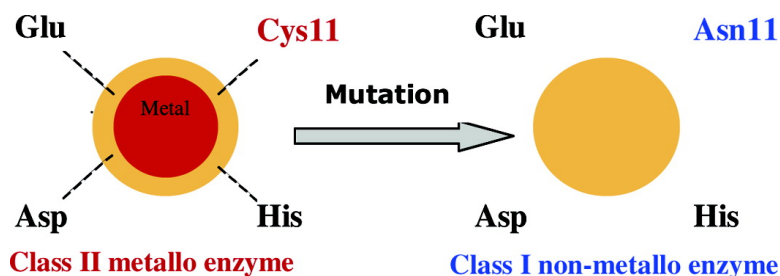Communication

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Conversion of *Aquifex aeolicus* 3-Deoxy-D-*manno*-octulosonate 8-Phosphate Synthase, a Metalloenzyme, into a Nonmetalloenzyme

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In this communication, we report the conversion of *Aquifex aeolicus* 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase (KDO8PS), a metalloenzyme, into a nonmetalloenzyme by a single amino acid mutation, namely, C11N. The result may provide insight into the evolutionary link between the two KDO8PS classes as well as the potential role of the metal and/or asparagines (N) in the catalytic mechanism.

KDO8PS catalyzes the aldol-type condensation of D-arabinose-5-phosphate (A5P) and phospho*enol*pyruvate (PEP) to form KDO 8-phosphate and inorganic phosphate.[1,2] This reaction constitutes the first committed step in the lipopolysaccharide biosynthetic pathway of Gram-negative bacteria.[3] On the basis of phylogenetic analysis, KDO8PSs have been separated into two classes differing primarily in their metal requirements.[4] Class I KDO8PSs (represented by *Escherichia coli*) are nonmetalloenzymes while class II KDO8PSs (represented by *A. aeolicus*) are metalloenzymes.[4] In the crystal structure of *A. aeolicus* KDO8PS, the four residues C11, H185, E222, and D233 were found to form the octahedral metal binding site.[5] Sequence alignment shows that three of these four residues (H185, E222, and D233) are absolutely conserved in ALL KDO8PSs, whereas C11 is absolutely conserved only in class II KDO8PSs. In class I KDO8PSs, C11 is replaced by an N that is absolutely conserved in the class I family. An overlay of the crystal structure of the nonmetallo *E. coli* KDO8PS with the metallo *A. aeolicus* KDO8PS demonstrates that all ligands are similarly juxtaposed in their respective active sites.[5]

On the basis of these observations, it is postulated that C and N are responsible for the difference in the metal requirement of the two KDO8PS classes; thus, it should be relatively straightforward to change the metal requirements for the enzymes. To test this hypothesis and gain mechanistic information concerning the two different KDO8PS classes, the C11 in *A. aeolicus* KDO8PS was mutated to an N. The conserved active site N26 in *E. coli* KDO8PS was mutated to a C.

The *A. aeolicus* KDO8PS mutations C11N, C11S, C11K, and C11G, as well as the *E. coli* KDO8PS mutant N26C, were constructed utilizing standard techniques.[6] The protein from these mutant constructs was isolated and purified by standard methods.[7] A sample of the "as isolated" protein from each of these mutants was divided into three aliquots. One portion was left untreated, the second portion was treated with a mixture of metal salts (Table 1) at 100 μM each for 2 h at 25 °C and then passed through a desalting column to remove excess metal ions, while the third portion was treated with 10 mM EDTA for 2 h at 25 °C and then dialyzed against metal-free buffer to remove EDTA. The amount of bound metal in each of the mutant and wild-type KDO8PSs was determined utilizing ICP-MS.[8] The results shown in Table 1 demonstrate that *A. aeolicus* C11N KDO8PS does not bind metals and that its activity is unaffected by treatment with EDTA. When individual metal salts were added to the assay mixture of the apo-

C11N KDO8PS, no metal ion tested caused an increase in enzyme activity (Supporting Information).

The enzymatic activities of the mutant proteins were measured using the Aminoff assay.[9] The results presented in Table 1 reveal that the *E. coli* N26C mutant KDO8PSs has minimal activity, while the *A. aeolicus* C11N mutant displays activity. The C11N protein was further kinetically characterized to compare its properties to that of the wild-type *A. aeolicus* KDO8PS.[10] To determine the kinetic constants, a continuous UV assay was used.[11]

