ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 9, pp. 1044-1048. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 9, pp. 1280-1285.

Simulated ^{18}O Kinetic Isotope Effects in Enzymatic Hydrolysis of Guanosine Triphosphate

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Received September 29, 2008
Revision received October 30, 2008

Abstract—We compare the computed on the base of quantum mechanical–molecular mechanical (QM/MM) modeling kinetic isotope effects (KIEs) for a series of the ^{18}O-labeled substrates in enzymatic hydrolysis of guanosine triphosphate (GTP) with those measured experimentally. Following the quantitative structure–activity relationship concept, we introduce the correlation between KIEs and structure of substrates with the help of a labeling index, which also aids better imaging of presentation of both experimental and theoretical data. An evident correlation of the computed and measured KIEs provides support to the predominantly dissociative-type reaction mechanism of enzymatic GTP hydrolysis predicted in QM/MM simulations.

DOI: 10.1134/S0006297909090132

Key words: kinetic isotope effect, GTP hydrolysis, p21Ras, Ras–GAP, EF-Tu

The chemical reaction of hydrolysis of guanosine triphosphate (GTP) by guanosine nucleotide binding proteins (G-proteins), leading to guanosine diphosphate (GDP) and inorganic phosphate (P_i), GTP + H_2O → GDP + P_i, constitutes one of the most important enzymatic processes in biology [1, 2]. Considerable efforts are undertaken to study details of the reaction mechanism, in particular, in order to learn how to affect the reaction route by site-specific mutagenesis. The vast majority of these works aimed to elucidate the mechanism of GTP hydrolysis by the p21Ras protein (called Ras below) and by the protein complex of Ras with the GTPase-activating proteins (GAPs) [3]. In the latter case, the ability of GAP to enhance the rate of GTP hydrolysis by Ras is known to play a key role in cellular signal transduction processes.

The associative and dissociative mechanisms of GTP hydrolysis in solutions and in proteins are usually discussed taking into consideration the nature of the transition state (TS) [4]. The fully dissociative TS for GTP hydrolysis is represented by an intermediate that shows bond cleavage between the γ-phosphate group and GDP. The fully associative TS is represented by a penta-coordinated intermediate that shows no β-γ bridge bond cleavage but significant amount of bond formation between the incoming lytic water molecule and the γ-phosphate of GTP. From the experimental side, it is not easy to distinguish between these two extremes, which results in a striking diversity of conclusions on this particular chemical reaction [3, 5-7].

One of the powerful experimental approaches to probe the structure of the TS in enzymatic reactions is to measure the kinetic isotope effect (KIE) [8]. By definition KIE is the ratio of the rate constants k referring to the light (L) and heavy (H) isotope species participating in the reaction. Following the transition state theory, an estimate of KIE through the free energy barrier ($\Delta G^{\#}$) differences can be considered:

$$\Phi = \frac{k^L}{k^H} \approx e^{-(\Delta G_L^{\#} - \Delta G_H^{\#})/RT}, \qquad (1)$$

Abbreviations: EF-Tu, elongation factor Tu; ES, enzyme–substrate complex; KIE, kinetic isotope effect; QM/MM, quantum mechanical–molecular mechanical method; TS, transition state; ZPE, zero point energies.

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that provides a theoretical basis to speculate on TS features from the kinetic data. Recently, an elegant method to measure KIEs, $\Phi(^{18}O) = k^{16}/k^{18}$, for Ras- and Ras–GAP-catalyzed hydrolysis of GTP substrates that are specifically labeled by ^{18}O at γ and β non-bridge oxygen atoms and the β-γ bridge oxygen has been developed [9, 10]. These substrates are listed in Table 1. The authors tentatively concluded [9] that the results of measurements of $\Phi(^{18}O)$ for the Ras-catalyzed GTP hydrolysis provided evidence in favor of the dissociative mechanism with a loose TS.

From the theoretical side, the results of simulations on GTP enzymatic hydrolysis based on different modeling tools also fail to show a consistent picture [11-17]. In a series of previous publications [16-19], we described an application of the *ab initio* type version of the combined quantum mechanics–molecular mechanics (QM/MM) theory for modeling enzymatic hydrolysis of GTP and adenosine triphosphate (ATP), the results of which better agreed with the dissociative type mechanism.

