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The O to S substitution in urea brings inhibition activity against thiocyanate dehydrogenase

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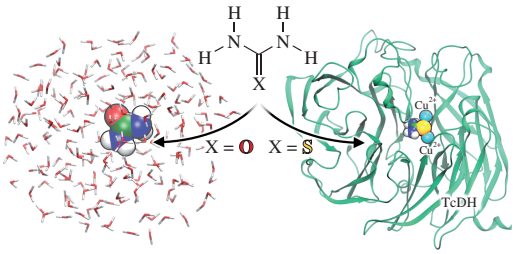
According to steady-state kinetic experiments, thiourea inhibits thiocyanate dehydrogenase TcDH, whereas urea does not. The QM/MM modeling combined with electron density analysis reveals the molecular mechanism of this process. For both compounds, interactions with bulk water molecules are similar, but upon binding to the active site of thiocyanate dehydrogenase a sulfur atom forms stronger coordination bonds with copper ions than an oxygen.



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Thiocyanate dehydrogenase or thiocyanate desulfurase (TcDH, EC 1.8.2.7), an enzyme recently isolated from the haloalkaliphilic sulfur-oxidizing *Thioalkalivibrio* bacteria, comprises a new type of a polynuclear catalytic center consisting of three copper ions.[1] It catalyzes a thiocyanate to cyanate conversion accompanied by the formation of elemental sulfur. This process occurs with the two-electron oxidation of the substrate resulting in temporary reduction of two of three active site copper ions from Cu^{2+} to Cu^{+}. Active site of this enzyme is quite small as it aims to accomodate a three-atom linear thiocyanate ion and a catalytic water molecule that initiates reaction. Preliminary studies demonstrated that among small inorganic ions composed of four or less atoms only cyanide and cyanate inhibit TcDH.[1] Up to now, all attempts to crystallize the TcDH with either of these molecules were unsuccessful. The available crystal structures carry only copper in the active site. Therefore, there is still a need to find other compounds that may act as competitive inhibitors and bind to the active site of TcDH. It is known that Cu^{2+} forms stable complexes with urea,[2,3] and sulfur analogs of the latter are even used as chemosensors to detect Cu^{2+}.[4]

Herein, we present a combined experimental and computational study of the TcDH inhibition by urea and thiourea. According to steady-state kinetic experiments, urea did not demonstrate any inhibitory activity, while thiourea did. We utilized molecular modeling to analyze equilibrium geometry configurations of the possible enzyme–inhibitor (EI) complexes and performed electron density analysis to evaluate the strength of interactions between urea/thiourea and the active site of TcDH as well as water molecules in solution.

We started with the experimental studies by determining inhibition potency of urea and thiourea.[†] Urea does not inhibit TcDH at concentrations up to 100 mM, while thiourea inhibits TcDH with the IC_{50} = 1 mM [Figure 1(*a*)]. We examined inhibition mode by analyzing dependencies of the reaction rate on substrate concentration at different concentrations of the inhibitor (thiourea) and plotting them in the Lineweaver–Burk and Dixon coordinates [Figure 1(*b*)–(*d*)]. The inhibition was found to be mixed, *i.e.* an inhibitor can be bound to both free enzyme and the enzyme-substrate complex. Linearization in Dixon coordinates, (v^{-1} *vs.* [I]) and ([S] v^{-1} *vs.* [I]) [Figure 1(*c*),(*d*)], allowed us to determine the inhibition constants corresponding to both competitive and uncompetitive mechanisms. Thiourea is preferably bound to the free enzyme (K_i = 0.6 mM), while

[†] *Experimental setup.* Experimental studies were performed with the TcDH enzyme that was prepared and purified as in ref. 1. The 100 μM TcDH activation was performed in the 25 mM borate buffer at pH 9.5 with the 3-fold excess of $CuCl_2$ at 4 °C for 12 h. The unbound Cu^{2+} ions were removed using dialysis against 25 mM borate buffer, pH 9.5. Enzyme activity in the thiocyanate oxidation reaction was measured on a Cary 100 spectrophotometer (Agilent Technologies). Cytochrome C from horse heart (Sigma, USA) (ε_{550} = 22500 M^{-1} cm^{-1}) was utilized as an electron acceptor in the corresponding saturating concentration. All experiments were performed at pH optimum 9.5 and 30 °C.[1] The reaction rates were measured in the following mixture: SCN^- (1 mM), cytochrome C (50 μM) and TcDH (10 nM) in borate buffer (25 mM) at pH 9.5. The reaction was initiated by addition of the enzyme. All kinetic measurements were performed in duplicate. Activity was evaluated as the amount of reduced electron acceptor (μM) per 1 min and 1 mg of the enzyme. Initial SCN^- concentrations were varied to obtain kinetic parameters. A nonlinear regression analysis according to the Michaelis–Menthen steady-state kinetic scheme was performed. The IC_{50} (half maximal inhibitory concentration) was measured for the selected potential inhibitors, urea and thiourea. Reaction conditions for the inhibition potency measurements were the same as discussed above with varying inhibitor concentration. The enzymatic activity against inhibitor concentration was plotted and the inflection point on the graph corresponded to the IC_{50} value. These calculations were performed using 'Quest Graph™ IC_{50} Calculator' (AAT Bioquest). The inhibition mechanism was determined according to the kinetic analysis in the Lineweaver–Burk coordinates (v^{-1} *vs.* $[S]^{-1}$). The inhibition constants, K_i and K_i', were determined according to the Dixon method; the (v^{-1} *vs.* [I]) and ([S] v^{-1} *vs.* [I]) dependencies were plotted at different substrate concentrations and the intersection point provides the K_i and K_i' values.

