ISSN 0027-1314, Moscow University Chemistry Bulletin, 2015, Vol. 70, No. 6, pp. 261–267. © Allerton Press, Inc., 2015.
Original Russian Text © A.A. Zakhariants, A.A. Poloznikov, D.M. Hushpulian, T.A. Osipova, V.I. Tishkov, I.G. Gazaryan, 2015, published in Vestnik Moskovskogo Universiteta. Khimiya, 2015, No. 6, pp. 329–335.

Benzimidazoles as Competitive Inhibitors of FAD-Monooxygenase

A. A. Zakhariants[a, d], A. A. Poloznikov[b, c, d], D. M. Hushpulian[a, d], T. A. Osipova[a], V. I. Tishkov[a, d, *], and I. G. Gazaryan[a, c]

[a] *Department of Chemistry, Moscow State University, Moscow, 119991 Russia*

[b] *D. Rogachev Federal Research and Clinical Center for Pediatric Hematology, Oncology, and Immunology, ul. Samora Mashela 1, Moscow, 117997 Russia*

[c] *W. M. Burke Medical Research Institute, Weill Cornell University Medical College, 785 Mamaroneck Ave, White Plains, NY, 10605 USA*

[d] *OOO Innovations and High Technologies MSU, ul. Tsimlyanskaya 16, Moscow, 109551 Russia*

**e-mail: vitishkov@gmail.com*

Received August 1, 2015

Abstract—Benzimidazoles are drugs which target tubulin and are widely used to treat intestinal parasites. Four benzimidazoles are tested with the well-characterized and commercially available bacterial *p*-hydroxybenzoate hydroxylase (PHBH), which belongs to the group of Class A FAD-monooxygenases, which also includes such enzymes as the FAD-monooxygenase domain of MICAL. PHBH is shown to be competitively inhibited by all four benzimidazoles (mebendazole, albendazole, fenbendazole, and oxibendazole) in the micromolar range in the hydroxylase reaction, but not in the non-physiological NADPH-dehydrogenase reaction of ferricyanide reduction. The inhibition pattern is consistent with benzimidazoles competing with *p*-hydroxybenzoate for the resting state of the enzyme, indirectly indicating the ordered mechanism of substrate binding. Modeling studies support the conclusions derived from steady-state kinetics.

Keywords: *p*-hydroxybenzoate hydroxylase, ferricyanide reduction, inhibition analysis, computer modeling, docking

DOI: 10.3103/S0027131415060073

Benzimidazoles, such as albendazole and fenbendazole, are antihelmintic medications inhibiting the formation of microtubules. In humans, they undergo oxidation and degradation catalyzed by FAD-dependent monooxygenases, which oxidize sulfur to sulfone. The human genome has more than 60 genes of FAD-dependent monooxygenases, most of which have unknown functions and have not been studied individually. A well-studied group is nonspecific monooxygenases, which degrade xenobiotics in parallel with cytochrome P-450. They feature a ping-pong type catalytic mechanism that includes FAD reduction with NADPH followed by oxygen binding and activation by FADH, finally resulting in xenobiotic oxidation [1].

Abbreviations: PHBH—*p*-hydroxybenzoate hydroxylase; MICAL—Molecule Interacting with CasL; FAD—flavin adenine dinucleotide; NADPH—nicotinamide adenine dinucleotide phosphate, reduced form; PHB—*p*-hydroxybenzoate.

Besides the nonspecific FAD-dependent monooxygenases which catalyze the oxidation reaction via the ping-pong mechanism, there exists another group, the so-called specific FAD-dependent monooxygenases. They play an important role in humans and their catalytic mechanism is different from the common "ping-pong" type. For instance, Class A FAD-dependent monooxygenases (in accord with the recently proposed classification [1]) are specific for various specified substrates. Only upon binding of a specified substrate, the enzyme does undergo a conformational change, providing an effective binding of NADPH followed by immediate FAD reduction. Thus, the catalytic mechanism includes the formation of a ternary complex. The rate constants for FAD reduction in the absence and in the presence of a specific substrate differ by five orders of magnitude (140000-fold [2]). It was of interest to study whether antihelmintic benzimidazoles behave as competitive substrates for specific monooxygenases like they do for nonspecific ones.

