Metals in membranes†

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In this *critical review* we discuss recent advances in understanding the modes of interaction of metal ions with membrane proteins, including channels, pumps, transporters, ATP-binding cassette proteins, G-protein coupled receptors, kinases and respiratory enzymes. Such knowledge provides a basis for elucidating the mechanism of action of some classes of metallodrugs, and a stimulus for the further exploration of the coordination chemistry of metal ions in membranes. Such research offers promise for the discovery of new drugs with unusual modes of action. The article will be of interest to bioinorganic chemists, chemical biologists, biochemists, pharmacologists and medicinal chemists. (247 references)

1. Introduction

Cell membranes typically consist of a lipid bilayer of thickness 7.5 to 10 nanometres,[1] with embedded proteins and peripheral proteins attached to the membrane surface. Membranes perform a variety of functions in cell biology. We focus here on their interactions with metal ions.

We are particularly interested in the design and mechanism of action of metallodrugs. Membrane proteins can potentially be involved in the transport of metal ions and sometimes their associated ligands through membranes and also may themselves be drug targets. The thermodynamics and kinetics of metal–ligand substitution and redox reactions will, in general, be highly dependent on the medium. Reactions of metal

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Table 1 The overall formation constants (log β_2) of dihalo complexes of silver in non-aqueous solvents (T = 296 K). From ref. 3

	$AgCl_2^-$		$AgBr_2^-$		AgI_2^-	
Solvent	Potentiometric	Voltammetric	Potentiometric	Voltammetric	Potentiometric	Voltammetric
DMSO[a]	11.9	12.1	11.7	12.3	13.1	13.2
Acetonitrile[a]	12.6	18.6	18.4	14.1	14.6	15.7
Methanol[b]	8.0	7.7	10.9	10.2	14.8	14.8
Acetone[c]	16.7	16.7	19.7	20.2	22.2	22.8
Nitroethane[a]	22.2	22.5	22.5	22.4	23.5	23.6

[a] Ionic strength maintained at 0.100F with $(C_2H_5)_4NClO_4$. [b] Ionic strength maintained at 1.00F with $LiClO_4$. [c] Ionic strength maintained at 0.100F with $LiClO_4$.

complexes within membranes may therefore differ significantly from those in media with higher dielectric constants, such as more aqueous extracellular fluids. The targeting of metals to membrane sites accompanied by control of their reactivity in these sites therefore presents considerable challenges.

Predictions of the preferred metal binding sites on potential ligands are often based on considerations of the 'hard' and 'soft' nature of metal ions and ligands.[2] The 'softer', more polarizable metal ions prefer 'soft' ligands. However, in general, the affinity of a metal ion for a ligand is dependent on the medium (the solvent) since the overall free energy change involves contributions from solvation of the metal ion, the ligand and the metal–ligand complex. As an example,[3] Table 1 shows data for the stability of Ag(I)–halide (X) complexes in different solvents:

$$Ag^+ + 2X^- \rightleftharpoons AgX_2^-;\ \beta_2 = [AgX_2^-]/[Ag^+][X^-]^2$$

Several features are apparent in Table 1. First for a given halide, the affinity shows a strong dependence on solvent. For $Ag(I)/Cl^-$ complexes, for example, the stability constants range from log β_2 = 8 in MeOH to 22 in $C_2H_5NO_2$. In some solvents, the discrimination between halides is high, *e.g.* MeOH log β_2 = 8 for Cl^-, 11 for Br^- to 15 for I^-, in accordance with the usual description of Ag(I) as a 'soft' metal ion, favoring the heavier halide. But in other solvents, *e.g.* $C_2H_5NO_2$, there is little discrimination (log β_2 values 22.2–23.6).

Therefore, the affinity of metal ions for ligands in non-aqueous media (lipid environments) may differ from that in aqueous media (extra- and intracellular environments). The thermodynamically-preferred binding sites for metal ions in membranes may not be readily predicted from current knowledge of metal–ligand stabilities, most of which have been determined for aqueous solutions. Seemingly poor ligands could bind tightly to metal ions in protein cavities with low dielectric constants.[4]

The dielectric constants for proteins can be considerably lower than that of water (ε = 80), and commonly have values of ε = 30–70, depending on the nature of the surrounding side-chains in protein cavities, perhaps as low as ε = 20, compared to ε = 2 for lipids.[5] Water is structured around hydrophobic protein side-chains and this enhances the effective forces between charged groups located in such environments.[6]

Understanding the interactions of metals with transmembrane proteins will aid the design of more effective metallodrugs and a range of examples is discussed in this review.

2. Channels, pumps and transporters

2.1. Channels

2.1.1. Aquaporins. Aquaporins, also known as water channels based on their function, are a family of integral membrane proteins, which facilitate the transport of water as well as glycerol (aquaglyceroporins), across membranes (Table 2).[7] At least 10 mammalian aquaporins are known and more than 350 others have been found in plants, microbes, invertebrates and vertebrates.[8] Compared with non-protein dependent diffusion, aquaporins increase water permeation through membranes by 10–100-fold with high selectivity. Positively-charged ions, including protons (H_3O^+), cannot pass through these water channels. Each aquaporin exists as a tetramer and each subunit contains its own pore with six membrane-spanning domains, which folds together to form a water channel (Fig. 1a). There are two tandem repeats in each subunit and each of them contains three transmembrane domains. Two highly conserved loops, B and E, contain the signature motif asparagine–proline–alanine (NPA). Both loops E and B are involved in forming an internal aqueous pore in each subunit.

Aquaporin-1[47] (AQP1, 28 kDa), from human red blood cells, has been intensely studied (Fig. 1b).[48] Its transmembrane domains TM1, 2, 4, and 5 are hydrophobic and two highly conserved residues Asn-76 and Asn-192 in the Asn–Pro–Ala (NPA) motifs can provide polar side-chains for hydrogen bonding to water (Fig. 1c).[7] In addition, the terminal parts of the pore-forming loops, B and E, each contain short α-helices which create a partial positive charge in the centre of the membrane. A size constriction region with 2.8 Å channel diameter is surrounded by residues Phe-56, His-180, Cys-189 and Arg-195. Arg-195 is conserved in all aquaporins and possesses a functionally important positive charge at the narrowest segment of the channel. His-180 is neutral at pH 7, but is protonated and becomes positively charged at acidic pH. Both positive Arg-195 and His-180, and the positive dipoles from the pore helices exhibit strong electronic repulsion against positive ions, *e.g.* protons, which provides the exquisite selectivity of the channel for H_2O over H^+.

Although, organomercury compounds were widely used as diuretics in the 20th century, the molecular basis for induction of diuresis was not understood. The use of mercurial diuretics ceased in about the 1950s. An example of such a diuretic is chlormerodrin.[49] Indeed Benga *et al*. suggested in 1986 that the binding of *p*-mercuribenzene sulfonate to band 4.5 and band 3 proteins from human erythrocytes leads to inhibition of water

Table 2 X-Ray structures for some metal-associated membrane proteins

Type	Protein	Source	Structural features	PDB code	Ref.
Aquaporin	AQP1	Human red blood cells	Tetramer, each unit: 6 transmembrane helices	1FQY	9
				1HW0	10
	AQP1	Bovine red blood cells		1J4N	11
	Aquaporin 0	Bovine eye lenses		1TM8	12
	Aquaporin Z	*Escherichia coli*		1RC2	13
	Aquaglyceroporin	*Escherichia coli*		1FX8	14
K(I) channel	KcsA	*Streptomyces lividans*	Tetramer, each unit: 2 transmembrane helices	1BL8	62
	KcsA	*Streptomyces lividans*		1K4C, 1K4D	15
	MthK	*Methanobacterium thermoautotrophicum*		1LNQ	65
	KirBac 1.1	*Burkholderia pseudomallei*		1P7B	16
	mGIRK1	*Mus musculus*		1N9P	17
	KvAP	*Aeropyrum pernix*	Tetramer, each unit: 6 transmembrane helices	1QRS, 1QRQ	66
P-type ATPase	Ca-ATPase (E1 state)	Rabbit sarcoplasmic reticulum	Three cytoplasmic domains: actuator, nucleotide binding and phosphorylation; 10 transmembrane helices	1EUL	85
		Rabbit sarcoplasmic reticulum		1VFP	18
		Rabbit sarcoplasmic reticulum		1T5S, 1T5T	19
	Ca-ATPase (E2 state)	Rabbit sarcoplasmic reticulum		1IWO	20
		Rabbit sarcoplasmic reticulum		1XP5	21
		Rabbit sarcoplasmic reticulum		1WPE, 1WPG	22
G-protein coupled receptor	Rhodopsin	Bovine rod outer segments	Seven transmembrane helices	1F88	23
				1HZX	175
				1L9H	24
				1U19	25
				1GZM	26
ABC transporter	BtuCD	*Escherichia coli*	Two membrane-spanning subunits for BtuC, each unit: 10 transmembrane helices	1L7V	122
Outer membrane receptor	FhuA	*Escherichia coli*	A transmembrane β-barrel of 22 β-strands	1FCP, 2FCP	124*a*
				1BY3, 1BY5	124*b*
	FhuA			1QFF, 1QFG	27
	FhuA			1FI1	28
	fepA			1FEP	125
	FecA			1KMO, 1KMP	126
	BtuB			1NQF, 1NQE, 1NQG, 1NQH	118
				1UJW	29
	FpvA	*Pseudomonas aeruginosa*		1XKH	30
Respiratory complex III	Cytochrome bc_1 complex	Bovine heart mitochondria	Dimer, each monomer: 11 subunits; 13 transmembrane helices	1QCR	229
				1BE3, 1BGY	31
		Yeast *Saccharomyces cerevisiae*	Dimer, each monomer: 9 subunits; 12 transmembrane helices	1EZV	32
				1KB9	33
		Yeast *Saccharomyces cerevisiae*		1KYO	34
	Cytochrome b_6f complex	*Mastigocladus laminosus*	Dimer, each monomer: 8 subunits; 13 transmembrane helices	1UM3	35
		Chlamydomonas reinhardtii	Dimer, each monomer: 8 subunits; 13 transmembrane helices	1Q90	36
Respiratory complex II	Fumarate reductase	*Escherichia coli*	4 subunits; 6 transmembrane helices	1FUM	37
				1FUM, 1KF6, 1L0V	38
		Wolinella succinogenes	Dimer, each monomer: 3 subunits; 5 transmembrane helices	1QLA,1QLB	39
	Succinate dehydrogenase	*Escherichia coli*	Trimer, each monomer: 5 subunits; 6 transmembrane helices	1NEK, 1NEN	40
Respiratory complex IV	aa_3-Cytochrome *c* oxidase	Bovine heart mitochondria	Dimer, 13 subunits; 28 transmembrane helices	1OCC	41
				2OCC, 1OCR	42
				1V54, 1V55	43
		Rhodobacter sphaeroides	4 subunits; 22 transmembrane helices	1M56, 1M57	44
	ba_3-Cytochrome *c* oxidase	*Thermus thermophilus*	3 subunits; 16 transmembrane helices	1EHK	45
	bo_3-Ubiquinol oxidase	*Escherichia coli*	4 subunits; 25 transmembrane helices	1FFT	46

transport.[50] The treatment of red blood cells (RBC) with *p*-chloromercuribenzoate (PCMB) can produce dramatic changes in RBC osmotic water permeability (up to 90%), which can be reversed by cysteine (competitively binds to Hg(II)).[51] The thiol group of Cys-189 of the human AQP1 may be the target site for mercurial diuretics. However, the possibility that Hg(II) binds to other side-chains in this channel can also be considered.

Arsenic trioxide (As_2O_3) is now an approved drug for the treatment of promyelocytic leukemia (the drug Trisenox). In aqueous solution near physiological pH (7.4), arsenic trioxide exists predominantly as the neutral trihydroxide $As(OH)_3$.[52,53] Arsenic trihydroxide uses the same translocation pathway as glycerol, and in particular the human aquaglyceroporin AQP9.[54] Interestingly, Lys but not Ala can substitute for the highly conserved Arg-219 residue of AQP9, but the

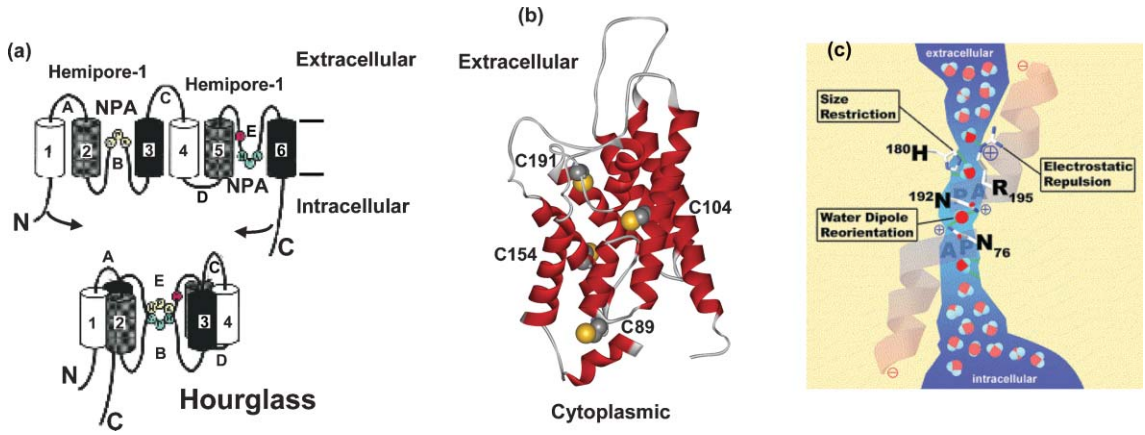


Fig. 1 (a) Hourglass model for AQP1 topology. Arrangement of loops B and E with highly conserved NPA motifs forms a single aqueous pathway through the AQP1 subunit. (b) Structure of the bovine AQP1. (c) Schematic diagram of the channel pore of AQP1. Water transports through the channel with high selectivity due to size restriction, electrostatic repulsion (H-180 and R-195) and partial positive dipoles of the short pore-lining α-helices and the two highly conserved asparagines (N-76 and N-192) in the signature NPA motifs at the centre of the channel. (a) Adapted from refs. 7 and 47. (b) Produced from pdb code 1J4N. (c) Reproduced from ref. 48 with permission.

substitution of Phe-56, which serves to orient water for H-bonding to Arg-219, has little effect on arsenic trihydroxide uptake. AQP9 expression increases 20-fold by fasting and is primarily expressed in leukocytes and the liver. There is concern that poor nutrition amongst people exposed to arsenic-contaminated drinking water may lead to increased arsenic uptake in the liver.[245]

2.1.2. Ion channels. Lipid membranes form barriers that separate cells from the outside.[55] While many charged and uncharged solutes, and even water, can slowly cross lipid membranes by a solubility-diffusion process, most life processes could not occur without more effective and regulated mechanisms for the passage of these molecules across cell membranes, for example when metabolic rates are high.[55] Ion channels are integral membrane proteins that form pores through the biological membranes and facilitate the diffusion of ions. Ions always flow down their electrochemical gradients through channels (passive transport). Many types of channels for Na(I), K(I) and Ca(II) have been identified. Access to the pore is gated and the gates open or close in response to the binding of a ligand outside or inside the cell for ligand-gated channels such as Ca(II),[56] G-proteins,[57] and glutamate,[58] or a change in membrane potential for voltage-gated channels.[59] Each ion channel is highly selective and recognizes only certain ions and allows them to pass through. An ion channel typically consists of multi-subunits in a homomeric protein, or several different subunits in a heteromeric protein.[55] Each unit contains a few transmembrane domains. X-Ray crystallographic studies have revealed the structures of several different K(I) channels[60] which can be related to their functions, including ion conduction, gating of the pore, and voltage-sensing. Therefore, we use K(I) channels as examples to illustrate the mechanism of permeation and selectivity of ion channels.

