Mechanism of Activation of an Immunosuppressive Drug: Azathioprine. Quantum Chemical Study on the Reaction of Azathioprine with Cysteine

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Abstract: Azathioprine is an important drug used in the therapy of autoimmune disorders and in preventing graft rejection. Its molecule is composed of two main moieties: mercaptopurine and imidazole derivative. It is an immunosuppressive agent whose biological activity results from its in vivo mercaptolysis mediated by a nucleophilic attack on the C(5i) atom of imidazole ring of the azathioprine molecule. Solvation model SM5.4 with the PM3 Hamiltonian have been applied to model the reaction of azathioprine with cysteine. The employed quantum mechanical method shed new light on the mechanism of the reaction of azathioprine with cysteine in aqueous solution. The obtained results indicated that the first step in the reaction most likely involves the nucleophilic attack of the COO^- of cysteine on the C(5i) atom of the imidazole ring of azathioprine, followed by a subsequent intramolecular attack of the SH group of the cysteine residue. It was shown that biogenic thiols such as glutathione or cysteine facilitate the first and crucial step of azathioprine metabolism, due to the presence of COO^-, SH, and NH_3^+ groups in their molecules.

Introduction

Azathioprine, 6-(1-methyl-4-nitroimidazole-5-yl)thiopurine (see Figure 1), is an immunosuppressive agent,[1−3] which is widely used in clinical treatment of autoimmune disorders as well as in prevention of graft rejection or graft-versus-host disease in organ and tissue transplantation.[4−8] Azathioprine acts on several modes in cellular immunity processes. It inhibits lymphocyte activation,[9] lymphocyte differentiation,[10] in vitro lymphocyte stimulation,[11,12] and in vitro mixed lymphocyte reaction[13] and it reduces the activity of natural killer lymphocytes.[14,15]

The azathioprine molecule contains two moieties: (i) 6-mercaptopurine and (ii) an imidazole derivative.[1,16] It is metabolized to the purine antagonist 6-mercaptopurine and to 5-substituted 1-methyl-4-nitro-5-thioimidazoles or -aminoimidazoles.[17−19] It was shown that the immunosuppressive action of azathioprine depends on the synergistic cooperation of relatively weak cytostatic effect of low doses of 6-mercaptopurine and the chemosensitizing effect induced by highly reactive imidazole derivatives.[20−22] The first step in transformation of azathioprine to biologically active products is a nonenzymatic reaction mediated by a nucleophilic attack on the C(5i) atom of the imidazole ring. This reaction in vivo and in vitro depends on the presence of glutathione, cysteine, other thiols or possibly proteins.[23−28]

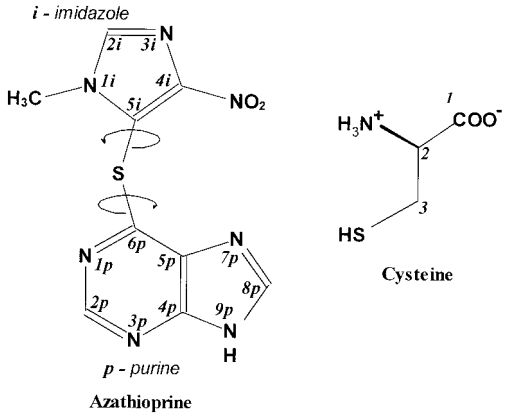

Figure 1. Formulas and numbering schemes for azathioprine and cysteine.

The importance of the cleavage of azathioprine, which is the first step in metabolism of this agent, stimulated our interest and prompted us to study the reaction of azathioprine with thiols and other nucleophiles by means of quantum chemistry. The gas-phase reaction of azathioprine with hydroxide anion was previously examined.[29] The results of ab initio and DFT calculations allowed us to propose the detailed mechanism of azathioprine's cleavage.[29] The OH^- attacks carbon C(5i) of the imidazole ring, so this carbon changes hybridization from sp^2 to sp^3. The intermediate is stabilized by the intramolecular hydrogen bond with the attacking OH^- as donor and azathio-

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(1) Elion, G. B.; Hitchings, G. H. Azathioprine. In *Handbook of Experimental Pharmacology*; Sartorelli, A. G., Johns, D. G., Eds.; Springer-Verlag: Berlin, 1975; Vol. 38, pp 404−425.
(2) Palace, J.; Rothwell, P. *Lancet* **1997**, *350*, 261.
(3) Elion, G. B. *Ann. N. Y. Acad. Sci.* **1993**, *685*, 401−407.
(4) Calne, R. Y.; Alexandre, G. P. J.; Murray J. E. *Ann. N. Y. Acad. Sci.* **1962**, *99*, 743−752.
(5) Murray, J. E.; Merrill, J. P.; Harrison, J. H.; Wilson, R. E.; Dammin, G. J. *New Eng. J. Med.* **1963**, *268*, 1315.
(6) Mukherjee, A. P. *Lancet* **1971**, *18*, 1350−1353.
(7) Alwall, N.; Halldorson, T.; Kjellstrand, C. M.; Lindholm., T.; Ahlstrom, C. G. *Acta Med. Scand.* **1972**, *192*, 455−463.

