Aldehyde Ferredoxin Oxidoreductase from the Hyperthermophilic Archaebacterium *Pyrococcus furiosus* Contains a Tungsten Oxo–Thiolate Center

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Abstract: X-ray absorption spectroscopy has been used to characterize the tungsten site of the aldehyde ferredoxin oxidoreductase from the hyperthermophilic organism *Pyrococcus furiosus*. The enzyme contains a tungsten oxo–thiolate center, with two W═O at 1.74 Å, approximately three W–S– ligands at 2.41 Å, and (possibly) a W–O or W–N ligand at 2.1 Å. The tungsten site of this enzyme is structurally quite similar to that of molybdenum in the relatively well-known molybdenum oxo–thiolate enzymes.

Introduction

Tungsten and molybdenum possess quite similar chemistries, yet, while molybdenum enzymes catalyze a variety of differenct reactions in diverse organisms,[1] until recently only a single tungsten enzyme had been identified.[2,3] Indeed, tungsten is perhaps best known in its role as a molybdenum antagonist. The apparent disparity between tungsten and molybdenum may be due to the low environmental abundance of tungsten [$<1 \times 10^{-6}$ ppm in seawater[4]], rather than to some inadequacy in a catalytic role. Tungsten, like iron and manganese,[5] may be rather more available in the extreme environments of shallow and deep sea hydrothermal vents,[6] which are inhabited by hyperthermophilic bacteria. These remarkable Archaebacteria grow optimally at temperatures near to, and even above, 100 °C.[7,8] Indeed, tungsten was shown to stimulate the growth of the marine hyperthermophile *Pyrococcus furiosus*,[9] and, recently, a tungsten containing enzyme which catalyzes the conversion of aldehydes to carboxylic acids was purified from this organism.[10] Aldehyde-ferredoxin oxidoreductase is a monomeric protein of approximate molecular weight 85 000 that contains approximately one tungsten, seven iron, and five sulfides. We present herein a tungsten L_{III}-edge EXAFS study of the tungsten site of this enzyme and show that the tungsten possesses an oxo–thiolate coordination, which is analogous to the most common structural type among the molybdenum enzymes.

Pyrococcus furiosus can grow at temperatures up to 105 °C by using a fermentative metabolism. Aldehyde ferredoxin oxidoreductase is thought to be an important component of this metabolic pathway.[11] The electrochemistry and EPR spectroscopy of this enzyme have previously been reported, and it has been suggested that it might contain a tungsten–iron–sulfur cluster, possibly with a WFe_3S_4 core.[10]

Experimental Section

Pyrococcus furiosus aldehyde ferredoxin oxidoreductase was prepared as previously described,[10] at a final tungsten concentration of 0.6 mM, in tris-HCl buffer at pH 7.0. X-ray absorption spectra were recorded at the Stanford Synchrotron Radiation Laboratory, on beam line VI-2, using a Si(111) double crystal monochromator. Eight 30-min scans were averaged, and samples were held at a temperature close to 4 K in an Oxford instruments CF204 liquid helium flow cryostat. XAFS spectra were measured as the fluorescence excitation spectra using a Stern-Heald-Lytle ion chamber detector[12] equipped with a slit assembly and appropriate thickness zinc oxide filters. The X-ray energy was calibrated with respect to the first inflection energy of a tungsten metal foil L_{III} edge, simultaneously recorded with the data, which was assumed to be 10207.0 eV. The EXAFS oscillations $\chi(k)$ were quantitatively analyzed by a curve-fitting procedure[13] to the approximate expression

$$\chi(k) = \sum_{i=1}^{n} \frac{N_i A_i(k,R_i)}{kR^2} \exp(-2R_i/\lambda_i(k,R_i)) \exp(-2\sigma_i^2 k_i^2) \sin\,[2kR_i + \phi(k,R_i)]$$

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Table I. EXAFS Curve-Fitting Results for *P. furiosus* Aldehyde Oxidoreductase[a]

interaction	N	R (Å)	σ^2 (Å^2)
W–O	2	1.74	0.0021
(Mo–O)	(2)	(1.70)	(0.0020)
W–S	3	2.41	0.0018
(Mo–S)	(3)	(2.42)	(0.0033)
W–O/N	1	2.16	0.0010
(Mo–O/N)	(1)	(2.06)	(0.0015)

