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Kinetic study of penicillin acylase from *Alcaligenes faecalis*

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Abstract **Penicillin acylase from *Alcaligenes faecalis* has a very high affinity for both natural (benzylpenicillin, $K_m = 0.0042$ mM) and colorimetric (6-nitro-3-phenylacetamidobenzoic acid, $K_m = 0.0045$ mM) substrates as well as the product of their hydrolysis, phenylacetic acid ($K_i = 0.016$ mM). The enzyme is partially inhibited at high benzylpenicillin concentrations but the triple SES complex formed still retains 43% of the maximal catalytic activity; the affinity of benzylpenicillin for the second substrate molecule binding site is much lower ($K_S' = 54$ mM) than for the first one. Phenylmethylsulfonyl fluoride was shown to be a very effective irreversible inhibitor, completely inactivating the penicillin acylase from *A. faecalis* in a few minutes at micromolar concentrations; this compound was used for enzyme active site titration. The absolute values of the determined kinetic parameters for enzymatic hydrolysis of 6-nitro-3-phenylacetamidobenzoic acid ($k_{cat} = 95$ s^{-1} and $k_{cat}/K_m = 2.1 \times 10^{-7}$ M^{-1} s^{-1}) and benzylpenicillin ($k_{cat} = 54$ s^{-1} and $k_{cat}/K_m = 1.3 \times 10^{-7}$ M^{-1} s^{-1}) by penicillin acylase from *A. faecalis* were shown to be highest of all the enzymes of this family that have so far been studied.**

Key words: Penicillin acylase (*Alcaligenes faecalis*); Enzyme kinetics

1. Introduction

Penicillin acylases (penicillin amidohydrolases, EC 3.5.1.11) are a group of enzymes that catalyze the selective hydrolysis of the relatively stable side chain amide bond in penicillins and cephalosporins while leaving the labile β-lactam ring intact. For many years the enzymes capable of performing this unique transformation (mainly penicillin acylase from *Escherichia coli*) have been widely studied as industrial biocatalysts for the modification of β-lactam antibiotics [1–3]. More recently, penicillin acylases have become the focus of interest from the viewpoint of fundamental enzymology. Their biosynthesis was extensively studied and a complex posttranslational processing pathway of the catalytically active heterodimer was elucidated [4–6]. Progress has been made in determining the amino acid sequence and subunit structure of several related penicillin and cephalosporin acylases and genes have been cloned from a number of species [6–8]. The crystal structure of the penicillin acylase from *E. coli* at 1.9 Å resolution has been reported and on the basis of this structure a principally new mechanism has been proposed [9]. Subsequently, a few more enzymes (aspartylglycosylaminase, proteosome) were demonstrated to involve the same mechanism [10,11].

There is quite limited and sometimes controversial information concerning the kinetic properties and specificity of penicillin acylases. The main complication in kinetic studies of most penicillin acylases is the very effective inhibition of the catalytic activity by the reaction product. Once the strong inhibition of penicillin acylase from *E. coli* by phenylacetic acid was recognized [12], the high affinity of this enzyme for its preferred substrates could be established. Consequently the K_m and k_{cat}/K_m values for a few previously characterized substrates (mainly benzylpenicillin) had to be corrected by factors of up to 1000 [13]. The substrate specificity of penicillin acylases has been hitherto studied mainly semiquantitatively or for only a very limited number of substrates [14–17]. Therefore early classifications of penicillin acylases based on their activity towards several natural penicillins (such as benzylpenicillin and phenoxymethylpenicillin) nowadays seem not very informative. It was recently recognized, for example, that benzylpenicillin, which was previously claimed to be the preferred substrate of the best studied penicillin acylase from *E. coli* [1–3], is not amongst the five most reactive substrates [18]. In contrast to aminoacylases (*N*-acylamino acid amidohydrolases, EC 3.5.1.14), penicillin acylase from *E. coli* was shown to be an effective biocatalyst for the enantioselective hydrolysis of a number of non-conventional amino acid derivatives such as *N*-acylated aminoalkylphosphonic acids [19], aminoalkylphosphonous acids [20], β- and γ-amino acids [21–24]. Penicillin acylases from different origin most probably also possess much wider substrate specificity than previously imagined but appropriate kinetic and specificity studies still have to confirm this suggestion. The fundamental question is whether the new catalytic mechanism proposed for *E. coli* enzyme operates in enzymes from other sources. It is quite likely that penicillin acylases are a group of enzymes that convert the same group of substrates but act according to several different mechanisms as was observed, for example, in the case of peptidases.