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prine's NO_2 group as the acceptor. However, ab initio as well as DFT methods indicated that isolated molecules of azathioprine and hydroxide anion are not stable. In the gas phase they spontaneously (without any activation energy) tend to convert into products: a thiopurine and an imidazole derivative.[29] In contrast, the experimental results showed that the alkaline hydrolysis of azathioprine in an aqueous solution of NaOH was a rather slow reaction of second order.[30] The conversion of azathioprine (concentration $= 5.87 \times 10^{-6}$ mol/L) at 37 °C in NaOH in 0.45 mol/L concentration was 8.4% after 0.5 h, 18.7% after 1 h, and 26.2% after 2 h.[28] In contrast, the reaction of azathioprine with biogenic thiols such as glutathione or cysteine was significantly faster.[27,28] The conversion of azathioprine (concentration $= 5.87 \times 10^{-6}$ mol/L) at 37 °C in pH 7.4 in the presence of cysteine in 1×10^{-3} mol/L concentration was 49.3% after 0.5 h, 74.9% after 1 h, and 94.4% after 2 h. Therefore, in this study, we focused on the reaction in aqueous solution with cysteine, as the main purpose of this work was to propose the mechanism of bioactivation of azathioprine.

Computational Methods

The solvation model SM5.4 with the PM3 Hamiltonian implemented in the AMSOL package[31] was employed to obtain geometries corresponding to substrates, products, and transition states and to asses their relative energies in the reaction of azathioprine with cysteine.

The Solvation Models (SMx) are semiempirical models which introduce into calculations the effects of solvents: water,[32−37] alkanes,[38,39] chloroform,[40] and other.[41] They were developed by Truhlar, Cramer, and their co-workers in the present decade.[42−45] In the SMx terms responsible for cavity formation, dispersion, solvent structure, and local field polarization are present.[36,46] The solvation energy is obtained via the usual approximation that solute treated at the quantum mechanical level is immersed in an isotropic, polarizable continuum representation of solvent. Therefore, the standard free energy of the solute in solution can be expressed as

$$G^0(\text{sol}) = G^0(\text{gas}) + \Delta G^0{}_S \tag{1}$$

where G^0(gas) is the gas-phase solute energy and $\Delta G^0{}_S$ is the free energy of solvation.

The solvation energy is written as

$$\Delta G^0{}_S = \Delta G_{ENP} + G_{CDS} \tag{2}$$

where G_{CDS}(cavitation−dispersion−solvent structure) is the contribution of the first solvation shell effects to the standard state free energy of

(8) Some recent examples: (a) Kahan, B. D. *Lancet* **2000**, *356*, 194−202. (b) Gleadle, J. M.; Mason, P. D. *Lancet* **2000**, *356*, 1768−1769. (c) Lewis, J. D.; Schwartz, J. S.; Lichtenstein, G. R. *Gastroenterology* **2000**, *118*, 1018−1024. (d) Kirschner, B. S. *Gastroenterology* **1998**, *115*, 813−821. (e) D'Haens, G.; Geboes, K.; Ponette, E.; Penninckx, F.; Rutgeerts, P. *Gastroenterology* **1997**, *112*, 1475−1481. (f) Neurath, M. F.; Wanitschke, R.; Peters, M.; Krummenauer, F.; Meyer Buschenfelde, K. H.; Schlaak, J. F. *Gut* **1999**, *44*, 625−628. (g) Thervet, E.; Anglicheau, D.; Toledano, N.; Houllier, A. M.; Noel, L. H.; Kreis, H.; Beaune, P.; Legendre, C. *J. Am. Soc. Nephrol*. **2001**, *12*, 170−176. (h) Wolfhagen, F. H.; van Hoogstraten, H. J.; van Buuren, H. R.; van Berge-Henegouwen, G. P.; Kate, F. J.; Hop, W. C.; van der Hoek, E. W.; Kerbert, M. J.; van Lijf, H. H.; Ouden, J. W.; Smit, A. M.; de Vries, R. A.; van Zanten, R. A.; Schalm, S. W. *J. Hepatol.* **1998**, *29*, 736−742. (i) Foster, B. J.; Bernard, C.; Drummond, K. N.; Sharma, A. K. *J. Pediatr*. **2000**, *136*, 370−375. (j) van der Dorpel, M. A.; Ghanem, H.; Rischen-Vos, J.; Man Veld, A. J.; Jansen, H.; Weimar, W. *Kidney Int.* **1997**, *51*, 1608−1612. (k) Mandler, R. N.; Ahmed, W.; Dencoff, J. E. *Neurology* **1998**, *51*, 1219−1220. (l) Palace, J.; Newsom-Davis, J.; Lecky, B. *Neurology* **1998**, *50*, 1778−1783. (m) Hoffmeyer, F.; Hoeper, M. M.; Spiekerkotter, E.; Harringer, W.; Haverich, A.; Fabel, H.; Niedermeyer, J. *Transplantation* **2000**, *70*, 522−525. (n) Opelz, G.; Dohler, B. *Transplantation* **2000**, *69*, 818−821. (o) Sandrini, S.; Maiorca, R.; Scolari, F.; Cancarini, G.; Setti, G.; Gaggia, P.; Cristinelli, L.; Zubani, R.; Bonardelli, S.; Maffeis, R.; Portolani, N.; Nodari, F.; Giulini, S. M. *Transplantation* **2000**, *69*, 1861−1867. (p) MacPhee, I. A.; Bradley, J. A.; Briggs, J. D.; Junor, B. J.; MacPherson, S. G.; McMillan, M. A.; Rodger, R. S.; Watson, M. A. *Transplantation* **1998**, *66*, 1186−1192. (q) Bergan, S.; Rugstad, H. E.; Bentdal, O.; Sodal, G.; Hartmann, A.; Leivestad, T.; Stokke, O. *Transplantation* **1998**, *66*, 334−339. (r) Amenabar, J. J.; Gomez-Ullate, P.; Garcia-Lopez, F. J.; Aurrecoechea, B.; Garcia-Erauzkin, G.; Lampreabe, I. *Transplantation* **1998**, *65*, 653−661. (s) Nicod, L. P. *Drugs* **1998**, *55*, 555−562. (t) Mccurry, K. R.; Parker, W.; Cotterell, A. H.; Weidner, B. C.; Lin, S. S.; Daniels, L. J.; Holzknecht, Z. E.; Byrne, G. W.; Diamond, L. E.; Logan, J. S.; Platt, J. L. *Hum. Immunol*. **1997**, *58*, 91−105. (u) Anstey, A.; Lear, J. T. *Biodrugs* **1998**, *9*, 33. (v) Shenoy, M.; Baron, S.; Wu, B.; Goluszko, E.; Christadoss, P. *J. Immunol*. **1995**, *154*, 6203−6208.

