Influence of metal ions on substrate binding and catalytic activity of mammalian protein geranylgeranyltransferase type-I

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Protein geranylgeranyltransferase type-I (GGTase-I) transfers a geranylgeranyl group from the prenyl donor geranylgeranyl diphosphate (GGPP) to the cysteine residue of substrate proteins containing a C-terminal CaaX-motif (a sequence motif of proteins consisting of an invariant Cys residue fourth from the C-terminus). The GGTase-I heterodimer contains one atom of zinc, and this metal is required for enzyme activity. In this regard, GGTase-I is similar to the related enzyme protein farnesyltransferase (FTase); the latter enzyme also requires Mg^{2+} for activity. The current studies were undertaken in an attempt to explore further the role of bivalent metal ions in the activity of GGTase-I. Surprisingly, we found that GGTase-I and FTase have different metal requirements. Specifically, in marked contrast to FTase, GGTase-I does not require Mg^{2+} for activity.

Direct binding assays, including a novel fluorescence-based technique, were employed to obtain quantitative information on the interaction of substrates with GGTase-I. Using these assays, we demonstrate that the Zn^{2+} in GGTase-I is required for peptide, but not for isoprenoid, substrate binding. Moreover, binding of GGPP protects GGTase-I from inactivation by zinc-chelating reagents; this protective effect is not seen with binding of peptide substrates. Metal substitution studies show that the Zn^{2+} in GGTase-I can be replaced by Cd^{2+} , and that the Cd form of GGTase-I has altered specificity with regard to utilization of both peptide and isoprenoid substrates. The significance of these findings in relation to proposed mechanisms for the GGTase-I reaction is discussed.

INTRODUCTION

Protein geranylgeranyltransferase type-I (GGTase-I) transfers a geranylgeranyl group from the prenyl donor geranylgeranyl diphosphate (GGPP) to a conserved cysteine residue fourth from the C-terminus of substrate proteins containing the so-called CaaX-motif [1–3]. Known targets of GGTase-I include most γ subunits of heterotrimeric G-proteins and many Ras-related small GTP-binding proteins, such as members of the Rac, Rap and Rho families [4–7]. Short peptides encompassing the CaaX motif of these substrates can also be recognized by the enzyme [2,3], and immobilization of such peptides has been used to produce affinity columns for isolation of the enzyme [8,9].

The properties of GGTase-I are similar to those of a related enzyme, protein farnesyltransferase (FTase), which also utilizes substrate proteins possessing a C-terminal CaaX motif [1,10,11]. The 'X' residue of this CaaX motif is, in general, responsible for recognition of particular substrate proteins by either FTase or GGTase-I; FTase prefers Met, Ser, Gln or Ala at this position while GGTase-I prefers Leu [1–3]. GGTase-I is distinct from the related enzyme protein geranylgeranyltransferase type-II (GGTase-II), also known as Rab geranylgeranyltransferase [11–13]. GGTase-II modifies small GTP-binding proteins of the Rab family, most of which contain two Cys residues at or near their C-termini [7,12,13].

Purified GGTase-I contains two subunits with molecular masses of 48 and 43 kDa, termed the α and β subunits respectively [8,9]. FTase also has an $\alpha\beta$ structure [10], and the α subunit of GGTase-I is identical with the α subunit of FTase [14,15]. cDNA clones of the α subunit have been isolated from rat, bovine brain and human cDNA libraries [16–18]. The mammalian α subunit has 377 amino acid residues and a calculated molecular mass of

44 kDa, and it shows about 30 % identity with its *Saccharomyces cerevisiae* counterpart encoded by a gene known as *ram2* [19,20]. cDNAs encoding the β subunits of mammalian GGTase-I and FTase have been isolated from rat and human libraries [15,17,21]. The rat GGTase-I β subunit has 377 amino acid residues and shares 31 % identity with the β subunit of rat FTase, which contains 437 residues [15,21]. The *S. cerevisiae* homologues of the mammalian GGTase-I and FTase β subunits have been identified as the products of the *cdc43/cal1* and *ram1/dpr1* genes respectively [15,16,18,21–23].