K(I) channels are tetrameric with fourfold symmetry and within the group there are two broad classes: the six-transmembrane-helix voltage-gated (K_v), and the two-transmembrane-helix inward-rectifier (K_{ir}) subtypes (Fig. 4a and 2a).[61] Subunits of the two-helix membrane-spanning variety appear to be shortened versions of their larger counterparts, as if they simply lack the first four membrane-spanning segments.[62] KcsA is a simple potassium channel containing only two transmembrane helices for each subunit (Fig. 2).[62,63] Four identical subunits form an inverted teepee or cone pore with the selectivity filter in its outer side. The selectivity filter is lined with the carbonyl groups of the peptide backbone, the amino acid residues of which form a highly conserved TXXTXGYG signature sequence.[61] The selectivity filter opens directly to the extracellular solution and exposes four carbonyl oxygen atoms which are directed straight out into solution in a ring surrounding the perimeter of the pore entrance.[63] The extracellular pore entrance is negative and attractive to K(I). These four carbonyl atoms assist the dehydration of K(I) at the extracellular entrance through electrostatic interactions. As shown in Fig. 2, there are four evenly-spaced layers of carbonyl oxygen atoms in the channel and a single layer of threonine hydroxyl oxygen atoms, which create four K(I) ion binding sites.[64] At all these sites, K(I) ions are surrounded by eight oxygen atoms, four above and four below each ion. The water-filled inner cavity is wider and lined with hydrophobic amino acids, which do not provide strong hydrogen-bonding donor or acceptor groups.[63] A K(I) ion, fully hydrated by eight discrete water molecules, is suspended at the centre of the cavity. However, the channel in the crystal structure of KcsA was in the closed state.

Recent studies on channels in the open state suggest a mechanism for gating.[65–67] The K(I) ion channels open in response to the initiating stimulus, ligand binding or membrane voltages (Figs. 3 and 4). Gating in K(I) ion channels is conferred through the attachment of a gating domain to the pore.[65] In ligand-gated channels, ligand-binding domains are attached to the pore near the membrane surface.[65] The basic function of these gating domains is to perform mechanical work on the ion conduction pore to change its conformation between closed and open states. The ligand-binding domains convert the free energy of ligand binding into mechanical

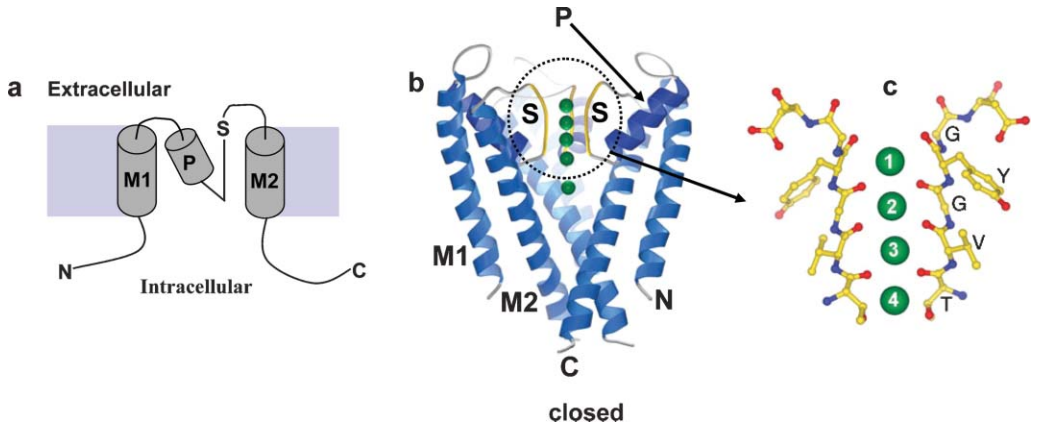


Fig. 2 Structure of the potassium channel. (a) A model for a subunit of tetrameric KcsA as an example of the K_{ir} potassium channel subtype. (b) Ribbon representation of the KcsA channel with the subunit closest to the viewer removed. Potassium ions (green spheres) bind at four locations in the selectivity filter (yellow) and in the water-filled cavity at the membrane centre (bottom ion). (c) Close-up view of the selectivity filter in ball-and-stick representation, with the front and back subunits removed. The four K^+ ions are numbered to indicate the location of binding sites in the filter; position 1 is closest to the extracellular solution and position 4 is closest to the cavity. The two transmembrane domains, the pore helix, and signature sequence for each subunit are labeled as M1 and M2, P, and S, respectively. (a) Adapted from ref. 61. (b) and (c) Reproduced from ref. 63 with permission.

work. The ligand-induced conformational changes result in opening of the inner helices, which line the ion pathway below the selective filter, to permit ion conduction. The inner helices bend at a point referred to as the glycine-gating hinge.[66] This glycine residue is conserved at a specific location in the inner helix of many K(I) ion channels.

Previous structures had relied on the use of antibodies to help crystallize the channels and this may have led to parts of the channel being tilted into an unnatural orientation. This controversy has recently been resolved by determination of the structure of the eukaryotic K_v channel, Kv1.2, from the *Shaker* family.[68] This channel contains an additional domain and a redox partner which is thought to stabilize the protein for crystallization. This new structure reveals the voltage sensor in a more upright orientation as well as its interaction with the pore.

All voltage-gating channels consist of six hydrophobic segments per subunit, S1–S6 (Fig. 4a).[66] Four subunits are identical in K(I) ion channels, whereas they are linked together as homologous domains in Na(I) and Ca(II) channels, surrounding a central ion-conduction pore. Four S4 segments for each channel are the main voltage-sensors with four to seven positive charges, mostly from arginine amino acid residues.[66,69] For the KvAP K(I) ion channel, the second helix in S3, S3b (two individual helices S3a and S3b are separated by an S3 loop) and S4 are packed together, forming a helix–turn–helix structure, called the voltage-sensor paddle (Fig. 4).[66] Three models have been proposed to describe the movement of the voltage sensor during activation.[246] The paddle was thought to move a large distance across the membrane from inside to outside when the channels open.[70] However, more recent evidence indicates that this model is incorrect.[246]

All K(I) channels show a selectivity sequence of K(I) ≈ Rb(I) > Cs(I), the order of increasing ionic radius (Table 3), whereas the permeability for the smallest alkali metal ions

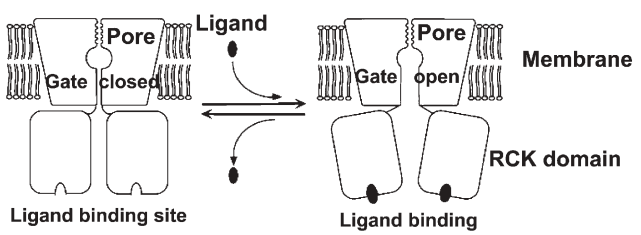


Fig. 3 Ligand-activated ion channel gating. The ligand receptor is usually located at the membrane surface on the extracellular side (top) in neurotransmitter-gated ion channels and on the intracellular side (bottom) in second-messenger-gated ion channels, as shown here. Ca^{2+} binding to the RCK (regulator of K^+ conductance) domains induces the gate to open. Adapted from ref. 65.

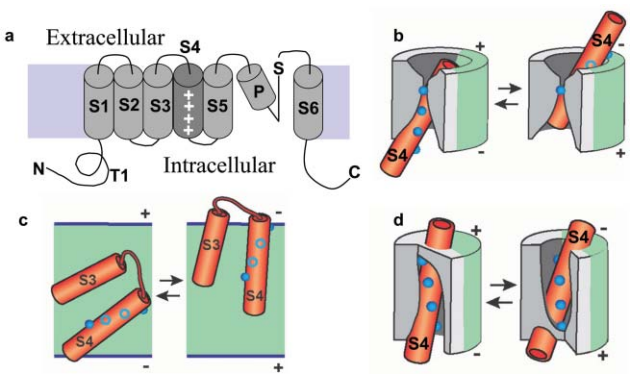


Fig. 4 Voltage-gated channel. (a) Transmembrane helices for one subunit are numbered S1–S6 in Kv channels. The pore helix, and signature sequence for each subunit are labeled as P and S, respectively. (b), (c) and (d) Models of voltage sensors of the potassium channels. (b) Helical screw model. (c) Paddle model. (d) Transporter model. Red cylinders represent the S3 and S4 helices as indicated in the pictures. Protein surrounding S4 is coloured grey. Lipid is coloured green. The blue spheres are the first four S4 arginines when appearing in the foreground, empty circles when appearing behind the cylinder. In each model, the S4 resting position is shown on the left, and the activated position on the right. (a) Adapted from ref. 61. (b), (c) and (d) Adapted from ref. 246.

Table 3 Ionic radii (Å) and hydration enthalpies (kJ mol^{-1}) for alkali metal ions[71]

	Li(I)	Na(I)	K(I)	Rb(I)	Cs(I)
Hydration enthalpy	531	416	334	308	283
Coordination number	Ionic radius				
4	0.59	0.99	1.37	—	—
6	0.76	1.02	1.38	1.52	1.67
8	0.92	1.18	1.51	1.61	1.74

Na(I) and Li(I) is immeasurably low.[62] K(I) is at least 10 000 times more permeant than Na(I), a feature that is essential to the function of K(I) channels. The selectivity filter accommodates dehydrated K(I) well, whereas the smaller Na(I) ions fit so loosely that the energy of the system is lower if they remain in aqueous solution.[61] In other words, tight binding of K(I) to the pore results in the high selectivity for K(I). The selectivity filter also has high selectivity for Rb(I) due to the similarity in its size to K(I). However, the strong binding can be overcome by ion–ion repulsion between close ions in the selective filter.[64] In addition, the conformational change reduces the binding affinity and achieves high conduction rates approaching the diffusion limit for the K(I) channels.[62,64]

Ion channels have been identified as significant therapeutic targets for various diseases. For example, 2-amino-6-(trifluoromethoxy)benzothiazole (Riluzole, Brand name Rilutek™) is a neuroprotective drug with anti-ischemic, sedative, and anti-epileptic properties and is effective in slowing the progression of amyotrophic sclerosis.[72] It may block sodium channels to prevent excess calcium influx into neurons. It also blocks certain calcium channels and may result in reduced calcium neurotransmitter release, which causes neuronal cell death in ischemic conditions. Protein toxins exhibit remarkably specific and selective interactions with a wide variety of ion channels.[73] Hanatoxin inhibits voltage-gated K(I) channels and grammotoxin inhibits voltage-gated Ca(II) channels. Both toxins inhibit their respective channels by interfering with normal operation of the voltage-dependent gating mechanism.

2.2. Ion pumps/transporters

2.2.1. P-type ATPase pumps. ATPase pumps ions across the cell membrane against their electrochemical potential gradients utilizing the energy of hydrolysis of ATP to ADP. The family of P-type ATPases consist of more than 50 membrane proteins including Na(I)/K(I)-, Ca(II)-, and Cu(II)-ATPases.[74] The catalytic cycle of P-type ATPases is characterized by the coupled reactions of cation translocation and ATP hydrolysis with the formation of aspartyl phosphate, a transient covalent intermediate, as a part of the reaction cycle.[75] The phosphorylation results in the enzyme changing its conformation from a state of high affinity for cation and nucleotide binding, E1, to a state of low affinity, E2. This transition coincides with cation translocation from the cytosolic to the lumenal side of the membrane. The release of the cation is followed by the hydrolysis of the aspartyl phosphate bond, and the return of the enzyme to the E1 state.

2.2.1.1. Na(I)/K(I)-ATPase. Na(I)/K(I)-ATPase was the first ion transporter and P-type ATPase to be discovered.[76] It is an integral membrane protein and responsible for the membrane potential of the cell, neuronal communication, and the osmotic regulation of the cell volume. In addition, the electrochemical Na(I) gradient is the driving force behind secondary transport systems. The sodium pump exists as a heterodimeric protein consisting of a catalytic α-subunit (~110 kDa) and a smaller, glycosylated β-subunit (~55 kDa).[77] The α-subunit spans the membrane 10 times, forming transmembrane domains M1 to M10 and contains the cation binding sites and the nucleotide binding and phosphorylation sites.[78] The β-subunit crosses the membrane only once and stabilizes the α-subunit and assists in its transport from the endoplasmic reticulum to the plasma membrane.[76] In addition, the β-subunit is important for ATP hydrolysis, ion transport, and the binding of inhibitors such as ouabain. For every molecule of ATP hydrolyzed, three Na(I) ions from the intracellular space and two K(I) ions from the external medium are exchanged across the membrane against concentration gradients.

ATP binding sites have been localized to the major cytoplasmic loop of the α-subunit with about 430 amino acid residues between transmembrane segments M4 and M5. Residues in the M4M5 loop thought to be involved in ATP binding include Lys-480, Lys-501, Gly-502, Asp-710, Asp-714 and Lys-719.[77] The binding sites for the three Na(I) and two K(I) ions have been identified by homology modeling (Fig. 5), based on the two atomic models of Ca(II)-ATPase (Ca(II)-bound form for Na(I), unbound form for K(I)).[79] There are three binding sites for Na(I) ions. Site I is formed entirely by side-chain oxygen atoms of residues on three helices (M5, M6 and M8). Site II is formed almost "on" the M4 helix with three main-chain carbonyls plus four side-chain oxygen atoms (Asp-811 and Asp-815 on M6 and Glu-334 on M4). Asp-815 is the only residue which coordinates to two Na(I) ions. Site III is unique to Na(I)/K(I)-ATPase and contiguous to site I. The carbonyls of Gly-813 and Thr-814 (M6), the hydroxyl of Tyr-778 (M5), and the carboxyl of Glu-961 (M9) contribute to this site. Two water molecules are also involved. Two K(I) ions have similar binding positions to those for Na(I) ions within the membrane plane. However, Ser-782 is involved in K(I) binding instead of Thr-814 in site I and Glu-334 coordinates to Na(I) directly but not K(I), although it may coordinate K(I) through a water molecule in site II. In addition, Asp-811, instead of Asp-815 (M6), and Glu-786 (M5) coordinate to both K(I) ions. All these changes result in a shift of the K(I) sites toward the extracellular side, by one turn of an α-helix for site II. These proposed binding sites from homology modeling are consistent with many biological and mutational results.

