Supporting Information.

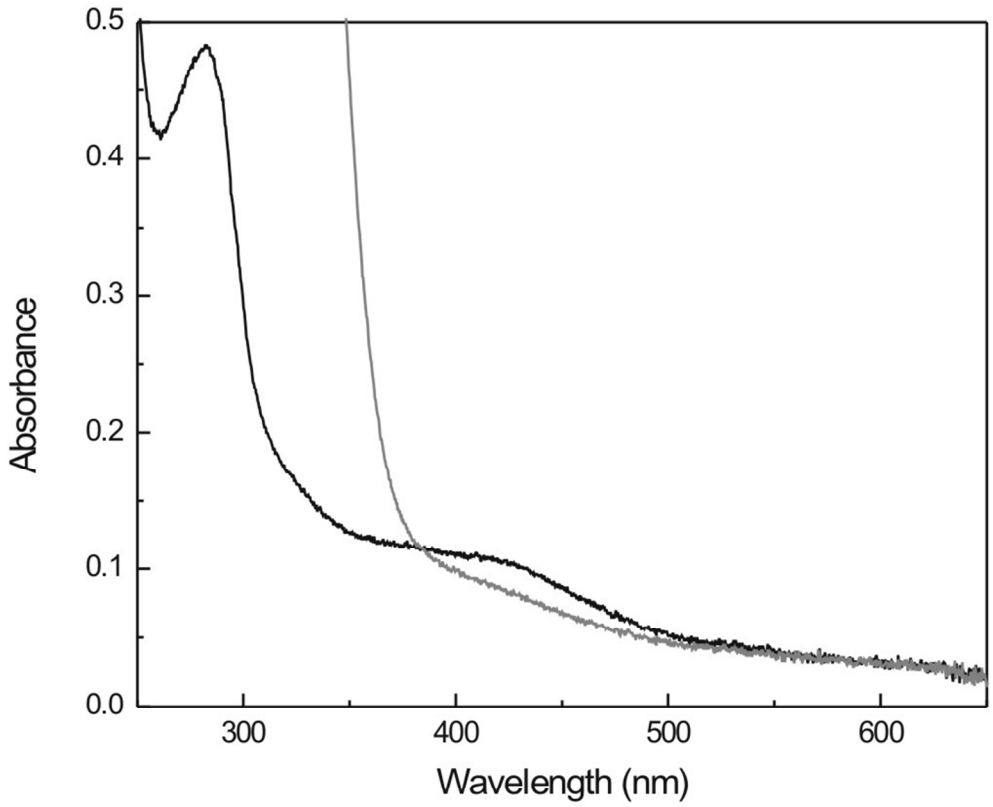

Figure S1. UV-visible absorption of as-isolated (—) and dithionite-reduced (—) IspH protein from *E. coli*. The peak at 330 nm is due to dithionite.

