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# Effect of oxidative stress on cystine transportation by $x\text{C}^-$ antiporter

Maryam Ghasemitearei<sup>1,2</sup>, Maksudbek Yusupov<sup>2</sup>, Jamoliddin Razzokov<sup>2</sup>, Babak Shokri<sup>1</sup> and Annemie Bogaerts<sup>2</sup>

<sup>1</sup> Physics Department, Shahid Beheshti University, G.C., Evin, Tehran 19839, Iran

<sup>2</sup> Research Group PLASMANT, Department of Chemistry, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium

E-mail: qasemi.maryam.mamh@gmail.com

Mar\_ghasemi@sbu.ac.ir

Maryam.Ghasemitearei@uantwerpen.be

## Abstract

We performed computer simulations to investigate the effect of oxidation on the extracellular cystine (CYC) uptake by the  $x\text{C}^-$  antiporter. The latter is important for killing of cancer cells. Specifically, applying molecular dynamics (MD) simulations we studied the transport of CYC across xCT, i.e., the light subunit of the  $x\text{C}^-$  antiporter, in charge of bidirectional transport of CYC and glutamate. We considered the outward facing (OF) configuration of xCT, and to study the effect of oxidation, we modified the Cys<sub>327</sub> residue, located in the vicinity of the extracellular milieu, to cysteic acid (CYO<sub>327</sub>).

Our computational results showed that oxidation of Cys<sub>327</sub> results in a free energy barrier for CYC translocation, thereby blocking the access of CYC to the substrate binding site of the OF system. The formation of the energy barrier was found to be due to the conformational changes in the channel. Analysis of the MD trajectories revealed that the reorganization of the side chains of the Tyr<sub>244</sub> and CYO<sub>327</sub> residues play a critical role in the OF channel blocking. Indeed, the calculated distance between Tyr<sub>244</sub> and either Cys<sub>327</sub> or CYO<sub>327</sub> showed a narrowing of the channel after oxidation. The obtained free energy barrier for CYC translocation was found to be 33.9 kJmol<sup>-1</sup>, indicating that oxidation of Cys<sub>327</sub>, by e.g., cold atmospheric plasma, is more effective in inhibiting the  $x\text{C}^-$  antiporter than in the mutation of this amino acid to Ala (yielding a barrier of 32.4 kJmol<sup>-1</sup>). The inhibition of the  $x\text{C}^-$  antiporter may lead to Cys starvation in some cancer cells, eventually resulting in cancer cell death.

## 1. Introduction

In recent years, cold atmospheric plasma (CAP) is frequently applied in biology and medicine [1]. Fields of application include the treatment of dental cavities [2], sterilization of various surfaces [3], cancer treatment [4], and wound healing [5]. Particularly, in the last decade, CAP has shown considerable anti-cancer capacity for various types of cancer cells [6]. CAP (or simply plasma) can selectively treat cancer cells, without (seriously) damaging normal cells [7-9], and up to now, cancer cells have not yet developed resistance against plasma treatment [10-13]. The generally accepted theory about the anti-cancer ability of plasma is based on oxidative stress [14]. The reactive oxygen species (ROS) formed in plasma, which can also be generated by some other traditional anti-cancer therapies, are believed to be responsible for apoptosis in cancer cells. The different cell cycle of healthy and cancer cells [15], the higher concentration of ROS and RNS (i.e., reactive nitrogen species) found in cancer cells [16], the higher expression of aquaporin channels in cancer cell membranes [17, 18], and the different lipid composition and cholesterol fraction in the membrane of healthy and cancer cells [19] are probably the reasons why cancer cells are more vulnerable to plasma treatment. Another theory suggests that plasma species are able to attenuate the antioxidant system of cancer cells [20]. Plasma oxidation of amino acids in proteins, especially oxidation of the thiol groups of Cys residues, can disturb the normal function of some antioxidant enzymes [21]. Indeed, the ROS-

induced protein modifications can alter the protein structure and disrupt their function [22]. In particular, a recent study on CAP oxidation of the human epidermal growth factor demonstrated that oxidation leads to a change in structural conformation of this protein, and in its binding affinity with the receptor [23].

In the present study, we will take another step towards the understanding of the selective anti-cancer capacity of plasma, based on the transmembrane protein  $xC^-$  antiporter. The  $xC^-$  antiporter is responsible for translocation of cystine (CYC, i.e., the negatively oxidized dimeric form of Cys [24]) from the extracellular milieu to inside the cell, and for sending out glutamate (Glu) from the intracellular fluid to outside the cell [25]. Inside the cell, CYC is rapidly modified to Cys that takes part in some synthesis reactions, especially in glutathione biosynthesis. Glutathione is a tripeptide thiol, consisting of Glu, Cys and Gly, which plays an important role in protecting cells against intracellular oxidative stress [25]. The  $xC^-$  antiporter is more essential for cancer cells than for healthy cells, as some cancer cells are unable to synthesize intracellular Cys [26]. For instance, lymphoma and leukemia cells do not have any endogenous Cys-synthetic ability, and hence, the CYC uptake from the extracellular milieu by  $xC^-$  antiporters is important for their viability. Thus, inhibition of this antiporter in cancer cells may lead to Cys starvation, and thus to a decrease of the intracellular glutathione concentration. The depletion of intracellular glutathione levels can subsequently inhibit the cell growth, thereby inducing apoptosis and reducing cancer cell drug resistance [25].