[a] These values gave the best-integer fit for the coordination number (N), as judged by comparing the sum of the squares of the differences between experimental and calculated curves. The accuracies of the EXAFS analysis are estimated to be ±0.02 Å for the mean interatomic distance (R) and ±20% for N and for the mean-square deviation in R (σ^2). The numbers in parentheses are those obtained for sulfite oxidase.[13]

where k is the photoelectron wave number, N_i is the number of i-type atoms at a mean distance R_i from the absorber atom (in this case tungsten), with a mean square deviation in R_i of σ_i. The summation is over all sets of equivalent atoms, n. The functions $A(k,R)$, $\lambda(k,R)$, and $\phi(k,R)$ are the curved-wave total EXAFS amplitude, photoelectron mean-free path, and total EXAFS phase functions, respectively. These were calculated by using the program ***feff*** of Rehr and co-workers.[14,15]

Results and Discussion

Figure 1 shows the tungsten L_{III}-edge EXAFS of the enzyme, together with the result of the curve-fitting analysis and EXAFS Fourier transforms. The EXAFS Fourier transform shows two intense peaks, at $R \cong 1.8$ and 2.4 Å, which are attributed to short

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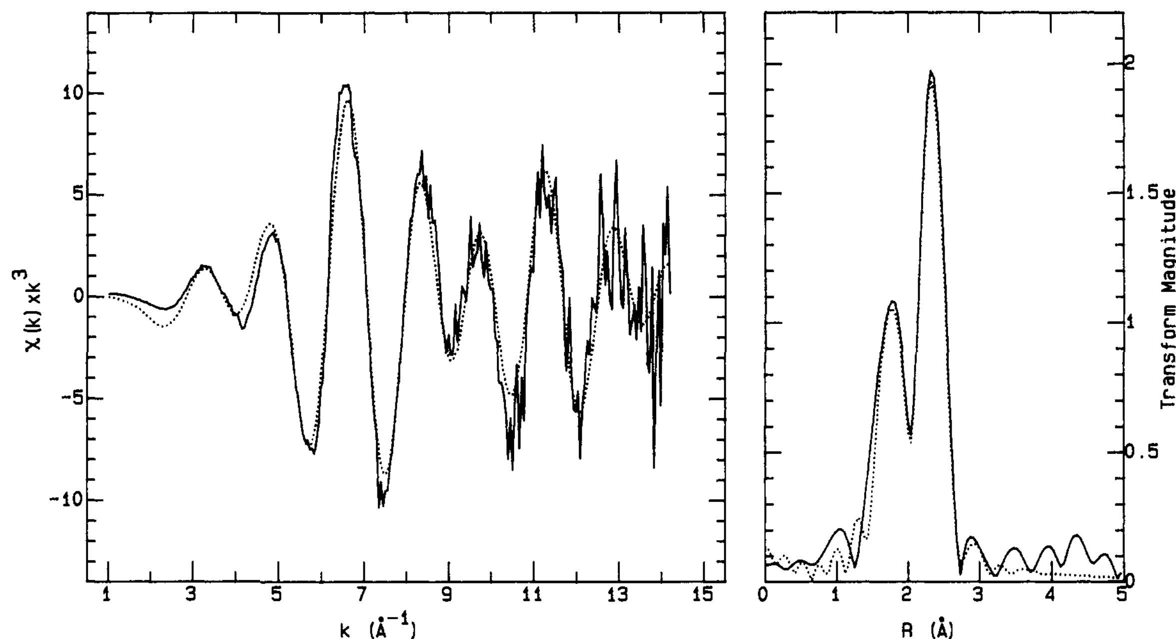

Figure 1. Tungsten L_{III} EXAFS data (solid lines) and curve-fitting result (broken lines) for *P. furiosus* aldehyde oxidoreductase. The left panel shows the EXAFS oscillations, and the right panel the EXAFS Fourier transforms (W–S phase-corrected).