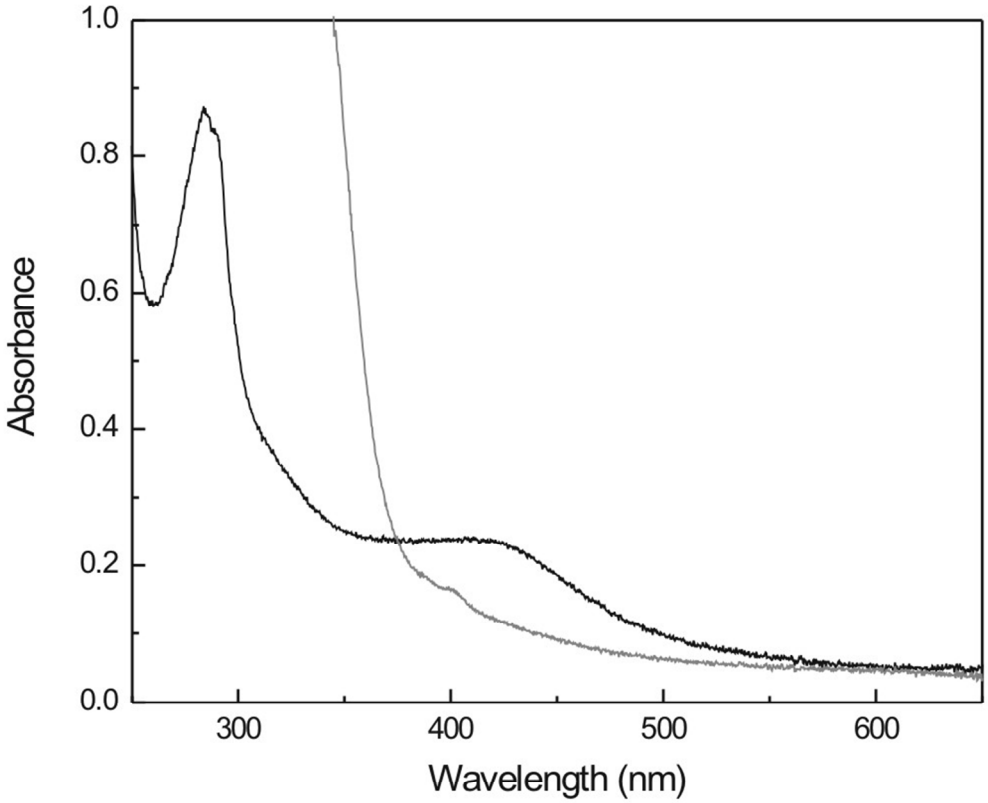

Figure S2. UV-visible absorption of as-isolated (—) and dithionite-reduced (—) IspH protein from *P. falciparum*. The peak at 330 nm is due to dithionite.

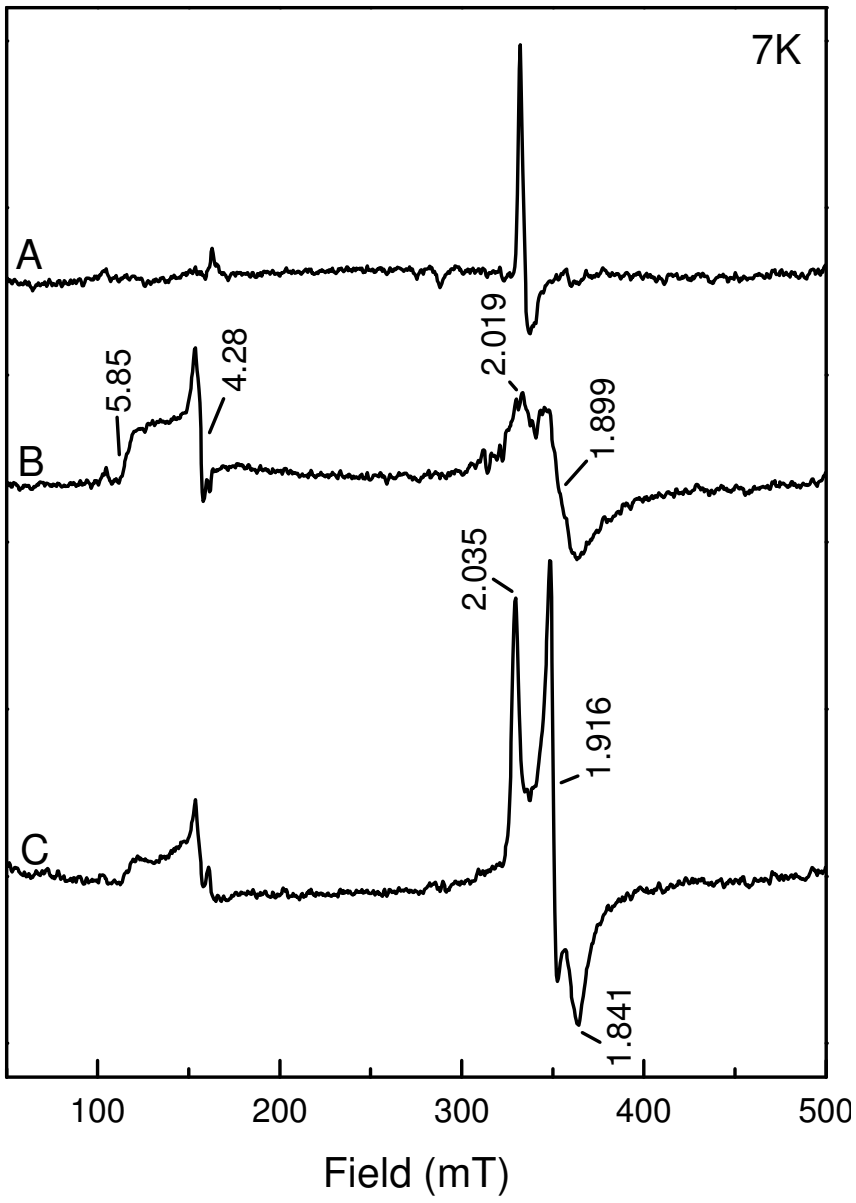

Figure S3. EPR spectra of IspH protein from *A. aeolicus*. (**A**) As-isolated; (**B**) Reduced with an excess dithionite; (**C**) Reduced with an excess dithionite in the presence of 20% ethylene glycol. IspH concentration in the samples, 0.36 mM; dithionite, 2 mM. EPR conditions: Microwave power (A) 2 mW, (B) 2 mW, and (C) 0.02 mW.

