Evidence for a Catalytic Role of Zinc in Protein Farnesyltransferase

SPECTROSCOPY OF Co²⁺-FARNESYLTRANSFERASE INDICATES METAL COORDINATION OF THE SUBSTRATE THIOLATE

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Protein farnesyltransferase (FTase) is a zinc metalloenzyme that catalyzes the addition of a farnesyl isoprenoid to a conserved cysteine in peptide or protein substrates. We have substituted the essential Zn²⁺ in FTase with Co²⁺ to investigate the function of the metal polyhedron using optical absorption spectroscopy. The catalytic activity of FTase is unchanged by the substitution of cobalt for zinc. The absorption spectrum of Co²⁺-FTase displays a thiolate-Co²⁺ charge transfer band (ε₅₂₀ = 1830 m⁻¹ cm⁻¹) consistent with the coordination of one cysteine side chain and also ligand field bands (ε₆₅₀ = 140 m⁻¹ cm⁻¹) indicative of a pentacoordinate or distorted tetrahedral metal geometry. Most importantly, the ligand-metal charge transfer band displays an increased intensity (ε₅₂₀ = 1830 m⁻¹ cm⁻¹) in the ternary complex of FTase-isoprenoid-peptide substrate indicative of the formation of a second Co²⁺-thiolate bond as cobalt coordinates the thiolate of the peptide substrate. A similar increase in the ligand-metal charge transfer band in a product complex indicates that the metal ion coordinates the thiolate of the peptide substrate in the presence of the isoprenoid.

Protein farnesyltransferase (FTase) catalyzes the transfer of the farnesyl group of farnesyl pyrophosphate (FPP) to a conserved cysteine residue of a protein substrate, including Ras proteins, nuclear lamins, and several proteins involved in cell signaling (1-4). Protein farnesylation mediates membrane association and, possibly, interactions with other proteins essential for the localization and function of these proteins (3, 4). One example in this regard is the requirement of farnesylation for the cell transforming ability of oncogenic Ras proteins (5); this result has stimulated an intense search for FTase inhibitors as potential anticancer drugs (6, 7). An increased understanding of the molecular mechanism of FTase should enhance the rational design of such FTase inhibitors.

FTase is a metalloenzyme that contains one zinc ion per α/β heterodimer that is essential for optimal activity (8, 9). Cross-linking and direct binding studies indicate that the zinc ion is required for the binding of protein but not isoprenoid substrates (8). Additionally, FTase containing Cd²⁺ substituted for Zn²⁺ has essentially normal catalytic activity, demonstrating that other metal ions can functionally substitute for the zinc (10). Chemical modification and site-directed mutagenesis studies have identified a conserved cysteine residue of FTase, Cys²⁹⁹, in the β subunit, as important for catalytic activity and zinc binding, suggesting that the thiolate of this residue may directly coordinate the zinc ion (11).

Although it is clear that the zinc ion in FTase is critical for activity, the precise function of the metal, particularly the question of whether the primary role of the zinc ion is structural or catalytic, is not yet known. Proposed catalytic functions for the zinc ion in FTase include increasing the nucleophilicity of the cysteine residue of the protein substrate (3, 8, 11, 12) and/or activating the diphosphate leaving group (11, 13, 14). Here we investigate the metal coordination polyhedron in FTase by substituting Co²⁺ for Zn²⁺, which does not change the catalytic activity of the enzyme. This substitution provides a useful spectroscopic probe of the geometry of the enzyme. The spectral data obtained indicate that the metal ion coordinates the thiolate of the peptide substrate in the presence of bound FPP.

EXPERIMENTAL PROCEDURES

Preparation of Apo-FTase—Recombinant Rat FTase was produced and purified as described (9). Apo-FTase was prepared by dialyzing the holo-enzyme (>1 mg/ml) for 24 h against 50 mM Tris-Cl, pH 7.8, 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Buffer 1) and 5 mM EDTA at 4°C followed by an additional 24 h dialysis against Buffer 1 containing 50 μM EDTA. After dialysis, the apo-FTase was concentrated to ~100 μM, flash-frozen, and stored at ~70°C.