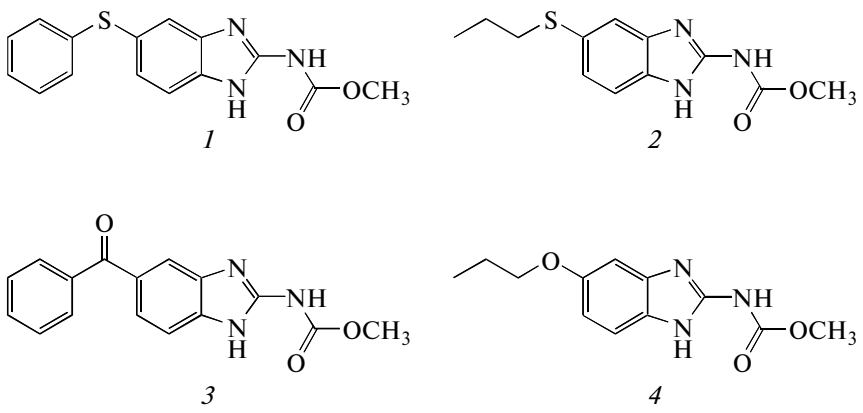


Fig. 1. The structures of the studied antihelmintic benzimidazoles: *1*—methyl-5-(phenylthio)-2-benzimidazole carbamate (fenbendazole); *2*—methyl-5-(propylthio)-2-benzimidazole carbamate (albendazole); *3*—methyl-*N*-(5-benzoyl-1*H*-benzimidazolyl-2) carbamate (mebendazole); *4*—methyl-(5-propoxy-1H-benzimidazolyl-2) carbamate (oxibendazole).

For this purpose, 4-hydroxybenzoate NADPH, oxygen oxidoreductase, commonly known as *p*-hydroxybenzoate hydroxylase (PHBH, EC1.14.13.2), from *Pseudomonas* sp., with the well-studied structure and catalytic mechanism [1–8], was chosen as a model specific FAD-dependent monooxygenase. PHBH inhibition was studied in the presence of four benzimidazoles, only two of which contained an easily oxidizable sulfur (albendazole and fenbendazole), whereas oxibendazole and mebendazole had no easily oxidizable groups in their structure (Fig. 1).

