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STRUCTURE OF MACROMOLECULAR COMPOUNDS

COPPER IONS' INFLUENCE ON THIOCYONATE DEHYDROGENASE PACKING AND CONFORMATION IN A CRYSTAL

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Abstract. The copper-containing enzyme thiocyanate dehydrogenase (TcDH) catalyzes oxidation of thiocyanate to cyanate and elemental sulfur. To date, the structures of two bacterial TcDHs (tpTcDH and pmTcDH) are known. Both enzymes are dimers and contain a trinuclear copper center in the active cite. An important difference between these enzymes is that in a crystal, the subunits of the tpTcDH dimer are in identical conformations, while the subunits of the pmTcDH dimer are in the different conformations – closed and open. To clarify the role of copper ions in changing the TcDH conformation, the structure of the apo-form of pmTcDH was established, in which both subunits of the dimer had the closed conformation. Soaking of apo-form crystals with copper led to the restoring of the trinuclear center and the conformational rearrangements of the subunits.

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INTRODUCTION

The copper-containing enzyme thiocyanate dehydrogenase (**TcDH**) is involved in nitrogen and/or energy metabolism in several sulfur-oxidizing microorganisms [1, 2], catalyzing the oxidation of thiocyanate to cyanate and elemental sulfur with the transfer of two electrons to an external acceptor [3]. The three-dimensional structure of this enzyme was first determined from the haloalkaliphilic bacterium *Thioalkalivibrio paradoxus* ARh1 **(tpTcDH)** [3]. Recently, atomic-resolution structures of the free enzyme and its complex with thiourea were determined for another representative of the class, from the bacterium *Pelomicrobium methylotrophicum* (**pmTcDH**). This enabled a precise characterization of the active site's structural details in various states and refinement of the initial stages of the catalytic reaction [4].

Both characterized enzymes share a similar structure and exist as dimers in both solution and crystal form [3, 4]. Each TcDH subunit adopts a seven-bladed β-propeller structure formed by antiparallel β-sheets. A substrate channel leads from the enzyme surface into the central cavity of the propeller, where the TcDH active site is located, containing three copper ions (Cu1–3) as cofactors. According to available structural data [4], the TcDH subunit can adopt an open conformation, where the active site is solvent-accessible, and a closed conformation, where the substrate channel is blocked from the solvent by a conserved proline residue. The mechanism underlying the transition between these conformations remains incompletely understood.

Despite the overall structural similarity, the tpTcDH and pmTcDH dimers exhibit a notable and intriguing difference: in tpTcDH, the subunit conformation within the dimer is identical across all determined structures, including those with point mutations [3, 5, 6]. Both subunits are always found either in the closed or open conformation. The closed conformation of tpTcDH subunits has been observed in only one structure (PDB ID: 6UWE), where an unidentified ligand was found in the enzyme's active site [3]. In contrast, pmTcDH dimers exhibit asymmetry: one subunit adopts the closed conformation, while the other remains in the open state [4]. However, no cooperative interaction between pmTcDH subunits was detected in kinetic experiments.

The growth of protein crystals and, consequently, the packing of molecules within them depend on various factors, including crystallization experiment parameters [7–10] and the properties of the crystallized molecule [11]. Ligand binding (such as cofactors or substrates) can also influence enzyme conformation. For instance, during the co-crystallization of an mRNA capping enzyme with guanosine triphosphate, one molecule in the asymmetric unit adopted a closed conformation while the other remained open [11]. Conversely, the crystallization of formate dehydrogenase from *Pseudomonas* sp. in complex with nicotinamide adenine

dinucleotide and azide resulted in crystals with a different space group, in which the enzyme molecules adopted a closed conformation compared to the open conformation observed in the apo form [12].

In this study, we investigated the effect of cofactors (copper ions) on the packing of pmTcDH molecules in the crystal and the conformation of dimer subunits.

METHODS AND MATERIALS

Isolation and Purification of pmTcDH. The expression of recombinant pmTcDH in *Escherichia coli* cells, as well as its isolation and purification, were carried out according to the method described in [4]. The resulting homogeneous pmTcDH preparation did not contain copper ions (apo form) and exhibited no enzymatic activity.

