The Thermostability of Nucleoside Phosphorylases from Prokaryotes. I. The Role of the Primary Structure of the N-terminal fragment of the Protein in the Thermostability of Uridine Phosphorylases

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Abstract—Mutant uridine phosphorylase genes from *Shewanella oneidensis* MR-1 (*S. oneidensis*) were constructed by site-directed mutagenesis and strains-producers of the corresponding recombinant (F5I and F5G) proteins were obtained on the basis of *Escherichia coli* cells. The mutant proteins were purified and their physicochemical and enzymatic properties were studied. It was shown that the N-terminal fragment of uridine phosphorylase plays an important role in the thermal stabilization of the enzyme as a whole. The role of the aminoacid (a.a.) residue phenylalanine (F5) in the formation of thermotolerance of uridine phosphorylases from gamma-proteobacteria was revealed.

INTRODUCTION

The thermal stability of protein structures implies their ability to perform their basic natural functions normally at elevated (often nonpermissive for the organism or host microorganism) temperatures. In the case of enzymes, this functional feature is the preservation of the structure of its active center in an unchanged form (within the limits for the manifestation of functional activity) or the ability for the protein to quickly restore its enzymatic activity when it returns to standard buffer, substrate, and temperature conditions. It should be noted that the thermal stability of enzymes is not always accompanied by a significant increase in the temperature optimum (T_{opt}) for the functioning of these proteins [1]. Moreover, the T_{opt} value cannot serve as a reliable sign indicating the thermal stability of the protein molecule as a whole [1, 2]. The cited research correctly stated that now the development of mathematical methods for reliable prediction of T_{opt} and thermal stability is significantly limited by the lack of data on the properties of mutant forms of the same class of proteins.

In fact, if we briefly summarize the computational methodological approaches to investigation of the thermal stability of proteins, we can identify the fol-lowing areas, among which purely theoretical ones dominate:

— development of computer algorithms that analyze hydrophobic and electrostatic interactions both in the proteins and the interactions of the protein with the components of the buffer systems in which they are located in terms of thermal stability, [3–6];

— creation of computer algorithms that analyze the physicochemical reasons for the manifestation of thermal stability in proteins [7, 8];

— comparison of the primary structures of proteins from mesophilic and thermophilic microorganisms for the determination of the patterns of formation of thermostable protein structures [9–11] and the role of individual amino acid residues in this process.

The above approaches to the mathematical analysis of protein structures for the prediction and artificial increase of their thermal stability are quite completely discussed in reviews [11–16].

However, the predictive potential of these algorithms must be checked every time using a wide range of rather laborious experimental approaches (including comparative X-ray diffraction analysis of the original and mutant forms of proteins), which leads to certain limitations with this important information in the scientific literature. Therefore, for the study of the

principles of the formation of thermostable proteins, it is important to select polypeptides of the same class and, by introduction of individual amino acid substitutions (point mutations) or extended protein fragments (hybrid proteins), to identify the contribution of these changes in making proteins resistant to temperature effects.

In this study, nucleoside phosphorylases (NP), enzymes of nucleoside catabolism that perform phosphorolysis of nucleosides to ribose-(deoxyribose)-1-phosphate and the corresponding heterocyclic base [17], were selected as such proteins. NP are revealed in the cells of almost all organisms and these proteins are the object of close attention of researchers, due to at least two reasons:

— elucidation of the role of these enzymes in the genesis, development, and the course of various pathological processes in mammalian cells (oncology, rheumatoid arthritis, gout, osteoarthritis, systemic scleroderma, etc.) [18−22];

— the high biotechnological potential of NP in the enzymatic synthesis of nucleoside derivatives used in practical medicine (antitumor and antiviral agents, inhibitors of cellular DNA replication, etc.) [23−28].

Many NP genes from various (including extremophilic) prokaryotic microorganisms have been cloned, their primary and spatial structures have been determined, and the enzymatic and physicochemical properties of the corresponding proteins have been studied [29−37].

