Biochemistry (Moscow), Vol. 68, No. 3, 2003, pp. 334-338. Translated from Biokhimiya, Vol. 68, No. 3, 2003, pp. 403-408.

Study of Nucleophile Binding in the Penicillin Acylase Active Center. Kinetic Analysis

M. I. Youshko, A. L. Bukhanov, and V. K. Švedas*

Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, 119992 Russia; fax: (095) 938-2422; E-mail: vytas@belozersky.msu.ru

Received February 28, 2002
Revision received March 20, 2002

Abstract—The influence of the external nucleophile (6-aminopenicillanic acid) on the kinetics of the penicillin acylase-catalyzed acyl transfer reactions was studied using a highly sensitive spectrophotometric assay. An adequate kinetic scheme is suggested based on kinetic analysis of the experimental dependencies of the k_{cat} and K_m values on the nucleophile concentration. The proposed kinetic scheme has been verified by a quantitative description of the above-mentioned experimental dependencies using the set of kinetic parameters obtained from independent experiments. Such an approach can be used for modeling of different penicillin acylase-catalyzed acyl transfer reactions.

Key words: penicillin acylase, nucleophile binding, kinetic analysis

Penicillin acylase (PA) is a well known enzyme exploited mainly for the biocatalytic production of β-lactam antibiotic nuclei: 6-aminopenicillanic (6-APA) and 7-aminodesacetoxycephalosporanic (7-ADCA) acids [1, 2]. Interest in the application of PA for the totally enzymatic production of new semi-synthetic penicillins and cephalosporins is now increasing significantly [3-6]. Consequently, the detailed investigation of the mechanism of PA-catalyzed acyl transfer reactions can play a very important role in understanding the general features of such enzymatic transformations and optimization of the biocatalytic conversions.

It is well known that PA-catalyzed acyl transfer reactions proceed *via* the formation of an acylenzyme intermediate [7-9]. The recent investigation of the kinetics of synthesis of enzymatic β-lactam antibiotics has also shown [10] that in the presence of external nucleophile (6-APA or 7-ADCA) the reaction proceeds with formation of an intermediate acylenzyme–nucleophile complex, which further can be either transformed to a target product P (synthetic route), or hydrolyzed with formation of a byproduct P_2 (hydrolytic route, see Scheme I). In particular, this means that an added nucleophile can bind near the PA active site and influence different stages of the enzymatic conversion. Recently published X-ray data characterizing the enzyme–substrate complex during PA-catalyzed hydrolysis of benzylpenicillin [11, 12] shows indeed that substrate binding induces a remarkable conformational change and leads to the formation of a so-called "β-lactam binding site". In terms of a kinetic scheme this means that an external nucleophile can bind, in principle, not only with an acylenzyme, but also with a free enzyme and an enzyme–substrate complex (Scheme IV).

Although the kinetic mechanism of PA-catalyzed acyl transfer to the added nucleophile (according to the "general" Scheme IV) was proposed for the first time in 1977 [13], there is still no reliable evidence able to prove or disprove this suggestion. This can be attributed mainly to the difficulties arising at attempts to analyze such a complicated kinetic scheme. Meanwhile, the investigation of the influence of external nucleophile on the different stages of enzymatic conversion is very important from both practical point of view and from the point of deeper understanding of the unique catalytic mechanism of PA-catalyzed acyl transfer reactions.

In the presented work the influence of the external nucleophile (6-APA) on the kinetics of the penicillin acylase-catalyzed acyl transfer reactions was studied in order to determine the "minimal" kinetic scheme for adequately describing the PA-catalyzed acyl transfer process in the presence of an external nucleophile.

Abbreviations: 6-APA) 6-aminopenicillanic acid; 7-ADCA) 7-aminodesacetoxycephalosporanic acid; NIPGB) D-2-nitro-5-[(phenylglycyl)amino]benzoic acid; PA) penicillin acylase; PMSF) phenylmethylsulfonyl fluoride.

* To whom correspondence should be addressed.

