Review–Hypothesis

Specificity and mechanism of action of metal ions in yeast enolase

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Yeast enolase requires one mole/subunit of an 'activating' metal ion (e.g. Mg^{2+}, Mn^{2+}) in 'conformational' sites. This enables substrate/product or analogues to be bound in distorted configurations. 'Nonactivating' metal ions (e.g. Ca^{2+}, Tb^{3+}) enable binding to occur but without the distortion. Activating metal ions appear to prefer octahedral coordination. Substrate/product or analogue binding enables one mole/subunit of additional, 'catalytic' metal ion to bind, which produces activity if the conformational metal ion is an 'activator'. Catalytic metal ion may bind directly to the carboxyl of substrate/product. We suggest that a molecule of water which is bound to conformational metal ion participates in actual bond breaking by donation or acceptance of protons to and from substrate/product. It is possible that metal ion-bound water is far more important in enzymatic catalysis that previously recognized.

Enolase *Specificity* *Yeast*

1. SIGNIFICANCE OF METAL IONS IN CATALYSIS

Approximately equal amounts of Ca^{2+} and Mg^{2+} are required for growth in such large quantities that they are not considered trace elements [1]. Inside cells, free Ca^{2+} concentrations are kept quite low, in the micromolar range. Free Mg^{2+} concentrations, on the other hand, fall in the millimolar range [2]. Hence, enzymes which require divalent metals for activity which are relatively weakly bound will employ Mg^{2+} as the physiological activator.

Over 2000 enzymes are known to exist [3]. Of these 23% require some structural or catalytic metal ion. Over 300, or 15%, use Mg^{2+}. Mg^{2+} is consequently the most common catalytic cofactor.

An increasing number of metal ion-dependent enzymes have been identified as requiring two equivalents of metal ions per active site [4]. Typically, one equivalent is bound to the protein and the other to an electron-rich substrate/product. Also typically, the metal ion bound to the protein does not interact directly with substrate/product [5]. Enolase from yeast is representative of these enzymes.

Enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) from yeast exhibits an absolute requirement for certain divalent cations for enzymatic activity, of which Mg^{2+} gives the highest activity. We will consider what the mechanism of action of Mg^{2+} is in this enzyme, and why the closely related Ca^{2+} produces no activity at all. We will first describe the pattern of metal ion binding by this enzyme.

2. CONFORMATIONAL METAL ION BINDING

As isolated, yeast enolase contains endogenous tightly bound Mg^{2+}. The enzyme may be stripped of its endogenous Mg^{2+} and almost any other metal ion substituted [4]. The enzyme binds one mole/subunit of Mg^{2+} [6], Tb^{3+} [7], Ca^{2+} [8], Ni^{2+}

Abbreviations: AEP, 3-aminoenolpyruvate-2-phosphate; TSP,D-tartronate semialdehyde-2-phosphate

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[8] Zn^{2+} (cf. [4]) and probably most other divalent metal ions. A conformational change results involving the tertiary but not the secondary structure of the enzyme [9]. Metal ion binding at this site is called conformational metal ion. There is no specificity for metal ions that produce activity, and the characteristics of the conformational change [8,10] are not related to the degree of enzymatic activity produced.

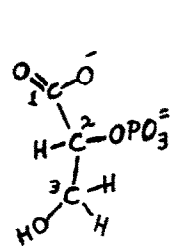
The coordination of conformational metal ions is distorted octahedral and the ligands are all oxyligands [7,9,10] including two or three carboxyl groups and two water molecules [11].

3. SUBSTRATE/PRODUCT OR ANALOGUE BINDING

The structures of substrate, product and some analogues are presented in fig.1.