(9) Drela, N.; Sinicka, E. *Arch. Immunol. Ther. Exp*. **1986**, *34*, 209−215.

(10) Gorski, A.; Korszak-Kowalska, A.; Nowaczyk, A.; Paczek, G.; Gaciong, L. *Immunopharmacology* **1983**, *6*, 259−266.

(11) Bähr, V.; Ullmann, U. *Eur. J. Clin. Microbiol*. **1983**, *2*, 568−570.

(12) Cook, J. D.; Lai, W.; McGrane, B. *Immunopharmacology* **1983**, *5*, 257−275.

(13) Sheldon, P. W.; Stratford, I. J. *Brit. J. Cancer* **1984**, *49*, 571−577.

(14) Duclos, H.; Maillot, M. C.; Galanaud, P. *Immunology* **1982**, *46*, 595−601.

(15) Prince, H. E.; Ettenger, R. B.; Dorey, F. J.; Fine, R. N.; Fahey, J. L. *J. Clin. Immunol*. **1984**, *4*, 312−318.

(16) Elion, G. B. *Science* **1989**, *244*, 41−47.

(17) Elion, G. B.; Callahan, S. W.; Bieber, S.; Hitchings, G. H.; Rundles, R. W. *Cancer Chemother. Rep*. **1961**, *14*, 93.

(18) Chalmers, A. H.; Knight, P. R.; Atkinson, M. R. *Aust. J. Exp. Biol. Med. Sci.* **1967**, *45*, 681−691.

(19) van Scoik, K. G.; Johnson, C. A.; Porter, W. R. *Drug Metab. Rev.* **1985**, *16*, 157−174.

(20) Sauer, H.; Hantke, U.; Wilmanns, W. *Arzneim.-Forsch./Drug Res*. **1988**, *38*, 820−824.

(21) Hemmens, V. J.; Moore, D. E. *Photochem. Photobiol.* **1986**, *43*, 257−262.

(22) Crawford, D. J. K.; Maddocks, J. L.; Jones, D. N.; Szawlowski, P. *J. Med. Chem*. **1996**, *39*, 2690−2695.

(23) de Miranda, P.; Beacham, L. M., III; Creagh, T. H.; Elion, G. B. *J. Pharmacol. Exp. Ther*. **1973**, *187*, 588−560.

(24) Chalmers, A. H. *Biochem. Pharmacol*. **1974**, *23*, 1891−1901.

(25) Ding, T. L.; Benet, L. Z. *J. Chromatogr.* **1979**, *163*, 281−288.

(26) Chrzanowska, M.; Hermann, T.; Gapinska, M. *Pol. J. Pharmacol. Pharm.* **1985**, *37*, 701−708.

(27) Chrzanowska, M.; Hermann, T. *Chem. Anal*. **1990**, *35*, 653−661.

(28) Chrzanowska, M.; Muszalska, I. *Chem. Anal*. **1996**, *41*, 103−111.

(29) Hoffmann, M.; Rychlewski, J. In *Quantum Systems in Chemistry and Physics: Advanced Problems and Complex Systems*; Hernandez-Laguna, A., et al., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; pp 233−240.

(30) Hermann, T.; Szajdak, L. *Chem, Anal*. **1984**, *29*, 429−434.

(31) Hawkins, G. D.; Giesen, D. J.; Lynch, G. C.; Chambers, C. C.; Rossi, I.; Storer, J. W.; Li, J.; Rinaldi, T. Z. D.; Liotard, D. A.; Cramer, C. J.; Truhlar D. G. AMSOL version 6.5.3, 1998. Based in part on AMPAC, version 2.1, by Liotard, D. A.; Healy, E. F.; Ruiz, J. M.; Dewar, M. J. S. and on EF by Jensen, F., University of Minnesota.

(32) Chambers, C. C.; Hawkins, G. D.; Cramer C. J.; Truhlar, D. G. *J. Phys. Chem.* **1996**, *100*, 16385−16398.