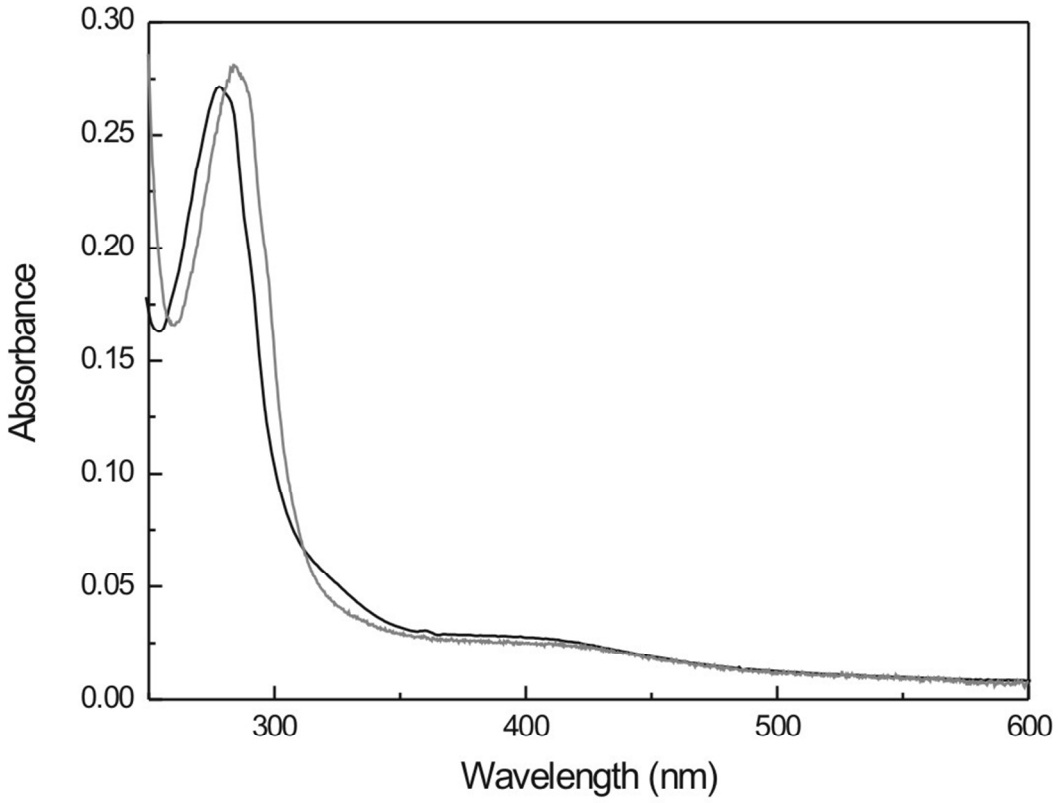

Figure S4. Absorption spectra of IspH protein from *P. falciparum* before the treatment with Chelex Resin (—) and after the treatment with Chelex Resin (—). Spectra were corrected for differences in concentration.

