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Computer-aided analysis of the interactions of glutamine synthetase with its inhibitors

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Abstract—Mechanism of inhibition of glutamine synthetase (EC 6.3.1.2; GS) by phosphinothricin and its analogues was studied in some detail using molecular modeling methods. Among three possible conformations of phosphinothricin in the active site of GS, this compatible with binding mode of methionine sulfoximine, determined recently by crystallography, was found to be energetically favored. Basing on these results eleven inhibitors of GS were docked into its active site. Taking into consideration that phosphinothricin acts as suicide inhibitor, which is due to phosphorylation by the enzyme, seven of studied analogues were additionally analyzed in their phosphorylated forms. All the inhibitor–enzyme complexes were evaluated quantitatively by using eight scoring functions implemented in Insight and Sybyl program packages and significant correlation between the obtained scores and experimental pK_i values was achieved. Computed surface charge distribution for five selected inhibitors in both free and phosphorylated forms and their comparison with electronic structure of enzymatic reaction transition state allowed us to determine important electronic features required to construct potent inhibitors of glutamine synthetase.

1. Introduction

Glutamine synthetase (EC 6.3.1.2; GS) is a key enzyme in nitrogen metabolism in bacterial and plant cells.[1,2] The enzyme catalyzes the conversion of glutamate to glutamine in the presence of ammonium ion with accompanied hydrolysis of ATP as an energy source. Inhibition of this enzyme in plants causes total impairment of nitrogen metabolism resulting in accumulation of toxic amounts of ammonia followed by plant death.[3–7] Thus, inhibitors of GS are good total herbicides.[8–11] Glutamine synthetase is also considered as a promising target for tuberculosis treatment.[12–18] *Mycobacterium tuberculosis* secretes GS into intercellular space in order to build polyglutamate/glutamine structures in cell wall. Inhibition of GS blocks cell wall biosynthesis and subsequently causes the death of pathogen. It is also known that GS plays a significant role in brain and the level of its activity is related to the development of diseases such as schizophrenia and Parkinson's,[19] Alzheimer's,[20] and Huntington's diseases.[21] In addition, the increased activity of glutamine synthetase was detected upon brain injury but the physiological meaning of this observation is not understood.[22]

Nowadays, the development of new inhibitors is often based on computer-assisted design process. Although the three-dimensional structure of the enzyme and mechanism of enzymatic reaction are most important for accurate design, the mode of action of already known lead compounds also has to be considered. Studies on the design and synthesis of glutamine synthetase inhibitors have quite long history.[2,23–27] Most of the known inhibitors are analogues of glutamate and replace this substrate in the active site of the enzyme. Some of them act as suicide inhibitors being, similarly to glutamate, phosphorylated by ATP.[28,29] Among several known inhibitors of GS, phosphinothricin (PPT) and methionine sulfoximine (MetSox) are the most potent ones and were used as leads in several projects.[30–33]

In this paper, several possible modes of binding of phosphinothricin and methionine sulfoximine to active site of glutamine synthetase have been proposed and carefully analyzed in respect to optimal complexation by the

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enzyme. Non-typical, suicide mode of the action of majority of known GS inhibitors makes computer-assisted design of new ones quite difficult. Therefore, a group of selected inhibitors was docked into active site of the enzyme and in cases when phosphorylation of inhibitor seemed to be possible, phosphorylated forms of these inhibitors were evaluated as well. Then modes of binding obtained for phosphorylated and non-phosphorylated species were compared by scoring inhibitor–enzyme complexes using eight scoring functions. Finally, five compounds were selected and their surface charge distribution was determined in both free and phosphorylated forms.

2. Results and discussion

Among several known inhibitors of glutamine synthetase 11 compounds with the wide range of inhibition constants were chosen (Fig. 1). All of them are simple structural analogues of glutamate and most probably they compete with this substrate for the active site of GS.

