Molecular Mechanisms of Enzyme Activation by Monovalent Cations

David W. Gohara and Enrico Di Cera

Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO 63104

Running title: Enzyme activation by M⁺s

To whom correspondence should be addressed: Enrico Di Cera, Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO 63104, USA, Tel.: (314) 977-9201; Fax: (314) 977-9206; E-mail: enrico@slu.edu

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Regulation of enzymes through metal ion complexation is widespread in biology and underscores a physiological need for stability and high catalytic activity that likely predated proteins in the RNA world. In addition to divalent metals like Ca²⁺, Mg²⁺ and Zn²⁺, monovalent cations often function as efficient and selective promoters of catalysis. Advances in structural biology unravel a rich repertoire of molecular mechanisms for enzyme activation by Na⁺ and K⁺. Strategies range from short-range effects mediated by direct participation in substrate binding, to more distributed effects that propagate long-range to catalytic residues. This review addresses general considerations and examples.

A large fraction of known proteins are metalloproteins (1) and regulation of enzyme activity through metal ion complexation is widespread in biology. When dealing with protein-metal complexes, examples that come to mind are the important role of divalent metals such as Fe²⁺ in redox cycles (2), Ca²⁺ in structural stability and signaling (3), or Zn²⁺ in catalysis (4). Less appreciated by biochemists is the key contribution that monovalent cations (M⁺s) such as Na⁺ and K⁺ often play in enzyme catalyzed reactions (5,6). Indeed, many enzymes in plants and the animal world utilize the abundance of Na⁺ in physiological fluids or K⁺ inside the cell as a source of chemical potential to broker substrate binding and catalysis (7,8). The large availability of Na⁺ and K⁺ makes high affinity unnecessary. The highest affinity reported for a protein-M⁺ interaction is in the 0.08-0.6 mM range (9), comparable to that measured for ion channels (10) and ATP-driven ion pumps (11). Likewise, ion selectivity is strictly not required for M⁺ activation. Yet some enzymes are capable of exquisite discrimination and are only active in the presence of K⁺ but not Na⁺, as pyruvate kinase (12), GroEL (9) and Rad51 (13), or Na⁺ but not K⁺, as β-galactosidase (14). Exactly how Na⁺ and K⁺ promote enzyme activation at the molecular level has long been a focus of investigation.

General considerations

M⁺ activation manifests itself as a hyperbolic increase in the rate of substrate hydrolysis that obeys the law of mass action (6). Replacement of M⁺ with a bulky alkyl ammonium such as choline serves as necessary control to establish that activation is specific and not due to changes in ionic strength. The independent Michaelis-Menten parameters $k_{cat}$ and $s = k_{cat}/K_m$ become of interest in the context of available structural information. In general, the midpoint of the hyperbolic dependence of $k_{cat}$ on [M⁺] measures the equilibrium dissociation constant for M⁺ binding to the enzyme-substrate complex. The midpoint of the dependence of $s$ on [M⁺] yields an approximate measure of the equilibrium dissociation constant for M⁺ binding to the free enzyme. Extent of activation and comparison of the midpoints of $k_{cat}$ and $s$ define the thermodynamic coordinates that link M⁺ and substrate recognition.

M⁺ complexation benefits enzyme-substrate interaction and catalysis in some general ways, independent of the specific mechanism of activation. M⁺ binding is typically associated with a large entropy cost required for ordering the site of complexation, as shown by Na⁺ binding to thrombin (15). The effect contributes a more favorable entropy balance when substrate binds to the M⁺-bound form compared to the M⁺-free form. M⁺ complexation also propagates entropic benefits to the entire structure of the enzyme by selecting more...
ordered and catalytically active conformations from an ensemble dominated by disordered and poorly active conformers. This is documented in clotting proteases (16,17), inosine monophosphate dehydrogenase (18), several α-amylases (19-21) and kinases (22,23), and is a determining factor of ion selectivity in the Streptomyces lividans K+ channel (10). ATP driven sequential switching between Na+ specific and K+ specific conformations drives ion transport in the Na/K-ATPase (11,24).

