

# Manganese in biological systems: Transport and function

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*PATAI'S Chemistry of Functional Groups; The Chemistry of Organomanganese Compounds (2011)*  
Edited by Zvi Rappoport, Online © 2011 John Wiley & Sons, Ltd; DOI: 10.1002/9780470682531.pat0540

## I. ABBREVIATIONS

<b>EDTA</b>	ethylene diamine tetraacetic acid.
<b>OEE</b>	oxygen evolution enhancer.
<b>OM</b>	outer membrane.
<b>PM</b>	plasma membrane.
<b>POX</b>	peroxide.
<b>PSI</b>	Photosystem I.
<b>PSII</b>	Photosystem II.
<b>ROS</b>	reactive oxygen species.
<b>SOD</b>	superoxide dismutase.
<b>TM</b>	thylakoid membrane.
<b>tMI</b>	transition metal ion.

## II. INTRODUCTION

Manganese import, transport, accumulation, sensing and control pathways are important for all organisms, and are of unique importance for oxygenic photosynthetic organisms due to their role in the catalysis of water oxidation by photosystem II (PSII) complexes. In this review we will describe general aspects of Mn biochemistry, its known roles in cellular processes and its mode of function in PSII. We will describe in detail different chemical mechanisms that have evolved to obtain and control Mn ions, focusing on cyanobacterial systems as an example of organisms with absolute Mn requirements that exist in a wide variety of environments. We will describe the steps and mechanisms by which Mn transport is performed under Mn deficient or replete conditions. Under deficient conditions a high affinity Mn transporter is expressed and becomes functional in the cyanobacterial plasma membrane. This ATP dependent transporter must overcome a number of mechanistic problems to perform its function, which is to bind Mn ions with high affinity and specificity at very low Mn concentrations in the possible presence of higher concentrations of similar metal ions. The Mn cluster of PSII functions within the cyanobacterial cell, yet studies suggest that it is assembled in the plasma membrane facing the periplasmic space rather than in the thylakoid membrane. The topology of the two processes, Mn transport and Mn cluster assembly, should therefore be regulated in order to insure efficient biogenesis and repair of PSII complexes. Based on the available results, we attempt to map the constraints imposed by Mn transport on PSII biogenesis events in cyanobacteria.

## III. TRANSITION METALS IN BIOLOGY

Biological viability is dependent on the availability of a large number of chemicals, many of which cannot be synthesized but must be obtained from outside the organism. Many of these essential chemicals, or nutrients, are needed at relatively minute concentrations and are thus collectively called 'micro-nutrients'. Among the micro-nutrients, one of the most important classes are the transition metal ions (tMI). The most prevalent tMIs in most organisms are Fe, Zn, Cu and Mn<sup>1</sup>. tMIs are essential for the function of many proteins, by facilitating redox or chemical group transfer reactions or by stabilizing protein structure. To satisfy the requirements for these metals, cells have numerous mechanisms for the solubilization and uptake of metals from the extracellular environment. Cells must, however, simultaneously protect themselves from the hazards inherent to the chemical characteristics of these metals. If cytosolic metal concentrations are not carefully regulated, spurious redox reactions can produce toxic free radicals. Therefore, each organism contains active

and passive transport systems that identify, bind and transfer tMIs from the outside into the necessary cellular compartments. Almost no free cytosolic tMIs exist, indicating that tMIs are sequestered into their final destination or stored by tMI storage proteins<sup>2</sup>. Excellent reviews have been published over the past 20 years that describe the biochemistry of tMI<sup>2-7</sup>, specifically that of Mn<sup>3,4</sup>. The biochemistry of tMIs cannot be easily described without including the proteins that bind and utilize them for their various activities.

## A. Manganese Characterization in Biological Systems

Mn is recognized as an absolutely required micro-nutrient, as reflected by a number of well-known diseases, disorders or syndromes that occur in organisms suffering from Mn deficiency<sup>8</sup> or uncontrolled excess<sup>9</sup>. The major enzymatic functionalities imparted by Mn on proteins are the ability to reduce reactive oxygen species in Mn-superoxide dismutase (MnSOD) and catalase, in electron transfer dependent catalysis (for instance in certain class I ribonucleotide reductases) and in the oxidation of water by Photosystem II (PSII). In the following section we will discuss issues pertaining to the sensing, transport and control of Mn as well as one of its most important roles in biology, in photosynthesis.

Mn is found in most terrestrial locations, comprising about 0.1% of the earth's crust, but found at concentrations of only about 1–10 nM in the earth's oceans<sup>10</sup>. The intracellular concentration is usually greater by one order of magnitude, but higher concentrations would induce potentially deleterious precipitation reactions with phosphate or carbonate anions. In its solvated form it is found as the stable Mn<sup>2+</sup> ion, and this is the state that is typically recruited and transported into the cell. Once in the cell, and depending on its role, it can be oxidized to other redox stable states. Mn is a versatile tMI in that it has the largest potential range of redox states (–3 to +7), however the actual stable redox states found in biological molecules are typically Mn<sup>2+</sup> and Mn<sup>3+</sup> (and Mn<sup>4+</sup> found in PSII). Both Mn<sup>2+</sup> and Zn<sup>2+</sup> are high spin ( $d^5$  and  $d^{10}$ , respectively), and thus do not exhibit the characteristic energies due to ligand field stabilization. Indeed, Mn and Zn are chemically quite similar and the specificity of binding by different proteins must take this into account. The redox potentials of the different Mn redox states is highly variable, but it is important to recognize that the  $E^0$  of the reduction of Mn<sup>3+</sup> to Mn<sup>2+</sup> (1.56 V) is more than twice that of the similar reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> (0.77 V)<sup>11</sup> and much higher than the reduction of Cu<sup>2+</sup> to Cu<sup>1+</sup>. Thus Mn has the chemical potential to serve in roles requiring the removal and transfer of highly energetic electrons. The actual redox state of a bound Mn ion will be quite different than in standard solutions and will be tuned by the protein environment, especially by the pKa's of nearby acids, by the presence or absence of bound oxygen species (including water) and by the presence of nearby hydrophobic residues.