0006-2979/03/6803-0334$25.00 ©2003 MAIK "Nauka/Interperiodica"

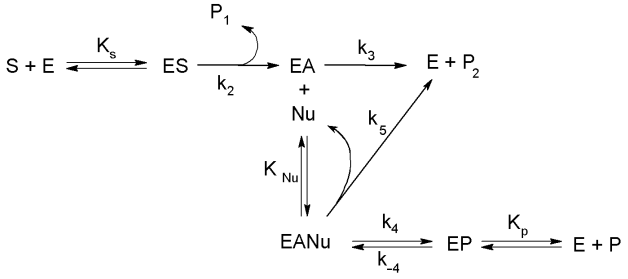
MATERIALS AND METHODS

Chemicals. The PA preparation was purified from *E. coli* ATCC 9637 according to [14]. Active site concentration in the purified enzyme preparation (30 μM) was determined by titration with phenylmethylsulfonyl fluoride (PMSF) as described earlier [7]. 6-APA and ampicillin were obtained from DSM (The Netherlands); D-2-nitro-5-[(phenylglycyl)amino]benzoic acid (NIPGB) was kindly donated by Dr. E. de Vries. Organic solvents (extra high purity) were from Kriokhrom (Russia). Other reagents and the components of the buffer systems were from Merck (Germany).

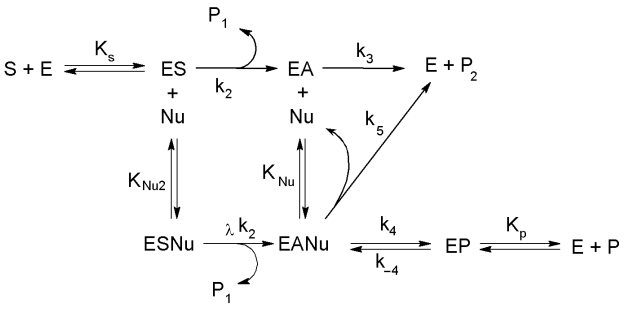
Determination of kinetic parameters of enzymatic NIPGB hydrolysis. Kinetic parameters (k_{cat} and K_m) of enzymatic conversion were determined by analysis of the dependence of initial reaction rates on the NIPGB concentration (10-200 mM). For that, the experimental data were approximated by nonlinear regression according to the Michaelis–Menten equation using Sigma Plot 2001 (SPSS, USA) software. The reactions of enzymatic hydrolysis were carried out in a thermostatted 500 μl cell of a spectrophotometer (Shimadzu UV-1601, Japan) in 0.01 M phosphate buffer, pH 7.5, 25°C, and initiated by adding 50 μl of an enzyme stock solution. The initial reaction rates (less than 2% of substrate conversion) were measured by accumulation of a colorimetric product (P_1) at 400 nm.

Study of the influence of 6-APA on the kinetic parameters of enzymatic NIPGB hydrolysis. The influence of an added nucleophile (6-APA) on the kinetics of the enzymatic conversion was studied by analysis of the dependence of kinetic parameters (k_{cat}^{eff} and K_m^{eff}) on the 6-APA concentration (0-100 mM). The initial reaction rates and the values of effective kinetic parameters in the presence of 6-APA were determined according to the procedure, described above. All measurements were performed in 0.01 M phosphate buffer, pH 7.5, 0.1 M KCl at 25°C.

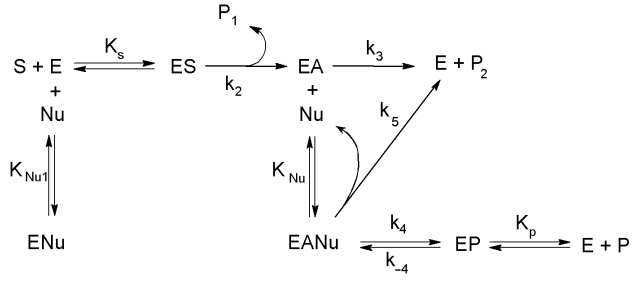
Kinetic Schemes and Equations



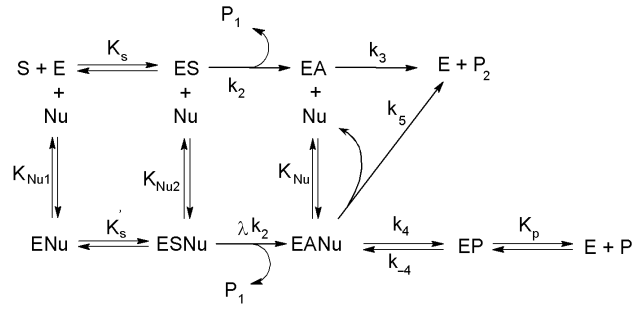
Scheme I. "Minimum" kinetic scheme of penicillin acylase-catalyzed acyl transfer to nucleophile. E is free enzyme, S is activated acyl donor, P_1 is a first reaction product being released on formation of an acylenzyme, Nu is an added nucleophile (antibiotic nucleus), P_2 is a product of acylenzyme hydrolysis, P is a product of acyl transfer to a nucleophile (a target antibiotic), ES is an enzyme–substrate complex, EA is an acylenzyme, EANu is an acylenzyme–nucleophile complex, and EP is an enzyme–product complex.



