STRUCTURAL AND FUNCTIONAL ANALYSIS OF BIOPOLYMERS AND THEIR COMPLEXES

MECHANISM OF THIOCYANATE DEHYDROGENASE FUNCTIONING BASED ON STRUCTURAL DATA

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Abstract. Thiocyanate dehydrogenase is enzyme catalyzing transformation of a thiocyanate ion into a cyanate ion with outcome of two electrons, two protons and a neutral atom of sulphur. Earlier structures of thiocyanate dehydrogenase from *Thioalkalivibrio paradoxus* were solved. Despite not perfect quality of the structures (twinning and pronounced anisotropy of the crystals, incomplete occupancy of the copper ions, absence of data for complexes with analogues of the substrate), there was suggested a mechanism of the enzyme functioning based on those structures. Recently at atomic resolution there have been solved structures of a gene-modified copy of relative enzyme from *Pelomicrobium methylotrophicum* for free protein and its complex with thiourea. In the new structures copper ions of the active site possess complete occupancy. In these structures it is possible to reliably identify two conformations of the protein molecule with opened and closed active sites. The new structural high resolution data also allowed us to determine the presence of the superposition of different states of the copper ions for each of the two conformations. In each state the copper ions have different oxidation degrees, different corresponding ligands and partial occupancies. The ion charges were determined according to the ions coordination. In the protein molecule with the closed active site the complexes with inhibitor (thiourea ion) and molecular oxygen are observed. The complex with thiourea allows us to model binding of thiocyanate ion to the enzyme molecule. Taking into account the changes of the structures in the opened and closed conformations, a mechanism of the attacking oxygen ligand activation is suggested. A new scheme of the enzymatic reaction is discussed.

Keywords: *X-ray analysis of proteins, conformational changes, multicopper enzymes, enzymatic reactions*

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INTRODUCTION

The structure of thiocyanate dehydrogenase from Thioalkalivibrio paradoxus (tpTcDH) has recently been solved [1]. Thiocyanate dehydrogenase catalyzes the reaction: $NCS^- + H_2O \rightarrow NCO^- + S^0 + 2H^+ + 2e^-$. The spatial structures of natural tpTcDH, its genetically engineered analog, and a number of mutant forms were determined [1, 2]. Most of the solved structures of tpTcDH (6G50, 6G5M, 6I3Q, 6UWE) belong to the spatial group P21 with approximately equal unit cell parameters a and b and a monoclinicity angle of approximately 120°, which suggests the possibility of twinning. In these structures, flat tetrameric enzyme molecules are arranged in planes perpendicular to the second-order axis, forming a layered structure in which tetrameric molecules in a layer are more strongly bonded than tetramers from different layers. This layered structure facilitates the formation of twins in crystals. This crystal modification makes it possible to collect diffraction data sets up to high resolution (1.45 Å), but crystal twinning significantly degrades the quality of diffraction data. In other crystalline modifications of tpTcDH (5OEX, 5F75, 6SJI), diffraction data can only be collected up to a resolution of 2 Å due to their high anisotropy. All tpTcDH structures were solved either from diffraction data from twin crystals or from crystals with high anisotropy of diffraction data. The quality of the data did not allow us to unambiguously interpret the structure of the active center of enzymes, which contains a superposition of states of atoms with partial populations. In addition, a partial population of copper ions of the active center was observed in all tpTcDH structures (for the Si3 ion, the population does not exceed 0.3).

The tpTcDH molecule is formed by a seven-bladed β-propeller, the blades of which are β-sheets of antiparallel chains. The active site of tpTcDH, containing a unique trinuclear cluster of copper ions, is located in the central part of the protein in a cavity formed by the β-sheets of the propeller. This cavity is closed on one side by the C-terminal region of the enzyme's polypeptide chain. Access to copper ions is possible only from the opposite side of the cavity. Topologically, the structure of the tpTcDH molecule is similar to the structure of the N-terminal domain of N ^{2} O-reductase [3]. In all solved tpTcDH structures, enzyme molecules form tetramers of two symmetrical dimers with very strong intermolecular contacts between subunits of the dimer and weaker contacts

between dimers. According to estimates by the PISA program [4], this dimeric structure is stable in solution. All tetramer subunits lie in the same plane.