There are two conformational states, E1 and E2 in the catalytic cycle of Na(I)/K(I)-ATPase (Fig. 6).[76] In the first step of the reaction sequence, the Na(I)/K(I)-ATPase opens towards the inside of the cell and exposes binding sites for three Na(I) ions and then Na(I)/K(I)-ATPase binds Na(I) and ATP in the E1 conformational state (step 1), during which phosphorylation at an aspartate residue occurs *via* the transfer of the γ-phosphate of ATP. In the second step, three Na(I) ions are occluded while the enzyme remains phosphorylated. In the third step, the ATPase changes its conformation to E2,

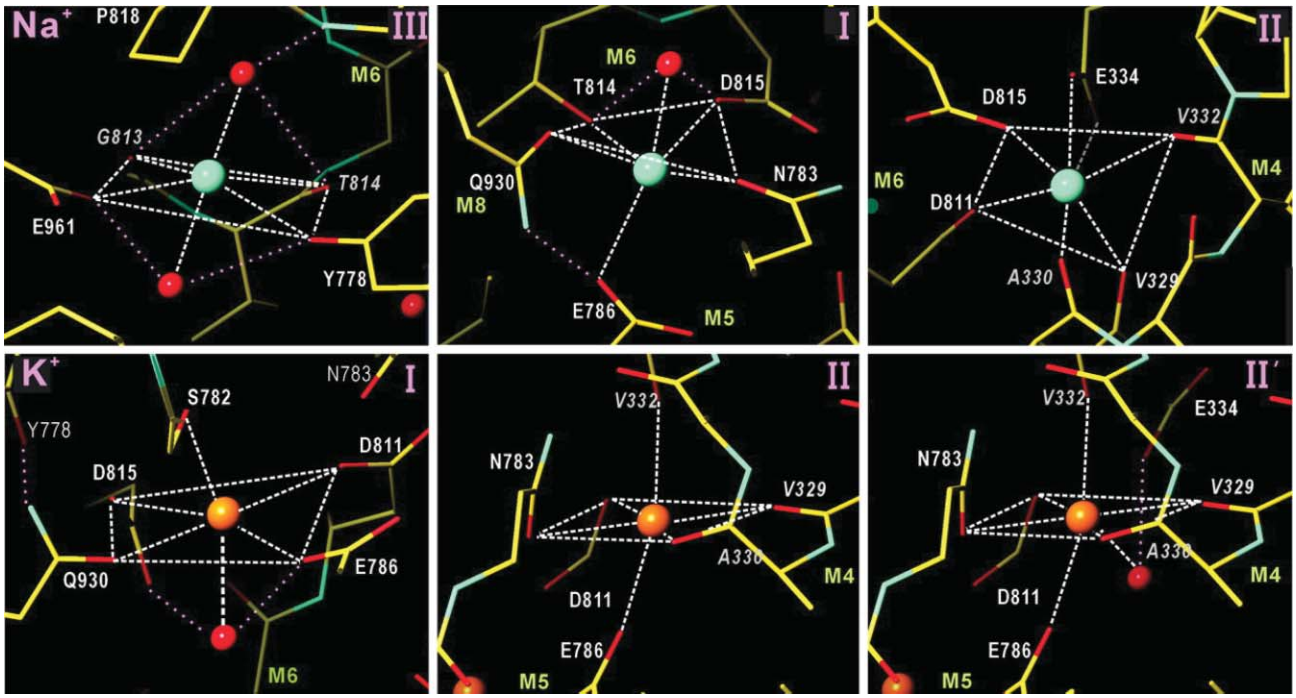


Fig. 5 Coordination geometry of Na^+ (3 sites) and K^+ (2 sites) in Na^+/K^+-ATPase. Spheres represent Na^+ (cyan), K^+ (orange), and water (red). Dotted lines in pink show some of the potential hydrogen bonds. The Roman numeral in the top right corner refers to the binding site discussed in the text. Site II′ for K^+ is the same as site II, except for the contribution of Glu-334 through a water molecule. Reproduced from ref. 79. Copyright 2002 National Academy of Sciences.

opening towards the outside, and, therefore, reduces the affinity to Na(I) ions and releases them. In the fourth step, the conformational change from E1 to E2 also results in exposure of binding sites for two K(I) ions and an increase in the binding affinity. Hence, two K(I) ions are selected. In the fifth step, binding of K(I) leads to dephosphorylation of the enzyme and to the occlusion of two K(I) cations. Finally, the K(I) ions are released into the cytosol after ATP binds to the enzyme.

Na(I)/K(I)-ATPase is an important target for therapy of hypertension and heart failure.[80] The steroid 17β-(3-furyl)-5β-androstane-3β,14β,17α-triol (PST 2238) is a new antihypertensive agent that may prevent cardiovascular complications associated with hypertension through the modulation of the Na(I)/K(I) pump function, and is undergoing phase II clinical studies.[81] PST 2238 displaces ouabain from its binding sites on Na(I)/K(I) ATPase without interacting with other receptors involved in blood pressure regulation or hormonal control.[82]

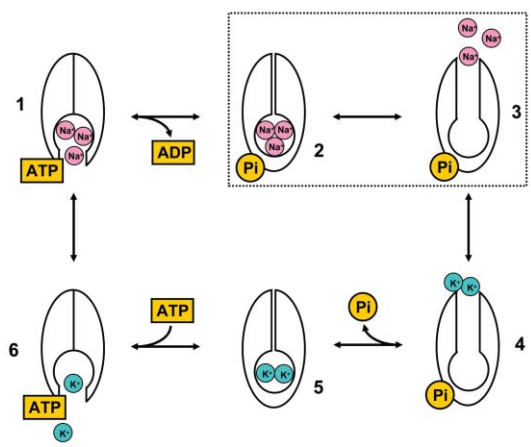


Fig. 6 Reaction cycle of Na(I)/K(I)-ATPase. The dashed box highlights the electrogenic steps of the catalytic cycle. Adapted from ref. 76.

2.2.1.2. Ca(II)-ATPase. The Ca(II)-ATPase from the sarcoplasmic reticulum is an integral membrane protein of 110 kDa and structurally and functionally the best characterized member of the P-type ion-translocating ATPase superfamily (Fig. 7).[83] It consists of 10 transmembrane helices (M1–M10), three cytoplasmic domains (A, actuator or anchor; N, nucleotide-binding; P, phosphorylation) and small lumenal loops. The three cytoplasmic domains are derived predominantly from two large cytoplasmic loops between transmembrane helices M2/M3 and M4/M5.[84] The latter loop forms the phosphorylation P domain, which sits directly on top of M4 and M5, and the nucleotide binding N domain, which is an insert within the P domain. These two domains are named for the ligands they carry, namely Asp-351, which forms the phosphoenzyme, and the site near Lys-492 that binds ATP. The third cytoplasmic loop, dubbed the transduction or actuator (A) domain, comprises the smaller M2/M3 loop as well as the N terminus.

There are two Ca(II) binding sites, termed sites I and II (Fig. 8a).[85] Ca(II) at site I is surrounded by the side-chain oxygen atoms of Asn-768 and Glu-771 on M5, Thr-799 and Asp-800 on M6 and Glu-908 on M8. Ca(II) at site II is coordinated by the main-chain carbonyl oxygen atoms of Val-304, Ala-305 and Ile-307 on M4 and the side-chain oxygen

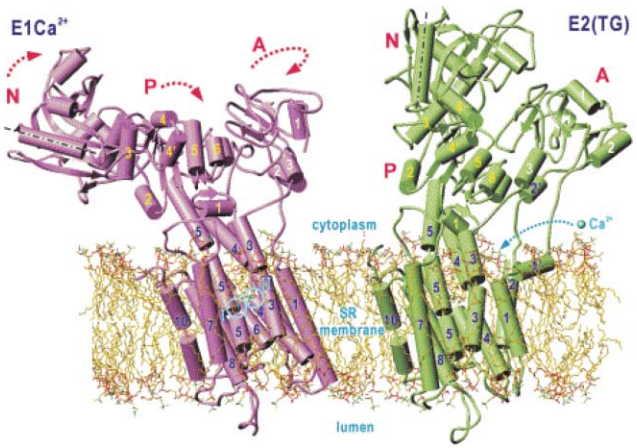


Fig. 7 E1Ca(II) and E2(TG) models of Ca(II)-ATPase. Cylinders represent α-helices, arrows β-strands. Two cyan spheres (circled also) in the transmembrane region of E1Ca(II) represent bound Ca(II) (sites I and II). Helices in the A and P domains and those in the transmembrane domain are numbered. Red arrows in E1Ca(II) indicate the direction of movement of the cytoplasmic domains during the change from E1Ca(II) to E2(TG). The cyan arrow in E2(TG) shows the proposed pathway for Ca(II) to enter into the binding cavity. Reproduced from ref. 83 with permission.

atoms of Asn-796, Asp-800 on M6 and Glu-309 on M4. Binding of the first Ca(II) to M5/M6/M8 induces a favorable configuration of M4, which facilitates binding of the second ion. Due to the flexibility of M4 and its direct link to the phosphorylation site, this conformational change of M4 produces important effects on ATPase function.

A comparison of the two conformations, E2(TG) (Ca(II)-free form), stabilized by the tight-binding sesquiterpene natural product inhibitor, thapsigargin, and E1Ca(II) (Ca(II)-bound form), for Ca(II)-ATPase reveals that the conformation E2(TG) has large changes relative to E1Ca(II) (Fig. 7).[84] In particular, the three cytoplasmic domains undergo large, rigid-body movements, which result in a compact cytoplasmic head that contrasts with the markedly open structure of E1Ca(II). However, the structure within individual cytoplasmic domains is largely unchanged. The transmembrane domain undergoes extensive deformations along most of its helices and the configuration of side-chains surrounding the calcium sites is also significantly different in the absence of these ions.

Active Ca(II) transport is achieved by changing the enzyme structure from E1 to E2 (Fig. 8b).[85] In E1, the ATPase has a high affinity for Ca(II) and ATP, and transmembrane Ca(II)-binding sites face the cytoplasm. Ca(II) binding and phosphorylation to E1P (where P indicates that the ATPase is phosphorylated) result in the conformational change from E1P to E2P. In E2, the ATPase has a low affinity for Ca(II) and ATP, and the binding sites face the lumen of the sarcoplasmic reticulum. The ATPase releases Ca(II) into the lumen of the sarcoplasmic reticulum for storage and dephosphorylates to E2. In each cycle, the ATPase can transport two Ca(II) per ATP hydrolyzed against a concentration gradient across the membrane.

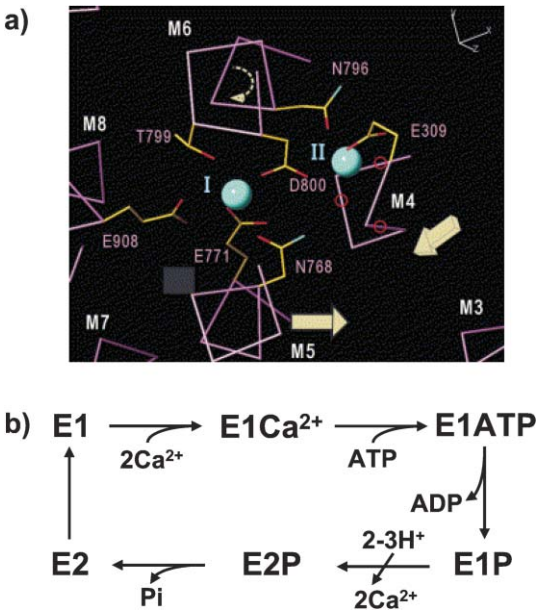


Fig. 8 (a) Ca-binding sites viewed from the cytoplasmic side. The arrows show the direction of movement in the E1Ca(II) to E2(TG) transition. Small red circles indicate approximate positions of carbonyl oxygens that contribute to the binding of Ca(II) in site II. (b) The bottom scheme shows a simplified reaction scheme toward the forward direction. Reproduced from ref. 83 with permission.

2.2.1.3. Cu(II)-ATPases ATP7A and ATP7B. The Cu(II)-ATPases, ATP7A and ATP7B, play an important role in the physiological regulation of the copper concentration in human cells.[86] They share about 70% amino acid identity.[87] Menkes disease is caused by various mutations or deletions in the gene *ATP7A*, which lead to copper deficiency.[88] In contrast, Wilson's disease is caused by copper accumulation, predominantly in the liver, brain, and kidneys, as a result of mutations in the *ATP7B* gene. Both ATP7A and ATP7B belong to the P_1-subfamily of cation-transporting P-type ATPases. Members of this subfamily commonly possess metal-binding sites in the N-terminal region of the protein, 8 transmembrane segments (6 segments before, and 2 segments after the ATP-binding domain, Fig. 9a), the conserved CP(C/H) motif in the transmembrane segment 6, and an SEHPL sequence located in the ATP-binding domain downstream of the putative DKTG phosphorylation site.

Both ATP7A and ATP7B have six repetitive sequences of approximately 70 amino acid residues at their N termini.[89] Each of these repeats contains a GMTCXXCXXXIE sequence motif and binds a copper cation in the +1 oxidation state.[89,90] Each copper atom is coordinated by two cysteines in a distorted linear geometry.[90] The ATP binding domain interacts with the N-terminal copper-binding domain in a copper-dependent manner.[91] Binding of copper to the N-terminal copper-binding domain decreases domain–domain interactions, which allows the ATP binding domain to adopt a new conformation with higher affinity for ATP.

In addition to the N-terminal copper-binding sites, there is at least one site in the membrane portion of the protein, surrounded by a sequence motif CPC and other conserved

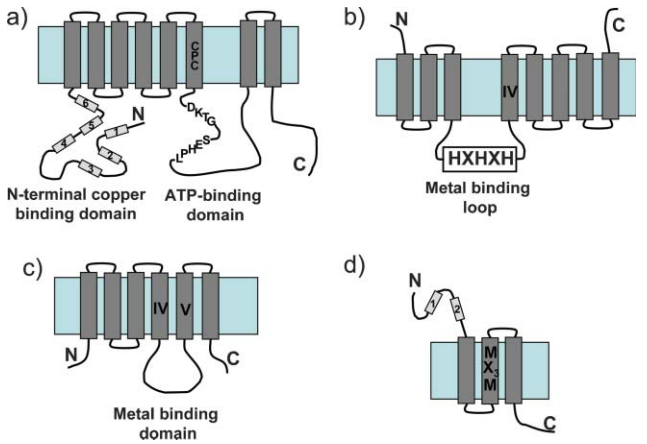


Fig. 9 Topological models of selected metal ion transporters. (a) ATP7A and ATP7B; (b) hZip; (c) ZnT1-4; (d) hCTR.

intramembrane residues.[92] Binding of copper to this intramembrane site is essential for copper-dependent catalytic phosphorylation of the ATPases by ATP.

Other members of the P_1-subfamily, or heavy-metal P-type ATPases, are the bacterial CopA and ZntA, which are involved in the transport of copper and zinc, respectively.[93] CopA and ZntA are highly homologous to ATP7A and ATP7B.

Recent evidence has indicated that expression of ATP7A and ATP7B can alter the sensitivity of cells to three platinum anticancer drugs, cisplatin, carboplatin and oxaliplatin.[94] It is still unclear if ATP7A and ATP7B are able to transport these three clinical drugs, and it remains possible that the effects of these proteins on drug sensitivity and accumulation are mediated indirectly through effects on other transporters or other detoxification mechanisms. The uptake, intracellular distribution, and efflux of the drug may be regulated in part by transporters and chaperones that evolved to manage copper homeostasis.

2.2.2. ATP-binding cassette (ABC) transporters. The ATP-binding cassette (ABC) transporter superfamily is a very large and diverse family of proteins which mediate active translocation of a wide range of chemically-diverse molecules across all cell membranes.[95] Analysis of various whole genome sequences has revealed that ABC transporters make up a large component of the encoded proteins (in the case of *Escherichia coli* $\sim$5%). The common structural features for this family are that each member contains a highly hydrophobic transmembrane domain (TMD) with 4 or 6 transmembrane spans, and a peripheral (cytosolic) nucleotide-binding domain (NBD) or nucleoside-binding fold. Most ABC proteins are primary pumps driven by ATP hydrolysis and transport a wide range of substrates including ions, sugars, lipids, peptides, pigments, xenobiotics, and drugs (including antibiotics).