In this paper we present the results of our kinetic studies of penicillin acylase from *Alcaligenes faecalis*, which has received scant attention in the literature and has only recently been shown to be an enzyme of high potential for enzymatic modification of β-lactam antibiotics [25].

2. Materials and methods

2.1. Materials

6-Nitro-3-phenylacetamidobenzoic acid and phenylmethylsulfonyl fluoride were obtained from Sigma, benzylpenicillin, potassium salt from Fluka, phenylacetic acid from Aldrich and the *N*-phenylacetylarginine enantiomers from the Russian-German joint venture 'Konstanta', Moscow. The penicillin acylase preparation of *A. faecalis* was kindly provided by Gist-Brocades, Delft, The Netherlands; its active site concentration was determined with phenylmethylsulfonyl fluoride using an earlier described method [26].

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2.2. Enzyme assay

Enzymatic hydrolysis of 6-nitro-3-phenylacetamidobenzoic acid was monitored continuously at 400 nm ($\varepsilon = 9500\ M^{-1}\ cm^{-1}$) with a Shimadzu UV-1601 or multichannel GEMSAEC (Electronucleonics, USA) spectrophotometer at 25°C at constant ionic strength of 0.1 M, pH 7.5 unless otherwise indicated. Continuous assay of enzymatic benzylpenicillin hydrolysis was performed using a registering Radiometer autotitrator RTS-22 by alkaline (KOH) titration of the phenylacetic acid in a buffer-free medium at 25°C and a constant ionic strength of 0.1 or 1 M KCl as indicated in the figure legends.

2.3. Determination of kinetic parameters

The kinetics of enzymatic hydrolysis were studied under standard assay conditions with an excess of substrate over the enzyme ($[S]_o \gg [E]_o$). The dependence of the initial rate on the substrate concentration was measured and the experimental data were analyzed by Lineweaver-Burk plots; kinetic parameters were determined by computer regression analysis (Sigma Plot). Substrate inhibition was analyzed according to the 'minimal' kinetic scheme of benzylpenicillin hydrolysis. K_m values for *N*-phenylacetylarginine enantiomers were determined from the competitive inhibition of colorimetric substrate hydrolysis at different concentrations of *N*-phenylacetylarginine derivatives. Maximum rates for the enzymatic hydrolysis of *N*-phenylacetylarginine derivatives were measured in independent experiments at saturating substrate concentrations ($10–15 \times K_m$); the concentration of the liberated arginine was monitored spectrophotometrically at 340 nm after derivatization with *o*-phthalaldehyde in the presence of mercaptoethanol as described earlier [27].

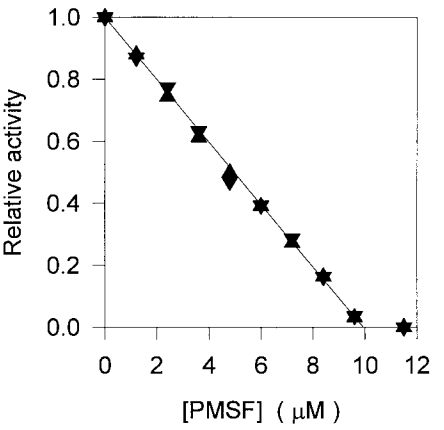

Fig. 1. Titration of penicillin acylase active sites by phenylmethylsulfonyl fluoride. The enzyme was incubated with different concentrations of phenylmethylsulfonyl fluoride in 0.01 M phosphate buffer pH 6.0, 0.1 M KCl. Residual catalytic activity was measured by colorimetric substrate (6-nitro-3-phenylacetamidobenzoic acid) (▲) and by benzylpenicillin (▼) at pH 7.5, 25°C, μ 0.1 M. The activity of penicillin acylase is presented in relative units as compared with the activity of enzyme before inactivation.