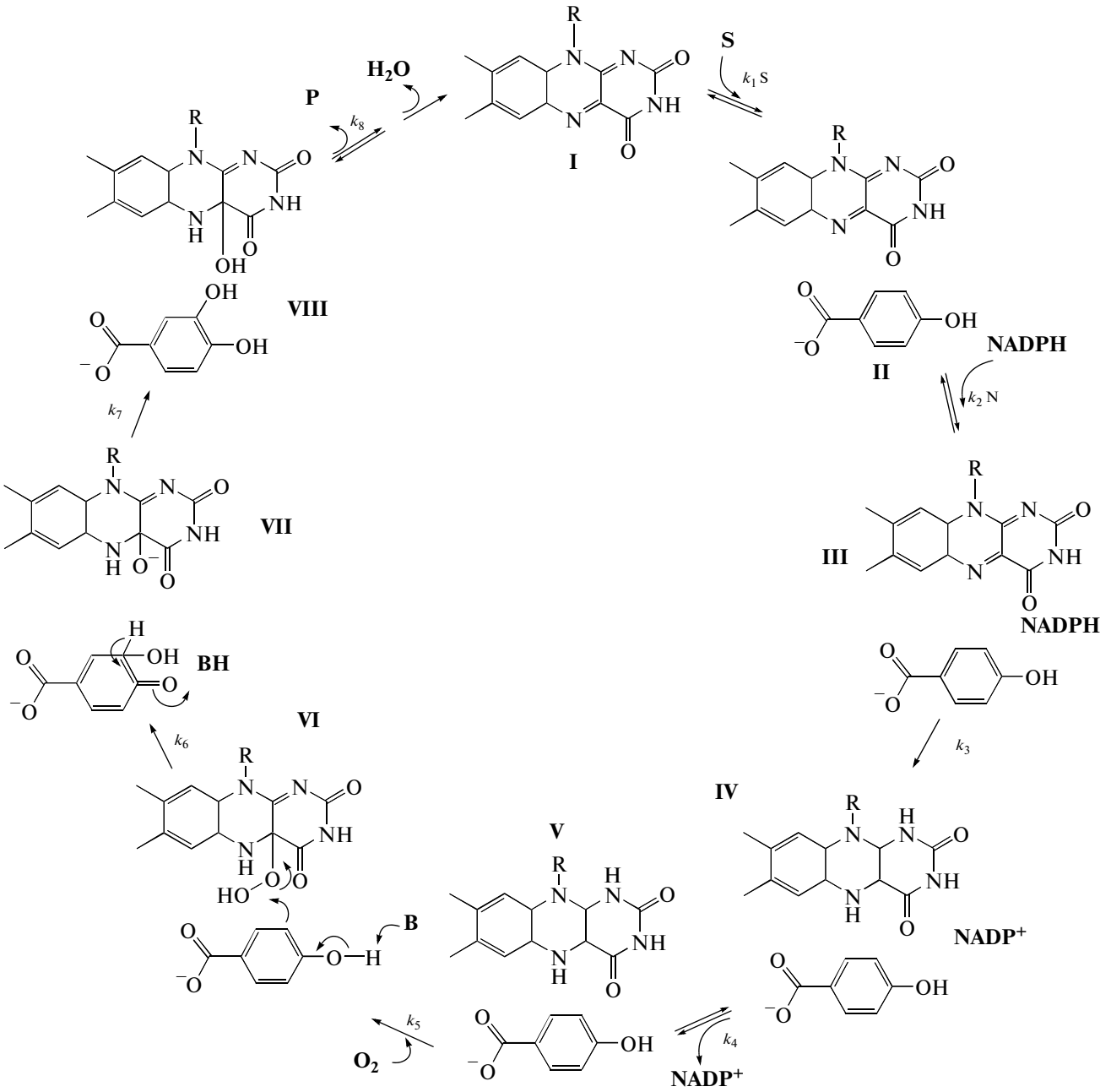
PHBH is a model enzyme for Class A specific FAD-dependent monooxygenases, which include the catalytic domain of MICAL, specific for actin [9] and playing an important role in cytoskeleton modification [10–12] both in axon navigation [13] and epithelial–mesenchymal transition [14].

MATERIALS AND METHODS

All reagents used in this work were purchased from Sigma (USA) and used without further purification. The solubility of the benzimidazoles was preliminarily studied: the optimized solubilization procedure included preparation of 50 mM stock solution in DMSO followed by its dilution with a 50% ethanol–water solution and then by the addition of a needed aliquot to the reaction mixture. The PHBH activity was measured in 50 mM potassium phosphate buffer, pH 7.5 The enzyme concentration was determined spectrophotometrically by FAD absorbance using the extinction coefficient $\varepsilon_{450} = 10.2$ mM^{-1} cm^{-1} [6]. The reaction rate was followed by NADPH consumption ($\varepsilon_{440} = 6.22$ mM^{-1} cm^{-1}) on a Spectramax M5 platereader (Molecular Devices, USA). The substrates' concentrations were varied in the following range: 25–100 μM NADPH, and 25–150 μM PHB. The reaction was initiated by the addition of NADPH. Ferricyanide (0.5 mM) and NADPH (50–150 μM) were used to measure the diaphorase (NADPH-dehydrogenase) activity. The activity was followed by monitoring both substrates (ferricyanide $\varepsilon_{420} = 1.04$ mM^{-1} cm^{-1}). The initial reaction rate (the maximum slope of the kinetic curve within the first three minutes) was taken as a measure of the enzyme activity. All experiments were performed in triplicate. Analysis of inhibition was performed in the Dixon plots. Modeling was performed with the Discovery Studio 2.5 software (Accelrys, USA) using two crystal structures of PHBH, one with the bound substrate (1PBE.pdb) and one with bound NADPH (1K0J.pdb).