The iron uptake systems are widely distributed among Gram-negative pathogenic bacteria, involving a periplasmic binding protein, a specific cytoplasmic permease, and an energy-supplying nucleotide binding protein.[96] Transport of iron from the periplasm into the cytosol is a classic active-transport process. Bacterial ABC transporters involved in the iron uptake systems include *Brachyspira hyodysenteriae* BitABC,[96] *Actinobacillus pleuropneumoniae* AfuABC,[97] *Haemophilus influenzae* HitABC,[98] *Neisseria meningitidis* FbpABC,[99] and *Serratia marcescens* SfuABC.[100]

2.2.2.1. Ferric-ion binding protein system FbpABC. We use FbpABC as an example to describe the mechanism of iron uptake in the pathogenic *Neisseria*. As shown in Fig. 10, there are three iron acquisition pathways in this system.[101]

In the first pathway, siderophores acquire iron from the host iron-binding proteins, transferrin and lactoferrin. To attain iron, many microorganisms secrete small molecular siderophores, which are specific Fe(III)-binding agents.[96] Although the pathogenic *Neisseria* do not produce siderophores, they possess a pathway for the uptake of iron-siderophore complexes produced by neighboring microbes.[102] The iron-siderophore complex is subsequently bound at the bacterial cell surface by an outer membrane receptor (labeled as R1), *e.g.* FetA, formerly designated FrpB.[103] The Fe-siderophore is transported through the outer membrane, energized by interaction with TonB from the TonB/ExbB/ExbD inner membrane complex.[101] The Fe-siderophore is transferred to a periplasmic siderophore-binding protein (labeled as P1) and then transported across the inner membrane by an inner membrane transport complex (labeled as C1).

In the second pathway, released intracellular haem iron present in the form of haemoglobin, haemoglobin-haptoglobin or free haem can be used directly as a source of iron for growth

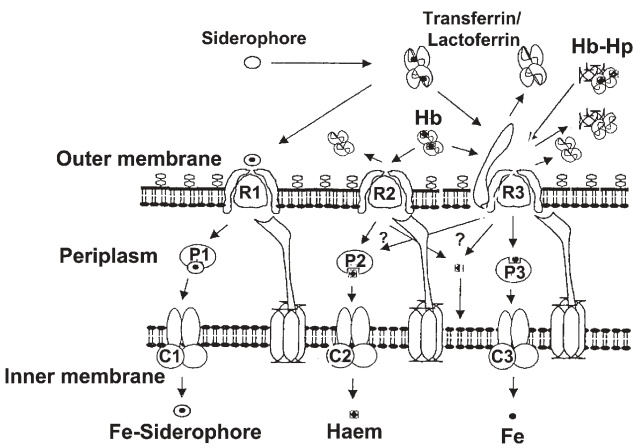


Fig. 10 Three iron acquisition pathways. The Fe-siderophore is transported in the first pathway. The proteins involved in the Fe-siderophore pathway are labeled as R1 for the outer membrane receptor, P1 for the periplasmic siderophore-binding protein, and C1 for the inner membrane transport complex. In the second pathway, Fe acquisition from Hb can be mediated either by an individual receptor protein, HmbR (labeled as R2), or by a bipartite receptor HpuA/B (labeled as R3). After transport across the outer membrane, the haem may bind to a periplasmic binding protein (labeled as P2) and be transported across the inner membrane by a transport complex (labeled as C2) or be incorporated directly by components present in the inner membrane. Fe(III) is transported in the third pathway. The proteins involved in this pathway are also labeled as R3 for the outer membrane receptor, P3 for the periplasmic iron-binding protein, FbpA, and C3 for the inner membrane transport complex, FbpB/C. Adapted from ref. 101.

through direct binding by specific surface receptors.[101] Fe acquisition from Hb can be mediated either by an individual receptor protein, HmbR (labeled as R2), or by a bipartite receptor HpuA/B (labeled as R3). After transport across the outer membrane, the pathway for haem is unknown. It may bind to a periplasmic-binding protein (labeled as P2) and be transported across the inner membrane by a specific inner membrane transport complex (labeled as C2), or be incorporated directly by components present in the inner membrane.

The third pathway involves direct iron binding from the extracellular host iron-binding proteins, transferrin and lactoferrin by a bipartite receptor (also labeled as R3), such as TbpA/B, or LbpA/B.[101,104] The iron is subsequently transferred to the periplasmic iron-binding protein, FbpA (labeled as P3) and shuttled across the periplasmic space. Finally it is transported across the inner membrane *via* FbpB/C (labeled as C3).

Upon iron starvation, *Neisseria* species express the 33.6 kDa protein FbpA in their periplasm to scavenge any iron which crosses the outer membrane. The gene encoding this FbpA belongs to an operon termed *FbpABC*, whose gene products have been proposed to behave as an ATP-binding cassette (ABC) transporter.[99] The crystal structure of HitA, an analog of FbpA from *H. influenzae* was the first periplasmic ferric-ion binding protein to have its crystal structure determined. This revealed that Fe(III) ion is bound by the phenolate oxygens of Tyr-195 and Tyr-196, an imidazole nitrogen from His-9, and a carboxylate oxygen from Glu-57, as well as two oxygens from phosphate and a water molecule, to give a pseudo-octahedral coordination in a closed-cleft conformation.[105] This binding site shows remarkably high binding affinity for ferric iron ($K_{eff} \sim 10^{20}$ M^{-1}). A similar binding mode has been proposed for Fe(III)-FbpA.[106] Recently, a new oxo-tri-iron cluster adduct of FbpA has been discovered.[107] FbpA can readily bind iron and other oxo-metal clusters[108,109] in an open protein cleft and these fragments of (hydr)oxo iron minerals could be involved in iron trafficking. FbpA itself may be able to sample the extracellular environment across the outer cell membrane. The strong binding of exogenous metal ions such as Ti(IV),[110] Zr(IV),[109] and Hf(IV),[108] could provide a basis for the design of novel antibiotics which act by blocking Fe(III) uptake.

FbpA homologs from the ferric transport systems of a number of other pathogens, *e.g. Mannheimia haemolytica*,[111] *Yersinia enterocolitica* YfuA, and *Serratia marcescens* SfuA,[112] and *Campylobacter jejuni* FbpA,[113] have been isolated and their structures determined. This has revealed subtle differences in the ferric ion binding ligands, *e.g.* the *C. jejuni* FbpA binding site contains only five protein ligands and no exogenous anion and can bind both ferrous and ferric ion substrates. It appears that the consecutive di-tyrosine motif is absolutely conserved and required for binding, but the other ligands can vary, as well as the coordination number of the iron ion. This has led to a classification system for >30 FbpAs from pathogenic bacteria.

In the bacterial ABC transporters, the cytoplasmic membrane protein and the nucleotide-binding domain (NBD) protein activities exist on separate polypeptides in the inner membrane.[96] It is thought that they associate, with the membrane protein acting as a permease and the other NBD protein providing energy for iron transport by ATP hydrolysis. In the FbpABC iron transport system, FbpB and FbpC are the cytoplasmic permease and the ATPase subunit, respectively, based on the amino acid sequence similarity to homologous proteins.[99]

2.2.2.2. Vitamin B_{12} transporter (BtuBFCD). A vitamin B_{12} uptake (*B twelve uptake*, Btu) system for Gram-negative bacteria is also well-known, and imports the organometallic cofactor into the cell through the outer membrane, periplasm and the cytoplasmic membrane of the cell envelope (Fig. 11a).[114–116] The outer membrane transporter for cobalamin in *E. coli* is BtuB, which couples with the TonB protein, comprising an inner membrane complex that provides energy for active transport.[117] BtuB consists of a β-barrel domain (137–594) and a hatch domain (6–132), connected by a linker (133–136) (Fig. 11b).[118] The 22-stranded β-barrel domain spans the outer membrane with extracellular loops and periplasmic turns. The hatch domain is formed around a core

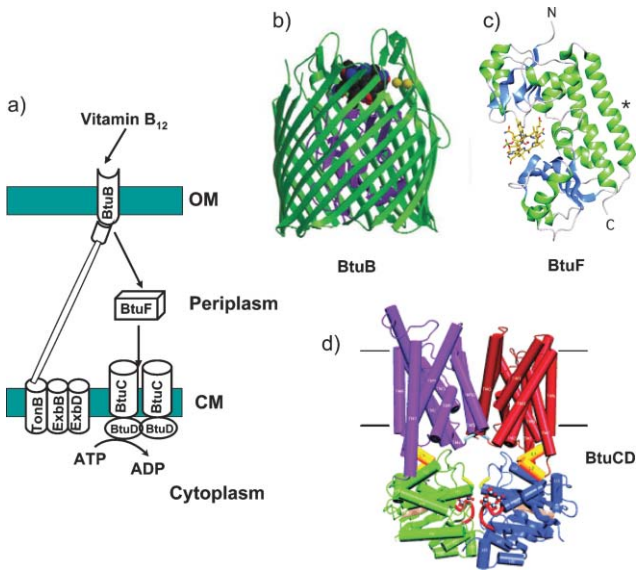


Fig. 11 (a) A vitamin B_{12} transport system in *Escherichia coli* involving the outer membrane protein BtuB, periplasmic protein BtuF and cytoplasmic membrane protein BtuCD. BtuB, which couples with the TonB protein, comprising an inner membrane complex that provides energy for active transport. BtuF is the periplasmic binding protein, which shuttles the substrate to the ABC transporter BtuCD. BtuCD consists of four subunits, two membrane-spanning permease BtuC subunits and two ATPase BtuD subunits. (b) Structure of BtuB in the bound state with the substrate B_{12} shown as side view. The β-barrel domains are shown in green; hatch domains, in purple. Bound calcium ions and bound vitamin B_{12} substrate are shown in space-filling format. (c) Structure of the periplasmic binding protein BtuF. The bound vitamin B_{12} is shown in ball and stick format. The N and C termini are labeled N and C, respectively. The α-helix, bridging the two lobes of the protein, is marked with an asterisk. (d) Structure of the ABC transporter, BtuCD for vitamin B_{12}. Two membrane-spanning BtuC subunits are shown in purple and red and two ABC cassette BtuD subunits are shown in green and blue. Helices are shown as cylinders and strands are shown as ribbons. (b) Reproduced from ref. 118 with permission. (c) Reproduced from ref. 120. Copyright 2002 National Academy of Sciences. (d) Reprinted with permission from ref. 122. Copyright 2002 AAAS.

of four β-strands with the Ton box located at residues 6–12. In the bound state, with the cyanocobalamin substrate, residues of two loops of the hatch domain, specially, residues 88–92, show extensive interaction with the substrate. Most of the interactions between the BtuB barrel domain and substrate are through the extracellular loops connecting β-strands 5–6 and 7–8. Additional interaction with cyanocobalamin is provided by residues in the loops of β-strands 17–18, 19–20 and 21–22, with the latter two loops contributing aromatic residues. Upon binding to the substrate, conformational changes occur in a loop of the hatch domain (residues 85–96), as well as the extracellular 19–20 loop and the periplasmic 2–3, 8–9 and 10–11 turns of the β-barrel domain. In addition, a substrate-induced conformational change in the Ton box region of BtuB occurs, which may lead to recognition by the TonB protein and recruit of TonB to the TonB box of this transporter.

The recent X-ray crystal structure[119] of BtuB in a complex with the C-terminal domain of TonB shows that the TonB box forms a β-strand that is recruited to an existing β-sheet of TonB. This interaction may induce a mechanical pulling force that drives transport and could be a target for drug design.

BtuF is the periplasmic binding protein, which shuttles the substrate to the ABC transporter BtuCD (Fig. 11c). It is composed of two structurally similar domains (lobes), each consisting of a central five-stranded β-sheet surrounded by helices.[120] The domains are connected by a single α-helix spanning the length of the protein. The substrate vitamin B_{12} is bound in a deep cleft between the two lobes of BtuF. The dimethylbenzimidazole (DMB)-binding surface of B_{12} interacts exclusively with residues in domain I in BtuF, while the catalytic surface interacts exclusively with domain II.[121]

The ABC transporter, BtuCD transports the B_{12} substrate across the cytoplasmic membrane, coupled with the hydrolysis of ATP. The complete transporter consists of four subunits, two membrane-spanning BtuC subunits and two ABC cassette BtuD subunits, to which a single BtuF monomer is thought to bind (Fig. 11d).[122] This complex can be formed in solution and is stable to size-exclusion chromatography. The BtuC subunits associate with the BtuD subunits. Each of the two BtuC comprises 10 transmembrane domains giving a total of 20 transmembrane helices, forming a translocation pathway for the substrate, which is closed to the cytoplasm by a gate region. During the transport process, conformational changes of the BtuD subunits, due to ATP binding and hydrolysis, are transmitted to the membrane-spanning domains BtuC, which causes the channel gate to open and allows B_{12} to enter the cell.

It is notable that the transport system for the cobalt complex vitamin B_{12} appears to involve exclusive recognition of the ligands for cobalt (the corrin ring and axial benzimidazole) without direct binding of the transport proteins to cobalt. Subsequent docking of B_{12} onto its enzyme target can involve cobalt coordination to amino acid side-chains (His).

2.2.2.3. ABC transporter systems FhuADBC, FepABCDG and FecABCDE. A number of other bacterial iron transport systems have been characterized. In general, there are also three steps for transport of ferric chelates, which involve an outer membrane receptor, a periplasmic-binding protein, and an inner membrane ATP binding cassette transporter (Fig. 12).

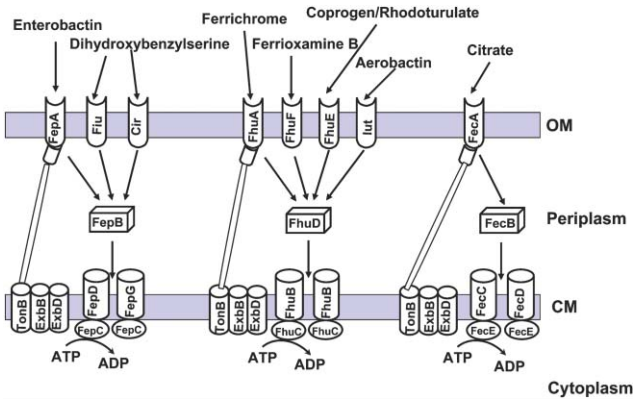


Fig. 12 Iron transport systems of *Escherichia coli.*

Outer transmembrane proteins in *E. coli*, such as FecA, FhuA and FepA for the active transport of the iron siderophores ferric citrate, ferrichrome, and enterobactin, respectively, need energy for transporting the ferric chelates into the periplasm, which is supplied by a complex which includes three membrane proteins: TonB, ExbB and ExbD.[123] Therefore, these outer membrane proteins are also known as TonB-dependent transporters. The X-ray crystal structures of FhuA,[124] FepA,[125] and FecA[126] indicate that their architecture is similar to that of BtuB with a common plug-barrel architecture: an N-terminal plug domain folds into a 22-stranded transmembrane β-barrel and obstructs the barrel lumen (Fig. 13). Interestingly, it has been shown recently that the N-terminal plug can fold independently of the β-barrel domain.[128] The ligand binding site is exposed to the external medium, whereas the TonB binding site is located at the periplasmic side of the receptor. A peptide motif near the N terminus is conserved among all TonB-dependent receptors: D7TITV in FhuA, D12TIVV in FepA, D81ALTV in FecA, which is called the "TonB box".[129] Binding of the ligand to TonB-dependent receptors induces conformational changes of the cork domain. For the receptor FhuA, a short α-helix ("switch-helix") following the TonB box, which is exposed to the periplasm, unwinds. The relocation of the switch-helix likely changes the position and accessibility of the TonB box on the periplasmic side of the receptor. The conformational changes, propagated from the ligand binding site to the periplasmic side, initiated by the binding of a siderophore near the extracellular surface of the protein, may result in the signal to induce the interaction between TonB-dependent siderophore transporters and the energy-transducer TonB. In order to acquire energy from the inner membrane protein complex ExbB/ExbD/TonB, the hydrophobic region at the N terminus (residues 1–32) anchors the TonB protein to the cytoplasmic membrane. Residues 12–32, which exist in an α-helical conformation, and contain four highly conserved residues (the so-called "SHLS-motif") play an important role in the interaction with the integral membrane protein ExbB. The transmembrane domain is followed by a periplasmic part with high proline content and a conserved C-terminal domain, each composed of ~100 amino acid residues. A recent X-ray structure of a complex between the TonB carboxyl-terminal domain and FhuA shows that TonB contacts and stabilizes FhuA's amino

