ISSN 1990-7508, Biochemistry (Moscow), Supplement Series B: Biomedical Chemistry, 2017, Vol. 11, No. 2, pp. 120–127. © Pleiades Publishing, Ltd., 2017.
Original Russian Text © D.S. Shcherbinin, M.Yu. Rubtsova, V.G. Grigorenko, I.V. Uporov, A.V. Veselovsky, A.M. Egorov, 2017, published in Biomeditsinskaya Khimiya.

The Study of the Role of Mutations M182T and Q39K in the TEM-72 β-Lactamase Structure by the Molecular Dynamics Method

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Received September 13, 2016

Abstract—Synthesis of β-lactamases is one of the common mechanisms of bacterial resistance to β-lactam antibiotics such as penicillins and cephalosporins. The widespread use of antibiotics resulted in appearance of numerous extended-spectrum β-lactamase variants or inhibitor-resistant β-lactamases. In TEM type β-lactamases mutations of 92 residues have been described. Several mutations are functionally important and they determine the extended substrate specificity. However, roles of the most so-called associated mutations, located far from the active site, remain unknown. We have investigated the role of associated mutations in structure of β-lactamase TEM-72, which contains two key mutations (G238S, E240K) and two associated mutations (Q39K, M182T) by means of molecular dynamics simulation. Appearance of the key mutations (in 238 and 240 positions) caused destabilization of the protein globule, characterized by increased mobility of amino acid residues. Associated mutations (Q39K, M182T) exhibited opposite effect on the protein structure. The mutation M182T stabilized, while the mutation Q39K destabilized the protein. It appears that the latter mutation promoted optimization of the conformational mobility of β-lactamase and may influence the enzyme activity.

Keywords: β-lactamase, TEM-1, TEM-72, mutations, thermoresistance, molecular dynamics
DOI: 10.1134/S1990750817020056

INTRODUCTION

The β-lactam antibiotics are the bactericide compounds that prevent synthesis of the bacterial cell wall [1]. In response to the effect of these antibiotics bacteria have elaborated various mechanisms determining their resistance. One of these mechanisms includes synthesis of β-lactamase enzymes hydrolyzing the β-lactam ring of antibiotics. Based on the amino acid sequence β-lactamases are subdivided into four molecular classes (A–D) [2]. The classes A, C, and D pool together serine-dependent β-lactamases, while class B enzymes are metal dependent and contain one or two zinc ions in their active sites.

The class A β-lactamases are the most common enzymes [3–6]. It is believed that these β-lactamases are derived from the penicillin-binding proteins that are natural targets of β-lactam antibiotics [7]. The widespread use of these antibiotics and selective selection of the resistant strains of β-lactamases are constantly evolutionized [3, 6]. Evolution occurs in the direction of extended substrate specificity and appearance of enzymes that hydrolyze not only penicillins and cephalosporins, but also a new type such as carbapenems. These widespread enzymes are known as extended-spectrum β-lactamases (ESBL).

Among the members of the molecular class A family the TEM-type β-lactamases are the most abundant [8, 9]. Currently, it comprises about 200 enzymes that represent the mutant forms of TEM-1 β-lactamase (the first discovered member of this family), which contain from one to seven amino acid substitutions [10, 11]. β-Lactamases of this type share a similar three-dimensional structure consisting of two subdomains (one α/β, and the other all α, Fig. 1) with the active site, located at the interface of the sub-domains [12]; the active site is restricted the conservative SDN- and Ω-loops. It is believed that catalysis involves five amino acid residues of the active site (S70, K73, S130, E166, and K234), which are responsible for the acylation-deacylation reaction, resulted in the cleavage of the amide bond of the β-lactam ring. The hydroxyl of S70 is the key group in this reaction.