Methionine sulfoximine (**1**) and phosphinothricin (**2**) are the best-known inhibitors of glutamine synthetase. It is well established that after binding to the enzyme they are phosphorylated by ATP and these forms act as irreversible inhibitors.[28,29] Recently published crystal structure of MetSox-P-GS complex was used as starting point for all calculations, as it is of the highest resolution of all already known GS structures and positions of all residues forming the active site are thus well determined.[38] Although the structure of MetSox-GS complex had been already published,[39] it was never deposited in Protein Data Bank, thus it was modeled (Fig. 2a) and compared with the crystal structure of MetSox-P-GS complex obtained by Krajewski et al. by X-ray crystallography (Fig. 2b). Obtained structure is consistent both with that measured by Liaw et al.[39] and available biochemical data indicating that only (*R*,*S*) diastereoisomer of **2** inhibits GS irreversibly.[40–44]

By analysis of crystal the structure of the PPT-GS complex,[45] it is possible to propose at least three conformations of phosphinothricin in the active site of the enzyme. Simple rotation of methylphosphinic group yields these conformers in which the methyl group is directed toward: (A) Arg368, (B) Glu335, and (C) metal ion n2 (Fig. 3). However, these structures could not be distinguished using published low-resolution X-ray PPT-GS structure.[45] It is the result of the fact that methyl group and oxygen atom are isoelectronic and within this low-resolution structure the electron density of the entire terminal moiety is rather featureless, thus giving no information on the conformation of this part of molecule.

It is known that phosphorylation of oxygen atom by ATP can occur only in proximity to metal ions (n1, n2) and Arg347, which polarize the γ-phosphate group of ATP.[2] Thus, conformation C has to be immediately rejected. The remaining two conformations were already proposed in the literature: conformation A that is analogous to conformation of MetSox proposed by Liaw et al.[39] and conformation B that was suggested for phosphinothricin by Gill and Eisenberg.[45] Moreover, various protonation states can be proposed for these two conformations. In conformation A, phosphinic group of phosphinothricin is not protonated and

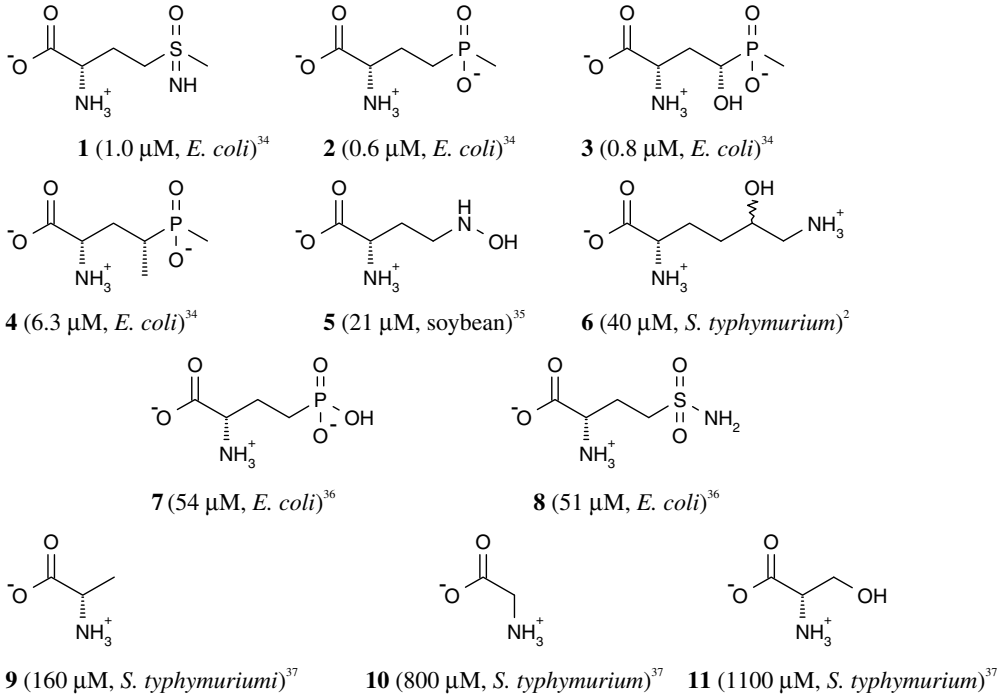

Figure 1. Structures of discussed inhibitors of GS and their inhibition constants reported in the literature. See above-mentioned references for further information.