In this paper, we show that the ^{18}O kinetic isotope effects in the reactions of GTP hydrolysis computed within the QM/MM based model are consistent with the results of experimental studies of KIEs.

METHODS OF INVESTIGATION

Theoretical methods. To estimate the values $\Phi(^{18}O)$, we computed vibrational frequencies for the stationary points on the potential energy surfaces corresponding to the enzyme–substrate complexes (ES), intermediates, and TS located previously [16, 17] for the Ras- and Ras–GAP-catalyzed hydrolysis by assuming various labeling schemes of GTP. These stationary points were obtained by using the flexible effective fragment QM/MM technique [20] with the Hartree–Fock wavefunctions and the LANL2DZdp_ECP basis set in the QM part including all phosphate groups of GTP, the catalytic water molecule, and a few neighboring molecular groups (Fig. 1). The protein environment (the MM part) was represented by effective fragments interacting with the quantum subsystem in an *ab initio* manner [21].

Here we use the simplest approach to estimate KIEs, which takes into consideration only the differences in zero point energies (ZPE) of reagents, transition states, and intermediates of the reaction. For the case of a single-stage chemical reaction in the protein matrix M

$$M\cdot(GTP + H_2O) \rightarrow M\cdot(GDP + P_i), \qquad (2)$$

we simplify Eq. (1) as follows:

$$\Phi(^{18}O) = \frac{k^{16}}{k^{18}} \approx F\, e^{-(\Delta U^{\#}_{16} - \Delta U^{\#}_{18})/RT}. \qquad (3)$$

Here $\Delta U^{\#}$ denotes the potential energy difference between TS and ES corrected for ZPE, and the pre-exponential factor F is a ratio of the corresponding partition functions that can be estimated through the (presumably major) contribution of the single reactive vibrational mode. In our QM/MM simulations, we observed such one-stage mechanism of triphosphate hydrolysis for ATP in myosin [18] and for GTP hydrolysis in the closed form of the elongation factor Tu (the EF-Tu protein) [19]. Below we show the results of ^{18}O KIE calculations for the EF-Tu protein.

In the Ras and Ras–GAP proteins [16, 17], GTP hydrolysis proceeds through the stage of formation of stable intermediates:

$$M\cdot(GTP + H_2O) \leftrightarrow M\cdot(\text{intermediate}) \leftrightarrow M\cdot(GDP + P_i).$$

In these cases the scheme to estimate KIEs should be properly generalized [8] by taking into account the contribution k_1^{16}/k_1^{18} from the first stage of breaking the $P_\gamma–O_{\beta\gamma}$ bond, the contribution $K_{eq}^{18} = k_1^{18}/k_{-1}^{18}$ from the equilibrium isotope effect, and the contribution k_2^{16}/k_2^{18} from the second stage of formation of inorganic phosphate from metaphosphate and lytic water. In Table 2 (see below), we show explicitly all these computed values for the GTP hydrolysis by the Ras–GAP protein complex as well as the total KIEs, which can be directly compared to the observed values. The results of calculations presented in Figs. 2 (lines *1* and *2*) and 3 and Table 2 refer to the temperature 300K.

Experimental methods. As discussed in references [9, 10], a double labeling, internal competition method to determine $\Phi(^{18}O)$ in reactions involving nucleotide triphosphates was recently developed. The substrate mixture is composed of ^{13}C-depleted nucleotide and nucleotide that is labeled with ^{18}O at sites of mechanistic interest and is also uniformly enriched with ^{13}C. The relative abundance of the labeled and unlabeled substrates or products is thereby reflected by the macroscopic carbon isotope ratio ($^{13}C/^{12}C$). The use of liquid chromatography, the chemical oxidation technique of carbon into CO_2, and the isotope ratio mass spectrometer allows direct determination of the isotope ratios of product and substrate and thus the high sensitivity of the procedure of KIE determination.

