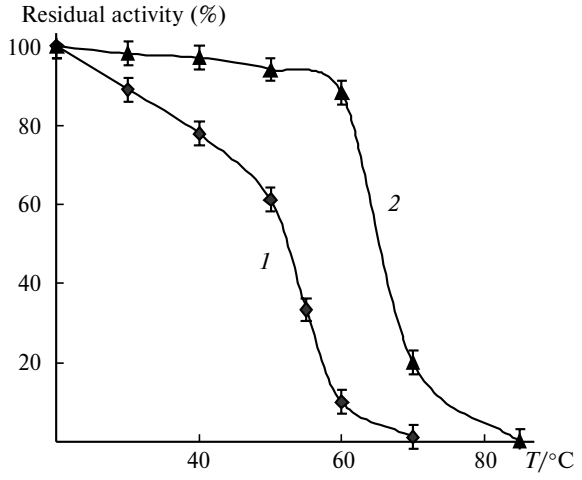

Fig. 4. Residual activities of the enzymes His_6-OPH (*1*) and F-Tyr-His_6-OPH (*2*) after 15-min incubation in a phosphate—carbonate buffer (pH 8.5, 50 mmol L^{-1}) at different temperatures.

Table 2. Kinetic parameters for the paraoxon hydrolysis catalyzed by F-Tyr-His_6-OPH and His_6-OPH*

Enzyme	K_m /μmol L^{-1}	k_{cat} /s^{-1}	k_{cat}/K_m /L mol^{-1} s^{-1}
F-Tyr-His_6-OPH	104±5	125±10	$(1.21\pm0.1)\cdot10^6$
His_6-OPH	10±0.5	5100±200	$(5.1\pm0.3)\cdot10^8$

* Phosphate—carbonate buffer (50 mmol L^{-1}), pH 10.5, 25 °C.

crease in the activity, which was less than 10% at 60 °C. Full inactivation of His_6-OPH was observed at 70 °C.

Determination of the kinetic parameters of paraoxon hydrolysis catalyzed by F-Tyr-His_6-OPH and His_6-OPH (Table 2) showed that the catalytic efficiency of the fluorine-containing enzyme is lower than that of the native enzyme. In the case of the modified protein, k_{cat} decreased 40-fold, while K_m simultaneously increased 10-fold. This might be due to configurational changes in the vicinity of the sorption site. The OPH molecule is known[28,29] to contain three hydrophobic sites, which affect the substrate binding and, hence, determine the enzyme specificity. The Tyr309 residue is directly involved in the formation of the hydrophobic pocket, which serves for binding of the hydrophobic part of the substrate. Probably, the replacement of the hydrogen atom by fluorine markedly changes the microenvironment of the sorption site, disturbs the hydrogen bond distribution, and loosens the hydrophobic interactions.

The obtained results are in good agreement with the results of computer analysis of the F-Tyr-His_6-OPH structure (Fig. 5), which attests to the possible stabilization of the protein globule through additional interactions of the fluorine atoms of the modified 3-F-Tyr156 and 3-F-Tyr248 residues with the positively charged nitrogen atoms of the Arg152 and Arg246 residues, respectively, as well as 3-F-Tyr292 and Lys294.

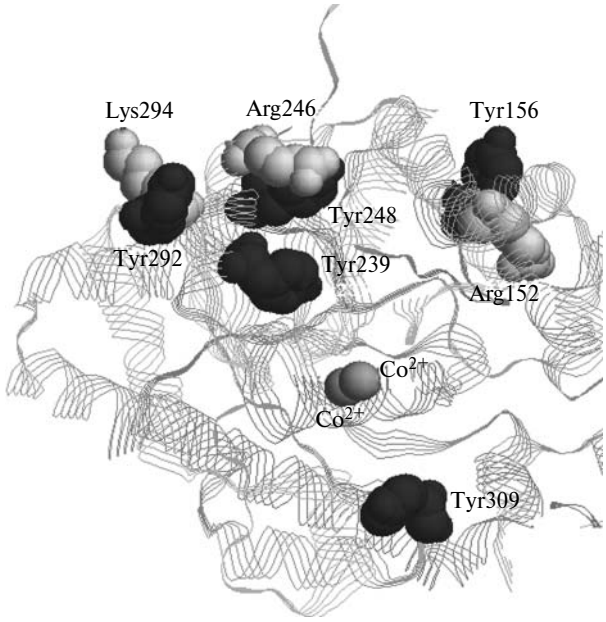

Fig. 5. Spatial structure of F-Tyr-His_6-OPH showing the localization of the 3-fluorotyrosine residues in the protein molecule.

The revealed catalytic properties of F-Tyr-His_6-OPH attest to an obvious stabilization of this modified enzyme over broad pH and temperature ranges with respect to the native enzyme.

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