Another difference between healthy and cancer cells is related to their extracellular pH environment. Cancer cells are able to upregulate anaerobic glycolysis, even in the presence of oxygen. Glycolysis is the pathway that converts glucose to pyruvate, which is useful to supply free energy to form high energy molecules, i.e., adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide bonded by a hydrogen atom (NADH). The anaerobic glycolysis stimulates the conversion of pyruvate to lactic acid [27]. To avoid acid-induced apoptosis, some transporters on the membrane of cancer cells export the accumulating acid (i.e., lactic acid) to the extracellular environment [28]. Hence, the extracellular pH in cancer cells becomes acidic. This acidic environment in cancer cells reduces the CYC uptake [29, 30]. In this regard, it is interesting to know how plasma, through oxidizing the extracellular amino acid residues of  $xC^-$  antiporter, influences the CYC uptake.

Because of the importance of CYC in cancer cell viability, the upregulation of  $xC^-$  antiporter has been demonstrated in some cancer cells, such as lymphomas, gliomas and pancreatic cancer cells [31, 32]. The main functional subunit of the  $xC^-$  protein is  $xCT$  (i.e., the so-called light subunit) that is responsible for bidirectional transport of CYC and Glu [25]. The heavy subunit of  $xC^-$  antiporter (i.e., 4F2hc) is responsible for holding the antiporter in the membrane. Figure 1 schematically illustrates the light and heavy subunits of  $xC^-$  antiporter. During the counter-transport (i.e., the import of CYC and export of Glu), the  $xCT$  subunit of  $xC^-$  antiporter can have three conformations, i.e., outward facing (OF), occluded (OCC) and inward facing (IF), the structures of which were modeled recently, and more information about their functions is given in [33].

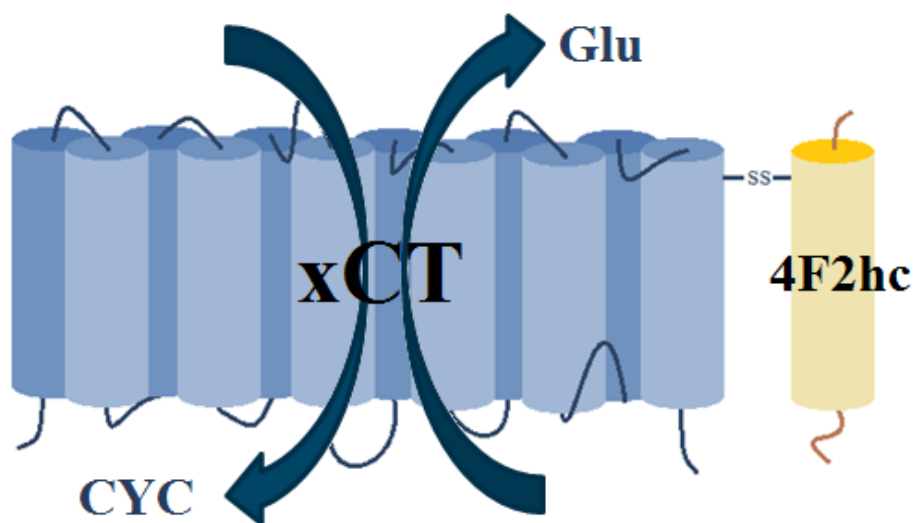


Figure 1. Schematic representation of the light ( $xCT$ ) and heavy ( $4F2hc$ ) subunits of  $xC^-$  antiporter.

One of the important amino acids of the xCT subunit is Cys<sub>327</sub>, which is not highly conserved, i.e., it is a main target for modification (e.g., mutation or oxidation). Experiments demonstrated that the mutation of this amino acid to Ala, Ser and Thr slightly reduces, whereas mutation to Leu even suppresses the transport function of xC<sup>-</sup> antiporter [34]. Cys<sub>327</sub> is highly reactive and located in the vicinity of the extracellular milieu, the protein channel and the substrate binding site in the OF state of the xCT subunit [34]. Thus, it is conceivable that during plasma treatment, this residue can be oxidized by plasma-generated species. The oxidative modification of Cys<sub>327</sub>, on the other hand, can also alter the channel conformation and hence the CYC uptake. In addition, Cys can be oxidized in different pathways at different pH conditions. Generally, the thiol group of Cys is easily oxidized by ROS and RNS [35]. In fact, because of the intracellular ROS, especially hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 5% of the Cys residues of cellular proteins are oxidized to sulfonic or cysteic acid [36]. The oxidation pathways of Cys are related to the structure of the oxidant and the reaction conditions [37], such as pH. Previous studies showed that in basic pH and in the presence of H<sub>2</sub>O<sub>2</sub>, Cys is oxidized to Cys sulfonic acid [37, 38]. In acidic pH, and in the presence of hydroxyl radical (OH<sup>•</sup>), H<sub>2</sub>O<sub>2</sub> and superoxide (O<sub>2</sub><sup>-</sup>), which are all generated by CAP, Cys is oxidized to cysteic acid [38]. These two kinds of oxidation are biologically irreversible reactions, while oxidation of Cys to CYC in neutral pH is a completely reversible reaction.