Based on the data on tpTcDH structures, a mechanism for the catalytic stage of the reaction has been proposed [1], which raises a number of questions. The main idea of this mechanism is that hydrolysis of the SCN **ion by a hydroxyl anion occurs in the active site of the enzyme,** but it does not explain how two similarly charged groups come together. The claim that the formation of two hydrogen bonds by a water molecule with histidine and glutamate residues can activate it, turning it into a hydroxyl anion, seems strange. A separate question concerns the positioning of the substrate molecule in the enzyme's active site by quantum chemical calculations in the absence of data on the structure of enzyme complexes with the substrate or its analogs. In addition, the proposed scheme does not include all reliably established data on the structure of the active site (in particular, about the presence of two different positions in the structure of the copper ion Cu2).

Copper ions, along with iron ions, play a determining role in biological processes involving binding, reduction, and activation of molecular oxygen. Additionally, they participate in the denitrification cycle during the reduction of nitrites and nitric oxide. The structures and spectroscopy of copper-containing proteins involved in electron transfer and catalysis of redox processes are discussed in detail in review [5]. The classification of copper centers in proteins, based on the analysis of copper ion coordination, is given in [6]. The coordination spheres of oxidized and reduced copper ions differ. Quantum-mechanical foundations of the structure of copper ion complexes can be found in [7]. Coordination of Cu(I) and Cu(II) ions with nitrogen- and oxygen-containing ligands has been studied for small molecules [8–10]. Copper ions Cu(I) can have coordination numbers of 2 (linear coordination), 3 (planar trigonal coordination) [7, 11], and 4 (tetragonal coordination) [12]. Complexes of copper ion Cu(II) can have coordination numbers of 4, 5, and 6. Copper Cu(II) complexes with a coordination number of 4 have a geometry close to a square planar [8]. Distortion toward tetragonal geometry is possible only due to steric contacts [10]. Copper Cu(II) complexes with a coordination number of 5 form a trigonal bipyramid [9]. Thus, there is a correspondence between the charge of the copper ion in the complex and the structure of its coordination sphere.

The presence of this pattern made it possible to assign charges to copper ions in laccase structures when studying the effect of enzyme reduction on its structure due to an increase in the absorbed dose of ionizing radiation by the crystal [13, 14]. In these studies, determining the structure of the coordination spheres of copper ions allowed not only to identify their charges but also to correctly interpret the structures of the active site in the presence of a superposition of several states of copper ions (with different oxidation states) together with their coordinating ligands.

The improvement of the diffraction quality of protein crystals can be achieved by using protein preparations with point substitutions of surface residues or preparations of related enzymes from other organisms for crystallization [15]. In the case of tpTcDH, the first approach did not yield results [2]. As part of the second approach, TcDH from *Pelomicrobium methylotrophicum* – pmTcDH was chosen as the object of study. The structures of the enzyme in its free state and the complex of the enzyme with the thiourea inhibitor were solved with atomic resolution. The structures have been deposited in the Protein Data Bank: PDB codes 8Q9X and 8Q9Y.

The aim of this work is to study the functioning mechanism of thiocyanate dehydrogenases, based on the interpretation of new high-resolution structures of pmTcDH active centers, taking into account the superposition of copper ion states in various oxidation degrees together with the corresponding ligands.

EXPERIMENTAL PART

A genetically engineered analogue of tpTcDH from P. methylotrophicum (pmTcDH) was developed at the A.N. Bach Institute of Biochemistry of the Russian Academy of Sciences, crystals were grown, and a set of diffraction data with a resolution of about 1 Å for the free form of the enzyme and a complex with its thiourea inhibitor was collected at a synchrotron in Japan. The complex with thiourea was obtained by soaking free enzyme crystals in a solution of 100 mM of thiourea for 5 minutes. The structures were solved by the molecular substitution method using the MOLREP program [16] based on the tpTcDH dimer model. The crystallographic refinement was performed using the REFMAC5 program [17]. In the final stages, the refinement was carried out taking into account the anisotropy of temperature factors. The values of partial atomic populations of the model were adjusted manually with the requirement of proximity of temperature factors of neighboring atoms. Both structures have been refined to a confidence factor of Rf = 10.8%. The structures were deposited in the bank of protein structures: 8Q9X (Varfolomeeva L.A., Polyakov K.M., Shipkov N.S., Dergousova N.I., Boyko K.M., Tikhonova T.V., Popov V.O. (2023) PDB 8Q9X: The structure of thiocyanate dehydrogenase from Pelomicrobium methylotrophicum with molecular oxygen at 1.05 Å resolution. doi: https://doi.org/10.2210/pdb8Q9X/pdb) and 8Q9Y (Varfolomeeva L.A., Polyakov K.M., Shipkov N.S., Dergousova N.I., Boyko K.M., Tikhonova T.V., Popov V.O. (2023) PDB 8Q9Y: The structure of thiocyanate dehydrogenase from Pelomicrobium methylotrophicum in complex with inhibitor thiourea at 1.10 Å resolution. doi: https://doi.org/10.2210/pdb8Q9Y/pdb).