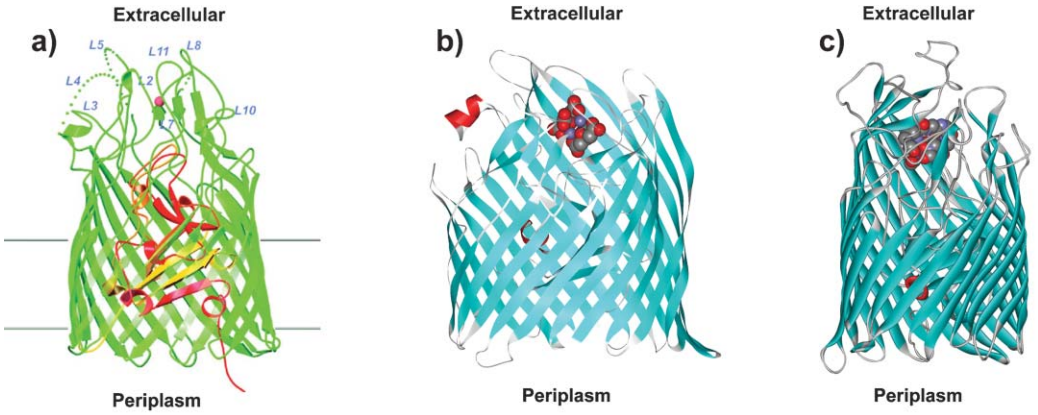
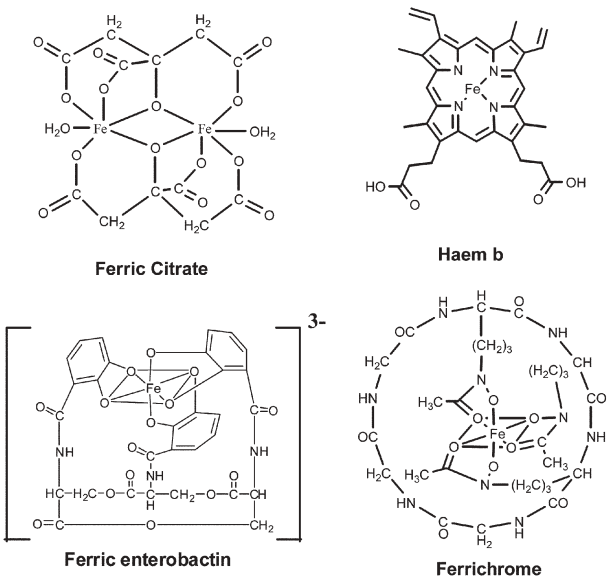


Fig. 13 (a) Ribbon diagram of FepA. The putative iron position of ferric enterobactin is indicated by the red sphere. Part of the barrel has been rendered transparent to reveal the N–terminal domain located in the channel. All long extracellular loops are labeled; disordered parts of the loops are indicated by dotted lines. Reproduced from ref. 125 with permission. (b) Ribbon diagram of FecA. The 22-stranded β-barrel is shown in blue and ferric citrate is shown in space-filling format. Produced from the pdb code 1PO3. (c) Ribbon diagram of FhuA. The barrel is colored blue and ferrichrome is shown in space-filling format. The extracellular space is located at the top and the periplasmic space is at the bottom. Produced from the pdb code 1FCP.

terminal residues.[130] In addition to its transport function, FecA acts as a regulator of transcription induction, and induces the transcription of the ferric citrate transport genes, *fecABCDE*, in the cytoplasm upon ligand binding.

There are two major classes of siderophores: hydroxamates and catecholates. Although *E. coli* itself secrets only one siderophore, the catecholate enterobactin, it can utilize siderophores from a variety of sources. The periplasmic protein FhuD can bind to a variety of hydroxamate siderophores including siderophore ferrichrome from FhuA, aerobactin from IutA, rhodoturulate and coprogen from FhuE, ferrioxamine B from FhuF, and further shuttles to the ABC transporter FhuBC (Fig. 12).[131] The N- and C-terminal domains of FhuD are connected by a 23-residue kinked α-helix.[132] The N-terminal domain (residues 27–141) consists of a twisted five-stranded parallel β-sheet and the C-terminal domain (residues 166–288) comprises a five-stranded mixed β-sheet. The siderophore is held in a shallow pocket lined with aromatic groups and Arg and Tyr side-chains bind to the hydroxamate moieties of this siderophore. The ABC transporter FhuBC consists of two permease subunits, and two peripheral ATPase subunits, FhuB, which attach to FepD and FepG separately and can hydrolyze ATP to generate the energy for transport of the siderophores across the inner membrane.[133] It further transports the substrate across the inner membrane.

FepB is another periplasmic protein, which can bind to catecholate siderophores, including enterobactin from FepA[134] and dihydroxybenzylserine from Cir and Fiu,[135] and further transport them across the periplasm (Fig. 12).[136] Once carried across the periplasm by FepB, the catecholate siderophores are delivered to the FepCDG complex, which is an ATP-dependent ABC transporter and can further transport the substrate across the inner membrane. FepCDG consists of two different permease subunits, FepD and FepG, and two identical ATPase subunits, FepC, which attach to FepD and FepG separately and can hydrolyze ATP to generate the energy for transport of the siderophores across the inner membrane.



The ferric citrate transport system consists of an outer transmembrane protein, FecA, a periplasmic protein, FecB and an ATP-dependent ABC transporter, FecCDE, which comprises two different permease subunits, FecC and FecD, and two identical ATPase subunits, FecE (Fig. 14).[137] FecA specifically binds to the ferric citrate and subsequently transfers to the periplasmic protein, FecB and shuttles across the periplasm to the ABC transporter, which further transports the substrate across the inner membrane (Fig. 12 and 14). "Ferric citrate" has complicated chemistry in solution.[138] Its structure depends highly on the pH and the Fe(III) : citrate ratio. Polynuclear species predominate at low Fe : citrate molar ratios (1 : 1 to 1 : 4), whereas at neutral and basic pH values mononuclear species are formed especially with excess citrate present. Many workers assume that a diferric dicitrate

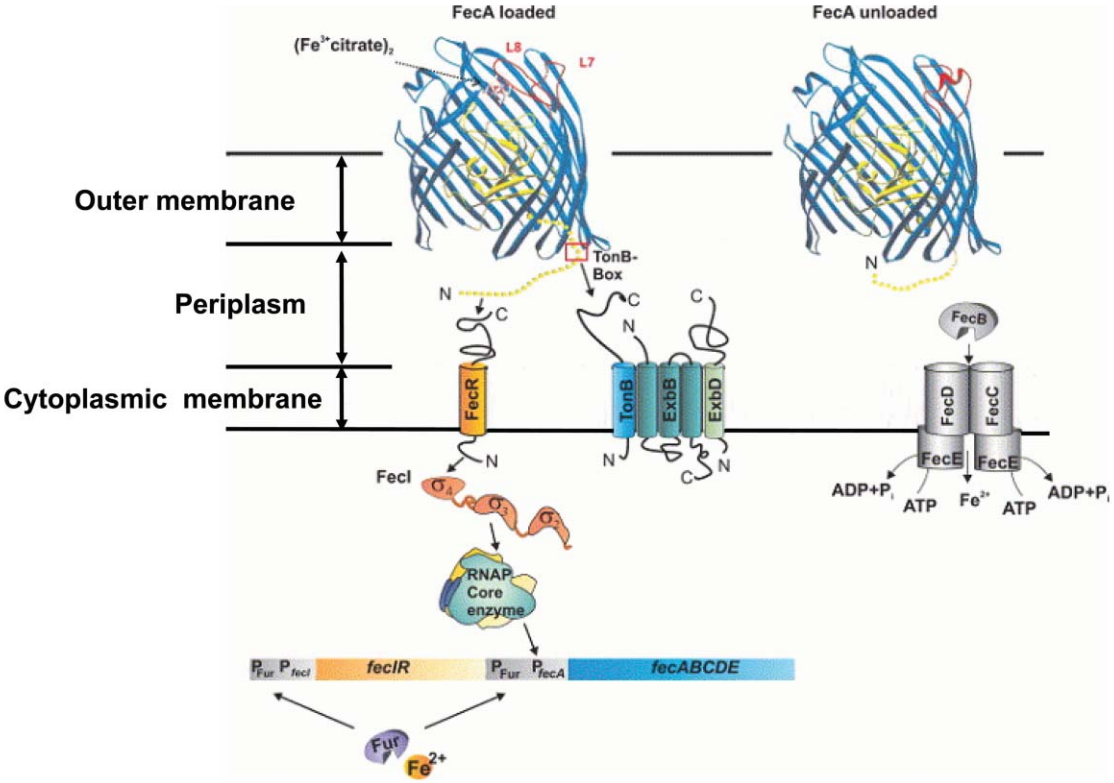


Fig. 14 Illustration of the regulatory activities (left side) and transport activities (right side) of the Fec system in *E. coli*. Adapted from ref. 137.

dimer is the species recognized by membrane transporters, but this requires further investigation.

Siderophore-drug conjugates are being developed for treatment as potential drugs since corresponding siderophores can be recognized and transported by certain transport systems.[139] Therefore, the conjugates may be selective and more effective.

2.2.3. Zip family of zinc transporters. Zinc transporters, Zrt1, Zrt2, and Irt1 were first identified in fungi and plants, and therefore this family was named as the ZIP family for **Z**rt/**I**rt-like **p**roteins.[140] The Zrt1, Zrt2 proteins transport zinc with high specificity in yeast, while the Irt1 protein transports iron as well as zinc, manganese, and cadmium in plants. There are 12 ZIP-encoding genes in humans, and many of these proteins are zinc transporters. Four Zip proteins, hZip1, hZip2, hZip3 and hZip4 have been characterized.[141,142] The ZIP proteins typically have eight membrane-spanning domains, one of which (domain four) contains fully conserved histidyl and glycyl residues in an amphipathic α-helix. This helix may provide an intramembrane metal ion binding site and aid the lining of the transmembrane channel that can serve as the transport pathway (Fig. 9b).[142,143] Irt1, Zrt1, and Zrt2 have a histidine-rich intracellular loop containing a possible binding motif HXHXH.[143] However, this binding motif occurs in the C-terminal hydrophilic tails, and in more than one place for some proteins. These structural motifs are hallmarks of the ZIP superfamily.

Fungal and human ZIPs work by different mechanisms.[141] Zinc uptake by hZip2 is energy-independent, while the yeast zinc transporters show a strict energy-dependence. Zinc uptake by hZip2 is stimulated by increased pH and treatment with bicarbonate. Zinc uptake could be driven by the high concentration of HCO_3^- in serum (10–25 mM) and by the concentration gradient of labile zinc across the plasma membrane.

2.2.4. Cation diffusion facilitator family (CDF). The cation diffusion facilitator family (CDF) contains the cobalt, zinc, and cadmium resistance protein CzcD in bacteria,[144] zinc resistance ZRC1 and cobalt transporter COT1 in yeast,[145,146] and the mammalian proteins ZnT1–7.[147] All ZnTs, with the exception of ZnT5, have six transmembrane domains, with NH_2 and COOH termini located on the cytoplasmic side of the membrane (Fig. 9c).[148] They also contain a conserved histidine-rich domain between transmembrane segments IV and V that, at least in the case of ZnT4, was shown to be capable of binding metal ions. ZnT5 is longer than the other ZnTs, but only its COOH-terminal portion displays homology to ZnT proteins. It appears to associate with secretory granules in pancreatic β cells and with the apical membrane of intestinal enterocytes.

2.2.5. Natural resistance-associated macrophage proteins (Nramp). The natural resistance-associated macrophage protein (Nramp) family consists of Nramp1, Nramp2, and yeast proteins Smf1, Smf2 and Smf3. Nramps are integral membrane glycoproteins composed of 12 predicted transmembrane domains.[149] Nramp1 transports M(II) cations, including Mn(II), Fe(II), and Co(II). It appears to play a role in macrophage antimicrobial function by extruding M(II) metal

ions (*e.g.*, Mn(II) and Fe(II)) from the phagolysosome, thereby depriving bacteria of metal ions that may be essential cofactors for bacteria-derived enzymes (*e.g.* superoxide dismutase) or required for bacterial growth. Two histidyl residues that reside in transmembrane helix 6 appear to be critical to normal function. His-267 and His-272 may both be involved in metal binding. In addition, genetic variation in the human *Nramp1* gene may be associated with cavitation in patients with tuberculosis.[150] Nramp2 is a transporter for Fe(II), Zn(II), Mn(II), Co(II), Cd(II), Cu(II), Ni(II) and Pb(II), and mediates metal transport coupling to the movement of H^+ down its electrochemical gradient.[151] The same mechanism for the cellular uptake of metals by Nramp2 has been proposed.[149] Mutations at *Nramp2* affect iron homeostasis and cause severe microcytic anemia.[152]

2.2.6. Copper transporters CTR and COPT. CTR family members, including CTR1–6, have been identified.[153,154] Among them, both CTR4 and CTR5 exist as components of a heteromeric complex that is essential for high affinity copper transport. CTR proteins contain three predicted transmembrane domains, and a series of clustered methionine residues in the hydrophilic extracellular domain and an MXXXM motif in the second transmembrane domain, which are important for copper uptake.[155] Two human proteins hCTR1 and hCTR2 have also been identified.[156] The hCTR1 and yeast CTR1 predicted transmembrane proteins are 29% identical; the human protein is smaller with only 190 amino acids as compared with 406 amino acids.[156,157] The model of hCTR1 also has a three-transmembrane segment with an extracellular amino terminus and an intracellular carboxyl terminus (Fig. 9d).[157] The amino-terminal domain of hCTR1 has been described as having two Met motifs; however, the first of these is MXMXXM, rather than the usual MXXMXM, and the second is unusually methionine-rich in having the sequence MMMMXM. hCTR1 also has a methionine-rich sequence at the extracellular boundary of the second putative transmembrane segment that may be important for function. Methionine sulfur is a relatively poor ligand for Cu(II) but binds more strongly to Cu(I).