Specific components of the mechanism of M+ activation may be identified from structural analysis. The locale for M+ binding pinpoints the origin of a transduction pathway that eventually influences residues of the active site and produces enhanced catalytic activity. The coordination shell of the bound M+ is composed mainly of O atoms from the protein backbone and water molecules. Six ligands are common for Na+ with average Na+-O distances of 2.4±0.2 Å, but K+ prefers six or seven ligands with average K+-O distances of 2.8±0.3 Å and 2.9±0.3 Å, respectively, due its larger ionic radius (Figure 1). M+-O distances and coordination optimize the ion specific valence of the binding site (25).

Activation is defined as Type I when M+ is in direct contact with substrate, or Type II when M+ binds to a separate site (5). Although M+ binding is necessary for activation, it is certainly not sufficient. The initial M+ binding event must be transduced into enhanced catalytic activity to produce a biological effect. Binding and transduction are mediated by the same locale in Type I activation where the bound M+ is a key determinant of substrate recognition. Dyol dehydratase (26) and pyruvate kinase (27) offer two relevant examples. Separation of M+ and substrate binding sites in Type II activation poses the additional challenge of identifying the pathway of transduction. Communication between M+ and active site may be traced to specific residues from inspection of the crystal structure, as in dialkylglycine decarboxylase (28), or may be long-range and less obvious to dissect as in thrombin (29).

**Type I activation**

In Type I activation, the M+ anchors substrate to the active site of the enzyme, often acting in tandem with divalent cations like Mn2+, Mg2+ or Zn2+. Numerous examples of this synergism as a staple of M+ activation cover structural effects that are local on the active site or extend to other regions of the enzyme. Typically, requirement for M+ is absolute. Two subgroups should be distinguished based on whether M+ is needed for substrate binding (Type Ia) or hydrolysis (Type Ib). Type Ia activation is associated with a value of $k_{cat}$ independent of [M+] and a value of $s$ that increases hyperbolically with [M+] (6). Diol and glycerol dehydratases are the simplest examples of Type Ia activation (26,30). The absolute requirement for K+ (31) is explained by the crystal structure bound to propanediol (Figure 2A): K+ is coordinated by five ligands from the protein and functions as bait for two hydroxyl O atoms of substrate (26,30,32). The activity of β-galactosidase is enhanced preferentially by Na+ over K+ (14) and synergy with Mg2+ secures substrate binding to the active site (33,34). Changes in $s$ and $k_{cat}$ for the hydrolysis of para-nitrophenyl-β-D-galactopyranoside are 16-fold and only 2-fold, respectively (35), suggestive of Type Ia activation. Far more common is Type Ib activation, where both $k_{cat}$ and $s$ increase hyperbolically with [M+] (6). This is the case reported originally for pyruvate kinase (36) and encountered in many other enzymes.

**Kinases**

Pyruvate kinase, an allosteric tetrameric enzyme of the glycolytic pathway catalyzing the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP, was the first reported enzyme to require K+ for catalytic activity (12), in addition with two Mn2+ or Mg2+. Structural biology reveals how the cations cooperate and enable substrate binding to the active site (27,37). Mn2+ anchors substrate via its carboxylate and phosphoester O atoms to the carboxylate O atoms of Glu242 and Asp266, while K+ increases electrostatic coupling of the phosphate group by screening the carboxylate O of Asp84. The critical role of K+ creates very favorable conditions for the transfer of the phosphate group from substrate to ATP. Although a convincing case can be made for the key role of K+ in the activation of pyruvate kinase, the strong preference of K+ over Na+ as an activator remains puzzling, given that the Na+-bound structure shows no significant changes in the architecture of the active site (38).