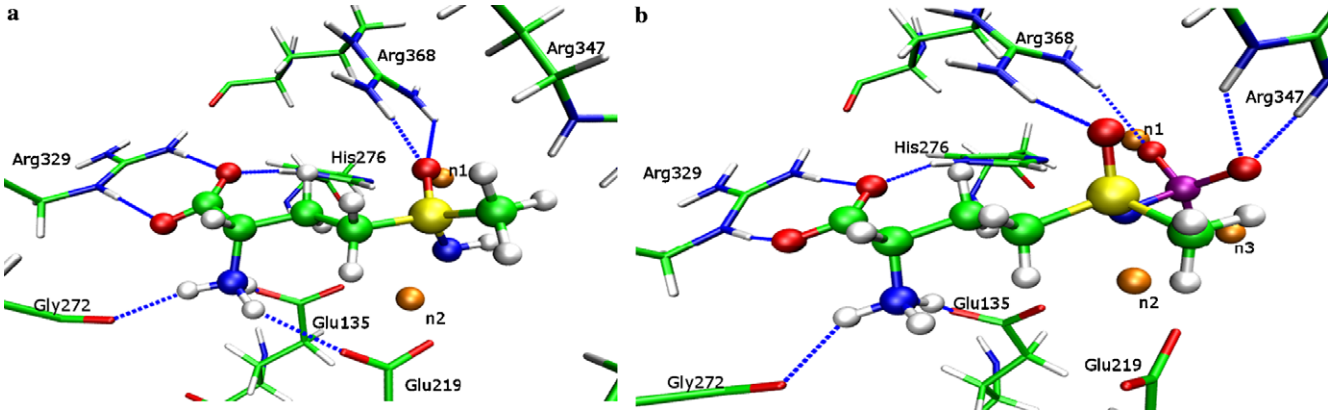

Figure 2. Optimized structure of MetSox-GS (a) and crystal structure of MetSox-P-GS (b) complexes.

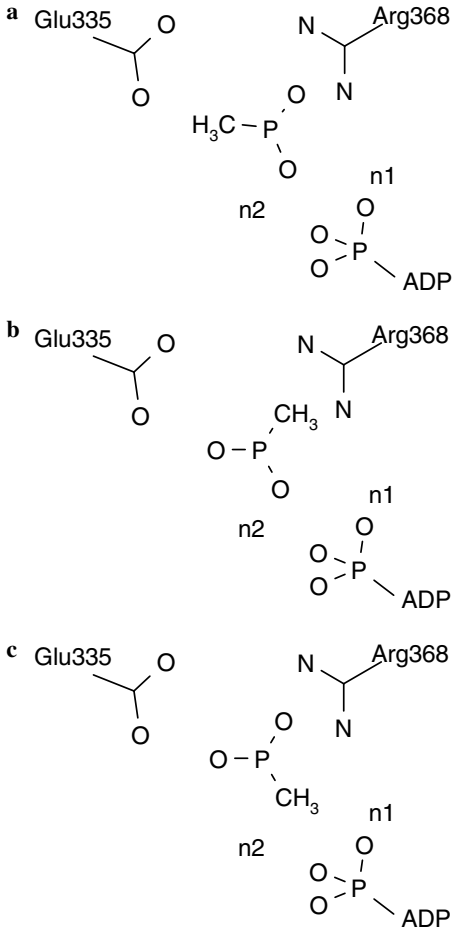

Figure 3. Schematic presentation of conformations A (a), B (b) and C (c) of PPT in active site (hydrogen atoms and rest of PPT molecule omitted for the clarity of picture).

therefore the inhibitor most closely reminds transition state of enzymatic reaction. According to Gill et al., in the case of conformation B two alternatives are possible in which either β-carboxylate group of Glu335 (conformation B1) or oxygen atom (Oε2) of phosphinothricin (conformation B2) is protonated.