This fact opens new possibilities in the systematic analysis of previously obtained experimental data in elucidation of the role of individual amino acid residues (a.a.) and extended parts of the polypeptide chain both, in the functioning of the enzymes and in the formation of thermotolerance in this class of proteins.

The aim of this study was the investigation of the role of the N-terminal part of the protein and, in particular, the highly conserved phenylalanine residue (F5) in uridine phosphorylases from mesophilic microorganisms in the formation of thermostable forms of these enzymes.

MATERIALS AND METHODS

The following reagents were used: Tris-hydrochloride (Tris-HCl), Tris base (Tris-OH), SDS-Na, agarose (Type I, Low EEO), EDTA, boric acid (Sigma, United States), ethidium bromide, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (Fluka, Switzerland). N,N'-methylene-bis-acrylamide, acrylamide − (Serva, Germany), tryptone, agar-agar and yeast extract Bacto - Difco (United States), ampicillin (Appli-Chem, Germany). Deoxyribonucleoside triphosphates (dNTPs) and protein molecular weight markers Unstained Protein Molecular Weight Marker (MBI Fermentas, Lithuania). Inorganic salts (Merck, Germany), chemically pure and high pure reagents (Russia).

DNA isolation, purification, restriction by endonuclease digestion, ligation of DNA fragments and transformation of *E. coli* cells with plasmids were performed according to [38].

Taq-polymerase and DNA ligase of T4 phage produced by MBI Fermentas (Lithuania) were used in accordance with the manufacturer's recommendations.

E. coli strains JM110 and C600ΔudpRecA- (*thi thrB leuB lacY supE tonA recA Tn10*) were provided by the All-Russian National Collection of Industrial Microorganisms (NRC Kurchatov Institute- GosNIIgenetika, Russia).

The source of recombinant SoUDP from *S.oneidensis* MR-1 was the previously obtained strain-producer of this enzyme [39].

The bacterial vector pSUDP [39] containing the full-length the *udp* gene from *S.oneidensis* MR-1 was used for the construction of mutant forms of the *udp* gene. After site-directed mutagenesis, according to [40−42], the genes of the mutant *udp* forms were cloned in the plasmid vector pTZ57R/T (Thermo Scientific, Lithuania).

The synthesis of oligodeoxyribonucleotides and DNA sequencing were carried out by Syntol (Russia) on a commercial basis. The structures of the oligodeoxyribonucleotides used in the study are presented in Table 1.

The polymerase chain reaction was carried out in an Eppendorf Mastercycler gradient amplifier (Eppendorf, Germany).

The isolation of plasmids was performed using the GeneJet™ Plasmid Miniprep Kit (MBI Fermentas, Lithuania).

E. coli cells containing the plasmid were cultivated for 16−18 h in glass tubes (or flasks) with LB medium (ampicillin, 150 µg/mL) at 37°C and 250 rpm in an Excella E25 shaker-incubator (New Brunswick Scientific, United States).

Isolation and purification of recombinant NP and their mutant forms were performed as described earlier for SoUDP [39].

Electrophoretic separation of proteins was carried out according to Laemmli [43].

The protein concentration was determined by Bradford method [44] using staining with the Bio-Rad Protein Assay reagent (Bio-Rad, United States). A solution of bovine serum albumin (Sigma, United States) was used as the standard.

The enzymatic activity of recombinant SoUDP and its mutant forms was determined in K-phosphate buffer according to [45, 46].

Table 1. The structures of synthetic oligodeoxyribonucleotides

Name	Structure 5'—3'	Function
F5Ipr	ATGGCTGATGTAATTCATTTAGG	Substitution of F5I in SoUDP
F5Irev	CCTAAATGAATTACATCAGCCAT	
F5Gpr	ATGGCCTGATGTAGGCCATTTAGG	Substitution of F5G in SoUDP
F5Grev	CCTAAATGGCCTACATCAGCCAT	
Shud1	TATAGAGCTCTGGCGTACTCCTTGTCGTC	Amplification of
Shud2	TATAGTCGACTTACGCGAGTAATTTCTTAGCT	DNA fragments during mutagenesis

The value of the Michaelis constants (K_M) for uridine and inorganic phosphate was determined as described previously [42].