3. Results and discussion

Although quantitative titration of the active site concentration is an obligatory step in determining absolute values of kinetic parameters of enzymatic reactions, it has so far been performed only for a rather limited group of enzymes [28,29]. A method based on the extremely effective stoichiometric inactivation by phenylmethylsulfonyl fluoride was earlier developed for active site titration of penicillin acylase from *E. coli* [26]. This method was later used for determination of active enzyme concentrations in highly and partially purified preparations of penicillin acylases from different strains of *E. coli, Bacillus megaterium* and *Kluyvera citrophila* [30–32] but was not effective for active site titration of penicillin acylases from *Streptoverticillium* sp. [33] and *Xanthomonas* sp. [13] or for some penicillin acylase mutants from *K. citrophila* [34] because of a very low (if any) sensitivity of first two enzymes to the inhibitor or due to the complicated course of inactivation of mutants.

We found that phenylmethylsulfonyl fluoride is a potent irreversible inhibitor of the penicillin acylase from *A. faecalis*, providing excellent possibilities for enzyme active site titration. Total inactivation of the penicillin acylase from *A. faecalis* takes place in a few minutes even at micromolar concentrations of the titrating agent. Activities of the enzyme to the colorimetric and natural substrates were titrated independently demonstrating that both compounds are converted by the same active center (Fig. 1).

The enzymatic hydrolysis of benzylpenicillin was studied with a wide range (0.001–1000 mM) of substrate concentrations (Fig. 2). The affinity of the penicillin acylase from *A. faecalis* for benzylpenicillin was found to be high ($K_m = 0.0038$ mM). At high substrate concentrations partial inhibition of catalytic activity was observed (Fig. 2) related to the binding of the second substrate molecule; the affinity of this second site for benzylpenicillin was more than four orders of magnitude lower ($K_S' = 54$ mM) than that of the first one and the triple SES complex formed still retained 43% of the maximal catalytic activity. A 'minimal' kinetic scheme is suggested in order to describe the experimental data obtained (Fig. 3).

The penicillin acylase-catalyzed conversion of colorimetric substrate was investigated under a wide range of experimental conditions (Fig. 4). The reaction was markedly inhibited at increasing phenylacetic acid concentrations (Fig. 4A). Initial rate analysis showed that phenylacetic acid is a strong competitive inhibitor; the corresponding inhibition constant was determined from the dependence of the effective K_m value on the concentration of inhibitor in the reaction mixture (Fig. 4B). It should be mentioned that penicillin acylase from *A. faecalis* is the most active enzyme in 6-nitro-3-phenylacetamidobenzoic acid and benzylpenicillin hydrolysis of all penicillin acylases characterized so far; its k_{cat}/K_m value of colorimetric substrate hydrolysis, for example, is 42 and 27 times higher

Table 1
Comparison of kinetic parameters for reactions catalyzed by penicillin acylases from *A. faecalis*, *E. coli* and *K. citrophila*

Source	Substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}\ s^{-1}$)	K_i^{PAA} (mM)	K_S' (M)	β
A. faecalis	Benzylpenicillin	54	0.0042	1.3×10^7		0.054	0.43
E. coli	Benzylpenicillin[a]	50	0.0038	1.3×10^7	0.034	0.23	0.67
K. citrophila	Benzylpenicillin[b]	50	0.015	3.3×10^6			0.45
A. faecalis	NIPAB	95	0.0045	2.1×10^7	0.016		
E. coli	NIPAB[c]	15	0.030	5.0×10^5			
K. citrophila	NIPAB[d]	23	0.030	7.7×10^5			
A. faecalis	*N*-Phac-L-Arg	37	0.032	1.2×10^6			
A. faecalis	*N*-Phac-D-Arg	1.1	3.1	3.5×10^2			

Experimental data from [12][a], [35][b], [36][c] and [32][d].