(33) Cramer, C. J.; Truhlar, D. G. *J. Am. Chem. Soc*. **1991**, *113*, 8305−8311.

(34) Cramer, C. J.; Truhlar, D. G. *Science* **1992**, *256*, 213−217.

(35) Cramer, C. J.; Truhler, D. G. J. *Comput. Chem.* **1991**, *13*, 1089−1097.

(36) Cramer, C. J.; Truhler, D. G. J. *Comput.-Aided Mol. Design* **1992**, *6*, 629−666.

(37) Hawkins, G. D.; Cramer, C. J.; Truhlar, D. G. *J. Phys Chem.* **1996**, *100*, 19824−19839.

(38) Giesen, D. J.; Storer, J. W.; Cramer, C. J.; Truhlar, D. G. *J. Am. Chem. Soc*. **1995**, *117*, 1057−1068.

(39) Giesen, D. J.; Cramer, C. J.; Truhlar, D. G. *J. Phys. Chem.* **1995**, *99*, 7137−7146.

(40) Giesen, D. J.; Chambers, C. C.; Cramer, C. J.; Truhlar D. G. *J. Phys. Chem.* **1997**, *101*, 2061−2069.

(41) Giesen, D. J.; Gu, M. Z.; Cramer, C. J.; Truhlar D. G. *J. Org. Chem.* **1996**, *61*, 8720−8721.

(42) Storer, J. W.; Giesen, D. J.; Cramer, C. J.; Truhlar, D. G. *J. Comput.-Aided Mol. Design* **1995**, *9*, 87−110.

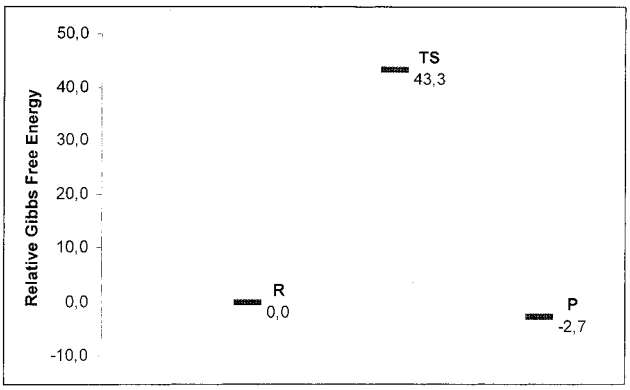
(43) Cramer, C. J.; Truhler, D. G. *J. Chem. Soc., Perkin Trans.* **1994**, *90*, 1799−1810.

(44) Giesen, D. J.; Cramer, C. J.; Truhlar, D. G. *J. Phys. Chem.* **1994**, *98*, 4141−4147.

(45) Liotard, D. A.; Hawkins, G. D.; Lynch, G. C.; Cramer, C. J.; Truhlar, D. G. *J. Comput. Chem.* **1995**, *16*, 422−440.

(46) Richards, N. G. J. In *Molecular Orbital Calculations for Biological Systems*; Sapse, A.-M., Ed.; Oxford University Press: New York, 1998; pp 11−48.

Chart 1. Relative Gibbs Free Energies (in kcal/mol) of Reactants (**R**), Transition State (**TS**), and Products (**P**) in Aqueous Solution for Case I: The SH Group of Cysteine Attacks the Imidazole Ring of Azathioprine



transfer and ΔG_{ENP}(electronic−nuclear−polarization) includes the change in the electronic and nuclear internal energy of the solute and the electronic polarization free energy of the solute−solvent system upon insertion of the solute into the solvent. Further, the first solvation shell term is expressed as:

$$G_{CDS} = \sum_k \sigma_k A_k \tag{3}$$

so that the assumption of the proportionality of solvent accessible surface and cavity energy is explicitly shown. In this equation σ_k is the atomic surface tension of atom k, and A_k, which is the solvent accessible surface area of atom k, is a function that depends on the van der Waals radius for the kth atom and the radius of the sphere encompassing an explicit solvent molecule. Obviously, solvent accessible area does not include regions that overlap with the cognate spheres computed for all other atoms surrounding the kth atom, and must therefore be reevaluated during each geometry optimization step to reflect changes in the molecular conformation.

The ΔG_{ENP} term, called the electrostatic term, can be written as

$$\Delta G_{ENP} = \Delta E_{EN} + G_P \tag{4}$$

where G_P represents the polarization free energy, and ΔE_{EN} is the change in the solute's internal free energy upon insertion in solution, approximated, as usual, as the change in the sum of electronic total energy and nuclear repulsion energy of the solute in going from the gas phase to solution.

