Reactivation of 3-Dehydroquinate Synthase by Lanthanide Cations

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Lanthanide cations, such as Eu^{3+}, have been increasingly used as spectroscopic probes for biological systems. However, since they are known to occur at only trace amounts in organisms, no biological role has so far been attributed to them.[1] Isomorphous substitution for octahedrally coordinated calcium ions which play noncatalytic roles in protein function in metalloproteins is well-known.[1] Substitution of lanthanide for zinc ions, however, would not be expected because of zinc's smaller ionic radius and its tendency to form a tetrahedral coordination state. In contrast to this view, we now report the novel binding and activation of a zinc metalloenzyme, 3-dehydroquinate (DHQ) synthase, by lanthanide cations Eu^{3+} and Sm^{3+}.

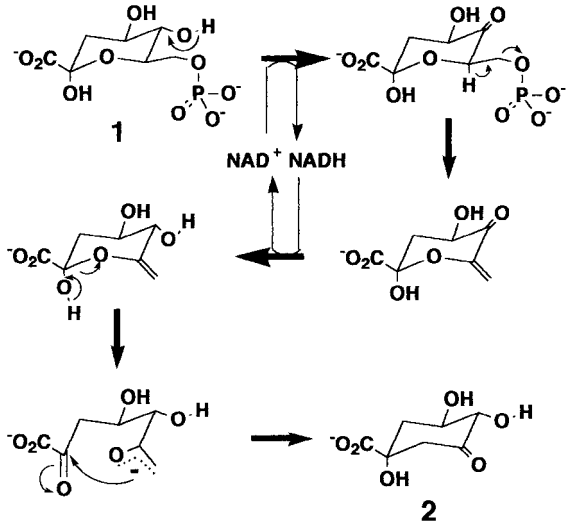
DHQ synthase is the second enzyme in the shikimate pathway that is ultimately responsible for the synthesis of aromatic amino acids and other ring-containing compounds.[2] DHQ synthase catalyzes the NAD^+-dependent conversion of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP, **1**) to DHQ (**2**) according to the mechanistic pathway given in Scheme 1.[3] Interest in this enzyme stems from its unusual ability to catalyze multiple reactions in its active site, namely an oxidation, a β-elimination, a reduction, a ring-opening, and an intramolecular aldol condensation.[3,4]

DHQ synthase has been demonstrated to require one divalent metal cation per monomer for activity, generally believed to be Zn^{2+} in vivo owing to its greater bioavailability[5] and increased affinity for NAD^+ in the case of the *Escherichia coli* enzyme.[6] On the basis of a number of factors:—(1) the sensitivity of DHQ synthase to the metal chelating agent ethylenediaminetetraacetic acid (EDTA); (2) the ability of substrate DAHP to protect the metal cation from chelation; (3) the enzyme's varying activities in the presence of different divalent metal cations; and (4) the influence of these cations on NAD^+ dissociation rates—Bender, Mehdi, and Knowles[6] proposed that the metal cation plays a central catalytic role throughout the enzyme mechanism.

As part of an effort to obtain an isomorphous complex of DHQ synthase with a metal of sufficiently high molecular weight for use in crystallographic studies, we discovered that the DHQ synthase domain from the *Aspergillus nidulans* AROM protein could also be activated by the trivalent lanthanide cations Eu^{3+} and Sm^{3+}. Recombinant enzyme was overproduced in *E. coli* and purified as previously described.[7,8] All buffers, substrates, and coupling enzymes used in this work were passed through Chelex resin (Sigma) to remove adventitious metal ions. Metal-free apoenzyme was reactivated[9] with Zn^{2+} or the lanthanide cations Eu^{3+} and Sm^{3+}. Metal content was confirmed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES).[10] Enzyme assays were carried out,[11] based upon established procedures.[6,8]

Under standard assay conditions, Eu^{3+} reactivated DHQ synthase to 59% and Sm^{3+} to 85% of the level observed for the