RESULTS AND DISCUSSION

Figure 1 illustrates a typical structure of the active site upon enzymatic GTP hydrolysis by GTPases like Ras and Ras–GAP in the geometric configuration of the ES. The GTP substrate is trapped by the protein matrix, in particular, with the help of magnesium cation. Coordination bonds of the magnesium ion to oxygen atoms of the γ- and β-groups of GTP as well as to oxygen

atoms of amino acid residues and water with typical distances between 1.8 and 2.2 Å are shown in Fig. 1 in dotted lines. The reaction starts by an in-line nucleophilic attack of a water molecule (Wat in Fig. 1) on the γ-phosphate group of GTP leading to stereochemical inversion of the configuration of the latter. Hydrogen bonds with appropriate amino acid residues (here, Gln and Thr) that help to orient the lytic water molecule (Wat) toward γ-phosphate of GTP are shown in dashed lines in Fig. 1. The two-headed black arrow in Fig. 1 illustrates the cleavage of the P_γ–$O_{\beta\gamma}$ chemical bond at the first (or single) stage of chemical transformations accompanying the route from ES to TS. The geometry configuration at TS (on the top of the potential energy barrier) corresponds to a planar arrangement of the $P_\gamma O_3$ group with the distances between P_γ and $O_{\beta\gamma}$ and between P_γ and oxygen of lytic water within the range 2.0-2.2 Å. The one-headed gray arrow in Fig. 1 shows an attack of hydroxyl from the lytic water on P_γ, which finally results in formation of P_i. The details of P_i formation as well as the fate of the proton from the lytic water differ in various enzymes [16-19], and they are not essential for the goals of the present paper.

What matters here is the relative significance of oxygen atoms from the γ- and β-groups of GTP. Apparently, the β-γ bridge oxygen plays the key role in the reaction consistent with the above scenario. Therefore, we can assume that labeling the β-γ bridge oxygen in the experiments with ^{18}O should have the greatest impact on the KIEs, while labeling β non-bridge oxygen atoms is also important but to somewhat lesser extent. Therefore, we suggest assigning the "labeling index" to the variously labeled GTP substrates. For this goal, we distinguish two options for labeling the β-γ bridge oxygen: if this atom is unlabeled, we consider the contribution to the labeling index as 0, and if it is labeled, then it is 2. Correspondingly, two options are distinguished for the β non-bridge oxygens: 0 if they are unlabeled, and 1 if they are labeled. In this definition, we can calculate the labeling index for each reacting species as specified in Table 1.