GGTase-I and FTase are both zinc metalloenzymes. Prolonged dialysis against chelating reagents inactivates both enzymes completely, and activity can be restored by adding Zn^{2+} [8,24]. Additionally, FTase requires Mg^{2+} for enzymic activity [8,24]. Analysis of both enzymes by atomic absorption spectroscopy revealed that GGTase-I and FTase contain one zinc atom per dimer [25,26]. While potential roles of the zinc atom in catalysis by these enzymes has not yet been defined, in the case of FTase available data indicate that the zinc is not required for isoprenoid substrate binding but is required for binding of the protein substrate [24]. Additionally, Mn^{2+} and Cd^{2+} were recently reported to substitute for Zn^{2+} in restoring activity to metal-depleted GGTase-I [27], but the properties of Mn^{2+} - and Cd^{2+} -substituted enzymes were not characterized. In terms of their kinetic mechanisms, both enzymes can bind either substrate in the absence of the second substrate, although binding of isoprenoid substrate first is the preferred (and perhaps obligatory in the case of FTase, see [28]) path to catalysis [26–29]. The precise chemical mechanism has not yet been defined for either enzyme, although recent reports have provided evidence for an electrophilic-type mechanism for FTase [30,31].

The current studies were undertaken in an attempt to explore

Abbreviations used: FTase, farnesyltransferase; FPP, farnesyl diphosphate; GGTase-I, geranylgeranyltransferase type-I; GGTase-II, geranylgeranyltransferase type-II; GGPP, geranylgeranyl diphosphate; CaaX, a sequence motif of proteins consisting of an invariant Cys residue fourth from the C-terminus; Ras-CVLL, H-Ras protein with a Leu-for-Ser substitution at the C-terminus; DTT, dithiothreitol.

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further the role of Zn^{2+} and Mg^{2+} in catalysis by GGTase-I. Surprisingly, we have found that GGTase-I and FTase have different metal requirements. Unlike FTase, GGTase I does not require Mg^{2+} for activity. This finding raised the question of whether the role of Zn^{2+} in GGTase-I was different from its role in FTase. Using direct-binding assays, we demonstrate that, similar to FTase, the presence of Zn^{2+} in GGTase-I is required for peptide, but not for isoprenoid, substrate binding. Moreover, binding of GGPP to GGTase-I protects the enzyme from inactivation by metal-chelating reagents, an effect not seen for binding of peptide substrates. We also confirm that Cd^{2+} can restore activity to metal-depleted GGTase-I, although the effect was not observed with either Mn^{2+} or several other bivalent metal ions. Furthermore, Cd-substituted GGTase-I has altered specificities with regard to utilization of both peptide and isoprenoid substrates compared with GGTase-I containing Zn^{2+} . The significance of these findings in relation to proposed mechanisms for the GGTase-I reaction is discussed.

EXPERIMENTAL

Preparation of metal-depleted GGTase-I and FTase

Purified recombinant GGTase-I and FTase (~1 mg of each) were dialysed for 24 h at 4 °C against 1 liter of 50 mM Tris/HCl (pH 7.8)/1 mM dithiothreitol (DTT)/5 mM EDTA, followed by an additional 24 h dialysis against the same buffer, except that the EDTA concentration was reduced to 0.1 mM. Removal of zinc from the dialysed enzymes (designated as apo-enzymes) was confirmed by assessing the zinc-dependence of their activities [8]. The prepared apo-enzymes were aliquoted, flash-frozen in liquid N_2 and stored at -80 °C until use.

Treatment of protein substrates to remove metal ions

Potential contaminating metals were removed from recombinant H-Ras (designated Ras-CVLS) and Ras-CVLL (a H-Ras protein with a C-terminal leucine-for-serine substitution), the protein substrates routinely used for FTase and GGTase-I respectively [8,26], by dialysis for 24 h against 20 mM Tris/HCl (pH 7.8)/100 mM KCl/1 mM DTT/30 μM GDP and 0.2 mM EDTA; the buffer was changed once during the period. The dialysed proteins were aliquoted, flash-frozen in liquid N_2 and stored at -80 °C until use.

Determination of GGTase-I and FTase activity

GGTase-I activity was routinely determined by the method described previously [26], except that MgCl_2 was omitted from the reaction mixtures unless otherwise noted. The standard reaction mixture contained 50 mM Tris/HCl (pH 7.8), 1 mM DTT, 20 mM KCl, 25 mM ZnCl_2 , 0.5 mM Zwittergent 3-14, 3 μM Ras-CVLL, 0.5 μM [^3H]GGPP (8 Ci/mmol) and 75 ng of purified recombinant GGTase-I in a final volume of 50 μl . FTase activity was determined as previously described [25] except that 0.5 mM Zwittergent 3-14 was included in the reaction mixture. The reaction mixture was the same as for GGTase-I except that the protein and isoprenoid substrates were Ras-CVLS and [^3H]farnesyl diphosphate ([^3H]FPP) respectively, and the mixture also contained 5 mM MgCl_2 unless otherwise noted. Assays for both enzymes were conducted at 37 °C for 15 min.

