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Structure and Function of Four Classes of the 4Fe-4S Protein, IspH

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Abstract

IspH, *E*-1-hydroxy-2-methyl-but-2-enyl 4-diphosphate reductase, is an essential enzyme in isoprenoid biosynthesis and an important drug/herbicide target. Using X-ray crystallographic, bioinformatics, mutagenesis/kinetics/stability and EPR results we show that organisms from different environments utilize one of four main IspH classes. The classes are based on the arrangement of the aromatic residues near the 4Fe-4S cluster and the presence or absence of N- and C-terminal extensions. They are: Class A, found primarily in anaerobic/microaerophilic bacteria; Class B, found in aerobic bacteria; Class C, found in cyanobacteria/plants; and Class D, found in apicomplexan parasites. Using mutagenesis, we show that the cluster-associated aromatic groups in Class A and a Class B IspHs enhance cluster oxidative stability. Y198A, F302A and a C-terminus truncation mutant of the Class B (*E. coli*) IspH have lower catalytic activity than wild-type protein when using methyl viologen as electron donor, but higher activity with dithionite as electron donor, due to ready access of the small reductant to the cluster, consistent with their increased oxygen/H₂O₂ sensitivity. F302A has the largest effect on the reaction rates and EPR studies indicate this residue affects Fe-S cluster structure. Similar effects on cluster stability are seen with Class A (F14A and Y98A) mutants, but effects on ET rates are smaller and there are no differences between the EPR spectra of mutant and wild-type proteins. Overall, the results are of general interest since they show, for the first time, that there are multiple IspH classes that have evolved to enable organisms to survive in diverse oxidative-stress environments.

Terpenes constitute the largest family of small molecule natural products and are biosynthetically derived from two universal precursors: dimethylallyl diphosphate (DMAPP, **1**, Chart 1) and isopentenyl diphosphate (IPP, **2**, Chart 1).¹ In most bacteria as well as in plants and apicomplexan parasites, these two C₅ building blocks are produced *via* the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway with both **1** and **2** being synthesized by the 2H⁺/2e⁻ reductive dehydroxylation of *E*-1-hydroxy-2-methyl-but-2-enyl 4-diphosphate (HMBPP, **3**, Chart 1).^{2, 3} This reaction is catalyzed by the 4Fe-4S cluster-containing enzyme IspH (also known as LytB—but not to be confused with the LytB found in *Streptococcus pneumoniae*) and due to the essential nature of the MEP pathway for survival—and its

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Notes

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SUPPORTING INFORMATION

Supporting Information available: Multiple Sequence Alignment of the NCD region of IspH, stability and kinetic results with EcIspHs and AaIspHs, correlations between kinetic results; *g* values of the EPR spectra.

absence in humans—IspH is thought to be an attractive drug target for infectious diseases such as malaria and tuberculosis,^{4, 5} as well as a target for herbicide development. In addition, HMBPP itself is a very potent "phosphoantigen"⁶ targeting $\gamma\delta$ T cells (containing the V γ 2V δ 2 T cell receptor) and activated $\gamma\delta$ T cells kill tumor cells, so IspH inhibitors also have the potential to be novel cancer immunotherapeutics.

IspH is a 4Fe-4S cluster-containing protein in which the cluster is bound (by Fe-S bonds) to 3 Cys residues (C13, C96, C197 in *E. coli*), the unique 4th Fe being directly involved in substrate binding and catalysis, and there have been numerous studies of its mechanism of action,⁷ based on which some potent inhibitors have been developed.^{7, 8} Both experimental and computational studies now generally agree on a reaction mechanism involving two consecutive one-electron transfer steps with a HIPIP (high-potential iron protein)-like [Fe₄S₄]³⁺-allyl anion intermediate.^{7, 9-12} The X-ray structures of IspH from *E. coli*¹³, *Aquifex aeolicus*¹⁴ as well as an N-terminal truncation mutant of *Plasmodium falciparum*¹⁵ have been obtained and show a similar "trefoil" arrangement of α/β domains with the Fe-S cluster bound at the center of the structure. However, both *E. coli* IspH (EcIspH) and *P. falciparum* IspH (PfIspH) also contain a C-terminal extension that is missing in *A. aeolicus* IspH (AaIspH), raising the question as to the role of this domain. This C-terminal extension is quite long (~25 residues) and appears as a "belt" across the proposed electron transfer (ET) surface of the protein, as discussed more below.

It has also been reported that PfIspH can form a relatively stable complex with *P. falciparum* ferredoxin (PfFd),¹⁶ and can readily convert HMBPP into DMAPP and IPP using reduced PfFd as the electron donor, while AaIspH does not use ferredoxin as efficiently.¹⁶ The binding between PfIspH and PfFd was then suggested, based on the results of a computational docking investigation, to be mediated by the C-terminal tail which was involved in several specific polar interactions that resulted in the 2Fe-2S cluster in PfFd being ~13 Å from the 4Fe-4S cluster.¹⁵ EcIspH was also able to utilize ferredoxin, as well as flavodoxin, as an electron shuttle partner.³ These studies suggest that the IspH ET partner binds to one surface of IspH, the surface that contains the belt residues. In other studies, AaIspH—which is tail-less—was shown to be catalytically active with dithionite, an inorganic reducing agent, but the reaction of EcIspH with dithionite was extremely slow.³ In both cases, the reactions were accelerated in the presence of the electron mediator methyl viologen, but the origins of these differences in catalytic activity have been unclear.