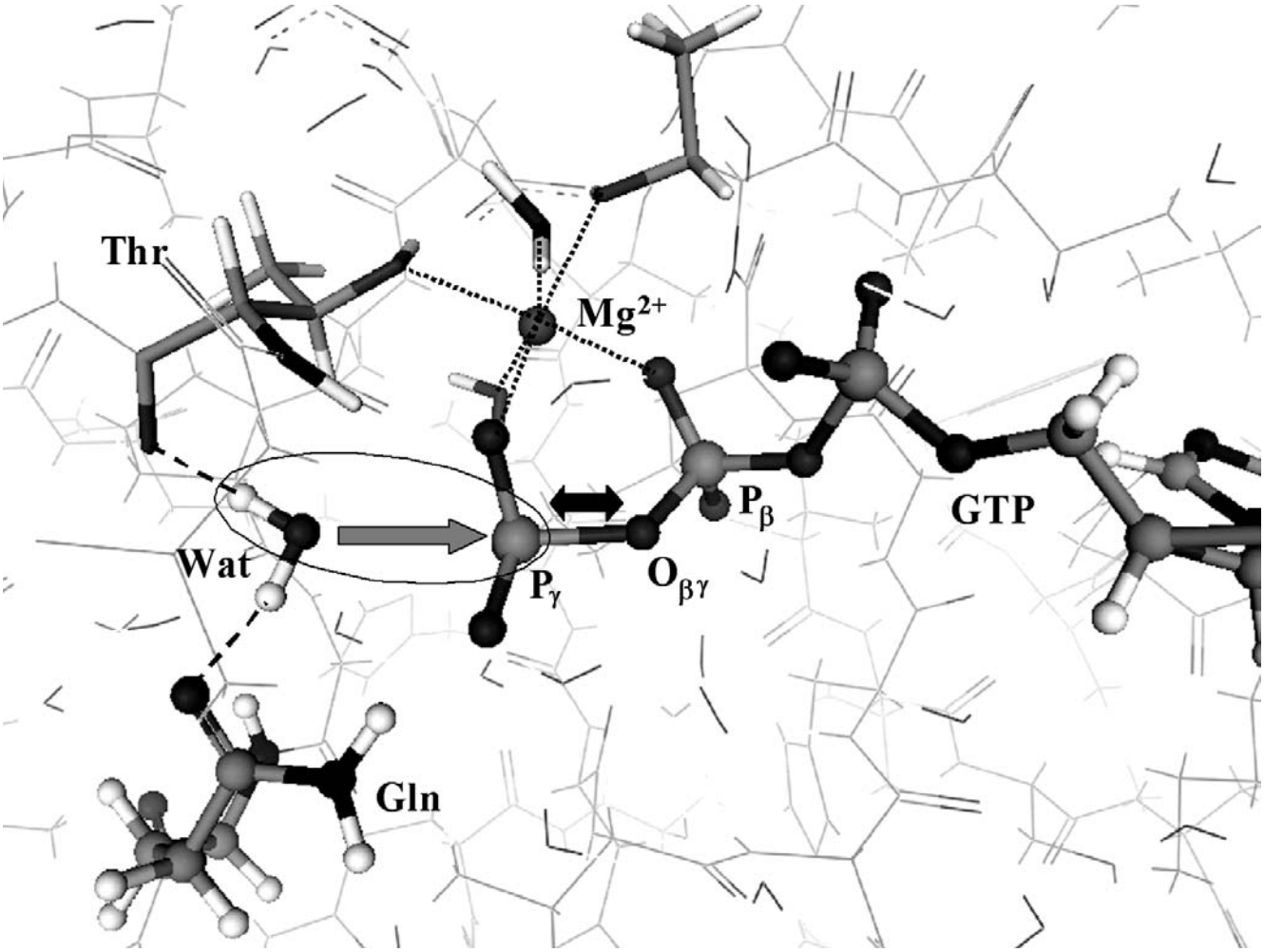
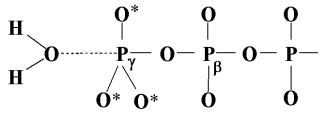
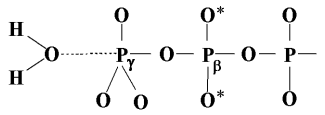
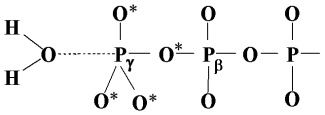
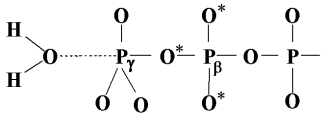


Fig. 1. Structure of the active site for GTP hydrolysis by Ras or Ras–GAP.

Table 1. Definition of "labeling index" for each reacting species studied in experimental papers [9, 10]

Reacting species	Notation used in ref. [9]	Labeling index
	$\gamma^{18}O_3$	0
	$\beta^{18}O_2$	1
	$\gamma^{18}O_4$	2
	$\beta^{18}O_3$	3

* ^{18}O-Labeled atoms.

The alternative notation which was used in papers [9, 10] for the labeled GTP substrates showed how many ^{18}O atoms were assigned to either β- or γ-groups: [$\gamma^{18}O_3$]GTP, [$\gamma^{18}O_4$]GTP, [$\beta^{18}O_3$]GTP, and [$\beta^{18}O_2$]GTP.

Remarkably, the experimentally measured [9, 10] Φ(^{18}O) plotted against such defined labeling index exhibit almost perfect linear dependence (see square symbols in Fig. 2). The lines (*3* and *4*) connecting these points are shown as visual aid only. Such presentation of the experimental data should be considered here as an application of the quantitative structure–activity relationship (QSAR) concept.

In Table 2, we show explicitly the contributions from both reaction stages as well as from the equilibrium relation to the isotope effect for GTP hydrolysis by the Ras–GAP protein complex.

In Fig. 2, we compare the measured [9] and calculated here ^{18}O KIEs for GTP hydrolysis by the Ras protein and by the Ras–GAP^{334} protein complex. Remarkably, the results of all calculations also exhibit almost perfect linear dependence of KIEs versus labeling index. As seen in Fig. 2, the computed values Φ(^{18}O) slightly deviate from the corresponding experimental estimates. Except for the compound [$\gamma^{18}O_3$]GTP with labeling index 0, the computed KIEs are larger than the experimental ones. On the contrary, the computed KIE for [$\gamma^{18}O_3$]GTP is underestimated, being even less than unity. It would be naive to expect better quantitative consent of the measured and calculated KIEs taking into account the simple way used to estimate these quantities (e.g. by Eq. (3)) as well as possible errors in QM/MM calculations of barrier heights and vibrational frequencies.