Preparation of dansyl-GCVLL-GGTase-1 complex

A 100 μl reaction mixture containing fluorescent peptide analogue dansyl-GCVLL (10 μM) was incubated with apo-GGTase-I (4 μM) for 5 min at 37 °C in 50 mM Tris/HCl (pH 7.8)/100 mM

KCl/1 mM DTT/0.2 % (v/v) octyl β -glucopyranoside (buffer A) containing either 50 μM EDTA or 50 μM ZnCl_2 as indicated in the legend of Figure 2(B). Following incubation, the dansyl-GCVLL-GGTase-I complex was separated from free peptide by chromatography through a 2.5 ml column of G-50 Sephadex that had been equilibrated with buffer A containing either EDTA or ZnCl_2 at the concentration used in the initial incubation. The column was eluted with repeated additions of 0.5 ml portions of the same buffer. The fluorescence intensities of each fraction were measured at 560 nm and 496 nm using an AmincoBowman Series 2 luminescence spectrometer. The excitation wavelength was 340 nm, and the halfwidths of the excitation and emission beams were 4 nm and 8 nm respectively. The sensitivity for the measurement was 800 V.

Measurement of the dissociation constant of the dansyl-GCVLL-GGTase-I complex

Both apo-GGTase-I and the same preparation after reconstitution with Zn^{2+} (0.2 μM of each), were incubated with increasing amounts of dansyl-GCVLL at room temperature in a final volume of 500 μl , and the increase in the fluorescence intensity at 496 nm was recorded. Values obtained were corrected for background values determined by incubating the peptide in the presence an identical quantity of BSA. Protein-bound fluorescence was determined and the results processed by the Klotz method, as described in [32], to obtain peptide-GGTase-I dissociation constants. The luminescence spectrometer parameters were the same as described above.

Preparation of GGPP-GGTase-I complex

Apo-GGTase-I (0.8 μM) was incubated with [^3H]GGPP (1.6 μM) at 37 °C for 5 min in 50 μl of 50 mM Tris/HCl (pH 7.8)/20 mM KCl/1 mM DTT/0.2 % (v/v) octyl β -glucopyranoside (buffer B) containing either 50 μM EDTA or 50 μM ZnCl_2 as described in the legend of Figure 3(A). Following incubation, samples was applied to a 2.5 ml Sephadex G-50 column that had been equilibrated at room temperature with buffer B containing either EDTA or ZnCl_2 as in the incubation. The column was eluted with repeated additions of 0.15 ml portions of the same buffer, and the radioactivity in each fraction was determined by liquid scintillation spectroscopy.

Measurement of dissociation constants of the GGPP-GGTase-I complexes

Apo-GGTase-I and the Zn-form (both at 13 nM) were incubated with increasing concentrations of [^3H]GGPP in 50 mM Tris/HCl (pH 7.8)/100 mM KCl/50 mM EDTA/0.2 % (v/v) octyl β -glucopyranoside in a final volume of 50 μl at room temperature for 15 min. Incubation mixtures were then subjected to vacuum filtration through nitrocellulose filters and the filters were then washed with 10 ml of ice-cold 50 mM Tris/HCl (pH 7.8)/100 mM KCl/1 mM EDTA/0.2 % (v/v) octyl β -glucopyranoside. Radioactivity retained on the filters was determined by liquid scintillation spectroscopy. Data were analysed as described above for peptide binding to obtain dissociation constants.

Metal chelation experiments

GGTase-I was incubated in 50 mM Tris/HCl (pH 7.8)/20 mM KCl and either 10 mM 1,10-phenanthroline (at 4 °C) or 50 mM EDTA (at 20 °C) both in the absence of substrates and in the presence of substrates and substrate analogues as noted in

Figure 4. At the indicated times, aliquots were withdrawn, diluted with 50 mM Tris/HCl (pH 7.8)/20 mM KCl/0.5 mM Zwittergent 3-14, and a portion containing 150 ng of the enzyme was assayed for GGTase-I activity by the method described above.