RESULTS AND DISCUSSION

The PHBH stepwise catalytic cycle presented in Scheme 1 includes ordered sequential binding of the substrates PHB (k_1S) and NADPH (k_2N); FAD reduction (k_3); NADP dissociation (k_4); reduced FAD oxidation with oxygen (k_5); PHB hydroxylation with the help of a base B in the enzyme active center (k_6); protonation and formation of FAD hydroxide with the help of the protonated form of base B (BH) in the enzyme active center (k_7); and finally, the release of the hydroxylated product and water (k_8).



Scheme 1. Full catalytic cycle of the reaction of *p*-hydroxybenzoate oxidation catalyzed by *p*-hydroxybenzoate hydroxylase from *Pseudomonas* sp. [1].

All of the studied benzimidazoles behaved as PHBH inhibitors when the reaction rate was measured by NADPH consumption. If they had been competitive substrates, NADPH would have been consumed on their oxidation and thus the reaction rate measured by NADPH consumption would not decrease. Since there is a significant drop in the reaction rate measured by NADPH consumption, one may conclude that benzimidazoles are true inhibitors, and not competitive substrates of PHBH as was commonly supposed before for albendazole and fenbendazole. Even in the case of their plausible oxidation by the enzyme, the oxidation products have to be considered as inhibitors. Moreover, mebendazole and oxibendazole, which do not have easily oxidizable sulfur in their structure like albendazole and fenbendazole do, behave as competitive inhibitors both with respect to the specific substrate and the cofactor NADPH (Fig. 2). It is worth noting that the value of the inhibition constant determined from the intersection point in the Dixon plot depends on whether the PHB substrate is varied at fixed cofactor concentration or the cofactor is varied