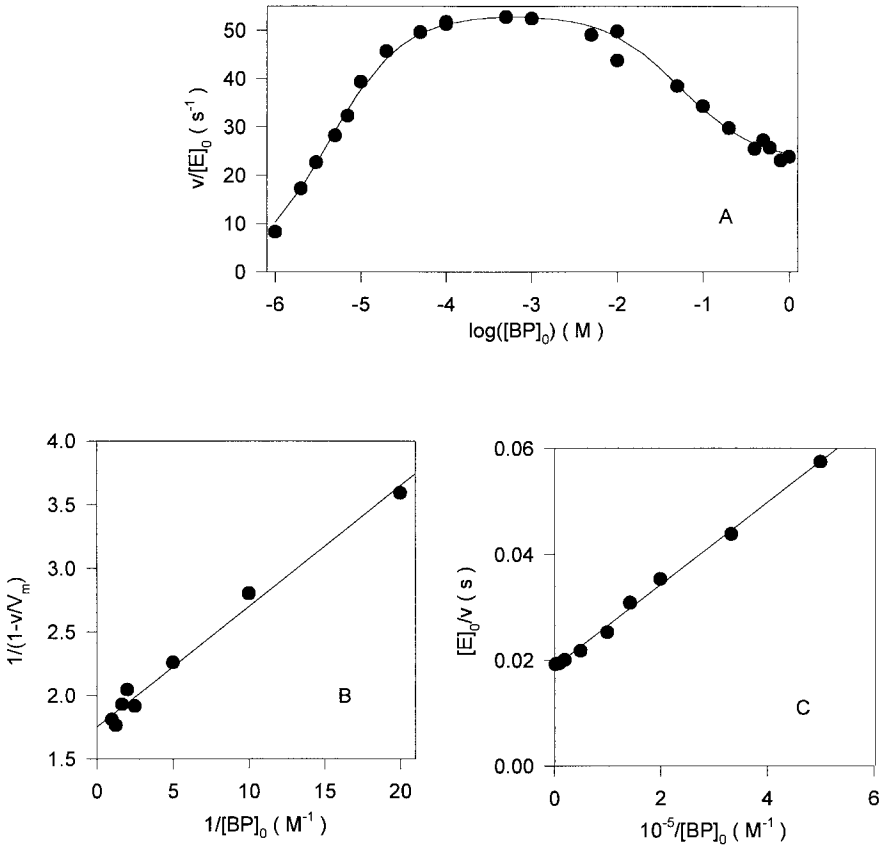

Fig. 2. Dependence of the initial rate of penicillin acylase-catalyzed benzylpenicillin hydrolysis on the initial substrate concentration (A). Experimental data are presented by circles, the curve was calculated according to the 'minimal' kinetic scheme (Fig. 3) and the equation $v/[E]_0 = (k_{cat}+\beta\times k_{cat}\times[BP]_0/K_S')\times[BP]_0/(K_M+[BP]_0+[BP]_0^2/K_S')$. The parameters of the equation responsible for substrate inhibition were determined from the right wing of the experimental curve A according to the linearized equation $1/(1-v/V_m) = 1/(1-\beta)+K_S'/(1-\beta)\times[BP]_0$ (B), K_M and k_{cat} values from Lineweaver-Burk plots of experimental data at low substrate concentrations (C). Experimental conditions: pH 7.5, 25°C, μ 1 M.

than the corresponding values for *E. coli* and *K. citrophila* enzymes, respectively (Table 1).

The penicillin acylase from *A. faecalis* is characterized by bell-shaped pH dependences of the kinetic parameters (Fig. 4C,D). The catalytic activity has a rather broad pH optimum (Fig. 4C) but the pH optimum for the k_{cat}/K_m value is much narrower demonstrating that the p*K* values of both ionogenic groups controlling catalytic activity are significantly changed due to substrate binding.

Our observations indicate that the catalytic mechanisms of the penicillin acylases from *A. faecalis* and *E. coli* are similar. The p*K* values of the 'acidic' and 'alkaline' ionogenic groups of both enzymes are rather close to each other (in the intervals 6.1–6.7 and 10.2–11.6) and depend on substrate binding. It is