Coordination of substrate coupled to ordering of the structure explains K+ activation in branched-chain α-ketoacid dehydrogenase (BCKD) kinase (22) and pyruvate dehydrogenase kinase (23). These mitochondrial serine protein kinases belong to the GHKL ATPases that also comprise gyrase, Hsp90, bacterial histidine kinase CheA and the DNA mismatch repair protein MutL involved in
DNA metabolism, protein folding and signal transduction. All crystal structures available for this class of enzymes show Mg$^{2+}$ bound to the $\text{O}^{\delta_1}$ atom of a conserved Asn and to the triphosphate moiety of ATP. K$^+$ sits on the opposite side of ATP relative to Mg$^{2+}$, rather than in cis as in pyruvate kinase, and bridges one O atom of the phosphate moiety of ATP to typically four protein atoms (Figure 2B). Comparison of the structures of BCKD kinase in the apo form and bound to ATP reveals how K$^+$ stabilizes an entire segment of the protein from His$^{302}$ to Phe$^{336}$ that is completely disordered in the apo form. Similar observations have been reported for pyruvate dehydrogenase kinase (23).

Pyridoxal kinase is a member of the ribokinase superfamily involved in the ATP-dependent phosphorylation of pyridoxal to provide pyridoxal-5'-phosphate (PLP), a widely used coenzyme. The enzyme requires K$^+$ and Zn$^{2+}$ as absolute cofactors (39) and the crystal structure reveals how K$^+$ assists formation of the enzyme-substrate complex through interactions with a negatively charged phosphate moiety (40).

**GroEL and Hsc70**

GroEL is an allosteric tetradecameric protein composed of two stacked heptamers that define a large central cavity when in complex with GroES (41). Activity of GroEL is influenced by Mg$^{2+}$ and has an absolute requirement for K$^+$ (9), as seen in pyruvate kinase. NH$_4^+$ and Rb$^+$ partially substitute for K$^+$, but Li$^+$, Na$^+$ or Cs$^+$ are poor activators. The crystal structure of GroEL bound to ATP reveals Mg$^{2+}$ and K$^+$ acting in tandem to assist binding of ATP to the protein (Figure 2C).

Two K$^+$ in tandem with Mg$^{2+}$ influence catalysis in the molecular chaperone Hsc70 (42), a member of the heat shock family of proteins involved in binding and release of polypeptides linked to ATP hydrolysis (43). Similar to GroEL and pyruvate kinase, ATPase activity of Hsc70 is optimal in the presence of K$^+$ and is minimal in Na$^+$ (44). Crystal structures of a fragment of Hsc70 retaining M$^+$ activation are available in the presence of Na$^+$ (45) and K$^+$ (42) and explain the functional difference between these M$^+$s, unlike the case of pyruvate kinase discussed above. K$^+$ provides optimal electrostatic coupling for the phosphate moiety of substrate and optimizes the register for docking in the enzyme active site and formation of the transition state. The function is assisted by a divalent cation, Mg$^{2+}$ in this case, that forms a $\beta_3\gamma$-bidentate complex with ATP favoring nucleophilic attack on the Py. The phosphate moiety of ADP is forced to clash within the active when K$^+$ is replaced by Na$^+$ (45,46).

**Phosphatases and aldolases**

Synergism between K$^+$ and Mg$^{2+}$ is also observed in fructose 1,6-bisphosphatase (47) and S-adenosylmethionine synthase (48). Fructose 1,6-bisphosphatase is a key enzyme of gluconeogenesis and catalyzes the conversion of $\alpha$-D-fructose 1,6-bisphosphate to $\alpha$-D-fructose 6-phosphate. The enzyme has an absolute requirement for Mg$^{2+}$, but the activity is further enhanced by K$^+$ and inhibited by Li$^+$ (49). K$^+$ anchors the substrate to the active site and assists the role of two neighboring Mg$^{2+}$ located in cis, as for other enzymes catalyzing phosphoryl transfer. K$^+$ replaces the guanidinium group of Arg$^{276}$ and polarizes the phosphate group for nucleophilic attack. The inhibitory role of Li$^+$ is due to replacement of one of the two Mg$^{2+}$ (47).

S-adenosylmethionine synthase catalyzes the formation of S-adenosylmethionine from ATP and Met and provides the most widely used methyl donor in biology. The enzyme has an absolute requirement for Mg$^{2+}$ and K$^+$ (50). Substantial crystallographic work has been carried out on this enzyme in complex with various substrates, cofactors and inhibitors (51). In the presence of an ATP analog and the substrate Met, the structure reveals two Mg$^{2+}$ and K$^+$ in the active site anchoring the phosphate moiety of the cofactor (48). The architecture is similar to that of pyruvate kinase and BCKD and explains the absolute requirement of K$^+$ for activation.

