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Binding of DNA Methyltransferase M.Ecl18 to Operator–Promoter Region Decreases Its Methylating Activity

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Abstract—The type II bifunctional DNA methyltransferase (MTase) Ecl18 that is able to control transcription of its own gene was studied kinetically. Based on initial velocity dependences from S-adenosyl-L-methionine (AdoMet) and target DNA and substrate preincubation assays, it is proposed that the enzyme apparently works by a rapid equilibrium ordered bi-bi mechanism with DNA binding first. By measuring the enzyme activity depending on DNA and AdoMet at different fixed concentrations of the operator sequence oligonucleotide, it was found that its binding has noncompetitive inhibitory effect on Ecl18 MTase activity.

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Key words: type II DNA methyltransferase, enzyme kinetics, noncompetitive inhibition

DNA methyltransferases (MTases) use S-adenosyl-L-methionine (AdoMet) as donor of methyl groups to transfer them to specific DNA sequences. In prokaryotic organisms they are mainly components of type II restriction–modification systems, protecting host cells from invasion of foreign genomes [1], but they can also exhibit autorepressor function by methylation of their own promoters (CfrBI and ScrFIA MTases [2-4]) or binding with promoters (EcoRII [5], MspI [6], and SsoII [7]). The latter three are bifunctional enzymes capable of recognizing at least two different DNA sequences specifically: one for methylation and one for binding. The binding usually decreases access of the RNA polymerase to the promoter of a corresponding gene and thus leads to reduction in its transcription. For example, SsoII MTase modifies the inner cytosine in the CCNGG sequence (where N = A, C, G, or T), and it tightly binds (K_d = 1.5·10^{-8} M) to 15 bp inverted repeat (5′-AGGACAAATTGTCCT-3′) within its own gene promoter, which allows this enzyme to be regulated autogenously [7]. An analog of M.SsoII, Ecl18 MTase [8] methylates the same CCNGG sequence and differs by only one amino acid residue exchange from SsoII MTase: isoleucine for methionine at position 56 (Ile56Met). Binding of M.Ecl18kI to its operator prevents RNA polymerase binding to the M promoter by steric exclusion, but this has no direct effect on its interaction with the R promoter [9].

While SsoII/Ecl18 DNA binding properties have been characterized in detail, its DNA methyltransferase function has not been studied yet. Neither was it shown for any bifunctional MTases of type II how binding to operator sequence would affect their MTase activity. The Ecl18 MTase belongs to the C5-cytosine DNA MTases family. The kinetic mechanisms for a number of enzymes of this group such as HhaI [10, 11], MspI [12], mouse DNMT1 [13], human DNMT1 [14], and mammalian DNMT3a [15] have been established. In general, they follow the ordered bi-bi mechanism with DNA binding first.

In this work our goal was to describe the kinetic parameters and mechanism of Ecl18 MTase. We found that this enzyme apparently works by a rapid equilibrium ordered bi-bi mechanism with DNA binding first. From kinetic studies, we have shown for the first time that operator binding has noncompetitive inhibitory effect on Ecl18 MTase activity. Thus, operator and methylated target sequence binding sites are not apparently overlapped

Abbreviations: AdoMet, S-adenosyl-L-methionine; DE, diethylaminoethyl; MTases, DNA methyltransferases; RE, restriction endonuclease.

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on the Ecl18 protein, and operator binding changes the protein conformation that leads to decrease in methylation activity.

MATERIALS AND METHODS

Chemicals. [^{3}H]Methyl-labeled AdoMet (15 Ci/mmol, 1 mCi/ml) was purchased from Amersham Biosciences (USA); DE filters were obtained from Sigma (USA); all other chemical reagents used were of the highest purity grade.

Methylation assay. To test Ecl18 DNA methyltransferase activity, we used DE-filters assay as described elsewhere [10-16]. The amount of enzyme that transferred 1 pmol of [^{3}H]methyl groups into DNA per minute with saturating concentrations of substrates at 37°C was taken as 1 U of enzyme activity.