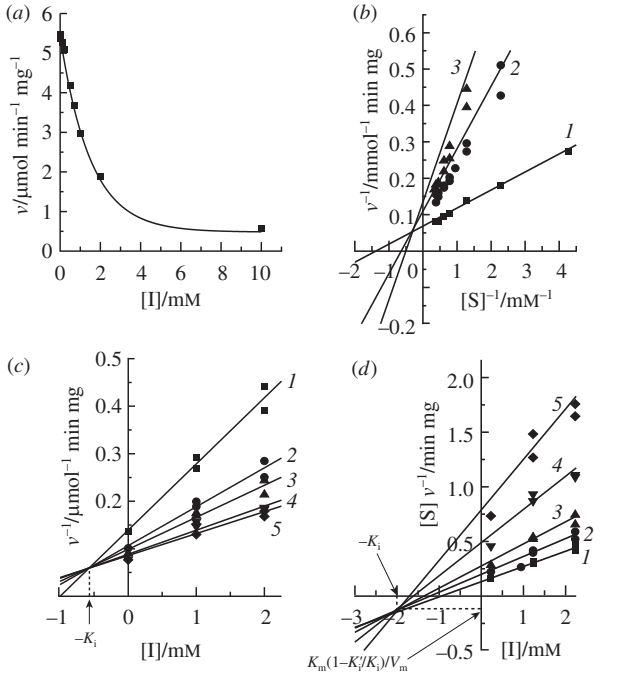

Figure 1 (*a*) The dependence of TcDH activity in the reaction of thiocyanate oxidation on thiourea concentration (substrate concentration is 1 mM). (*b*) Lineweaver–Burk plot of TcDH activity on the substrate concentration (v^{-1} *vs.* $[S]^{-1}$) at different thiourea concentrations (mM): (*1*) 0, (*2*) 1 and (*3*) 2. Dixon plots of the TcDH activity on the thiourea concentration (*c*) (v^{-1} *vs.* [I]) and (*d*) ([S] v^{-1} *vs.* [I]) at different substrate concentrations (mM): (*1*) *1*, (*2*) 2, (*3*) 3, (*4*) 6 and (*5*) 10.

binding to the ES complex is about 3 times weaker (K_i^i = 2 mM). Both values are approximately the same as the Michaelis constant for thiocyanate (1 mM).[1]

This stimulated us to study molecular mechanism that underlies different inhibition potencies of urea and thiourea as these compounds differ in only one atom.

Binding efficiency is determined by lowering the standard Gibbs free energy of the system upon formation of the EI complex compared with the enzyme (E) and inhibitor (I) in the unbound states in bulk solution. This can be written as the following equation:

$$E_{aq.} + I_{aq.} = EI_{aq.}$$

The standard Gibbs free energy $\Delta_r G^o$ of complex formation can be written as

$$\Delta_r G^o = \mu^{o*}(EI) - \mu^{o*}(E) - \mu^{o*}(I),$$

where asterisk denotes that asymmetrical reference system was chosen to describe a thermodynamic properties of solution, *i.e.* the infinitely diluted solution of E, I or EI in water. The standard chemical potential can be written *via* partial molar properties, standard enthalpy ($\bar{H}^o_i$) and entropy ($\bar{S}^o_i$) of the *i*-th component at the selected temperature *T*:

$$\mu_i^{o*} = \bar{H}^o_i - T\bar{S}^o_i$$

The standard Gibbs free energy of complex formation is then

$$\Delta_r G^o = \bar{H}^o(EI) - T\bar{S}^o_i(EI) - [\bar{H}^o(E) - T\bar{S}^o_i(E) + \bar{H}^o(I) - T\bar{S}^o_i(I)].$$

To understand the origin of different binding affinity of urea and thiourea, we compared this equation written for both compounds. We assume that the entropic contributions $\bar{S}^o(EI)$ and $\bar{S}^o(I)$ are similar for both urea and thiourea as they differ only in one atom. Then the difference of compound binding is due to the value of $\bar{H}^o(EI) - \bar{H}^o(I)$. This quantity depends on the interactions of the compound with the solvent molecules and with the active site of TcDH. We can reformulate the problem as follows. The EI complex stability increases with the increase of differences between the strength of interactions in EI and interactions of the compound of interest and water molecules.