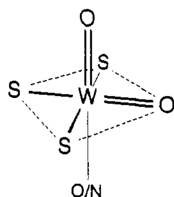

Figure 2. Proposed structure for the tungsten site of aldehyde oxidoreductase. Note that no information is available from the present work upon the geometry of the site; octahedral geometry is assumed, as this is very common for W^{VI}.[16,17]

W–O and to W–S interactions, respectively. No W–Fe interactions were observed, indicating that the previous suggestion[10] that the enzyme contains a W–Fe–S cluster is not correct. A best-integer curve-fitting analysis of the EXAFS data[13] gave the fit shown in Figure 1 and the parameters given in Table I. The data could only be adequately fitted with two short oxygen and 3–4 sulfur ligands. The W–O bond length of 1.74 Å clearly indicates that this is a terminally bound oxygen or oxo ligand (i.e., W=O).[16,17] Furthermore, the presence of two such oxo ligands strongly suggests that the tungsten is present in the formal oxidation state W^{VI}, as no dioxo tungsten compounds are known for any other oxidation state. In agreement with this the W L_{III} X-ray absorption edge spectrum (not illustrated) shows a strong bound state transition, with a peak at approximately 10210.8 ev. Such an intense transition is expected for the L_{III} (and L_{II}) edges of high oxidation state tungsten species, as transitions from the $2p_{3/2}$ to the vacant 5d orbitals are dipole-allowed. The fit was not improved by attempts to include a W=S (at ca. 2.18 Å), and we conclude that no such ligands are present. A slight, but significant, improvement in the fit was found when a long W–O/N interaction was included (Table I). The value for σ^2 for the W=O interaction is a little larger than might be expected on the basis of model compound data and probably indicates a slight inequivalence of the two W=O ligands, which, for W^{VI}, would be expected to be arranged cis to one another[16,17] (Figure 2). The presence of W^{VI} in our samples, which were prepared in the presence of 5 mM sodium dithionite, suggests that the W^{V-VI} midpoint potential probably lies below about −500 mV (vs SHE), which is within the chemically expected range.[16,17] Moreover, it also supports the hypothesis that the tungsten is part of the catalytic site, as the potential for aldehyde to carboxylic acid oxidations is typically about −560 mV (pH 7, 25 °C).[18]

Only one other tungsten enzyme has been characterized; the formate dehydrogenase from *Clostridium thermoaceticum*.[2,3,19,20] Preliminary EXAFS data from reduced samples [possibly a mixture of W^{IV}, W^{V}, and W^{VI} (see ref 20)] of this enzyme have been reported.[19] Although the low signal to noise of the data prevented any detailed analysis (Cramer, S. P., personal communication), they suggest the presence of W–S, but no W=O ligands, at least in the oxidation state(s) investigated. As suggested by Cramer et al.,[19] it is possible that the *C. thermoaceticum* formate dehydrogenase may have a tungsten site analogous to that of the assimilatory molybdenum-containing nitrate reductases, such as the *Escherischia coli* enzyme.[21] EPR signals, which probably originate from W^{V}, have also been reported for the *C. thermoaceticum* formate dehydrogenase.[20]

Dioxo Mo^{VI} is a very common structural motif in molybdenum enzymes,[1] the most thoroughly investigated examples being sulfite oxidase (see ref 13 and references therein) and desulfo xanthine oxidase (see ref 1 and references therein). These enzymes possess molybdenum oxo–thiolate active sites, with two of the thiolate ligands hypothesized to originate from the dithiolene side chain of a pterin cofactor.[1] The EXAFS curve-fitting results for the sulfite oxidase molybdenum site[13] are compared to those for the tungsten site of aldehyde ferredoxin oxidoreductase in Table I. It is apparent that the coordination of the metals in the two enzymes is very similar, which suggests that the tungsten enzyme may well contain a pterin cofactor. Indeed, in recent experiments such a cofactor has been identified; it appears to be similar, although not identical, to the molybdenum pterin cofactor.[11] A tungsten pterin cofactor also seems to be present in the *C. thermoaceticum* formate dehydrogenase.[2,3] This cofactor may also be part of yet another, as yet uncharacterized, tungsten-dependent

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enzyme in this organism, which catalyzes the oxidation of aldehydes to carboxylic acids.[22]

It remains to be established, however, exactly why certain acetogenic eubacteria and hyperthermophilic marine archaebacteria use tungsten rather than molybdenum as an essential catalytic component of specific oxidoreductase enzymes, in apparent contrast to the remainder of the microbe world.