Class II aldolases like fructose-1,6-bisphosphate aldolase (52) and tagatose-1,6-bisphosphate aldolase (53) use a similar strategy for optimization of substrate docking by pairing Na$^+$ with Zn$^{2+}$. Notably, tagatose-1,6-bisphosphate is the only example where the coordination shell of Na$^+$ involves a cation-π interaction.

**Recombinases**

Repair of double-stranded DNA breaks or stalled replication forks and homologous gene recombination involves several M$^+$ activated members of the recombinase superfamily. Activity of bacterial RecA, archaeal RadA or archaeal and eukaryal Rad51 depends on ATP and Mg$^{2+}$, but also requires K$^+$ in the case of human and yeast. In the Rad51 homolog from *Methanococcus voltae* requirement for K$^+$ is absolute (13). The crystal
structure of MvRadA has been solved in the presence of an ATP analog and Mg$^{2+}$, with and without K$^+$ (13,54). The structures reveal a typical arrangement of Mg$^{2+}$ and two K$^+$ in the active site that polarize the P$_\gamma$ of ATP. Each K$^+$ bridges an O atom from the P$_\gamma$ and a carboxylate from the protein, but also makes extensive contacts at the dimer interface formed upon assembly of the MvRadA filament that explain the absolute requirement for K$. Notably, binding of K$^+$ in the active site produces long-range conformational ordering of the putative single-stranded DNA binding domain, establishing a link between M$^+$ binding and selection of functionally active conformations.

**Type II activation**

In Type II activation, M$^+$ binds to a site not in direct contact with substrate and enhances enzyme activity through conformational transitions. Unlike Type I activation, the requirement for M$^+$ is less stringent. Measurements of $k_{cat}$ and $s$ as a function of [M$^+$] document a hyperbolic increase in both parameters that is difficult to distinguish from Type Ib activation without independent insight from structural biology (6). A relevant example is the large (>100-fold) K$^+$-induced increase in activity from a minuscule baseline level reported for inosine monophosphate dehydrogenase from *Tritrichomonas foetus* (55). In general, Type II activation poses challenges to structural interpretation as the underlying mechanism becomes more difficult to resolve the greater the spatial separation between M$^+$ and residues of the active site.

**Ribokinase and a path to Type II activation**

Ribokinase breaks the typical K$^+$-Mg$^{2+}$ tandem of kinases obeying Type I activation and sequesters K$^+$ in a $\beta$-turn adjacent to the active site, but separated from solvent and substrate (56) (Figure 3A). The same arrangement is used by aminomimidazole riboside kinase (57). Lack of information on the structure of the apo form makes it difficult to identify the structural determinants responsible for enhanced catalytic activity. A recent structural analysis of ribokinase from *Vibrio cholerae* shows significant conformational changes induced by Na$^+$ binding that also acts as a preferred activator (58). It is tempting to speculate that these enzymes may represent end-points of an evolutionary pathway where coordination of the phosphate moiety of ATP transitions from direct binding to M$^+$ (Type I activation) to complete separation from the M$^+$ (Type II activation). A possible intermediate in this transition is MutL, an enzyme whose broad M$^+$ specificity has so far eluded structural interpretation (59).