Scheme II. Kinetic scheme of penicillin acylase-catalyzed acyl group transfer to nucleophile with formation of an enzyme–substrate–nucleophile complex. All the designations in Scheme II correspond to those given for Scheme I and ESNu refers to an enzyme–substrate–nucleophile complex. Coefficient λ represents the ratio of acylation rates in enzyme–substrate and enzyme–substrate–nucleophile complexes.



Scheme III. Kinetic scheme of penicillin acylase-catalyzed acyl group transfer to nucleophile with formation of an enzyme–nucleophile complex. All the designations in Scheme III correspond to those given for Scheme I and ENu refers to an enzyme–nucleophile complex.



Scheme IV. "General" kinetic scheme of penicillin acylase-catalyzed acyl group transfer to nucleophile. All the designations correspond to those given for Schemes I-III.

Equations, describing the kinetics of product P_1 accumulation according to the Schemes I-IV (Eqs. (1)-(4), respectively) were obtained at the steady-state assumption for EA and EANu at $[S]_0 >> [E]_0$, [P], $[P_1]$, $[P_2]$.

$$\frac{d[P_1]}{dt}=\frac{\dfrac{k_2\{k_3K_{Nu}+[Nu](k_4+k_5)\}}{\{k_3K_{Nu}+[Nu](k_4+k_5)\}+k_2(K_{Nu}+[Nu])}[E]_0[S]_0}{K_S\dfrac{\{k_3K_{Nu}+[Nu](k_4+k_5)\}}{\{k_3K_{Nu}+[Nu](k_4+k_5)\}+k_2(K_{Nu}+[Nu])}+[S]_0}, \quad (1)$$

$$\frac{d[P_1]}{dt}=\frac{\dfrac{k_2(1+\lambda[Nu]/K_{Nu2})\{k_3K_{Nu}+[Nu](k_4+k_5)\}}{(1+[Nu]/K_{Nu2})\{k_3K_{Nu}+[Nu](k_4+k_5)\}+k_2(1+\lambda[Nu]/K_{Nu2})(K_{Nu}+[Nu])}[E]_0[S]_0}{K_S\dfrac{k_3K_n+[Nu](k_4+k_5)}{(1+[Nu]/K_{Nu2})\{k_3K_{Nu}+[Nu](k_4+k_5)\}+k_2(1+\lambda[Nu]/K_{Nu2})(K_{Nu}+[Nu])}+[S]_0}, \quad (2)$$

$$\frac{d[P_1]}{dt}=\frac{\dfrac{k_2\{k_3K_{Nu}+[Nu](k_4+k_5)\}}{\{k_3K_{Nu}+[Nu](k_4+k_5)\}+k_2(K_{Nu}+[Nu])}[E]_0[S]_0}{K_S\dfrac{\{k_3K_{Nu}+[Nu](k_4+k_5)\}(1+[Nu]/K_{Nu1})}{\{k_3K_{Nu}+[Nu](k_4+k_5)\}+k_2(K_{Nu}+[Nu])}+[S]_0}, \quad (3)$$

$$\frac{d[P_1]}{dt}=\frac{\dfrac{k_2(1+\lambda[Nu]/K_{Nu2})\{k_3K_{Nu}+[Nu](k_4+k_5)\}}{(1+[Nu]/K_{Nu2})\{k_3K_{Nu}+[Nu](k_4+k_5)\}+k_2(1+\lambda[Nu]/K_{Nu2})(K_{Nu}+[Nu])}[E]_0[S]_0}{K_S\dfrac{\{k_3K_{Nu}+[Nu](k_4+k_5)\}(1+[Nu]/K_{Nu1})}{(1+[Nu]/K_{Nu2})\{k_3K_{Nu}+[Nu](k_4+k_5)\}+k_2(1+\lambda[Nu]/K_{Nu2})(K_{Nu}+[Nu])}+[S]_0}. \quad (4)$$