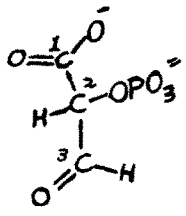
Conformational metal ion must be present for substrate/product or substrate analogue binding [6,13,14]. No catalysis occurs upon binding substrate/product [6,13]. It is believed that the substrate/product of enolase, like substrates of most enzymes [15], are bound in distorted configurations [8,13,16] provided the conformational metal ion is an 'activating' metal ion [8] capable of producing some degree of enzymatic activity (if present in sufficient concentration). These include Mg^{2+}, the physiological cofactor, most of the first row divalent transition metal ions and Cd^{2+}; Ca^{2+} and Tb^{3+} produce binding but no distortion [7,13] and no activity. The interaction between substrate/product and bound conformational metal ion is the level at which specificity effects occur.

This specificity is absolute [8]; all activating metal ions produce similar spectroscopic changes in the product analogue AEP while similar spectroscopic changes in the product analogue AEP while nonactivating ones produce no changes. With activating conformational metal ions, binding of AEP is accompanied by marked red shifts in its absorption, increases in absorption intensity and appearance of a strong CD signal [8]. These effects were interpreted as indicating the AEP was bound so its $C_2 = C_3$ double bond was twisted. TSP shows similar changes and the same specificity

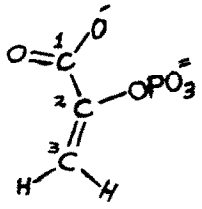


SUBSTRATE

(2-PHOSPHOGLYCERATE)

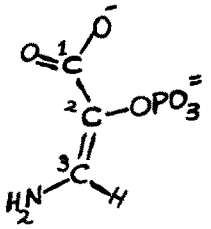


TSP

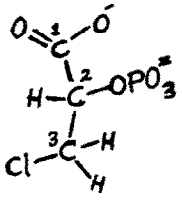


PRODUCT

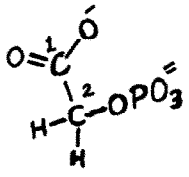
(PHOSPHOENOLPYRUVATE)



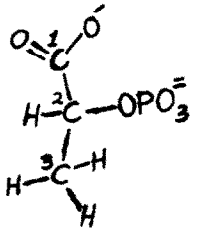
AEP



3-CHLOROLACTIC ACID PHOSPHATE



GLYCOLIC ACID PHOSPHATE



LACTIC ACID PHOSPHATE

Fig.1. Substrates and competitive inhibitors of enolase. The structures of TSP (D-tartronate semialdehyde-2-phosphate) and AEP (3-aminoenolpyruvate-2-phosphate) and presumed ones, from their syntheses [12]. The numbers beside the carbons are used to identify them.

[12,16]; it is believed to enolize on the enzyme. Substrate/product ^{31}P-NMR resonances shift in the presence of conformational Mg^{2+} but not Ca^{2+} [13], suggesting distortion of the bond [17] between the phosphorus and the rest of the molecules occurs with Mg^{2+}.

Examination of the properties of activating and nonactivating metal ions shows only a possible correlation between activity and preference for octahedral geometry in complexes (table 1) [18].

Substrate/product or analogue binding further distorts the ligand geometry around conformational metal ions. Conformational Co^{2+} and Cu^{2+} experience rhombic distortions of their geometries on substrate/product or analogue binding [10,23].

4. CATALYTIC METAL ION BINDING

Kinetic studies of metal ion activation confirm an ordered sequential mechanism [6,11]: conformational metal ion permits substrate/product binding which in turn permits additional metal ion binding. This additional metal ion binding produces catalysis if activating conformational metal ions are involved, thus termed catalytic metal ion.

One additional mole per subunit of Mg^{2+} [6,23], Zn^{2+} [4] or Co^{2+} [23] is bound in the presence of substrate/product. Oxy-ligands, in an octahedral environment, again appear to be coordinated to catalytic metal ion [9]. It has been suggested that one or more of the ligands comes from substrate/product [23]. There is little specificity for an activating metal ion at this stage. Additional Ca^{2+} binds in the presence of substrate/product [23]. Brewer and Ellis [13] suggested that catalytic metal ion coordinated directly to the phosphate and carboxyl group of substrate/product.