**Dialkylglycine decarboxylase**

Dialkylglycine decarboxylase is a PLP-dependent enzyme capable of both decarboxylation and transamination. Activity depends on K$, with Na$^+$ producing modest enhancement (60). The enzyme is composed of four identical subunits, each containing a PLP binding domain, N-terminal and C-terminal domains. Active sites in the tetramer are close to each other and formed by residues from both monomers of a tightly assembled dimer. The resulting tetramer is formed by two such dimers associated symmetrically. Crystal structures of the enzyme solved in the presence of K$^+$ and Na$^+$, with PLP bound to the active site, reveal the mechanism of M$^+$ activation and the need for K$^+$ over Na$^+$ (28,61). K$^+$ binds to O$_{\delta}$1 of Asp$^{307}$, O$_{\gamma}$ of Ser$^{80}$ and the carbonyl O atoms of Leu$^{78}$, Thr$^{303}$ and Val$^{305}$ near the dimer interface where PLP binds. A water molecule completes the octahedral coordination shell (Figure 3B). When Na$^+$ is bound to this site, a water molecule replaces Thr$^{303}$ and Ser$^{80}$ in the coordination shell around the smaller M$. The O$_{\gamma}$ of Ser$^{80}$ relocates and causes the phenyl ring of the active site residue Tyr$^{301}$ to adopt a conformation no longer favorable for substrate binding. This is a simple and elegant example of how the architecture of the M$^+$ binding site is precisely tailored for K$^+$ and not Na$^+$ to optimize communication with nearby residues of the active site. A similar strategy is used by other PLP-dependent enzymes like Ser dehydratase (62), tryptophanase and tyrosinase (63,64), for which K$^+$ is absolutely required for activity and Na$^+$ acts as a poor activator.

**Dehydrogenases**

Two K$^+$ binding sites have been identified in BCKD. One site controls binding of thiamine diphosphate and the other, also found in pyruvate dehydrogenase (65), stabilizes the quaternary structure (66). The BCKD catalytic machine is a member of the highly conserved mitochondrial $\alpha$-ketoacid dehydrogenase complexes including the BCKD complex (BCKDC), the pyruvate dehydrogenase complex (PDC) and the $\alpha$-ketoglutarate dehydrogenase complex (67). The BCKDC contains multiple copies of BCKD, as well
as a dihydrolipoyl transacylase, the BCKD kinase and phosphatase. Activity of BCKD and BCKDC is abolished by phosphorylation of Ser\textsuperscript{202}, which promotes an order-disorder transition in the phosphorylation loop of BCKD (68). BCKDC utilizes the entire repertoire of K\textsuperscript{+} binding sites found in BCKD (66) and its kinase (22). The crystal structure of BCKD bound to thiamine diphosphate shows two K\textsuperscript{+} binding sites in crucial positions, with one separated from cofactor and substrate that is most likely responsible for enzyme activation. The second K\textsuperscript{+} site has a structural role and maintains the tetrameric assembly of BCKD (66). This second site is also found in pyruvate dehydrogenase, where the first K\textsuperscript{+} is constitutively replaced by a pair of H-bonds (65).

**Trp synthase**

Among enzymes utilizing PLP-mediated catalysis, Trp synthase has been studied in great detail both structurally and kinetically (69). Trp synthase is a tetramer with the subunits arranged in a linear αββα fashion. The α subunit catalyzes cleavage of IGP to G3P and indole, which is then tunneled to a neighboring β subunit that catalyzes condensation of indole with L-Ser to give L-Trp. The enzyme requires Na\textsuperscript{+} or K\textsuperscript{+} for optimal catalysis (70,71). M\textsuperscript{+} coordination increases catalytic activity 30-fold by affecting the distribution of intermediates along the ground and transition state. Crystal structures of Trp synthase bound to Na\textsuperscript{+} or K\textsuperscript{+} show that the M\textsuperscript{+} does not contact substrate or PLP and binds the β subunit near the tunnel which shuttles the indole for complexation with L-Ser (72). Binding to the active site in the α subunit displaces Na\textsuperscript{+} from its site in the β subunit through an allosteric communication involving the salt-bridge between Asp\textsuperscript{56} in the α-subunit and Lys\textsuperscript{167} in the β subunit. (73). When Na\textsuperscript{+} is bound (Figure 3C), Asp\textsuperscript{305} in the β subunit assumes two possible orientations, one in contact with Lys\textsuperscript{167} and the other rotated away from this residue. In the K\textsuperscript{+} structure, Lys\textsuperscript{167} flips 180° and engages Asp\textsuperscript{56} in the α subunit, thereby establishing a critical communication within the αβ dimer. Changes are propagated to the tunnel that is partially blocked by the bulky side chains of Phe\textsuperscript{280} and Tyr\textsuperscript{279} in the Na\textsuperscript{+} form, but is more open in the K\textsuperscript{+} form. Significant changes are confirmed by more recent structures solved in the presence of Na\textsuperscript{+} and Cs\textsuperscript{+} (74).