Based on these considerations, in this study, we investigate the effect of Cys<sub>327</sub> oxidation to cysteic acid on the transport of CYC across native and oxidized xCT, by applying umbrella sampling (US) simulations. For this purpose, we use the OF conformation of the xCT subunit, which is the first conformation of the xCT subunit (see above); thus, it has access to the extracellular milieu and is completely closed from the intracellular fluid.

## 2. Computational details

### *Model systems*

The model system of the native OF conformation is composed of the OF protein embedded in a fully-hydrated palmitoyl-oleoyl-phosphatidylcholine (POPC) bilayer. This system was used in our previous study and a detailed explanation of its preparation is given there [33] as well as in the Supplementary Information. Briefly, the protein sequence (FASTA) of the human xCT [39] subunit was extracted from the NCBI website (<https://www.ncbi.nlm.nih.gov/>). To construct the OF conformation of xCT, the OF open face of AdiC (PDB ID: 3OB6 [40]) antiporter with similarity 24.83% was chosen as a template, applying the SWISS-MODEL server [41], since the 3D structure of this system was not available in the protein data bank. Subsequently, the OF protein was incorporated into the fully-hydrated POPC lipid bilayer, applying the CHARMM GUI web server [42, 43]. The orientation of the model protein into the lipid bilayer surrounding it was determined by the OPM [44] database (<http://opm.phar.umich.edu/>). To build the oxidized OF system, we modified Cys<sub>327</sub> of the native OF protein to cysteic acid (CYO<sub>327</sub>), applying the GaussView software version 5.0.8 [45].

### *Molecular dynamics simulation protocols*

Molecular dynamics (MD) simulations were carried out using the GROMACS 5.0.4 package [46, 47]. Both the native and oxidized OF systems were prepared as follows. The systems were initially energy minimized, applying the steepest descent algorithm. Subsequently, they were equilibrated for 500 ns in the isothermal-isobaric (NPT) ensemble at 310 K and 1 atmosphere. These simulations were repeated three times by changing the values of the initial velocities of the atoms in the system in order to ensure that all three structures of each system are consistent. The Nose-Hoover thermostat [48] in combination with a coupling constant of 1 ps and the semi-isotropic Parrinello-Rahman barostat [49] with a compressibility of  $4.5 \times 10^{-5} \text{ bar}^{-1}$  and a coupling constant of 5 ps were applied to equilibrate the systems. The cut-off distance of both the electrostatic and van der Waals interactions was chosen as 12 Å. Electrostatic interactions were treated by the particle mesh Ewald method [50, 51] and long-range dispersion corrections were applied for both energy and pressure. The net positive charges of both systems were neutralized by adding chloride ions. Overall, both systems were composed of 202 POPC lipids, the native or oxidized OF protein and 15000 water molecules covering them from top

(extracellular part) and bottom (intracellular part), see Supplementary Fig. S2. Both systems were simulated under periodic boundary conditions and the dimension of these systems was  $\approx 105 \times 105 \times 95 \text{ \AA}^3$ . A time step of 2 fs was used in all simulations, and the CHARMM36m force field was applied to describe the interatomic interactions in the systems in combination with the TIP3P water model [52]. The CHARMM-type force field parameters for CYC and cysteic acid (i.e.,  $\text{CYO}_{327}$  residue, see above) of the oxidized OF system were derived as follows. Similar to the methods applied by Spoel et al. [53], to obtain the force field parameters of CYC and  $\text{CYO}_{327}$ , the Gaussian16 software [54] and the CHARMM general force field (CGenFF) program [55] were implemented. Specifically, to optimize the structures and compute the partial charges, the Gaussian software was used by applying DFT calculations, including the B3LYP functional with standard 6-311G\* basis set. It should be noted that according to literature, cysteic acids are formed in proteins as a result of ROS or RNS interactions, and they mostly contain negative charges [36]. Therefore, we also used the negatively charged  $\text{CYO}_{327}$  in our simulations. The CGenFF program was applied to obtain the parameters for bonds, angles and dihedrals. Finally, the parameters were again renewed by adding the Gaussian-made partial charges to CGenFF-made parameters. The details of the partial charges of the residues in acidic pH of the oxidized OF system are presented in the Supplementary Information. The funnel radius analysis and the US simulations (see below) were applied after equilibration of both systems for 500 ns. Note that this equilibration time was sufficient, as the calculated root mean squared deviations (RMSDs) of both the native and oxidized OF systems showed a convergence after 400 ns (see Supplementary Fig. S3). The non-bonded (i.e., Coulomb + van der Waals) interaction energies between some amino acid residues of both systems were computed using the *gmx energy* tool of GROMACS.

#### ***Calculation of the funnel radii***

We applied the HOLE program [56] to calculate the profiles of the funnel radii across the channels of both the native and oxidized OF systems. This program is useful to visualize and analyze the pore dimension of protein structures, such as ion channels and transporters. It computes the largest radius of protein channels along the channel vector (i.e., z direction in our case), without overlapping with the van der Waals surface of any atoms. Around 60 configurations were extracted from the last 50 ns of the equilibration simulations (see above) to obtain the average funnel radii along the z direction (i.e., transporter axis) for both native and oxidized OF systems.