In order to analyze in detail the proposed conformations, they were modeled starting from a recently published high-resolution crystal structure of the MetSox-P-GS complex.[38] Conformation A (Fig. 4a) seems to be the most favorable due to several reasons. First, it is similar to MetSox-GS complex if considering hydrogen bond network, charge distribution, and the value of LUDI scoring function.[46–52] Second, the value of LUDI scoring function for this structure is much higher than for other conformations (1140 vs 946 and 881 for conformations A vs B1 and B2, respectively). Third, the protonation states of phosphinothricin and active site residues are consistent with their pK_a values (while for conformations B1 and B2 are not).[53] Fourth, the structure of PPT-P-GS complex (Fig. 4b) based on this structure is highly analogous to the published MetSox-P-GS structure.

Basing on the obtained structures of PPT-GS and MetSox-GS complexes, the remaining set of chosen inhibitors (compounds **3**–**11**) was docked into the active site of glutamine synthetase. The proposed modes of binding of compounds **9**, **10**, and **11** are consistent with low-resolution crystal structures of their complexes with the enzyme.[37] The minimized structures were scored with functions from LUDI program[46–52] (LUDI1, LUDI2, and LUDI3) and from Sybyl program[54] (D_SCORE, PMF_SCORE, G_SCORE, CHEMSCORE, and F_SCORE). Relations between obtained scores and negative logarithm of inhibition constant (pK_i) are presented in Figure 5. It is clear that correlation between scores and pK_i is good for most of the functions with the exception of D_SCORE and CHEM_SCORE functions, which gave somewhat poorer correlations. Correlation was done using kinetic data obtained by various laboratories and by application of various assay methods. Therefore, there is a possibility of non-compatibility of these experimental data. In order to check how a potentially incorrectly analyzed structure may influence R^2 value, this value was also calculated omitting a point, which deviated from the analyzed pattern (outlier). In this case, no dramatic change of R^2 value was observed.

Considerable structural analogy of compounds **3**–**5**, **7**, and **8** with phosphinothricin and methionine sulfoximine allows assuming that they are also phosphorylated in the active site of glutamine synthetase and most probably these forms are actual inhibitors of the enzyme.

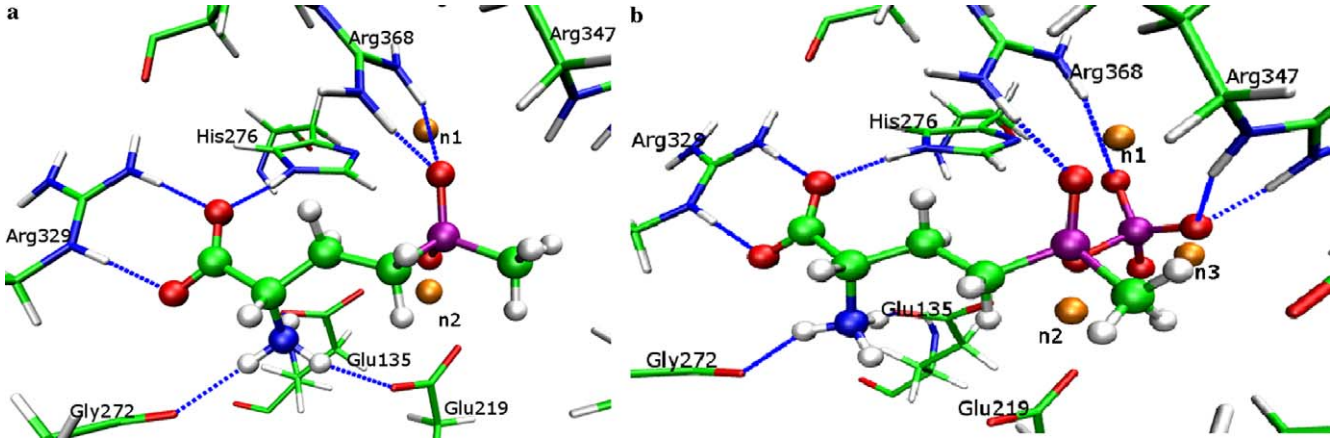

Figure 4. Optimized structures of PPT-GS (a) and PPT-P-GS (b) complexes.

