Biological Iron−Sulfur Cluster Assembly. Detection of Kinetic Intermediates by Time-Resolved Fluorescence Spectroscopy

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Protein-bound iron−sulfur clusters display a remarkable diversity of functional chemistry, including electron-transfer,[1] catalytic,[2] structural,[3] and sensory.[4] An important facet of protein cluster chemistry is the mechanism of assembly of the Fe−S prosthetic center, which is also of functional relevance for the understanding of iron regulatory proteins.[5] Previously, we have monitored Fe−S cluster assembly and backbone folding for native *Chromatium vinosum* high potential iron protein (HiPIP) by use of $^{1}H-^{15}N$ heteronuclear single quantum coherence (HSQC) spectroscopy and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry experiments.[6] These studies suggest that the 4Fe−4S cluster assembles through a key structural intermediate (**I**, Scheme 1) that already possesses the tertiary structural elements associated with the native protein. In this paper, we further develop this model by detecting earlier kinetic intermediates and rate constants for several key steps on the reaction pathway by use of the intrinsic protein fluorescence.[9]

Figure 1 shows the emission spectra obtained from the apo and native forms of HiPIP, which have not previously been reported, as well as the emission from intermediate **I**. A decrease in fluorescence occurs after addition of inorganic sulfide to a solution of **I**, as a result of 4Fe−4S core formation. HiPIP contains three Trp residues and one Tyr.[10] Tyr emission at ∼300 nm is efficiently quenched by neighboring Trp residues[11] and is not observed in the emission spectrum of either native or apo protein. The Trp residues show an emission maximum at >350 nm for apo and intermediate states at pH 8, and there is evidence of overlapping bands that arise from the three Trp's in the protein. A blue shift of ∼13 nm is observed for native, relative to apo, HiPIP. Control experiments taken in the absence of protein show no significant fluorescence in the observation range.

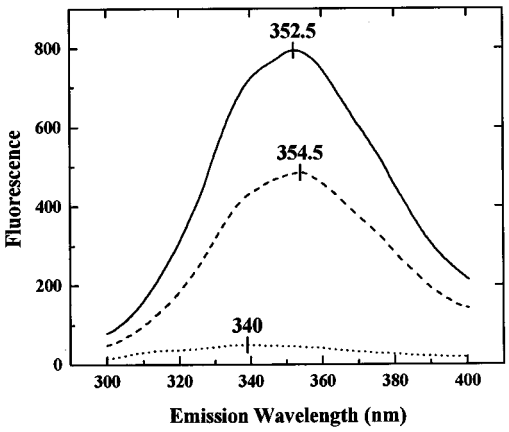
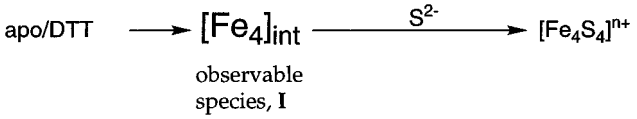


Figure 1. Fluorescence spectra for holo (dotted line), apo (solid line), and intermediate (dashed line) HiPIP species recorded from 300 to 400 nm with excitation at 280 nm. Fluorescence spectra were taken on a Perkin-Elmer LS 50B luminescence spectrometer, with 3.3 μM protein in 0.1 M Tris pH 7.8 and 0.44 mM DTT. The intermediate spectrum was collected 30 min after addition of 33 μM Fe^{2+} to the apo solution. Native and apo were prepared as previously described.[6]

Scheme 1



By use of these and other distinct emission characteristics, the kinetic profile for HiPIP cluster assembly has been evaluated by time-resolved fluorescence methods. The rapid initial drop in fluorescence is lost in the mixing time of a routine fluorescence experiment, and so, this time regime was further monitored by stopped-flow experiments. Both time domains are shown in Figure 2, with fitted rate constants and errors in the legend. Binding of iron to apo-HiPIP (probably to one or more cysteine residues) results in an initial rapid quenching of the Trp fluorescence. Further reductions in fluorescence intensity most likely reflect the binding of additional iron centers and intramolecular quenching mechanisms arising from structural changes as protein residues begin to form a more compact structure.[8c] A subsequent increase in intensity is attributed to the exclusion of water molecules as the cluster intermediate develops and the protein folds around it,[11] thereby establishing tertiary structure.[6]

Following addition of Fe^{2+} and exogenous thiol (DTT) to apo-HiPIP, the fluorescence traces could best be fit to a model of consecutive reactions (summarized in Scheme 2),[12] where k_1, k_2, k_3, and k_4 represent the rate constants from the fastest phase to the slowest phase. This model, and the kinetics of intermediate formation measured by these fluorescence experiments, is consistent with the folding pathway to intermediate **I** previously characterized by NMR and mass spectrometric measurements made in our laboratory.[6] It is not possible to discount reversibility in early steps of the pathway, and so, the reported k's are apparent rate constants.