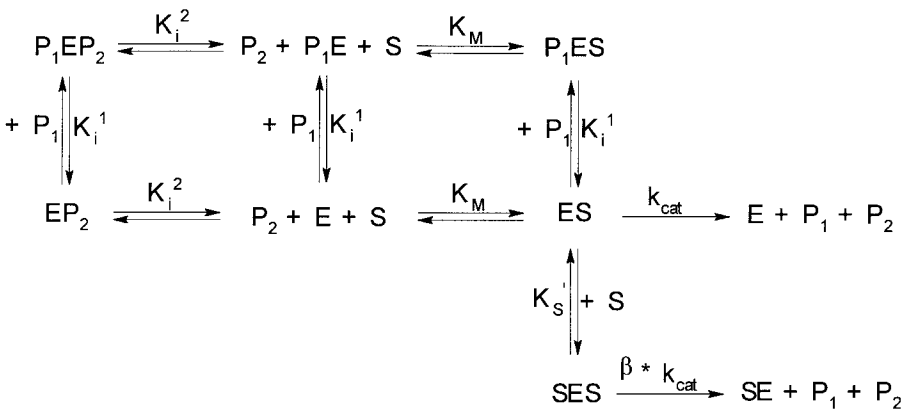

Fig. 3. 'Minimal' kinetic scheme of penicillin acylase-catalyzed hydrolysis. E, penicillin acylase; S, benzylpenicillin; ES, enzyme-substrate complex; P_1 and P_2, products of enzymatic hydrolysis (6-aminopenicillanic and phenylacetic acid); EP_1, EP_2 and P_1EP_2, corresponding enzyme complexes with products; SES, partially active enzyme complex with two substrate molecules; P_1ES, catalytically inactive triple complex of enzyme with substrate and 6-aminopenicillanic acid.