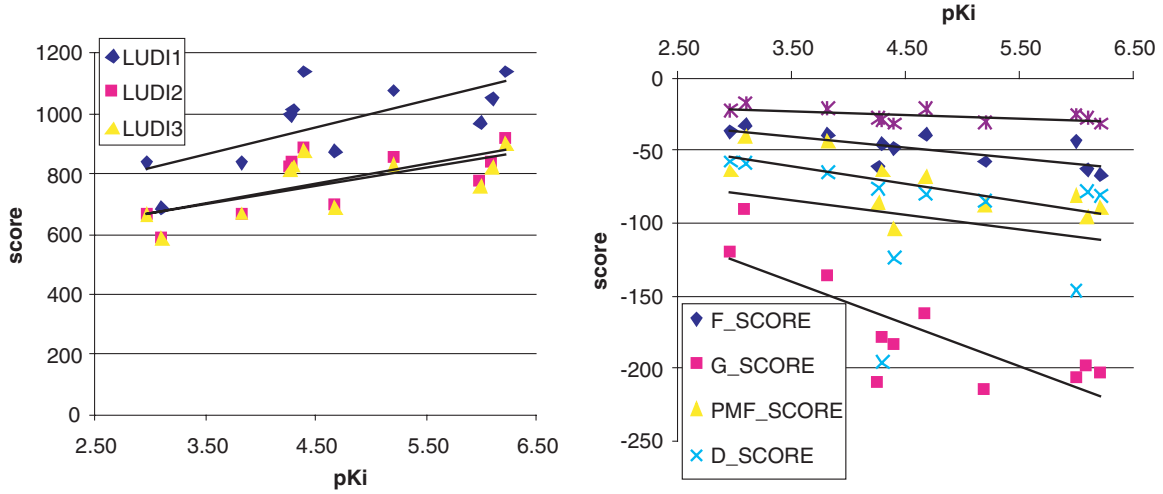

Figure 5. Correlations between applied scores and pK_i for non-phosphorylated GS inhibitors.

Therefore, structures of GS complexes with phosphorylated inhibitors were additionally analyzed. They were obtained by modification of docked inhibitors and subsequent energy minimization. The modeled structures were scored with the same set of functions as applied in the previous step. Correlation between these scores and pK_i presented in Figure 6 shows significant agreements between theoretical and experimental data.

Combination of R^2 values found for these correlations for series of non-phosphorylated and phosphorylated inhibitors is presented in Table 1. For both groups of inhibitors R^2 values indicate that most scoring functions produce reasonable results. R^2 values of phosphorylated inhibitors were higher for most functions than those found for non-phosphorylated ones. Also the mean R^2 value was higher for phosphorylated species. This significant correlation between experimental data and obtained scores strongly supports correctness of both the used approach and of the choice of the conformation A of PTT as the most favorable. Moreover, these results suggest that analysis of phosphorylated inhibitors is more accurate and might be helpful when designing new potential inhibitors of GS.

In order to perform more precise analysis of electronic structure of glutamine synthetase inhibitors, compounds with similar three-dimensional structure but exhibiting varying inhibition constants (compounds **1**, **2**, **5**, **7**, and **8**) were chosen for computation of electrostatic potential $\Phi(r)$ on molecular surface of each of them by means of ab initio approach (Fig. 7). Similar electrostatic potential distribution was found for the part of molecule identical with glutamate in all studied cases. Obviously, electrostatic potential of the fragment of glutamate γ-carboxylate mimetic is crucial for complexation of inhibitor in the active site and the studied inhibitors varied in this respect. Compounds **1** and **2** have similar electronic structure of this fragment—two atoms have negative potential, while one group is neutral. Because compounds **1** and **2** are the most active known inhibitors of GS, their charge distribution should be claimed optimal.