This paper presents an interpretation of the electron density in the active centers of these structures. In all structures, states with a superposition of copper ions and the corresponding ligands with partial occupancies are identified. In all states of the active center, the coordination of copper ions with partial occupancies is determined, which allows their charges to be determined. The figures are made using the CCP4MG program [18].

RESEARCH RESULTS

General structure of pmTcDH

The independent part of the unit cell contains a dimeric pmTcDH molecule. The pmTcDH dimer (unlike the tpTcDH dimer composed of two equivalent subunits) is formed by two non-equivalent subunits (Fig. 1). The conformations of the subunits in the dimer are different. In one subunit of the dimer, the access for solvent molecules to the active site of the enzyme is open ("open" conformation), while in the second subunit, the active site is inaccessible to solvent molecules ("closed" conformation). The superposition of the structures of the subunits in the pmTcDH dimer is shown in Fig. 2. The structures of the subunits of the pmTcDH dimer are similar; the subunits can be superimposed by coordinates of all CA atoms with a root-mean-square deviation of 0.53 Å. Significant differences are observed only for three regions of the polypeptide chain (251-265, 362-367, and 394-401), where the differences in the positions of their CA atoms are about 1 Å when the subunits are superimposed. Excluding these regions from the structural comparison reduces the root-mean-square deviation to 0.35 Å. These fragments of the polypeptide chain participate in the formation of substrate channels and active sites of the subunits (Fig. 2).

Fig. 1. The pmTcDH dimer from the structure of the complex with thiourea. The subunits in the "closed" and "open" conformations are shown as ribbon models on the right and left, respectively. The copper ions of pmTcDH are shown as spheres. The thiourea molecule is presented as a ball-and-stick model.

Fig. 2 . Superposition of the structures of pmTcDH subunits in the "closed" and "open" conformations. The course of the polypeptide chain of the subunit in the "closed" conformation is shown in a light tone. The loops of the subunit in the "open" conformation, whose positions differ in the subunits in the "closed" and "open" conformations, are shown in dark tones. The positions of copper ions for the subunit in the "closed" conformation are shown as spheres. The thiourea molecule is shown as a ball-and-stick model. View from the entrance to the active site.

The simultaneous presence of subunits in "open" and "closed" conformations within one crystal suggests that the energies of these states are close. It should be noted that the structure of the subunits is identical for the chain fragments participating in the formation of contacts between subunits in the dimer, therefore contacts between subunits in the dimer cannot be the reason for the difference in their structures. Subunits in "closed" and "open" conformations have significant differences in intermolecular contacts. However, it is not possible to link changes in the conformation of subunits with intermolecular contacts in the crystal (areas with significant difference in structures do not participate in intermolecular interactions in the crystal). Based on this, it can be assumed that the conformations of the subunits are related to their internal structure. The key residues responsible for stabilizing the conformations of the subunits are Pro256 and Glu253. The side chain of the Pro256 residue in the "closed" conformation overlaps the substrate channel. The atoms of this residue are located in the place of water atoms from the substrate channel of the enzyme in the "open" conformation. The side chain of Glu253 stabilizes the structure of the "open" and "closed" conformations through hydrogen bonds (see below).

In general, the structure of the active site of pmTcDH is similar to the structures of the active site of tpTcDH. However, it should be noted that the occupancy of all three copper ions of the pmTcDH active site is close to complete, and in the active site of the "closed" subunit, a thiourea ion and molecular oxygen are localized for the complex with thiourea and the free enzyme, respectively. It is also important to emphasize that the amino acid residues involved in the coordination of copper ions in the active site do not change their position when the conformation of the subunits changes. The structure of the active site is examined in detail below.