In our group, we recently discovered that some IspHs from anaerobes form "Rosetta stone" hybrids with other proteins¹⁷ and the IspH domains in these and many other obligate anaerobic bacteria lack the C-terminal tail region seen in EcIspH, suggesting that the presence of this region might be related to oxygen tolerance and indeed, IspH is one of the few essential proteins in *E. coli* that are air-sensitive.¹⁸ Here, we sought to investigate the structure and function of a broad range of IspHs—proteins from anaerobic and aerobic bacteria, plants as well as parasitic protozoa—in order to see how different organisms might protect their clusters from oxidative damage, in addition to how residues on the ET side (as opposed to the substrate-binding side) of the protein might affect catalytic activity.

EXPERIMENTAL PROCEDURES

General

All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) unless otherwise indicated. Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Restriction enzymes were from New England Biolabs (Beverly, MA). DNA purification kits were from Qiagen (Valencia, CA). LC-MS analyses were performed at the Metabolomics Center at the University of Illinois, Urbana-Champaign.

Purification of EcIspHs

E. coli IspH and truncation mutant Ec291 were cloned from *E. coli* strain K-12 into the pET-28a (+) (Novagen, WI) vector. EcF302A and EcY198A mutants were generated with a QuickChange site-directed mutagenesis kit (Agilent, CA). Sequence-verified plasmids containing the corresponding IspHs were co-transformed into *E. coli* BL21 (DE3) (Novagen, WI) together with an *isc* expression system-encoding plasmid (for 4Fe-4S cluster incorporation). For protein production, recombinant *E. coli* BL21 (DE3) cells were grown in 3 liters of LB media at 37 °C. Cells were induced by adding 100 mg/L cysteine, 1 g/L arabinose and 0.1 mM FeCl₃ at an OD₆₀₀=0.5 for *isc* expression, followed by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD₆₀₀=0.8, for IspH expression. Expression was continued at 25 °C for 24 hrs. Cells were then harvested and frozen at -80 °C. Purification of wild-type and mutant IspHs was performed at 4 °C in an anaerobic chamber (Coy Lab, Grass Lake, MI) with an O₂ level < 1 ppm. Briefly, cell pellets were suspended in 30 mL of pH 8.0, 150 mM NaCl, 50 mM Tris/HCl buffer containing benzonase, protease inhibitor, and 2 mg/mL lysozyme. The suspension was sonicated, then centrifuged to remove cell debris. The clear cell lysate was loaded onto a gravity column containing 3 mL of Hispur™ Ni-NTA resin (Thermo Fisher Scientific, NY), followed by washing with 50 mL of pH 8.0, 150 mM NaCl, 50 mM Tris/HCl, 35 mM imidazole buffer. His-tagged IspH was eluted using 20 mL of pH 8.0, 150 mM NaCl, 50mM Tris/HCl, 250 mM imidazole buffer. The elute was then concentrated and desalted by using a PD-10 column (Agilent, CA).

Purification and Reconstitution of AaIspH

AaIspH mutants AaF14A and AaY98A were cloned from an AaIspH-containing pQE60 plasmid (modified to harbor a Tet induction system)² with the QuickChange site-directed mutagenesis kit. Recombinant *E. coli* BL21(DE3) cells were grown in 2 liters of LB media and induced with 200 μM anhydrotetracycline at an OD₆₀₀=0.8. Expression was continued at 25 °C for 24 hrs. Cells were then harvested and frozen at -80 °C. Purification of AaIspHs were the same as for the EcIspHs. However, since Fe₄S₄ cluster incorporation into AaIspHs was less efficient than with EcIspH, we used the cluster reconstitution steps for AaIspHs that we described previously.¹⁷

Enzyme Kinetics

All kinetic assays were performed in an anaerobic chamber with O₂ levels < 1 ppm. For enzyme kinetics assays using the methyl viologen radical cation as the reducing agent, 1 mL

reaction mixture containing 50 nM IspH, 80 μ M sodium dithionite, 1 mM methyl viologen and HMBPP (0-70 μ M) was incubated at 26 °C. The absorbance of the reaction mixture at 606 nm was monitored over a period of 2 min in order to follow the disappearance of the methyl viologen radical cation. K_m and k_{cat} were derived from the Michaelis–Menten equation by using OriginPro 2015 software (OriginLab, Northampton, MA). For enzyme kinetics assays using dithionite as the reducing agent, 100 μ L of reaction mixtures containing 5 μ M EcIspH or 3 μ M AaIspH, 0.75 mM HMBPP and 3 mM dithionite were incubated at room temperature and quenched at 3, 10 or 30 min by adding 100 μ L acetonitrile. The mixtures were centrifuged to remove any precipitates and then analyzed by LC-MS. HPLC conditions were as follows: flow rate, 250 μ L/min; gradient, 25 mM NH_4HCO_3 , 20 mM N, N-dimethylhexylamine (DMHA, ion-pairing agent), pH=7.5 to 80% MeOH, 2 mM DMHA.

Electron Transfer Rate

Electron transfer rates between the IspHs and dithionite were measured by using the stopped-flow technique with an Applied Photophysics (Beverly, MA) model SX20 spectrometer. A final concentration of 40 μ M IspH and dithionite at 0.5 mM, 1 mM, 2 mM or 3 mM were mixed in a 20 μ L chamber. The reaction was monitored over a period of 5 min for EcIspHs or 10 s for AaIspHs. The ET reaction rate, as indicated by the disappearance of absorption at 415 nm for the $[\text{Fe}_4\text{S}_4]^{2+}$ cluster, was fitted by using first-order kinetics, and the pseudo-first-order rate constants were plotted against $[\text{dithionite}]^{0.5}$ to obtain the second order rate constants. The equilibrium constant for dissociation of $\text{S}_2\text{O}_4^{2-}$ (K_1) was taken to be 1.4×10^{-9} M.¹⁹

Iron Content Measurement

The iron contents of all IspHs were measured by using a phenanthroline colorimetric method as described elsewhere.²⁰ The proteins were desalted to remove any adventitious iron present, prior to analysis.