Reconstitution of apo-GGTase-I with bivalent metal ions

Apo-GGTase-I (4 μ l at 1.2 mg/ml) was diluted 1:1 with a solution containing each of the metal chlorides examined (CoCl_2 , MnCl_2 , NiCl_2 , CdCl_2 , HgCl_2 or ZnCl_2), all at 166 μ M, for 5 min at 37 °C. The free-metal concentration of the resultant reconstitution reactions was 33 μ M. The reconstitution solutions were then diluted in 50 mM Tris/HCl (pH 7.8)/20 mM KCl/1 mM DTT/50 M EDTA and assayed for GGTase-I activity; the EDTA was included in the dilution buffer to prevent exogenous metals (e.g. Zn^{2+} contamination of buffers) from influencing enzyme activity.

Prenylation of peptide substrates

To assess prenylation of synthetic peptides, the peptides indicated in the legend of Figure 5 (4 μ M) were incubated with either Cd- or Zn-GGTase-I (both at 150 ng) in the standard reaction mixture for 15 min at 37 °C. Following incubation, an aliquot of the reaction mixture was spotted on a silica-gel thin-layer plate (Si250-PA, Baker) and developed for 3 h in n-propyl alcohol/ammonium hydroxide/water (6:3:1, by vol.) as described [33]. After drying, TLC plates were sprayed with EN3HANCE (Dupont) and exposed to X-ray film for detection of radiolabelled reaction products.

Miscellaneous methods and materials

Both the GGTase-I and FTase used in this study were recombinant proteins produced by infection of Sf9 cells with recombinant baculoviruses and purified from cell extracts as described previously [25,26]; both enzymes were > 95 % pure as judged by SDS/PAGE analysis. Ras-CVLS and Ras-CVLL were purified from bacterial expression systems as described [2]. [$1\text{-}^3\text{H}$]GGPP (66.6 Ci/mmol) and [$1\text{-}^3\text{H}$]FPP (66.6 Ci/mmol) were obtained from American Radiolabeled Chemicals. The stock solutions of [^3H]FPP and [^3H]GGPP (20 μ M) were prepared in 20 mM Tris/HCl containing 3 mM Zwittergent 3-14 to facilitate manipulation of the isoprenoid [26]. Peptides were synthesized on an Applied Biosystems Synergy Synthesizer and HPLC-purified prior to use. The dansyl-GCVLL peptide was a gift from Dr. S. Kerwar (Lederle Laboratories, Pearl River, NY, U.S.A.). Ultrapure Tris(hydroxymethyl)-aminomethane, potassium chloride, manganese(II) chloride tetrahydrate and magnesium chloride were obtained from Fluka. Zinc chloride was obtained from Fisher Scientific. Cadmium chloride (99.99 %), cobalt chloride (99.999 %), nickel chloride (99.999 %) and mercury chloride (99.999 %) were obtained from Aldrich. 1,10-Phenanthroline was obtained from Sigma, and a stock solution of 500 mM was prepared in 50 % ethanol. All water used in this study had a resistivity of $\sim 18 \text{ M}\Omega/\text{cm}$. Protein was routinely analysed by the Bradford method [33a] using a commercial dye preparation (Bio-Rad) and BSA as the standard.

RESULTS

GGTase-I does not require Mg^{2+} for enzymic activity

Previous studies have indicated that metal-depleted FTase (apo-FTase), prepared from enzyme purified from tissues, required

Figure 1 Metal requirements of FTase and GGTase-I

(A) Reconstitution of apo-FTase and apo-GGTase-I activities by bivalent metals. Apo-FTase (○, ●) and apo-GGTase-I (▽, ▼) (75 ng of each), prepared by extensive dialysis against EDTA as described in the Experimental procedures section were incubated in the assay mixture in the presence of the indicated amounts of ZnCl_2 either with (●, ▼) or without (○, ▽) 1 mM MgCl_2 . EDTA (14 μ M) was present in the assay mixture. (B) Effect of added MgCl_2 and EDTA on activities of FTase and GGTase-I holoenzymes. FTase (○, ●) or GGTase-I (▽, ▼) (75 ng of each) that had not been subjected to metal depletion was assayed in the presence of the indicated amounts of either MgCl_2 (●, ▼) or EDTA (○, ▽). For the experiments in both (A) and (B), assays were conducted as described in the Experimental procedures section, and metal-depleted Ras-CVLS and Ras-CVLL were used as the protein substrates for FTase and GGTase-I respectively. Data shown represent the mean of duplicate determinations from a single experiment, which is representative of two such experiments.