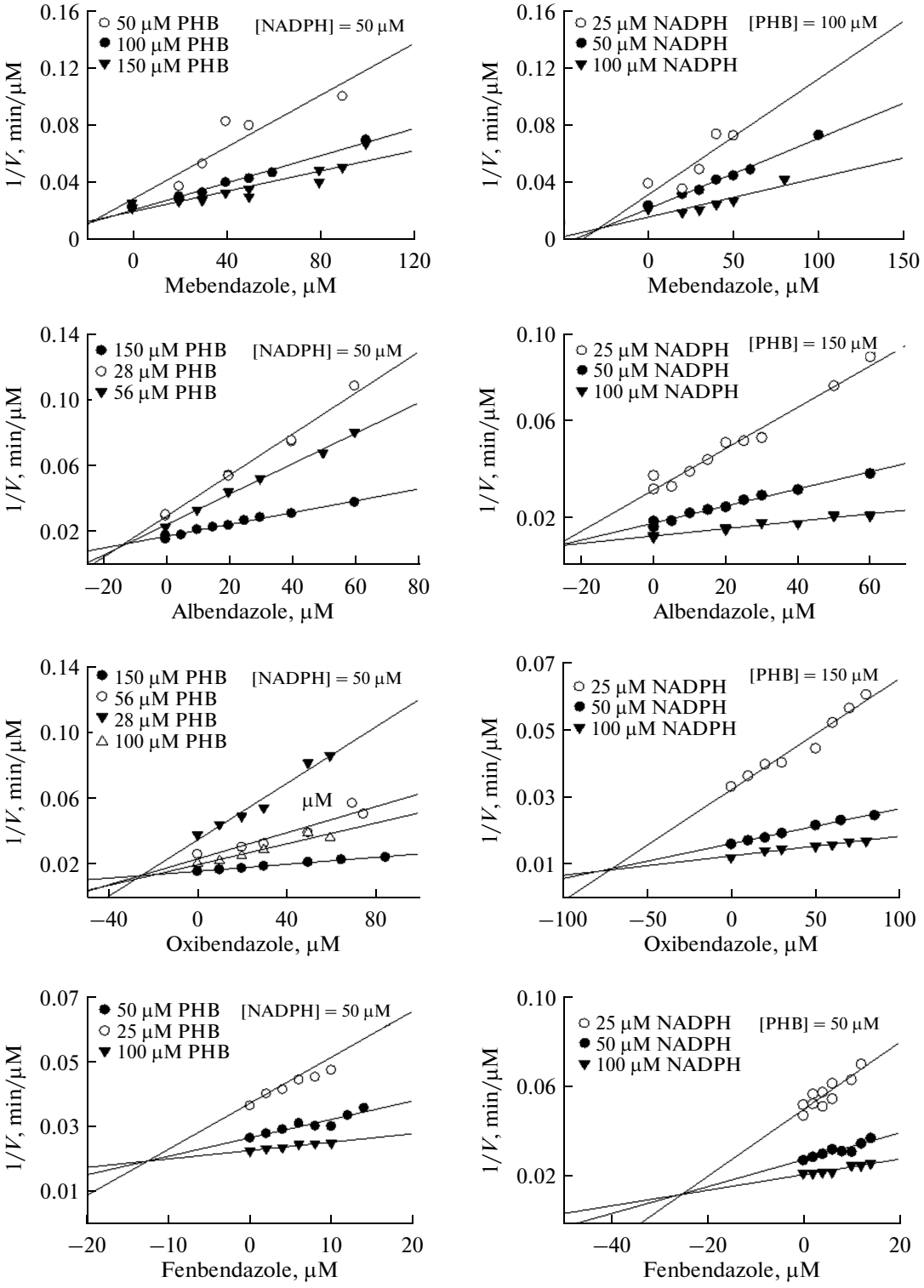
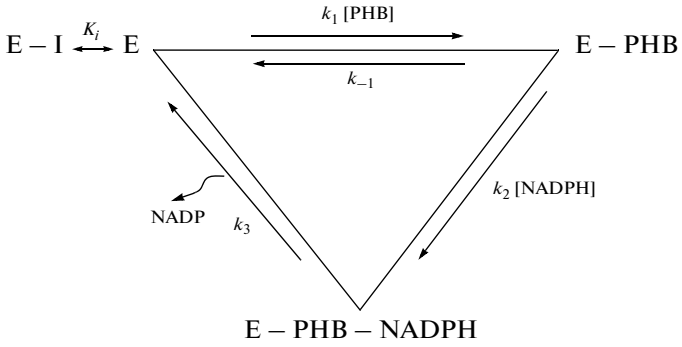


Fig. 2. Dixon plots for benzimidazole inhibition of PHB oxidation catalyzed by PHBH. Left, varied PHB at fixed NADPH concentration; Right, varied NADPH at fixed PHB concentration.

at fixed concentration of the specific substrate (Fig. 2). The inhibition constant determined at the fixed NADPH is 2–3 times better (smaller) than that determined at the fixed PHB. When the steady-state mechanism for the PHBH-catalyzed reaction was studied in the 1970s, it was classified as a random-ordered ternary complex mechanism [2]. If one supposes that benzimidazoles compete for the resting enzyme both