Mn<sup>2+</sup> is the hardest Lewis acid of the four major tMIs, and will prefer hard ligands in its coordination sphere, such as the negatively charged oxygen atoms of aspartate, glutamate, tyrosinate, the polar oxygen atoms of asparagine, glutamine or small solvated ions such as HO<sup>–</sup> or O<sub>2</sub><sup>–</sup> (or H<sub>2</sub>O which is an intermediate strength Lewis base). The imidazole nitrogen atoms of histidine are considered ligands of borderline hardness and are also found in many cases as appropriate ligands for Mn<sup>2+</sup><sup>11</sup>. The involvement of histidine residues indicates that binding of Mn<sup>2+</sup> within a protein imparts significant changes in its chemical properties as compared to the ion in solution. Mn<sup>2+</sup> coordination geometry is usually square pyramidal or trigonal bipyramidal for coordination number = 5; octahedral geometry is also observed with coordination number = 6 and occasionally 4-coordinated complexes with tetrahedral geometry exist. As we shall describe below, many of the characteristics of Mn binding, in functionally different protein families, are actually quite similar. Rather small variations in the first and second shells surrounding the Mn impart whether its role will be in electron transfer, in reduction of substrates or in transport.

## B. Transition Metals in Photosynthesis

In the photosynthetic apparatus, tMIs are required as cofactors in electron transport processes<sup>12</sup>. Among these are Fe cofactors such as Fe-S clusters, cytochromes and non-heme Fe, Cu in plastocyanin and Mn in Photosystem II. As a result of the absolute importance of photosynthesis for cyanobacteria, algae and plants, the demand for these tMIs far exceeds that required by other non-photosynthetic organisms. The transition from non-oxygenic to oxygenic photosynthesis was an extremely successful step from an evolutionary standpoint<sup>13</sup>, however it resulted in a *ca* 100 times higher internal Mn requirement<sup>14</sup>. Nevertheless, oxygenic photosynthesis is not without its risks. Transporting, accumulating and assembling tMIs into functional cofactors are particularly dangerous tasks when photosynthetic processes are concerned. The photosynthetic apparatus performs some of the most extreme enzymatic reactions, in energetic terms<sup>15</sup>. Consequently, reactive oxygen species can be formed in the course of normal photosynthetic activity. Hot spots for oxygen radical production include antenna complexes, PSII and photosystem I (PSI) (reviewed in Reference 16). In order to overcome inherent dangers from radical chain reactions between transition metals and intermediates in photosynthetic catalysis, the handling of these metals is tightly regulated<sup>16,17</sup>. The evolutionary selection of manganese as a cofactor in PSII water oxidation processes is not a trivial one. Although Mn ions can donate up to 6 electrons, only a limited number of enzymes utilize Mn as a cofactor<sup>17</sup>. Current hypotheses on the origins of water oxidation revolve around the utilizations of bicarbonate-manganese adducts as templates for the evolution of the water splitting enzyme<sup>18</sup>.

## C. Redox Active Mn Enzymes

As described above, Mn serves as the active tMI center in many redox active centers. While each enzyme has its own unique structural characteristics that enable performance of all of its required functionalities, we can reduce our discussion here to the basic facets that are common to all: (i) the requirements for stable and specific binding of Mn; (ii) establishment of the conditions required for efficient electron transfer to and from the bound Mn; (iii) obtaining the correct redox potential for performance of the activity; and (iv) providing structural attributes that allow for useful substrate binding and product release. Of the enzymes that use Mn as its major cofactor, we will use the rather ubiquitous Mn superoxide dismutase (MnSOD) as a general example.

Superoxide dismutases are enzymes that function to catalyze the conversion of a superoxide radical to oxygen and hydrogen peroxide, thus protecting the cell against toxic products of cellular respiration. These enzymes carry out catalysis at near diffusion controlled rate constants via a general mechanism that involves the sequential reduction and oxidation of the metal center, with the concomitant oxidation and reduction of superoxide radicals. The enzymes are classified according to their metal ion in the active site. The catalytically active metal can be copper, iron, manganese or nickel. Structures of the different SOD families have recently been reviewed in depth elsewhere<sup>19</sup>, and we will thus limit our discussion here to questions relevant to Mn chemistry.