In the TEM type β-lactamases, mutations of 92 amino acid residues have been described. It has been known that mutations at four positions (E104,

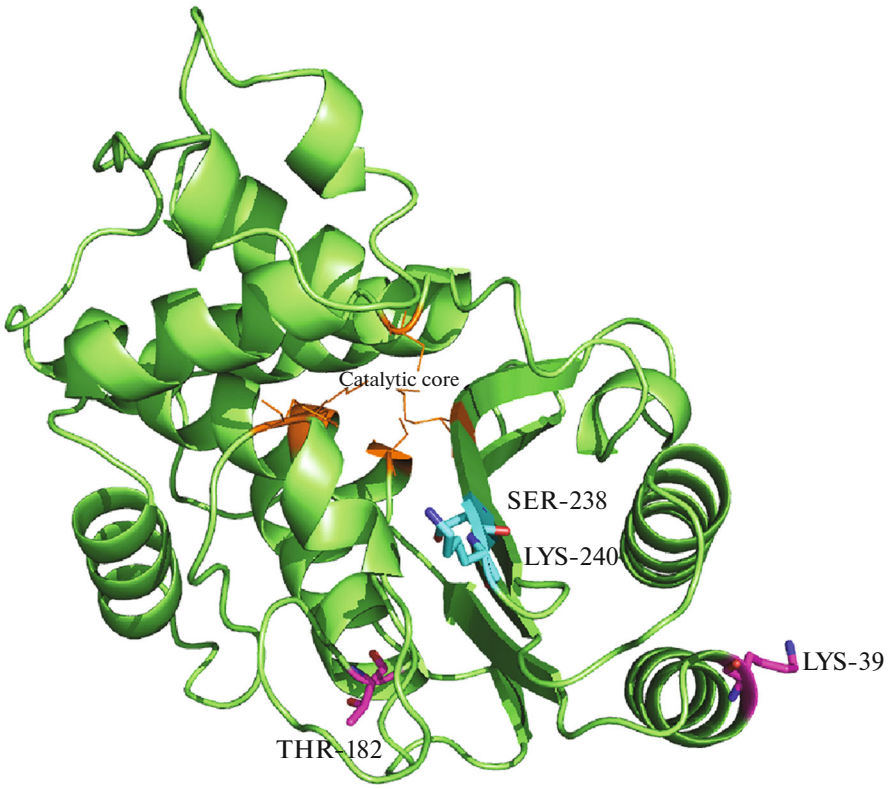

Fig. 1. Spatial structure of β-lactamases TEM-1 and TEM-72. Solid lines indicate residues that differ in β-lactamase TEM-72: S238, K240 (functional mutation), K39 and T182. The thin lines show the catalytic site residues.

R164S, R164H, G238S, E240K) are functionally (key) important and they are responsible for extended substrate specificity. These amino acid substitutions occur near the active site and contribute to an increase in its size so that it could fit to the large hydroxyimino group of cephalosporins. However, the role of so-called associated mutations located apart from the active site remains unknown. The "fees" for the expanded substrate specificity in ESBL consisted in decreased thermodynamic stability of the protein globule. Previously, a stabilizing effect on the associated mutation M182T on the TEM variants with functional mutations G238S and M69I has been recognized [13–15]. The study of the effect of other mutations on the changes in the thermal stability of β-lactamases attracts much interest. One of such mutations includes the substitution Q39K, which is common, and occurs in enzymes of different phenotypes. For example, this mutation is present in the β-lactamase TEM-72, along with two key mutations (G238S, E240K), extending substrate specificity, and the associated mutation M182T.

Known mutations not necessarily result in structural changes of β-lactamases; this suggests that their impact on the catalytic properties of this enzyme are mediated changes in the protein mobility. Detection of these changes may be well detected by the method of molecular dynamics simulations. Previously, using this method, it was shown that single point mutations could lead to changes in the mobility of different sites in TEM-1 β-lactamases [16], and in β-lactamase of the SHV family (belonging to the same molecular class) [17].

The aim of this study was to investigate the role of associated mutations in the structure of β-lactamase TEM-72 containing two key mutations (G238S, E240K) and two associated mutations (Q39K, M182T) on the molecular dynamics of the β-lactamase enzyme. The molecular dynamics calculations have been carried out at two temperatures (300 K and 400 K) for the structures of β-lactamases TEM-1, TEM-72 and TEM-72 with inverse substitutions K39Q and T182M.