On the electron density maps in the region of the N-terminal part of the "closed" subunit of the enzyme, a copper ion is localized, as shown in Fig. 2. The ion is coordinated in a square planar arrangement by the N-terminal nitrogen atom of Gly43, the nitrogen atoms of the main and side chains of the His44 residue, and the side chain of the His376 residue of the adjacent "closed" subunit.

General Structure of pmTcDH Active Site

As in tpTcDH, the active site of pmTcDH is located in the central cavity of the β-propeller. In general, the active site of pmTcDH is similar to the active site of tpTcDH. The high quality of the diffraction data made it possible to establish new important features in the pmTcDH structures. Unlike the tpTcDH structures, the positions of three copper ions with full occupancy are localized in the active site of pmTcDH. The structures of the active sites in the "closed" and "open" conformations differ. In the active sites of subunits in the "closed" conformation, a thiourea molecule and molecular

oxygen are localized in the structures of the complex with thiourea and the free enzyme, respectively. It should be noted that the position of the side chains of histidine residues coordinating copper ions does not change when the conformation of the subunits changes (H171 and H346 for the first copper ion, H100 and H493 for the second, H402 and H447 for the third). The high quality of the electron density allows not only to reliably establish several positions of the copper ions in the active site, but also to determine the structure of the coordination spheres of copper ions with partial occupancy (and thereby determine the charges of these ions). This made it possible to interpret the structures of the active site of enzyme subunits in the "closed" and "open" conformations as a superposition of two states of copper ions with partial occupancies and their corresponding ligands. This interpretation is made both for the structure of the complex with thiourea and for the structure of the free enzyme. Below is a detailed examination of the structure of the enzyme active sites in various states.

Structure of pmTcDH Active Site for Complex with Thiourea

The structure and electron density of the active site of the subunit of the complex with thiourea in the "closed" conformation are shown in Fig. 3 *a* . In this case, the substrate channel is blocked. In the "closed" conformation of the active site of the pmTcDH subunit, two states can be distinguished.

The occupancy of the first state is $q = 0.7$ (Fig. 3 *b*). In this state, a thiourea molecule is localized in the active site. The Cu1(II) ion is coordinated in a square planar manner by four ligands - atoms NE2 H171, NE2 H346, OD1 D279, and N2 of thiourea (here and further, the oxidation state of copper ions is indicated in parentheses). The Cu2(II) ion is coordinated by five ligands in a trigonal bipyramidal manner (atoms ND1 H493, NE2 H100, S and N1 of thiourea, a water molecule). The Cu3(I) ion is coordinated by three ligands in a planar triangular manner - atoms NE2 H402, ND1 H447, S of thiourea. The N1 atom of thiourea forms a hydrogen bond with H101.

The occupancy of the second state is 0.3 (Fig. 3 *c*). In this state, the inhibitor is absent from the active site. The Cu1(I) ion is linearly coordinated by atoms NE2 H171 and NE2 H346. The D279 residue does not participate in the coordination of the Cu1 ion. The Cu2(I) ion is coordinated by three ligands in a planar triangular manner - atoms ND1 H493, NE2 H100, and a water molecule. The Cu3(I) ion is linearly coordinated by atoms NE2 H402 and ND1 H447. In the active site, two water molecules are located in place of the thiourea molecule. One water molecule W is located at the position of the N1 atom of thiourea and forms a hydrogen bond with H100 (see below). The second water molecule is located at the position of the sulfur atom of thiourea.

In the "closed" conformation of the pmTcDH subunit, the substrate channel is closed by residue P256. The side chain of E253 forms a hydrogen bond with residue K68, which helps to stabilize the "closed" conformation.

Fig. 3. Active site of the pmTcDH subunit complex with thiourea in the "closed" conformation. *a* - Complex model and electron density (2 *F* obs - *F* calc) for levels 1σ and 5σ. First state (*b*) and second state (*c*) of the active site of the pmTcDH subunit in the "closed" conformation. Active site amino acid residues and thiourea are represented as a ball-and-stick model. Water molecules are shown as small spheres. Copper ions in oxidation states II and I are shown as large spheres. The "attacking" water molecule is designated as W. Coordination bonds and hydrogen bonds with residues H101 and K68 are shown by dashed lines.