The actual binding site of the metals is quite similar, with four ligating residues (His and/or Asp residues) and a bound solvent molecule (water or hydroxide ion) forming a trigonal bipyramidal coordination (Figure 1)<sup>19</sup>. However, despite this similarity, SOD activity requires exquisite matching between the protein environment, which is maintained mostly by second and third shell residues, and the bound tMI. Although the folding and the active site of MnSOD and FeSOD are nearly identical, the replacement of the Mn in *E. coli* MnSOD by Fe inactivates the enzyme. This is most likely due to protein induced changes in the reduction potential ( $E^\circ$ ), which is appropriate for Mn catalysis but not

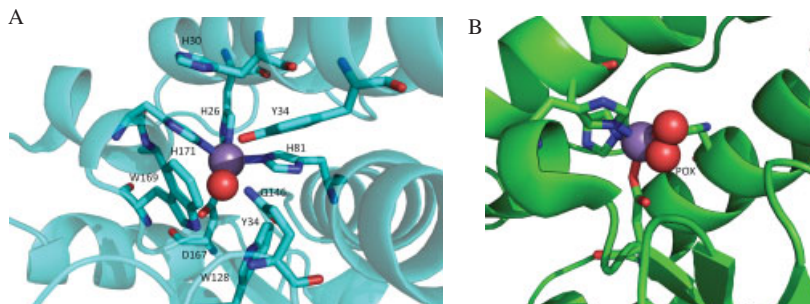


FIGURE 1. The active site of *E. coli* MnSOD (PDB code 2VEW). (A) Bound Mn (pink sphere) with water (red sphere) at ligating position 5. The ligating residues and the second shell of residues that are important for activation and ligand binding are depicted in sticks. (B) MnSOD (PDB code 3K9S) with a bound peroxide molecule (POX, red spheres) replacing the fifth Mn (pink sphere) coordinating ligand

for Fe. Apparently residues in the second coordination sphere strongly affect  $E^\circ$ , thus insuring the effectiveness of the enzyme activity and its specificity<sup>20</sup>.

Crystallography is the major source of high resolution information on MnSOD, although other methods have been utilized<sup>21,22</sup>. Crystallography cannot always provide a definitive visualization of the wild-type active site with bound substrate(s) in pre-reaction binding modes, due to fast reaction rates (nearly diffusion-rate limiting in SOD), crystal lattice effects and the presence of crystal stabilization solutions that are far from the physiological surroundings of the enzyme. Recently, the structure of the MnSOD from *E. coli* was determined with bound peroxide molecule (POX)<sup>23</sup>. This structure actually shows four different views of the same enzyme, since each monomer in the asymmetric unit was trapped in a somewhat different state. The overall conclusion is, however, that the POX molecule replaces the coordinating water molecule at the fifth position in a side-on orientation. Binding of a POX at this position inhibits substrate binding and thus inactivates the enzyme, suggesting that position 5 is where the superoxide substrate binds during the catalysis. The POX binding site is lined by second shell residues (Tyr34, Gln143 and Trp169) which create a hydrogen bond network. It is likely that this bonding network supports proton transfer that is not just critical for catalysis, but also facilitates product release and substrate binding<sup>24</sup>. To conclude, enzymatic activity of the MnSOD is maintained not only by the presence of the correct metal ion at the active site but also by the presence of the second shell residues that provide the specific environment for the catalysis. These residues enable the specificity of the enzyme, which the binding site by itself is unable to provide.

An interesting observation is that in some bacterial species, the accumulation of intracellular Mn can alleviate the stress of reactive oxygen species (ROS) in a MnSOD independent and Mn concentration dependent manner<sup>25,26</sup>. This has been attributed to the direct interaction between  $Mn^{2+}$  ions, imported by energy-dependent import systems (see below). Attempts to link the presence of Mn with other components (proteins, nucleic acids, phosphate ions, etc.) have not yet revealed a mechanism for free-Mn detoxification. In addition, it is not clear how unbound Mn can exist in the cell, which is certainly in opposition to other measurements showing that high intracellular concentrations of  $Mn^{2+}$  will precipitate with important anions. As will be described below, it has been shown that cyanobacteria perform uptake of large amounts of Mn into their outer membrane while all of the intracellular Mn appears to be bound to target proteins.

## IV. Mn TRANSPORT IN CYANOBACTERIA

Mn<sup>2+</sup> cannot freely penetrate into the intracellular compartments through the plasma membrane. Transport of Mn<sup>2+</sup> is performed by a variety of proteins, depending on organism and cell type<sup>17, 27, 28</sup>. Each system has its own unique characteristics, especially the source of energy for transport: ATP in the case of ATP binding cassette (ABC) transporters<sup>29</sup> and P-type ATPases<sup>17, 30</sup> or chemical gradients in the case of ZIP (Zrt and Irt related proteins)<sup>31, 32</sup> or Nramp (natural resistance-associated macrophage protein)<sup>27, 33</sup> transporters. All transporters require some attributes that are similar to those of the Mn dependent enzymes described above—specific Mn binding (although less specific transporters are also known) that is stable enough to promote directional movement across the membrane but that provides fast release as well. Due to the scope of this review, we will focus on one transporter system, the high-affinity Mn ABC transporter in cyanobacteria. Mn is not considered as a limiting factor for primary photosynthetic productivity (unlike Fe), however its concentration is in the low nM range in open oceans<sup>34</sup> and in some fresh bodies of water<sup>35</sup>. For comparison, the Mn concentration in standard bacterial growth media like BG11<sup>36</sup> or A+<sup>37</sup> is in the  $\mu$ M range. In the analysis of Mn transport and homeostasis in cyanobacteria we should consider two conditions; the Mn deficient condition prevailing in open water bodies and the Mn sufficient condition existing for brief periods following aeolian dust depositions<sup>38, 39</sup> and in culture media.