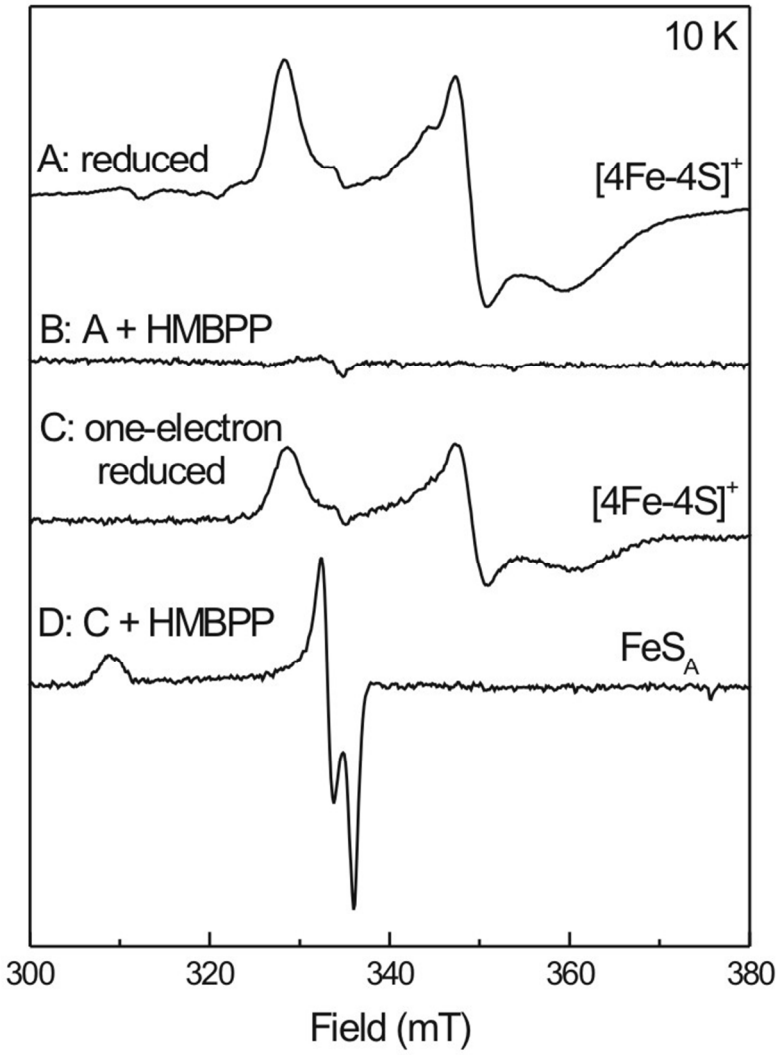

Figure S5. Effect of HMBPP on the [4Fe-4S]$^{+}$ signal of *A. aeolicus* IspH. (**A**) Reduced IspH with a 10x excess of dithionite. (**B**) Sample A after addition of HMBPP and incubation of 8 sec at RT before freezing in cold ethanol (200 K). (**C**) One-electron reduced IspH. (D) Sample C after addition of HMBPP and incubation for 8 sec before freezing. EPR conditions: Microwave power (A) 0.2 mW, (B) 0.2 mW, (C) 0.2 mW, and (D) 2 μW.