In the structure of the complex with thiourea in the "open" conformation, two states of the enzyme active site can also be distinguished. In these structures, the substrate channel is open, and only oxygen ligands are observed in the active site. The occupancy of the first state is 0.35 (Fig. 4 *a*). The Cu1(II) ion is coordinated by five ligands in a trigonal bipyramidal arrangement formed by the atoms NE2 H171, NE2 H346, OD2 D279, and two water molecules. The Cu2(II) ion is also coordinated by five ligands in a trigonal bipyramidal arrangement formed by the atoms ND1 H493, NE2 H100, NZ K68, and two water molecules. The Cu3(I) ion is coordinated by three ligands in a planar triangular arrangement: the atoms NE2 H402, ND1 H447, and a water molecule. In the "open" conformation, three oxygen ligands are present at the site of the thiourea inhibitor in the complex structure. One water molecule is located at the position of the N1 atom of thiourea, the second at the position of the S atom, and the third at the position of the N2 atom.

The occupancy of the second state is 0.65 (Fig. 4 *b*). The Cu1(I) ion is linearly coordinated by the atoms NE2 H171 and NE2 H346. The Cu2(I) ion is coordinated by three ligands in a planar triangular arrangement - the atoms ND1 H493, NE2 H100, and a water molecule. The Cu3(I) ion is linearly coordinated by the atoms NE2 H402 and ND1 H447.

Fig. 4. The first state (*a*) and the second state (*b*) of the active site of the pmTcDH subunit in the "open" conformation of the complex structure with thiourea. The amino acid residues of the active site are represented as a ball-and-stick model. Water molecules are shown as small spheres. Copper ions in oxidation states II and I are shown as large spheres. The "attacking" water molecule is designated as W. Coordination bonds and hydrogen bonds with water molecule W are shown by dashed lines.

In the "open" conformation of the enzyme, the active site is accessible to the solvent from the outside, and residue P256 does not prevent the binding of water molecules in the substrate channel. The side chain of E253 is turned away from K68 and, along with H101, forms a hydrogen bond with water molecule W.

Structure of the active site of the free pmTcDH enzyme

The quality of the electron density also allows to identify in the structures of the free enzyme two states in both "open" and "closed" conformations of the subunit, including copper ions with partial occupancy and corresponding ligands.

The model of the active site structure of the subunit in the "closed" conformation is shown in Fig. 5. The first state includes molecular oxygen and three copper ions with corresponding ligands with occupancy $q = 0.7$. The Cu1(II) ion is coordinated by four ligands (NE2 H171, NE2 H346, OD1 D279, and a water molecule) in a square planar arrangement. The Cu2(II) ion is coordinated by five ligands in a trigonal bipyramidal arrangement. The coordination includes atoms ND1 H493, NE2 H100, O of molecular oxygen, two water molecules (including W). The Cu3(I) ion is coordinated by three ligands (NE2 H402, ND1 H447, O of molecular oxygen) in a planar triangular arrangement.

The occupancy of the second state is 0.3 (Fig. 5). The Cu1(I) ion is linearly coordinated by atoms NE2 H171 and NE2 H346. The Cu2(I) ion is coordinated by three ligands in a planar triangular arrangement - atoms ND1 H493, NE2 H100, and a water molecule. The Cu3(I) ion is linearly coordinated by atoms NE2 H402 and ND1 H447.

Fig. 5. Active site of the free pmTcDH subunit in "closed" conformation. Amino acid residues of the active site and water molecules are presented as ball-and-stick models. Copper ions in oxidation states II and I are shown as large spheres. The "attacking" water molecule is labeled W. Coordination bonds and hydrogen bonds with residues H101 and K68 are shown as dashed lines.

The structures of the free enzyme in the "open" conformation are almost completely identical to the structures of the complex with thiourea in the "open" conformation, and their descriptions are not provided.