both Zn^{2+} and Mg^{2+} to restore activity [8,24]. We repeated this analysis using recombinant FTase and obtained essentially identical results (Figure 1A). In the absence of Mg^{2+} , addition of Zn^{2+} alone to apo-FTase does not restore activity, however inclusion of 1 mM Mg^{2+} in addition to Zn^{2+} fully restores activity. Surprisingly, when the same experiment was performed using apo-GGTase-I, it was found that Zn^{2+} alone could restore activity of this enzyme (Figure 1A). In the absence of Mg^{2+} , GGTase-I activity was fully restored by inclusion of 20 μ M Zn^{2+} in the assay. Much less Zn^{2+} was required to restore GGTase-I activity in the presence of 1 mM Mg^{2+} ; however the difference in Zn^{2+} requirement in the presence and absence of Mg^{2+} can be attributed to the presence of $\sim 14 \mu$ M EDTA in the assay reactions, rather than a direct affect of Mg^{2+} on the reaction (see below).

The distinct metal requirements of GGTase-I and FTase were confirmed using the holo-enzymes in assays conducted in the presence of varying amounts of EDTA and Mg^{2+} (Figure 1B). Inclusion of EDTA in the assay mixtures does not remove the

Figure 2 Influence of zinc on peptide substrate binding to GGTase-I

(A) Fluorescence enhancement accompanying dansyl-GCVLL binding to GGTase-I. The fluorescence emission spectra of dansyl-GCVLL alone and the dansyl-GCVLL-GGTase-I complex (both at $0.1 \mu\text{M}$) are shown. (B) Gel-filtration analysis of the formation of the dansyl-GCVLL-GGTase-I complex. A mixture of dansyl-GCVLL ($10 \mu\text{M}$) and apo-GGTase-I ($4 \mu\text{M}$) was incubated either in the presence of $50 \mu\text{M}$ EDTA (◆) or $50 \mu\text{M}$ ZnCl_2 (■). Specificity was assessed by also performing the binding analysis in the presence of $100 \mu\text{M}$ tetrapeptide inhibitor CVFL (●). Mixtures were loaded onto a G-50 Sephadex column, and eluted as described in the Experimental procedures section. Fractions of 0.5 ml were collected and measured for fluorescence intensity at $\lambda = 560 \text{ nm}$. (C) Equilibrium binding analysis of dansyl-GCVLL binding to GGTase-I. The indicated amounts of dansyl-GCVLL were incubated with either apo-GGTase I (■) or Zn-GGTase-I (◆) (both at $0.2 \mu\text{M}$) at room temperature for 10 min in a final volume $500 \mu\text{l}$. The fluorescence intensity at $\lambda = 496 \text{ nm}$ was measured. Specificity was assessed by replacing the GGTase-I with the same amount of BSA (●); the difference curve between Zn-GGTase-I and BSA is also shown (▼). The fluorescence of samples was measured as described in the Experimental procedures section. For (B) and (C) the data shown represent the means of duplicate determinations from a single experiment, which is representative of two such experiments.

zinc from either GGTase-I or FTase during the 15 min assay (results not shown). Inclusion of EDTA in the FTase assay completely abolishes its activity, and activity can be restored, in a dose-dependent fashion, by the addition of Mg^{2+} (Figure 1B). In contrast, neither EDTA nor Mg^{2+} significantly affects the activity of GGTase-I under identical assay conditions (Figure

1B). These results indicate that FTase requires Mg^{2+} for activity while GGTase-I does not. Similar results were also obtained when these experiments were repeated using FTase and GGTase-I purified from bovine brain (results not shown), indicating that the effects are not due to some altered property of the recombinant enzymes.