with the substrate and the cofactor, in this case, the experimentally determined inhibition constant should not depend on which substrate is fixed and which is varied. Subsequent detailed studies on the PHBH mechanism, which included mutagenesis and crystallization of the mutant forms with the substrate and product, allowed the enzyme mechanism to be clarified (Scheme 1). It was unequivocally shown that the conformation change necessary to optimize the position of the FAD flavin ring for the effective interaction with NADPH takes place only upon binding of PHB [1, 3, 8]. It was shown later that the rate-limiting step in PHBH catalysis was dissociation of the NADP product at the end of the reductive half-reaction [3]. This process is depicted in Scheme 1 as transition IV $\longrightarrow$ V with the rate constant k_4. Oxygen binding and PHB oxidation are not rate limiting (k_5, k_6, k_7, k_8 steps in Scheme 1). Taking into account all the experimental evidence, the enzyme catalytic cycle can be represented by a scheme where PHB is reversibly bound at the first step (k_1, Scheme 2), followed only then by NADPH binding (k_2, Scheme 2), resulting in immediate FAD reduction (the rate constant can be determined from transition kinetics only for the enzyme mutants with the significantly compromised activity towards FAD reduction), followed by NADP dissociation (k_3), FAD oxidation, and substrate hydroxylation in the third position. The proposal regarding the necessity of $NADP^+$ dissociation comes from the consideration of the structural restrictions necessary for PHB oxidation [1]. Since the rate constants for the oxidative half-reaction are much higher than the rate-limiting step for the overall reaction, $NADP^+$ dissociation, and since the oxygen concentration is constant under the steady-state conditions, to simplify the kinetic scheme, the NADP dissociation, substrate oxidation, and product release steps have been combined into one, which has the rate-limiting constant equal to k_3.



Scheme 2. Simplified catalytic cycle of PHBH: E – enzyme, I – inhibitor.

In this simplified variant, the rate equation in the presence of an inhibitor, I, capable of binding to the resting form of the enzyme, E, can be presented by two versions of the variables' separation (either substrate or cofactor concentration) to make the interpretation of the Dixon plots easier (S = [PHB], N = [NADPH).

At the fixed NADPH concentration

$$\frac{1}{\nu} = \left(\frac{k_{-1} + k_2 N}{k_2 N}\right)\left(\frac{1}{k_1 S}\right)\left(1 + \frac{i}{K_i}\right) + \frac{1}{k_2 N} + \frac{1}{k_3}, \quad (1)$$

the abscissa of the intersection point in the Dixon plot is

$$i = -K_i; \quad (1a)$$

at the fixed PHB concentration and introducing its dissociation constant as K_S

$$\frac{1}{\nu} = \left(\frac{1}{k_1 S}\right)\left(1 + \frac{i}{K_i}\right) + \frac{1}{k_2 N}\frac{K_S}{S}\left(1 + \frac{S}{K_S} + \frac{i}{K_i}\right) + \frac{1}{k_3}, \quad (2)$$

where $K_S = \frac{k_{-1}}{k_1}$, the abscissa of the intersection point in the Dixon plot is

$$i = -K_i\left(1 + \frac{S}{K_S}\right). \quad (2a)$$

It is evident from the analysis of equations (1), (2) that the intersection point under the conditions of the fixed NADPH corresponds to the true value of the inhibition constant (1), (1a), whereas at the fixed PHB concentration the inhibition constant depends on the fixed concentration of PHB (2), (2a), namely, on the ratio of PHB concentration to its enzyme binding constant K_S. The latter was determined as 43 μM at pH 6.6 in [2] via monitoring the significant changes in the enzyme spectrum upon PHB binding. Taking into account the range of PHB concentrations used in this work (50–150 μM) and the increase in the binding constant upon rising pH (in this case to pH 7.5), the 2–3 fold increase in the inhibition constant under the conditions of fixed PHB compared to the conditions with fixed NADPH corresponds to the magnitude of the [S]/K_S factor. Thus, based on the steady-state kinetics data, one may assume that benzimidazole binding interferes with PHB binding. This conclusion is also supported by the absence of any inhibition by these benzimidazoles in the nonspecific reaction of ferricyanide reduction by NADPH, catalyzed by PHBH (like all other enzymes of this class) and proceeding via a ping-pong mechanism (results not shown).