The conformational metal ion determines whether any degree of activity will occur. The catalytic metal ion controls the rate of the enzymatic reaction [8].

There is a good correlation between maximum activity produced by the first row transition metal ions and their crystal field stabilization energies. Sinha and Brewer [21] suggested that this occurred because the change from substrate to product involved a change in coordination geometry of catalytic metal ions.

Table 1

Some chemical properties of divalent metal ions

Ion	Activator?	pH opt.	Max. Act.	ΔH hydration (kJ/mol)	log (hydrolysis constant)[a]	log (exchange rate)	Favored coordination number
Mg^{2+}	yes	7.8	(100)	−1921	11.44	5.2	6
Cr^{2+}	yes[b]	?	?	?	–	8.3	6? (distorted)
Mn^{2+}	yes	8.1	41	−1841	10.59	6.7	6
Fe^{2+}	yes	7.4	10	−1946	9.5	6.2	6
Co^{2+}	yes	≥8.0	20	−1996	10.20	5.6	4/6
Ni^{2+}	yes	7.2?	2	−2105	9.86	4.3	4/6
Cu^{2+}	yes[c]	6.9	13	−2100	7.6	8.5	6 (distorted)
Zn^{2+}	yes	7.8?	75	−2046	8.96	7.5	5/6
Cd^{2+}	yes	8.8	9	−1807	10.08	8.4	6
Be^{2+}	no	–	–	−2494	5.4	2.1	4
Ca^{2+}	no	–	–	−1577	12.85	8.6	7/8
Sr^{2+}	no	–	–	−1443	13.29	8.8	8/9?
Ba^{2+}	no	–	–	−1305	13.47	9.3	8/9?
Hg^{2+}	no	–	–	−1824	3.70	9.4	2/4
Pb^{2+}	no	–	–	−1481	7.7	?	?

[a] $M^{2+} + H_2O \rightleftharpoons MOH^+ + H^+$ [19].
[b] E. Westhead, personal communication
[c] From [21].

The data were otherwise obtained from [4,16,18,20,21,22]