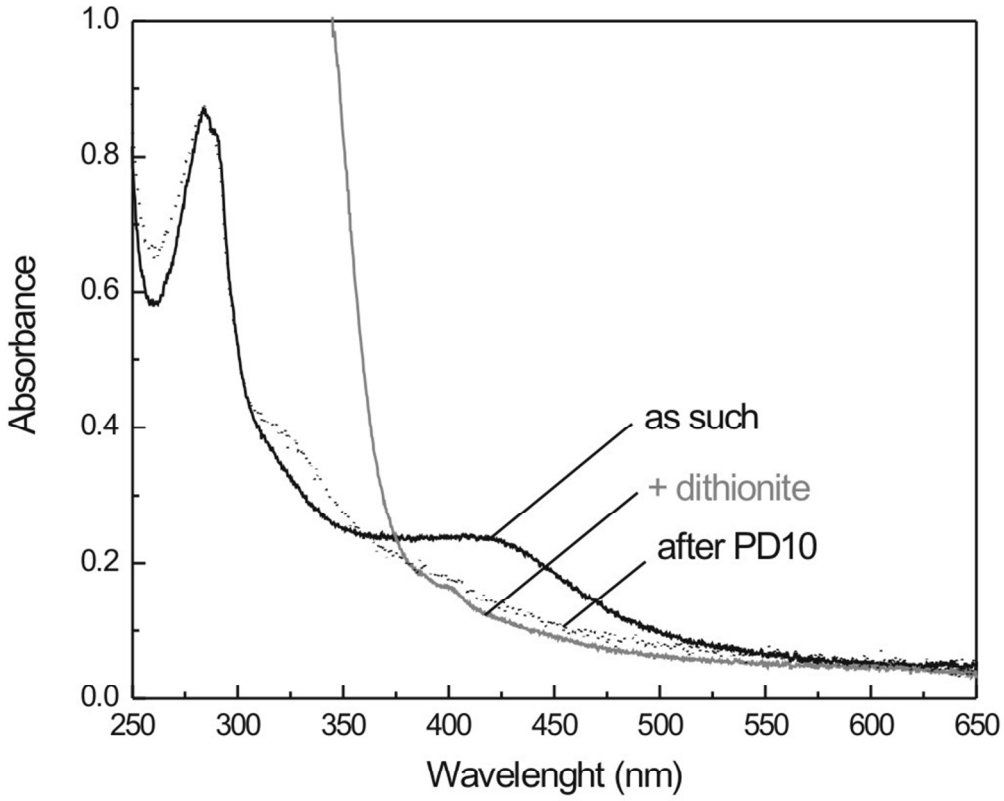

Figure S6. UV-visible absorption of as-isolated (—), dithionite-reduced (—), and desalted (····) IspH protein from *P. falciparum*. The peak at 330 nm is due to dithionite.

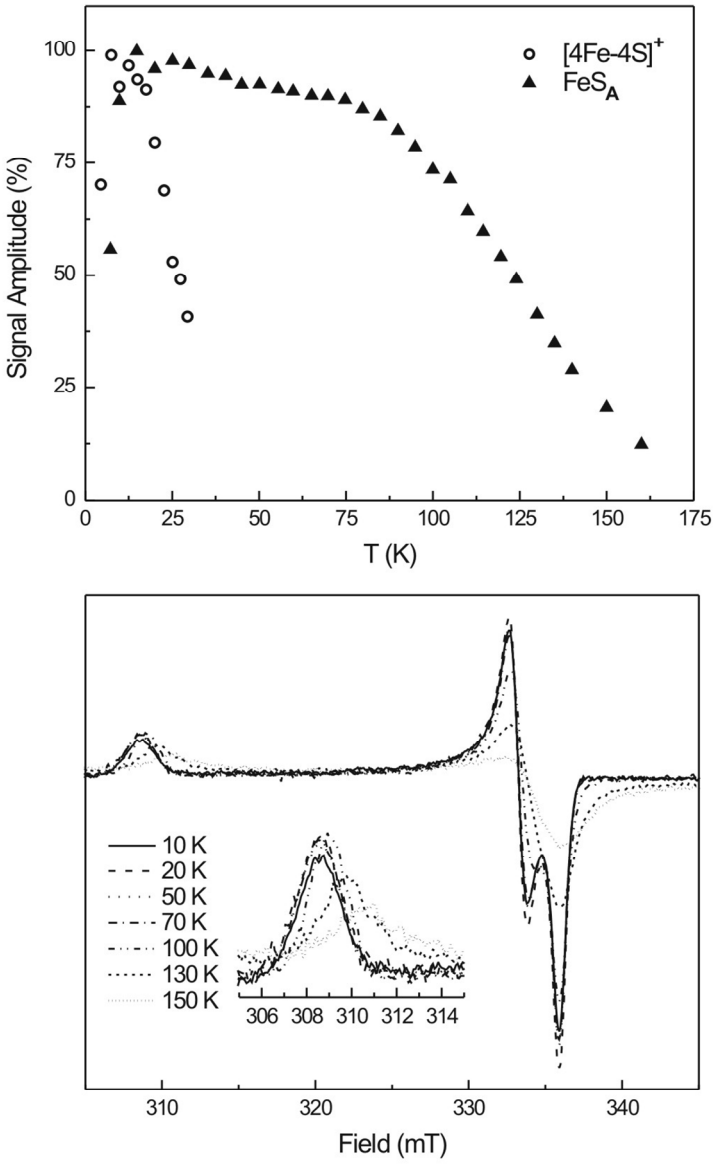

Figure S7. Curie plots for the EPR signals in IspH from *A. aeolicus*. (**A**) Normalized signal intensity (I_n) as a function of the temperature for the $[4Fe\text{-}4S]^+$ signal (O) and the FeS_A species (□). (**B**) Overlay of the normalized spectra of the FeS_A species.