Preparation of Metal-free Substrates—Recombinant H-Ras was purified from a bacterial expression system as described (1, 17). Residual metals in the H-Ras preparation were removed by dialysis against Buffer 1 containing 50 μM EDTA and 30 μM guanosine 5'-diphosphate at 4°C for 24 h. Metal ions in the [³H]farnesyl diphosphate (³H-FPP) (American Radiolabeled Chemicals) were removed by incubation of the solution with Chelex-100 resin (Sigma) for 5–10 min. The final preparation of ³H-FPP (20 μM, 8 Ci/mmol) was stored at ~18°C.

Metal Reconstitution Experiments—FTase was reconstituted with different divalent metals (Zn²⁺, Co²⁺, Cd²⁺, or Ni²⁺) by incubating the apo-enzyme (150 μg/ml) with 60 μM of the respective metal salt (atomic absorption grade, Aldrich) in Buffer 1 and 50 μM EDTA for 5–10 min on ice before dilution into the assay mixture. For reactions containing diethiothreitol (DTT), the reducing agent (1 mM) was added to the apoenzyme prior to the addition of the metal.
RESULTS AND DISCUSSION

Co2+-FTase Is Catalytically Active—Previous metal substitution experiments indicated that Zn²⁺ and Cd²⁺ restore activity to apo-FTase, whereas Ni²⁺, Mn²⁺, Hg²⁺, and Cd²⁺ do not (10). Because Co²⁺ can generally substitute for Zn²⁺ in other zinc metalloenzymes (15), we tested the activity of the Co²⁺-FTase in an assay with TCEP substituted for DTT. DTT catalyzes the formation of Co³⁺ from Co²⁺ in the presence of oxygen while TCEP reacts more slowly (22, 23). Under these conditions, the activity of Co²⁺-FTase is comparable with that of Zn²⁺-FTase (Fig. 2). This restoration of activity by Co²⁺ is not due to trace contamination of Zn²⁺ in the assay solutions for the following reasons. First, the high concentration of FTase employed in this reconstitution experiment (0.16 μM) made it very unlikely that Zn²⁺-FTase could form quantitatively in the assay. Second, the addition of 1 mM DTT to apo-FTase before the addition of metal had no effect on the activity of Zn²⁺-FTase, but abolished 75% of the activity in the presence of Co²⁺ (Fig. 2), and third, the addition of Ni²⁺, which binds EDTA with an affinity greater than either Zn²⁺ or Co²⁺ (24), does not restore the activity of apo-FTase (Fig. 2).

Absorption Spectra of Co²⁺-FTase—Substitution of Co²⁺ into the zinc binding site of proteins provides a useful spectroscopic probe of the composition and geometry of the metal polyhedron (15, 16). Because the high catalytic activity of Co²⁺-FTase indicates that the metal binding site in the enzyme is not significantly disturbed by this replacement, spectral information obtained from Co²⁺-FTase is both structurally and catalytically relevant. Indeed, the optical absorption spectrum of Co²⁺-substituted FTase (Fig. 3A, solid line) displays characteristic features of the spectra of many Co²⁺-substituted zinc enzymes (15, 16). The low wavelength absorbance (below 450 nm) is assigned to a ligand-metal charge transfer (LMCT) band indicative of sulfur-to-cobalt charge transfer resulting from thiolate coordination (16). Furthermore, the intensity of the absorption shoulder at 320 nm (ε320 = 1030 m⁻¹ cm⁻¹) is consistent with one thiolate ligand; the extinction coefficient of LMCT bands is normally 900-1300 m⁻¹ cm⁻¹ per cobalt-thiolate bond (25). Consistent with this assignment, an essential cysteine in the β subunit of FTase, Cys299, has been identified that exhibits the characteristics of a zinc ligand (11). In addition, the intensity of the ligand field absorption bands that are observed in the low energy region of the spectrum (ε560 = 140 m⁻¹ cm⁻¹; ε615 = 100 m⁻¹ cm⁻¹) is indicative of a pentacoordinate or distorted tetrahedral metal geometry (16). Removal of the magnesium ion from the solution did not alter the absorption spectrum (data not shown).