DISCUSSION OF RESULTS

New data on the structure of the TcDH active site can be summarized as follows. The enzyme complex with thiourea can serve as a model of the transition state that occurs during nucleophilic attack on the carbon atom of thiocyanate. Based on the structure of the enzyme complex with thiourea, we can suggest how the substrate (thiocyanate ion) binds in the active site of pmTcDH (Fig. 6). The sulfur atom of thiocyanate binds in the enzyme's active site similarly to the sulfur atom of thiourea. In this position, the sulfur atom can form two coordination bonds with copper ions Cu2 and Cu3. The nitrogen atom of thiocyanate corresponds to the nitrogen atom of the thiourea amino group from the coordination sphere of the Cu1 ion and forms a coordination bond with it. The water molecule W, located near the position of the nitrogen atom of the second amino group of thiourea, may play the role of the attacking group.

Fig. 6. Superposition of active sites of the thiourea complex structure in "closed" and "open" conformations. The "open" conformation structure is shown in light tone. The structure fragment corresponding to the "closed" conformation is highlighted in dark tone. Copper ions in structures in "open" and "closed" conformations in oxidation states II and I are shown as large spheres (dark and light tone, respectively). The thiourea molecule is shown as a ball-and-stick model. The "attacking" water molecule is designated as W. Hydrogen bonds are shown by dashed lines. View from the entrance to the active site. The Pro256 side chain in the closed conformation blocks access to the enzyme active site.

In the structures of the free enzyme and the complex with thiourea, it has been established that the subunits in the pmTcDH dimer exist in two different states. In the "open" conformation, there is free solvent access to the active site of the enzyme. In the "closed" conformation, the side chain of residue P256 blocks the substrate channel, breaking the chain of hydrogen-bonded water molecules. These two states are present simultaneously in a single crystal. Complexes of the enzyme with thiourea and molecular oxygen were found only for subunits in the "closed" conformation (Fig. 3 *a* , Fig. 5). The successful formation of a complex with thiourea by the method of soaking crystals of the free enzyme suggests that thiourea is able to penetrate even into the closed substrate channel of the subunit in the "closed" conformation through thermal motion and bind there. It should be emphasized that binding of the thiourea ion in the active site of the subunit in the "open" conformation is not observed, although penetration into the active site under these conditions is significantly facilitated.

In the pmTcDH structures, as in the tpTcDH structure [1], the oxygen ligand W, located near the nitrogen atom of the second thiourea amino group (Fig. 6), can play the role of an attacking group in the enzymatic reaction. In the "open" conformation of both enzyme structures, this water forms two hydrogen bonds with residues E253 and H101 (Fig. 6). In the structures in the "closed" conformation, it forms a hydrogen bond only with H101 (Fig. 3 *in* , Fig. 5). In the activation of the attacking water, the main role belongs to residue E253. This residue occupies two different positions in the structures of "open" and "closed" conformations (Fig. 6). In the "closed" conformation, the E253 side chain forms a hydrogen bond with the side chain of residue K68. When the enzyme subunit transitions to the "open" conformation, the E253 side chain breaks the hydrogen bond with the side chain of residue K68. Moreover, during the breaking of this bond, the proton remains with lysine. E253 in the deprotonated state turns toward the "attacking" water W and forms a hydrogen bond with it through its proton (Fig. 6). When this hydrogen bond is formed, the proton transfers to E253, and the "attacking" water W turns into a hydroxyl anion. In this activated state, OH $^{\text{is capable}}$ of attacking the substrate. It can be assumed that after the substrate is converted into the product with the participation of the hydroxyl anion, the protonated E253, when changing to the "closed" conformation, returns to K68 and forms a hydrogen bond through its proton (Fig. 6).

To determine the role of copper ions in the enzymatic reaction, let's consider the changes in the structures surrounding the copper ions of the active center when their oxidation states and subunit conformations change. The Cu1 ion does not change its position either depending on the oxidation state or the change in subunit conformation (Fig. 6). Upon its reduction, only the number of coordinating ligands changes, making it similar to the T2-center of laccases [14]. Taking into account the binding of thiourea by the enzyme, it can be assumed that the role of this copper ion is to bind the nitrogen atom of the substrate through a coordination bond. The coordination sphere of the Cu3 ion in all states of the copper cluster of pmTcDH corresponds to the reduced state (Fig. 3 *b,c* , Fig. 4 *a* , *b* , Fig. 5). These considerations allow us to conclude that the role of the ion is reduced to the correct positioning of the substrate molecule in the active center through the formation of a coordination bond with the sulfur atom, and the ion does not participate in electron transfer during the enzymatic reaction. The Cu2 copper ion is connected to the external electron acceptor by an electron transport chain that includes the H493 residue (one of the residues coordinating the Cu2 ion) and three water molecules linked by hydrogen bonds. This chain is revealed in all solved structures of the pmTcDH active center. The coordination sphere of the Cu2 ion undergoes the most complex rearrangements during the closing of the substrate channel and oxidation-reduction. The Cu2 ion can occupy three positions in the active center of pmTcDH: one reduced and two oxidized (Fig. 6). It can be assumed that the