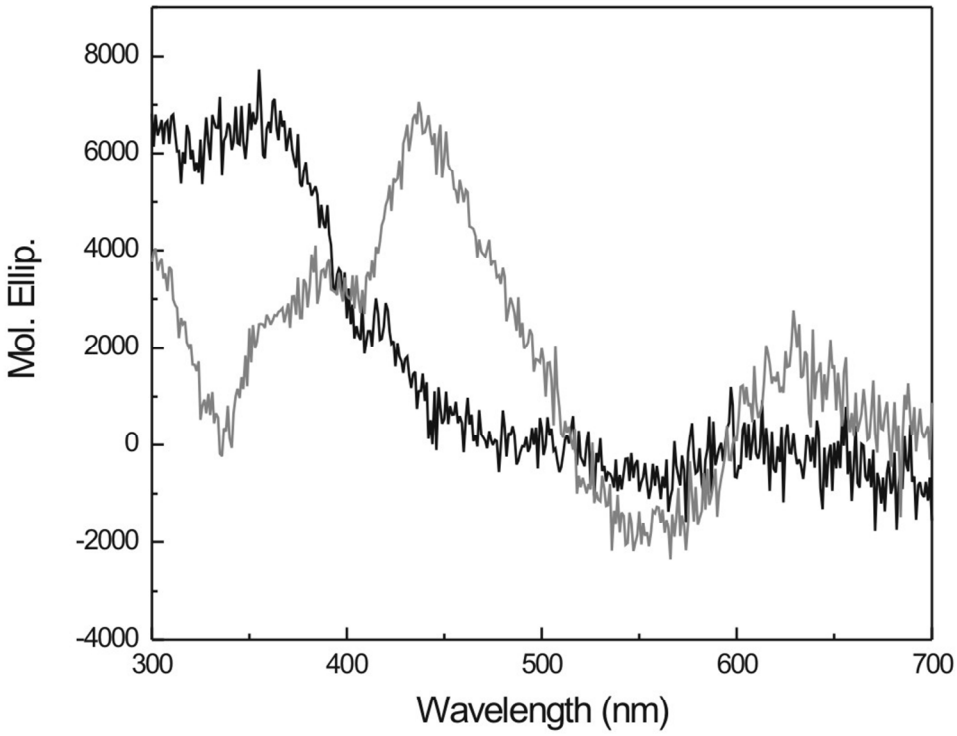

Figure S8. Circular dichroism spectra of reconstituted IspH from *A. aeolicus*.

(**—**) As-isolated enzyme. (—) Enzyme in the presence of HMBPP. IspH concentration was 0.6 mM. HMBPP concentration was 4.76 mM.

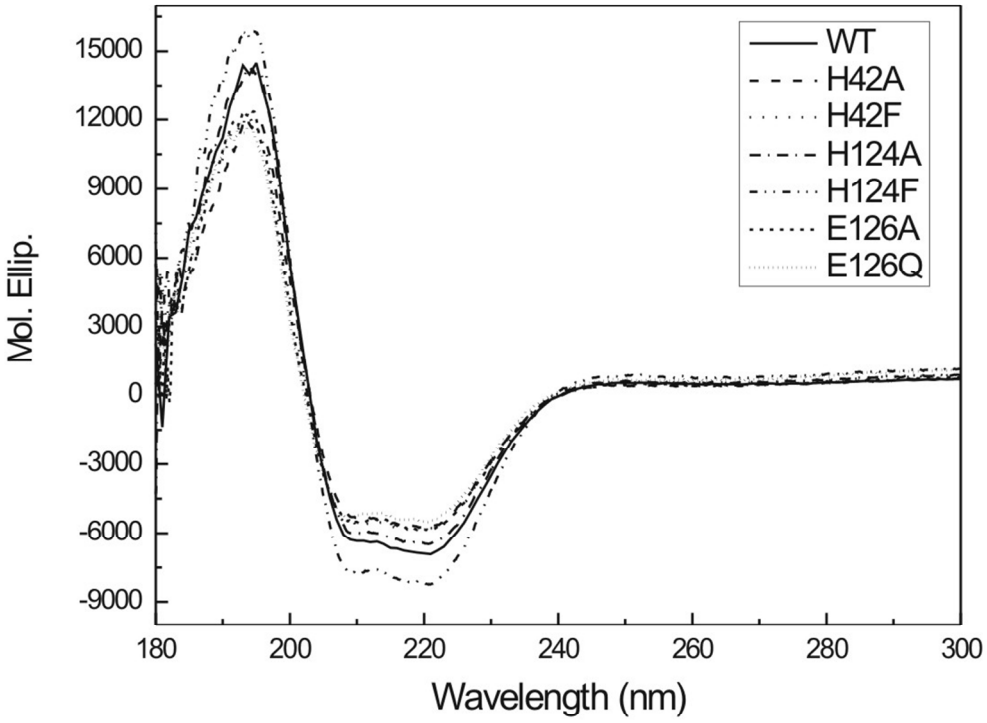

Figure S9. Circular dichroism spectra of WT and mutant IspH proteins from *A. aeolicus*.