RESULTS AND DISCUSSION

To perform a quantitative characterization of the influence of an added nucleophile on the kinetics of PA-catalyzed acyl transfer reactions, the dependence of the kinetic parameters of the enzymatic hydrolysis of NIPGB on the added nucleophile (6-APA) concentration has been investigated. Choosing this particular "chromogenic" substrate allowed us to determine the kinetic parameters (k_{cat} and K_m) of investigated reactions using fast and highly sensitive spectrophotometric assay (i.e., by measuring the accumulation of a chromophore (P_1) released during the acylation of the enzyme).

Kinetic analysis of a "minimum" kinetic Scheme I showed that the accumulation of the P_1 product is described by a trivial Michaelis–Menten dependence (Eq. (1)), where

$$k_{cat}=\frac{k_2\{k_3K_{Nu}+[Nu](k_4+k_5)\}}{\{k_3K_{Nu}+[Nu](k_4+k_5)\}+k_2(K_{Nu}+[Nu])}, \quad (5)$$

$$K_m=K_S\frac{\{k_3K_{Nu}+[Nu](k_4+k_5)\}}{\{k_3K_{Nu}+[Nu](k_4+k_5)\}+k_2(K_{Nu}+[Nu])}. \quad (6)$$

Based on Scheme I, one can see that while both parameters of the Michaelis–Menten equation are complex nonlinear functions of the added nucleophile concentration, the so-called "second order rate constant" (or specificity constant) in this case does not depend on the nucleophile concentration:

$$\frac{k_{cat}}{K_m}=\frac{k_2}{K_S}. \quad (7)$$

The experimental data, in contrast, clearly show that both constants (k_{cat} and K_m) and their ratio are complex functions of the 6-APA concentration (Figs. 1-4).

To discriminate the kinetic schemes adequately describing the PA-catalyzed acyl transfer to 6-APA, it makes sense to systematically consider all potential reaction routes presented in the kinetic Schemes I-IV.

Scheme I is the simplest; here the binding of a nucleophile with a free enzyme and an enzyme–substrate complex is not considered. In this case, as shown above, the specificity constant should not depend on the nucleophile concentration (Eq. (7)). One can conclude (see Fig. 3 and Eq. (7)) that this scheme does not correspond to the experimental data.

In Scheme II the binding of an added nucleophile with an enzyme–substrate complex is included. In this case, the expression for the specificity constant should be as follows:

$$\frac{k_{cat}}{K_m}=\frac{k_2(1+\lambda[Nu]/K_{Nu2})}{K_S}. \quad (8)$$

As it follows from Eq. (8), the dependence of the specificity constant on the nucleophile concentration in this case should be linear, which does not correspond to the experimentally observed hyperbolic trend (see Fig. 3).

Scheme III describes the situation when a nucleophile binds with free enzyme, but not with enzyme–substrate complex. For this scheme, the dependence of the specificity constant on the 6-APA concentration should be a hyperbolic decay function (Eq. (9)), which is in agreement with the experimental data (Fig. 3):

$$\frac{k_{cat}}{K_m}=\frac{k_2}{K_S(1+[Nu]/K_{Nu2})}. \quad (9)$$

Scheme IV is the most general one, considering a nucleophile binding with free enzyme, enzyme–substrate complex, and acylenzyme intermediate. In this case, the dependence of the specificity constant on the nucleophile concentration is also hyperbolic:

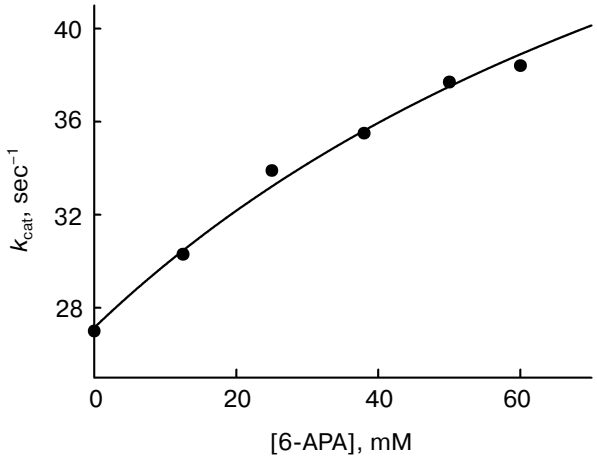


Fig. 1. Dependence of the catalytic constant of NIPGB hydrolysis on the 6-APA concentration. Dots represent the experimental data, solid line the results of modeling. Reaction conditions: pH 7.5, 0.1 M KCl, 25°C.