In Fig. 3 we compare the computed KIEs for the stage of cleavage of the P_γ–$O_{\beta\gamma}$ bond in GTP for three enzymatic systems considered here: Ras, Ras–GAP^{334},

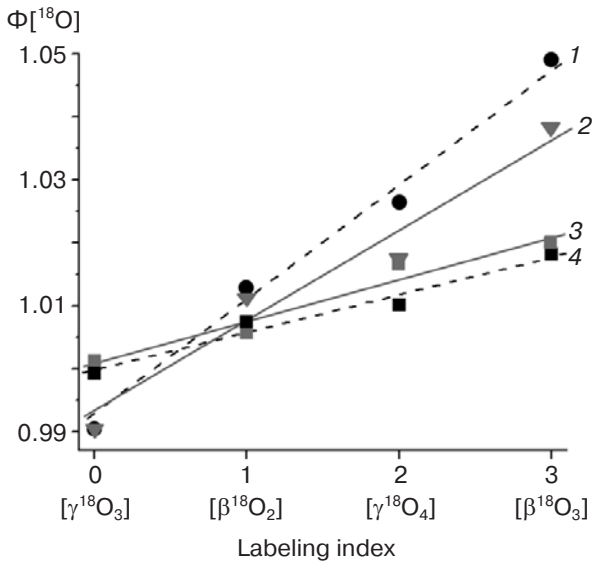


Fig. 2. Plot of calculated (*1*, *2*) and measured (*3*, *4*) KIEs for GTP hydrolysis. Black symbols and dashed lines (*1*, *4*) refer to the results for the Ras protein. Gray symbols and solid lines (*2*, *3*) refer to the results for the Ras–GAP protein complex.

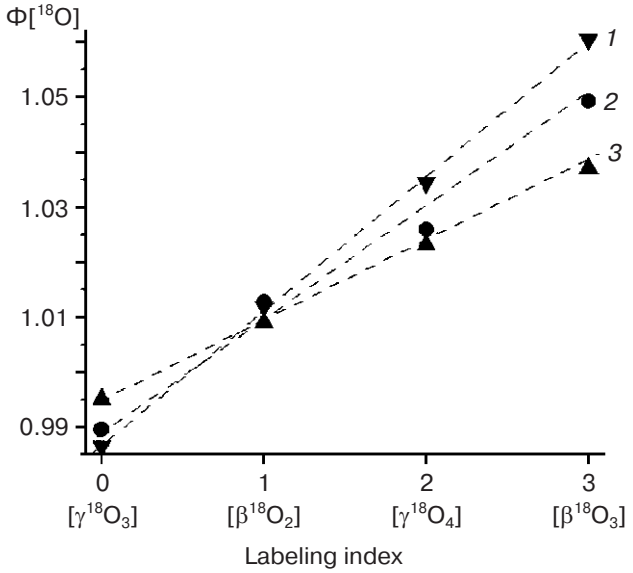


Fig. 3. Plots of calculated KIEs for the first stage of GTP hydrolysis (cleavage of the P_γ–$O_{\beta\gamma}$ bond) by EF-Tu (*1*), Ras (*2*), and Ras–GAP^{334} (*3*).

Table 2. Contributions to the isotope effect for the two-stage hydrolysis reaction of GTP by the Ras–GAP protein complex

Labeling index	0 [$\gamma^{18}O_3$]	1 [$\beta^{18}O_2$]	2 [$\gamma^{18}O_4$]	3 [$\beta^{18}O_3$]
KIE for the first stage, k_1^{16}/k_1^{18}	1.0011	1.0118	1.0289	1.0394
KIE for the second stage, k_2^{16}/k_2^{18}	0.8944	0.9104	0.8926	0.9086
Equilibrium isotope effect, $K_{eq}^{18} = k_1^{18}/k_{-1}^{18}$	1.1074	1.1107	1.1398	1.1426
Summary	0.9904	1.0112	1.0174	1.0382

and EF-Tu. Apparently, all three systems share the same property upon isotope labeling of GTP substrates. It should be noted that the role of isotopic substitution at the γ-phosphate group of GTP would be more important if the associative-type mechanism in GTP hydrolysis dominates. In the latter case, we would not observe the linear dependence of ^{18}O KIEs on the labeling index as defined above.