Acknowledgment. We thank Drs. E. I. Stiefel and C. L. Coyle for helpful discussions and the Staff of SSRL for their assistance. SSRL is funded by the DOE (Contract DE-AC03-82ER-13000, Office of Basic Energy Sciences, Division of Chemical Sciences) and NIH (Biotechnology Resource Program, Division of Research Resources). This research was supported in part by a DOE Grant (F609-88ER13901 to M.W.W.A.).

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Structural Effects in the Recognition of DNA by Circular Oligonucleotides

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Abstract: It was recently reported that certain pyrimidine-rich circular DNA oligomers can bind strongly and specifically to purine-rich DNA or RNA strands by forming bimolecular triple helical complexes.[1–3] In this study are investigated the effects of structural variations on the strength of binding for this new class of nucleotide-binding ligand. The number of loop nucleotides (nt) which is optimum for bridging the two binding domains of a circle is examined. Comparing loop sizes of 3, 4, 5, 6, and 10 nt, the optimum number of nucleotides in a loop is found to be five for the sequences studied. In order to test the method of construction and the ability of these compounds to bind sites of varied length, we attempted to synthesize circles of varied size. Circles over the size range 24–46 nt were successfully constructed. Varying the target site length shows that oligomers of four, eight, twelve, and eighteen nucleotides can be complexed strongly by circles, with melting temperatures (T_m) 17° to >33 °C higher at pH 7.0 than the corresponding Watson–Crick duplexes of the same length. Also studied is the effect of the covalently closed circular structure in comparison to linear oligomers having the same sequence; it is shown that a covalently closed circle has considerably higher binding affinity than do three different "nicked" circles (linear oligomers) which contain the same bases. The high binding affinities of these circles are thus attributed to the entropic benefit of preorganization. Finally, the ability of such circles to bind to complementary sites within longer oligomers, the ends of which must pass beyond the loops of a circle, is confirmed by melting studies with synthetic target strands 36 bases in length.

Introduction

A common theme in the field of molecular recognition is the idea that macrocyclic molecules are efficient at recognizing and binding specific substrates. Researchers have become skilled at constructing cyclic synthetic hosts for noncovalent binding of complementary-shaped guest molecules and ions.[4–7] Among the earliest examples of cyclic synthetic hosts were the crown ethers, which chelate metal ions with high affinity and selectivity.[8] Macrocyclic structures can give host molecules strong advantages over their linear analogues. One such advantage is strength of binding, due in large part to the entropic benefit of preorganization.[9] A second advantage is specificity of recognition, which is the result of defined cavity size and shape.[10] Although it is certainly true that some elegant noncyclic structures can possess these properties as well,[11] the use of macrocyclic structure remains an important strategy in this field.

There are also many naturally occurring examples of circular molecules which function as ligands for guest species. For example, the cyclic siderophores act to bind and transport iron,[12] and many other natural ionophores, such as valinomycin[13] and nonactin,[14] are macrocyclic as well. The cyclodextrins, which are circular oligosaccharides, have been studied intensely for their efficient binding of hydrophobic guest molecules inside their central cavity.[15] Several cyclic oligopeptides have also been characterized; examples are the antibiotics echinomycin and triostin A, which bind to specific sequences of duplex DNA.[16] Interestingly, of the three common natural biopolymers—saccharides, peptides, and nucleotides—only the first two have been represented in the known family of cyclic host molecules.

We recently found, however, that cyclic oligonucleotides can also act as efficient hosts for molecular recognition. It was demonstrated that circular DNA oligomers can bind strongly and sequence specifically to single strands of DNA and RNA by forming triple helical complexes.[1,2] Circular oligomers were shown to bind their complements six orders of magnitude more strongly than standard Watson–Crick complementary oligomers at pH 7.0 and are completely resistant to degradation by exonucleases.[1] It was subsequently found that these circular oligomers are less tolerant of mismatches in the target sequence than are standard linear oligomers, and thus show higher sequence selectivity in binding.[2] Because of the similarities in structure, binding strength, and selectivity to the crown family of compounds, these circular ligands were referred to as "crown nucleotides".[1]

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