A plausible reaction mechanism, that is consistent with earlier observations by Rabinowitz et al.,[13] is illustrated in Scheme 2, which shows an elaborated version of the assembly pathway described in Scheme 1. Consistent with our previous study,[6] steps k_1 and k_2 in the fast kinetic phase show a clear dependence on

(1) (a) Knaff, D. B.; Hirasawa, M. *Biochim. Biophys. Acta* **1991**, *1056*, 93. (b) Beinert, H.; Holm, R. H.; Münck, E. *Science* **1997**, *277*, 653.

(2) (a) Flint, D. H.; Allen, R. M. *Chem. Rev.* **1996**, *96*, 2315. (b) Emptage, M. H.; Kent, T. A.; Kennedy, M. C.; Beinert, H.; Munck, E *Proc. Natl. Acad Sci. U.S.A.* **1988**, *80*, 4674. (c) Holm, R. H.; Kennepohl, P.; Solomon, E. I. *Chem. Rev.* **1996**, *96*, 2239.

(3) Thayer, M. M.; Ahern, H.; Xing, D.; Cunningham, R. P.; Tainer, J. A. *EMBO J.* **1995**, *14*, 4108.

(4) (a) Hidalgo, E.; Bollinger, J. M., Jr.; Bradly, T. M.; Walsh, C. T.; Demple, B. *J. Biol. Chem.* **1995**, *270*, 20908. (b) Gaudu, P.; Weiss, B. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 10094.

(5) Hentze, W.; Kuhn, L. C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8175.

(6) Natarajan, K.; Cowan, J. A. *J. Am. Chem. Soc.* **1997**, *119*, 4082.

(7) (a) Clark, P. L.; Liu, Z.; Zhang, J.; Gierasch, L. M. *Protein Sci.* **1996**, *5*, 1108. (b) Kotik, M.; Radford, S. E.; Dobson, C. M. *Biochemistry* **1995**, *34*, 1714. (c) Denton, M. E.; Rothwarf, D. M.; Scheraga, H. A. *Biochemistry* **1994**, *33*, 11225.

(8) (a) Agoshe, V. R.; Shastry, M. C. R.; Udgonkar, J. B. *Nature* **1995**, *377*, 754. (b) Kim, D.; Kim, C.; Park, C. *J. Mol. Biol.* **1994**, *240*, 385. (c) Itzhaki, L.; Evans, P. A.; Dobson, C. M.; Radford, S. E. *Biochemistry* **1994**, *33*, 5212.

(9) Fluorescence spectroscopy has been extensively applied to the study of protein folding pathways, either by use of the intrinsic protein fluorescence[7] or proteins labeled with fluorescent labels.[8] However, in these examples, the assembly of a metalloprosthetic center was not involved in the protein folding process.

(10) Cowan, J. A.; Lui, S. M. *Adv. Inorg. Chem.* **1998**, *45*, in press.

(11) Freifelder, D. *Physical Biochemistry. Applications to Biochemistry and Molecular Biology*, 2nd ed.; W. H. Freeman and Co.: San Francisco, CA, 1982; pp 532−570.

(12) Rodiguin, N. M.; Rodiguina, E. N. In *Consecutive Chemical Reactions*; Schneider, R. F., Ed.; Van Nostrand: Princeton, NJ, 1964; pp 6−23.

(13) Sweeney, W. V.; Rabinowitz, J. C. *Annu. Rev. Biochem.* **1980**, *49*, 139.