presence of structures with the reduced copper ion Cu2(I) is due to the absence of an electron acceptor in the enzyme crystals. The position of the Cu2(I) ion coincides in pmTcDH subunits in "closed" and "open" conformations in all structures (free enzyme and complex with thiourea). Upon oxidation, the Cu2 ion can shift from its reduced state in two opposite directions. In the "open" subunit, it shifts toward the K68 side chain (Fig. 4), and in the "closed" one, it shifts toward the sulfur atom in the complex with thiourea (Fig. 3 *b*) or oxygen in the free enzyme (Fig. 5). The distance between these positions of the Cu2 ion is approximately 2 Å. This indicates the key role of the Cu2 ion in the enzymatic reaction. The change in subunit conformation from "open" to "closed" is accompanied by a rearrangement of the coordination sphere of this ion upon its reduction. It is this ion in the reduced state that can participate in the transfer of electrons from the substrate to the external acceptor. In one of its oxidized states in the "closed" conformation, it coordinates the sulfur atom of thiourea in the complex structure or one of the atoms of molecular oxygen in the free enzyme.

Taking into account all the details of the pmTcDH structure in all observed states of the active center in the "open" and "closed" conformations, the mechanism of the catalytic reaction can be formulated as follows (Fig. 7).

Fig. 7. Proposed mechanism of the pmTcDH catalytic cycle in the $NCS^{+} H_2O \rightarrow NCO^{+} S^{0} + 2H^{+} + 2e^{-}$ reaction. Key coordination bonds are shown with light lines. Covalent bonds are shown with black lines. Dashed lines indicate hydrogen bonds. Oxidized and reduced copper ions are shown as large spheres. Small short arrows show the displacement of the Cu2 copper ion when the oxidation state changes. Long arrows show electron transfer, as well as the movement of molecules in the active center. Open and closed boxes denote "open" and "closed" conformations of the hbTcDH molecule, respectively.

At the beginning of the catalytic cycle (stage *a*), the enzyme molecule is in an "open" conformation. The Cu1(II) and Cu2(II) ions are oxidized, and the Cu3(I) ion is in a reduced state. The Cu1 and Cu3 ions are coordinated by two water molecules. The third water molecule ("attacking" water W, Fig. 3 *c*) forms a hydrogen bond with residues E253 and H101. The amino acid residue K68 coordinates the copper ion Cu2. Due to electrostatic attraction, the SCN^{-} (substrate) enters the active site, which is in an "open" conformation. Upon entering the active site, the SCN ion reduces the copper ion Cu2(II), which is part of the electron transport chain, to Cu2(I). The binding of the neutral SCN· radical is accompanied by its substitution of two water molecules coordinating the Cu1 and Cu3 ions. The reduction of the Cu2 ion causes its displacement from K68 and a change in its coordination sphere

(to a trigonal planar coordination) with the loss of its coordination with K68. It is this change in Cu2 coordination that initiates the transformation of the "open" conformation of the active site to the "closed" one. This change in the subunit conformation leads to the breaking of the hydrogen bond between E253 and the "attacking" water W and the displacement of its side chain in the protonated state (explained below) towards K68. In the "closed" conformation, E253 forms a hydrogen bond with K68 through this proton, which compensates for the loss of the coordination bond of K68 with the copper ion in the open conformation of the subunit.