Peptide substrate binding to GGTase-I requires Zn^{2+}

The presence of Zn^{2+} in FTase has been shown to be required for cross-linking of the protein substrate Ras-CVLS to the enzyme [24], but direct binding of the protein substrate to FTase was not examined and no quantitative data were reported. To examine this property directly with GGTase-I and to obtain quantitative data, we developed a direct binding assay to measure peptide substrate binding to the enzyme. The dansylated peptide, dansyl-GCVLL, is a substrate of GGTase-I [34]. Binding of dansyl-GCVLL peptide to GGTase-I resulted in a ~ 10 -fold enhancement in its fluorescence intensity and a shift from 560 nm to 496 nm in its emission maximum (Figure 2A). This property allowed the use of fluorescence enhancement as a direct assay to measure peptide binding. To determine the Zn^{2+} requirement for binding of the dansylated peptide, apo-GGTase-I was incubated with dansyl-GCVLL in the presence of either EDTA or Zn^{2+} . Enzyme-bound peptide was then separated from free peptide by gel filtration and the fluorescence of each fraction determined (Figure 2B, and see also Figure 2A). The results from this analysis revealed that GGTase-I reconstituted with Zn^{2+} binds substantially more of the dansylated peptide than does the apo-enzyme. The specificity of dansyl-GCVLL binding to GGTase-I was demonstrated by conducting the binding assay in the presence of the tetrapeptide CVFL, an inhibitor of GGTase-I that is competitive with protein substrates [26]. Inclusion of the inhibitory peptide in the reaction reduced the binding of the dansylated peptide to essentially the same low level as that observed when using apo-GGTase-I (Figure 4B).

In order to quantify the binding affinity of the dansylated peptide substrate to GGTase-I, a direct method, exploiting the fluorescence enhancement noted above, was used. Titration of both apo-GGTase-I and Zn-GGTase-I with increasing amounts of dansyl-GCVLL led to an increase in fluorescence at 496 nm (Figure 2C, and see also Figure 2A). In the presence of apo-GGTase-I, the increase in fluorescence is quite low and is linearly related to peptide concentration; similar enhancement was seen when apo-GGTase-I was replaced by BSA, indicating that this is a non-specific response. In the presence of increasing concentrations of Zn-GGTase-I, however, the fluorescence intensity is increased in a saturable fashion that levels off with a slope parallel to that obtained in the presence of BSA and apo-GGTase-I (Figure 2C). The plot of the difference between the fluorescence enhancement obtained using Zn-GGTase-I versus BSA reveals saturable binding of the dansylated peptide substrate, with a calculated dissociation constant of $0.11 \mu\text{M}$. These results confirm the Zn^{2+} requirement for binding of the peptide substrate to GGTase-I and provide a direct assessment of peptide-binding affinities to the enzyme.

GGPP binding to GGTase-I is not markedly influenced by Zn^{2+}

FPP binding by FTase does not require either Zn^{2+} or Mg^{2+} [24]. To test the metal requirements for GGPP binding by GGTase-I, [^3H]GGPP was incubated with apo-GGTase-I in the presence of either EDTA or Zn^{2+} and enzyme-bound isoprenoid was

Figure 3 Influence of zinc on GGPP binding to GGTase-I

(A) Isolation of the GGPP–GGTase-I complex by gel filtration. Apo-GGTase-I ($0.8 \mu\text{M}$) was incubated with [^3H]GGPP ($1.6 \mu\text{M}$) in the presence of either $50 \mu\text{M}$ EDTA ($\circ$) or $50 \mu\text{M}$ ZnCl_2 ($\bullet$). Specificity was assessed by replacing the GGTase-I with the same amount of BSA (∇). Mixtures were loaded onto a G-50 column and eluted as described in the Experimental procedures section. Fractions of 0.15 ml were collected and their radioactivity determined. (B) Equilibrium binding analysis of GGPP binding to GGTase-I. Apo-GGTase I ($\circ$) and Zn-GGTase I ($\bullet$) (both at 13 nM) were incubated with the indicated amounts of [^3H]GGPP at room temperature for 15 min . Enzyme-bound isoprenoid was then determined using the filter binding described in the Experimental procedures section. For both (A) and (B) the data shown represent the means of duplicate determinations from a single experiment, which is representative of two such experiments.

separated from free isoprenoid by gel-filtration chromatography. The results of this analysis are shown in Figure 3A. The quantity of enzyme-bound GGPP was essentially the same regardless of whether Zn^{2+} was included in the binding reaction. In the control experiment, replacement of GGTase-I with BSA resulted in no detectable binding of [^3H]GGPP, demonstrating the specificity of the observed binding. These results demonstrate that Zn^{2+} is not required for GGPP binding by GGTase-I.