Crystallization. For crystallization, the apo form of pmTcDH was used at a concentration of 11.5 mg/mL in 25 mM MOPS buffer, pH 7.5, containing 150 mM NaCl.

Crystallization was performed using the vapor diffusion method (hanging drop variant) in 24-well plates (VDX, Hampton Research, USA) at 15°C. For this, 1 μL of the protein solution was mixed with 1 μL of the precipitant solution, and 500 μL of the precipitant solution was added to the well. Crystals of the pmTcDH apo form were obtained under the following conditions: 0.1 M HEPES, pH 7.5, 21% PEG 8000, 8% ethylene glycol.

Restoration of the Trinuclear Copper Center in the Crystal. Copper ion incorporation into the active site of the pmTcDH apo form in the crystal was performed by soaking with copper (II) and copper (I) ions. For soaking with copper (II) ions, pmTcDH apo crystals were incubated for ~24 hours or longer in the crystallization solution containing 1 mM $CuCl_2$. For soaking with copper (I) ions, the pmTcDH apo crystals were incubated for 12 hours in the crystallization solution containing 1 mM $CuCl_2$ and 1 mM sodium ascorbate.

X-ray Data Collection. Diffraction data sets for pmTcDH apo crystals and pmTcDH soaked with copper (II) ions (**pmTcDH^{Cu2+}**) were collected at the BL17U1 beamline (SSRF, China), while data for pmTcDH soaked with copper (I) ions (**pmTcDH^{Cu+}**) were collected using an XtaLAB Synergy-S diffractometer (Rigaku, Japan) at the N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences. Prior to data collection, crystals were soaked for 5–10 seconds in the crystallization solution supplemented with 20% ethylene glycol as a cryoprotectant. Data collection was conducted at 100 K.

Data processing for the pmTcDH apo and pmTcDH^{Cu2+} structures was performed using the XDS and XSCALE programs [13], while processing for pmTcDH^{Cu+} was carried out using CrysAlis^{Pro} 1.0.43 (Rigaku, Japan). Data collection statistics are presented in Table 1.

Structure Solution and Refinement. Structure solution and refinement were performed using the CCP4 software suite [14]. All structures were solved by molecular replacement using the MOLREP program [15], with the pmTcDH holo subunit (PDB ID: 8Q9X) as the search model [4]. Refinement was carried out using REFMAC5 [16] and COOT [17]. For pmTcDH^{Cu2+} and pmTcDH^{Cu+}, copper ion B-factors were refined anisotropically in the final refinement cycles. Structure refinement statistics are presented in Table 1. Crystal contact analysis was performed using the PDBePISA service [18]. Structural superposition based on all aligned Cα atoms was conducted using LSQKAB [19].

DISCUSSION OF RESULTS

The Subunits of the Apoform Protein Dimer Exhibit Identical Conformation

Crystals of the apoform pmTcDH were obtained under crystallization conditions previously used for growing crystals of the enzyme's holoform. However, the space group and the composition of the asymmetric unit of these crystals differed. The space group of the apoform crystals corresponds to the centered group $C222_1$ (Table 1), unlike the primitive $P2_12_12_1$ for the holoform crystals [4]. Additionally, in the first case, the asymmetric unit of the unit cell contained not a dimer but three enzyme molecules (Fig. 1a): a dimer (subunits A and B) and one subunit (subunit C) of the second dimer with a symmetry axis coinciding with the crystallographic one.

All molecules of the enzyme apoform in the asymmetric unit of the unit cell exhibit the same closed conformation (Table 2). Residue P256, located on the flexible loop 251–266, restricts solvent access to the active site of all three subunits (Fig. 1b), consistent with the closed conformation observed in the holoform structure of pmTcDH (Table 2). In the latter, during the transition to the open conformation, residue P256 in the loop shifts, exposing the active site to the external solvent and substrate (Fig. 1c). Notably, in the closed conformation of both the apo- and holoforms, the flexible loop 251–266 is stabilized by a larger number of hydrogen bonds with adjacent regions of the polypeptide chain (13 bonds) compared to the open holoform conformation (9 bonds). Hydrogen bonds between residue pairs E253 and K68, E253 and Y129, G257 and Y397, and E259 and H346 are only observed in the closed conformation. Residues Q347 and F401, which are crucial for conformational transitions between open and closed states [5], are well-ordered in the apoform structure, adopting positions corresponding to the closed holoform conformation.