MATERIALS AND METHOS

The spatial structures of β-lactamases TEM-1 and TEM-72 were obtained from the Protein Data Bank (PDB) (PDBid 1ZG4 and 3P98, respectively) [18, 19]. Preliminary preparation of structures was performed using the Sybyl 8.1 software package, which was also used for virtual introduction of reverse substitutions K39Q and T182M in β-lactamases TEM-72. This was performed in order to assess the contribution of each

mutations in the properties of the enzyme protein, containing amino acid substitutions that extend its substrate specificity.

Molecular dynamics simulations were performed using the Gromacs-5.1 software package. Parameterization of atomic structures was carried out using the Amber99-SB force field. The system was solvated by explicit solvent (water TIP3P model) and was neutralized using Na^+ ions. At the first stage sequential minimization of the system was performed in vacuum and in a solvent (for 150000 steps). The next stage included sequential heating of the system up to 300 K or 400 K and pressure increase with NVT and NPT ensembles. The molecular dynamics was performed for 25 ns with a 2 fs-time step.

Trajectories were analyzed using the Gromacs-5.1 software package, VMD-1.9.1 and principal component analysis (PCA). Molecular dynamics trajectories were analyzed by the root mean square deviation (RMSD), root mean square fluctuation (RMSF) of amino acid residues, the number of hydrogen bond formed between the enzyme subdomains and stability of the elements of the secondary structure of the protein globule by the DSPP program. Simulations were performed on an IBMC computation server (48 cores, 8 Tesla S2050).

RESULTS

The crystal structures of β-lactamases TEM-1 and TEM-72 insignificantly differ from each other (the RMSD value is about 0.68 Å) (Fig. 1). The key (functional) amino acid substitutions at positions 238 and 240 of the β-lactamases are located in the ligand binding region of the active site; this results in extended substrate specificity. The amino acid residues at positions 39 and 182 are located at a considerable distance (15–20 Å) from the active center of this enzyme. The residue 39 is localized on the N-terminal helix of the α/β-subdomain, while the residue 182 is localized in one of helices of the other subdomain; it is located on the opposite side of the protein globule (versus the active site of the enzyme).

Since the spatial structures of the β-lactamases TEM-1 and TEM-72 are characterized by minor differences, the effect of distant mutations located far from the active site but affecting the enzymatic properties should be obviously determined by the change in the motions of some regions of the protein globule. Therefore, analysis of the functional role of associated amino acid substitutions was performed by molecular dynamics simulations.

Molecular dynamics simulations were performed for the following structures of β-lactamases: TEM-1, TEM-72, TEM-72 with reverse amino acid substitution K39Q (TEM-$1_{238,240,182}$), TEM-72 with reverse amino acid substitution T182M (TEM-$1_{238,240,39}$) and TEM-72 with two reverse amino acid substitution K39Q and T182M (TEM-$1_{238,240}$) (this variant corresponds to the TEM-1 structure with two substitutions extending substrate specificity). Two temperatures were used: 300 K and 400 K. This resulted in 5 molecular dynamics trajectories for different β-lactamase modifications at each temperature (300 K and 400 K).

Figure 2 shows changes in the RMSD values of amino acid residues during the dynamics. It could be seen that the structure of these proteins remained stable. At 300 K, the RMSD values reached the plateau after 4 ns and represented 1.3–1.4 Å (Fig. 2a). No principal differences in the mobility of the studied structures have been detected at 300 K. The only difference at this temperature was found in the RMSD values for β-lactamases TEM-72 with simultaneously introduced reverse substitutions K39Q and T182M (TEM-$1_{238,240}$) which were higher within the first ten ns than in TEM-1, and then the values became equal.