Since analysis of phosphorylated forms of compounds **1**–**5**, **7**, and **8** as GS inhibitors seems to be more accurate, electrostatic potential on electrostatic charge isosurfaces was also computed for these forms as well (Fig. 8). Phosphorylated compounds **1** and **2** (**1-P** and

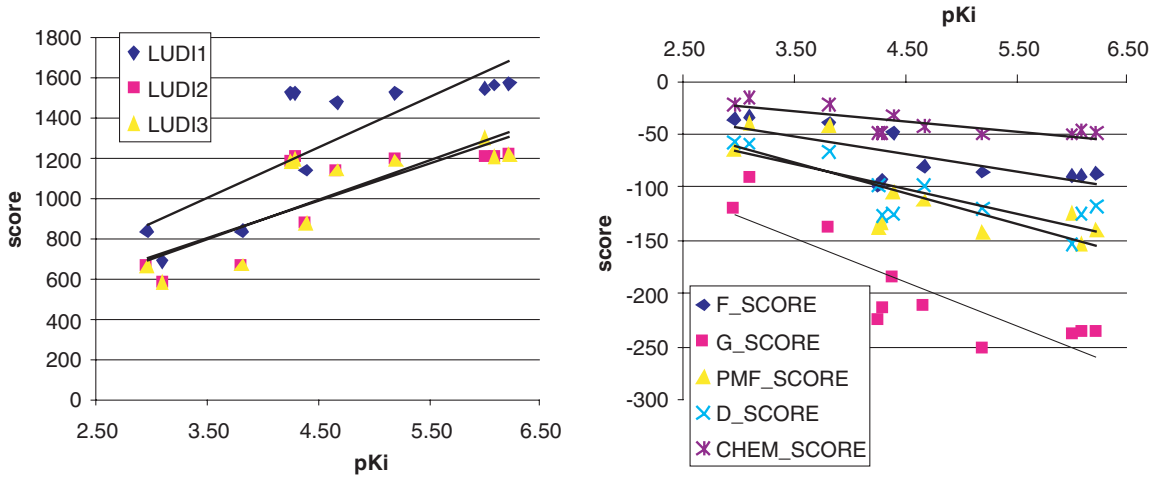

Figure 6. Correlations between various scores and pK_i for compounds **6**, **9**–**11** and phosphorylated compounds **1**–**5**, **7**, and **8**.

Table 1. R^2 values for correlations dependences between different scores and experimental pK_i for selected inhibitor–enzyme complexes

Function	R^2 for non-phosphorylated inhibitors (without one outlier)	R^2 for phosphorylated inhibitors (without one outlier)
LUDI 1	0.50 (0.65)	0.67 (0.76)
LUDI 2	0.49 (0.58)	0.66 (0.75)
LUDI 3	0.46 (0.57)	0.69 (0.77)
D_SCORE	0.07 (0.29)	0.67 (0.76)
PMF_SCORE	0.45 (0.64)	0.63 (0.72)
G_SCORE	0.66 (0.79)	0.74 (0.81)
CHEM_SCORE	0.36 (0.48)	0.65 (0.74)
F_SCORE	0.49 (0.71)	0.53 (0.69)
Mean value	0.43 (0.59)	0.65 (0.75)

2-P, respectively) have very similar electrostatic potential distribution. Comparison of electrostatic potential on surfaces of compounds **1-P**, **2-P**, **5-P**, **7-P**, and **8-P** would lead to the conclusion that there are two important electronic features required for strong binding: negative potential on atoms respective to Oε1 atom of PPT and lack of negative potential in position of methyl group of PPT. This is well seen in the cases of compound **5**, which does not fulfill the first requirement and compounds **7** and **8**, which do not fulfill the second, features that correlate well with their lower inhibitory potency.

Additionally, electrostatic potential on molecular surface of transition state of enzymatic reaction was computed (Fig. 8). Significant similarity of this distribution with those found for compounds **1-P** and **2-P** confirms that **1** and **2** might be considered as optimal transition state analogues of this reaction. This finding strengthens the accuracy of proposition that PPT binds to the enzyme as conformer A since phosphorylation of PPT in conformation A in the active site yields (*R*,*R*) diastereoisomer, while in conformation B1 or B2 (*R*,*S*) diastereoisomer. Only (*R*,*R*) diastereoisomer (presented in Fig. 8) is electrostatically equivalent to transition state and therefore only conformation A seems to be correct.