Despite the absence of copper ions, the active site of the apoform pmTcDH is well-structured (Fig. 2a): all active site residues adopt a single conformation and are clearly visible in the electron density map. Interestingly, copper ions Cu1 and Cu3 are absent from the active site. However, at the position of ion Cu2, there is a peak in the electron density map (Fig. 2b) that cannot be explained

Table 1. Statistics of data collection and refinement of the structures of the apo form of pmTcDH, pmTcDH^{Cu2+} and pmTcDH^{Cu+}

Station	Apoform pmTcDH	pmTcDH^{Cu2+}	pmTcDH^{Cu+}
	BL17UM, SSRF, China	BL17UM, SSRF, China	XtaLAB Synergy-S, Rigaku
Space group	$C222_1$	$C222_1$	$C222_1$
a, *b*, *c*, Å	97.99, 101.95, 276.17	97.80, 101.75, 276.35	98.09, 101.99, 276.80
Wavelength, Å	0.9792	0.9792	1.5418
Resolution, Å	50.00–1.45 (1.50–1.45)	50.00–1.80 (1.85–1.80)	23.71–2.00 (2.03–2.00)
Number of independent reflexes	243938 (23220)	125833 (9819)	93349 (4421)
Repeatability	13.1 (12.6)	6.7 (6.9)	13.0 (10.3)
$I/\sigma(I)$	20.3 (2.6)	12.7 (2.0)	14.2 (4.3)
Completeness of data, %	99.5 (98.8)	98.8 (98.2)	99.6 (96.5)
R_{meas}, %	7.1 (105.5)	11.0 (121.7)	15.6 (58.9)
$CC_{1/2}$, %	99.9 (82.8)	99.8 (67.9)	99.7 (82.7)
R_{cryst}, %	16.7	16.8	17.5
R_{free}, %	19.6	21.7	22.2
Standard deviations			
Bond lengths, Å	0.016	0.014	0.018
Bond angles, deg	2.259	2.261	2.319
Number of non-hydrogen atoms			
Protein	10914	10858	10826
Solvent	1315	699	652
Copper ions	3	10	9
Total average *B*-factor			
Average *B*-factor for protein	19.1	29.5	18.2
Average *B*-factor for solvent	28.1	34.4	19.0
Average *B*-factor for copper ions	17.9	26.7	14.8
PDB code	8Z75	8Z76	8Z77

Note: Values in parentheses are for high-resolution data.