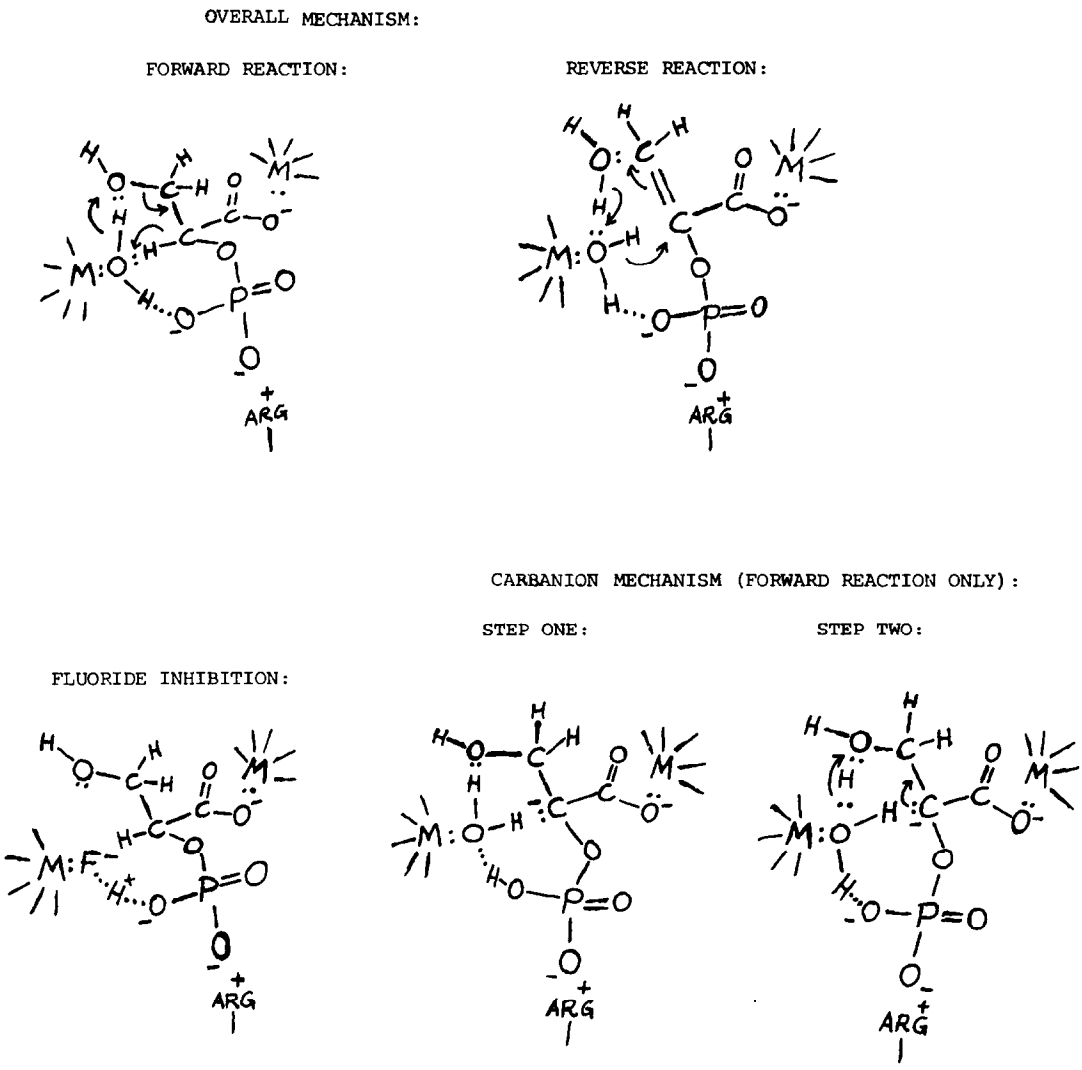


Fig.2. Hypothetical mechanisms of action of conformational metal ion (charge signs on metal ions omitted) in yeast enolase. The conformational metal ion is to the left and the catalytic metal ion is to the right.

5. MECHANISM OF ACTION OF METAL IONS: HYPOTHESIS

The basic hypothesis explaining the function of conformational metal ion was developed by Nowak et al. [11]. Their hypothesis is that the hydroxyl on C_3 of the substrate coordinates directly with the conformational metal ion. The hypothesis is based on distances calculated from measurements of ^{31}P- and ^{1}H-NMR relaxation rates. The measurements were made in the presence of conformational Mn^{2+} only, using slowly reacting substrate analogues and competitive inhibitors.

However, attempts to show direct interaction between conformational Cu^{2+} and Ni^{2+} and the amino group of AEP using various spectroscopic techniques have not been successful [23]. There are theoretical reasons for objecting to a direct interaction also: generally, methanolic hydroxyls are much poorer ligands to metal ions than water molecules [18]. This would be unfavorable to replacement of bound water by the hydroxyl group on C_3 of the substrate. And it is hard to explain

why AEP binds so much more strongly than the substrate [12]: the amino group of AEP would hardly interact strongly with Mg^{2+}.

However, these difficulties can be skirted if we modify the hypothesis of Nowak et al. [11] so that the interaction occurs between a molecule of metal ion-bound water and the substrate/product (fig.2). Catalysis involves transfer of protons to and from the bound water molecule, and the substrate.

Donation of a proton to the hydroxyl on C_3 of the substrate would produce a hydroxide ion on the metal ion. There is no evidence suggesting that the conformational metal ion functions in the hydrolyzed state [16], and no correlation between the p*K* of metal ion hydrolysis and any facet of enzymatic activity (table 1). Hence, a replacement proton must be obtained from the medium or from another source. We suggest further that the replacement comes ultimately from the proton on C_2-two of the substrate, completing a catalytic cycle involving a molecule of metal ion-bound water.