### A. Mn Transport under Deficient Conditions: The MntABC Transporter

Transport of transition metals through biological membranes requires active transport. It is not currently known how organisms actively transport the correct ions at the proper concentrations, since at least some of the known transporters can recognize and deal with multiple ions (IRT1, for example<sup>40</sup>). Currently, only one transporter that exhibits both high affinity and specificity for Mn has been identified and structurally characterized—the MntABC transporter from cyanobacteria<sup>41</sup>, a member of the ABC transporter family<sup>42</sup>. Transporters from this family are ubiquitous, having roles in both import and export. In bacteria, ABC transporters function mainly in transport across the plasma membrane<sup>42</sup> (the locations of proteins discussed in this review are depicted in Figure 2).

MntABC was identified in a mutant screen that took advantage of the glucose sensitive strain of *Synechocystis* sp. PCC 6803<sup>43</sup>. In this strain, only mutants with impaired photosynthetic abilities can survive on plates containing glucose in the light. This method has yielded a number of interesting mutants, including one that was mapped to the *mntCAB* operon encoding for the 3 subunits of the MntABC transporter<sup>41</sup>. The mutant exhibited low oxygen evolution rates and could be rescued by the addition of excess Mn. The function of MntABC mutants was further studied by <sup>54</sup>Mn transport assays<sup>44</sup>. A difference in the transport rate between wild type and mutant cultures was observed only under Mn deficient conditions, leading the authors to suggest that MntABC is a high affinity transporter which is expressed when Mn is scarce. As with all ABC importers, the MntABC has three components, the MntA cytoplasmic nucleotide binding domain (NBD), the MntB transmembrane permease and the MntC solute binding protein (SBP) found in the periplasmic space. The SBP recognizes and binds the substrate tightly, releasing it into the permease which transports it across the membrane. Energy for cycling the components and for the conformational changes required is provided by ATP binding to the NBD, followed by its hydrolysis and the subsequent unbinding of ADP and P<sub>i</sub>. The question that arises is that, since the MntABC system is only expressed at very low Mn concentrations, and since similar ABC importers lack tMI specificity, how does the MntABC system ensure that it is not saturated by other tMIs (especially Zn), thus preventing Mn transport?

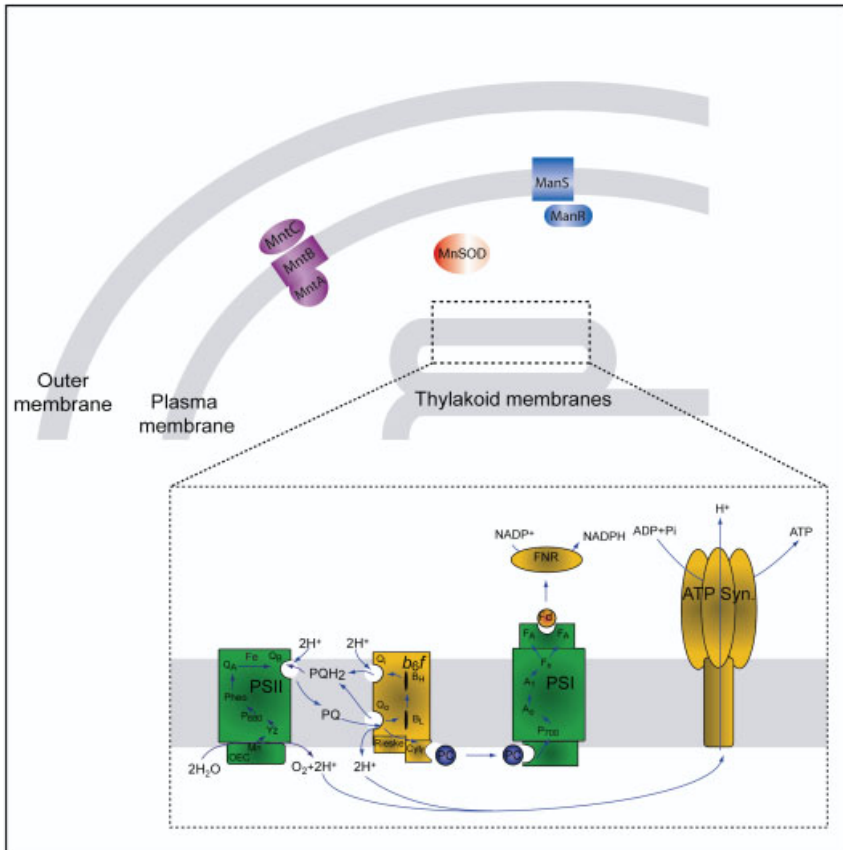


FIGURE 2. Topology of a cyanobacterial cell, the location of Mn enzymes and electron transport chain components. **Top:** Membrane architecture of a cyanobacterial cell with internal thylakoid membrane and external plasma and outer membranes. The locations of the Mn enzymes ManS/ManR, MnSOD, MntABC are noted. **Bottom:** The organization of the protein complexes involved in the electron transport chain in the thylakoid membranes of cyanobacteria, PSII, Cytochrome  $b_6/f$  PSI and ATP synthase

The structure of the periplasmic binding protein, MntC, was resolved by X-ray crystallography (PDB code 1XVL), which discovered a unique and surprising feature<sup>45</sup>. While the overall sequence similarity with other tMI SBPs is not high (20–30%), the overall structure (Figure 3) was expected (and later found) to be quite similar to other SBPs. When phases were obtained, it was revealed that the asymmetric unit of the crystal contained two forms of the protein; two monomers contained an oxidized disulfide bond while in the third monomer the cysteines were reduced. Mn was shown to bind tightly to the protein with oxidized disulfide bond, but was released upon disulfide reduction. The existence of a redox active cysteine pair in a periplasmic solute binding protein was unexpected, and immediately hinted that this system may be under the control of other redox active components. We suggested a regulatory role for this feature, changing the protein from