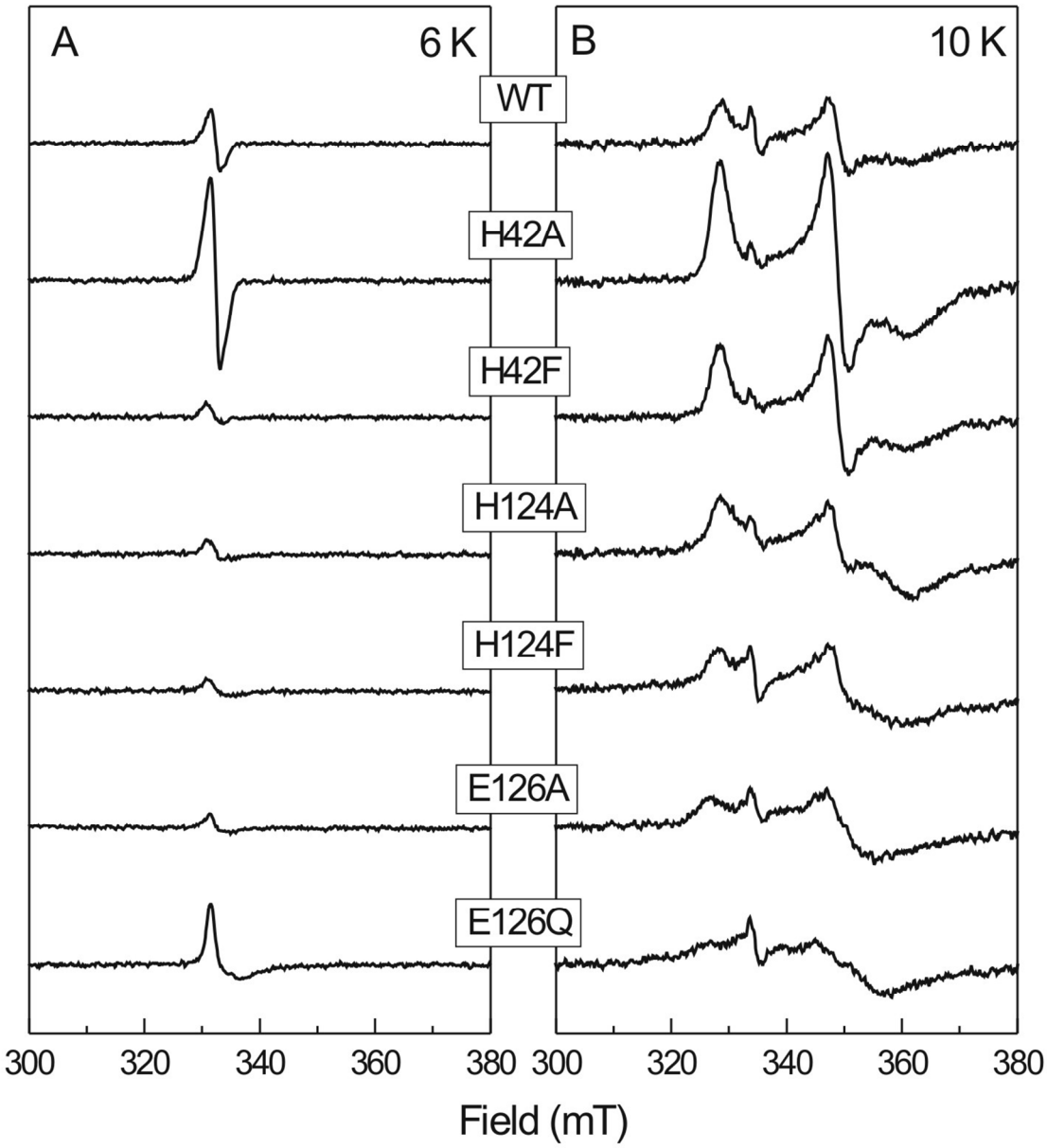

Figure S10. EPR spectra of WT and mutant IspH proteins from *A. aeolicus*. (**A**) Enzyme as isolated. (**B**) Enzyme in the presence of dithionite. EPR conditions: Microwave power: Panel A, 2 mW; Panel B, 0.2 mW.