#### ***Umbrella sampling (US) simulations***

To compute the potentials of mean force (i.e., free energy profiles (FEPs)) of CYC translocation across both the native and oxidized OF systems, the US method was employed. In both structures, the initial position of CYC was chosen in the extracellular milieu, i.e., at -3.9 nm distance from the center of mass (COM) of the protein (i.e., in the z position close to the aperture of the protein channel). CYC was then pulled against the COM of both systems along the z direction with a harmonic biasing force constant of  $1000 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{nm}^{-2}$  and pulling rate of  $0.01 \text{ nm} \cdot \text{ps}^{-1}$ . After pulling CYC for 1100 ps, 98 windows (i.e., 49 for the native and 49 for the oxidized system), separated by a distance of  $\leq 0.1 \text{ nm}$ , were extracted for the US simulations for both the native and oxidized structures. In this manner, the sampling windows spanned the whole protein channel, starting from the extracellular milieu and ending at the end of the protein channel, where the channel is closed from the intracellular milieu. Subsequently, the US simulations were run for 20 ns and the last 15 ns of the trajectory was used to obtain the FEPs. To construct the FEPs the periodic version of the weighted histogram analysis method (WHAM) [57] was applied. Errors associated with the sampling were computed by the bootstrapping method. At last, the final FEP of each system was obtained by averaging over four independently calculated FEPs. These FEPs were calculated using four configurations (for each system) derived from the last 50 ns (i.e., taken at 470, 480, 490 and 500 ns) of the equilibration simulations. In total,  $98 \times 4 = 392$  US simulations were performed to obtain the FEPs for each system.

### **3. Results and Discussion**

#### ***Funnel radii analysis***

Figure 2A compares the profiles of the averaged funnel radii, i.e., the largest radii of the pore channels without overlapping with the van der Waals surface of any atom, of the native and oxidized OF systems along the z direction (i.e., across their channels). It is clear that the channel of the native OF system is fully open for CYC passage to the substrate binding site ( $z \approx -0.5$ – $0.5$  nm), while it is completely closed in the oxidized OF structure, preventing the CYC access to the substrate binding site. The latter can be seen by looking at the funnel radius for the negative values on the transporter axis, which drops to 0.1 nm around  $-0.8$  nm of the protein channel, which is too small for CYC to pass, while the funnel radius of the native OF system is large enough (i.e.  $\sim 0.37$  nm) in this region. The closure of the pore in the oxidized OF system blocks the access of CYC to the substrate binding site. Therefore, the extracellular CYC is not able to translocate towards the xCT substrate binding site (see also the US results below).

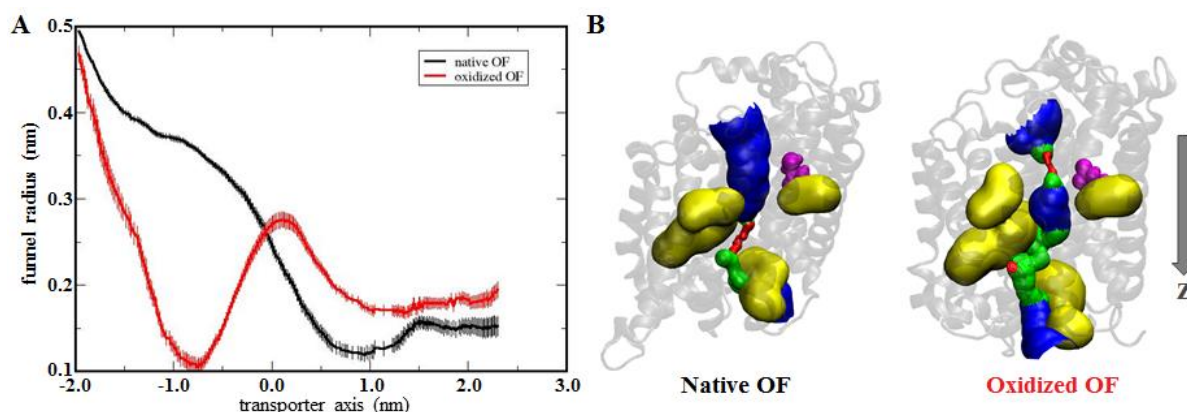


Figure 2. (A) Profiles of the funnel radii of the native (black) and oxidized (red) OF structures along the main axis ( $z$  direction), averaged over the 60 different profiles for each system. (B) Illustration of the changing size (pore radius) of the channels for native (left) and oxidized (right) OF structures, as well as the position of Cys<sub>327</sub> (left) and CYO<sub>327</sub> (right) relative to the protein channel (magenta color). Red color indicates that the pore radius is too tight for a water molecule, whereas green shows that there is room for a single water molecule, and blue is used where the radius is double the minimum for a single water molecule. The residues involved in the substrate binding site are illustrated in yellow color. The rest of the protein is shown in light gray Newcartoon view.