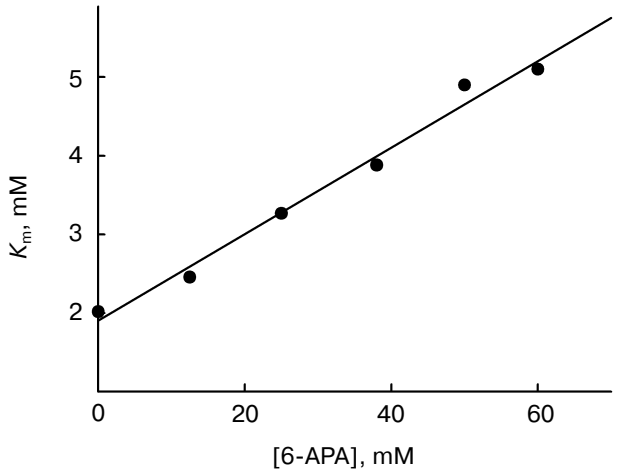


Fig. 2. Dependence of the Michaelis constant of NIPGB hydrolysis on the 6-APA concentration. Dots represent the experimental data, solid line the results of modeling. Reaction conditions: pH 7.5, 0.1 M KCl, 25°C.

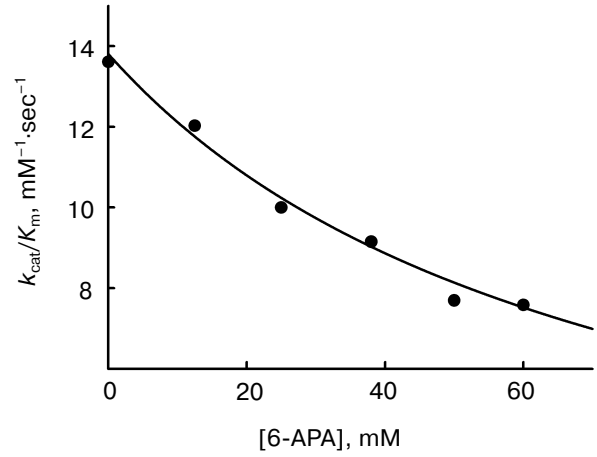


Fig. 3. Dependence of the specificity constant of NIPGB hydrolysis on the 6-APA concentration. Dots represent the experimental data, solid line the results of modeling.

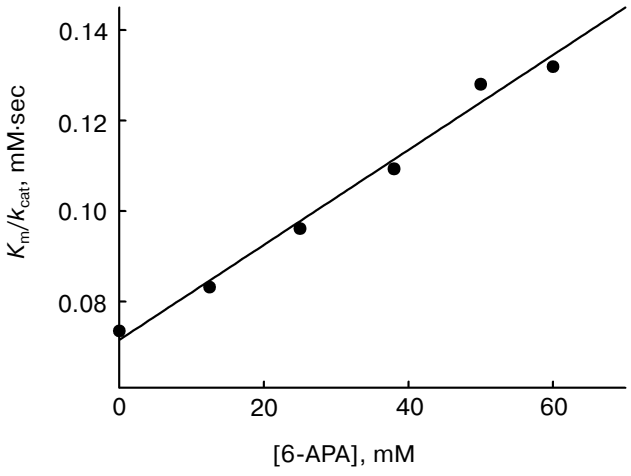


Fig. 4. Dependence of the reciprocal specificity constant of NIPGB hydrolysis on the 6-APA concentration. Dots represent the experimental data, solid line the results of modeling.