3. Conclusions

Detailed picture of binding mode of potent organophosphorus herbicide, phosphinothricin, in the active site of glutamine synthetase was obtained by means of molecular modeling. Despite the fact that crystal structure of PPT-GS complex was solved, inhibitor–enzyme interactions were not fully determined. The results described herein represent the first detailed computer-assisted analysis of this problem—the molecular basis of PPT inhibition was explained by combining all experimental information available and computational results. All these data strongly support the thesis that structure A (Fig. 4a) is the actual conformation of PPT in GS active site. The most important factors confirming the correctness of the proposed enzyme–inhibitor complex structure are as follows: (a) stronger net of hydrogen bonds formed by this conformer; (b) much higher scores found for this one over other evaluated structures; (c) computed scores correlating satisfactorily with experimental kinetic data found for all studied compounds; (d) similarity of conformation A with the conformation of MetSox in GS active site; (e) location of one of the oxygen atoms near n2 metal ion thus allowing phosphorylation of PTT by ATP; (f) good accordance with ionization constants for PPT and γ-carboxylate of glutamate (Glu335) in physiological conditions; (g) finding that charge distribution of phosphorylated phosphinothricin is very close to that found for enzymatic reaction transition state. The interactions of phosphinic moiety with n2 metal ion and Arg368 are the most crucial for PPT binding. Thus, similarity of the phosphorylated phosphinothricin to the enzymatic reaction transition state is the key element of its high binding affinity. Moreover, the same mode of binding of methionine sulfoximine deriving from both experimental and computational data strongly supports our hypothesis.

Modeling of other selected inhibitor-GS complexes and their evaluation with several scoring functions gave good correlation between experimental data and computations, which once more confirms correctness of the used approach. Higher correlation for series of

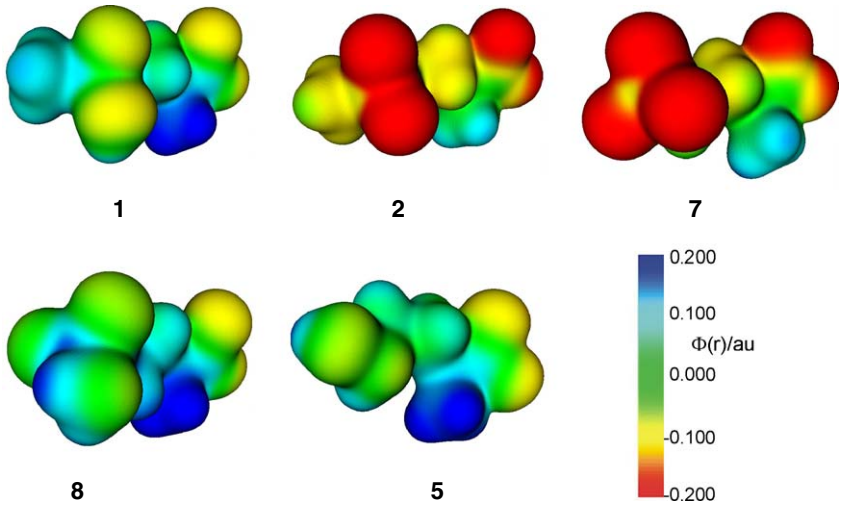

Figure 7. Electrostatic potential mapped onto electron charge density isosurfaces of selected inhibitors.