IspH Stability

200 μ L solutions (50 mM Tris/HCl, 150 mM NaCl, pH=7.5) containing 40 μ M IspH, with or without 400 μ M of ligand (**3** or **4**)¹⁷ were incubated in air at room temperature (22 °C). The absorbance of the 4Fe-4S cluster at 415 nm was monitored over 10 hrs. A similar set of experiments were also performed in the presence of 100 μ M H_2O_2 . In addition, we used a qualitative plate assay in order to illustrate the loss of the brown color associated with cluster degradation.

Electron Paramagnetic Resonance

All samples were prepared in an anaerobic chamber with an O_2 level <1 ppm. 20% glycerol was added to all samples as a glassing agent. Mixtures of 0.3 mM IspH (mutant or wild-type), 6 mM dithionite and in some cases, 3 mM **4**, were incubated for 15 min and then frozen in liquid nitrogen. CW-EPR experiments were performed at X-band (9 GHz) using a Varian E-122 spectrometer with an Air Products (Allentown, PA) liquid helium cryostat.

Typical parameters for EPR experiments were as follows: temperature, 10 K; power, 1 mW; modulation amplitude, 0.5 mT; scan field: 335±80 mT.

Sequence Similarity Network

The sequence similarity network for the IspH family proteins was that we generated previously,¹⁷ and is colorized here to indicate the Class A, B, C and D IspHs.

RESULTS AND DISCUSSION

IspH Active Site Structures

The X-ray crystallographic structures of three IspHs have been reported.¹³⁻¹⁵ In early work, we reported the structure of IspH from the thermophile *Aquifex aeolicus*.¹⁴ Then, the structure of the *E. coli* protein was reported,¹³ followed later¹⁵ by that of the *Plasmodium falciparum* protein (minus its N-terminus). None of these three proteins crystallized with intact 4Fe-4S clusters, although these are seen in ligand-bound *E. coli* structures. Three views of the reported structures of each protein are shown in Figure 1A-C. In each case there is the basic "trefoil" arrangement of three α/β domains that surround a central Fe-S core. In functional IspHs the (oxidized) cluster is $[\text{Fe}_4\text{S}_4]^{2+}$ which is bound to 3 conserved Cys: C13, C96 and C197 (we use the *E. coli* numbering system since most structures have been of this protein). The 4th, unique Fe is involved in ligand binding, in catalysis. The results of nuclear resonance vibrational spectroscopy (NRVS) indicate that 3 waters bind to this Fe in the oxidized protein.²¹ This oxidized form of IspH is relatively sensitive to oxidants, however, losing one Fe, but is stabilized by ligands such as HMBPP, as well as by some inhibitors. The question then arises as to how IspHs might in general stabilize their 4Fe-4S cluster. And how might IspHs from different organisms, subjected to different oxidative stresses, effect cluster stabilization? In addition: are protein residues on the ET side of the protein directly involved in catalysis?

As can be seen by comparing the *A. aeolicus* IspH structure (Figure 1A; PDB ID 3DNF)¹⁴ with that of *E. coli* IspH (Figure 1B; PDB ID 3KE8)¹³ and *P. falciparum* IspH (Figure 1C; PDB ID 4N7B),¹⁵ both EcIspH and PfIspH contain a long (~25 residue) C-terminal extension. This C-terminal tail, highlighted in green, is above the trefoil plane, on the opposite side to the HMBPP substrate-binding pocket, shown in pink in the EcIspH-HMBPP structure (Figure 1B), and is also present in the PfIspH structure (Figure 1C). In space-filling models the C-terminal extension clearly appears as a belt or strap across one face of the protein, above the first and third α/β domains. This location suggests that the C-terminal tail is not involved in substrate binding but rather, as suggested by Rekittke et al.,¹⁵ is involved in electron transfer and perhaps, in protecting the cluster from oxygen or other reactive oxygen species (ROS) and potentially, reactive nitrogen species (RNS). In AaIspH (Figure 1A), the Fe-S cluster is relatively solvent exposed. Expanded views of the cluster and belt region of both EcIspH-HMBPP and PfIspH are shown in Figure 1D and 1E and gives clues as to the role of some of the residues in this domain. In EcIspH (Figure 1D), there are two salt bridges that link the belt with the trefoil plane: R296 forms a salt bridge with E205, and E298 with R203. In PfIspH, only the latter interaction is present (Figure 1E). Note that the

Fe-S cluster in EcIspH is [4Fe-4S] and that the HMBPP substrate is bound, while in PflIspH the cluster is [3Fe-4S], due to loss of one Fe during crystallization.

What is particularly interesting is that in both structures, there are two aromatic amino-acid residues that are very close to the more solvent-exposed side of the cluster—the proposed ET surface. In EcIspH (PDB ID 3KE8),¹³ Y198 has 3.8 Å (Cε2-S4), 4.4 Å (OH-S4) contacts with (or, more accurately, distances to) the cluster, while F302 has two longer distances, both at 5 Å (Cε1-S4 and Cζ-S4). The central "core" of all IspHs, as noted previously, has three conserved Cys, and Y198 is adjacent the third Cys, C196. So one of the aromatic residues is in the trefoil core IspH structure (as found in e.g. AaIspH) while the second is in the C-terminus extension. In the PflIspH structure (Figure 1E), Y414 (corresponding to Y198 in EcIspH since PflIspH has an N-terminal plastid-targeting domain) has a 3.8 Å (Cε1-S3) distance to the cluster (PDB ID 4N7B)¹⁵ while W520 (in the C-terminus extension) has a 4.4 Å (Cη2-S3) contact distance. While these distances are long compared to bond distances, they obviously show that in both EcIspH as well as in PflIspH, two aromatic amino-acids are close to the clusters, suggesting that in both cases they could be involved in electron transfer.¹⁵ In addition, these aromatic groups could provide steric protection against oxygen or other, reactive oxygen/nitrogen species. In the case of AaIspH, the C-terminus extension is absent but there are still two aromatic amino-acids close to the cluster, F14 and Y98, with cluster distances in the 4.4-4.6 Å range (PDB ID 3DNF).¹⁴ A superposition of the aromatic groups in all three IspHs near the ET side of the cluster is shown in Figure 1F. In each of the three proteins there are, therefore, two aromatic amino-acids that have cluster distances of, on average, ~4.3 Å. We hypothesize that these residues and structural arrangements observed might represent distinct motifs in different organisms, tailored perhaps to the oxygen levels (or ROS/RNS) in the organism's environment—as well as being involved in ET.