Table 1. The Relative Energies (in kcal/mol) of Reactants (**R**), Transition State (**TS**), and Products (**P**) in the Gas Phase and in Aqueous Solution for Case I: The SH Group of Cysteine Attacks the Imidazole Ring of Azathioprine

	reactants (**R**)	transition state (**TS**)	products (**P**)
ΔE(gas)	0.0	38.4	−4.5
relative zero point correction	0.0	0.4	−0.1
relative thermal correction to Gibbs free energy	0.0	2.8	−0.8
ΔG(reaction in gas phase)	0.0	41.2	−5.3
ΔG(solvation)	−46.6	−44.5	−44.1
ΔG(reaction in solution)	0.0	43.3	−2.7

The polarization free energy of a molecule insertion in a medium of dielectric constant ϵ is expressed in the generalized Born approximation for a multicentered system as

$$G_P = -\frac{1}{2}\left(1 - \frac{1}{\epsilon}\right)\sum_{k,k'} q_k q_{k'} \gamma_{kk'} \tag{5}$$

where k and k' are atomic centers, q_k is the partial charge on atom k, and $\gamma_{kk'}$ is a Coulomb integral. The Coulomb integrals $\gamma_{kk'}$ ($k \neq k'$) are modeled such that they reduce to the self-Coulomb integrals $\gamma_{kk'}$ when the distance $R_{kk'}$ between the identical atom tends to zero, and they are asymptotic to $1/R_{kk'}$, where $R_{kk'}$ is the interatomic distance between k and k', at large distances. The expression for Coulomb integral $\gamma_{kk'}$ is

$$\gamma_{kk'} = \frac{1}{\sqrt{\left[R_{kk'}^2 + \alpha_k \alpha_{k'}\left(\exp\left(\frac{-R_{kk'}^2}{d_{kk'}\alpha_k\alpha_{k'}}\right) + C_{kk'}\right)\right]}} \tag{6}$$

where a_k is an effective atomic radius, and $d_{kk'}$ is an empirically optimized constant. The $C_{kk'}$ was introduced in the SM1−SM3 models to empirically correct for certain trends in experimental data. However, in the SM5 family of solvation models, $C_{kk'}$ is equal to zero.

In this work we were using the SM5.4 aqueous model,[32] in which the solute Hamiltonian is modeled using NDDO molecular orbital theory (AM1 or PM3) with class IV atomic charges from the CM1A and CM1P charge models.[42] In these CM1A and CM1P charge models,

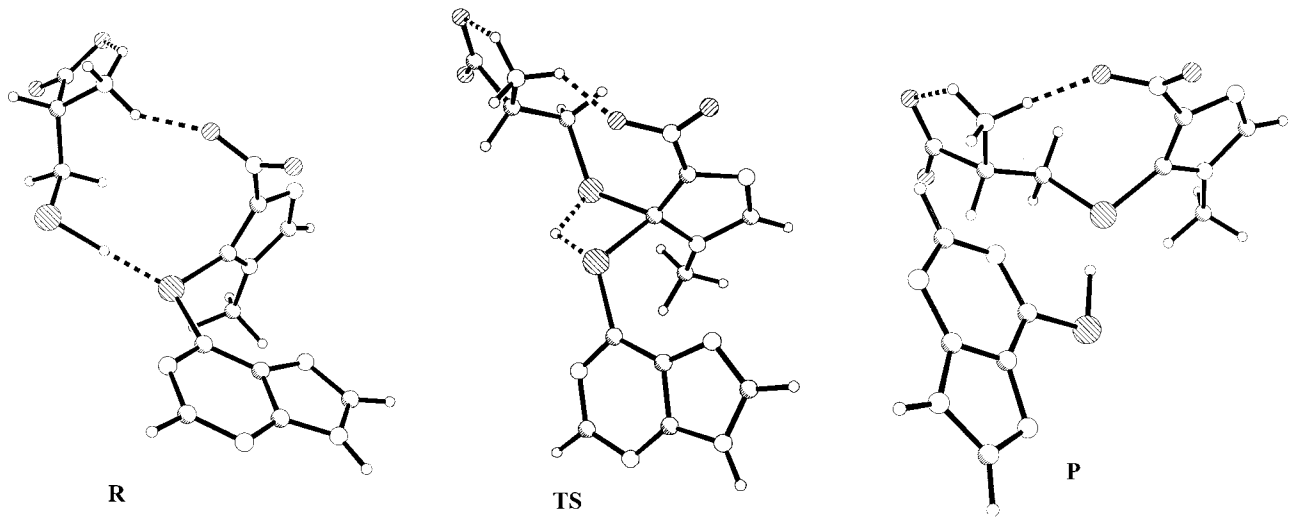
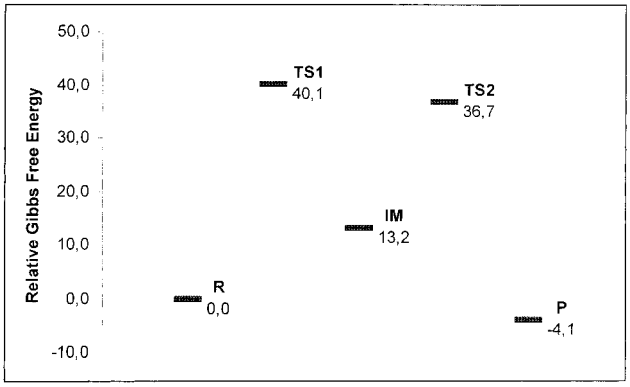


Figure 2. Perspective view on reactants (**R**), transition state (**TS**), and products (**P**) of the reaction of azathioprine with cysteine: attack of the SH group of cysteine. In the transition state both sulfur atoms are linked to the C(5i) carbon atom of the imidazole ring of azathioprine. The hydrogen atom initially bound in the SH group of cysteine is almost equidistant to the sulfur atoms of cysteine and thiopurine. As the reaction proceeds the C(5i)−S(cysteine) distance decreases, whereas the C(5i)−S(thiopurine) bond length increases. Parallelly, the H−S(cysteine) bond becomes longer, whereas the H−S(thiopurine) distance decreases. The transition state is stabilized by the hydrogen bond with NH_3^+ as a donor and O_2N as an acceptor.