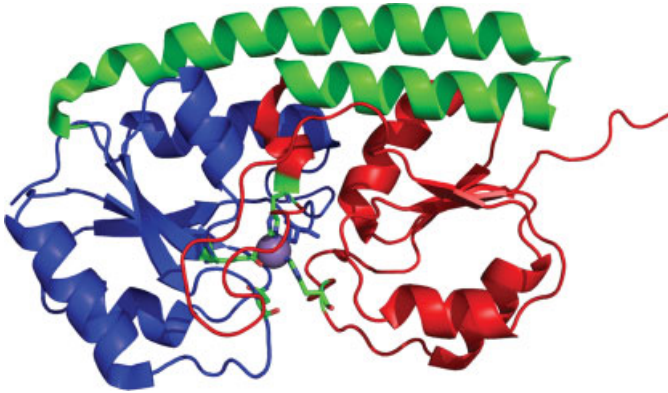


FIGURE 3. MntC (PDB code 1XVL, subunit A) with bound Mn ion (pink sphere). The protein backbone is depicted with cartoon ribbons colored from N to C termini according to the spectrum (blue to red). The active site residues are depicted in stick form

an active (facilitating transport) into an inactive state depending on the redox state of the periplasm. Support for the feasibility of such a mechanism comes from a recent work by Singh and coworkers that demonstrates the function of a plasma membrane protein involved in disulfide bond formation in the periplasmic space of cyanobacteria<sup>46</sup>.

In the context of this review, the most important facet of the MntC structure is its ability to describe in structural terms the requirements for the differentiation between Mn and other similar ions. We have recently improved the resolution of the MntC structure to 2.7 Å and determined the structure of a site specific mutant R116A<sup>47</sup>. The improved structure includes additional solvent molecules in the vicinity of the Mn binding site, enabling us to provide a better description of which structural facets are required for Mn selectivity. The immediate ligands are identical to that of non-specific tMI transporters such as PsaA<sup>48</sup>: two histidines, a glutamic acid and an aspartic acid residue (Figure 4). These residues can potentially provide between 4 to 6 lone pair interactions with the tMI (depending on whether the acids are positioned in a bidentate or monodentate orientation). In the *S. pneumoniae* Zn/Mn binding protein, PsaA, all four ligating residues are equidistant from the tMI and the geometry is a distorted pyramid. In the *T. pallidum* Zn/Mn binding protein, TroA (in which the glutamic acid residue is replaced by a third histidine), the tMI–ligand distances are more than 0.5 Å longer and the geometry is more tetrahedral, however the Asp ligating residue may contribute two ligating oxygen atoms<sup>49, 50</sup>. In the *Synechocystis* 6803 Zn binding protein ZnuA, three histidine residues ligate the ion with a very tightly bound water molecule serving as the fourth ligand, in a nearly perfect tetrahedral geometry<sup>51</sup>. The MntC subunits with an oxidized disulfide bond hold the Mn ion very tightly with the two acid ligands at distances of less than 1.9 Å, and the two histidines at about 2.2 Å, forming a highly distorted tetrahedron. In the subunit with reduced disulfide, the two acidic residues move more than 0.5 Å away and indeed Mn is loosely bound to MntC with reduced disulfide bonds. We have analyzed the possible source of this tight binding and found a number of possible reasons. Other homologous SBPs have a conserved DPH motif preceding the two first His ligating residues that have been suggested as being important for proper positioning. In MntC the sequences are EVH and NPH, respectively. The DPH motifs are proposed to facilitate the optimal position of the two histidine tMI ligands<sup>48</sup>, however there may be an additional role in controlling the electronic environment and thereby the  $pK_a$  of the histidine N $\epsilon$ 2 atom. Perhaps more



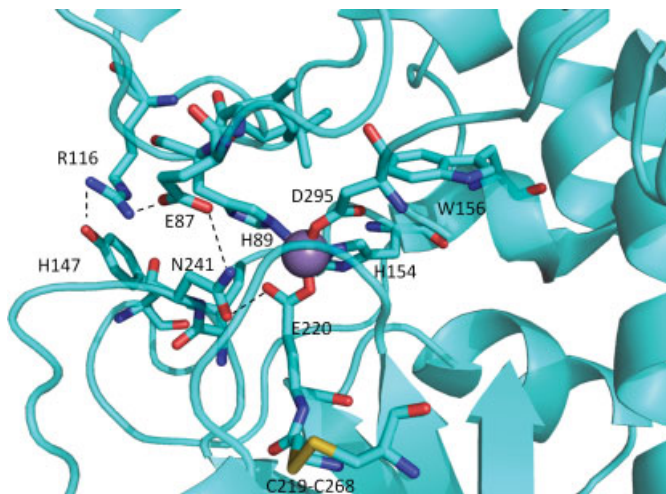


FIGURE 4. MntC active site with bound Mn ion (pink sphere). Residues important for Mn binding specificity and protein structure integrity are shown in stick figures. Black dashed lines show polar interaction network centered on Arg116, locking the proteins N and C terminal domains

important are additional second and third shell residues that are different between MntC and other tMI SBPs. The MntC second shell residues are less polar, while the third shell residues are more polar, including the potentially positively charged Arg116. This residue contributes two interesting aspects to the binding site. On the one hand, the putative exit to the active site becomes rather positive—which may repel a disbound Mn from leaving the active site (Figure 5). In addition, the Arg116 residue forms the center of a network