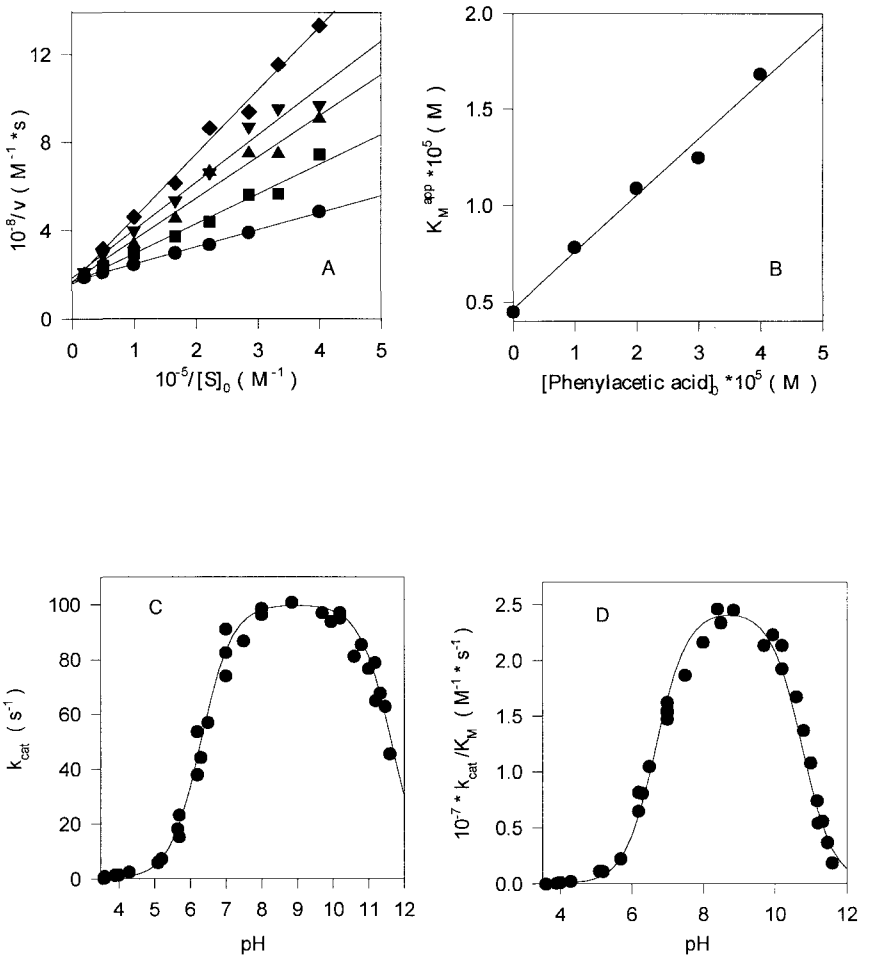

Fig. 4. Initial rate analysis of colorimetric substrate (6-nitro-3-phenylacetamidobenzoic acid) hydrolysis and penicillin acylase inhibition by phenylacetic acid (A). Determination of the competitive inhibition constant for phenylacetic acid (PAA) from the dependence of the apparent K_M values (determined from A) on phenylacetic acid concentration according to the equation $K_M^{app} = K_M \times (1+[PAA]_0/K_i^{PAA})$ (B). pH dependences of catalytic activity (k_{cat}) (C) and second-order rate constant (k_{cat}/K_M) (D) for penicillin acylase-catalyzed hydrolysis of 6-nitro-3-phenylacetamidobenzoic acid. Curves were calculated according to the equations $k_{cat} = k_{cat}^0/(1+[H^+]/K_a'+K_b'/[H^+])$ and $k_{cat}/K_M = (k_{cat}/K_M)^0/(1+[H^+]/K_a+K_b/[H^+])$, where $k_{cat}^0 = 100\ s^{-1}$, $pK_a' = 6.3$, $pK_b' = 11.6$, $(k_{cat}/K_M)^0 = 2.45\times10^7\ M^{-1}\ s^{-1}$, $pK_a = 6.7$, $pK_b = 10.8$. Experimental conditions: 25°C, μ 0.1 M.

clear, however, that in both cases at least two more groups are involved in catalysis in addition to the single amino acid residue suggested in the mechanism based on the crystal structure of the *E. coli* enzyme [9]. Moreover, a positively charged third residue which is responsible for chiral discrimination of the substrate, as shown recently for the *E. coli* enzyme [18], could be a fourth important component of the active center of penicillin acylase from *A. faecalis*, as this enzyme also exhibits high stereospecificity (ratio of the second-order rate constants for enzymatic hydrolysis of L- and D-enantiomers of *N*-phenylacetylarginine is more than 3000; Table 1). In conclusion, the active centers of both the *A. faecalis* and *E. coli* enzymes are similar but differ at least in their detailed organization especially in the subsite responsible for interaction with the leaving group of the substrate. It would be quite interesting also to compare the binding sites of the acyl group. Such a comparison could lead to a better understanding of the extremely effective (but somehow different for both penicillin acylases) recognition of the acyl moiety. Further studies on the substrate specificity and stereospecificity of the enzyme from *A. faecalis*, which are in progress, are expected to be useful for clarifying the unique mechanism of action of this group of penicillin acylases.

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References

[1] Vandamme, E.J. (1980) in: Economic Microbiology (Rose, A.A., Ed.), Vol. 5, pp. 467–522, Academic Press, New York.
[2] Abbott, B.J. (1976) Adv. Appl. Microbiol. 20, 203–257.
[3] Shewale, J.G. and Sivaraman, H. (1989) Proc. Biochem. 24, 146–154.
[4] Virden, R. (1990) Biotechnol. Genet. Eng. Rev. 8, 189–218.
[5] Bruns, W., Hoppe, J., Tsai, H., Bruning, H.J., Maywald, F., Collins, J. and Mayer, H. (1985) J. Mol. Appl. Genet. 3, 36–44.
[6] Choi, K.S., Kim, J.A. and Kang, H.S. (1992) J. Bacteriol. 174, 6270–6276.
[7] Schumacher, G., Sizmann, D., Haug, H., Buckel, P. and Bock, A. (1986) Nucleic Acids Res. 14, 5713–5727.