A more quantitative measurement of GGPP binding to GGTase-I was performed by an equilibrium-binding method. Apo-GGTase-I and Zn-GGTase-I were incubated with increasing amounts of [^3H]GGPP, and the bound GGPP was determined by a filter-binding method (see the Experimental procedures section). The results shown in Figure 3(B) reveal that there is essentially no difference in the binding affinity of GGPP to either apo-GGTase-I or Zn-GGTase-I; analysis of the data yielded dissociation constants of 7.3 nM and 8.9 nM for apo-GGTase-I and Zn-GGTase-I respectively. Therefore, there is no appreciable effect of Zn^{2+} on the dissociation constant of the GGPP–GGTase-I complex. Table 1 summarizes the binding constants determined for interaction of both isoprenoid and peptide substrates of

Table 1 Dissociation constants of substrate complexes with GGTase-I

Enzyme form	K_d	
	Dansyl-GCVLL	GGPP
Apo-GGTase-I	$> 13 \mu\text{M}$	7.3 nM
Zn-GGTase-I	$0.11 \mu\text{M}$	8.9 nM

Figure 4 Inactivation of GGTase-I by metal-chelating reagents

(A) Inactivation by 1,10-phenanthroline. Holo-GGTase-I ($14 \mu\text{M}$) was incubated with 10 mM phenanthroline either in the absence of substrates (∇ , control) or in the presence of $50 \mu\text{M}$ GGPP ($\bullet$) or $50 \mu\text{M}$ TKCVIL ($\blacktriangle$) at 4°C . At the indicated times, aliquots were withdrawn, diluted with 50 mM Tris/HCl ($\text{pH } 7.8$)/ 20 mM KCl/ 0.5 mM Zwittergent 3-14/ 1 mM MgCl_2 , and 150 ng of enzyme was assayed for activity. Data shown represent the mean of four determinations from two separate experiments. (B) Inactivation by EDTA. Incubations were conducted as in (A) except that the GGTase-I concentration in the preincubation was $3 \mu\text{M}$ and phenanthroline was replaced by 50 mM EDTA. The incubation temperature was 20°C . For both (A) and (B), the standard assay described in the Experimental procedures section was used. Data shown represent the means of duplicate determinations from a single experiment which is representative of three such experiments.

GGTase-I with both apo and Zn^{2+} -containing forms of the enzyme.

Inactivation of GGTase-I by metal-chelating reagents

Incubation of GGTase-I with 10 mM 1,10-phenanthroline, a commonly-used Zn^{2+} -chelating reagent, led to a time-dependent inactivation of the enzyme (Figure 4A). Addition of Zn^{2+} to the

phenanthroline-inactivated enzyme fully restored its activity (results not shown), indicating that the inactivation is due to depletion of Zn^{2+} from the enzyme. Given the Zn^{2+} requirement for GGTase-I in binding peptide substrates, we considered it likely that binding of peptide substrate to the enzyme might protect it from inactivation by phenanthroline, particularly if the -SH group of the Cys residue of the peptide directly coordinated to the metal atom, as has been suggested for FTase [24]. To test this hypothesis, GGTase-I was incubated with 1,10-phenanthroline in the absence or presence of either the peptide substrate TKCVIL or the isoprenoid substrate GGPP, and the enzyme activity was assessed. However, rather than reduce the rate of inactivation, inclusion of the peptide substrate slightly increased the rate of inactivation by the chelating reagent, while, conversely, inclusion of GGPP markedly reduced the rate of inactivation (Figure 4A). When the data were fitted to a first-order kinetic equation, the inactivation rate constant in the absence of either substrate was determined to be 0.53 h^{-1} , while the presence of the peptide substrate increased the inactivation rate constant 2-fold to 1.0 h^{-1} and GGPP reduced it 4-fold to 0.14 h^{-1} . To examine the specificity of these effects, two related compounds that are not substrates of GGTase-I, the peptide TKSUIL and the isoprenoid FPP, were used in the same type of experiment; neither of these compounds had an appreciable effect on the inactivation process (results not shown). However, two inhibitors of GGTase-I that are competitive with substrates, the isoprenoid 3-aza-GGPP and the peptide CVFL, had similar effects on the inactivation process as the substrates GGPP and TKCVIL respectively (results not shown).

One concern with the preceding experiments was that the hydrophobic phenanthroline interacted directly with GGTase-I in such a way as to influence the release of the bound metal ion. To provide confirmation for the finding that GGPP protects GGTase-I from inactivation by chelating agents, we also performed the studies using EDTA as the chelating reagent. EDTA-mediated inactivation of GGTase-I closely resembled that mediated by phenanthroline (Figure 4B), and again addition of Zn^{2+} to the inactivated enzyme fully restored its activity (results not shown). The overall results of this experiment were similar to those for phenanthroline, in that inclusion of GGPP markedly reduced the inactivation of GGTase-I while the hexapeptide substrate TKCVIL had relatively little influence on the process. Additionally, incubation with the peptide substrate alone (i.e. in the absence of any additional chelating agent) did not influence the inactivation of the enzyme (results not shown). Taken together, these results of the experiments shown in Figure 4 indicate that GGPP binding, but not that of a peptide substrate, dramatically reduces the dissociation of the zinc from GGTase-I.