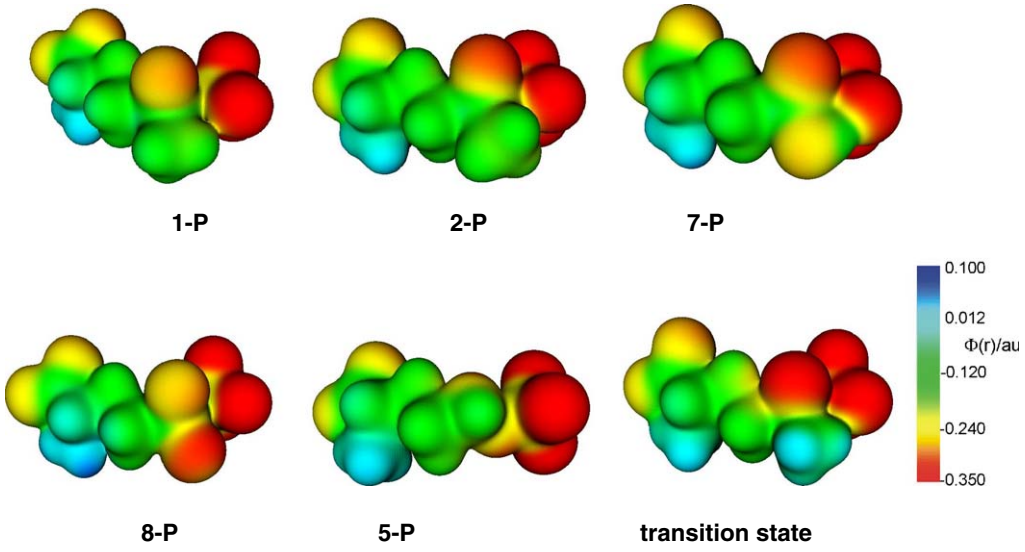

Figure 8. Electrostatic potential mapped onto electron charge density isosurfaces of selected phosphorylated inhibitors.

phosphorylated inhibitors suggests that they should be analyzed in this form whenever any experimental data suggest or prove phosphorylation. Thus, such an analysis demonstrates that the approach using simple scoring functions for examination of inhibitors reacting within the active site, gives reliable results at least in the case of glutamine synthetase and might be used for the evaluation of structures designed de novo.

Finally, required features of optimal GS inhibitor elucidated from this study might be defined as: (a) inhibitor has to be phosphorylated in the GS active site and (b) the phosphorylated form of the inhibitor has to be structurally and electronically similar to the transition state of this reaction. Furthermore, the results presented in this paper were successfully used for the computer-aided design of novel, potent inhibitors of GS,[55,56] and thus proved usefulness of this computational technique.

4. Experimental

Crystal structure of MetSox-P-GS (*M. tuberculosis*) complex[38] obtained from Protein Data Bank[57] (refcode 2BVC) was used as starting point for all calculations and all residue numbering is according to this structure. The hydrogen atoms were added using Insight 2000 program (Accelrys).[58] The protonation states of the amino acid side-chain residues were set up for pH 7.0. All calculations by means of molecular mechanics were done using Discover program with cff97 force field and conjugate gradient minimizer.[59] Minimizations were done up to energy change 0.02 kcal/mol. In order to obtain starting structures of analyzed inhibitor–enzyme complexes, MetSox-P in the MetSox-P-GS complex was modified using Builder module resulting in the desired inhibitor. The structure of inhibitor–enzyme complex was minimized in two steps. First, positions of hydrogen atoms were optimized and second, positions of all atoms of

active site residues and inhibitor were minimized. Minimized structures were scored using LUDI1, LUDI2, and LUDI3 functions from LUDI program[46–52] deriving from Insight package, whereas D_SCORE, PMF_SCORE, G_SCORE, CHEMSCORE, and F_SCORE functions were implemented in Sybyl program.[54]

Ab initio quantum chemical calculations of the electrostatic potential surfaces ($\Phi(r)$) and charge density isosurfaces ($\rho(r)$) were performed using Gaussian 03 program.[60] B3LYP method with 6-31++G(d, p) basis set was used. Used structures derived from molecular mechanics calculations of the inhibitor–enzyme complex. Graphics (Figs. 7 and 8) was prepared using script[61] in The Visualisation Toolkit,[62] by mapping electrostatic potential $\Phi(r)$ onto 0.01 e/a_0^3 charge density isosurfaces.

Acknowledgments

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