A reliable approach to quantify the strength of interactions is the QTAIM theory.[5] It is widely used in both gas and condensed phases for calculated and experimentally determined electron densities.[6–10] The idea is to study the topology of electron density of the system and find its critical points over spatial coordinates. Among them, there are bond critical points (BCPs) that lie on bond paths connecting the interacting atoms. The electron density features at BCPs characterize these interactions. Here we use value of the electron density ρ as a simple criterion to quantify the interaction strength. The larger is ρ at BCP, the more pronounced (or stronger) is the interatomic interaction. This criterion was successfully utilized earlier to explain the experimentally observed selectivity of calix[4]arenes to the certain alkali metal cations.[11]

We obtained four model systems.‡ Two of them are the EI complexes with urea (EU) and thiourea (ET) and other two ones are the same compounds in bulk water. Figure 2 demonstrates parts of TcDH active site that include thiourea, T, [Figure 2(*a*)] or urea, U, [Figure 2(*b*)] and the adjacent groups. The sulfur atom forms coordination bonds with both Cu^{2+}(2) and Cu^{2+}(3), whereas oxygen atom interacts with only one copper cation, Cu^{2+}(3). Such location of the thiourea sulfur atom is similar to that of

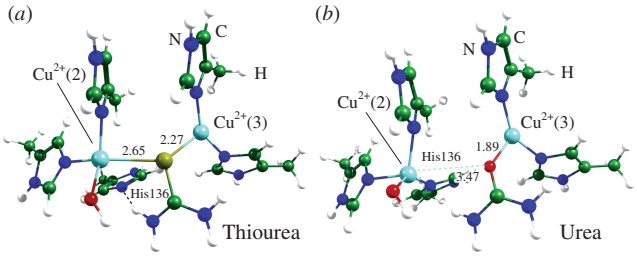

Figure 2 (*a*) Thiourea and (*b*) urea in the active site of TcDH. A hydrogen bond is shown by black dashed line. Distances are shown in Å.

‡ *Computational protocol.* The model systems of the enzyme–inhibitor complexes were derived from the ES complex of the TcDH with the thiocyanate from ref. 1. The substrate molecule was substituted by urea or thiourea to obtain the equilibrium geometry configurations of the EI complexes. The combined quantum mechanics/molecular mechanics method was utilized with the description of the QM subsystem at the PBE0/6-31G**[13] level and MM subsystem with the full-atom force fields, AMBER[14] for protein and TIP3P[15] for water molecules. The QM subsystem comprised an inhibitor, urea or thiourea, three Cu^{2+} ions, and their coordination shells including His135, His206, Asp314, His381, His437, His482, and His528, the catalytic His136 and Glu288, other residues that form hydrogen bonds in the active site (Lys103, Gln156, Asp529, Arg544), and water molecules, similarly to that in ref. 1. To model interactions of the selected compound with bulk water, we prepared the model system composed of urea/thiourea and 199 water molecules. Eight of them that form hydrogen bonds with the solute together with a molecule of urea/thiourea were treated at the QM(PBE0/6-31G**) level and the rest of the system was described with the TIP3P force field parameters. All QM/MM calculations were performed using the NWChem program.[16] The electron densities at the stationary points were calculated taking into account contributions from the partial atomic charges of the MM subsystem to the one-electron part of the QM Hamiltonian. No restraints or constraints were imposed on the system. The convergence criteria were the following: 1.5×10^{-5}, 1×10^{-5}, 6×10^{-5} and 4×10^{-5} a.u. for maximum and RMS energy gradients and maximum and RMS Cartesian coordinate changes, respectively. Electron density analysis was performed in the Multiwfn program.[17] The coordination/hydrogen bonds energies between ligands and copper ions/water molecules are evaluated according to the Espinosa empirical equation: $E = 0.5\,V(r)$, where the $V(r)$ is a potential energy density calculated at the corresponding bond critical point.[12] PDB files with equilibrium geometry configurations of all considered model systems can be found at ZENODO: https://doi.org/10.5281/zenodo.4284073.