There are 4 Major IspH Structural Classes

We next sought to see how the general structural observations noted above might be extended and perhaps generalized to different organisms, to determine if there are clearly-defined IspH structural classes. To do this we first carried out a sequence alignment²² of a broad range of bacterial, plant and apicomplexan IspHs, Figures 2, S1.

The conserved IspH C-terminal tail is mainly found in aerobic bacteria and in protozoan parasites, with aerobic bacteria containing a conserved F302 and the protozoan parasites, a corresponding W302 (using the EcIspH numbering system). When 1389 IspHs (all IspHs, with <80% homology, from the database) were aligned, 617 (44%) were found to contain the C-terminal domain with conserved Phe or Trp. C-terminal tail-less IspHs were almost all from anaerobic/microaerophilic bacteria and photosynthetic organisms—cyanobacteria and green plants (Figure 2). However, instead of the C-terminal extension, the IspHs of cyanobacteria and green plants have large N-terminal extensions²³. In plants, there is also a plastid targeting sequence (that is not required for activity and is absent in the cyanobacteria). Moreover, there is a highly conserved residue, Y72* (we use the *Arabidopsis thaliana* numbering system here to be consistent with the literature, the * indicating a different numbering system to that used for the bacteria) in the N-terminal domain, Figure

S1, that is essential for catalytic activity. This Tyr has been proposed to be involved, in chloroplasts, in protecting IspH from high oxidative stress.²³ However, since only a computational model of a plant IspH is available,²⁴ it is not possible to know whether or not this Tyr residue is close to the 4Fe-4S cluster. Nevertheless, when all of the above structural and bioinformatics data is considered, it leads to the conclusion that there are actually four major classes of IspH that we shall call Classes A-D. A graphical representation of this proposal is shown in Figure 3A.

The simplest structural organization we shall call Class A and is that found in e.g. *A. aeolicus* in which there is just the basic trefoil "core" IspH structure reported earlier¹⁴ — with no N or C-terminal extensions—and the aromatic amino acids are F14, Y98, adjacent the 1st and 2nd conserved Cys, Figure 2. Class B IspHs are characterized by addition of the conserved C-terminal domain (CCD), Figure 3, and the aromatics are the core Y198 (adjacent the 3rd conserved Cys) (Figure 2), and Y302, in the CCD domain. Class C IspHs are those found in plants and cyanobacteria. They adopt the Class A core structure, but lack the CCD, and have instead a conserved N-terminal domain (NCD)²³ with a highly conserved Y72*. These IspHs are expected to be under great oxidative stress (during photosynthesis) and the proposal that the NCD contributes to oxidative stability is an attractive one. Class D IspHs are those found in apicomplexan parasites such as *P. falciparum*, a malaria parasite, *Toxoplasma gondii*, the causative agent of toxoplasmosis, and *Babesia bovis*, which causes babesiosis. Class D IspHs have the basic Class B structure, Y/W198 together with a CCD (F302 or W302), plus, there is also an NCD, just as found in plants, in addition to a plastid (apicoplast) targeting domain. As can be seen in the structural alignment in Figure S1, the highly conserved Y72* seen in plants is also present (as Y or W) in the apicomplexan parasites, which are also expected to be under oxidative stress (ROS/RNS) when inside mammalian host cells.

In earlier work¹⁷ we reported using a Cytoscape analysis²⁵ that there were ~9 different IspH clusters, based purely on amino-acid sequences. However, essentially all (96%) of these IspHs are now seen to belong to one of the four "superclasses" discussed above, as shown in Figure 3B (gold=Class A; blue=Class B; green=Class C and red=Class D). The exception is the grey cluster iii (4%) comprised of firmicutes, mostly *Bacillus* and *Geobacillus* spp. which have just 1 aromatic residue (Y14) near the cluster, and a CCD without aromatic residues.

The results described above indicate, then, that there are 4 major classes of IspH that can be distinguished based on the presence of aromatic amino-acid side-chains near the [4Fe-4S] cluster; the presence or absence of a C-terminal extension (containing an aromatic amino-acid near the cluster); and/or an N-terminal extension (with a highly conserved Y72*). We thus next sought to see how mutations of some of the putative key residues discussed above, known to be located near the 4Fe-4S clusters, might affect catalytic activity, as well as oxidative stability, since it appeared that these Phe/Tyr/Trp residues could be involved in ET activity (they are located near the cluster, on the ET side of the protein) and in their absence, the clusters appeared to be relatively solvent exposed. Since, to date, there are no structures of complete Class C or Class D proteins (i.e. with the C/N-terminal domains), we focused on the Class A and B proteins, and in Class B, the C-terminal chain extension, the CCD. So,

only the NCD was not investigated, and indeed studies of the complete NCD in plants will be challenging since the intact proteins are expected to target plastid membranes and to date, none have been successfully expressed and isolated—yet alone crystallized—although the cyanobacterial IspHs may be more tractable.