[8] Barbero, J.L., Gonzalez-de-Buitrago, G., Mendes, E., Perez-Aranda, A. and Garcia, J.L. (1986) Gene 49, 69–80.
[9] Duggleby, H.J., Tolley, S.P., Hill, C.P., Dodson, E.J., Dodson, G. and Moody, P.C.E. (1995) Nature 373, 264–268.
[10] Brannigan, J.A., Dodson, G., Duggleby, H.J., Moody, P.C.E., Smith, J.L., Tomchik, D.R. and Murzin, A.G. (1995) Nature 378, 416–419.
[11] Seemuller, E., Lupas, A. and Baumeister, W. (1996) Nature 382, 468–470.
[12] Berezin, I.V., Klyosov, A.A., Nys, P.S., Savitskaya, E.M. and Švedas, V.K. (1974) Antibiotiki (Rus.) 19, 880–887.
[13] Švedas, V.K. (1988) in: Advances in Scientific Technology ser. Biotechnology (Nys, P.S. and Mitin, Yu.V., Eds.), Vol. 7, 147 pp. [14]#Waldmann, H. (1989) Tetrahedron Lett. 30, 3057–3058.
[15] Fuganti, C., Rosell, C.M., Servi, S., Tagliani, A. and Terreni, M. (1992) Tetrahedron: Asymmetry 3, 383–386.
[16] Baldaro, E., D'Arrigo, P., Pedrocchi-Fantoni, G., Rosell, C.M., Servi, S., Tagliani, A. and Terreni, M. (1993) Tetrahedron: Asymmetry 4, 1031–1034.
[17] Zmijewski, M.J., Briggs, B.S., Thompson, A.R. and Wright, I.G. (1991) Tetrahedron Lett. 32, 1621–1622.
[18] Švedas, V.K., Savchenko, M.V., Beltser, A.I. and Guranda, D.F. (1996) Ann. NY Acad. Sci. 799, 659–669.
[19] Solodenko, V.A., Kasheva, T.N., Kukhar, V.P., Kozlova, E.V., Mironenko, D.A. and Švedas, V.K. (1991) Tetrahedron 47, 3989–3998.
[20] Solodenko, V.A., Belik, M.Y., Galushko, S.V., Kukhar, V.P., Kozlova, E.V., Mironenko, D.A. and Švedas, V.K. (1993) Tetrahedron: Asymmetry 4, 1965–1968.
[21] Soloshonok, V.A., Kirilenko, A.G., Fokina, N.A., Shishkina, I.P., Galushko, S.V., Kukhar, V.P., Švedas, V.K. and Kozlova, E.V. (1994) Tetrahedron: Asymmetry 5, 1119–1126.
[22] Soloshonok, V.A., Kirilenko, A.G., Fokina, N.A., Kukhar, V.P., Galushko, S.V., Švedas, V.K. and Resnati, G. (1994) Tetrahedron: Asymmetry 5, 1225–1228.
[23] Soloshonok, V.A., Galaev, I.Yu., Švedas, V.K., Kozlova, E.V., Kotik, N.V., Shishkina, I.P., Galushko, S.V., Rozhenko, A.B. and Kukhar, V.P. (1993) Bioorg. Khim. 19, 467–473.
[24] Margolin, A.L. (1993) Enzyme Microb. Technol. 15, 266–280.
[25] De Vroom, E. and van der Mey, M. (1995) European patent 0638649 A2.
[26] Švedas, V.K., Margolin, A.L., Sherstiuk, S.F., Klyosov, A.A. and Berezin, I.V. (1977) Bioorg. Khim. 3, 546–553.
[27] Švedas, V.K., Galaev, I.Yu., Borisov, I.L. and Berezin, I.V. (1980) Anal. Biochem. 101, 188–195.
[28] Fiedler, F., Seemuller, U. and Fritz, H. (1984) In: Methods of Enzymatic Analysis (Bergmeyer, H.U., Ed.), Vol. 5, 3rd edn., pp. 297–314, Verlag Chemie, Weinheim.
[29] Knight, C.G. (1995) Methods Enzymol. 248, 85–101.
[30] Siewinski, M., Kuropatwa, M. and Szewczuk, A. (1984) Hoppe-Seyler's Z. Physiol. Chem. 635, 829–837.
[31] Mahajan, P.B. (1984) Appl. Biochem. Biotechnol. 9, 537–552.
[32] Martin, J., Slade, A., Aitken, A., Arche, R. and Virden, R. (1991) Biochem. J. 280, 659–662.
[33] Borisov, I.L., Markaryan, A.N., Oreshina, M.G., Penzikova, G.A., Galaev, I.Yu., Bartoshevich, Yu.E. and Švedas, V.K. (1984) Biochimiya 49, 519–526.
[34] Prieto, I., Martin, J., Arche, R., Fernandez, P., Perez-Aranda, A. and Barbero, J.L. (1990) Appl. Microbiol. Biotechnol. 33, 553–559.
[35] Alvaro, G., Fernandez-Lafuente, R., Rosell, C.M., Blanco, R.M., Garcia-Lopez, J.L. and Guisan, J.M. (1992) Biotechnol. Lett. 14, 285–290.
[36] Kasche, V., Haufler, U., Markowsky, D., Melnyk, S., Zeich, A. and Galunsky, B. (1987) Ann. NY Acad. Sci. 501, 97–102.