Figure 2B illustrates the protein channel of the native and oxidized OF systems, and how its size varies along the  $z$  direction, which is shown in different colors (i.e., red, green and blue). The red color corresponds to very tight parts of the channels (i.e., channel radius below 0.115 nm), where even a single water molecule cannot permeate (i.e., the passage of CYC is totally blocked). The parts with green color indicate that the channels have enough space for passage of a single water molecule, but CYC still cannot translocate (i.e., channel radius between 0.115 and 0.230 nm). Hence, only in the blue parts of the channels (i.e., radius above 0.230 nm) the passage of CYC is possible. It is obvious from Fig. 2B that the substrate binding site is not accessible from the extracellular milieu in the oxidized OF system, whereas it is fully accessible in the native OF system. The substrate binding site consists of residues that form temporary bonds with the substrate (i.e., CYC) and play an important role in guiding the substrate into the protein channel. The binding sites on proteins are critical parts of signal transduction pathways [58]. In our previous study we found that Arg<sub>135</sub>, Arg<sub>396</sub>, Arg<sub>126</sub>, Lys<sub>198</sub> and Arg<sub>340</sub> are the most important residues in the substrate binding site of the OF state of the xCT subunit, as they strongly bind to CYC [33]. These amino acids are illustrated in Fig. 2B by the yellow color, and they are accessible to CYC only from the protein channel. Therefore, if the channel becomes closed, they will not be accessible for CYC from the extracellular milieu. This is due to oxidation of Cys<sub>327</sub> to CYO<sub>327</sub> that leads to conformational changes in the channel, thereby affecting the CYC permeation (see next section).

### Conformations of the native and oxidized OF structures

We found from previous section that the channel of the oxidized OF system is completely closed from the extracellular milieu, which is the result of Cys<sub>327</sub> oxidation to CYO<sub>327</sub>. Monitoring of the amino acids around the closed part showed that the rearrangements in the side chains of Tyr<sub>244</sub> and CYO<sub>327</sub> residues have a critical role in closure of the OF channel. Figure 3 shows the positions of Tyr<sub>244</sub> and Cys<sub>327</sub> (Fig. 3A), as well as Tyr<sub>244</sub> and CYO<sub>327</sub> (Fig. 3B) of the native and oxidized OF channels, respectively. The comparison of Fig. 3A and B clearly shows that the rotation of the Tyr<sub>244</sub> side chain and the change in orientation of the CYO<sub>327</sub> side chain towards Tyr<sub>244</sub> leads to a closure of the channel. Indeed, the calculated distance between the alcohol group of Tyr<sub>244</sub> and the sulfur atom of CYO<sub>327</sub>, (i.e., ~8.2 Å, see Table 1) was found to be shorter than that between the alcohol group of Tyr<sub>244</sub> and the sulfur atom of Cys<sub>327</sub> (i.e., ~15.5 Å, see Table 1), resulting in a narrowing of the channel after oxidation took place.

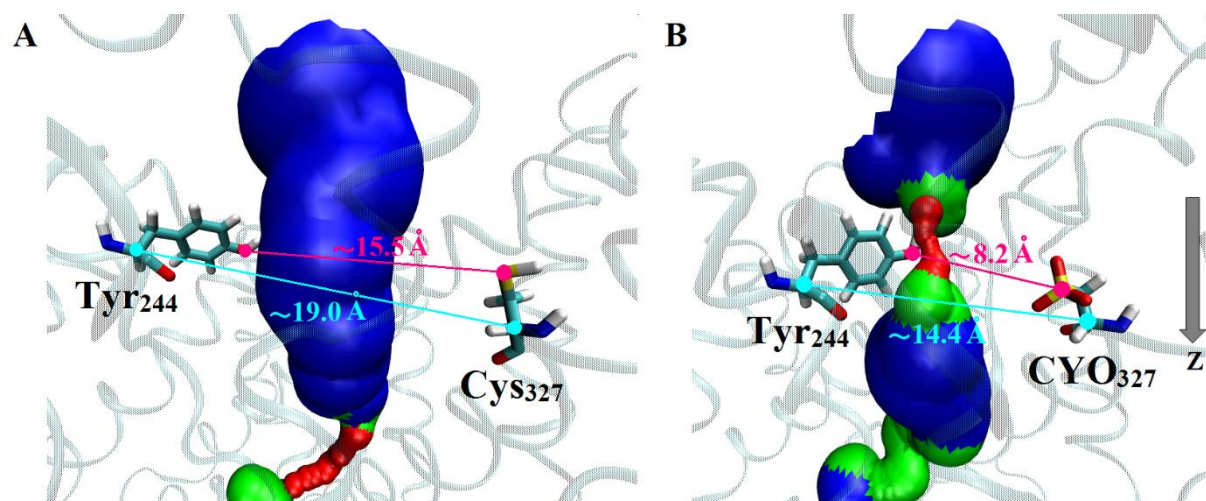


Figure 3. Orientations of Tyr<sub>244</sub> and Cys<sub>327</sub> (A) and Tyr<sub>244</sub> and CYO<sub>327</sub> (B) in the channels of the native (A) and oxidized (B) OF proteins, indicating the distance between the alcohol group of Tyr<sub>244</sub> and the sulfur atom of Cys<sub>327</sub> (~15.5 Å) (A), and between the alcohol group of Tyr<sub>244</sub> and the sulfur atom of CYO<sub>327</sub> (~8.2 Å) (B), and hence the narrowing of the channel upon oxidation.