Determination of kinetic parameters. Methylation assays were carried out for 5 min to determine initial velocity dependence. In a series of otherwise identical reactions containing 5 nM Ecl18 MTase and 4 μM [^{3}H]methyl-labeled AdoMet, the concentration of DNA was varied from 10 to 500 nM. To determine the value of K_m^{DNA}, DNA concentration was established at which the enzyme velocity was half maximal [17]. In a similar way, experiments were carried out by varying the concentration of [^{3}H]methyl-labeled AdoMet in the range of 50 to 2000 nM with DNA concentration fixed at 2 μM keeping other reaction conditions identical to the prior K_m^{DNA} assay. K_m^{AdoMet} was calculated as AdoMet concentration at which the enzyme velocity was half maximal. The k_{cat} for Ecl18 MTase was calculated as the ratio of maximal velocity to the enzyme concentration used. Kinetic studies were performed using oligonucleotides containing a single Ecl18 site (underlined): Ecl18_MS_for: 5′-GAAGATGGGAGGCCGGGGAAAC-3′; Ecl18_MS_rev: 5′-GTTTCCCCGGCCTCCCATCTTC-3′. All enzyme activity data were taken as the average of at least triplicate measurements. For all [^{3}H]methyl-labeled AdoMet dilutions 0.2 M H_2SO_4 was used.

Initial dependence of velocity on DNA and AdoMet concentrations. Methylation assays were carried out to define the initial velocity dependence on DNA at various fixed [^{3}H]methyl-labeled AdoMet concentrations and *vice versa*. In a series of otherwise identical reactions containing 5 nM Ecl18 MTase and different fixed concentrations of [^{3}H]methyl-labeled AdoMet (100, 200, 500, and 1000 nM), the concentration of DNA was varied from 10 to 100 nM. Similarly, by using a number of fixed DNA concentrations (10, 20, 50, and 100 nM) the initial velocity dependences were obtained in the range of 50-500 nM for [^{3}H]methyl-labeled AdoMet concentrations. The data were presented as Lineweaver–Burk double-reciprocal plots of DNA on AdoMet and *vice versa* [17]. All of the data were the average of at least triplicate measurements.

Preincubation studies. Preincubation experiments were carried out as described elsewhere [11, 12] using 20 nM Ecl18 MTase, 2 μM oligonucleotide DNA, and 4 μM [^{3}H]methyl-labeled AdoMet. Aliquots were withdrawn at 10, 20, 30, 45, 60, 90, and 120 sec time points, denatured at 95°C, and the product formation was analyzed by standard DE-filter assays [10-16].

Operator inhibition studies. The Ecl18 MTase activity was studied depending on its binding with oligonucleotide containing the operator sequence (underlined): Ecl18_Oper_for-1: 5′-ATC AAA ACA GGA CAA ATT GTC CTA AAA CCA A-3′; Ecl18_Oper_rev: 5′-T TGG TTT TAG GAC AAT TTG TCC TGT TTT GAT-3′. In a series of otherwise identical reactions containing 5 nM Ecl18 MTase, 4 μM AdoMet and different fixed concentrations of the operator oligonucleotide (0, 0.1, 0.5, and 1 μM), the concentration of DNA was varied from 10 to 100 nM. Similarly, by using reactions containing 5 nM Ecl18 MTase, 2 μM DNA with various fixed concentrations of the operator oligonucleotide (0, 0.1, 0.5, and 1 μM), the concentration of AdoMet was varied from 50 to 500 nM. The data were presented as Lineweaver–Burk double-reciprocal plots [17].

Data analysis. All enzyme activity data were calculated as an average of at least triplicate determinations. Data points were collected, plotted as Lineweaver–Burk double-reciprocal plots, and fitted to weighted linear regressions. The Michaelis–Menten equation was used for all studies described here [17].

RESULTS AND DISCUSSION

Determination of kinetic parameters. In the experiments carried out with varying concentrations of one substrate while keeping the second substrate at excessive constant concentration, K_m^{AdoMet} and K_m^{DNA} values were calculated as concentrations at which the enzyme velocity was half maximal (Fig. 1). K_m^{AdoMet} was found to be 277.8 nM, and K_m^{DNA} was 58.7 nM. These values are about 20-30-fold higher than usual K_m values of the bacterial C5-cytosine methyltransferases, which are usually in the nanomolar range. For example, K_m^{AdoMet} and K_m^{DNA} values for HhaI [10, 11] are 14.5 and 2.3 nM, and for MspI [12] they are 13.5 and 2.28 nM, respectively. By these parameters, Ecl18 MTase is closer to Eco29kI (1.1 and 0.55 μM [16]), mammalian DNMT1 (0.63 and 0.36 μM [15]), and DNMT3a (0.52 and 0.25 μM [15]) MTases and some exocyclic MTases such as KpnI (0.56 and 0.15 μM [18]). From comparison of the two Ecl18 K_m constants obtained in our experiments, we conclude that it has 4.7-fold higher affinity to DNA than to AdoMet. A similar proportion of affinity was observed in the cases of HhaI and MspI MTases.