Comparison of the experimental and theoretical (obtained in terms of kinetic Schemes I-IV) dependencies of the kinetic parameters of PA-catalyzed acyl transfer reactions in the presence of an external nucleophile

Experimental dependence	k_{cat} on [Nu]	K_m on [Nu]	k_{cat}/K_m on [Nu]	K_m/k_{cat} on [Nu]
Type of curve	hyperbolic	linear	hyperbolic decay	linear
Scheme I	+	—	—	—
Scheme II	+	—	—	—
Scheme III	+	+	+	+
Scheme IV	+	—	+	—

$$\frac{k_{cat}}{K_m} = \frac{k_2 (1+\lambda[Nu]/K_{Nu2})}{K_S (1+[Nu]/K_{Nu1})} . \qquad (10)$$

Thus, both kinetic Schemes III and IV adequately describe the primary experimental data. For the further discrimination of these two schemes let us consider an equation reciprocal to the specificity constant, i.e., K_m/k_{cat}. From Eqs. (11) and (12) one can see that this theoretical dependence should be linear in the case of Scheme III (Eq. (11)), and hyperbolic in the case of Scheme IV (Eq. (12)):

$$\frac{K_m}{k_{cat}} = \frac{K_S (1+[Nu]/K_{Nu1})}{k_2} , \qquad (11)$$

$$\frac{K_m}{k_{cat}} = \frac{K_S (1+[Nu]/K_{Nu1})}{k_2 (1+\lambda[Nu]/K_{Nu2})} . \qquad (12)$$

The experimental data on the dependence of K_m/k_{cat} on the nucleophile concentration, provided in Fig. 4, clearly shows that this dependence is linear and, therefore, only Scheme III can adequately describe the experimental data under the investigated conditions. The results of analysis performed for the discrimination of Schemes I-IV are summarized in the table.

For the validation of the suggested "minimum" kinetic Scheme III the quantitative description of the above-mentioned experimental dependencies using kinetic parameters of the PA-catalyzed acyl transfer reactions obtained earlier [10, 14] has been performed. As one can see from Figs. 1-4, the application of Scheme III described the experimental data using a set of parameters obtained from the independent kinetic experiments quite satisfactorily. This means that proposed kinetic scheme can be used as a powerful tool for the modeling of different penicillin acylase-catalyzed acyl transfer reactions.

This work was financially supported by the Russian Foundation for Basic Research (grants 00-04-48658, 01-04-06614, and 01-04-06621). We also thank DSM for donated reactants.

REFERENCES

1. Matsumoto, K. (1993) *Bioprocess Technol.*, **16**, 67-88.
2. Shewale, J. G., and Sivaraman, H. (1989) *Proc. Biochem.*, **24**, 146-154.
3. Berezin, I. V., Margolin, A. L., and Švedas, V. K. (1977) *Dokl. Akad. Nauk SSSR*, **235**, 961-964.
4. Kasche, V. (1986) *Enzyme Microb. Technol.*, **8**, 4-16.
5. Bruggink, A., Roos, E. C., and de Vroom, E. (1998) *Org. Proc. Res. Dev.*, **2**, 128-133.
6. Moody, H. M., and Boesten, W. H. J. (1998) *Process for Preparation of Ampicillin*, International Patent Application, WO98/56946 to DSM.
7. Švedas, V. K., Margolin, A. L., Sherstyuk, S. F., Klyosov, A. A., and Berezin, I. V. (1977) *Bioorg. Khim.*, **3**, 546-553.
8. Konecny, J., Schneider, A., and Sieber, M. (1983) *Biotechnol. Bioeng.*, **25**, 451-467.
9. Kasche, V., Haufler, U., and Zollner, R. (1984) *Hoppe-Seyler's Z. Physiol. Chem.*, **365**, 1435-1443.
10. Youshko, M. I., and Švedas, V. K. (2000) *Biochemistry (Moscow)*, **65**, 1367-1375.
11. Alkema, W. B. L., Hensgens, C. M. H., Kroezinga, E. H., de Vries, E., Floris, R., van der Laan, J.-M., Dijkstra, B. W., and Janssen, D. B. (2000) *Protein Eng.*, **13**, 857-863.
12. McVey, C. E., Walsh, M. A., Dodson, G. G., Wilson, K. S., and Brannigan, J. A. (2001) *J. Mol. Biol.*, **313**, 139-150.
13. Klyosov, A. A., Margolin, A. L., and Švedas, V. K. (1977) *Bioorg. Khim.*, **5**, 654-661.
14. Youshko, M. I., Shamolina, T. A., Guranda, D. F., Sinev, A. V., and Švedas, V. K. (1998) *Biochemistry (Moscow)*, **63**, 1104-1109.