IspH Tail and Aromatic Residues Affect IspH Catalytic Activity

We first investigated the cluster-associated residues in EcIspH since EcIspH is the only IspH that has known structures containing intact 4Fe-4S clusters, and ligands. We made three mutants: a "truncation mutant" Ec291 containing only residues 1-291, i.e. without the C-terminal tail (residues 292-316); EcF302A, with the mutation in the CCD, and EcY198A, in which the mutation is near the third conserved Cys that binds to the 4Fe-4S cluster. All EcIspHs were produced with the 4Fe-4S cluster bound by co-expressing IspH with an *isc* expression system (for cluster insertion), and had a dark brown coloration and an Fe content that was very similar to that we find with wild-type IspH, Table 1. We then determined the kinetic behavior of the three mutants by using a methyl viologen-based assay², Figure 4. In this assay, Figure 4A, HMBPP reduction is determined spectrophotometrically *via* the oxidation of the methyl viologen radical. k_{cat} and K_{m} values derived from Figure 4A are given in Table 1. The activity of Ec291 was significantly diminished as compared to wild-type IspH, with only ~7% activity found. EcF302A also exhibited low activity, ~30% that of the wild-type enzyme, but EcY198A was just as active as wild-type. The loss of activity was mainly due to a significant decrease in k_{cat} , while K_{m} changed by less than a factor of two. This K_{m} observation was not unexpected since none of the mutations were close to the substrate binding site, Figure 1. We then investigated the HMBPP reduction kinetics of these three mutants using this time, dithionite as the reducing agent and an HPLC-based end-point assay to measure the time course of the reaction, Figure 4B. The initial conversion rates, k_{DT} , derived from Figure 4B, are shown in Table 1. In contrast to the results obtained in Figure 4A, Ec291 exhibited a much faster (~10-fold) k_{DT} than did wild-type IspH. Moreover, EcF302A was ~5-fold more active than was wild-type, and EcY198A had about the same activity as wild-type. These results, while surprising, are consistent with the report that "tail-less" AaIspH converts HMBPP using dithionite at a faster rate than does EcIspH.³ And since the mutants did not change the K_{m} of IspH significantly, the changes in catalytic activity seemed likely to originate from differences in the ET rates. We thus next investigated the ET rates using a stopped-flow technique, and correlated the results with other kinetic properties.

Dithionite uses the $[\text{SO}_2^-]$ radical as the (one-electron) reductant with the following mechanism and kinetics:¹⁹

The pseudo-first order rate constants, k_{obs} , from the reactions between IspH and excess dithionite were measured by monitoring the decrease of the absorbance of the oxidized

4Fe-4S cluster at 415 nm. The second-order kinetic constants, k_2 , were then determined by plotting k_{obs} against the square root of the dithionite concentration and dividing the slope of this plot by the square root of the dissociation constant of dithionite, K_1 . As shown in Figure 4C and Table 1, the reaction rate between Ec291 and dithionite was $>5\times$ faster than with wild-type IspH, and the ET rate for the EcF302A mutant was $3\times$ faster than wild-type, with EcY198A again having about the same reaction rate as with wild-type protein. These results indicate that removing the tail region of EcIspH increases the ET rate between the enzyme and dithionite. Moreover, these k_2 ET rate results correlate (Figure S2A) with the catalytic activity results k_{DT} . Figure 4B, using dithionite as the reducing agent, indicating that the differences in the ET rates are the basis for the correlation observed. The observed ET rates (k_{obs}) were, however, $\sim 3\times$ smaller than the overall reaction rates (k_{DT}) at the same dithionite concentration, Figure 4B, C and Table 1. This observation is surprising, but a possible explanation is that in the ET experiments (k_{obs}), the Fe_4S_4 cluster is bound to three waters²¹ but in actual catalysis (k_{DT}), the Fe_4S_4 cluster is bound to the substrate, which can stabilize the cluster. The ΔG for the ET reaction may thus be lower in the latter case, resulting in an increased rate of the ET reaction.²⁶

There is also an interesting anti-correlation between k_2 and k_{cat} using methyl viologen, Figure S2B. The fact that tail-less EcIspH was reduced more rapidly by dithionite than was wild-type IspH, but more slowly than wild-type with methyl viologen, is again surprising. However, a likely explanation is that since dithionite (or $[\text{SO}_2^-]$) is a very small species, it can very readily react with the cluster when the IspH tail region of IspH is removed and the Fe-S cluster is solvent exposed. The methyl viologen radical cation is, however, much larger and more hydrophobic than $[\text{SO}_2^-]$, and ET may require an ET mediator on the IspH tail—one of the aromatic amino-acids—to facilitate binding and electron transfer. Such a process would be similar to the reaction that occurs inside cells, where the C-terminal tail has been proposed to be critical for binding (or reaction) between ferredoxin and PflIspH.¹⁵ Such an exposure of the cluster would also be expected to affect cluster stability, as discussed below.

The IspH Tail and Aromatic Residues Affect IspH Stability Towards Oxygen/Reactive Oxygen Species

Since the tail-less EcIspH reacted with dithionite more rapidly than did the wild-type protein, it seemed likely that other small molecules, such as oxygen and other reactive oxygen (or nitrogen) species would also have ready access to the cluster, resulting in cluster degradation. That is, the presence of the CCD or other cluster-associated aromatics might be expected to enhance IspH stability, under oxidizing conditions. We therefore tested the stability of wild-type and mutant IspHs upon exposure to air. All wild-type as well as IspH mutants were stable for months inside a glovebox without significant loss of Fe-S cluster content. However, upon exposure to air, mutant stability decreased, Figures 5 and S3. Without any "exogenous" cluster ligand present (endogenous water is assumed), the cluster in Ec291 degraded very rapidly and there was some protein precipitation, while wild-type and the two EcIspH point mutants reacted at similar rates (Figure 5A). In the presence of the HMBPP (**3**) substrate, however, the stability of the wild-type enzyme increased and a significant amount of $[\text{4Fe-4S}]$ cluster was still present even after a 12 hr exposure to air (Figures 5B, C). The EcY198A and EcF302A mutants had intermediate stability while