Further temperature increase from 300 K to 400 K was accompanied by the increase in the RMSD values by 0.5–0.7 Å for all five examined structures (Fig. 2b) and all variants of this enzyme remained stable. Stability of the secondary structures was confirmed using the DSSP program (data not shown). The lowest RMSD value was obtained for β-lactamase TEM-1; introduction of two functional mutations (TEM-$1_{238,240}$) caused an increase in the RMDS value of 0.5 Å. This suggests that introduction of functional mutations induced destabilization of the β-lactamase structure and increased mobility of the protein globule at higher temperature. Additional mutation M182T (TEM-$1_{238,240,182}$) led to a decrease in the RMSD value; this is consistent with the well-known feature of this mutation to restore the stability of TEM-type β-lactamases to thermal inactivation [20]. However, introduction of Q39K mutations (TEM-$1_{238,240,39}$) in the β-lactamase variant with functional mutations had an opposite effect: the RMSD value increased and higher RMSD peaks were observed. The RMSD value for β-lactamase TEM-72 (containing the four mutations) had a middle position between the variants of TEM-$1_{238,240,182}$ and TEM-$1_{238,240,39}$; it was characterized by smooth peaks, indicating the mutual influence of associated mutations. The results indicate the multidirectional effect of associated mutations on the protein globule behavior at high temperatures.

Since changes in mobility of the protein globules of the studied variants of β-lactamases were observed at 400 K, further analysis was carried out at this temperature. For β-lactamases with different variants of amino acids substitutions, fluctuations of heavy atoms (RMSF) were calculated during the molecular dynamics. Figure 3 shows that introduction of functional mutations (TEM-$1_{238,240}$) resulted in the expected increase in the overall mobility of residues compared to TEM-1 (an averaged difference in the values was 0.5–0.7 Å, but for some residues it reached