Krammer et al. [59] determined that the rotameric state of Trp<sub>202</sub> in the AdiC transporter structure was crucial for occlusion of the OF to OCC state. Their study stressed that the OF state of the AdiC conformation without proper rotation of the Trp<sub>202</sub> side chain cannot occlude completely. This amino acid is equivalent to Tyr<sub>244</sub> in the xCT subunit (i.e., at the same position as Tyr<sub>244</sub> in the amino acid sequence). Thus, we believe that Tyr<sub>244</sub> plays an important role in channel closure, which was the case in our study.

Table 1 illustrates the averaged distance between the backbones (i.e., alpha carbons) and side chains of Tyr<sub>244</sub> and Cys<sub>327</sub> for the native OF state and of Tyr<sub>244</sub> and CYO<sub>327</sub> for the oxidized OF state of the xCT subunit.

Table 1. Averaged distance between the backbones (i.e., C<sub>α</sub>-C<sub>α</sub>) and sidechains (i.e., S-OH) of Cys<sub>327</sub> and Tyr<sub>244</sub> in the native OF state and of CYO<sub>327</sub> and Tyr<sub>244</sub> in the oxidized OF proteins.

residues	C <sub>α</sub> -C <sub>α</sub> (Å)	S-OH (Å)
Cys <sub>327</sub> -Tyr <sub>244</sub> (native OF)	19.04 ± 0.03	15.47 ± 0.04
CYO <sub>327</sub> -Tyr <sub>244</sub> (oxidized OF)	14.44 ± 0.02	8.15 ± 0.02

It is obvious that after oxidation of Cys<sub>327</sub> to CYO<sub>327</sub> the overall distance between these residues and Tyr<sub>244</sub> decreases, which is most clear for S-OH (i.e., drop by about a factor two). This again indicates that Cys<sub>327</sub> oxidation in the OF structure leads to conformational changes in the channel, resulting in closure of this channel and hence blockage of the CYC uptake.

### Analysis of the interaction energies

Table 2 illustrates the non-bonded (i.e., Coulomb + van der Waals) interaction energies between Cys<sub>327</sub>-Tyr<sub>244</sub>, Cys<sub>327</sub>-rest of the system and Tyr<sub>244</sub>-rest of the system in the native OF system, as well as between CYO<sub>327</sub>-Tyr<sub>244</sub>, CYO<sub>327</sub>-rest of the system and Tyr<sub>244</sub>-rest of the system in the oxidized OF system.

*Table 2. Non-bonded (i.e., Coulomb + van der Waals) interaction energies calculated for Cys<sub>327</sub>, CYO<sub>327</sub> and Tyr<sub>244</sub> of the native and oxidized OF systems, with each other and with the rest of the system. Note that the interaction energies between Cys<sub>327</sub>-Tyr<sub>244</sub> and CYO<sub>327</sub>-Tyr<sub>244</sub> correspond to the native and oxidized OF structures, respectively. The same applies to Cys<sub>327</sub>-rest of the system and CYO<sub>327</sub>-rest of the system.*

protein	Cys <sub>327</sub> /CYO <sub>327</sub> -Tyr <sub>244</sub> (kJ/mol)	Cys <sub>327</sub> /CYO <sub>327</sub> -rest (kJ/mol)	Tyr <sub>244</sub> -rest (kJ/mol)
native OF	≈ 0	-5.1 ± 0.7	-82.2 ± 2.0
oxidized OF	≈ 0	-160.0 ± 6.2	-100.6 ± 1.3

It is obvious that after oxidation of Cys<sub>327</sub> to cysteic acid (CYO<sub>327</sub>), the interaction energy becomes more negative (i.e., stronger interaction) in all cases, although the change in energy for CYO<sub>327</sub>-Tyr<sub>244</sub> (second column in Table 2) was not significant. The latter is probably because the distance between CYO<sub>327</sub> and Tyr<sub>244</sub> is still relatively long (~ 8.2 Å; see Fig. 3B and Table 1), which is not enough to make a H-bond, although this H-bond interaction for the S-H system is inherently weaker [60]. On the other hand, the interaction energy between CYO<sub>327</sub> and the rest of the system, attributed to the attractive (namely, Coulomb) interaction of three oxygen atoms in the side chain of CYO<sub>327</sub> with the surrounding amino acids, was much stronger than the energy between Cys<sub>327</sub> and the rest of system (third column). In general, the movement and rotation of the side chain of Tyr<sub>244</sub> and a change in the orientation of the side chain of CYO<sub>327</sub> results in a rearrangement of the surrounding amino acids, bringing Tyr<sub>244</sub> and CYO<sub>327</sub> in the vicinity of some new amino acids, allowing to make H-bonds with some of them. These mediator amino acids are Ala<sub>60</sub>, Gly<sub>61</sub>, Ala<sub>145</sub>, Phe<sub>146</sub> and Lys<sub>198</sub>, resulting in H-bonds with all of them, while they did not have any H-bonds with Cys<sub>327</sub> and Tyr<sub>244</sub> prior to Cys<sub>327</sub> oxidation. Moreover, some other amino acids, such as Phe<sub>63</sub>, Ile<sub>64</sub>, Thr<sub>139</sub>, Arg<sub>148</sub>, Tyr<sub>149</sub>, Ser<sub>330</sub> and Met<sub>331</sub>, make H-bonds with both CYO<sub>327</sub> and Tyr<sub>244</sub>, while they had H-bonds with only the backbone of Cys<sub>327</sub> before oxidation. Specifically, among all mentioned amino acids, Ala<sub>60</sub>, Gly<sub>61</sub> are found to be the most important ones because of two reasons. Firstly, they are in the vicinity of the closed parts of the protein channel (cf. red color part in Fig. 3B). Secondly, they did not have any H-bonds with Cys<sub>327</sub> and Tyr<sub>244</sub> before oxidation. The positions of Ala<sub>60</sub> and Gly<sub>61</sub> relative to the channels of both the native and oxidized OF proteins are illustrated in the Supplementary Fig. S4. Moreover, the distances between these two amino acids and other residues are given in Table S2, together with the explanation about their interactions (see the Supplementary Information). It is important to mention that the distance of Ala<sub>60</sub> and Gly<sub>61</sub> with CYO<sub>327</sub> partially increased, whereas their distances with Tyr<sub>244</sub> decreased noticeably. In general, in the oxidized OF system, these four amino acids (Ala<sub>60</sub>, Gly<sub>61</sub>, CYO<sub>327</sub> and Tyr<sub>244</sub>) moved toward each other, making the channel more narrow, eventually blocking the protein channel in this area.