The thermal stability of proteins was determined according to [1, 47]. T_{50}-the temperature value at which a 50% decrease in protein enzymatic activity was observed at the specified temperature, was used as an indicator of thermal stability. [48].

The quaternary structure of recombinant NP was confirmed by analytical gel filtration on a Tricorn 10/300 column with a Superdex 200 sorbent using an AKTA FPLC instrument (GE Healthcare, UK) as described previously [42]. Gel Filtration Calibration Kits (GE Healthcare Life Sciences, UK) and recombinant SoUDP from *S. oneidensis* MR-1 [39] were used as marker proteins.

The primary structure of the isolated recombinant proteins was additionally confirmed by MALDI-TOF/TOF-mass spectrometric analysis of their tryptic hydrolysates.

The construction of spatial structures was carried out using the PyMol program (www.pymol.org).

Statistical processing of the results of a series of measurements was carried out using the StatPlus2007 program (http://analystsoft.com).

RESULTS AND DISCUSSION

The study of the nature of the formation of thermostable proteins is now one of the most important areas of protein engineering, due both to the desire to elucidate the molecular basis for the formation and functioning of thermotolerant proteins and the practical goal of using enzymes for biocatalysis in the synthesis [32, 49] of various organic compounds (green chemistry), including modified nucleosides [23, 24, 37, 50]. The use of enzymatic synthesis of these compounds has a number of advantages in comparison with the chemical variant of their preparation: the almost complete elimination of toxic organic reagents and solvents, as well as a high level of stereo- and regioselectivity. The latter is practically unattainable in chemical synthesis and significantly complicates the isolation of the target isomer from the reaction mixture.

For the increase in the solubility of the starting compounds and the yield of the target substance, it is often necessary to carry out enzymatic synthesis at an elevated temperature [28, 51]. Accordingly, the catalyst protein itself must be resistant to elevated temperatures, which can be achieved by introducing multiple or point amino acid substitutions into the structure of the catalyst protein, which improve or even change its substrate specificity [52] and also increases its thermal stability [1, 5, 36, 48, 53].

The comparative analysis of the primary structures of UDP from various microorganisms (Fig. 1) was based on the alignment of functional areas, for example, PGDP, which is part of the phosphate binding site [1, 54] of the enzyme. This analysis showed that the primary structure of uridine phosphorylases contained an invariant histidine residue involved in the formation of the active center of the enzyme [54–56]. This residue is located in the poorly structured N-terminal region of the polypeptide chain (Figs. 1, 2), which is also preceded by a phenylalanine residue invariant in uridine phosphorylases from mesophiles (Figs. 1a; 2). The role of the histidine residue (H8) in the operation of UDP from *E. coli* was investigated and it was found that the substitution of H8N almost completely abolished the enzymatic activity of this protein [55]. These data were largely confirmed in [56].

Unfortunately, there are practically no data on the spatial organization of uridine phosphorylases from thermophilic microorganisms in the scientific literature, which complicates a direct comparative crystallographic analysis of the structures of these proteins in the mesophile–thermophile series. However, if we assume the common structure of active centers in nucleoside phosphorylases [42, 56], then the invariant histidine residue of UDP from thermophiles (Fig. 1b) can perform binding function of the carbohydrate residue of the nucleoside in the active site of the enzyme. It should be noted that in UDP from thermophilic microorganisms, this residue (Fig. 1b) is preceded by a significantly longer compared with uridine phosphorylases from mesophiles, N-terminal fragment of the polypeptide chain.