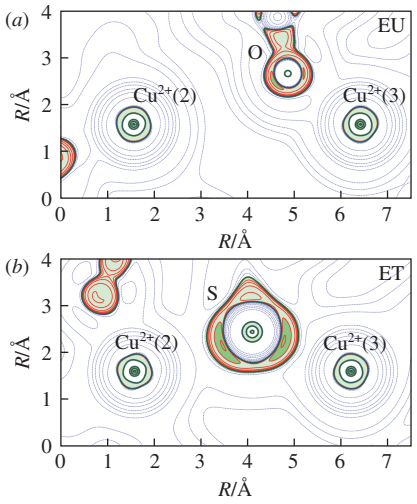

Figure 3 The 2D maps of Laplacian of the electron density, $\nabla^2\rho(\mathbf{r})$, in the plane of $Cu^{2+}(3)$, S/O and $Cu^{2+}(2)$ for (*a*) EU and (*b*) ET. Contour lines are $\pm(2;4;8)\times10^n$ a.u., $-2 \leqslant n \leqslant 1$, blue dashed contour lines indicate the electron density depletion areas $[\nabla^2\rho(\mathbf{r}) > 0]$ and red solid lines identify the electron density concentration $[\nabla^2\rho(\mathbf{r}) < 0]$, green solid line is $\nabla^2\rho(\mathbf{r}) = 0$. The area with $\nabla^2\rho(\mathbf{r}) < 0$ is colored in light green and the electron lone pairs on the oxygen atom in EU $[\nabla^2\rho(\mathbf{r}) < -4]$ and on the sulfur atom in ET $[\nabla^2\rho(\mathbf{r}) < -0.2]$ are highlighted green.

thiocyanate in the ES complex. However, in the ES complex the $Cu^{2+}(2)$–S bond is elongated (3.40 Å) compared with the ET complex. The coordination surrounding of $Cu^{2+}(2)$ is different in ET and EU. In ET complex, it has planar square configuration that is the most preferable for Cu^{2+} cation among those with the coordination number of four. In EU complex, the $Cu^{2+}(2)$ ligands form a less preferable tetrahedral coordination sphere. Also, formation of the S–$Cu^{2+}(2)$ coordination bond results in the conformational change of the His136 side chain and formation of the N(His136)–H(thiourea) hydrogen bond that additionally stabilizes the ET complex.

To further analyze interactions between copper cations and an oxygen (urea) or a sulfur (thiourea) atoms, we plotted 2D maps of the Laplacian of electron density in the plane of $Cu^{2+}(2)$–S/O–$Cu^{2+}(3)$ (Figure 3). In both complexes, the distances between copper ions are similar and equal to 4.6 Å in ET and 4.8 Å in EU. However, much larger size of sulfur atom facilitates formation of two coordination bonds. Both of them are formed due to the interactions of copper cations with the electron lone pairs of a sulfur atom.

Table 1 summarizes the data on the electron density values at bond critical points found for the interactions of urea/thiourea with the active site of TcDH [ρ_{prot}]. It is larger in ET complex compared with the EU complex. Both compounds form similar hydrogen bond networks in water solution and the overall interactions between them and water molecules are similar ($\rho_{aq.}$), being slightly weaker for the thiourea. The $\Delta\rho$ values are calculated as the differences between the sums of electron densities at BCPs in the EI complexes and for the compounds surrounded by water molecules. The interactions of thiourea with the active site of TcDH is stronger than those with water molecules in solution, whereas the opposite is observed for urea. This explains formation of the ET complex and absence of inhibitory potency of urea. Additionally, we calculated energies of corresponding coordination and hydrogen bonds according to the Espinosa equation (see Table 1).[12] Hydrogen bonds in solution are slightly stronger in case of urea that is in line with the ρ values at the BCPs. In the active site of TcDH the coordination bonds strengths is more than twice larger if a thiourea molecule is bound.

Table 1 Sums of electron densities at BCPs calculated for interactions of urea/thiourea in the active site of TcDH (ρ_{prot}) and in water solution ($\rho_{aq.}$) and their differences ($\Delta\rho$). The energies of coordination/hydrogen bonds between ligands and copper ions/water molecules were calculated according to the Espinosa equation.[12]

Compound	ρ_{prot}/a.u.	$\rho_{aq.}$/a.u.	$\Delta\rho$/a.u.	E_{prot}/ kcal mol^{-1}	$E_{aq.}$/ kcal mol^{-1}	ΔE/ kcal mol^{-1}
Thiourea	0.22	0.18	0.04	164	42	122
Urea	0.15	0.19	–0.04	68	46	22

To conclude, we have demonstrated that O to S substitution in urea is crucial for the formation of stable EI complex. Urea does not inhibit the TcDH, while thiourea has an IC_{50} value of 1 mM. Molecular modeling revealed that it is due to formation of stable coordination bonds between copper ions of the active site and sulfur atom of thiourea and an additional hydrogen bond. Also, the planar square coordination sphere of a copper ion $Cu^{2+}(2)$ found in the ET complex is more preferable than tetrahedral one in the EU complex.

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