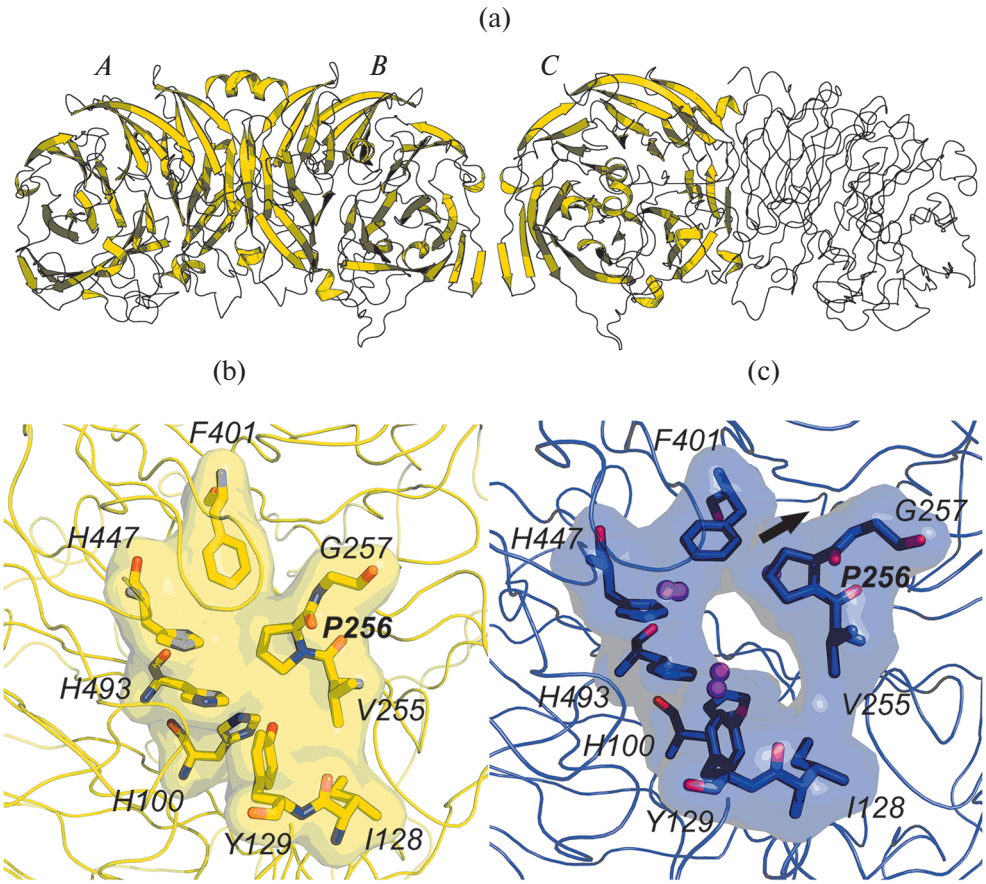

Fig. 1. Three subunits A, B, C from the independent part of the unit cell (secondary structure is shown) and the symmetrical subunit C (secondary structure is not shown) of the apo form of pmTcDH (PDB ID: 8Z75) (a). The position of the P256 residue in the closed conformation of the subunit of the apo form of pmTcDH (PDB ID: 8Z75) (b) and the open conformation of the holo form of pmTcDH (PDB ID: 8Q9X) (c). The arrow shows the direction of movement of the P256 residue during the opening of the substrate channel. The surface is shown for the amino acid residues forming the walls of the substrate channel. Copper ions of the active site are shown as spheres.

Table 2. Comparison of the apo form subunits of pmTcDH, pmTcDH^{Cu2+} and pmTcDH^{Cu+} with the closed apo form subunit and with the closed and open holo form subunits (PDB ID: 8Q9X)

Structure	Subunits	Standard deviation, Å		
		Closed subunit apo form	Closed holoform subunit	Open subunit of holoform
Apoform pmTcDH	A		0.26	0.59
	B	0.22	0.23	0.62
	C	0.17	0.25	0.61
pmTcDH^{Cu2+}	A	0.21	0.29	0.52
	B	0.25	0.21	0.61
	C	0.29	0.26	0.43
pmTcDH^{Cu+}	A	0.39	0.41	0.38
	B	0.26	0.20	0.57
	C	0.42	0.39	0.42

Note: The values of the standard deviation (Å) are given when superimposed by the Cα atoms. The names of the subunits that adopt a closed conformation are indicated in italics.