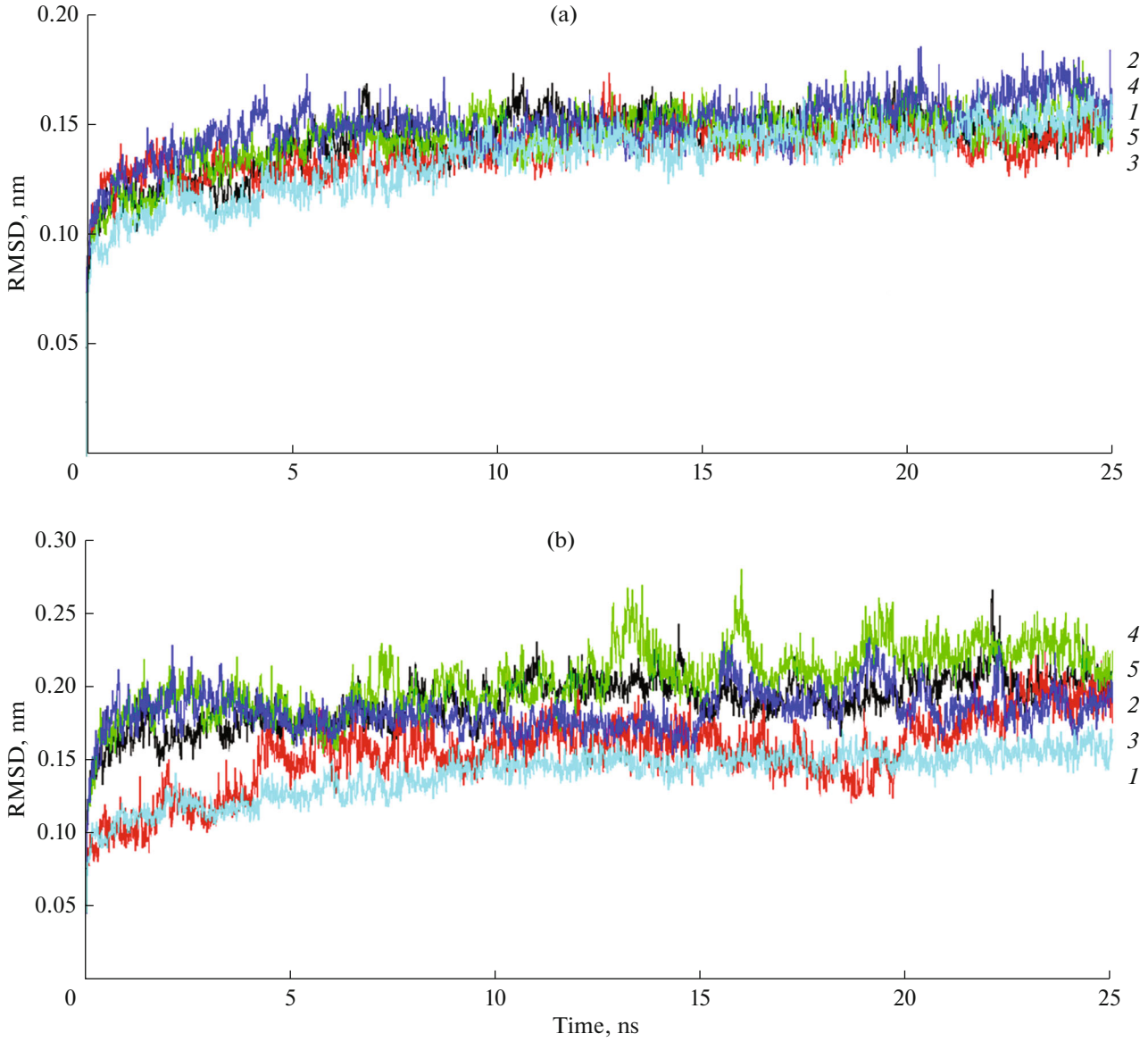

Fig. 2. Changes in RMSD values for heavy atoms of β-lactamase during molecular dynamics at 300 K (a) and 400 K (b). TEM-1—*1*, TEM-$1_{238,240}$—*2*, TEM-$1_{238,240,182}$—*3*, TEM-$1_{238,240,39}$—*4*, TEM-72—*5*.

2 Å) (Fig. 3a). This also pointed to the fact that these mutations destabilized the protein globule and extended substrate specificity of this enzyme. However, additional mutation of the residues 182 (TEM-$1_{238,240,182}$) did not result in significant reduction in the mobility of β-lactamase amino acid residues, except the site 172–179 (C-terminus of the Ω-loop) (Fig. 3b). The effect of other associated residue 39 (TEM-$1_{238,240,39}$) was more complex (Fig. 3c). This additional mutation insignificantly increased mobility of amino acid radicals of the whole protein, except two sites, where the mobility was lower. The first site (as in the case of the introduced mutation to position 182) included residues 170–178, and the second site included residues 215–219 (the loop connecting H9 and H10 [21]). However, the presence of two associated mutations (TEM-72) resulted in an increase in the RMSF values for the site 170–178 (C-terminus of the Ω-loop) and the loop Met155–163Asp. Comparison of the RMSF values for TEM-1 and TEM-72 showed that the amino acid residues in TEM-72 were more mobile than in TEM-1 (Fig. 3d). In this case, the differences in mobility of several residues forming the Ω-loop in TEM-72 reached 2 Å, but in the region of the catalytic residue (166) differences in mobility were absent.

Collective movements inside protein globules of the studied variants of TEM-type β-lactamases were