The k_{cat} value of Ecl18 MTase is 0.144 sec^{-1} for oligonucleotide DNA with a single recognition site, and

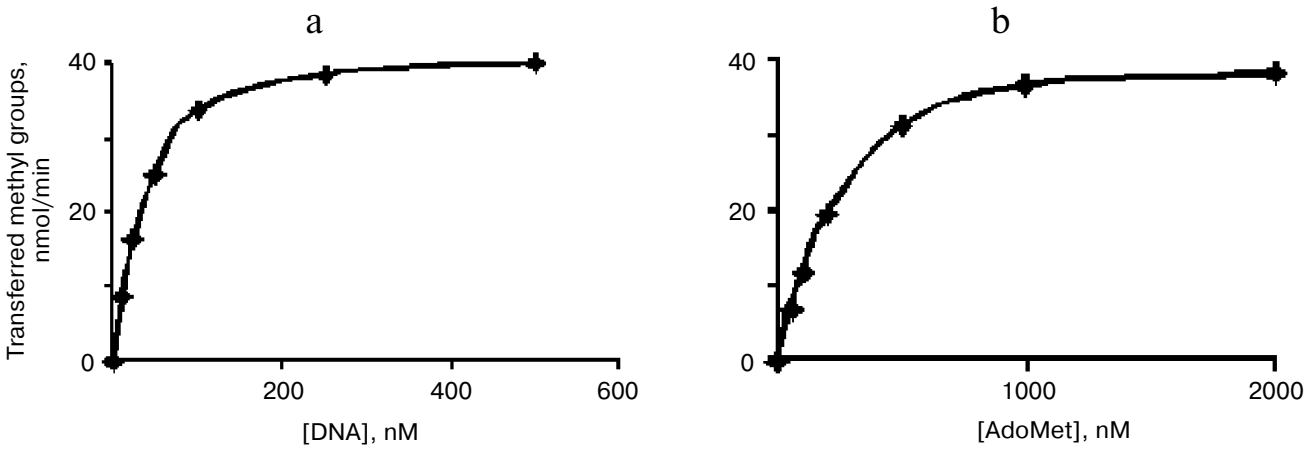

Fig. 1. Dependence of Ecl18 MTase catalytic reaction on DNA (a) and AdoMet (b) concentration. a) Ecl18 MTase concentration was fixed at 5 nM, and AdoMet concentration was fixed at 4 μM. DNA concentration was varied from 10 to 500 nM. b) Each reaction mixture contained 5 nM Ecl18 MTase, 2 μM target oligonucleotide DNA, and varying concentrations of AdoMet (50-2000 nM).