Absorption Spectra of Co²⁺-FTase Binary Complexes—The addition of FPP to Co²⁺-FTase to form the binary complex has little influence on the optical spectrum (Fig. 3A, dotted line), except that the extinction coefficient of the ligand field bands decreases (ε560 = 110 m⁻¹ cm⁻¹). This diminution suggests a slight alteration in the geometry of the metal polyhedron, perhaps caused by a protein conformational change or interaction of the pyrophosphosphate group of FPP with Co²⁺. Additionally, the spectrum of Co²⁺-FTase with a bound FPP analogue (compound Ia, see Fig. 1) is virtually identical to the spectrum of E-FPP (Fig. 3, A and B), suggesting that FPP and Ia bind to the
enzyme in a similar manner. Finally, the shape of the spectrum of Co\(^{2+}\)-FTase in the presence of the peptide substrate TKCVIM is indistinguishable from that of Co\(^{2+}\)-FTase alone, although the extinction coefficients of both the ligand field and the LMCT bands increase slightly upon the addition of peptide (<20%; data not shown). The marginal influence of peptide binding on the absorption spectrum suggests that cobalt does not coordinate the thiolate of the peptide substrate in the binary complex.

**Absorption Spectra of Ternary Complexes of Co\(^{2+}\)-FTase**—To probe the environment of the metal binding site in the ternary complex, we used the aforementioned FPP analogue (compound Ib), which binds to FTase but is not utilized in catalysis to form an inactive but stable ternary complex mimic. The spectrum of the Co\(^{2+}\)-FTase-Ib-TCVKIM ternary complex (Fig. 3B) exhibits quite striking differences from the absorption spectrum of either the E-Ia or E-FTase binary complex. Most importantly, the intensity of the LMCT band at 320 nm essentially doubles (\(\epsilon_{320} = 1830 \text{ M}^{-1} \text{ cm}^{-1}\)), indicating the formation of a second Co\(^{2+}\)-thiolate bond, consistent with coordination of the thiolate of the peptide substrate with Co\(^{2+}\) in this ternary complex.

**FIG. 3.** Optical absorption spectra of Co\(^{2+}\)-FTase and its binary and ternary complexes. A, absorption spectrum of Co\(^{2+}\)-FTase (solid line); Co\(^{2+}\)-E-FPP complex (dotted line) formed by the addition of 70 \(\mu\text{M}\) FPP, and Co\(^{2+}\)-E-FTase (dashed line) formed by the addition of 91 \(\mu\text{M}\) TKCVIM to the Co\(^{2+}\)-E-FPP complex. B, absorption spectra of Co\(^{2+}\)-Ia, complex (dotted line) formed by the addition of 70 \(\mu\text{M}\) Ia, to Co\(^{2+}\)-FTase and the ternary Co\(^{2+}\)-FTase-Ia-TCVKIM complex formed by the addition of either 22 \(\mu\text{M}\) (dashed line) or 110 \(\mu\text{M}\) (solid line) of TKCVIM to FTase-Ia. C, influence of the addition of increasing concentrations of TKCVIM peptide on the observed extinction coefficient at 320 (closed circle) and 640 nm (open circle) as the peptide bound to Co\(^{2+}\)-FTase-Ia to form the E-Ia-FTase ternary complex. Saturation occurs when the concentration of added peptide equals the concentration of Co\(^{2+}\)-FTase-Ia.

Furthermore, the increased intensity of the ligand field absorption bands at higher wavelengths in the E-Ia-TCVKIM complex compared with those in the E-Ia binary complex is also consistent with additional coordinating thiolates (15, 27–29). Finally, the increased extinction coefficient of the d-d transition maximum at 635 nm (\(\epsilon_{635} = 310 \text{ M}^{-1} \text{ cm}^{-1}\)) is typical of a cobalt binding site with tetrahedral geometry (30). The observed extinction coefficients at both 320 and 640 nm increase linearly with the concentration of added peptide before saturating at a peptide concentration equal to the enzyme concentration (Fig. 3C), providing compelling evidence that (i) the peptide binds stoichiometrically to the E-Ia complex and (ii) the increased extinction coefficient observed upon addition of peptide reflects the spectrum of the E-Ia-TCVKIM complex and not the absorption of nonspecific Co\(^{2+}\)-TCVKIM complexes.