**Thrombin**

The stimulatory effect of Na\textsuperscript{+} on the activity of some clotting factors has been known for a long time (75-77). A simple structure-function link identifies the presence of Tyr\textsuperscript{225} near the Na\textsuperscript{+} binding site (29) as a necessary determinant of Na\textsuperscript{+} activation in the entire family of trypsin-like proteases (78). Na\textsuperscript{+} binding shifts the pre-existing equilibrium of the trypsin-fold between active and inactive conformers and produces specific changes that promote substrate binding and catalysis (16,17), rigidify the structure (79) and increase thermal stability (80). The Na\textsuperscript{+} binding site is located >15 Å away from residues of the catalytic triad within loops that control the primary specificity of the enzyme (Figure 4). The structural determinants of this long-range communication offer an instructive example of allosteric control that has eluded X-ray structural biology (81) and even NMR measurements (79), unlike the cases of dialkyglycine decarboxylase or Trp synthase. A clear separation of roles exists between residues responsible for Na\textsuperscript{+} binding and those transducing this event into enhanced catalytic activity in thrombin and other clotting proteases. Site-directed mutagenesis and linkage analysis are in this case of critical importance to arrive at the mechanism of Na\textsuperscript{+} activation.

The bound Na\textsuperscript{+} in thrombin is octahedrally coordinated by two backbone O atoms from Arg\textsuperscript{211} and Lys\textsuperscript{224} and four buried water molecules anchored to the side chains of Asp\textsuperscript{189}, Asp\textsuperscript{211} and the backbone O atoms of Gly\textsuperscript{223} and Tyr\textsuperscript{184} (Figure 4A). Mutagenesis of residues in immediate proximity to the site, such Asp\textsuperscript{189} (Figure 4B), results in significantly (>10-fold) reduced Na\textsuperscript{+} affinity and weakened activation (81). Other mutations do not affect Na\textsuperscript{+} binding, yet abrogate Na\textsuperscript{+} activation (Figure 4B). They involve residues strategically positioned along the corridors of communication between the Na\textsuperscript{+} site and active site: Asp\textsuperscript{221} supports one of the waters in the coordination shell (81), The backbone N atom of Asn\textsuperscript{143} makes an important H-bond interaction with the backbone O atom of Glu\textsuperscript{192} that ensures a correct architecture of the Glu\textsuperscript{192}-Gly\textsuperscript{193} peptide bond organizing the oxyanion hole (82), and Ser\textsuperscript{195} is a member of the catalytic triad (83). Asp\textsuperscript{221} functions as the initial reporter of the bound Na\textsuperscript{+} and transmits information to the neighbor Cys\textsuperscript{191}-Cys\textsuperscript{220} disulfide bond that splits the signal toward the active site along the Cys\textsuperscript{191}-Asp\textsuperscript{211} and Ser\textsuperscript{214}-Cys\textsuperscript{220} corridors. Additional positive contributions to the Na\textsuperscript{+} effect come from Asp\textsuperscript{204} that stabilizes the fold by H-bonding to the new N-
terminus generated after zymogen activation, and Trp^{215} involved in the pre-existing equilibrium between active and inactive forms of the enzyme. A negative contribution comes from Ser^{214} that H-bonds to the catalytic Asp^{102}. Removal of the side chain of Ser^{214} significantly enhances the Na^{+} effect. The end-point of transduction along the two corridors is the rotamer of the catalytic Ser^{195} itself, as assessed by the complete loss of Na^{+} activation in the S195T mutant of thrombin and other clotting proteases like activated protein C and factor Xa (83). The Thr replacement constrains mobility of the Oγ nucleophile within the active site. The Na^{+} effect of thrombin is of dynamic origin and will definitely require further investigation.