Chart 2. Relative Gibbs Free Energies (in kcal/mol) of Reactants (**R**), Transition States (**TS1**) and (**TS2**), Intermediate (**IM**) and Products (**P**) in Aueous Solution for Case II: The COO− Group of Cysteine Attacks First the Imidazole Ring of Azathioprine



charges are obtained from one electron density matrix by a semiempirical linear mapping. These charges have two major advantages. First, they make up for errors intrinsic to replacing a continuous charge distribution by a set of distributed point charges because the mapping from which they are obtained is chosen to minimize errors in the physical observables predicted from point charges. Second, they make up for deficiencies in the semiempirical wave function from which they are obtained because the parametrizations are chosen to minimize deviations from experiment.

During recent years the solvation models have proven their usefulness in various types of calculations.[47] Thus, we have decided to utilize the SM5.4 method to examine the reaction of azathioprine cysteine in aqueous solution. All calculations were performed with the AMSOL6.5.3 package[31] on a Cray J916 in the Poznan Supercomputing and Networking Center.

Table 2. The Relative Energies (in kcal/mol) of Reactants (**R**), Transition States (**TS1**, and **TS2**), and Intermediate (**IM**), as Well as Products (**P**) in the Gas Phase and in Aqueous Solution, for Case II: The COO^- Group of Cysteine Attacks First the Imidazole Ring of Azathioprine

	R	TS1	IM	TS2	P
ΔE(gas)	0.0	37.5	0.2	32.7	−0.1
relative zero point correction	0.0	−1.2	1.2	0.7	0.7
relative thermal correction to Gibbs free energy	0.0	3.3	5.6	5.7	1.4
ΔG(reaction in gas phase)	0.0	40.8	5.8	38.5	1.3
ΔG(solvation)	−38.7	−39.4	−31.3	−40.5	−44.1
ΔG(reaction in solution)	0.0	40.1	13.2	36.7	−4.1

Results and Discussion

Earlier ab initio and DFT calculations for the reaction of the isolated molecules of azathioprine and OH^- anion[29] as well as experimental data[17,30] demonstrated that the hydrolysis of azathioprine is mediated by nucleophilic attack on the C(5i) carbon atom of the imidazole ring. (See Figure 1.) The experimental data showed that reaction of azathioprine with a strong nucleophilic agent (OH^- anion) is slower[28,30] (26.2% converion of azathioprine after 2 h in 37 °C, OH^- concentration = 0.45 mol/L) than reaction with thiols which naturally occur in blood, that is with glutathione or cysteine (94.4% conversion