The hypothesis is chemically realistic. One or both of the two lone-pair electrons on a coordinated water oxygen may interact with a metal ion [18]. One pair may be normally available for hydrogen bonding to the proton on C_2 of the substrate, or perhaps the electrons may be made available because of the interaction with the phosphate of the substrate.

Nowak et al. [11] suggested that substrate or analogue 'immobilized' one water molecule on conformational Mn^{2+}. They suggested this involved formation of a hydrogen bond with the phosphate of the substrate. The interaction with the phosphate might be responsible for distortion of the bond between the phosphorus and the carbon chain [13] but would correspondingly draw the coordinated water away from the metal ion, this being reflected in the observed rhombic distortion of the ligand geometry about the conformational metal ion [10,23].

In the reverse reaction, a water molecule would have to move into the appropriate orientation between C_3 of PEP and the metal-bound water to produce a transfer to the reverse effect.

Catalysis would occur most efficiently if it involved the concerted transfer of the substrate proton to the water and a water proton to the substrate hydroxyl (metal ion-coordinated H_3O^+ is never produced). Of course, it should be understood that any proton transfers occur at a reasonable rate only when catalytic metal ion also binds. The function of the catalytic metal ion is discussed below. However, there are reasons to believe the mechanism is not quite so straightforward (see below).

This scheme obviously places a premium on the orientation of one metal-bound water molecule and hence on the ligand geometry about the metal ion. This is consistent with the observed specificity (table 1).

Inorganic phosphate and fluoride may inhibit catalysis by replacing the critical water molecule [24]. The link with inorganic phosphate must then be reestablished by incorporation of a proton between the metal-bound fluoride and the phosphoryl oxygen. A net uptake of protons is observed [23]. (The inhibition by fluoride is observed in the presence of substrate also, but exhibits a relatively slow onset [25]. This may reflect the time required for the fluoride to replace the immobilized water molecule.)

The hypothesis seems to accommodate all the data available. The stronger interactions with TSP-enolate and AEP [12] can be explained as being partly due to hydrogen bond formation with the conformational metal-bound water. It also explains why no direct interaction between conformational Cu^{2+} and AEP or between conformational Ni^{2+} and AEP [23] were found, and why addition of substrate/product to the Tb^{3+}-enzyme did not result in loss of Tb^{3+}-bound water [7]. The large ^{31}P-NMR shift observed with conformational Co^{2+} could result from linkage through Co^{2+}:O-H:O-P [13]. And binding of substrate/product to the enzyme-conformational metal ion complex would not be charracterized by significant changes in proton equilibria [23].

Stubbe and Abeles [26] showed that the substituent on C_3 of some substrate analogues (chloro-, fluoro-, methyl, etc.) had a dramatic effect on the rate of breakage of the bond between C_2 and hydrogen. There may be some interaction between the substituent on C_3 and the conformational metal ion-bound water molecule, perhaps some orienting effect, that facilitates proton transfer from C_2.

The hypothesis also explains why the 3-chlorolactic acid phosphate reacts more slowly than the substrate [26]. Although Cl^- is a better

leaving group than OH^-, it is also a much worse proton acceptor. Similarly the fluoro derivative reacts somewhat faster than the chloro-, although the C-F bond is stronger than a C-Cl bond, since F^- is a much better proton acceptor (weaker acid) than is Cl^-. In other words, the leaving group from C_3 should be protonated.

An apparent objection to the hypothesis also comes from the work of Stubbe and Abeles [26]. They obtained kinetic evidence that the hydroxyl bond breakage and the C_2 proton bond breakage were not concerted. They also found that the enzyme could catalyze exchange of the proton on C_2 of an analogue which has no C_3 (glycolic acid phosphate).

In addition, it is generally accepted that the enolase reaction proceeds through a carbanion (C_2 proton off first) mechanism [20,26,27]. However, the hypothesis presented can easily accommodate such a process (fig.2). The proton from C_2 adds to the water molecule while the proton which bridges the conformational metal ion-bound water and the phosphate is transferred to the phosphate. Thus, the phosphate acts as a kind of reservoir or overflow receptor for excess protons from the water molecule.