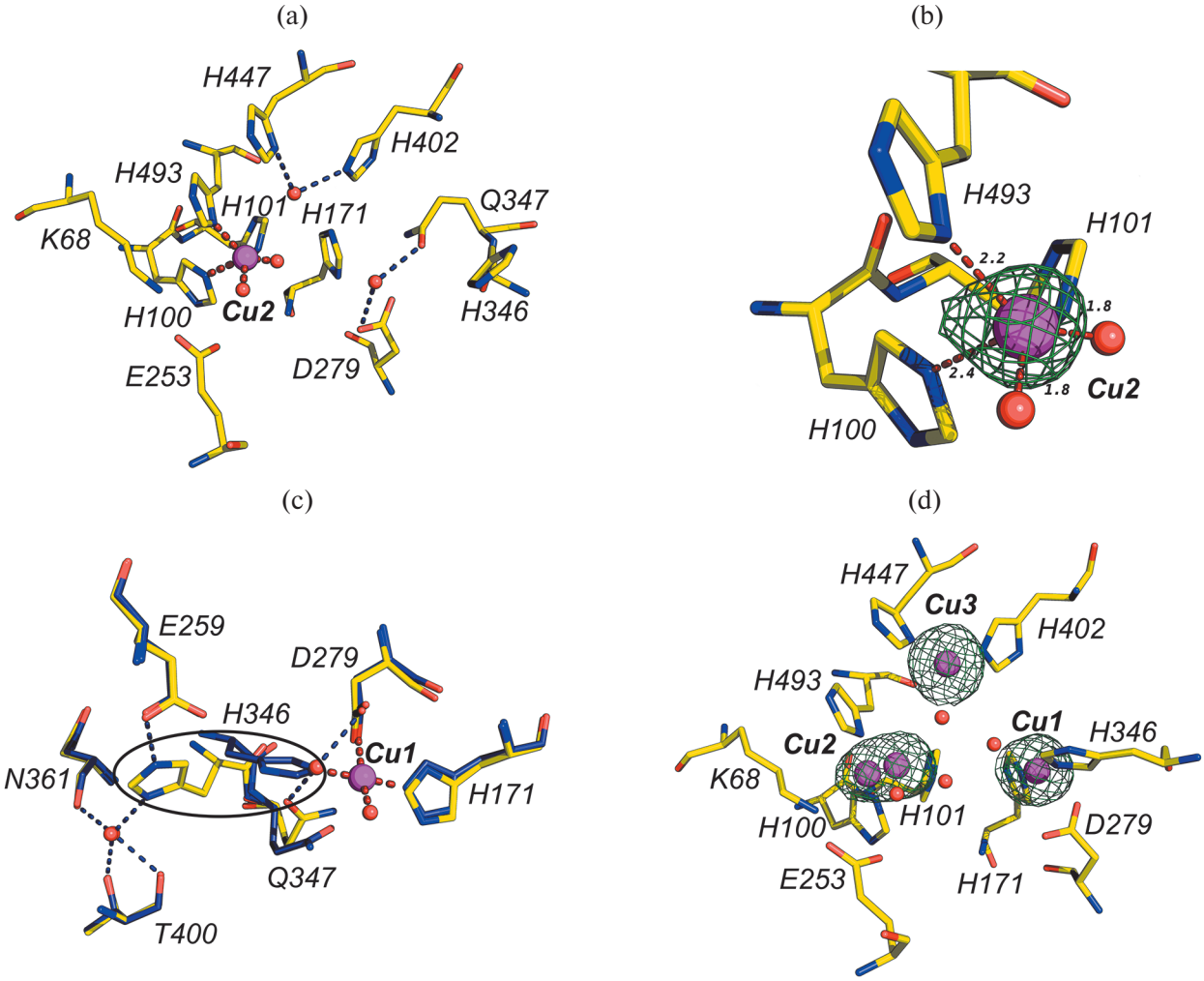

Fig. 2. Active center of the apo form of pmTcDH (PDB ID: 8Z75) (a) before infusion of crystals with copper ions. The maximum in the difference electron density map at the 3σ level is described by the copper ion Cu2 with an occupancy of 0.2, the corresponding coordination distances are given in Å (b). Superposition of the structures of the apo form (PDB ID: 8Z75) and holo form (PDB ID: 8Q9X) showed (c) that in the absence of the Cu1 ion, the H346 residue changes its position and forms hydrogen bonds with the E259 residue and a water molecule. Reduction of the trinuclear copper center of pmTcDH^{Cu2+} after infusion of crystals with copper (2+) ions (PDB ID: 8Z76): maxima in the difference electron density map at the 3σ level are shown at the positions of the Cu1, Cu2, Cu3 ions (d). Copper ions and water molecules are large and small spheres, respectively; coordination and hydrogen bonds are thick and thin dotted lines, respectively.

by solvent due to the short distance to surrounding ligands. This peak was interpreted as a copper ion with an occupancy of 0.2. The presence of Cu2 in the active site is likely due to the enzyme's ability to bind a small amount of copper ions during protein expression in *E. coli* cells. In the apoform, the conformation of amino acid residues coordinating Cu2 and Cu3 ions corresponds to that in the holoform (Fig. 2a), while the absence of Cu1 results in the displacement of residue H346 to an alternative position (Fig. 2c). H346 is firmly stabilized in this position by a hydrogen bond network directly involving E259 from the flexible loop 251–266 and residues N361, T400 through a shared water molecule.