The C11N mutant enzyme exhibits a temperature optima of 90 °C compared to 95 °C for wild-type *A. aeolicus* KDO8PS. C11N is catalytically more efficient ($k_{cat}/K_m = 53 \times 10^{-3}$ s^{-1} μM^{-1} at 60 °C) and binds PEP much tighter ($K_m^{PEP} = 12$ μM at 60 °C) compared to the wild-type *A. aeolicus* KDO8PS ($k_{cat}/K_m = 2.7 \times 10^{-3}$ s^{-1} μM^{-1}; $K_m^{PEP} = 155$ μM at 60 °C). While the KDO8PS C11N mutant displays many of the characteristics of the thermostable metallo family, the kinetic parameters for the C11N *A. aeolicus* KDO8PS ($K_m^{A5P} = 30$ μM and those above) are more similar to the kinetic values of the nonmetallo *E. coli* enzyme ($K_m^{A5P} = 30$ μM, $K_m^{PEP} = 19$ μM, $k_{cat} = 6.8$ s^{-1}) than to the wild-type metallo *A. aeolicus* KDO8PS values.[7] The change in the kinetic parameters for PEP, but not for A5P, is not unexpected since the amino acid modified resides in the PEP binding site and not the A5P binding site.[5]

The metal binding and enzymatic activities of the C11 control mutants of *A. aeolicus* KDO8PS, namely C11G, C11S, and C11K, were as predicted. The C11G mutant enzyme did not bind metal because of the missing key metal ligand and had no activity since it had neither a metal at the active site nor an asparagine at the C11 position. Replacing the sulfur of the cysteine metal ligand with an oxygen[12] in the C11S mutant again resulted in a catalytically inactive KDO8PS that did not bind a metal ion. Since it has been speculated that the γ-nitrogen of the class I N26 may interact with the negatively charged carboxylate moiety of PEP at the active site to adjust the angle of the carboxylate π-electron system with the carbon=carbon double bond π-electron system to facilitate the anti-Michael addition to C-3 of PEP,[13,14] we constructed and tested the C11K mutant for both enzymatic activity and its ability to bind divalent metal ions. While an unlikely metal binding ligand, the ω-nitrogen of the C11K KDO8PS has the potential to be positively charged at the active site and might assist in aligning the orbital angles by interaction with the carboxylate anion as observed for the γ-nitrogen of the N26 of wild-type *E. coli* KDO8PS. The C11K mutant does not bind metal as expected and is inactive.

The data presented herein demonstrate that the mutation of a single conserved amino acid, cysteine, in a metallo KDO8PS to an asparagine found in nonmetallo KDO8PS results in a catalytically functional enzyme. Examination of the crystal structure of wild-type *A. aeolicus* KDO8PS[6] reveals that the four metal binding ligands are positioned solely on one side of the metal ion, whereas the other side of the metal is facing the substrate PEP. This suggests

Table 1. Metal Analysis of KDO8P Synthase

samples		zinc	cadmium	manganese	copper	cobalt	nickel	magnesium	iron	sp. activity
		Molar Equivalent Metal/Enzyme Subunit								Units/mg
A. aeolicus wild-type	as isolated	0.51	—[a]	—	0.02	—	—	0.09	0.06	1.88
	all[b]	0.12	0.36	0.04	0.24	0.04	0.04	0.11	0.08	1.98
	apo[c]	0.02	—	—	—	—	—	0.05	0.02	0.08
A. aeolicus C11N	as isolated	0.04	—	—	0.02	—	—	0.04	—	1.47
	all	0.08	0.03	0.03	0.08	0.04	0.04	0.05	0.06	1.40
	apo	0.02	—	—	0.01	—	—	—	—	1.65
A. aeolicus C11S	as isolated	0.02	—	—	0.02	—	—	—	—	0.11
	all	0.09	0.02	0.02	0.14	0.02	0.03	0.03	0.07	0.04
	apo	0.01	—	—	—	—	—	—	—	0.12
A. aeolicus C11K	as isolated	0.05	—	—	—	—	—	—	—	0.07
	all	0.06	—	—	—	—	—	0.04	—	0.22
	apo	0.02	—	—	—	—	—	—	—	0.15
A. aeolicus C11G	as isolated	0.07	—	—	0.01	—	—	—	—	0.08
	all	0.09	0.02	—	0.01	0.02	—	0.02	0.02	0.09
	apo	0.02	—	—	—	—	—	—	—	0.04
E. coli wild-type	as isolated	0.02	—	—	0.02	—	—	0.04	0.01	12.72
	all	0.06	0.02	0.02	0.35	0.03	0.03	0.08	0.03	0.80
	apo	0.02	—	—	—	—	—	0.07	0.02	12.28
E. coli N26C	as isolated	0.12	—	—	0.10	—	—	0.07	0.04	0.07
	all	0.17	0.13	0.05	0.54	0.04	0.06	0.10	0.12	0.03
	apo	0.02	—	—	—	—	—	0.05	0.03	0.17
	blank[d]	—	—	—	—	—	0.02	—	—	