Na/K-ATPase
A similar interplay between long-range conformational transitions and direct pathways of communication is observed in the Na/K-ATPase, a ubiquitous ATP driven ion pump within the family of P-type ATPases (11,24,84). Na^{+} and K^{+} bind at sites separate from ATP binding and phosphorylation that requires Mg^{2+} as a cofactor. During a catalytic cycle of ATP hydrolysis, the pump switches from the K^{+}-specific E_{2} form to the Na^{+}-specific E_{1} form by adjusting the ligation distances and coordination of the M^{+}s at the same set of sites and changing their orientation rather than moving the M^{+}s form one site to another. In the E_{2} form two dehydrated K^{+} are bound and orientation is toward the extracellular phase. In the alternative E_{1} form bound to ATP the geometry is suitable for coordination of three dehydrated or partially dehydrated Na^{+} ions and the sites are oriented toward the cytoplasm. Communication between the M^{+} binding sites and the site of phosphorylation in the P domain is mediated by long range conformational transitions the involve the transmembrane domain of the ATPase.

Evolutionary origins
Structural biology also provides a framework to understand the evolutionary origin of M^{+} activation. Widespread occurrence of enzymes activated by M^{+}s in plants and the animal world underscores a physiological need for stability and high catalytic activity that likely predated proteins in the RNA world. Mg^{2+} stabilizes tRNA structures and assists phosphoryl transfer reactions in ribozymes (85,86). However, RNA catalysis may have required M^{+}s to broaden its chemical repertoire (87), as suggested by the architecture of the rRNA of the large ribosomal subunit from the archaeon *Halocarcula marismortui* (88). In small ribozymes, like the hammerhead, hairpin and Varkud satellite, M^{+}s are sufficient to stimulate catalysis even in the absence of divalent metal ions and stabilize a catalytically competent conformation (89-91). With the emergence of proteins, stability in high temperatures or salinity became key to extremophiles and revealed the thermodynamic benefit of a more ordered structure for catalysis. The formyltransferase of the archaeon *Methanosarcina kandleri* utilizes high concentrations of K^{+} for activity and thermostability (92). Na^{+} binding sites have been reported in archaeal dehydrogenases (93,94) and aldehyde ferredoxin oxidoreductase of the hyperthermophile *Pyrococcus furiosus* (95). The architecture of these sites has been retained during evolution (6). Carbonic anhydrase of the halophilic alga *Dunaliella salina* carries an added loop for specific Na^{+} binding that confers stability and resistance to high salinity (96). The loop is strikingly similar to the Na^{+} binding loop of thrombin (6), an enzyme emerged much later from the deuterostome lineage and that utilizes Na^{+} not only for stability but also for optimal physiological function.

Conclusion
Much has been learned on the structural determinants of enzyme activation by M^{+}s. We currently understand several mechanisms to promote catalysis and how the biological abundance of Na^{+} and K^{+} has been strategically utilized during evolution. Much remains to be learned from the subtlety of M^{+} activation in some systems. Structural biology pinpoints likely players, but their interconnectedness may be complex and often involves more distributed, dynamic properties of the protein. That explains why switching M^{+} specificity (15,97) or engineering M^{+} activation de novo in protein scaffolds devoid of such property (98) requires a large number of amino acid substitutions. Achieving high activity by mimicry M^{+} activation is no simpler, as shown by nature’s unique success with actin (99) and murine thrombin (100). Engineering proteins for optimal catalysis may benefit a great deal from increased attention to the structural determinants of M^{+} activation.

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CONFLICT OF INTEREST
The Authors declare no conflict of interest.