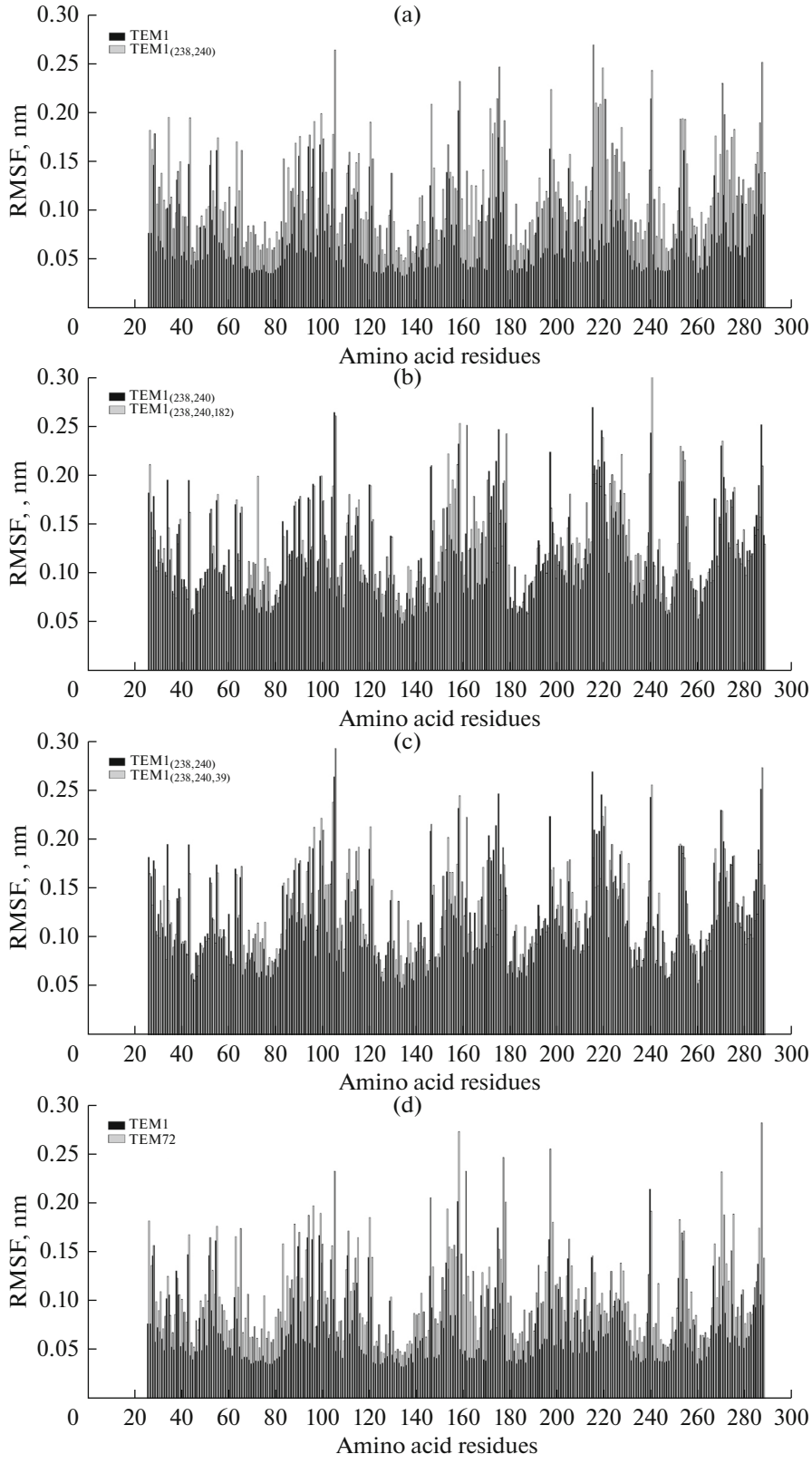

Fig. 3. Plots of the β-lactamase RMSF values at 400 K: (a) TEM-1 and TEM-$1_{238,240}$; (b) TEM-$1_{238,240}$, and TEM-$1_{238,240,182}$; (c) TEM-$1_{238,240}$ and TEM-$1_{238,240,39}$; (d) TEM-1 and TEM-72.