CTR1 is a major protein associated with the uptake of the anticancer drug cisplatin in yeast,[158] and there is evidence that CTR1 mediates cisplatin uptake in yeast and mammals.[159] Methionine sulfur is a strong ligand for Pt(II) and its high *trans* effect can lead to labilization of the ammine ligands in cisplatin.[160] Deletion of the yeast *CTR1* gene results in increased cisplatin resistance and reduced intracellular accumulation of cisplatin. Degradation and internalization of CTR1 enhances survival of wild-type yeast cells exposed to cisplatin and reduces cellular accumulation of the drug. Mouse cell lines lacking one or both mouse *mCTR1* alleles exhibit increased cisplatin resistance and decreased cisplatin accumulation in parallel with *mCTR1* gene dosage. CTR1 might serve as a channel that facilitates uptake of the drug.[159] It appears, therefore, that transporters and chaperones that normally mediate Cu homeostasis also directly or indirectly modulate the accumulation of cisplatin in some cell lines,[161] but not in others.[162]

COPT1–5 belong to a five-member family of Cu transporters.[163] COPT1 is a highly hydrophobic protein with 169 amino acid residues and three putative transmembrane domains, which shows sequence homology to the copper transporter CTR1 from yeast.[164] The first 44 residues display significant homology to the methionine- and histidine-rich copper binding domain of the bacterial copper-binding proteins.[165]

2.2.7. Iron regulated-transporter-1 (Ireg1). The iron regulated-transporter-1 (Ireg1), also known as ferroportin or metal transporter protein-1 (MTP1), is the only member of the SLC40 transporter family.[166] The predicted structure shows that it possesses 9 transmembrane α-helices with the N-terminus on the intracellular side of the membrane and the C-terminus on the extracellular side. Ferroportin has been identified as the basolateral iron transporter of the duodenal enterocyte, which exports iron and loads it onto serum transferrin.[167] The peptide hormone hepcidin, secreted by the liver, regulates cellular iron efflux by binding to ferroportin and inducing its internalization and degradation, leading to a decrease in release of iron into the blood stream.[168] Mutation of the gene that encodes ferroportin leads to an iron overload disorder, ferroportin disease.[169] Improved understanding of how the metabolism of iron is regulated by hepcidin may aid development of treatments for chronic anemia and hemochromatosis.[168]

2.2.8. Bacterial magnetosomes. Magnetotactic bacteria are prokaryotic aquatic microbes that were originally isolated from mud samples more than 30 years ago (Fig. 15).[170] They can navigate along the Earth's magnetic field and the molecular mechanism that allows them to orientate themselves in this manner has long been the subject of intrigue.[171] The best studied magnetotactic bacteria are *Magnetospirillum gryphiswaldense* and *M. magneticum* and transmission electron microscopy of these organisms has revealed the presence of unique intracellular structures, "magnetosomes", which comprise magnetic crystals surrounded by a lipid bilayer membrane ~3–4 nm thick. These crystals contain either iron oxide magnetite (Fe_3O_4) or iron sulfide greigite (Fe_3S_4). Little is known about the mechanism by which these particles are synthesized and the metal transport systems involved, however, recent molecular biological studies have begun to unravel this complex biomineralization pathway. The magnetosome membrane (MM) is crucial in the biological control of mineral formation and recent proteomic studies have identified at least 18 proteins present in the MM fraction from *M. gryphiswaldense*.[172] The *mam* genes encoding these proteins have been localized to a 35-kbp region of the bacterial chromosome "magnetosome island" containing three gene clusters that have been sequenced. Approximately half of the encoded proteins show homology to proteins of currently unknown function, and the others have similarity to haem and iron binding proteins. One in particular is predicted to be a CDF protein, which has been shown to be a heavy metal ion transporter (see 2.2.4 above). All CDF proteins contain six putative transmembrane-spanning domains, but it remains to be elucidated what exact role they play in the biomineralization process. At present it appears that the magnetosome formation is dependent on the arrangement of protein complexes present in the magnetosome membrane. It will be interesting to explore

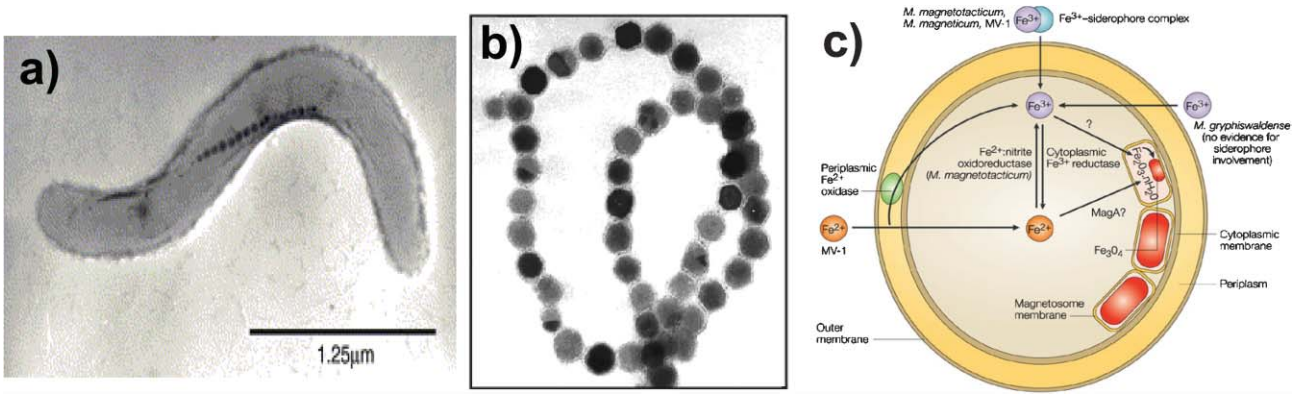


Fig. 15 (a) Electron micrograph showing *Magnetospirillum magneticum* AMB-1. Reproduced from ref. 171b with permission. (b) Electron micrographs of isolated, untreated magnetosome particles from *Magnetospirillum gryphiswaldense*. Reproduced from from ref. 172 with permission. (c) Schematic of possible reactions leading to magnetitie biomineralization in known, cultured species of magnetotactic bacteria. Reproduced from ref. 171a with permission.

the function of each of the proteins and discover the molecular details of the metal transport pathways in these unusual organisms.

3. G-protein coupled receptors (GPCRs)

Transmembrane receptors are also integral membrane proteins. Binding of signaling molecules to the receptor on one side of the membrane initiates a response on the other side. These receptors play important roles in cellular communication and signal transduction. It has been suggested that all GPCRs function as dimers.[173]

G-coupled receptors with 7 transmembrane helices are an important superfamily of membrane receptors with the N-terminal sequences located on the extracellular side and C-terminal sequences on the intracellular side. Over 800 genes are known to encode such proteins,[174] but to date there is high-resolution X-ray structural information only for the detergent-solubilized light receptor bovine rhodopsin (Rho).[175] Members of this superfamily include receptors for many hormones, neurotransmitters, chemokines and calcium ions, as well as sensory receptors for various odorants, bitter and sweet taste, and even photons of light. G-coupled receptors are thought to be protein targets involved in $\sim$40% of all therapeutic interventions.

G proteins are made up of three subunits, α, β, and γ.[176] G proteins are inactive in the GDP-bound state. After binding to its ligand, the activated receptor induces a conformational change in the associated G protein α-subunit leading to release of GDP followed by binding of GTP.[177] GTP binding leads to dissociation of Gα·GTP from the Gβγ subunits.[176] Both the GTP-bound α-subunit and the released βγ-dimer can modulate several cellular signaling pathways. These include stimulation or inhibition of adenylate cyclases and activation of phospholipases, as well as regulation of potassium and calcium channel activity. In addition, human immune deficiency virus (HIV) utilizes G protein-coupled chemokine receptors as cofactors for entry into cells,[178] and SDF-1/CXCR4 interactions regulate the mobilization of hematopoietic stem cells.[179] In this review, we concentrate on the aspects related to metals.

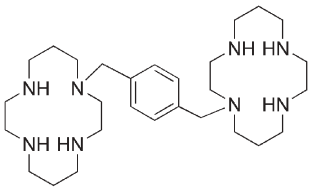
G protein-activated inward rectifier K(I) (GIRK) channels are activated by binding of Gβγ.[180] Four subunits are identified for mammal GIRK1 to GIRK4, which are identical (homotetramer) or different (heterotetramer). The primary binding sites of GIRK channels for Gβγ are in both intracellular C- and N-terminals.[181]

G-protein also can activate phospholipase C, which hydrolyzes phosphoinositol bisphosphate (PIP_2) to cytosolic inositol-1,4,5-triphosphate (InsP3) and membrane-bound diacylglycerol (DAG).[182] The second messenger InsP3 binds to its receptor, a ligand-gated calcium channel spanning the endoplasmic reticular membrane, causing release of calcium into the cytosol from intracellular stores. Calcium binds to the protein calmodulin, and the Ca–calmodulin complex activates a number of processes. In addition, Gβγ can also activate directly G-protein-responsive Ca(II) channels to facilitate diffusion through ion channels.[176]

G-protein also can activate protein kinases through different pathways. In this review, we use a G-protein regulated cyclic AMP signaling pathway as an example. The Gα/GTP subunit of the G-protein activates a membrane bound adenylate cyclase which further catalyzes the cyclization of ATP to cAMP.[183] cAMP acts as a second messenger and activates protein kinase A, which can transfer a phosphate from ATP to another protein kinase, thus initiating a signal cascade. The phosphorylation occurs in the presence of Na(I).

Several G-coupled receptors have been identified as cofactors for HIV-1 entry into the host cells.[184] The co-receptor CCR5 mediates entry of viruses characterized as macrophage tropic or dual tropic,[185] and the co-receptor CXCR4 has been shown to mediate entry of T-cell-tropic or dual-tropic strains.[178a,184] Therefore, both CCR5 and CXCR4 are targets for anti-HIV inhibitors. The drug AMD3100, in which two cyclam (1,4,8,11-tetraazacyclotetradecane) units are bridged by an aromatic phenylenebis(methylene) linker, is a highly potent and selective anti-HIV-1 and HIV-2 agent[186] and blocks HIV-1 entry by binding to the co-receptor CXCR4.[187] It was selected as a clinical candidate, but after phase II trials, significant cardiac side-effects led to its withdrawal from further development as an anti-HIV agent.[188] There is

continued clinical interest in the ability of AMD3100 to mobilize hematopoietic progenitor cells from marrow to peripheral blood,[189] and AMD3100 is in clinical use as the drug "Mozobil".



AMD3100

Cyclams bind strongly to Zn(II), and relatively rapidly at (micromolar) concentrations close to those in the blood.[190] AMD3100 may therefore acquire Zn(II) in the blood. After incorporation of Zn(II), AMD3100 contains additional binding sites through which it can interact with the carboxylate (and perhaps histidine) side-chains of the co-receptor CXCR4.[191] The affinity of AMD3100 for the co-receptor CXCR4 is enhanced 7–50-fold by complexation to the transition metal ions Cu(II), Zn(II) and Ni(II).[192] The strength of binding to the co-receptor correlates with the anti-HIV activity of cyclams. Zn(II) together with acetate binding induces an unique folded configuration for AMD3100, *cis*-V.[191] This involves coordination of Zn(II) to acetate on one side of the macrocycle and double H-bonding between two cyclam NH groups and the two oxygens of acetate on the opposite face. The *cis*-V configuration may be very important for the anti-HIV activity of cyclams and is stabilized by binding to specific Asp and Glu carboxylate side-chains of the co-receptor CXCR4 *via* metal coordination and H-bonding. Hydrophobic interactions between the periphery of the macrocycle and protein side-chains may also be important for entry into the hydrophobic cavities of the membrane protein. Our studies show that Cu-cyclam interacts with specific tryptophan residues of lysozyme (Trp-62, Trp-63, and Trp-123).[193] Two major binding sites for both Cu-cyclam and Cu_2-xylyl-bicyclam on lysozyme were detected by X-ray crystallography. In the first site, Cu(II) in one cyclam ring of Cu_2-xylyl-bicyclam adopts a *trans* configuration and is coordinated to a carboxylate oxygen of Asp-101, whereas for Cu-cyclam two ring NH groups form H-bonds to the carboxylate oxygens of Asp-101, stabilizing the unusual *cis* (folded) cyclam configuration. For both complexes in this site, a cyclam ring is sandwiched between the indole side-chains of two tryptophan residues (Trp-62 and Trp-63). In the second site, a *trans* cyclam ring is stacked on Trp-123 and H-bonded to the backbone carbonyl of Gly-117. We have recently carried out similar studies on the interaction of Ni-cyclam and Ni_2-xylyl-bicyclam with lysozyme and found similar binding sties to those for the copper cyclams.[194] Interestingly, octahedral *trans*-III configurations can slowly convert to square-planar *trans*-I configurations in aqueous solution and for Ni^{II}-xylylbicyclam, a mixture of *cis*-V and *trans*-I configurations can exist in aqueous solution. In a model of the human CXCR4 co-receptor, there is a pocket in which *trans* and *cis* configurations of metallobicyclam can bind by direct metal coordination to (Asp, Glu) carboxylate side-chains, by cyclam-NH···carboxylate H-bonding, together with hydrophobic interactions with tryptophan residues (Fig. 16).[193] These studies provide a structural basis for the design of macrocycles that bind stereospecifically to G-coupled and other protein receptors, and suggest that configurationally-restricted metallomacrocycles may be highly effective CXCR4 inhibitors.

The 18-residue cationic polypeptide T22 ([Tyr,^{5,12} Lys^{7}]-polyphemusin II), an analogue of polyphemusin II isolated from the hemocyte debris of American horseshoe crabs (*Limulus polyphemus*), also exhibits strong anti-HIV-1 activity.[195] T22 has an antiparallel β-sheet structure maintained by two disulfide bridges, and contains two antiparallel repeats of Cys–Tyr–Arg–Lys–Cys. T22 specifically inhibits the entry of T cell line-tropic HIV-1 into target cells mediated by CXCR4.[195] Zn(II) coordination to this peptide leads to a fourfold increase in its anti-HIV activity.[196]

Zinc ions also bind with high affinity to and modulate G-protein coupled receptors such as the tachykinin NK1 and NK3 receptors,[197] the β_2-adrenergic receptor,[198] melanocortin MC1 and MC4 receptors.[199] The melanocortin (MC) receptors are regulated by agonists, the melanocortins, *e.g.* adrenocorticotropic hormone, and α-, β-, and γ-melanocyte-stimulating hormone (MSH), and by agonists, the agouti and agouti-related proteins.[200] They are widely distributed both in the periphery and in the central nervous system.[201] The physiological functions of the MC3 and MC4 receptors are in appetite regulation and metabolic control.[199] Zn(II) can function both as a partial agonist and as an enhancer or potentiator for the peptide agonists for the MC1 and MC4 receptors. Zn(II) modulates the melanocortin receptors positively towards increased signaling through three different mechanisms: (1) direct agonism; (2) enhancement of the endogenous agonist function; and (3) inhibition of the natural inverse agonist.[202] Mutational mapping of the Zn(II) binding site in the MC1 receptor has indicated that Cys-271 in extracellular loop 3 and possibly Asp-119 at the extracellular end of TM-III, which are both conserved among all MC receptors, are parts of the

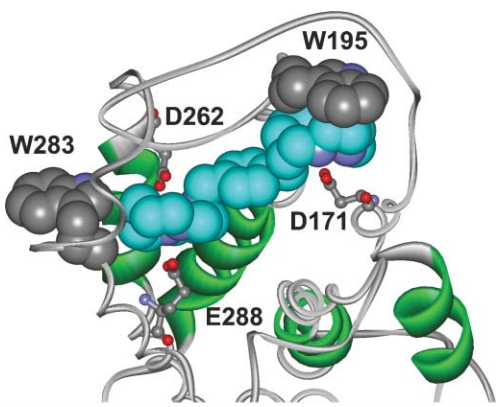


Fig. 16 Model of the zinc complex of the anti-HIV drug AMD3100 bound to the CXCR4 co-receptor. One of the cyclam rings in a *trans* configuration is stacked on Trp-195 and its Zn is bound to Asp-171. The other cyclam ring has a folded configuration (*cis*) and is close to Trp-283; its Zn is bound to Asp-262, and two ring NH groups are H-bonded to the oxygens of Glu-288 Adapted from ref. 193.

site.[199,202] The ligand-binding crevice, located between TM-III, -IV, -V, -VI, and -VII in the MC receptors is easily approached by the ligands since TM-IV connects directly into TM-V *via* an extremely short extracellular loop 2, consisting of only one or two residues, whereas extracellular loop 3 is constrained by a putative disulfide bond between Cys-265 and Cys-273 (Fig. 17).