An analysis of crystal contacts in the structures of the apo- and holoform pmTcDH revealed no correlation between the number of crystal contacts and the conformation of molecules in the asymmetric unit of the unit cell.

Incorporation of Copper Ions into the Active Site Induces Conformational Changes

To restore the complete trinuclear copper center, the apoform pmTcDH crystals were soaked with copper ions. A fully occupied trinuclear copper center, where Cu1, Cu2, and Cu3 ions have full occupancy, was achieved by soaking the crystals with both copper (II) ions, pmTcDH^{Cu2+} (PDB ID: 8Z76, Fig. 2d), and copper (I) ions, pmTcDH^{Cu+} (PDB ID: 8Z77). However, in the case of copper (II) ions, a longer soaking time was required, likely due to the higher energy cost associated

with removing the hydration shell of the copper (II) ion during binding in the enzyme's active site. It should be noted that copper proteins often exhibit selectivity towards the oxidation state of copper ions during their activation in crystals [20, 21]. For instance, in the case of tpTcDH, the complete copper center structure can only be achieved by treating the crystals with copper (I) ions [3], which is often accompanied by structural artifacts.

Each of the three subunits from the asymmetric unit in the structures of pmTcDH^{Cu2+} and pmTcDH^{Cu+} was superimposed with the structures of the apoform subunit and the closed and open holoform subunits (Table 2). The results showed that the incorporation of copper ions into the active site in the crystal induces conformational changes in the apoenzyme molecules.

When the crystals were soaked with copper (II) ions, the flexible loop 251–266 adopted a closed conformation in dimer subunits A and B, while subunit C exhibited two positions for this loop (Fig. 3a). When soaked with copper (I) ions, the loop 251–266