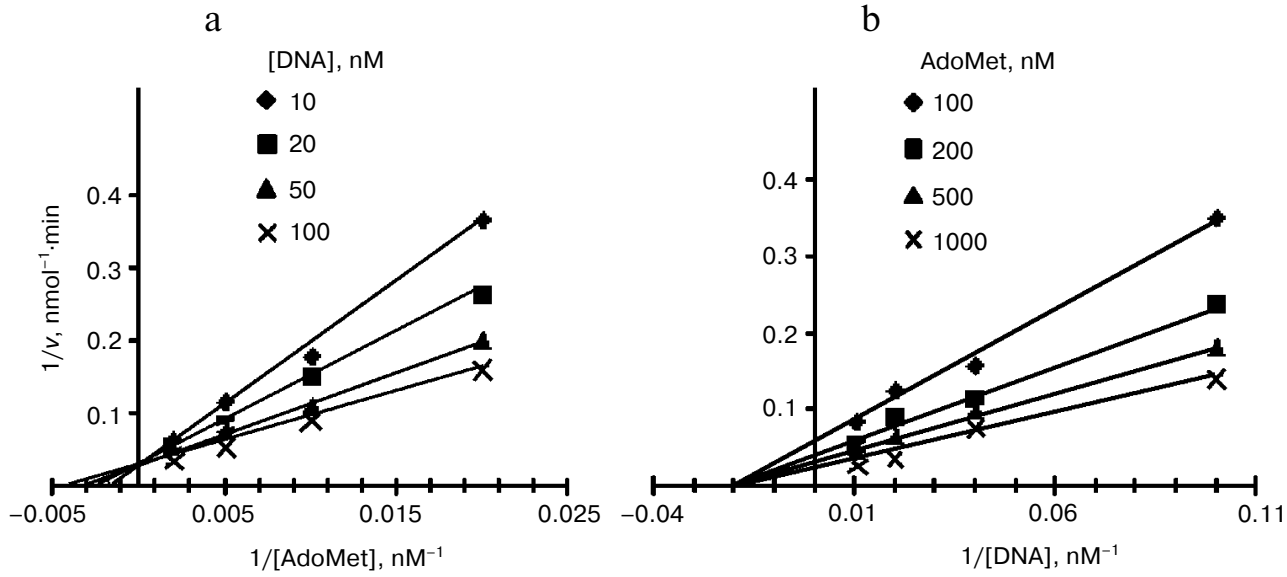

Fig. 2. Double-reciprocal plot of Ecl18 activity at fixed DNA and varying AdoMet concentrations (a) and *vice versa* (b). a) In otherwise identical reactions containing 5 nM Ecl18 MTase and different fixed DNA concentrations (10, 20, 50, 100 nM), the concentration of [^{3}H]methyl-labeled AdoMet was varied in the 50-500 nM range. b) In otherwise identical reactions containing 5 nM Ecl18 MTase and different fixed concentrations of [^{3}H]methyl-labeled AdoMet (100, 200, 500, 1000 nM), the concentration of DNA was varied from 10 to 100 nM.

this is comparable with the k_{cat} values for HhaI MTase (0.022 sec^{-1} [10, 11]), MspI (0.056 sec^{-1} [12]), Eco29kI (0.047 sec^{-1} [16]), BamHI (0.175 sec^{-1} [19]), and EcoRI (0.124 sec^{-1} [20]). The k_{cat}/K_m^{DNA} value for Ecl18 MTase is $2.45 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$, which gives high preference for methylation of its cognate sequence against nonspecific DNAs.

Dependence of initial velocity on DNA and AdoMet concentrations. The effect of different concentrations of DNA and AdoMet on the initial velocity was determined by varying DNA at fixed AdoMet concentrations and *vice versa* (Fig. 2). The Lineweaver–Burk double-reciprocal plots gave a number of intersecting lines on both graphs. These results allow discrimination between ping-pong and sequential mechanisms, because the lines are not parallel, in favor of the latter [17]. As depicted in Fig. 2a, in the case of varying DNA at fixed AdoMet concentrations, the lines intersected in quadrant IV. On the other hand, in the case of variation of AdoMet at fixed DNA concentrations (Fig. 2b), the lines intersected at the $1/v$ axis, which is characteristic for first substrate dependence in the rapid equilibrium ordered mechanism [17]. The

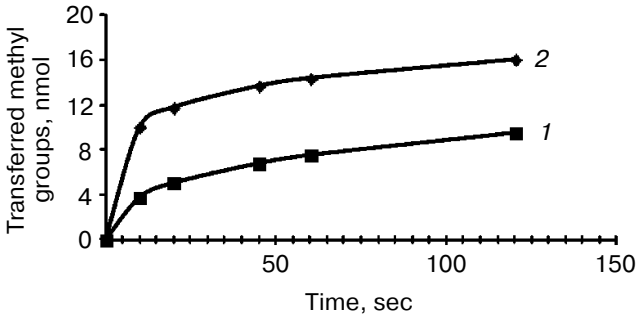

Fig. 3. Preincubation analysis of Ecl18 MTase. Methylation reactions were carried out in methylation buffer containing 20 nM Ecl18 MTase, 2 μM target oligonucleotide DNA, or, alternatively, 4 μM [^{3}H]methyl-labeled AdoMet. Methylation reactions were started by the addition of DNA to a solution containing enzyme and AdoMet ([E, AdoMet] + DNA) (*1*), or by the addition of AdoMet to a solution containing enzyme and DNA ([E, DNA] + AdoMet) (*2*).

ordered kinetic mechanism with DNA binding first has been shown to be typical of the C5-cytosine MTases family [10-15].