Zn(II) complexes with the synthetic ligands 1,10-phenanthroline and 2,2′-bipyridine, as well as their analogs, can also act as agonists on MC receptors. These receptors are potential drug targets, especially in the control of food intake and for treatment of erectile dysfunction.[199,202] The Zn complex of 5-chloro-1,10-phenanthroline is not only twofold more potent than free Zn(II), but also has a higher efficacy, reaching 167% of the maximally Zn(II)-induced cAMP production in the MC1 receptor.[199] Such Zn complexes could be developed as drugs, targeting the MC receptors through Zn(II). However, Zn(II) complexes have different potencies and efficacies towards MC receptors. Thus, it is likely that Zn(II) complexes can also be developed to target selectively different MC receptors.[202]

The β_2-adrenergic receptor (β_2AR) mediates adrenergic responses in both the central nervous system and the sympathetic nervous system.[198*a*] Physiological concentrations of Zn(II) increase agonist affinity and enhance cAMP accumulation stimulated by submaximal concentrations of the βAR agonist isoproterenol. There are two Zn(II) binding sites in β_2AR, one primarily affecting agonist binding and the other primarily affecting antagonist binding. Zn(II) binding to a high affinity site (IC_{50} of $\sim$5 μM) enhances agonist affinity and agonist-stimulated cAMP accumulation. Zn(II) binding to a low-affinity site (IC_{50} of $>$500 μM) inhibits antagonist binding, yet slows antagonist dissociation. The effects of Zn(II) on agonist binding and cAMP accumulation occur at concentrations of Zn(II) that may be achieved within a synapse. Thus, Zn(II) may be a physiological modulator of β_2AR function.

Mutagenesis studies have localized the binding site responsible for the positive allosteric effect of Zn(II) on agonist affinity to the third intracellular loop, consisting of Cys-265 and His-269 on the cytoplasmic extension of TM6 and Glu-225 on the cytoplasmic extension of TM5 (Fig. 18).[198*b*] Zn(II) may form a bridge between TM5 and TM6 and alter the position of TM5 relative to TM6. Thus, Zn(II) binding approximates or stabilizes the conformational changes induced by agonists since agonist binding leads to movement of TM6 relative to TM5.

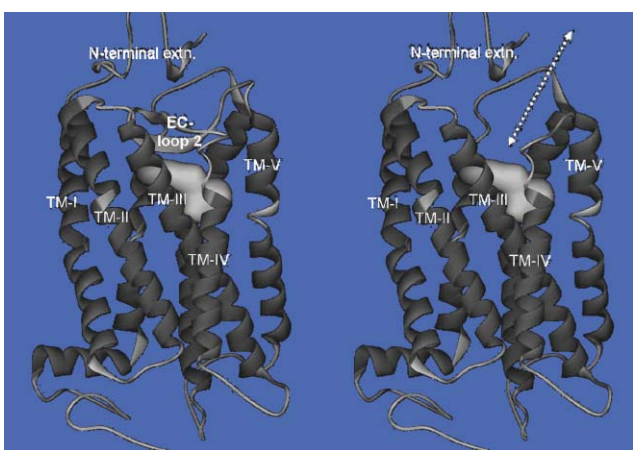


Fig. 17 Comparison of two models of a prototype rhodopsin-like 7TM receptor (left panel) and a melanocortin MC receptor (right panel) built over the X-ray structure of rhodopsin. The surface of the ligand of rhodopsin, 11-*cis* retinal chromophore, is shown as a white "cloud" in the middle of both models. Due to the extremely short extracellular loop 2 in the MC receptors, the main ligand-binding crevice, *i.e.*, the location of the 11-*cis* retinal chromophore, is easily accessible for their ligands. The receptors are seen from the lipid membrane with TM-IV in front. Adapted from ref. 202.

4. Enzymes

4.1. Receptor kinases

There are two classes of receptor kinases, receptor tyrosine kinases (RTKs), and receptor serine/threonine kinases (RSTKs). Signals are transmitted across the membrane from outside the cell to inside. Ligand binding to the extracellular domain causes receptor dimerization, which triggers mutual transphosphorylation of two catalytic domains, leading to activation of the intracellular kinase domains.[203] The activated kinases then phosphorylate substrate proteins within the cell, resulting in transduction of the signals.[204] Most enzymes that catalyze phosphoryl transfer using ATP as the phosphate source use Mg(II) as a cofactor.

4.1.1. Receptor tyrosine kinases. RTKs play an important role in the control of most fundamental cellular processes including the cell cycle, cell migration, cell metabolism and survival, as well as cell proliferation and differentiation.[205] All receptor kinases contain an N-terminal extracellular ligand binding domain, a single membrane-spanning region, a cytoplasmic kinase catalytic domain, as well as additional regulatory sequences. These receptor kinases catalyze transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosines on target proteins.[206] Members of the insulin receptor family of

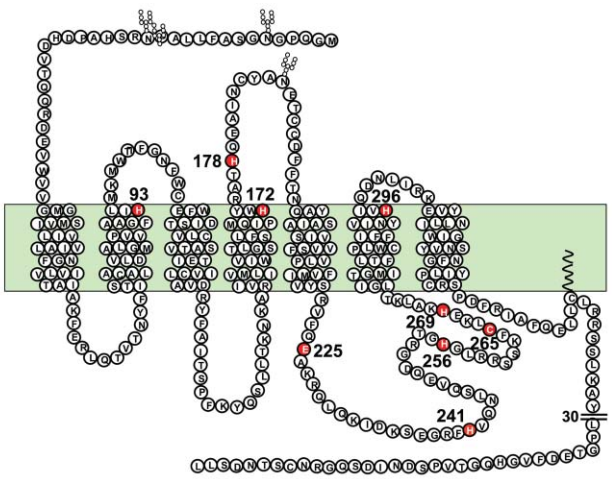


Fig. 18 Sequence of human β_2-adrenergic receptor. The residues mutated to identify the Zn(II) binding sites are highlighted. Adapted from ref. 198*b*.

RTKs are disulfide linked dimers of two polypeptide chains forming an $\alpha_2\beta_2$ heterodimer.[205] Insulin binding to the extracellular domain of the insulin receptor induces a rearrangement in the quaternary heterotetrameric structure that leads to increased autophosphorylation of the cytoplasmic domain. The other known RTKs, *e.g.* receptors EGF (epidermal growth factor) and PDGF (platelet derived growth factor) are monomers and ligand binding induces dimerization of these receptors resulting in autophosphorylation of their cytoplasmic domain. However, active insulin receptor and monomeric RTKs are both dimeric and have similar signaling mechanisms.

The phosphorylated insulin-receptor further activates the downstream substrates and induces signal transduction. Insulin-receptor signaling involves two major pathways: the mitogen-activated protein (MAP) kinase and the PI3-K (Fig. 19).[207] Under certain circumstances, each pathway can cross-activate the other. The MAP kinase pathway is activated by the binding of Grb2 to Tyr-phosphorylated SHC or IRS *via* its SH2 domain. Grb2 is pre-bound to mammalian Son-of-Sevenless (mSOS), a nucleotide exchange protein that catalyzes the exchange of GDP for GTP on Ras (a small GTPase protein); this results in activation of Ras. The prenylated form of Ras binds the inner leaflet of the plasma membrane and, on activation, binds the NH_2-terminal region of Raf, recruiting Raf to the plasma membrane. Ras–Raf interaction displaces the 14-3-3 proteins that are bound to Raf and allows the phosphorylation of Raf by a number of (Ser/Thr) kinases, thus disinhibiting Raf kinase. Raf-1 activates a dual-specificity kinase, MEK1, by phosphorylating two regulatory Ser residues. In turn, MEK1 activates extracellular signal-regulated kinase (ERK)-1 and ERK2 by phosphorylating regulatory Tyr and Thr residues. Activated ERKs mediate the growth-promoting effects of insulin by phosphorylating transcription factors such as Elk-1, leading to the induction of genes.

The metabolic response to insulin is primarily mediated *via* the PI3-K pathway.[207] Following the association of the p85/p110 complex of PI3-K with IRS, PI3-K activity results in production of phosphatidylinositol 3,4,5-phosphate (PIP_3). PIP_3 binds to the PH domains of PI3-K-dependent kinase

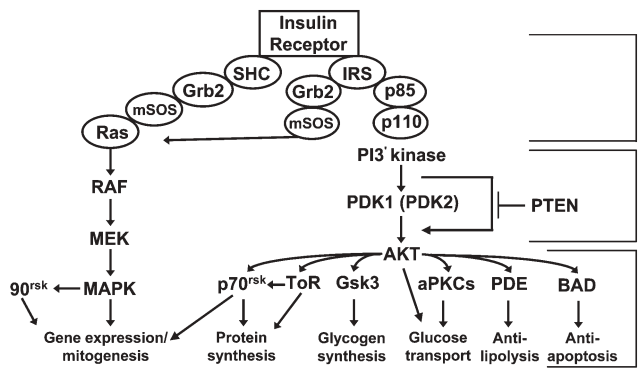


Fig. 19 Two major insulin receptor signaling pathways, the Ras/Raf/MAP kinase and PI3-K. The activated insulin receptor initially binds the proteins, SHC and IRS, and they subsequently further activate downstream substrates. Adapted from ref. 207.

(PDK)-1 and Akt (protein Ser/Thr kinase B). This binding leads to the activation of PDK1, which in turn phosphorylates and activates Akt, resulting in a large variety of biological actions.

Vanadyl VO^{2+} ions and vanadates $[VO_4]^{3-}$ and some vanadium(IV) and vanadium(V) complexes, such as bis(maltolato)oxovanadium(IV) and peroxovanadium(V) compounds, are of medical interest for the treatment of diabetes as insulin-mimetic agents.[208] These vanadium compounds have been shown to lower plasma glucose levels, increase peripheral glucose uptake, improve insulin sensitivity, decrease plasma lipid levels, and normalize liver enzyme activities in a variety of animal models of both type I and type II diabetes.[209]

Several potential sites in the insulin-signaling pathway, including both receptor and post-receptor mechanisms, have been proposed for the insulin-like effects of vanadium compounds.[210] The peroxovanadium compounds activate the insulin receptor kinase (IRK) by inhibiting an IRK-associated protein, tyrosine phosphatase, which prevents IRK autoactivation in the absence of insulin, and leads to activation of the whole MAP kinase cascade.[211]

A natural low molecular weight chromium-containing oligopeptide is thought to play an essential role in glucose metabolism in relation to insulin action.[212] It has been shown to interact with insulin-stimulated kinase receptors of isolated adipocytes.[213] The activation occurs at the active site of the β-subunit of the receptor. A trinuclear cation $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ has been synthesized, which has a similar activation mode to the natural oligopeptide.[214] Potentially it could be used in the treatment of adult-onset diabetes. Intriguingly, tungstate (WO_4^{2-}) also exhibits anti-diabetic activity.[215]‡

4.1.2. Receptor serine/threonine kinases. The transforming growth factor-β (TGF-β) superfamily regulates a broad range of biological processes including cell growth, differentiation, development, tissue repair, and apoptosis.[216] Based on structural and biological similarities, this superfamily can be divided into four major subfamilies, the Mullerian inhibitory substance (MIS) family, the inhibin/activin family, the bone morphogenetic protein (BMP) family, and the TGF-β family. Type I and type II serine/threonine kinase receptors are directly involved in signaling by TGF-β superfamily members.[217] The cytokine ligands initially bind to their corresponding type II receptors, after which the type I receptors are recruited into the signaling complex. The type II receptor phosphorylates the type I receptor, which is activated and, in turn, phosphorylates specific Smad proteins, which then transduce the ligand-activated signal to the nucleus.[216] Smad regulates target gene transcription either by directly binding

‡ *Footnote added at proof.* A 3.1 Å X-ray crystal structure of a molybdate ABC transporter ($ModB_2C_2$) from *Archaeoglobus fulgidus* in a complex with its binding protein ModA has now been reported.[247] ModB subunits form a large cavity, a potential pathway for molybdate translocation through the membrane, separated from the extracytoplasmic space by a gate beneath the interface with ModA. Intriguingly, the structures of *A. fulgidus* ModA with bound molybdate or tungstate contain an oxyanion binding site with octahedral coordination for Mo and W, in contrast with the tetrahedral coordination previously observed for *E. coli* and *A. vinelandii* ModA.

DNA sequences, or by complexing with other transcription factor or co-activators. These kinases are metal related. For example, Mg(II) is essential for kinase activation as discussed below.

4.1.3. Metal catalysis of phosphorylation. The two classes of receptor kinases, RTKs and RSTKs, activate their substrates by phosphorylation and induce signal transduction, which involves further phosphorylation by the intracellular non-receptor tyrosine and serine/threonine kinases. The protein kinases require essential Mg(II) ions for catalysis under physiological conditions. In general, in addition to forming the substrate $MgATP^{2-}$, activation of the protein kinase also requires binding of free Mg(II) as an activator since this stimulation occurs at concentrations of Mg(II) in excess of that necessary to form the substrate, $MgATP^{2-}$.[218] Therefore, Mg(II) plays a dual role in activating these protein kinases. The activated kinase catalyzes a phosphoryl-transfer process from the phosphate-donating substrate $MgATP^{2-}$ to an hydroxyl group in a phosphate-accepting protein. Two well-ordered Mg(II) ions have been found in the activated insulin receptor tyrosine kinase in a complex with a peptide substrate and an ATP analog.[219] Mg1 is coordinated in octahedral geometry by six oxygen atoms: two from each of the β- and γ-phosphates, two from amino acid residues, Asn-1137 and Asp-1150, and two from water molecules (Fig. 20). Mg2 is surrounded by seven oxygen atoms: the β-phosphate oxygen shared with Mg1, both carboxylate oxygens of Asp-1150, and four water molecules. The second Mg(II) activates the insulin receptor-like kinase by increasing its affinity for ATP–Mg(II).[218]

4.2. Respiratory enzyme complexes

In the inner mitochondrial membrane in the respiratory chain, electrons are passed along a series of respiratory enzyme complexes: complex I (NADH:ubiquinone oxidoreductase), complex II (succinate:ubiquinone oxidoreductase), complex III (cytochrome bc_1 complex), and complex IV (cytochrome *c* oxidase) (Fig. 21).[220] These electrons are generated from NADH (reduced nicotinamide adenine dinucleotide), produced by oxidation of nutrients such as glucose, and are ultimately transferred to molecular oxygen. The passage of electrons between these complexes releases energy that is stored in the form of a proton gradient across the membrane and is then used by ATP synthase to make ATP from ADP (adenosine 5′-diphosphate) and phosphate.

Complex I is one of the largest known membrane protein complexes.[221] The bovine enzyme contains about 46 subunits with a molecular mass of about 980 kDa, whereas the bacterial enzyme contains 13–14 subunits with a molecular mass of 550 kDa.[222] This complex catalyzes the transfer of two electrons from NADH to ubiquinone-10 and translocates about 4 protons across the membrane to produce a membrane potential and proton gradient for ATP synthesis.[221,223] Complex I has an L-shaped structure containing two major segments, the peripheral domain and the membrane domain.[224] Whereas most of the membrane domain is embedded in the lipid bilayer of the inner-mitochondrial membrane (or the bacterial cytoplasmic membrane), the peripheral segment protrudes into the matrix side of the inner-mitochondrial membrane (or the cytoplasmic phase in bacteria). A typical complex I/NDH-1 contains eight iron–sulfur centres including two binuclear [2Fe–2S] centres (N1a and N1b) and six tetranuclear [4Fe–4S] centres (N2, N3, N4, N5, and N6).[225] The NDH-1 in certain bacteria contain an additional tetranuclear cluster (designated N1c) and N4 containing two tetranuclear clusters.