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**FIGURE LEGENDS**

**Figure 1. M⁺ coordination in proteins.** M⁺ and water coordination with O atoms participating in interactions for each coordination number from 4 (blue), 5 (red), 6 (green), 7 (purple) to 8 (orange). All structures annotated as containing Na⁺ (4,838) or K⁺ (1,534) in the RCSB as of May 2016 were analyzed. Interactions related by symmetry were included (>1% of all observations). A single ionic bond equals one observation. O atoms within ligating distance represent the vast majority (79,567 total), followed by N atoms (5,711), K⁺ (1,030), S atoms (302), with the remaining observations coming from other atom types. Water was the most commonly observed ligating residue (23,304) followed by Asp (7,144), Thr (5,613), Glu (5,289), Ser (4,497) with all remaining observations coming from other protein residues or ligands. (A) Na⁺-O coordination. The average bond distance across all coordination numbers is 2.4±0.2 Å when only distances between 2.0-2.7 Å are considered (31,675), and 2.6±0.3 Å for the entire range (47,892). For coordination numbers 4 and 5, there is a secondary peak around 2.75 Å, suggesting possible misidentification of water molecules as Na⁺. (B) K⁺-O coordination. The average bond distances for coordination numbers 6 and 7 are 2.8±0.2 Å and 2.9±0.3 Å, respectively, over the entire range (20,494). (C) Water-O coordination. The coordination of all crystallographic waters in the data set used for Na⁺ and K⁺ analysis (1,067,258) were calculated for comparison. For coordination number 4, shoulders around the peak correspond approximately to distances of 2.7 Å and 3.2 Å. The 2.7 Å shoulder is consistent
with the secondary Na+-O peaks observed for coordination numbers 4 and 5, suggesting stronger peaks in electron density maps and longer bond distances may refer to water molecules.

**Figure 2. Type I activated enzymes.** (A) Diol dehydratase (PDB ID 1DIO) has K⁺ (yellow sphere) coordinated by five ligands from the protein and acting as bait for the two hydroxyl O atoms of substrate propanediol. (B) BCKD kinase (PDB ID 1GJV) shown with substrate, relevant residues, K⁺ (yellow sphere) and Mg²⁺ (green sphere) bound to the Oδ1 atom of a conserved Asn and to the triphosphate moiety of ATP. (C) GroEL (PDB ID 1KP8) shown with substrate, relevant residues, K⁺ (yellow sphere) and Mg²⁺ (green sphere). Nucleophilic attack on the Pγ of ATP is mediated by Asp52.

**Figure 3. Type II activated enzymes.** (A) Ribokinase (PDB ID 1GQT) shown with substrate, relevant residues and Cs⁺ (yellow sphere) that plays a functional role analogous to K⁺. The bound M⁺ is sequestered from solvent and contact with substrate, the ATP analog phosphomethylphosphonic acid adenylate ester (ACP). (B) Dialkylglycine decarboxylase (PDB ID 1DKA) shown with substrate, relevant protein residues and K⁺ (yellow sphere). When Na⁺ replaces K⁺ in the site, a structural rearrangement brings the Oγ of Ser⁸⁰ in conflict with the phenyl ring of Tyr³⁰¹ that adopts a new conformation incompatible with substrate binding. (C) Trp synthase (PDB ID 1BKS) shown with substrate, relevant protein residues and Na⁺ (yellow sphere) that binds to the β subunit, away from substrate and PLP, but near the tunnel that shuttles the indole for complexation with L-Ser.

**Figure 4. Molecular mechanism of Na⁺ activation in thrombin.** (A) Structural determinants of Na⁺ activation in thrombin (PDB ID 1SG8). Shown are the Na⁺ (yellow sphere) coordination shell with water (red spheres) and relevant protein residues. Na⁺ binding is detected by Asp¹⁰² and Asp²²¹ and then channeled through the corridors Cys¹⁹¹-Asp¹⁹⁴ and Ser²¹⁴-Cys²²⁰ to the catalytic residues Asp¹⁰² and Ser¹⁹⁵. The rotamer of Ser¹⁹⁵ is the end point of the Na⁺ effect, as demonstrated by the properties of the S195T mutant in panel B. The spatial separation of key residues responsible for transduction of the Na⁺ effect (arrows) underscores the likely contribution of backbone dynamics and overall conformational changes. (B) Contribution to Na⁺ activation of thrombin from residues in the two corridors Cys¹⁹¹-Asp¹⁹⁴ and Ser²¹⁴-Cys²²⁰ connecting the Na⁺ site to the catalytic residues Asp¹⁰² and Ser¹⁹⁵ (see also panel A; thrombin has no residue 218). Three residues are of particular importance, as their mutation has no effect on Na⁺ affinity but abrogates Na⁺ activation (81-83): Asp²²¹ supports one of the waters in the coordination shell, Asn¹⁴³ stabilizes the functional conformation of the backbone N atom of Gly¹⁹³ in the oxyanion hole, and Ser¹⁹⁵ is a member of the catalytic triad.
Figure 4.