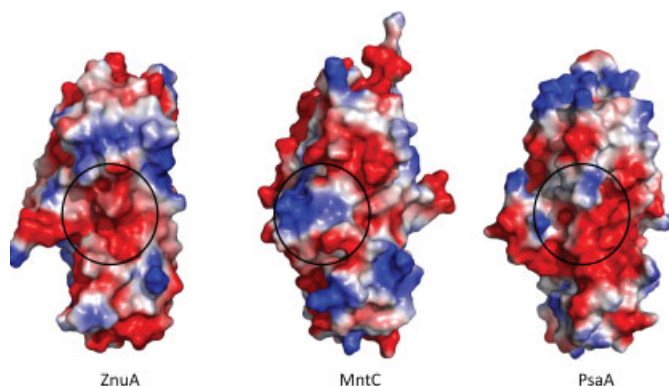


FIGURE 5. Surface electrostatic potential of three transition metal ion solute binding components of ABC transporters: ZnuA (1PQ4, left), MntC (1XVL, middle) and PsaA (1PSZ, right) tMI SBPs. All potentials are depicted at the same level with blue and red representing the positive and negative potentials, respectively. Black ovals show the general surface enclosing the tMI binding site which is about 10–12 Å deeper into the protein's interior

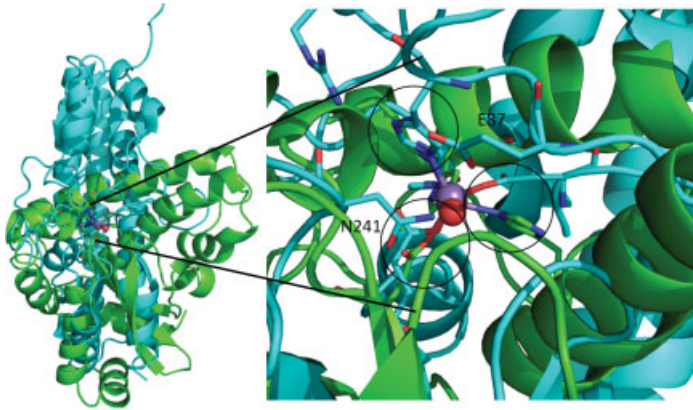


FIGURE 6. Superposition of the *E. coli* MnSOD (3K9S, green) and MntC (1XVL, cyan). Panel A shows the overall lack of similarity between the two proteins. Panel B shows an enlargement of the active site around the bound Mn ion (pink sphere). Four of the five MnSOD ligating residues overlap almost perfectly with the four ligating residues of the MntC, three of the ligating sites are identified by the black circles, with the fourth site located behind the Mn ion. The fifth MnSOD ligand is a water molecule (red sphere). In the MntC, Asn241 and Glu87 partially obstruct this position, perhaps preventing the MntC from uncontrolled activity on ROS

of contacting residues with Glu87, Tyr147 and Asn241, locking the N-terminal and C-terminal domains (Figure 4). This network extends further to the active site itself with Asn241 forming a potential hydrogen bond with the Mn ligating residue Glu219 that is immediately adjacent to the disulfide bond<sup>45</sup>. We recently determined the structure of the R116A mutant MntC and, as expected, the loops that surround the active site became extremely disordered, although there was still a bound tMI in the active site. Further studies will be required to determine the identity of the bound tMI in the R116A mutant. We can conclude that Mn is tightly bound to the active site and that the second and third shells of residues ensure specificity, affinity and a potential chemical controlling step that can either induce release of bound Mn, or prevent binding. The second and third shells also prevent the MntC from becoming a SOD by not providing the necessary protein environment (Figure 6).

We have recently successfully crystallized the MntB permease component of the MntABC transporter system described here. We are in the process of crystal improvement towards structure determination. We hope that this structure will reveal the mechanism of Mn unbinding from the MntC protein, and whether the permease has any novel Mn recognizing elements along the pathway of transfer through the membrane.

## B. Mn Transport under Sufficient Conditions: Mn Accumulation in an Outer Membrane Bound Pool

Measurements of <sup>54</sup>Mn transport rate have clearly demonstrated that, in addition to MntABC, other transport systems function in cyanobacteria<sup>44</sup>. While Mn transport was severely inhibited in  $\Delta mntC$  mutants under Mn deficient conditions, no inhibition could be measured under Mn sufficient conditions. Furthermore, the Mn uptake rate, as a function of Mn concentration, exhibited a biphasic behavior with increasing transport rates into

the millimolar concentration range, both in wild type and in  $\Delta mntC$  mutant cells.  $V_{max}$  values could not be reached even in the presence of 2 mM  $Mn^{4+}$ .

Further analysis of Mn uptake under sufficient conditions<sup>52</sup> found that early logarithmic growth phase *Synechocystis* 6803 cells have the ability to bind large amounts of Mn from fresh BG11 media. Fractionation of *Synechocystis* cells indicated that most of the bound Mn is associated with the outer membrane<sup>52</sup> but the face of the membrane to which Mn is bound remains to be determined. X-ray absorption spectroscopy verified that this pool contains  $Mn^{2+}$  and examination of the extended X-ray absorption fine structure indicated that the Mn interacts with the membrane. However, the nature of the binding species could not be resolved using this technique<sup>52</sup>. Recent work from the Robinson group suggested that a protein from the cupin family, MncA, is the major Mn binding protein in the periplasm of *Synechocystis* 6803 cells<sup>53</sup>. The relation of this protein to Mn mass storage was not further investigated.