Ec291 degraded just as rapidly as with the unliganded protein. The stability of the different IspHs correlated with their reactivity with dithionite, Figure S2C, as anticipated. We then carried out a second set of experiments with a second ligand **4** that we previously found enhanced cluster stability, as well as with **3** and **4** in the presence of 100 μ M H₂O₂, Figure 5C and Figure S3. Similar results were obtained in the H₂O₂ exposure experiments to those obtained with oxygen exposure. Thus, addition of **3** or **4** increases cluster stability and this can be attributed to these ligands binding to the unique, 4th Fe in the cluster, preventing attack—from the substrate side—by ROS. However, oxygen and ROS can still attack from the "top" or ET side of the cluster, and here we see a difference in stability between the mutants with stability increasing from Ec291 to EcF302A to EcY198A to wild-type, with F302 playing the major role in cluster protection, Figures 5B, C.

These results and those of our EPR studies (discussed more below) indicate that the HMBPP (**3**) and **4** ligands can bind to Ec291 just as they do to wild-type IspH. However, the Fe-S cluster in Ec291 is more susceptible to attack by oxygen because the cluster is more solvent exposed, while in wild-type IspH, the cluster is protected by the C-terminal tail as well as by Y198. As noted above, all apicomplexan parasites have a conserved Trp instead of a Phe in their CCD tail region due, we propose, to the fact that these parasites reside inside mammalian cell where oxidative stress from ROS (and RNS) is likely to be very high, and the larger Trp may provide an increased steric barrier against attack by these species. Taken together with the kinetic results, it thus appears that the CCD and aromatic residues near the 4Fe-4S cluster (both F302 and Y198) significantly affect cluster stability, and in the case of F302, catalytic activity. The question then arises as to whether such aromatic residues affect activity and stability of the naturally "tail-less" Class A IspHs.

Effects of Cluster Associated Aromatic Residues in AaIspH

AaIspH, from *Aquifex aeolicus*, contains F14 and Y98 near the 4Fe-4S cluster, Figure 1, so we prepared AaF14A and Y98A mutants. The catalytic activity of AaIspH and its mutants were measured as described above, with methyl viologen radical or dithionite as the reducing agents. As shown in Table 1 and Figure S4, the mutations have similar effects in both dithionite and methyl viologen assays: AaY98A ~2.2x and AaF14A ~1.6x faster than wild-type. Both sets of kinetics results correlate well with the ET rates between the AaIspHs and dithionite, Figures S5A, B, in which we plot k_{cat} or k_{DT} for AaIspH catalysis against the ET rate constant (k_2) of the AaIspHs. The small increase in reactivity of AaY98A and AaF14A with dithionite can be explained by a steric effect, as we proposed for EcIspHs. Notably, with wild-type AaIspH, the conversion rate of the enzyme using the methyl viologen radical as the reducing agent was only ~3x larger than when using dithionite, Table 1, while with wild-type EcIspH, methyl viologen was >100x more efficient than was dithionite, and with Ec291, methyl viologen was less efficient than dithionite: these results again indicate a critical role for the C-terminal domain in EcIspH in transferring electrons between the methyl viologen radical and the Fe-S cluster.

In contrast to these relatively small effects on catalytic activity, F14 and Y98 in AaIspH have major effects on cluster stability with respect to oxygen/ROS. In the absence of any exogenous ligand, AaIspH and the two mutants decomposed in air at similar rates, Figure

S6A, rates that were larger than with the *E. coli* homologs (which contain the tail). The presence of HMBPP (**3**) improved the stability of all AalSpHs, Figure S6B, and the decay rates were more similar to those of EcSpH and were also well-correlated with the ET rates measure with dithionite, Figure S5C. The effect of nearby aromatic residues on Fe-S cluster stability have also been observed in other 4Fe-4S systems. In a HIPIP ferredoxin,²⁷ a Tyr residue in close proximity to the 4Fe-4S cluster was demonstrated to stabilize the cluster upon exposure to air, and the redox potential of the 4Fe-4S cluster was not affected by mutating the Tyr. Instead, steric effects were proposed to be the main reason for the differences in stability, as we propose here for SpH.

Interestingly, the 4Fe-4S cluster protein IspG (GcpE) is even less stable to oxidants than is SpH.^{28, 29} This protein functions as a dimer and each monomer has 2 domains: a 4Fe-4S domain that binds to O₃ in the 2-*C*-methyl-D-erythritol-2,4-*cyclo*-diphosphate (MEcPP) substrate, and a TIM barrel domain that binds to the MEcPP diphosphate. The structure exhibits a hinge-bend motion and in the open form (that is, in the absence of substrate/intermediate/product/inhibitor ligands) the cluster is rapidly degraded by oxygen, and in cells by methyl viologen which generates ROS,³⁰ this instability arising we propose from the lack of any nearby aromatics in the 4Fe-4S cluster, unlike the situation with SpH.