[a] Value < 0.01. [b] Enzyme as isolated (100 μM) was incubated with a mixture of metal salts (100 μM) in 20 mM Tris buffer (pH 7.5) at 25 °C for 2 h and then applied to a FAST desalting column to remove excess metal salts from the protein−metal complex. The protein fraction was subjected to the discontinuous enzyme assay, protein assay, and metal analysis. [c] EDTA-treated enzyme without metal reactivation. [d] A solution of all the metal salts (100 μM each), without enzyme, was subjected to gel filtration under identical conditions as used for the enzyme-containing samples. The fraction corresponding to the elution volume of protein samples was collected as the background for analysis. Values presented represent metal concentration divided by the average protein concentration determined for all other samples.

that the function of the metal may be to orient the PEP in a manner similar to that discussed for the function of the γ-nitrogen of the nonmetallo N26. In addition to the loss of the chelating sulfur atom, the exchange of an N for the C at position 11 likely destroys the geometry necessary for proper metal binding, and thus the mutant enzyme does not bind metal. Results from in silico mutation, utilizing the Swiss-PdbViewer software, suggest that the functional amide group of N11 fills the hole left from the loss of the metal. The amide's carbonyl oxygen appears to be capable of hydrogen bonding to both E222 and D233, which might help maintain the active site geometry; further, the γ-nitrogen of N11 is positioned to interact with PEP similar to that predicted for the N26 of *E. coli* KDO8PS. In other words, the amide group of the N in the C11N mutant may substitute for the function normally performed by the metal. Regardless of the exact role(s), our results demonstrate that the active site amino acid residue occupying position 11 in *A. aeolicus* KDO8PS plays a major role in catalysis directly by interaction with the PEP moiety or indirectly by furnishing a ligand for metal binding needed for catalytic activity. The inability to convert the nonmetallo KDO8PS to a metal-binding, enzymatically active enzyme suggests that there must be additional secondary changes in enzyme structure between class II and class I KDO8PSs besides the single-site amino acid substitution. Potentially, N26C *E. coli* KDO8PS lacks the proper active site geometry for efficient metal binding as compared to wild-type *A. aeolicus* KDO8PS. These same changes may also account for the increased catalytic efficiency (k_{cat}/K_m) observed in the nonmetallo class I enzymes compared to the metallo class II. Indeed, the k_{cat} value for the nonmetallo C11N *A. aeolicus* KDO8PS lies between that of the two classes. Efforts are underway to obtain the structures of the *A. aeolicus* C11N KDO8PS as well as the *E. coli* N26C KDO8PS in the presence of

substrates to understand the more subtle structural changes. The results of these structural studies should provide insight into the catalytic mechanism of both the metallo and nonmetallo KDO8PS, in particular the role and function of the metal and asparagines.

Supporting Information Available: Experimental details, sequence alignment of KDO8PS, and table of C11N specific activity after incubation with different metals. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Levin, D. H.; Racker, E. *J. Biol. Chem.* **1959**, *234*, 2532−2539.
(2) Ray, P. H. *J. Bacteriol.* **1980**, *141*, 635−644.
(3) Raetz, C. R. *Annu. Rev. Biochem.* **1990**, *59*, 129−170.
(4) Birck, M. R.; Woodard, R. W. *J. Mol. Evol.* **2001**, *52*, 205−214.
(5) Duewel, H. S.; Radaev, S.; Wang, J.; Woodard, R. W.; Gatti, D. L. *J. Biol. Chem.* **2001**, *276*, 8393−8402.
(6) Weiner, M. P.; Costa, G. L.; Schoettlin, W.; Cline, J.; Mathur, E.; Bauer, J. C. *Gene* **1994**, *151*, 119−123.
(7) Salleh, H. M.; Patel, M. A.; Woodard, R. W. *Biochemistry* **1996**, *35*, 8942−8947.
(8) Duewel, H. S.; Woodard, R. W. *J. Biol. Chem.* **2000**, *275*, 22824−22831.
(9) Aminoff, D. *Biochem. J.* **1961**, *81*, 384−392.
(10) Duewel, H. S.; Sheflyan, G. Y.; Woodard, R. W. *Biochem. Biophys. Res. Commun.* **1999**, *263*, 346−351.
(11) Howe, D. L.; Sundaram, A. K.; Wu, J.; Gatti, D. L.; Woodard, R. W. *Biochemistry* **2003**, *42*, 4843−4854.
(12) Park, O. K.; Bauerle, R. *J. Bacteriol.* **1999**, *181*, 1636−1642.
(13) Li, Y.; Evans, J. N. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4612−4616.
(14) Shumilin, I. A.; Bauerle, R.; Kretsinger, R. H. *Biochemistry* **2003**, *42*, 3766−3776.

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