We further performed a detailed analysis of the conformational changes together with these important amino acids, by aligning the native and oxidized structures of OF (see Supplementary Fig. S5 and S6 and explanations therein). Specifically, we studied the positions of the transmembrane (TM) domains (i.e., TM1, TM3, TM5, TM6 and TM8), which contain the above mentioned amino acids. In general, we found that among the TM domains, TM1, TM5 and TM6 moved towards TM8, which resulted in a change of the channel size. This subsequently led to a dramatic increase of the interaction energy between CYO<sub>327</sub> and other amino acids (including Ala<sub>60</sub>, Gly<sub>61</sub> and Tyr<sub>244</sub>) surrounding this residue. This is summarized in Fig. 4, illustrating the positions of four important amino acids (i.e., Cys<sub>327</sub>/CYO<sub>327</sub>, Tyr<sub>244</sub>, Ala<sub>60</sub> and Gly<sub>61</sub>) by superimposing the structures of the native and oxidized OF systems. Hence, we can conclude that the reorientation of these amino acids, and thereby the movement of the TM domains to which they belong, led to a narrowing of the channel after oxidation (cf. also Fig. 2 and 3), resulting in a blocking of the access of CYC to the substrate binding site of the OF system.



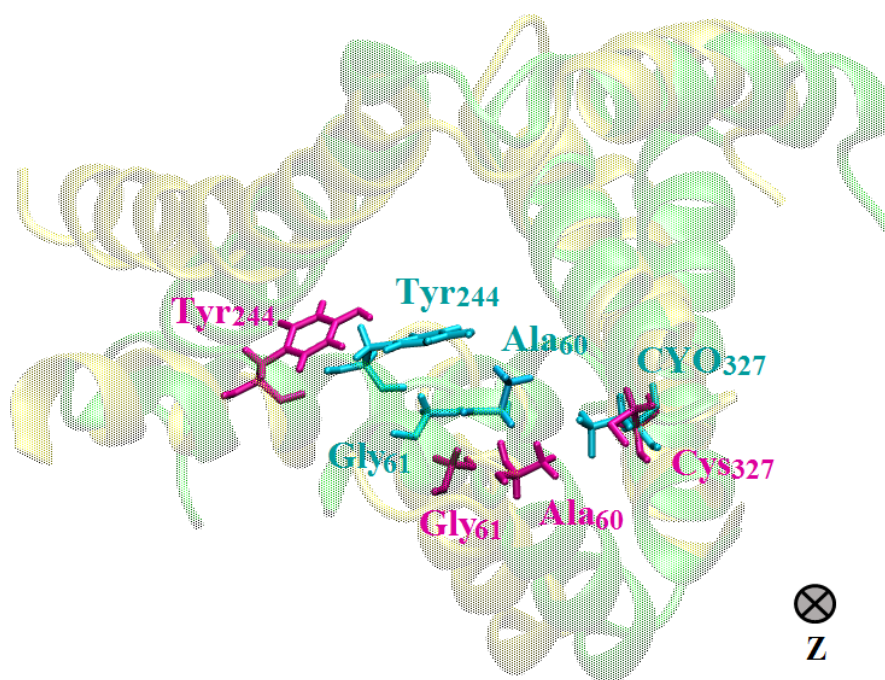


Figure 4. Superimposed structures of the native (yellow) and oxidized (green) OF from top view, together with some important amino acids (i.e., Cys<sub>327</sub>/CYO<sub>327</sub>, Tyr<sub>244</sub>, Ala<sub>60</sub> and Gly<sub>61</sub>) depicted in magenta (native system) and cyan (oxidized system) colors.

#### Free energy profiles (FEPs)

The FEPs of CYC translocation across the channels of both the native and oxidized OF systems are compared in Fig. 5.