Aromatic Residues Affect the [Electronic] Structure of the [4Fe-4S] Cluster

In order to investigate possible interactions between the [4Fe-4S] cluster and ET-side residues in SpH, we obtained EPR spectra of each of the SpHs. It is important to note that, to date, there have been no reported SpH X-ray structures containing intact 4Fe-4S clusters, oxidized or reduced, in the absence of exogenous ligands, although NRVS²¹ indicates 3 waters bind to the oxidized protein—consistent with previous Mössbauer results.³¹ As shown in Figure 6A and Table S1, reduced wild-type EcSpH exhibits an axial (9 GHz) EPR spectrum with *g* values of [2.025, 1.899, 1.876] and a *g* of 0.149. In the case of Ec291 in which F302 (and other) residues are absent, the spectrum is rhombic, with *g* values of [2.007, 1.907, 1.797] and a *g* of 0.21. The rhombic spectrum has a line shape that is similar to that of a [2Fe-2S] cluster,³² although the possibility that the signal actually arises from such a species seems remote given that all spectra were taken at 10 K; the mutants had similar iron contents as did wild-type, and the acetylene inhibitor **4** bound to all four reduced SpHs. The Ec291 EPR spectrum shows that at least one residue post-291, in the tail, must be responsible for the differences between the spectra of wild-type SpH and the Ec291 mutant, but does not indicate which residue/residues are responsible. However, since the spectrum of reduced EcF302A is also different to wild-type and is very similar to that of Ec291, the spectroscopic differences between wild-type EcSpH, Ec291 and EcF302A must be due, at least in part, to the presence of F302 in the wild-type protein (otherwise the wild-type and EcF302A spectra would be the same). The spectrum of reduced EcY198A is also very similar to that of EcF302A and Ec291, but not wild-type, meaning that both of the aromatic amino-acid residues that are close to the [4Fe-4S] cluster can have similar effects on the structure of the Fe₄S₄ clusters that result in the spectral changes observed. After binding to compound **4**, however, the spectra of all four systems are essentially the same and are characteristic of typical π -ligated 4Fe-4S clusters, Figure 6B, reported previously.³³ Whether the spectral changes that are seen in the ligand-free proteins are due to a direct

cluster interaction with the aromatic rings or a more indirect, protein-mediated effect, or changes in water ligation, is not known, but in either case, it is of interest that there are in fact aromatic ring effects on cluster structure, with both aromatic residues stabilizing the EcIspH cluster from ROS.

In the case of AaIspH, the effects of mutating the aromatic residues to AaF14A and AaY98 were quite small, Figure 6C, Table S1. In the absence of any exogenous ligand, lineshapes and g -values were very similar with g values of [2.022, 1.903, 1.842; AaIspH], [2.023, 1.904, 1.856; AaF14A] and [2.021, 1.898, 1.849; AaY98A], Figure 6C. The only differences were very slight changes in g_3 and a line broadening of g_3 that gave AaY98A a more axial lineshape. The broadening was more obvious in the ligand-bound spectra, Figure 6D, leading to a slightly different lineshape for AaF14A and AaY98A, although their g values were identical to wild-type, [2.097, 2.014, 1.997]. The line-broadening is likely caused by a larger g -strain, due perhaps to increased heterogeneity in protein conformations caused by the mutations. In addition, AaY98A exhibited a larger signal for a higher spin species, at $g \sim 5$, as compared to AaIspH wild-type and AaF14A. The $g \sim 5$ signal is likely due to an $S=7/2$ species, as suggested previously.¹⁷ The EPR results show, therefore, that there are clear differences between the Class A (AaIspH) and Class B (EcIspH) proteins. In the Class B protein EcIspH there are interactions between F302 and the 4Fe-4S cluster that result in changes in cluster structure, consistent with a role for this residue in ET and catalysis where, as can be seen in Table 1, the difference in k_{cat}/K_m between wild-type EcIspH and Ec302A is large. In contrast, the differences between the k_{cat}/K_m values for AaIspH and its mutants are all small, Table 1, consistent with the lack of significant differences in their EPR spectra, Figure 6. However, in both the Class A and Class B protein, the aromatics provide cluster protection from O₂ and other reactive oxygen species.

CONCLUSIONS

The results presented here are of interest for several reasons. Using the results of X-ray crystallography and bioinformatics we show that there are four main classes of IspH proteins, each of which have the side-chains of (at least) two aromatic amino-acids in close proximity to the electron transfer (as opposed to the substrate-binding) surface of the protein. These residues confer oxidative stability as well as in some cases, enhance catalytic activity. Class A IspHs are found primarily in anaerobic/microaerophilic bacteria and have F14, Y98 close to the 4Fe-4S cluster. Class B are found in most aerobic bacteria and have Y198 together with F302 close to the cluster, with F302 being located in a conserved C-terminal extension. Class C are found in green plants and cyanobacteria and contain the Class A motif in addition to incorporating an N-terminal domain with an essential Y72*. Class D are found in apicomplexan parasites and contain the Class B motif with its C-terminal extension, together with the N-terminal domain found in plants. Site-directed mutagenesis results indicate that mutagenesis of F302 (to alanine) in Class B proteins, or removal of the entire conserved C-terminus "tail", significantly changes catalytic activity with different electron donors, differences that originate from changes in electron transfer rates. The effects on catalysis of mutagenesis of F14 and Y98 in the Class A protein from *A. aeolicus* are smaller. Both sets of cluster-associated aromatic amino-acid residues are crucial for 4Fe-4S cluster stability in the presence of atmospheric oxygen, as well as other reactive

oxygen species. Based on the results of EPR spectroscopy, we propose an interaction between Y198, F302 and the cluster in EcIspH, a Class B protein, but no aromatic interaction in the Class A protein, AaIspH, consistent with the effects of these residues on catalytic activity. The 4 diverse classes of IspH can thus be categorized based on the types of aromatic amino-acids on the ET side of the 4Fe-4S cluster together with the presence or absence of C and N-terminal extensions, and the occurrence of these 4 IspH classes is clearly related to the nature of the organism's environment—from anaerobic/microaerophilic bacteria to aerobic bacteria to photosynthetic organisms to intracellular protozoan pathogens. Overall, the results are thus of broad general interest since they provide intriguing new insights into the structure and function of an important class of proteins that are both drug as well as herbicide targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DMAPP	dimethylallyl diphosphate
IPP	isopentenyl diphosphate
HMBPP	<i>E</i> -1-hydroxy-2-methyl-but-2-enyl 4-diphosphate
CCD	C-terminal conserved domain
NCD	N-terminal conserved domain
ROS	reactive oxygen species
EPR	electron paramagnetic resonance
ET	electron transfer

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Figure 1.