N-terminal	Microorganism	GenBank

(a)

MSKSDV**FH**LGLTKNDLQGATLAIV**PGDP**DRV *Escherichia coli* — NC_000913
MSKSDV**FH**LGLTKNDLQGATLAIV**PGDP**ERV *Klebsiella pneumoniae* — CDO16248
MPQSDV**FH**LGLTKADLQGATLAIA**PGDP**ERV *Erwinia pyrifoliae* — CAY72523.1
MADV**FH**LGLTKAMLDGATLAIV**PGDP**ERV *Shewanella oneidensis* MR-1 — GQ294526
MSKSDV**FH**LGLTKNDLQGATLAIV**PGDP**ERV *Klebsiella aerogenes* — NZ_LR134475
MSKSDV**FH**LGLTKNDLQGAQLAIV**PGDP**ERV *Salmonella typhimurium* — NC_003197.2
MTKTV**FH**LGVTEADLNGATLAII**PGDP**ARV *Vibrio cholerae* — AKB06100.1

(b)

MSERLKSASRPESEEGRL**YH**LQVKPGDVSRYILL**PGDP**DRV *Desulfurococcus amylolyticus* — AFL66855*
MERGKRLSSASAPVDESGRV**YH**LGVKPGDVSKYVLL**PGDP**GRV *Staphylothermus marinus* — WP_011838765.1*
MVKALKSASTPETEEGRQ**YH**LEVKPGDVSRYVLL**PGDP**GRV *Thermosphaera aggregans* — WP_013129600.1*
MYSPPRFMRRLLFAFYKAYDKVMEMKFVSADRPQTEEGYQ**YH**IACKPGDVARYVLL**PGDP**ERV *Thermococcus barophilus* — ALM74274.1*
MGDESLRSAARPEGEGGLQ**YH**LRVRRGDVARYVLL**PGDP**ERT *Aeropyrum pernix* — WP_131160395*
MPGPRRTGGLAVTGEKVRAREPSGSGGLQ**YH**IKCRPGDVAPTVLL**PGDP**ERV *Thermofilum pendens* — WP_011752903*

Fig. 1. The functional alignment of the primary structures of the N-terminal parts of uridine phosphorylases from various micro-organisms. The fragment of the polypeptide chain involved in the formation of the binding site of the inorganic phosphate ion is highlighted in yellow [1, 42], and invariant amino acid residues **FH** and **YH** in uridine phosphorylases from mesophilic (a) and thermophilic (b) microorganisms, respectively are highlighted in red. (*Thermophilic microorganisms.)
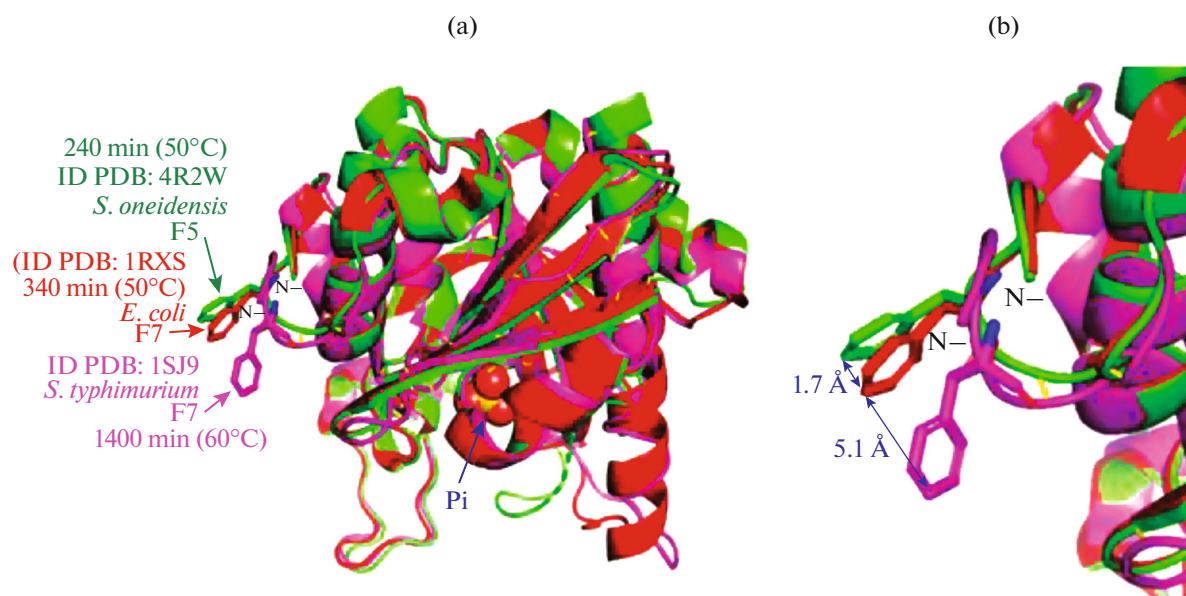
Fig. 2. The alignment of spatial structures (subunit A) of UDP monomers (a) from *S. oneidensis* MR-1 (PDB ID: 4R2W, green color), *E. coli* (PDB ID: 1RXS, red color), *S. typhimurium* (PDB ID: 1SJ9, turquoise color). For enzymes, time (T_{50}) of their thermal semi-inactivation is shown [1]. Pi is an inorganic phosphate ion in the active site of the enzyme. An enlarged representation of the spatial arrangement of phenylalanine residues (b). The arrows indicate the mutual distances of the side radicals.