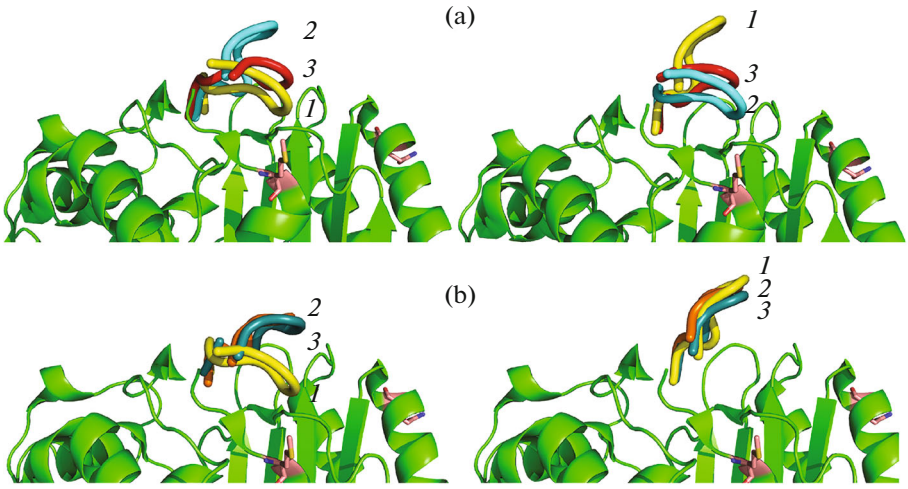

Fig. 4. Mobility of the loop Asn170–Arg178. (a) TEM-72—*1*, TEM-$1_{238,240,39}$—*2*; TEM-$1_{238,240,182}$—*3*. (b) TEM-72—*1*, TEM-1—*2*, TEM-$1_{238,240}$—*3*.

analyzed using PCA. PCA results showed that molecular dynamics was accompanied by the slide-type displacement of the regions formed by α-helices relative to β-sheet. In this case, displacements of the first and the second regions versus the β-sheet were oppositely directed (one clockwise, the other one anti-clockwise); this results in "twist" of the molecule. Such twist of β-lactamase was also found earlier [21, 22]. The most pronounced differences in the collective movements of the mutant forms of β-lactamase TEM-72 were observed for the region including Asn170–Arg178. This region is the C-terminal part of the Ω-loop, which is considered to be important for binding of substrates and their hydrolysis. The Ω-loop is often subdivided into three independent parts: weakly mobile, structured N-terminal (R161–W165), central (E166–N170), representing a helix containing the catalytic residue E166 and the C-terminal part (E171–R178). Figure 4 shows mobility of the loop containing Asn170–Arg178. In β-lactamase TEM-1 it moved from the semi-open to the open state. Introduction of the functional mutations G238S and E240K (TEM-$1_{238,240}$) had no influence on the movement behavior of this part of the Ω-loop. The introduction of additional replacement M182T (TEM$1_{238,240,182}$) froze loop motion in the closed state, and the residues of this loop began to contact with the main globule. On the contrary, introduction of the substitution Q39K to β-lactamase TEM-$1_{238,240}$ (TEM-$1_{238,240,39}$) increased the amplitude of oscillation of the site from a close to the open conformation. This behavior of the loop was also preserved in β-lactamase TEM-72. Previously, it was noted that this fragment of the Ω-loop can perform large fluctuations; in some cases, this site can interact with the main protein globule, filling the cavity between the Ω-loop and the main globule of the protein [23].

DISCUSSION

The class A β-lactamases have a rigid protein globule, which is well-structured and optimized for hydrolysis of the peptide bond in the β-lactam ring. The use of broad-spectrum antibiotics and β-lactamase inhibitors resulted in appearance of numerous variants of these enzymes. Currently, about 200 variants of TEM-type β-lactamases are known; they have been isolated from clinical strains, which include ESBL phenotypes and inhibitor-resistant forms, and different from the β-lactamase TEM-1 by substitutions of one or more amino acid residues [10, 24]. It has previously been shown that appearance of the ability to hydrolyze cephalosporins or resistance to inhibitors is accompanied by a reduction in the thermodynamic stability of this enzyme [25]. For example, substitution of the residue 238 not only extends the range of hydrolyzable substrates but also reduces thermal inactivation temperature by 5 degrees [16]. Simultaneously, β-lactamases often have so-called associated mutations, which do not affect the extended substrate specificity or resistance to inhibitors. It is believed that some of these associated mutations contribute to the proper folding of the protein chain or increase the globule stability, decreased due to key (functional) mutations [13, 26, 27]. Currently, two such mutations, acting as a global suppressor (compensatory mutations that stabilize functionally modified enzyme), M182T and L201P, are known [13, 28]. However, the functional importance of many other associated mutations remain unknown. In this study we have investigated the variant of β-lactamase TEM-72 containing in addition to functional mutations (G238S and E240K) and suppressor mutation M182T, the mutation Q39K with unknown function. It was originally discovered in β-lactamase TEM-2, where it did not affect the spectrum of hydrolyzable substrates [29].