Scheme 1



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(9) Metal-free apoenzyme was prepared based upon an established protocol[8] by dissolving 20 μM DHQ synthase in Chelex-treated 50 mM MOPS−NaOH, pH 7.5, containing 200 μM EDTA. The solution was incubated at 4 °C for 1 h and then chelator and metal were removed by ultrafiltration with a Centricon-30 tube (Amicon) by concentrating the retained material to one-fifth of its original volume and then diluting to the original volume with Chelex-treated 50 mM MOPS−NaOH, pH 7.5 (lacking EDTA). This procedure was repeated four times to yield a final concentrated solution of 75 μM apo-DHQ synthase. Reactivation by metal cations was effected by dilution of a 10-μL aliquot of the concentrated apoenzyme into 500 μL of Chelex-treated 50 mM MOPS−NaOH, pH 7.0, containing 50 μM zinc chloride, samarium chloride, or europium chloride followed by a 30 min incubation at 4 °C.

(10) For ICP-AES, apo-DHQ synthase or Zn^{2+}, Eu^{3+} and Sm^{3+} holoenzymes were prepared in Chelex-treated phosphate buffered saline (Sigma), pH 7.5. Adventitious metal cations were removed by passing samples through a Hi-trap desalting column (Pharmacia). To identify a potential second metal binding site, reconstituted Zn^{2+}-holoenzyme was incubated in the presence of 300 μM metal cation, and the sample was passed through the desalting column as before prior to ICP-AES analysis. In addition, ICP-AES analysis of metal content of the enzyme assay reaction mixture,[11] in the presence and absence of added metal ions, was also performed to confirm the low level of adventitious zinc contamination under assay conditions and the presence of each lanthanide in solution.

(11) For the coupled assay, reactions were carried out in 50 mM MOPS−NaOH, pH 7.0, 30 μM DAHP, 40 μM NAD^+, 50 μM EDTA (which does not inactivate DHQ synthase in the presence of substrate[8]), 50 μM metal chloride (if required), and 20 μg mL^{-1} *Mycobacterium tuberculosis* type-II 3-dehydroquinase (purified by using an established protocol[12]). After equilibration at 25 °C for 5 min, DHQ synthase was added to a concentration of 1.5 μM and the production of 3-dehydroshikimate was monitored at 234 nM (effective ϵ_{234} = 9850 M^{-1} cm^{-1}).[8] Control experiments indicate that 1.5 μM DHQ synthase is maximally reactivated by 50 μM zinc or lanthanide chloride. For inhibition studies, reconstituted Zn^{2+}-holoenzyme was assayed in the presence of 300 μM metal chloride. Control experiments indicated that these concentrations of metal cations did not substantially inhibit the *M. tuberculosis* type-II dehydroquinase coupling enzyme.

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Table 1. Activities of DHQ Synthases Reconstituted with Metal Cations

	V_{max}(cation)/V_{max}(Zn^{2+})	
cation	*A. nidulans*[a]	*E. coli*[b]
Zn^{2+}	1.00	1.00
Co^{2+}	1.25	1.89
Fe^{2+}	0.18	
Ni^{2+}	0.16	0.43
Cd^{2+}		0.23
Mn^{2+}		0.19
Cu^{2+}		0.17
Eu^{3+}	[c]0.59	
Sm^{3+}	[c]0.85	

[a] Reference 8 unless otherwise specified. [b] Reference 6. V_{max}(cation)/V_{max}(Zn^{2+}) for Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} = 0. [c] This study.

reconstituted Zn^{2+}-holoenzyme (Table 1). Assay of the apoenzyme in the absence of metal cations gave a 5−6% residual rate. This corresponded to a concentration of less than 0.2 μM zinc compared to 1.5 μM enzyme in the assay buffer as determined by ICP-AES. ICP-AES analysis of assay solutions containing enzyme, following the subsequent addition of 50 mM lanthanide chlorides to the assay buffer,[13] confirmed the presence of each lanthanide and showed no increase in the amount of zinc (less than 0.2 μM). These results were consistent with ICP-AES analysis of apo-DHQ synthase, which indicated a fractional saturation level for zinc of only 9.1%. In comparison, ICP-AES confirmed the substitution of the various metal cations in the reconstituted holoenzymes with a marginally greater fractional saturation for zinc (92%) compared to europium (81%) and samarium (88%).

