

## Metalloenzymes: Native Co-factor or Experimental Artifact?

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There has been much interest in the biochemical characterization of metalloenzymes, owing in part to past successes in targeting metalloenzymes for the development of therapeutic agents. It is estimated that ~10% of approved drugs target metalloenzymes, [1] such as carbonic anhydrase, matrix metalloproteases, and angiotensin-converting enzyme [2-5]. Inhibitors of metalloenzymes typically contain a metal-binding group that targets the catalytic metal ion, and therefore, inhibitor affinity is affected by changes to the catalytic metal ion. Consequently, the development of therapeutically effective inhibitors requires that the biologically relevant form(s) of the enzyme be identified to ensure that *in vitro* activity of an inhibitor is a good indicator of its potential *in vivo* efficacy.

Since multiple metal ions are often capable of serving as effective co-factors for a given enzyme *in vitro*, identification of the native co-factor is largely based on the identity of the metal ion that copurifies with the protein of interest. For known metalloenzymes, zinc is the predominant co-factor observed owing to its many desirable properties [6]. However, over the last several years there has been an increasing number of zinc-dependent enzymes reclassified as cambialistic or iron-dependent enzymes, including histone deacetylase 8 (HDAC8), UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase (LpxC), S-Ribosylhomocysteinase (LuxS), methionine aminopeptidase (MetAP), N-acetyl-1-D-myo-inositol-2-amino-2-deoxy- $\alpha$ -D glucopyranoside deacetylase (MshB), and peptide deformylase (PDF) [7-13]. One reason for these findings is that traditional protein expression and purification protocols favor the incorporation of zinc over the oxygen sensitive metal ion Fe (II). Isolation of the Fe (II)-form of the enzyme often requires expression in chemically defined media and purification under anaerobic conditions to prevent the oxidation of Fe (II) to Fe (III), dissociation from the enzyme, and replacement with zinc. This begs the question, is zinc truly the predominant biological co-factor or is this artifact arising from the experimental conditions that are used?

One factor that likely plays an important role in governing co-factor selection is the environment of the enzyme. The protein LuxS was initially proposed to function as a Zn<sup>2+</sup>-dependent enzyme; however, due to the instability of the native protein under aerobic conditions additional studies were carried out that ultimately demonstrated LuxS from *E. coli* and *Bacillus subtilis* to be an Fe<sup>2+</sup>-dependent enzyme [10]. The initial discoveries of bound zinc co-factors were experimental artifacts attributed to the purification of the enzyme under aerobic conditions. While LuxS is an example of an enzyme that seemingly prefers a single metal ion co-factor across species, there are also examples of enzymes that exhibit species-specific co-factors. For example, the enzyme PDF from *E. coli* uses Fe<sup>2+</sup> as co-factor, while same enzyme from the Fe-limited organism *Borrelia burgdorferi* uses Zn<sup>2+</sup> as its co-factor [13,14]. Similarly, the native co-factor for MetAP from *E. coli* is Fe<sup>2+</sup>, while the native co-factor for human MetAP is proposed to be Mn<sup>2+</sup> [11,15]. These examples illustrate that the metal ion environment of the enzyme, specifically metal ion availability, makes an important contribution to the co-factor preferences of metalloenzymes, and therefore, should be taken into account when designing experiments with the purpose of identifying the native co-factor. The occurrence of species-specific co-factors is not limited to

metallohydrolases. Superoxide dismutase (SOD) is an example of a metalloenzyme that utilizes Mn<sup>2+</sup>, Fe<sup>2+</sup>, or Mn/Fe as native co-factors across different species [16-20]. (Note: The cited references offer just a small sampling of the research examining SOD co-factor preferences). These detailed studies on SOD reinforce the important contributions that metal ion availability and experimental conditions have on identifying the native co-factor(s) preferences of metalloenzymes.

In addition to metalloenzymes that prefer a single co-factor as described above, there are also increasing numbers of cambialistic enzymes that can utilize multiple co-factors *in vivo* has been identified. An early example of a cambialistic enzyme is SOD, which can utilize Mn/Fe as its co-factors [20]. More recently, the metal-dependent deacetylase LpxC from *E. coli* was shown to be a cambialistic enzyme that utilizes Fe/Zn co-factors *in vivo* [8,9]. Interestingly, the Fe/Zn co-factor preferences observed for LpxC mirrored the Fe/Zn metal ion content of the cell lysate, suggesting that LpxC is able to switch co-factors in response to changing environmental conditions. The ability to utilize multiple co-factors may be an advantageous feature for metalloenzymes as it would enable the enzyme to adapt to dynamic metal ion environment(s) in order to preserve enzyme function under different conditions. The metal-dependent deacetylase MshB appears to be another example of a Fe/Zn cambialistic enzyme. MshB prefers Fe<sup>2+</sup> as a co-factor under anaerobic conditions, as well as under aerobic conditions in the absence of zinc, while MshB prefers Zn<sup>2+</sup> under aerobic conditions in the presence of zinc [12]. These findings may have interesting biological implications in light of the metal ion environment mycobacteria, as the vacuoles of infected macrophages switch between an Fe-rich environment and Zn-rich environment during the course of infection [21]. The ability to switch co-factors under changing environmental conditions is likely an important feature for metalloenzymes that carry out critical cellular functions, as it preserves enzyme function under changing environmental conditions.

Recently, there have been an increasing number of detailed studies on metalloenzymes resulting in the identification of metalloenzymes with species-specific co-factors, as well as cambialistic enzymes. These studies have shown that co-factor incorporation into metalloenzymes is sensitive to the experimental conditions that are used and illustrate that if studies are not carried out under proper conditions incorrect co-factor identification can occur. Therefore, one must exercise caution when interpreting results from experiments that are carried out under a single condition. Additionally, findings from these studies suggest that

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the metal environment plays an important role in the native co-factor preferences of metalloenzymes, and therefore, it must be taken into account when designing experiments with the purpose of identifying native co-factors. Consequently, detailed studies that examine co-factor preferences under multiple conditions provide a more accurate understanding of the biologically relevant form(s) of the enzyme and offer insights into possible mechanisms of enzyme regulation and environmental adaptation.

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