We also formed the ternary complex using authentic FPP and an analog of a peptide substrate, B581 (compound Ib, see Fig. 1), that cannot be used in catalysis. The spectrum of the resultant Co\(^{2+}\)-FTase-FPP-Ia ternary complex retains the significant spectral features of the Co\(^{2+}\)-FTase-Ia-TCVKIM complex described above, including the increase in the LMCT band (\(\epsilon_{1900} = 1900 \text{ M}^{-1} \text{ cm}^{-1}\)) and changes in the shape and extinction coefficients of the ligand field absorption bands (\(\epsilon_{660} = 150 \text{ M}^{-1} \text{ cm}^{-1}\); \(\epsilon_{610} = 170 \text{ M}^{-1} \text{ cm}^{-1}\); \(\epsilon_{560} = 150 \text{ M}^{-1} \text{ cm}^{-1}\)) (data not shown). This similarity suggests that the observed spectral properties are general features of Co\(^{2+}\)-FTase ternary complexes rather than unique to the inactive E-Ia-TCVKIM complex. Taken together, these spectral data provide direct evidence that the cysteine thiol(s) of the peptide substrate directly coordinates Co\(^{2+}\) in these ternary complexes.

**Absorption Spectrum of the Co\(^{2+}\)-FTase Product Complex**—Because the equilibrium constant for the formation of farnesylated peptide is very large (26), the reaction of FTase-FPP with one equivalent of the peptide substrate should produce a stoichiometric amount of product bound to FTase. Furthermore, under these conditions the enzyme should not turnover or release the farnesylated peptide. We therefore used this method to prepare the Co\(^{2+}\)-FTase-farnesylated-peptide product complex and recorded its spectrum (Fig. 3A). Examination of the spectrum of the product complex reveals features that are distinct from those of either the binary or ternary complexes described above. The LMCT band in the product complex displays a distinctive shoulder at 320 and 370 nm (\(\epsilon_{320} = 1600 \text{ M}^{-1} \text{ cm}^{-1}\) and \(\epsilon_{370} = 650 \text{ M}^{-1} \text{ cm}^{-1}\)). However, as observed in the ternary complexes, the extinction coefficient at 320 nm in the product complex is significantly greater than...
that in the Co^{2+}-FTase-FPP binary complex, indicative of additional sulfur-cobalt coordination in the product complex. On the other hand, the shape and extinction coefficient ($\epsilon_{1cm} = 130$ M$^{-1}$ cm$^{-1}$) of the ligand field absorption band resembles the Co$^{2+}$-E-FPP binary complex rather than the ternary complexes. These spectral changes also increase linearly upon the addition of limiting TKCVM to the E-FPP complex and saturate when one equivalent of the peptide is added (data not shown), indicating that the spectral changes are due to an interaction with Co$^{2+}$-FTase. This is a surprising result because metal coordination by a thioether in a zinc metalloenzyme has only been implicated in one previous case, the DNA repair protein Ada (12, 31, 32). However, an interaction between a thioether sulfur and Co$^{3+}$ has been observed in Co$^{3+}$-substituted azurin (33), and ligand-metal charge transfer has been detected between a thioether sulfur and Co$^{3+}$ in model compounds (34). The observed spectral changes in the product complex suggest that the thioether moiety of the farnesylated peptide product interacts with Co$^{2+}$ in the enzyme-product complex.

**Transient Absorbance of Active and Inactive Ternary Complex**—To verify that the increase in the LMCT band observed in the Co$^{2+}$-FTase coordinates the thiolate of one cysteine residue with either a pentacoordinate or distorted tetrahedral geometry. In the ternary complex, the peptide thiol binds to the metal and is activated to attack the carbon of farnesylpyrophosphate as a nucleophile. In the product complex, the sulfur of the farnesylated peptide remains coordinated with the metal. $R$, farnesyl group; FPP, farnesyl pyrophosphate; CVIM, tetrapeptide Cys-Val-Ille-Met; E, FTase.

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