Release of Mn from the outer membrane pool could be achieved by incubation with EDTA at concentrations higher than 2 mM. High EDTA concentrations can poke holes in the outer membrane of Gram-negative bacteria in addition to chelating released transition metal ions<sup>54</sup>. Using this method it was possible to establish that in early log phase cells, an excess of  $ca\ 1 \times 10^7$  atoms/cell can be stored in the outer membrane bound pool, approximately 10 times the Mn concentration inside the plasma membrane. Along with the growth of the culture, Mn from the outer membrane pool is gradually utilized, thus keeping the internal Mn concentration constant<sup>52</sup>. This mode of transport is not affected by inactivation of MntABC proteins<sup>52</sup>. Maintaining a Mn concentration gradient of such large proportions requires energy. Indeed, accumulation of Mn in this outer membrane pool was found to be coupled to photosynthetic activity<sup>52</sup>. However, the mechanism by which photosynthetic processes in the thylakoid membrane supports Mn accumulation on the outer membrane is still unknown.

### C. Mn Sensing and Regulation of Transport

In order to regulate different Mn transport pathways, cyanobacteria require a Mn sensor. Ogawa and coworkers<sup>55</sup> and Yamaguchi and coworkers<sup>56</sup> have independently identified the components of such a system. A mutant of the ManS sensory kinase was identified in a screen that utilized the *MntCAB* promoter linked to a reporter gene<sup>55</sup>. Microarray analysis of mutants in genes coding for two-component regulators has uncovered, in addition to ManS, the response regulator ManR<sup>56</sup>. The ManS/R system represses the expression of the *mntCAB* operon and its inactivation results in constitutive transcription of its mRNA. Interestingly, inactivation of either *manS* or *manR* did not significantly affect the transcription of any other genes apart from those of the *mntABC* operon<sup>56</sup>.

The ManS protein, like many other two-component sensors, is a plasma membrane embedded protein. The sensor domain of the protein is in the periplasm and the kinase domain is in the cytoplasm<sup>55,56</sup>. The topology of the protein should have an effect on its mode of sensing. Lowering the external Mn concentration will result in the depletion of the outer membrane bound pool, which will activate the ManS/R system in advance of an internal Mn deficiency.

## V. Mn AND PSII FUNCTION

### A. Assembly of the Mn Cluster: Sequence of Events

The assembly of the  $Mn_4$ -Ca cluster occurs for the first time during *de novo* synthesis of PSII complexes and then repeatedly throughout the photodamage repair cycle (reviewed

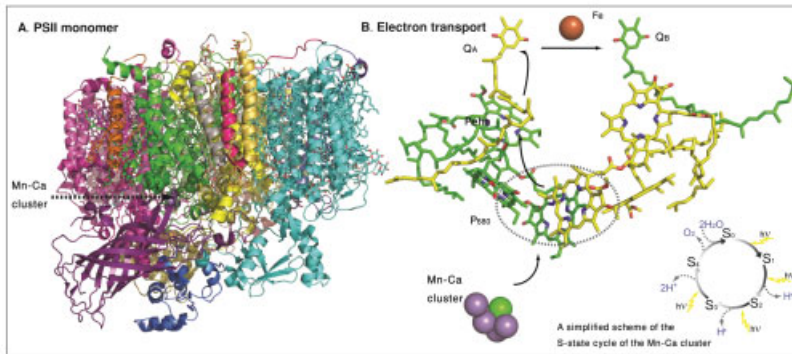


FIGURE 7. Structure of PSII and the electron transport chain within it. (A) Structure of a PSII monomer (based on the PDB file 3BZ1<sup>89</sup>). The location of the Mn-Ca cluster is indicated. (B) Stripped down view of the electron transfer chain cofactors. Cofactors attached to the D1 or D2 protein are colored green or yellow, respectively. The arrows represent the direction of electron transfer and a simplified scheme of the S-state cycle<sup>90–94</sup> is included at the bottom

in Reference 57). Considering the function of this cluster (Figure 7), it stands to reason that the assembly process will be tightly regulated and that stop-gap measures will be put into place to avoid its untimely activation. So far, two control mechanisms have been identified.

The D1 subunit of PSII protein is translated as a precursor containing a C'-terminal extension. Cleavage of the C' terminus, a process that is catalyzed by the CtpA protease<sup>57–59</sup>, trims the D1 to the correct size and position for contributing to the ligation of the Mn<sub>4</sub>-Ca cluster. While the C' terminal extension can be found in the vast majority of photosynthetic organisms, replacement of pD1 with a mature D1 does not result in any observable problems in the assembly process<sup>60</sup>. Nevertheless, in mixed culture competition experiments between *Synechocystis* 6803 containing either pD1 or mature D1, cells containing pD1 prevailed<sup>60</sup>.

Psb27, a protein which is not a part of the mature photosystem, was found to be attached to monomeric PSII centers lacking Mn and extrinsic proteins<sup>61, 62</sup>. These complexes lacked oxygen evolution capacity and had impaired forward electron transfer on the acceptor side<sup>63</sup>. Studies suggest that transient Psb27-PSII assemblies play a role in the assembly and repair cycles of PSII.

Based on these studies we can suggest the following order of events: The pD1 protein is inserted into a partially assembled PSII core complex. After cleavage of its C' terminus by CtpA, the mature D1 protein can attach the Mn cluster. It is not clear whether the cluster is assembled sequentially from the ligation of individual Mn atoms, or whether the entire complex is assembled and then transferred to PSII by a Mn chaperon. Psb27 prevents Mn attachment by associating with the luminal side of PSII, blocking the docking sites of the extrinsic proteins<sup>61, 62</sup>. This prevents immature binding and interference with the sequence of the photosystem assembly process. Following the completion of the D1 processing step, Psb27 is detached<sup>62, 64</sup> and the extrinsic donor side proteins can bind.