Order of binding of the substrates. To further confirm the order of the interaction of the substrates with Ecl18 MTase, we performed substrate preincubation assays. The enzyme was preincubated with the labeled AdoMet, and the reaction was started by addition of DNA; or *vice versa*, the enzyme was preincubated with the target DNA, and the reaction was started by addition of labeled AdoMet. In both experiments the final concentrations of DNA, AdoMet, and enzyme were identical. As seen from Fig. 3, binary enzyme–DNA complex gave higher rates of methylation, and thus was more catalytically active than enzyme–AdoMet complex, if it occurs. The data confirm DNA binding prior to AdoMet, which was shown by initial velocity dependence experiments (Fig. 2b).

Operator inhibition studies. Ecl18 MTase is a bifunctional enzyme able to bind to its own gene promoter region [8, 9]. But it has not yet been shown either for SsoII/Ecl18 or for any bifunctional MTases of type II how binding to operator sequence would affect MTase activity. To address this question in the case of Ecl18, we measured its activity at varying concentrations of DNA (Fig. 4a) and AdoMet (Fig. 4b) at different fixed concentrations of the oligonucleotide carrying the operator sequence. The data presented by the Lineweaver–Burk double-reciprocal plots gave a number of intersecting lines on both graphs on the 1/[S] axis in quadrant IV. The activity of the enzyme decreased with increase in operator sequence concentration. The character of the lines intersecting in Fig. 4 allows considering the operator sequence as a noncompetitive inhibitor of Ecl18 MTase by both substrates [17]. This means that the operator-binding site on the enzyme is different from the binding sites of both target DNA and AdoMet, and binding with the operator leads to a Ecl18 conformation that is less favorable for methylation of the target DNA.

Recently in work on SsoII MTase, which is different from Ecl18 by only one Ile56Met substitution, it was shown by sequential affinity binding with two types of modified DNA ligands that initial binding of the *N*-terminal SsoII MTase region with the operator alters the enzyme conformation, thus decreasing the efficiency of its interaction with the methylation site sequence [21]. Our kinetic data support the model of the enzyme interactions with the target and regulatory DNAs proposed by those authors [21]. However, as demonstrated, binding to operator depends on the region responsible for methylation, because its deletion almost completely prevents the

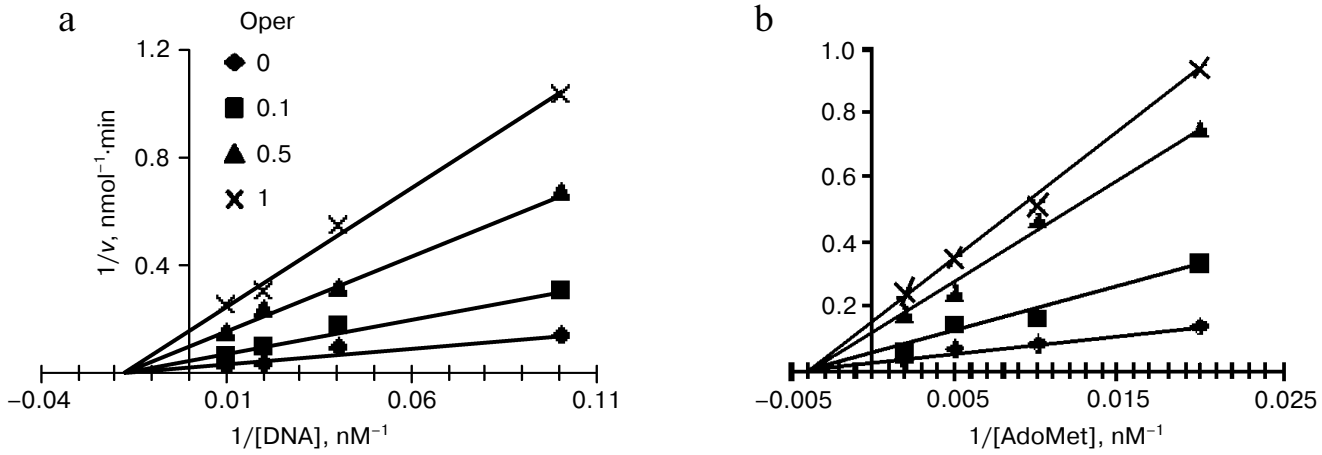

Fig. 4. Double-reciprocal plot of Ecl18 activity depending on operator oligonucleotide at fixed AdoMet and varying target DNA concentrations (a) and *vice versa* (b). a) In otherwise identical reactions containing 5 nM Ecl18 MTase, 4 μM AdoMet, and different fixed concentrations of the operator oligonucleotide (0, 0.1, 0.5, and 1 μM), the concentration of target DNA was varied from 10 to 100 nM. b) In otherwise identical reactions containing 5 nM Ecl18 MTase, 2 μM target DNA, and different fixed concentrations of the operator oligonucleotide (0, 0.1, 0.5, and 1 μM), the concentration of AdoMet was varied from 50 to 500 nM.

ability of the protein to interact with the regulatory sequence [22].