This fragment can play a significant role in the stabilization of the secondary structure of the N-terminal region of the UDP polypeptide chain, providing a stable and most favorable for catalysis conformational state of the considered histidine residue. Such a peculiar protective function of this fragment of the polypeptide chain can also manifest as the increased stability of uridine phosphorylases from thermophiles under temperature exposure.

The comparative analysis of the primary structures of the N-terminal parts of UDP from mesophilic and thermophilic microorganisms (Fig. 1) also shows that the considered histidine residue in mesophiles is preceded by a phenylalanine residue, while in thermophiles it is preceded by a tyrosine residue. Considering the higher hydrophilicity of the tyrosine residue compared to the phenylalanine residue, as well as its location in close proximity to the functionally important

histidine residue, it can be assumed that this particular residue plays a significant role in imparting thermal stability of uridine phosphorylases. It should also be noted that in according to the results of the analysis carried out in [9], the surface of proteins from thermophilic microorganisms is, to a greater extent, compared to mesophiles, enriched with charged amino acid residues. These data also drew attention to the considered hydrophobic phenylalanine residue located on the surface of uridine phosphorylases and involved in the direct contact with the solution surrounding the protein (PDB ID: 4R2W, 1RXS, 1SJ9, 6EYP, 4NY1 etc.).

The assumptions made above about the role of the structure of the N-terminal parts of UDP in thermal stabilization are largely based on nucleoside phosphorylases previously obtained and studied by the authors, in which point and extended amino acid substitutions were carried out in this class of homologous proteins [1, 41]. The analysis of the summary results presented in the cited studies showed that the structure of the N-terminal parts of nucleoside phosphorylases is one of the factors that largely determines the thermal stability of this class of enzymes.

In addition, in [1] it was found that thermal stability (T_{50}) of native uridine phosphorylases changes in *S. typhimurium* $\geqslant$ *E. coli* > *S. oneidensis* MR-1 series. The X-ray diffraction data of the crystalline forms of these UDP are known and a comparative analysis of their spatial structures was performed (Fig. 2).

A significant difference in the spatial arrangement of the phenylalanine residue in the composition of the N-terminal parts of the considered nucleoside phosphorylases should be mentioned (Fig. 2). Such changes in the location of the phenylalanine residue can affect the properties of the protein in the solution, including its thermal stability, which was quite convincingly noted in a review [57]. For uridine phosphorylases a certain correlation between the specific activity [42] and thermal stability [1] of these enzymes, depending on the primary structure of the analyzed fragment of the polypeptide chain of proteins was noted (Fig. 2).

The above analysis of the literature data predetermined the need for an experimental investigation of the role of the phenylalanine residue (F5) in the thermal stabilization of bacterial uridine phosphorylases, using the example of UDP from *S. oneidensis* MR-1.

For this purpose, in the structural part of the uridine phosphorylase gene from *S. oneidensis* MR-1, the phenylalanine (F5) codon was substituted by the glycine (F5G) and isoleucine (F5I) codons by site-directed mutagenesis [42] with the use of synthetic primers (Table 1). The choice of the type of substituting amino acid residues was predetermined by the following reasons: the isoleucine residue has a relatively hydrophobic side radical, which partially models the properties of a phenylalanine residue with the aromatic side radical, while the glycine residue is completely devoid of a side radical.