A prediction for the order of binding of the donor side proteins can be derived from biochemical and structural studies. The major extrinsic protein, PsbO, is attached to the luminal side of CP47<sup>65–67</sup>, followed by PsbV, which binds to CP43<sup>65–68</sup>, and PsbU, which interacts with CP43, CP47, PsbO and PsbV<sup>69</sup>. In *Synechocystis* 6803 the donor

side assembly process culminates with the binding of PsbQ. PSII complexes containing PsbQ were more active and stable as compared with PSII units lacking this protein<sup>14, 64</sup>. PsbP was found to be associated only with a fraction of PSII complexes and is not believed to be a functional component of the donor side, but rather to have a regulatory role<sup>70</sup>.

Binding of the Mn ions and extrinsic proteins to the donor side of PSII are required but not sufficient for the function of PSII. The binding is accompanied by a series of light driven Mn oxidation events necessary for the correct functional assembly of the Mn-Ca cluster in a process termed as photoactivation<sup>71</sup>.

## B. Assembly of the Mn Cluster: Topology

The structure of cyanobacterial cells is more complex than the structure of other prokaryotic cells. On top of a periplasmatic and intracellular space, defined by the outer (OM) and plasma membrane (PM), which are common to all Gram-negative bacteria, cyanobacteria contain a third luminal space inside the thylakoid membrane (TM). Thylakoid membranes take up a large fraction of the intracellular space, creating a maze that stands in the way of transport inside the cell<sup>72, 73</sup>. From an evolutionary standpoint, thylakoid membranes are considered the descendants of the photosynthetic membranes of the non-oxygenic photosynthetic purple bacteria<sup>74</sup>. However, while in purple bacteria the photosynthetic membranes invaginate from the plasma membrane<sup>75</sup>, in cyanobacterial thylakoids there is no clear evidence for such a connection<sup>72, 73</sup>.

While it is clear that PSII functions in the thylakoid membranes, there are conflicting reports as to the site of its biogenesis. Smith and Howe<sup>76</sup> were able to detect chlorophyll and D1 proteins in PM membranes of *Synechococcus sp.* PCC 7942. In this study, membranes were isolated by sucrose density gradient centrifugation. Using a two-phase separation technique<sup>77</sup>, Zak and coworkers were able to detect D1, Cytochrome *b*<sub>559</sub> and CtpA, but not CP43, CP47 or psbO in PM membranes of *Synechocystis* 6803<sup>78</sup>. The PSII proteins present in the PM preparation formed a complex that migrated as one band in native gels. Based on these data they suggested that the site of PSII biogenesis is in the plasma rather than in the thylakoid membrane. This hypothesis represented a departure from the accepted view of the location of PSII assembly in the chloroplast thylakoids of eukaryotic organisms<sup>79</sup>. In a later work using the same preparation technique<sup>80</sup>, it was demonstrated that a limited extent of charge separation can be measured in OM partial PSII assemblies. The concentration of Mn attached to PM membranes was found to be *ca* 20% that of the TM on a chlorophyll basis<sup>80</sup>. The lack of stable charge separation and Mn in the PM is not surprising considering the role of the CP47 and CP43 extra-membranal loops in stabilizing the oxygen evolving complex<sup>81</sup>. Jansen and coworkers<sup>82</sup> combined the two techniques, performing sucrose gradient density centrifugation followed by two-phase separation, and were still able to detect D1, D2 and cytochrome *b*<sub>559</sub> in PM preparations of *Synechocystis* 6803. In a recent study from the same group<sup>83</sup>, PM right-side out and inside out vesicles were isolated from *Synechocystis* 6803. The additional fractionation step revealed heterogeneity with respect to protein distribution within the PM. D1 and pD1 were enriched in inside out as compared to right-side out vesicles on a total protein basis.

While the results of the studies mentioned above suggest a PM PSII assembly pathway, other studies did not detect chlorophyll in PM. These include work on membranes isolated by sucrose gradient centrifugation from *Synechococcus* 7942<sup>84</sup>, *Synechocystis* 6714<sup>85, 86</sup>, or by hyperspectral confocal fluorescence imaging of *Synechocystis* 6803 cells<sup>87</sup>. Peschek and coworkers<sup>88</sup>, analyzing membrane fractions isolated from *Synechococcus* 7942 cells, could detect precursors in the chlorophyll biosynthesis pathway but very little or no chlorophyll in the PM.

These reports seem conflicting but they are not mutually exclusive. It is possible that PSII assembly takes place in different topological locations during *de novo* synthesis or during repair, when Mn concentrations are high or low or in response to other environmental cues. Any attempt to further resolve Mn transport routes and PSII assembly pathways must take into account the variability imposed by changes in the bioavailability of Mn. While it is hard to control transition metal bioavailability, and even harder to precisely control the physiological status of the cells, it is nevertheless essential that these parameters will be measured and reported so that experimental results from different growth conditions and from different cyanobacterial strains may be compared.

## VI. ACKNOWLEDGMENTS

This work was supported by the Israeli Science Foundation (grant No. 1168/07 to N.K.) and the US–Israel Binational Science Foundation (grant 2005179 to N.A.). We gratefully thank the staff of the European Synchrotron Radiation Facility for their assistance in the collection of diffraction data.

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