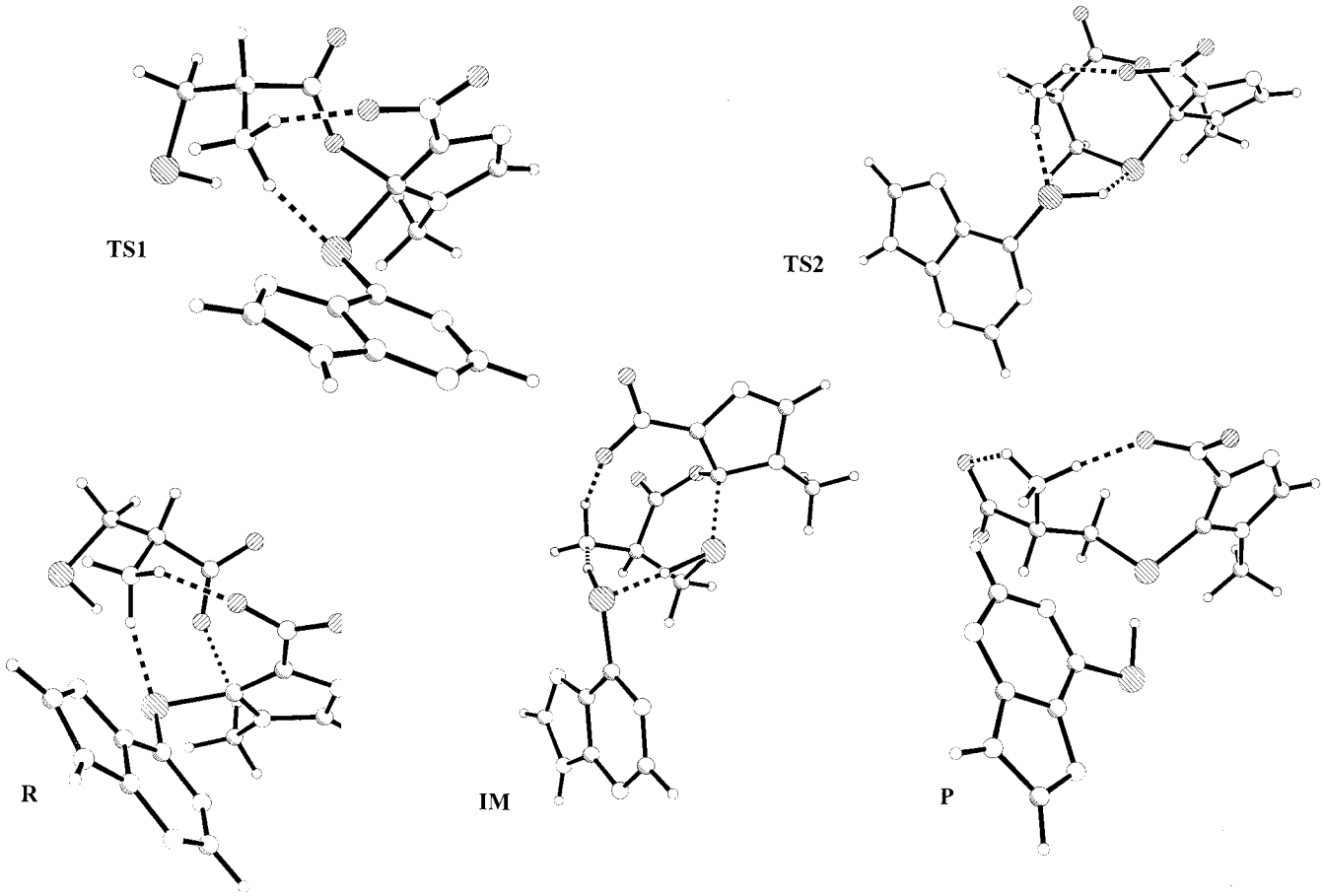


Figure 3. Perspective view on reactants (**R**), transition states (**TS1** and **TS2**), intermediate (**IM**), and products (**P**) of the reaction of azathioprine with cysteine: initial attack of the COO^- group of cysteine. The first transition state is stabilized by hydrogen bonds $NH_3^+\cdots O_2N$ and $NH_3^+\cdots$ S(thiopurine). The C(5i) carbon atom of the imidazole ring is in sp^3 hybridization in both the transition states and intermediate. In the second transition state of the COO^- group is leaving the imidazole ring, while SH group of cysteine attacks the C(5i) carbon atom.

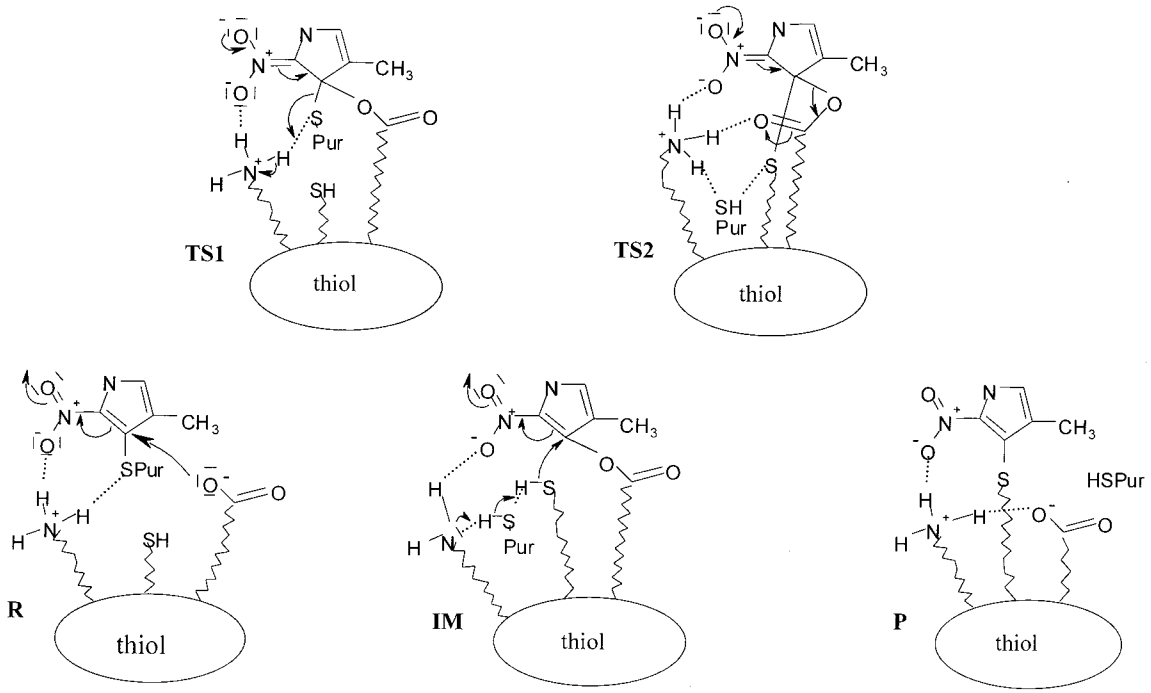


Figure 4. Mechanism proposed for bioactivation of azathioprine by biogenic thiols such as cysteine or glutathione.

of azathioprine after 2 h in 37 °C, cysteine concentration = 1×10^{-3} mol/L).[28] These thiols, in physiological pH, possess both SH and COO^- moieties, which may mediate a nucleophilic attack on the azathioprine molecule.

Reaction of Azathioprine with Cysteine. (a) Nucleophilic Attack of the SH group of Cysteine. The results of calculations for the reaction of azathioprine with cysteine in aqueous solution in which the SH group of cysteine attacks the azathioprine molecule are presented in Chart 1. This chart presents Gibbs free energy of reactants, transition state, and products plotted against reaction coordinate. Gibbs free energy in solution was calculated as a sum of energy of isolated molecules, zero point energy, rovibrational and thermal correction to Gibbs free energy calculated in the gas phase, and Gibbs free energy of solvation calculated with the SM5.4 method. The relative energies of reactants, transition state, and products are presented in Table 1.