The resulting mutant forms of the *udp* gene were cloned in pTZ57R/T vector (Thermo Scientific, Lithuania) and the nucleotide sequence of the target genes were confirmed by sequencing. *E. coli* C600Δudp cells were transformed with recombinant plasmids (see Materials and Methods). The accumulation of mutant UDP forms was assessed using denaturing polyacrylamide gel electrophoresis (Fig. 3).

As can be seen in Fig. 3, the target mutant SoUDP forms accumulate in *E. coli* cells under heterologous expression conditions in a significant amount: on electrophoresis pattern they are presented as a major band with an approximate molecular weight of 27.5 kDa. Mutant SoUDP forms were isolated and purified according to [39], after which they were transferred to a 5.0 mM Tris-HCl solution, pH 8.0 using ultrafiltration (PM30 membrane, Millipore, United States) and stored at −70°C.

Data on the properties of a number of nucleoside phosphorylases, including their thermostable and mutant forms, have been published in the literature [1, 36, 37, 58].

The thermal stability of these proteins was studied not only using various buffer systems, but also often in the presence of at least one substrate (for example, inorganic phosphate) [58, 59]. However, it was previously shown [1, 60] that nucleoside phosphorylases are thermally stabilized in the presence of substrates. Moreover, the presence of a foreign protein (e.g., BSA [60]), also increases the resistance of nucleoside phosphorylases to temperature. At the same time, the effectiveness of such stabilization is hardly predictable both for the original form of the studied proteins and for mutants based on them. Certain aspects of conformational changes in bacterial uridine phosphorylases upon binding to substrates were described by us earlier in [61]. In order to exclude the influence of the substrate on the thermal stabilization of the studied SoUDP and its mutant forms (Fig. 4), in this study the enzymes were incubated in a similar way [1, 62] in a 20 mM Tris-HCl buffer, pH 8.0.

The results shown in Figs. 2 and 4 indicate that the spatial arrangement of the side radical of the phenylalanine residue can affect the conformation of the low structured N-terminal region, as a result of which its accessibility to the buffer surrounding the protein probably changes and these changes are expressed as an increase in the thermal stability of the recombinant mutant forms of the studied SoUDP. At the same time, conformational changes in the N-terminal fragment may be insignificant, but have a significant effect on the resistance of the protein to thermal effects [32, 57].