IspH structures. (A) X-ray structure and space filling model of AaIspH (PDB ID 3DNF). (B) X-ray structure and space filling model of EcIspH bound to substrate HMBPP (**3**) (PDB ID 3KE8). (C) X-ray structure and space filling model of PfIspH (without the N-terminal domain; PDB ID 4N7B). (D) EcIspH tail region. R296 and E298, in green, form salt bridges with E205 and R203, respectively. F302, in cyan, and Y198, in yellow are the two aromatic residues near the Fe-S cluster. (E) PfIspH tail region. E515, in green, forms a salt bridge with R419. W520, in pink, and Y414, in yellow, are the two aromatic residues near the Fe-S cluster (that correspond to Y198 and F302 in EcIspH). (F) Alignment of the Fe-S cluster regions in three IspHs, with the aromatic residues near the clusters highlighted. Shown in cyan are Y198 and F302 from EcIspH, light blue are F14 and Y98 from AaIspH and purple are Y414 and W520 from PfIspH. Green ribbons in the cartoons and space-filling models indicate the C-terminal region. Pink spheres: HMBPP. Yellow/orange spheres: Fe-S cluster.

Figure 2. Multiple sequence alignment of IspHs from different species showing the aromatic residues near the Fe-S cluster. Sequence alignment was performed with Multiple Sequence Comparison by Log-Expectation (MUSCLE, <http://www.ebi.ac.uk/Tools/msa/muscle/>). See SI for the full names of the organisms.

Figure 3.

Four classes of IspH. (A) A schematic illustration of different IspH classes based on the nature of the aromatic amino-acids near the 4Fe-4S cluster and the presence or absence of conserved N-terminal (NCD) and C-terminal (CCD) extension domains. (B) The sequence similarity network of IspH family proteins showing the distribution of different classes of IspHs among various species. Major organism clusters are: (i) gammaproteobacteria; (ii) alphaproteobacteria; (iii) firmicutes; (iv) firmicutes; (v) bacteroidetes; (vi) actinobacteria; (vii) cyanobacteria and plants; (viii) apicomplexa and (ix) desulfobacterales (an order of deltaproteobacteria). The nine clusters are those reported¹⁷ and are colored here as gold=Class A; blue=Class B; green=Class C and pink=Class D.

Figure 4.

Kinetic properties of EcIspH wild-type and mutant proteins used. (A) Michaelis–Menten plots of HMBPP reduction using methyl viologen radical as the electron donor for various EcIspHs. (B) Time course of HMBPP reduction by various EcIspHs using dithionite as electron donor. 100% conversion corresponds to 150 turnovers. (C) Plot of k_{obs} against $[dithionite]^{0.5}$ used to calculate the second order reaction rate constant for electron transfer reaction between EcIspHs and dithionite.

Figure 5.

Air sensitivity of the 4Fe-4S cluster in EcIspHs under different reaction conditions. (A) Fraction of 4Fe-4S cluster in IspHs upon exposure to air. Precipitation in Ec291 caused an apparent increase of absorption in the later part of the curve. (B) Same as (A), in the presence of a 10-fold excess of HMBPP (**3**). (C) IspH plate assay: after an overnight exposure to air, the brown color of the 4Fe-4S cluster was still present in wild-type IspH and EcY198A, but not in Ec291 or EcF302A.

Figure 6.

9 GHz CW-EPR spectra of EcIspH and AaIspH. (A) Spectra of reduced EcIspHs without any added ligand. (B) Spectra of reduced EcIspHs with a 10-fold excess of compound **4**. (C) Spectra of reduced AaIspHs without any added ligand. (D) Spectra of reduced AaIspHs with a 10-fold excess of compound **4**.

Chart 1.
IspH substrate (3), products (1, 2) and inhibitor (4).

Table 1

Kinetic parameters and iron contents of the IspHs used in this study.

	K_m , μM ^a	k_{cat} , min^{-1} ^a	k_{cat}/K_m , $\text{min}^{-1}\mu\text{M}^{-1}$ ^a	k_{DT} , min^{-1} ^b	$k_{obs, 3\text{mM DT}}$, min^{-1} ^c	$k_{2,[E], [SO_2]}$, $\text{min}^{-1}\mu\text{M}^{-1}$ ^d	k_{decay} , min^{-1} ^e	[Fe] content
EcIspH	15±3	285±20	16±3	2.3	1.3±0.1	0.86±0.04	3.7 E-4	3.6
Ec291	23±5	21±2	0.78±0.16	28	6.9±0.2	4.8±0.2	4.0 E-3	3.3
EcF302A	27±8	83±9	2.6±0.7	9.2	4.0±0.1	2.7±0.1	1.7 E-3	3.4
EcY198A	17±2	265±10	13±1	3.0	1.5±0.1	0.90±0.05	7.4 E-4	3.6
AaIspH	2.7±0.8	72±6	23±6	25	38±3	26±1	4.4 E-4	3.6
AaF14A	3.7±1.1	128±11	29±8	44	67±1	45±2	7.8 E-4	3.7
AaY98A	3.6±1.3	170±20	40±12	56	82±2	58±3	2.3 E-3	3.5

^a Measured with methyl viologen as reducing agent at 27°C.^b Initial turnover rates with dithionite as reducing agent at 23°C.^c The observed pseudo-first order ET rate constant at 25°C with 3 mM dithionite, the same concentration used in the enzyme activity assay using dithionite as reducing agent.^d Second-order rate constants of the reaction between IspH and dithionite at 25°C.^e The cluster degradation rate constant of IspH+3 in air at 23°C.