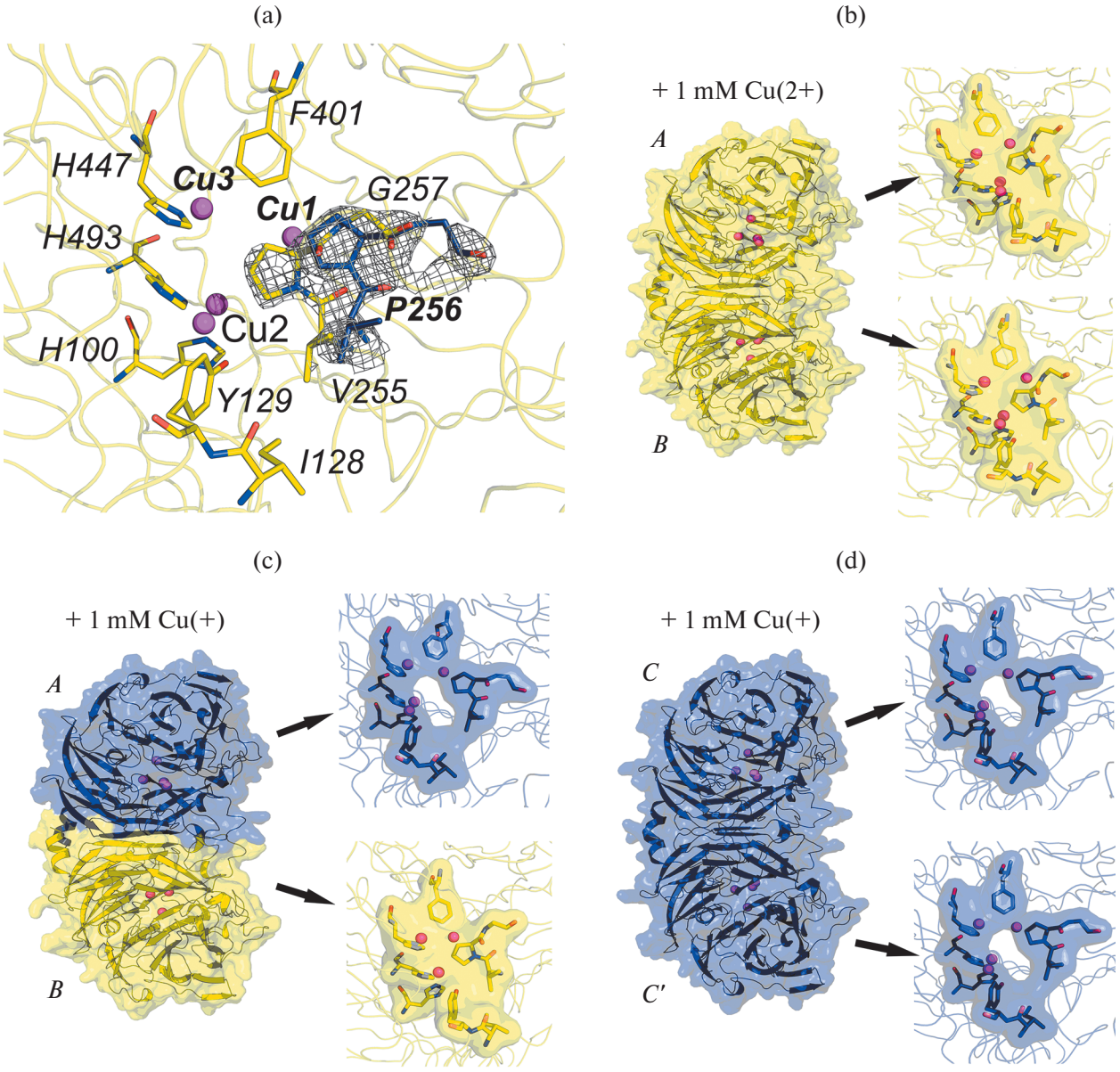

Fig. 3. Two positions of the P256 residue on the difference ($2F_o - F_c$) electron density map, corresponding to the closed and open conformations, in the active site of subunit C of the pmTcDH^{Cu2+} structure after crystal infusion with copper (2+) ions (PDB ID: 8Z76) (a). Conformations of subunits of the pmTcDH dimer after crystal infusion with copper ions: two closed subunits A and B in the pmTcDH^{Cu2+} structure (PDB ID: 8Z76) (b), open A (top) and closed B (bottom) subunits in the pmTcDH^{Cu+} structure (PDB ID: 8Z77) (c), two open C subunits and a symmetrical C in the pmTcDH^{Cu+} structure (PDB ID: 8Z77) (d). Panels b–d show insets depicting the residues that form the substrate channel.

in subunit B remained in a closed position, while in subunits A and C, the loop transitioned to an open conformation. It is noteworthy that residues Q347 and F401, which also influence the transition between open and closed conformations, were poorly defined in the electron density maps of both copper-soaked structures.

The obtained structural data allowed characterization of the conformational states of the pmTcDH dimer with a complete trinuclear copper center, where both dimer subunits can be closed (subunits A and B in the pmTcDH$^{Cu^{2+}}$ structure, Fig. 3b), adopt different conformations (A and B in the pmTcDH$^{Cu^{+}}$ structure, Fig. 3c), or be open (C in the pmTcDH$^{Cu^{+}}$ structure, Fig. 3d). Based on the obtained data, it can be concluded that crystal contacts do not restrict conformational transitions of pmTcDH subunits, as subunits A and C, which form more contacts, demonstrate greater flexibility of the loop 251–266 and residues Q347 and F401 compared to subunit B in both pmTcDH$^{Cu^{2+}}$ and pmTcDH$^{Cu^{+}}$ structures.

Thus, the results of this structural study of the apoform pmTcDH and the restoration of the enzyme's complete copper center by soaking crystals with copper ions, along with previously obtained data for the holoform [4], suggest that copper ions in the active site influence the packing of enzyme molecules in the crystal and their preferred conformation. Symmetric contacts do not restrict transitions between the enzyme's closed and open conformations. In the absence of cofactors in the active site, pmTcDH molecules adopt the most rigid — closed — conformation.

CONCLUSION

It has been shown that the packing of pmTcDH molecules in the crystal and the conformation of enzyme subunits depend on the presence of copper ions in the active site. In the apoform, both subunits of the dimer adopt a closed conformation. The complete trinuclear copper center in the pmTcDH structure can be restored by soaking the crystals with either copper (II) or copper (I) ions. The incorporation of copper ions into the enzyme's active site during soaking induces conformational transitions between closed and open subunit conformations.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTERESTS

The authors of this work declare that they have no conflicts of interest.

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