Molecular dynamics performed at normal temperature (300 K) did not cause any noticeable changes in the initial variants of β-lactamases TEM-1 and TEM-72, as well as in the variants containing only functional mutations (TEM-$1_{238,240}$) and the variants also containing one associated mutation (TEM-$1_{238,240,182}$ and TEM-$1_{238,240,39}$). Differences were observed only during molecular simulation performed at higher temperature (400 K). During molecular dynamics of the studied TEM variants mutual location of catalytic residues remained unchanged over the whole trajectory even at the elevated temperature (400 K). Taking into consideration that hydrolysis of antibiotics by β-lactamases is a diffusion-limited process [30], this may indicate that changes in the catalytic activity of the mutant forms of this enzyme is determined by the change in the efficiency of substrate binding at the active site due to changes in mobility or conformation of residues involved in substrate translocation to the active site or determining structural adaptation of the active site to the structure of particular substrates.

Analysis of protein mobility showed that the introduction of only functional mutations (TEM-$1_{238,240}$) resulted in destabilization of the protein structure (Figs. 1 and 2). Additional introduction of associated mutation in position 182 (TEM-$1_{238,240,182}$) caused an expected decrease in mobility of the protein globule, whereas mutation in position 39 (TEM-$1_{238,240,39}$) caused further destabilization of the globule. Combination of all four mutations in β-lactamase TEM-72 leads to the variant, in which the protein globule demonstrated higher mobility as compared to the TEM-1 enzyme.

Analysis of the collective movements of the investigated variants of β-lactamases showed that the α-helical regions of globule perform oppositely directed rotational movement. However, introduction of various mutations had no influence on these movements. Earlier rotational movement were observed in molecular dynamics simulations of TEM-1 [21] and it was suggested that such movement could affect the conformation and mobility of the Ω-loop.

The most pronounced differences in movement of the studied variants were found in the C-terminal part of the Ω-loop. The functional mutations (TEM-$1_{238,240}$) had no influence on loop movement, while associated mutations changed its behavior. The mutation M182T (TEM-$1_{238,240,182}$) decreased loop mobility and its fixation in the close state, while the mutation Q39K (TEM-$1_{238,240,39}$) increased the oscillation amplitude. The combination of these two mutations in β-lactamase TEM-72 retained the loop behavior similarly to the β-lactamase TEM-$1_{238,240,39}$. Previously it has been shown repeatedly that the position of the C-terminal region of the Ω-loop and its mobility significantly affect activity of β-lactamases [31–35]. Therefore, the observed change in the loop behavior during molecular dynamics can influence the enzyme activity.

Thus, the influence of the investigated mutations on the structure of β-lactamases TEM-1 may be realized as follows. The appearance of mutations at positions 238 and 240 not only extends their substrate specificity, but also decreases the thermodynamic stability of the protein. The mutation M182T stabilizes the structure of the protein globule, but simultaneously decreases catalytic activity due to reduced conformational mobility of the protein sites responsible for substrate transport and binding in the active site. Appearance of the mutation Q39K, which is probably destabilizing, causes reverse relaxation of the protein structure and this can possibly increase in the catalytic activity of this enzyme.

CONCLUSIONS

The role of two associated mutations in β-lactamase TEM-72 has been investigated by the molecular dynamics method. It has been shown that the appearance of the key (functional) mutations in positions 238 and 240 leads to destabilization of the protein globule. Associated mutations covering residues 39 and 182 cause opposite effects on the protein structure. The mutation M182T increases stability of the protein globule during heating, while the mutation Q39K is destabilizing. The latter mutation seems to be necessary for optimization of the conformational mobility of β-lactamase, and may affect the efficiency of substrate cleavage.

ACKNOWLEDGMENTS

This study was supported by the Russian Science Foundation (project no. 15-14-00014).

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Translated by A. Medvedev