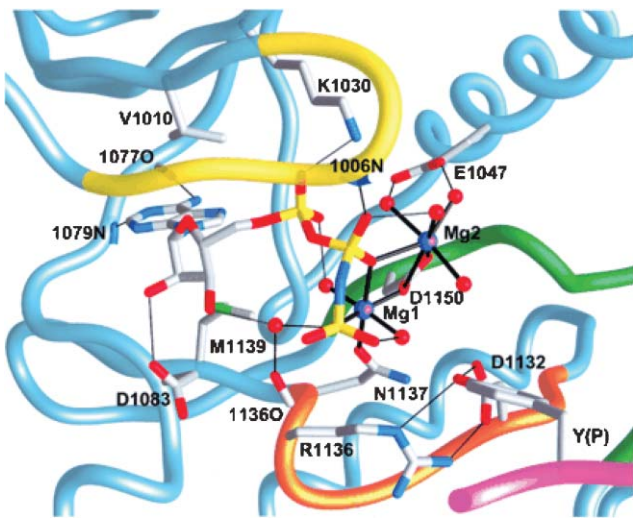


Fig. 20 Insulin receptor tyrosine kinase. Activation requires two Mg(II) ions in complex with a peptide substrate and an ATP analog. Sulfur atoms are in green and Mg(II) ions are in purple. Selected hydrogen bonds are shown as thin black lines and bonds to Mg(II) ions are shown as thick black lines. Reproduced with permission from ref. 219.

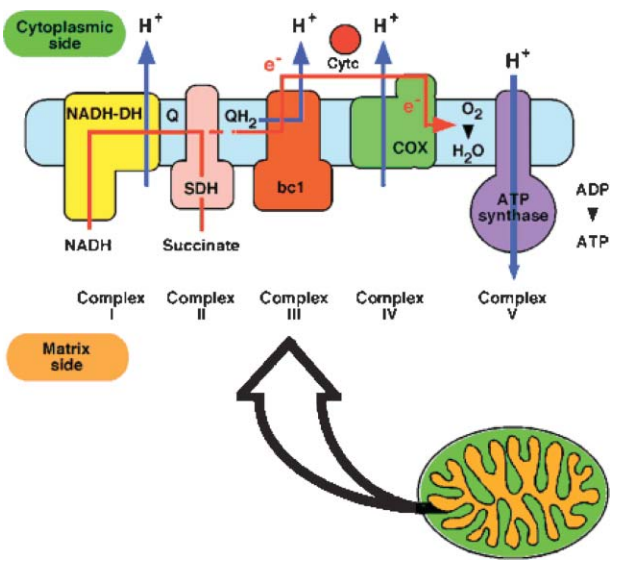


Fig. 21 Enzymes of the mitochondrial inner membrane involved in the respiratory chain: NADH-dehydrogenase (yellow), succinate dehydrogenase (pink), cytochrome bc_1 (red), and cytochrome oxidase (green), which form the electron transfer chain to O_2. With the exception of SDH, these enzymes translocate protons across the membrane. The proton gradient is used by ATP synthase (purple) to make ATP. Reprinted with permission from ref. 220. Copyright 1999 AAAS.

In the respiratory chain, complex II oxidizes succinate to fumarate and passes the electrons directly into the quinone pool.[226] Complex II is an integral membrane protein consisting of four subunits.[227] The largest subunit is the FAD (flavin adenine dinucleotide)-containing flavoprotein subunit (Fp). The dehydrogenase catalytic portion of complex II is formed by Fp and an iron–sulfur protein subunit (Ip) containing three different clusters: [2Fe–2S], [4Fe–4S] and [3Fe–4S]. The small hydrophobic subunits, two cytochrome *b* subunits, anchor the catalytic portion to the membrane and are also required for electron transfer to quinones.

Complex III is a membrane protein complex in the middle section of the respiratory chain, which oxidizes ubiquinol and reduces the small soluble cytochrome *c* (Fig. 22).[228] Part of the free energy released from the reactions is used to generate a transmembrane gradient in the electrochemical potential of the hydrogen ion, which serves as the energy source for a number of other processes including adenosine triphosphate (ATP) synthesis. The mitochondrial complex is a homodimer consisting of 11 subunits, but only three of them carry the redox centres.[220] The key subunits are cytochrome *b*, which has eight transmembrane helices with two haems sandwiched between helices B and D; a membrane-anchored FeS protein (ISP) carrying a Rieske-type centre (Fe_2S_2), and a membrane-anchored cytochrome c_1.

According to the Q cycle mechanism, ubiquinol is oxidized at site Q_o, near the protochemically positive side (high proton electrochemical potential) of the inner mitochondrial membrane, and ubiquinone is reduced at site Q_i, near the protochemically negative side of the membrane (Fig. 23).[229] One electron is transferred from ubiquinol to the Rieske iron–sulfur centre, and subsequently to cytochrome c_1 and cytochrome *c*. The newly generated reactive ubisemiquinone then reduces the low-potential cytochrome *b* haem (b_L). The reduced b_L rapidly transfers an electron to the high-potential

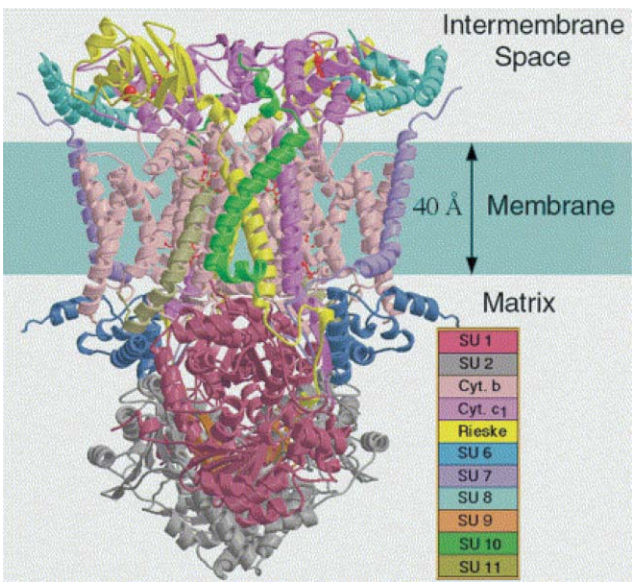


Fig. 22 The overall structure of a vertebrate cyt. bc_1 complex (complex III), a homodimer with 11 subunits. Reproduced from ref. 228 with permission.

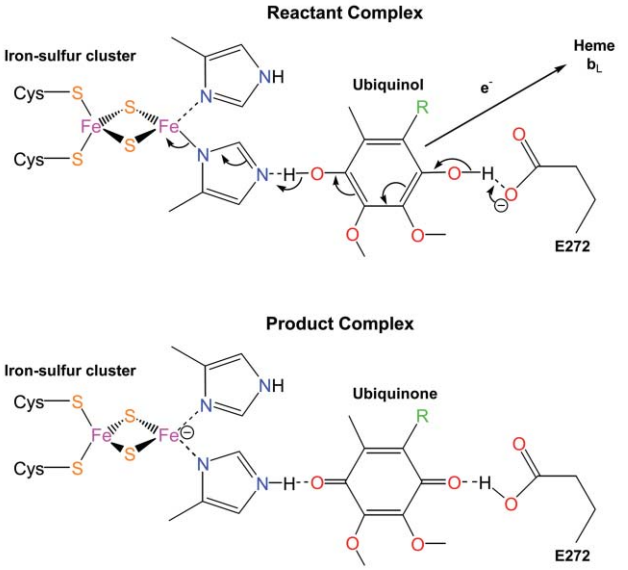


Fig. 23 Reactant and product complexes at the Q_o site in complex III of the mitochondrial electron transport chain. Adapted from ref. 228.

cytochrome *b* haem (b_H), which is located near the opposite side of the membrane. An ubiquinone or ubisemiquinone bound at the Q_i site then oxidizes the reduced b_H. Proton translocation is the result of deprotonation of ubiquinol at the Q_o site and protonation of the reduced ubiquinone at the Q_i site. Ubiquinol generated at the Q_i site is re-oxidized at the Q_o site.

Complex IV is the terminal oxidase of cell respiration, reducing O_2 to water *via* a mechanism coupled with a proton pumping process.[230] The mammalian cytochrome *c* oxidase enzyme is a large multicomponent membrane protein comprising 13 different subunits, four redox-active metal sites (haems *a* and a_3, Cu_A and Cu_B), three redox-inactive metal sites (Mg(II), Zn(II) and Na(I)) and several different classes of phospholipids. The catalytic cycle of complex IV is shown in Fig. 24.[231,232] During each cycle of reduction of O_2 to H_2O, the enzyme pumps four protons across the membrane, while another four protons are consumed to make two H_2O at the active site.

Agents targeted to mitochondria which lead to the breakdown of mitochondrial membrane potentials and increased membrane permeabilization have potential as anticancer drugs.[233] Au(I) thiolate complexes such as the tetraacetylthioglucose Au(I) phosphine complex auranofin are well known as anti-arthritic drugs, and some Au(I) phosphine derivatives exhibit anticancer activity *in vivo*.[234] The antimitochondrial lipophilic cationic tetrahedral Au(I) phosphine complex[235] $[Au(dppe)_2]Cl$ was selected for preclinical anticancer trials, but failed due to severe cardiotoxicity.[236] Anticancer Au(III) porphyrins also target mitochondria and interfere with membrane potentials.[237]

As an antioxidant defense, the mitochondrial thioredoxin system maintains mitochondrial proteins in their reduced state to avoid cell apoptosis.[238] New evidence indicates that the anti-arthritic drug auranofin[239,240] and some Au(I) carbene complexes may target mitochondrial thioredoxin reductase and

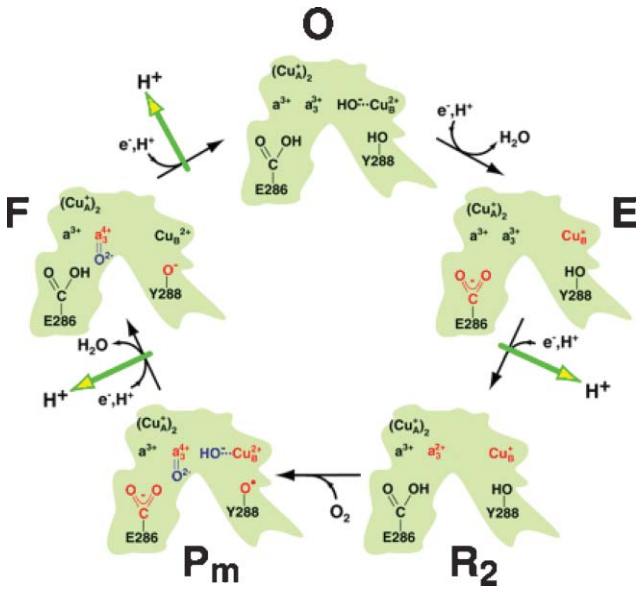


Fig. 24 The catalytic cycle of complex IV of the mitochondrial electron transport chain. O: The oxidized state of the enzyme, in which all four metal centres are oxidized; E: The one-electron reduced haem/copper centre; R_2: The two-electron reduced binuclear centre, which reacts with O_2; P_m: Product of the reaction of O_2 with the two-electron reduced enzyme (R_2). The O–O bond is cleaved in this state. This requires four electrons, since the oxidation state of each oxygen atom is reduced from 0 to −2. One electron comes from the oxidation $Cu_B(I) \rightarrow Cu_B(II)$; and two electrons come from the haem a_3 iron, Fe(II) → Fe(IV); and it is postulated that the fourth electron comes from the active-site tyrosine in H-284–Y-288, which forms a neutral radical. One of the oxygen atoms is associated with the ferryl state of haem a_3 (Fe(IV)O) and the other is associated with Cu(II)(OH). F: The presumed tyrosyl radical is reduced back to tyrosine (or tyrosinate). Reproduced from ref. 231. Copyright 2002 National Academy of Sciences.

disrupt mitochondrial transmembrane potentials, induce mitochondrial permeability transitions, and release cytochrome *c*.[241] Although Au(I) is a 'soft' metal ion with a high affinity for 'soft' thiolate sulfur, X-ray crystallography shows that the anti-arthritic complex [Et_3PAuCl] forms a Et_3PAu–His adduct with the enzyme cyclophilin, despite the presence of four cysteines in the protein.[242] This is an example of how ligand preferences for metal ions can be greatly affected by their environment. In this case, reactions were carried out by soaking cyclophilin crystals with the gold complex.

5. Conclusions and perspective

Our understanding of interactions between membrane proteins and metal ions has increased considerably over the past few years and it is clear that such knowledge can be used to aid drug design. Indeed the long-established use of organomercurials as diuretics has now been correlated with the ability of these compounds to bind to aquaporins and inhibit the transport of water through these channels.[49,50] Moreover the new anti-leukemia drug arsenic trioxide, which is largely the neutral molecule $As(OH)_3$ at physiological pH, enters cells *via* the membrane protein aquaglyceroporin, the glycerol transporter.[54] Such knowledge will aid the design of new drugs that take advantage of natural uptake pathways.

The selectivity of membrane transporters for ions depends on ion size and thermodynamic preferences for binding ligands. Ion channels are known for Na(I), K(I) and Ca(II), and for K(I) provide 8-coordinate binding sites composed of backbone carbonyl and side-chain hydroxyl oxygens.[64] Ion pumps and transporters can couple the transport of Na(I), K(I), Ca(II) and Cu(II) to ATP hydrolysis, and can switch protein conformations using ATP binding and protein phosphorylation.

ATP binding cassette (ABC) transporters such as those used for iron uptake by many Gram-negative pathogenic bacteria, consist of an outer membrane receptor, a periplasmic binding protein and an inner membrane ATP binding protein. Since the receptors recognize specific complexes of metal ions (*e.g.* siderophores), they are potential drug targets. However, much work remains to be done to elucidate the actual forms of complexes of metal ions that are transported *via* these cassette proteins. Ferric citrate (handled by the Fec system), for example, has a complicated structure which can consist of mono-, di- or polynuclear species, depending on the pH and Fe : citrate ratio.

High affinity copper transport proteins containing methionine-rich sequences have recently been associated with the uptake of cisplatin.[158,159] Pt(II) is known to have a high affinity for methionine sulfur. G-protein coupled receptors (GPCRs) have a negatively-charged electrostatic surface directed towards the exterior of the cell membrane and are therefore strong potential targets for metal ions, Such interactions have been little studied to date. The affinity of the antiviral and stem-cell-mobilising drug AMD3100, xylylbicyclam, for its target site CXCR4 is enhanced by 7–50-fold by binding to Cu(II), Ni(II) or Zn(II). In models,[191,193] metal ions bound in the macrocyclic rings of xylylbicyclam can coordinate to Asp and Glu carboxylate groups and ring NH groups can form H-bonds to side-chains. In addition there are possible hydrophobic interactions between the indole rings of Trp residues and the methylene backbone of bicyclam. It should be possible to design new generations of metal complexes which bind specifically to different GPCRs based on such interactions. Constraining metal macrocycle configurations so as to optimize the configuration for receptor binding may lead to new generations of antiviral, stem-cell-mobilizing, and other drugs. Some success has already been achieved with strapped macrocycles.[243]

Zn(II) binds strongly to the GPCRs tachykinin, β-adrenergic and melanocortin (MC) receptors. Zn(II) complexes (*e.g.* with 1,10-phenanthroline as a ligand) can act as agonists and provide a basis for drug design.[199,202] Similarly Zn(II) may be a physiological mediator of the function of the β-adrenergic receptor and this needs further elucidation. Cysteine and histidine side-chains in loops and membrane helices are likely to play key roles in agonist-induced conformational changes in the receptor. Several vanadium complexes have the potential to lower blood plasma glucose levels and act as anti-diabetic agents.[208,209] This behavior may arise from an ability to inhibit phosphorylation of the insulin receptor, but needs further investigation. The mitochondrial respiratory enzyme system is

a potential target for metallodrugs including cationic Au(I) phosphine complexes and Au(II) porphyrins.[239–241,244]

These examples will provide a stimulus for the further exploration of the chemistry of metal ions in membranes and offer promise for the discovery of drugs with novel modes of action.

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