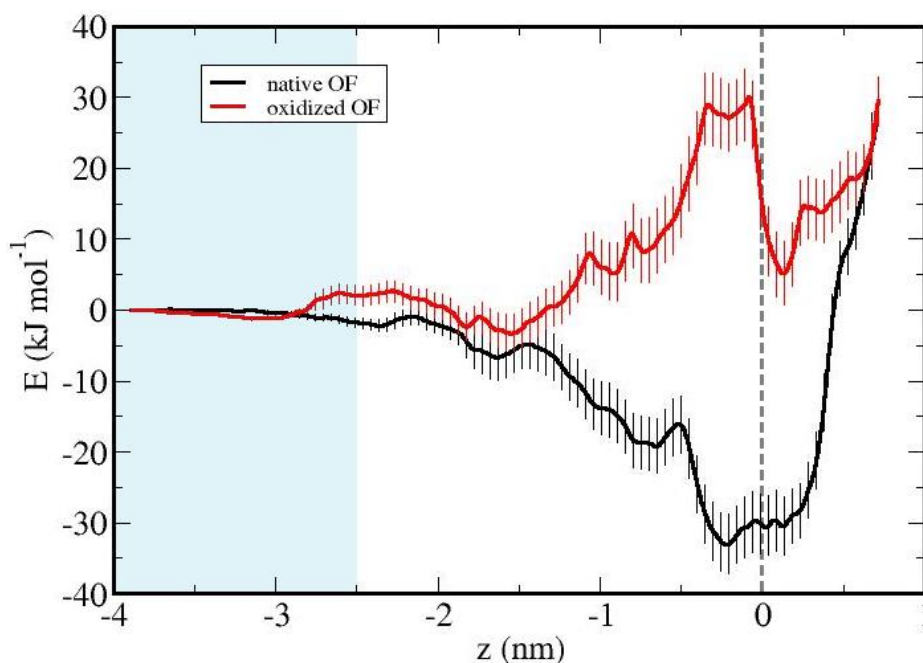


Figure 5. Free energy profiles for the translocation of CYC across the native and oxidized OF structures. The position of the center of mass (COM) is indicated by the gray dashed line. The light blue color corresponds to the extracellular water phase.

It is clear that oxidation of Cys<sub>327</sub> to CYO<sub>327</sub> and thus the reorientation of this and other residues around the substrate binding site, including Tyr<sub>244</sub>, creates a free energy barrier of 33.9 kJ mol<sup>-1</sup> for

CYC translocation, making the CYC penetration to the substrate binding site more difficult than in the native OF state. In our previous study we found that the free energy barrier of CYC translocation through the modified OF state (i.e., Cys<sub>327</sub> modification to Ala<sub>327</sub>) was 32.4 kJ mol<sup>-1</sup>. Hence, the barrier becomes even higher in the oxidized OF system. Our results thus indicate that oxidation of Cys<sub>327</sub> to CYO<sub>327</sub>, which can be caused by cold atmospheric plasma (CAP), is more effective than mutation of this amino acid to Ala in inhibition of the xC<sup>-</sup> antiporter.

## 4. Conclusions

We investigated the effects of Cys<sub>327</sub> oxidation in acidic pH on CYC translocation across the OF conformation of the xCT subunit of the xC<sup>-</sup> antiporter, which might be important as a future cancer therapy target by using CAP. Indeed, in cancer cells an enhanced transport of CYC across the xC<sup>-</sup> antiporter is observed, and this protects the cancer cells from intracellular oxidative stress. If the CYC uptake is inhibited by oxidation of some amino acid residues of the xCT subunit via CAP, this can reduce the cellular protection from oxidative stress, as well as reduce cancer cell drug resistance, eventually leading to cancer cell death.

We applied US simulations to study the permeation of CYC across the native and oxidized OF systems, based on Cys<sub>327</sub> oxidation to cysteic acid (CYO<sub>327</sub>). The free energy profiles of CYC translocation across the native and oxidized OF structures showed that oxidation of Cys<sub>327</sub> to CYO<sub>327</sub> makes the CYC transportation more difficult, creating a barrier of 33.9 kJ mol<sup>-1</sup>, thereby leading to a decrease in the CYC permeation rate. Comparison of the funnel radii profiles of both the native and oxidized OF states showed that oxidation leads to closure of the protein channel from the extracellular milieu, so that the substrate binding site is not accessible anymore from outside of the cell. By monitoring the amino acids around the closed part of the oxidized OF state, we found that oxidation results in a rearrangement of the amino acids around the oxidized Cys<sub>327</sub> in the protein channel. Among these amino acids, Tyr<sub>244</sub> is the most effective one on closure of the protein channel. This amino acid becomes closer to CYO<sub>327</sub> by rotating its side chain, whereas CYO<sub>327</sub> also changes its orientation towards Tyr<sub>244</sub>, together making the channel narrower. These rearrangements of the amino acid residues form H-bonds with some neighboring amino acids, such as Ala<sub>60</sub> and Gly<sub>61</sub>.

This study is important, since it elucidates the effect of oxidation of Cys<sub>327</sub> on closure of the protein channel, thereby making CYC uptake by the xCT subunit of the xC<sup>-</sup> antiporter more difficult. This oxidation can happen as a result of e.g., CAP application to cancer cells at acidic pH. Hence, this study might explain the possible mechanism of CYC starvation in cancer cells, through inhibition of the xC<sup>-</sup> antiporter, which results in a depletion of the intracellular level of glutathione, and consequently, in inhibition of cell growth, inducing apoptosis and reducing the cancer cell drug resistance.

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