In summary, we suggest a QSAR-type correlation between the structure of the labeled GTP substrates and the measured ^{18}O KIEs. We obtain very similar correlation between the structure of the labeled GTP substrates and the simulated ^{18}O KIEs. Since the latter have been obtained for the theoretically established reaction mechanism, we have strong reasons to believe that the experimental data are also consistent with the predominantly dissociative type reaction mechanism of enzymatic GTP hydrolysis. A surprisingly good agreement of the experimental and theoretical plots shown in Fig. 2 provides support to the qualitative conclusions of this work.

This study was partially supported by a grant from the Russian Foundation for Basic Research (project No. 07-03-00060) and by Program No. 10 from the Division of Chemistry and Material Sciences of the Russian Academy of Sciences.

REFERENCES

1. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) *Nature*, **348**, 125-131.
2. Sprang, S. R. (1997) *Annu. Rev. Biochem.*, **66**, 639-672.
3. Li, G., and Zhang, X. C. (2004) *J. Mol. Biol.*, **340**, 921-932.
4. Mildvan, A. S. (1997) *Proteins: Struct. Funct. Genet.*, **29**, 401-416.
5. Aqvist, J., Kolmodin, K., Florian, J., and Warshel, A. (1999) *Chem. Biol.*, **6**, 71-80.
6. Wittinghofer, A. (2006) *Trends Biochem. Sci.*, **31**, 20-23.
7. Kotting, C., Blessonohl, M., Suveydis, Y., Goody, R. C., Wittinghofer, A., and Gerwert, K. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 13911-13916.
8. Cleland, W. W. (2005) *Arch. Biochem. Biophys.*, **433**, 2-12.
9. Du, X., Black, G. E., Lecchi, P., Abramson, F. P., and Sprang, S. R. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 8858-8863.
10. Du, X., Ferguson, K., and Sprang, S. R. (2008) *Anal. Biochem.*, **372**, 213-221.
11. Glennon, T. M., Villa, J., and Warshel, A. (2000) *Biochemistry*, **39**, 9641-9651.
12. Shurki, A., and Warshel, A. (2004) *Proteins: Struct. Funct. Bioinf.*, **55**, 1-10.
13. Cavalli, A., and Carloni, P. (2002) *J. Am. Chem. Soc.*, **124**, 3763-3768.
14. Heesen, H. T., Gerwert, K., and Schlitter, J. (2007) *FEBS Lett.*, **581**, 5677-5684.
15. Topol, I. A., Cachau, R. E., Nemukhin, A. V., Grigorenko, B. L., and Burt, S. K. (2004) *Biochim. Biophys. Acta*, **1700**, 125-136.
16. Grigorenko, B. L., Nemukhin, A. V., Topol, I. A., Cachau, R. E., and Burt, S. K. (2005) *Proteins: Struct. Funct. Bioinf.*, **60**, 495-503.
17. Grigorenko, B. L., Shadrina, M. S., Topol, I. A., Burt, S. K., and Nemukhin, A. V. (2007) *Proteins: Struct. Funct. Bioinf.*, **66**, 456-466.
18. Grigorenko, B. L., Rogov, A. V., Topol, I. A., Burt, S. K., Martinez, H. M., and Nemukhin, A. V. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 7057-7061.
19. Grigorenko, B. L., Shadrina, M. S., Topol, I. A., Collins, J. R., and Nemukhin, A. V. (2008) *Biochim. Biophys. Acta (Proteins and Proteomics)*, **1784**, 1908-1917.
20. Nemukhin, A. V., Grigorenko, B. L., Topol, I. A., and Burt, S. K. (2003) *J. Comput. Chem.*, **24**, 1410-1420.
21. Gordon, M. S., Freitag, M. A., Bandyopadhyay, P., Jensen, J. H., Kairys, V., and Stevens, W. J. (2002) *J. Phys. Chem. A*, **105**, 293-307.