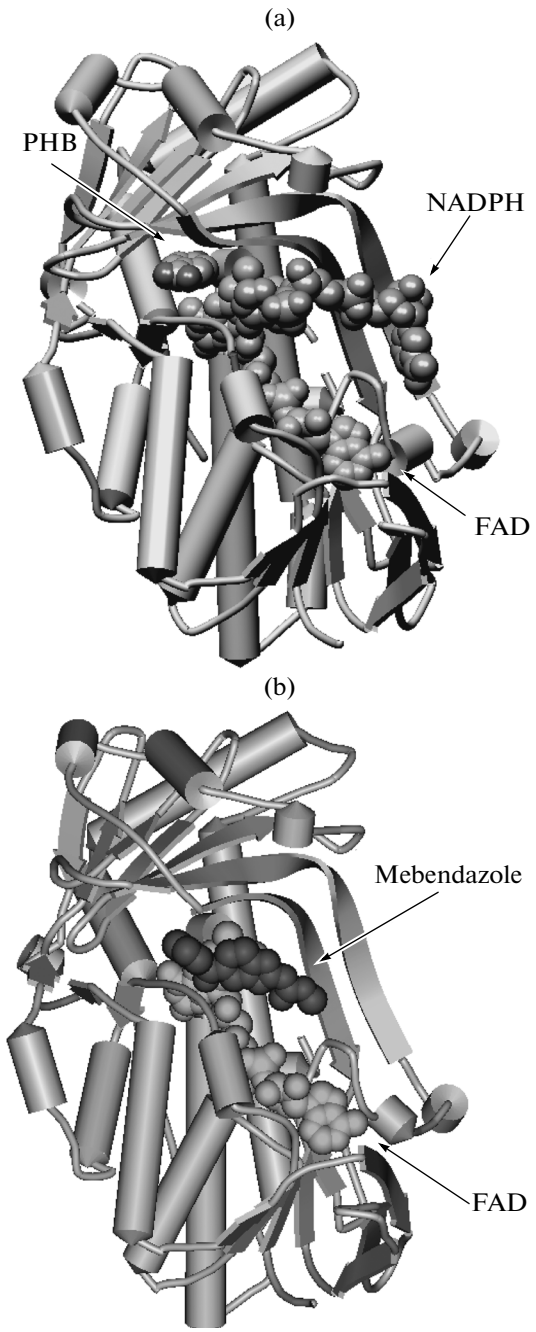


Fig. 3. Modeling of mebendazole binding to PHBH: (a) superimposed structures of PBE1 and 1K0J showing the position of substrates and FAD in the enzyme molecule; (b) docking of mebendazole in the binding site of NADPH: screening of the substrate channel leading to the PHB binding site.

Before analyzing the results of computer modeling, we would like to highlight one point that is essential for understanding the situation. Namely, despite the long and fruitful study of PHBH, there is no reported crystal structure of native PHBH with NADPH, whereas there are multiple successfully produced and resolved versions of the native (1PBE.pdb) and mutant enzyme complexes with the bound PHB. The only available structure with the bound NADPH was obtained and resolved for the Arg220Gln mutant of PHBH (1K0J.pdb) [7]. The most intriguing feature of the above structure is the position of the NADPH molecule with respect to the FAD molecule: exactly the opposite of what could be logically predicted based on the data generated by all spectral measurements on native and mutant enzyme forms and observation of the charge transfer complex upon NADPH binding to the native enzyme. When speculating on the conformational changes to the enzyme in the course of catalysis, it should be supposed either that the real position of NADPH does not correspond to the crystal structure of the mutant discussed above, but is positioned in an opposite orientation, as discussed in [1], or that the enzyme molecule undergoes unbelievable conformational changes to bend the nicotinamide portion of the NADPH molecule towards the FAD flavin ring, as discussed in [3].

To show the positions of PHB, FAD, and NADPH in one structure, the PHB molecule from the 1PBE structure, with the preserved coordinates with respect to FAD and the protein molecule, was transferred to the 1K0J structure. As seen in Fig. 3a, to bind in the cavity of the active center, PHB must enter via a channel directed perpendicularly to the flavin ring. Despite the fact that the NADPH binding mode in the actual catalytic cycle may not correspond to its binding mode in the 1K0J crystal structure, the principal moment for modeling is the position of adenine in the molecule of the bound cofactor. The docking of the benzimidazole inhibitors was performed using the docking sphere centered at the adenine residue in the NADPH molecule in the 1K0J1 structure. An example of mebendazole docking is shown in Fig. 3b, which clearly demonstrates that the benzyl portion of mebendazole partially screens the channel heading to the PHB binding site. Thus, the analysis of the binding mode of benzimidazoles in PHBH makes it possible to understand why benzimidazoles containing the benzimidazole ring stereo-equivalent to the adenine ring in NADPH compete with PHB more than with NADPH (whose binding in the real catalytic act may be exactly opposite to the one shown in the crystal structure) and interfere with the PHB binding, thus making the enzyme transition into the conformational state corresponding to FAD activation with respect to NADPH impossible.