In a second concerted step, the proton on the phosphate is transferred back to the water oxygen while another proton on the water is transferred to the hydroxyl of the substrate. This 'two step' mechanism would disconnect the proton abstraction and hydroxyl removal reactions.

Evidence from ^{31}P-NMR measurements leads to the suggestion that a proton or metal ion interacts more strongly with the phosphate when catalytic metal ion binds [13]. If the agent for the shift observed is a proton, this must come from a protonated species such as a water molecule since there is no evidence for proton equilibria associated with substrate/product or catalytic metal ion binding [23].

In some circumstances, for example, when glycolic acid phosphate is bound, a proton can add back to the carbanion from the medium while the abstracted proton is lost from the metal ion-bound water, producing exchange of C_2 protons. Such exchange would be expected to be of low efficiency and is [26].

The measured distance between the proton on C_2 of substrate analogues and conformational Mn^{2+} was too great for the interaction we are proposing [11]. Consequently, we must also propose that the distance shortens considerably when catalytic metal ion is present. Otherwise, we are assuming that no major changes in structure of the complex occur when catalytic metal ion binds. There is no evidence for major changes in the environment of conformational metal ion produced by catalytic metal ion binding.

Nowak et al. [11] could not measure distances to catalytic metal ion in the presence of conformational metal ion using NMR. Brewer and Ellis [13] suggested, partly from some qualitative effects of Co^{2+} on ^{31}P-NMR spectra of substrate/product, that catalytic metal ion coordinated directly to the phosphate of substrate/product and possibly to the carboxyl group as well. They pointed out that electronic rearrangements at C_2 and C_3 would occur more readily if electron density on the carboxyl and phosphate was tied up, most simply by direct coordination with a metal ion. In other words, catalytic metal ion makes C_2 more acidic.

The question of whether the catalytic metal ion coordinates directly to the phosphate of the substrate/product has not been settled as yet. It might coordinate directly only to the carboxyl group of the substrate/product, as is indicated in fig.2. The phosphate has two negative charges when bound to the enzyme. One may be neutralized by interaction with an arginyl group (cf. [4] and the other by hydrogen bonding to the critical water molecule on conformational metal ion [11].

If the inner coordination sphere of catalytic metal ion includes the carboxyl group from substrate/product, then interconversion between substrate and product might produce a change in the coordination geometry.

The overall rate of the enolase reaction is correlated with the readiness of an octahedral complex of any of the activating transition metal ions to convert to tetrahedral geometry [18,21]. Since there is no evidence for tetrahedral coordination of conformational or catalytic metal ions under any circumstances [9,10,23], we interpret the correlation as suggesting that, if a direct interaction between catalytic metal ion and substrate/product exists, the geometry changes, if only slightly, when substrate converts to product. Hence, the greater the energy required to produce this change in catalytic metal ion geometry, the lower the overall

activity [21].

The suggestion that the substituent on C_3 interacts not with metal ion but with water coordinated to metal ion agrees with current thinking about metal ion function in another enzyme, glyoxalase I [28]. Rosevear et al. [28] obtained a series of intermolecular distances from NMR relaxation measurements which showed that a metal ion-bound water molecule interacted with a critical carbonyl of the substrate.

Metal ion-dependent catalysis must often involve substrate/product interaction with a molecule of metal ion-bound water rather than the metal ion itself because the substrate/product would otherwise have to replace metal ion-bound water (or another ligand) or else the metal ion would have to change coordination number. Either circumstances would probably require considerable free energy. Metal ion-bound water can perform both acid- and base-catalyzed reactions [29], without having to replace the water molecule or change it to hydroxide ion.

Hammes [29] has pointed out the advantages of concerted acid-base reaction mechanisms in catalysis. Since acid and base catalysis occur in some step of most enzymatic reactions and since metal ions are such common cofactors, it is hoped that the suggestion about the mechanism of their action in this paper will focus and stimulate further investigations in this field.

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