In conclusion, we have calculated kinetic parameters of the enzyme (K_m^{DNA} = 58.7 nM, K_m^{AdoMet} = 277.8 nM, and k_{cat} = 0.144 sec^{-1}), proposed a kinetic mechanism of its action (rapid equilibrium bi-bi mechanism with DNA binding before AdoMet), and defined inhibitory effect of regulatory sequence on its MTase activity.

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REFERENCES

1. Wilson, G. G. (1991) *Nucleic Acids Res.*, **19**, 2539-2565.
2. Beletskaya, I., Zakharova, M., Shlyapnikov, M., Semenova, L., and Solonin, A. (2000) *Nucleic Acids Res.*, **28**, 3817-3822.
3. Zakharova, M., Minakhin, L., Solonin, A., and Severinov, K. (2004) *J. Mol. Biol.*, **335**, 103-111.
4. Butler, D., and Fitzgerald, G. F. (2001) *J. Bacteriol.*, **183**, 4668-4673.
5. Som, S., and Friedman, S. (1993) *EMBO J.*, **12**, 4297-4303.
6. Som, S., and Friedman, S. (1997) *J. Bacteriol.*, **179**, 964-967.
7. Karyagina, A., Shilov, I., Tashlitskii, V., Khodoun, M., Vasil'ev, S., Lau, P., and Nikolskaya, I. (1997) *Nucleic Acids Res.*, **25**, 2114-2120.
8. Denjmukhametov, M., Brevnov, M., Zakharova, M., Repyk, A., Solonin, A., Petrauskene, O., and Gromova, E. (1998) *FEBS Lett.*, **21**, 233-236.
9. Protsenko, A., Zakharova, M., Nagornykh, M., Solonin, A., and Severinov, K. (2009) *Nucleic Acids Res.*, **37**, 5322-5330.
10. Wu, J., and Santi, D. (1987) *J. Biol. Chem.*, **262**, 4778-4786.
11. Lindstorm, J., Flynn, J., and Reich, N. (2000) *J. Biol. Chem.*, **275**, 4912-4919.
12. Bhattacharya, S., and Dubey, A. (1999) *J. Biol. Chem.*, **274**, 14743-14749.
13. Flynn, J., and Reich, N. (1998) *Biochemistry*, **37**, 15162-15169.
14. Bacolla, A., Pradhan, S., Roberts, R., and Wells, R. (1999) *J. Biol. Chem.*, **274**, 33011-33019.
15. Yokochi, T., and Robertson, K. (2002) *J. Biol. Chem.*, **277**, 11735-11745.
16. Nikitin, D., Mokrishcheva, M., and Solonin, A. (2007) *Biochim. Biophys. Acta*, **1774**, 1014-1019.
17. Roberts, D. V. (1977) in *Enzyme Kinetics*, Cambridge University Press, London-New York.
18. Bheemanaik, S., Chandrashekaran, S., Nagaraja, V., and Rao, D. (2002) *J. Biol. Chem.*, **278**, 7863-7874.
19. Kang, Y., Lee, H., Noh, M., Cho, N., and Yoo, O. (1995) *Biochem. Biophys. Res. Commun.*, **206**, 997-1002.
20. Reich, N., and Mashhoon, N. (1991) *Biochemistry*, **30**, 2933-2939.
21. Romanenkov, A., Kisil, O., Zatsepin, T., Yamskova, O., Karyagina, A., Metelev, V., Oretskaya, T., and Kubareva, E. (2006) *Biochemistry (Moscow)*, **71**, 1341-1349.
22. Fedotova, E., Protsenko, A., Zakharova, M., Lavrova, N., Alekseevsky, A., Oretskaya, T., Karyagina, A., Solonin, A., and Kubareva, E. (2009) *Biochemistry (Moscow)*, **74**, 85-91.