At the second stage (stage b), the subunit is in a closed conformation. The copper ion Cu1(II) is oxidized, while Cu2(I) and Cu3(I) are reduced. The substrate in the form of a neutral radical $SCN^{\cdot}$ is bound in the active site so that its nitrogen atom replaces a water molecule from the coordination sphere of Cu1(II) and forms a coordination bond with Cu1(II), while the sulfur atom replaces the second water molecule and forms a coordination bond with the Cu3(I) ion. The position of the substrate's sulfur atom corresponds to the position of the sulfur atom in the complex with the thiourea molecule. The Cu2(I) ion transfers its electron to an acceptor through the transfer chain (see above). The oxidation of the Cu2 ion leads to its further movement from K68 to the sulfur atom of the substrate. The breaking of the hydrogen bond between the side chain of residue E253 and the water molecule W during the conformation switch of the subunit leads to the protonation of glutamate (see above) and the transformation of this water molecule into an attacking hydroxyl ion (OH^-).

At the third stage of the reaction (stage c), the oxidized Cu2(II) ion is five-coordinated by atoms: ND1 H493, NE2 H100, S from SCN, and two oxygen ligands (one of them is OH $^{\text{ion in}}$ position W). The OH ion is connected to H101 via a hydrogen bond. The substrate's sulfur atom has two coordination bonds with Cu2(II) and Cu3(I), and the substrate's nitrogen atom forms a coordination bond with Cu1(II). The interactions of the substrate's sulfur atom with the copper ions Cu2(II) and Cu3(I) and its nitrogen atom with the Cu1(II) ion weaken the covalent bonds in the substrate molecule. The position of the OH ion allows to carry out an attack on the carbon atom in the SCN radical according to the equation $OH + SCN \rightarrow NCOH + S^-$.

At the fourth stage (stage d), the S ion coordinates copper ions Cu2(II) and Cu3(I). The product molecule HOCN is bound in the active site. The nitrogen atom of this molecule coordinates the Cu1(II) ion and its oxygen atom forms a hydrogen bond with H101. The S ion reduces Cu2(II) to Cu2(I) and becomes a neutral sulfur atom. This is possible in the presence of an acceptor in the electron transport chain. The reduced Cu2(I) returns to its middle position (planar trigonal coordination: two histidine residues and a water molecule).

The fifth stage (stage e) includes the release of an electron from Cu2(I) to the electron transport chain and the displacement of Cu2 to the K68 side chain. The binding of the HOCN molecule remains unchanged. It should be noted that the localization of reduced Cu2(I) in structures is only possible in the absence of an electron acceptor. In the presence of an electron acceptor, the states in stages b and e become short-lived intermediates.

In the sixth stage (stage f), the Cu2(II) ion returns to its initial position, where it is coordinated by the K68 residue. The change in coordination of the Cu2 ion initiates the transition of the subunit to an "open" conformation. When the coordination bond between Cu2(II) and NZ K68 is restored, a proton is released. This is the first proton described in the reaction equation. The position of the E253 side chain changes. The hydrogen bond between E253 and K68 breaks. After its rupture, E253 remains in a deprotonated state. After the conformational change of the subunit, three molecules from the surrounding solution replace the neutral reaction products (S and HOCN) in the active site. Two of them enter the coordination spheres of the Cu1 and Cu3 ions. The third becomes the "attacking" water W. It forms two hydrogen bonds with E253 and with H101. E253 forms a hydrogen bond with this water molecule while in a deprotonated state. The reaction ends with the dissociation of the HOCN molecule into NCO^{-} and proton H^{+} (the second proton in the reaction) outside the active site in the solution.

CONCLUSION

The structures of the free enzyme and the pmTcDH complex with its inhibitor thiourea were solved with atomic resolution. In the crystal, the enzyme dimer consisted of subunits in "closed" and "open" conformations, differing in the accessibility of the active site to the solvent. Atomic resolution made it possible to precisely establish the coordination of copper ions in the unique trinuclear copper center of pmTcDH. Based on theoretical principles and the obtained structural data, it was possible to assign a charge to each copper ion, which allowed the identification of several states of the enzyme's copper cluster. Based on these states, the main stages of the catalytic process of pmTcDH were proposed. This mechanism describes an electrochemical reaction that is only possible in the presence of an external electron acceptor. The detailed mechanism of electron transfer and the protonation states of oxygen ligands in the active site (with the exception of the W ligand) requires further quantum-mechanical calculations.

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ETHICS DECLARATION

This work was performed without involving humans or animals as research subjects.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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