The quaternary structure of a number of enzymes, including nucleoside phosphorylase, is present in solution as homooligomers (dimers, trimers, hexam-
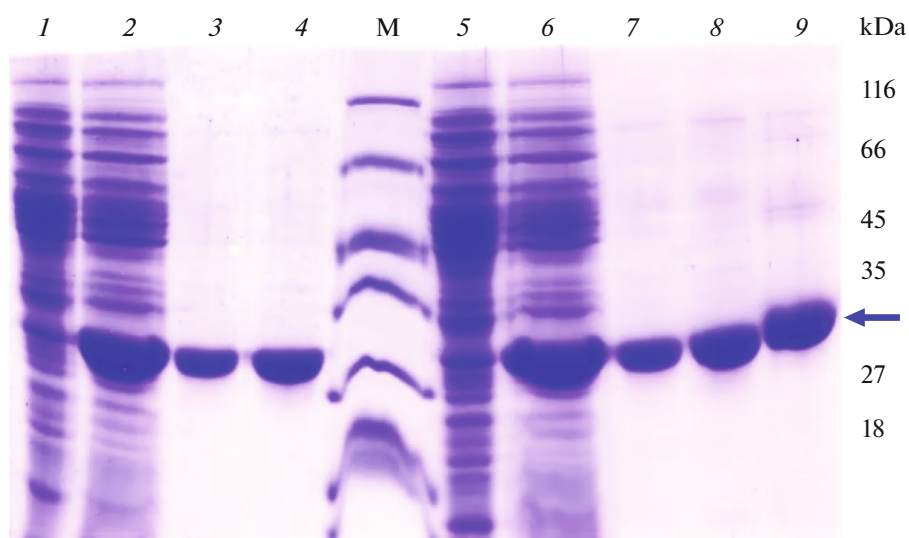
Fig. 3. An electrophoretic analysis of recombinant proteins in *E. coli* C600Δudp cells in 12.5% PAGE with SDS-Na. M—protein molecular weight markers (116, 66, 45, 35, 27, 18 kDa); *1, 2, 5, 6*—proteins of soluble fractions of the recipient strain and producer strains obtained after disruption of *E. coli* cells by ultrasound; *3, 4, 7, 8*—purified recombinant enzymes: F5I, F5G, 3 and 5 μg, respectively; *9*—SoUDP from *S. oneidensis* MR-1, 5 μg. Target recombinant proteins are indicated by an arrow.
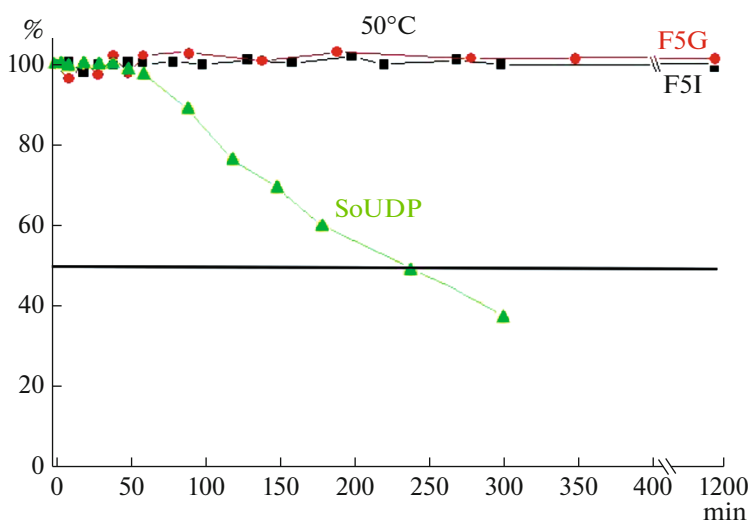
Fig. 4. A comparative analysis of the thermal stability of uridine phosphorylase (*%*) from *S. oneidensis* MR-1 (SoUDP) and its mutant (F5G and F5I) forms. Each point on the graph corresponds to the average value of three independent experiments.

ers). This type of oligomerization is important not only for the manifestation of enzymatic activity of these proteins, but also for the increase of their thermal stability [6, 63, 64]. In this regard, the retention of the hexameric quaternary structure by the mutant SoUDP forms was experimentally confirmed by the method of analytical gel filtration (Table 2).

The decrease in affinity (K_M) in mutant forms of uridine phosphorylase to substrates compared to the original wild-type form deserves attention (Table 2). At the same time, this change was accompanied by a significant increase in the thermal stability of mutant proteins (Table 2, Fig. 4). This fact coincides with the earlier conclusions about the decisive role of the architectural structure of the phosphate-binding region in the acquisition of NP thermal stability [1]. Now, crystalline forms of the studied mutant proteins have been obtained and their X-ray diffraction analysis is being carried out, which can significantly clarify the molecular nature of the observed phenomenon.

Thus, based on a comparative analysis of the literature data and the experimental results obtained in this

Table 2. Comparative characteristics of properties of SoUDP and its mutant forms

Protein	T_{opt}, °C	T_{50}, 50°C, min	T_{50}, 60°C, min	K_M		V_{yield}, mL
				Urd, mM	Pi, mM	
SoUDP	60 ± 4	240 ± 4	14 ± 2	0.49 ± 0.05	5.5 ± 0.3	13.85 ± 0.05
F5I	58 ± 3	Stable 1200 ± 9	15 ± 3	0.77 ± 0.04	9.3 ± 0.2	14.03 ± 0.04
F5G	61 ± 2	Stable 1200 ± 10	18 ± 2	0.84 ± 0.08	8.9 ± 0.1	13.98 ± 0.06

study, the important role of the individual F5 amino-acid residue and the entire N-terminal fragment of the polypeptide in the formation of the thermal stability of uridine phosphorylases was revealed.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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