Zn^{2+} is capable of both reactivating *E. coli* DHQ synthase activity and inhibiting it at high concentrations. This inhibition is thought to be mediated through a second metal binding site on the enzyme.[6] To determine whether this potentially regulatory feature is conserved in the *A. nidulans* DHQ synthase domain we reconstituted the enzyme with Zn^{2+} and then assayed in the presence of excess Zn^{2+} or lanthanide cations.[11] It was observed that all three metal cations, Zn^{2+}, Eu^{3+}, and Sm^{3+}, reduced enzyme activity to 31−35% of the level of the reconstituted Zn^{2+}-holoenzyme. Furthermore, we find that incubation of the Zn^{2+}-holoenzyme in high metal concentrations[10] yields fractional saturation of the second site, which appears greater for zinc (50%) compared to europium (35%) and samarium (36%).

The data presented clearly indicate that the lanthanide cations Eu^{3+} and Sm^{3+} are capable of activating DHQ synthase to levels approaching the Zn^{2+}-holoenzyme and, in fact, are superior to other divalent cations which have been studied with the exception of the cobalt cation[6,8] (Table 1). General characteristics of lanthanides include adoption of coordination numbers greater than 6 (usually 8 to 9) and a preference to bind "hard" bases such as oxygen and fluorine, rather than "soft" bases such as nitrogen, phosphorus, and sulfur.[13] In contrast, Zn^{2+} tends to adopt a tetrahedral or pentacoordinate state in proteins with a preference for nitrogen and sulfur ligands although oxygen ligands are also frequently observed.[14] The ability of Eu^{3+} and Sm^{3+} to bind and function in the active site of DHQ synthase suggests a certain flexibility in the metal binding site although full coordination of the lanthanide cations may include some chloride ligands present in the salts used for reconstitution. Other ligands could potentially include groups such as oxygen arising from active site residues and water molecules. The ability of the DAHP to protect the metal cation from chelation by EDTA[6,8] also suggests that some coordination positions may be occupied by groups from the substrate itself. Previously observed metal-dependent variations in NAD^+ dissociation rates[6] also indicates the potential for a structural interaction between the metal and dinucleotide. In any case, the ability of Eu^{3+} and Sm^{3+} to catalyze the variety of reactions (Scheme 1) implicated to involve the zinc cation[6] is intriguing and merits further investigation.

In addition, we have shown that the *A. nidulans* DHQ synthase domain has two metal binding sites which may bind Zn^{2+}, Eu^{3+} or Sm^{3+}, albeit with a reduced fractional saturation for the lanthanide cations compared to zinc. Nevertheless, enzyme activation studies indicate that the addition of excess lanthanide cations to DHQ synthase inhibits activity to a similar extent as that of excess zinc. This suggests that the fractional saturation of the second metal binding is not directly proportional to the fractional enzyme inhibition, possibly suggesting an allosteric response. The binding of Zn^{2+} to the inhibitory site may be a potentially useful switch in vivo since the enzyme is used in the multistep shikimate pathway present in plants, bacteria, and microbial eukaryotes.[15] Although flux through the pathway may be regulated by intracellular concentrations of aromatic amino acids,[15] sensitivity to intracellular zinc concentrations may also assist in maintaining appropriate levels of biosynthesis.

In conclusion, the serendipitous discovery of lanthanide activation of DHQ synthase has stimulated more questions about the nature of the active site of this enzyme and the catalytic properties of lanthanides in general. The substitution of Eu^{3+} and Sm^{3+} for Zn^{2+} raises the possibility of using lanthanide cations for generating metalloproteins for crystallographic studies involving multiple anomalous dispersion phasing techniques.[16] Furthermore, the established use of lanthanides as bioprobes may also allow utilization of other spectroscopic techniques (reviewed by Cotton[13]) to develop a better understanding of the precise role the metal cation plays in the reaction mechanism of DHQ synthase.

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