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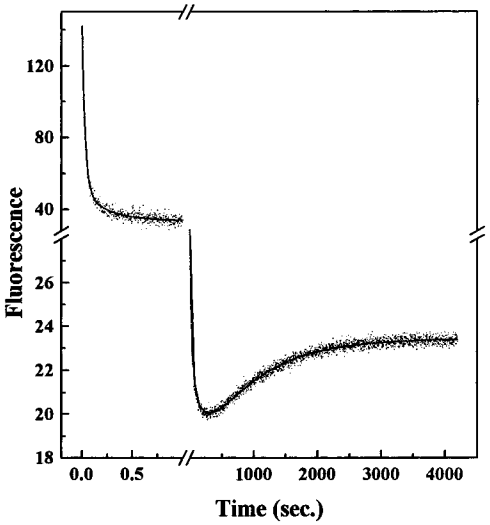
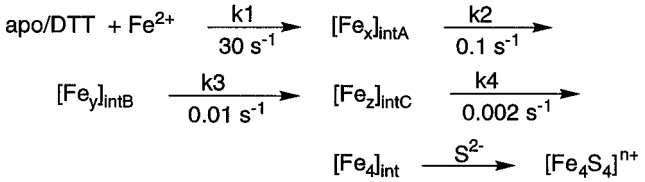


Figure 2. Time-dependent fluorescence traces after addition of Fe^{2+} to a solution of apo-HiPIP in the presence of DTT. Rate constants k_1 (30 ± 8 s^{-1}), k_2 (0.10 ± 0.02 s^{-1}), k_3 (0.010 ± 0.002 s^{-1}), and k_4 (0.0020 ± 0.0004 s^{-1}) (from the fastest phase to the slowest phase) were obtained by fitting the traces to a model for a series of consecutive reactions.[12] Stopped-flow measurements were made on an Olis 1000 RSM apparatus with excitation at 280 nm, and the emission was collected using a 10-nm band-pass filter centered at 355 nm. One syringe was filled with 20 μM apo-HiPIP in 10 mM Tris (pH 8.0) and 0.9 mM DTT, while the other syringe contained 0.2 mM $FeCl_2$ in the same buffer. Routine fluorescence was done as described in the legend for Figure 1, with solution conditions as used for the stopped-flow experiment and detection of the emission wavelength at 352 nm.

[DTT] (Figure 3A, Supporting Information), suggesting a requirement for exogenous thiol to promote formation of the initial intermediates $[Fe_x]_{intA}$ and $[Fe_y]_{intB}$ by providing additional coordination to the iron center(s). Later steps are not dependent on [DTT]. In the slower phases, changes in fluorescence reflect further protein conformational changes in accommodating the newly formed cluster. As expected, the rate constants k_1, k_2, and k_3 increase with increasing $[Fe^{2+}]$ (Figure 3B, Supporting Information), since iron is obviously involved in promoting the formation of cluster intermediates. The possibility of reversibility for these steps precludes a detailed assessment of iron stoichiometry in each step. The reaction step (k_4) does not depend on $[Fe^{2+}]$ and may reflect internal ligand rearrangement. Reaction rates for steps up to the formation of intermediate **I** are independent of inorganic sulfide.

Scheme 2



Martin et al. have reported a cluster-driven protein rearrangement for *A. vinelandii* Fd I.[14] Our time-resolved fluorescence study described here indicates that protein folding is also driven by cluster assembly and that cluster assembly is the rate-limiting process,[7,8] reflecting the greater complexity of the assembly reaction. In this paper, we have further defined the reaction pathway leading to the protein-bound 4Fe−4S cluster in vitro. It is likely that in vivo assembly follows a similar pathway, although other proteins and enzymes will be utilized for delivery of the iron[15] and sulfur constituents, for example, delivery of sulfide from cysteine by sulfur transferases.[16] In this work, we have detected several kinetic intermediates and found their relative rates of formation to decrease stepwise by over 1 order of magnitude. The consequent buildup of each species will favor future spectroscopic characterization.

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Supporting Information Available: Figures showing dependence of rate constants on $[Fe^{2+}]$ and [DTT] (2 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(14) Martin, A. E.; Burgess, B. K.; Stout, C. D.; Cash, V. L.; Dean, D. R.; Jensen, G. M.; Stephens, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 598.
(15) Fu, W.; Jack, R. F.; Morgan, T. V.; Dean, D. R.; Johnson, M. K. *Biochemistry* **1994**, *33*, 13455−13463.
(16) (a) Flint, D. H.; Tuminello, J. F.; Miller, T. J. *J. Biol. Chem.* **1996**, *271*, 16053. (b) Flint, D. H. *J. Biol. Chem.* **1996**, *271*, 16068. (c) Zheng, L.; Dean, D. R. *J. Biol. Chem.* **1994**, *259*, 18723.