The results of the SM5.4 calculation show that in aqueous solution the reaction of thiolysis of azathioprine by cysteine is slightly exoenergetic. The products are of almost 3 kcal/mol lower energy than the reactants. The energy of activation is high, over 43 kcal/mol. The solvation effects favor slightly reactants over products and transition state. Figure 2 presents structures of reactants, transition state, and products. In the transition state the SH group of cysteine attacks the imidazole ring of azathioprine, so that the attacked carbon atom is in sp^3 hybridization. The NH_3^+ group of cysteine is attracted by the NO_2 group of azathioprine (which bears negative charge). In the transition state the proton from the SH group is in the middle of transfer from the sulfur of cysteine to the sulfur of thiopurine. All in all in the reaction of thiolysis the carbon atom of the imidazole ring of the azathioprine molecule is attacked by the SH group of cysteine. This leads to a transition state in which the C(5i) atom of the imidazole ring is in sp^3 hybridization and electron pairs are being shifted toward the NO_2 group. The negative charge of the NO_2 group is stabilized due to attraction of the NH_3^+ group of the attacking cysteine. Moreover, as the reaction proceeds, the proton from the attacking SH group is being transferred to the sulfur atom of thiopurine.

(b) Nucleophilic Attack of COO^- group of Cysteine. Chart 2 presents relative Gibbs free energies of reactants, transition states, intermediate, and products of the reaction of azathioprine with cysteine initiated by nuclephilic attack of the carboxylic group of cysteine. The relative energies of reactants, transition states, and products are presented in Table 2, whereas Figure 3 shows perspetive views on these structures.

The first transition state is stabilized by a net of hydrogen bonds: NH···ON, NH···SC, SH···S C, which lower the energy of activation barrier from over 43 kcal/mol for SH attack to almost 40 kcal/mol for COO^- attack. Further, in the reaction, the thiopurine moiety separates from the active complex TS1, and the reaction proceeds toward the intermediate (**IM**). In this form, the cysteine moiety is bent such that its SH group is about to attack the C(5i) atom of the imidazole ring, to which this cysteine's OOC moiety is attached. As may be expected, in transition state **TS2**, the tail-SH group of cysteine attacks intramolecularly, the head-C(5i) atom to which the COO group of cysteine is attached. Such a transition is facilitated by the presence of thiopurine. The sulfur atom of thiopurine mediates in proton transfer from the SH group of cysteine to its NH_2 group. Thus the energy of transition state **TS2** is roughly 37 kcal/mol in relation to reactants and the activation barrier from the intermediate (**IM**) is about 23.5 kcal/mol significantly less than the activation barrier of the **TS1**.

Conclusions

The undertaken quantum mechanical studies allowed us to shed new light on the crucial step of azathioprine transformation to precursors of biologically active compounds: thiopurine and imidazole derivative. The reaction of azathioprine with cysteine is facilitated by the presence of COO^-, SH, and NH_3^+ groups in the cysteine molecule. These groups are deeply involved in lowering the activation barrier of the reaction: stabilization of transition states and proton transfer. Thus, most likely the first

(47) Hoffmann, M.; Rychlewski, J. In *New Trends in Quantum Systems in Chemistry and Physics*; Maruani, J., et al., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; Vol. 2, pp 189−210.

step in the reaction involves the nucleophilic attack of COO^- of cysteine on the C(5i) atom of the imidazole ring of azathioprine. This step is followed by a subsequent nucleophilic attack of the SH group of cysteine on the C(5i) atom of the imidazole ring, which causes the COO^- group to leave. Thus, the cysteine acts in the reaction with azathioprine not only as a reactant but also as a catalyst of the reaction. Therefore, it can be concluded that biogenic thiols, glutathione or cysteine, facilitate the first and crucial step of azathioprine metabolism, due to the presence of COO^-, SH, and NH_3^+ groups in their molecules. The obtained results allow us to postulate the detailed general mechanism presented in Figure 4 for the reaction of azathioprine with biogenic thiols. The reactants form a supramolecular structure stabilized by two hydrogen bonds with a protonated amino group of a biogenic thiol as a donor and S and NO_2 groups of azathioprine as acceptors. Nucleophilic attack of the COO^- group of the biogenic thiol leads to a transition state (**TS1**) that gains stabilization from hydrogen bonding NH···O_2N and NH···SPur. The next step of the reaction involves proton transfer from the NH_3^+ group of the biogenic thiol to the SPur moiety and after the cleavage of the C(5i)−S bond results in the intermediate (**IM**). This structure is stabilized by the cooperative hydrogen bond network S(biogenic thiol)−H···S(thiopurine)−H···N(biogenic thiol)−H···O_2N. Then, the nucleophilic attack of the sulfur atom of the biogenic thiol takes place leading to the transition state (**TS2**). The **TS2** state is stabilized by hydrogen bonds: NH···SH···S, NH···O_2N, and NH···OOC. The following cleavage of the C(5i)−OOC bond leads to products of the reaction.

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