In conclusion, the inhibitory analysis performed in this work gives additional evidence for the ordered sequential mechanism of substrate binding for Class A FAD-monooxygenase. This is the same class of enzymes that includes the FAD-monoxygenase domain of MICAL: enzyme activation is observed only upon F-actin binding. Given the fact that MICAL is responsible for actin transformation and benzimidazoles inhibit tubulin rearrangement, the discovery of inhibitory action of benzimidazoles on

the Class A monooxygenase model enzyme, PHBH, raises the question of the possible effect of benzimidazoles on the catalytic domain of MICAL and thus, on actin transformation, in addition to their established effect on tubulin. The ongoing research in this laboratory on the recombinant FAD-monooxygenase domain of MICAL will answer this question in the nearest future.

ACKNOWLEDGMENTS

The work was carried out under financial support of Russian Foundation for Basic Research (project no. 13-04-01909).

REFERENCES

1. Crozier-Reabe, K. and Moran, G.R., *Int. J. Mol. Sci.,* 2012, vol. 13, p. 15601.
2. Husain, M. and Massey, V.*, J. Biol. Chem.,* 1979, vol. 254, p. 6657.
3. Entsch, B., Cole, L.J., and Ballou, D.P., *Arch. Biochem. Biophys.*, 2005, vol. 433, p. 297.
4. Hosokawa, K. and Stanier, R.Y., *J. Biol. Chem.*, 1966, vol. 241, p. 2453.
5. Howell, L.G., Spector, T., and Massey, V., *J. Biol. Chem.*, 1972, vol. 247, p. 4340.
6. van Berkel, W., Westphal, A., Eschrich, K., Eppink, M., and de Kok A., *Eur. J. Biochem.,* 1992, vol. 210, p. 411.
7. Wang, J., Ortiz-Maldonado, M., Entsch, B., Massey, V., Ballou, D., and Gatti, D.L., *Proc. Natl. Acad. Sci. U. S. A.*, 2002, vol. 99, p. 608.
8. Westphal, A.H., Matorin, A., Hink, M.A., Borst, J.W., van Berkel, W., and Visser, A.J., *J. Biol. Chem.*, 2006, vol. 281, p. 11074.
9. Zucchini, D., Caprini, G., Pasterkamp, R.J., Tedeschi, G., and Vanoni, M.A., *Arch. Biochem. Biophys.*, 2011, vol. 515, p. 11.
10. Hung, R.J., Pak, C.W., and Terman, J.R., *Science,* 2011, vol. 334, p. 1710.
11. Hung, R.J., Spaeth, C.S., Yesilyurt, H.G., and Terman, J.R., *Nat. Cell Biol.*, 2013, vol. 15, p. 1445.
12. Hung, R.J., Yazdani, U., Yoon, J., Wu, H., Yang, T., Gupta, N., Huang, Z., van Berkel, W., and Terman, J.R., *Nature*, 2010, vol. 463, p. 823.
13. Lee, B.C., Peterfi, Z., Hoffmann, F.W., Moore, R.E., Kaya, A., Avanesov, A., Tarrago, L., Zhou, Y., Weerapana, E., Fomenko, D.E., Hoffmann, P.R., and Gladyshev, V.N., *Mol. Cell*, 2013, vol. 51, p. 397.
14. Lundquist, M.R., Storaska, A.J., Liu, T.C., Larsen, S.D., Evans, T., Neubig, R.R., and Jaffrey, S.R., *Cell*, 2014, vol. 156, p. 563.

The article was translated by the authors.