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**Title:** The Key to the Extraordinary Thermal Stability of P. furiosus Holo-Rubredoxin: Iron Binding-Guided Packing of a Core Aromatic Cluster Responsible for High Kinetic Stability of the Native Structure

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**Abstract:** Pyrococcus furiosus rubredoxin (PfRd), a small, monomeric, 53 residues-long, iron-containing, electron-transfer protein of known structure is sometimes referred to as being the most structurally-stable protein known to man. Here, using a combination of mutational and spectroscopic (CD, fluorescence, and NMR) studies of differently made holo- and apo-forms of PfRd, we demonstrate that it is not the presence of iron, or even the folding of the PfRd chain into a compact well-folded structure that causes holo-PfRd to display its extraordinary thermal stability, but rather the correct iron binding-guided packing of certain residues (specifically, Trp3, Phe29, Trp36, and also Tyr10) within a tight aromatic cluster of six residues in PfRd's hydrophobic core. Binding of the iron atom appears to play a remarkable role in determining subtle details of residue packing, forcing the chain to form a hyper-thermally stable native structure which is kinetically stable enough to survive (subsequent) removal of iron. On the other hand, failure to bind iron causes the same chain to adopt an equally well-folded native-like structure which, however, has a differently-packed aromatic cluster in its core, causing it to be only as stable as any other ordinary mesophile-derived rubredoxin. Our studies demonstrate, perhaps for the very first time ever that hyperthermal stability in proteins can owe to subtle differences in residue packing vis a vis mesostable proteins, without there being any underlying differences in either amino acid sequence, or bound ligand status.

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