Reconstitution of apo-GGTase-I activity with Cd^{2+}

As noted in the Introduction, both Cd^{2+} and Mn^{2+} , in addition to Zn^{2+} , have been reported to restore activity to metal-depleted GGTase-I [27]. Since our finding that the binding of the isoprenoid substrate can influence the dissociation of Zn^{2+} from GGTase-I (see above) suggested that the metal ion and isoprenoid substrate can influence each other, we wanted to assess the substrate specificity parameters of enzymes containing metals other than Zn^{2+} . Our first experiments were designed to confirm the finding that other bivalent metals could in fact substitute for Zn^{2+} in supporting GGTase-I activity. To circumvent problems with metal contamination of solutions employed in the reconstitution experiments, a relatively high concentration of apo-GGTase-I ($\sim 7 \mu\text{M}$) was used for in the reconstitution

Table 2 Reconstitution of apo-GGTase I by bivalent metals

Apo-GGTase-I ($4.8 \mu\text{g}$) was incubated with the indicated bivalent metals (all as the chloride salts) as described in the Experimental procedures section. Following incubation, enzyme activity was determined in the absence of any additional metal ions. Data shown represent the means of duplicate determinations from a single experiment, which is representative of two such experiments.

Metal in reconstitution	Activity ($\text{nmol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$)
None	4.6
Zn^{2+}	88.0
Co^{2+}	5.9
Mn^{2+}	7.6
Ni^{2+}	5.2
Cd^{2+}	85.0
Mg^{2+}	4.9

protocol, and the enzyme obtained was assayed in the presence of $50 \mu\text{M}$ EDTA. Using this method, we found that only Zn^{2+} and Cd^{2+} could restore activity to apo-GGTase I, while Co^{2+} , Mn^{2+} , Ni^{2+} and Hg^{2+} could not (Table 2). We cannot rule out the possibility that Mn^{2+} could in fact replace the zinc, but that its binding was so weak that it was removed in the subsequent dilution in the presence of EDTA. Nonetheless, the Cd^{2+} result reported previously was confirmed, and allowed us to assess the specificity parameters of this form of the enzyme (Cd-GGTase-I). The Cd-GGTase-I produced had a specific activity essentially equal to that of Zn-GGTase-I under our standard assay conditions (Table 2).

Substrate specificity of Cd-GGTase-I

The specificity of substrate utilization by Cd-GGTase-I was initially examined using our standard substrates for the GGTase-I and FTase reactions, namely FPP and GGPP as the isoprenoid and Ras-CVLL and Ras-CVLS as the protein substrates. Cd-GGTase-I was similar to Zn-GGTase-I in its ability to catalyse geranylgeranylation of Ras-CVLL, and also, like Zn-GGTase-I, its ability to catalyse farnesylation or geranylgeranylation of Ras-CVLS was quite low (Figure 5A). However, compared with Zn-GGTase I, Cd-GGTase-I was found to have markedly increased ability to utilize FPP as a substrate for farnesylation of Ras-CVLL (Figure 5A), indicating that the identity of the metal can indeed influence isoprenoid utilization by the enzyme.

We also examined the specificity of Cd-GGTase-I using peptide substrates. In these analyses, enzymic activity was assessed by detecting production of radiolabelled prenylated peptide which can be detected by TLC. Three tetrapeptides were chosen for this study. Two of these peptides, CVFL and CVFM, are dead-end substrates for GGTase-I and FTase respectively [26,33], while the third peptide, CAIL, consists of a CaaX sequence which is known to be a good substrate for GGTase-I [1]. Both the Zn and Cd forms of GGTase-I effectively utilized CAIL as a substrate (Figure 5B), as expected. Surprisingly, however, both CVFL and CVFM were also efficiently geranylgeranylated by Cd-GGTase-I, but not by Zn-GGTase-I (Figure 5B). Densitometric quantification of the radiolabelled products visualized by autoradiography revealed that geranylgeranylation of CVFL and CVFM by Cd-GGTase-I was 7-fold higher than by Zn-GGTase-I (Figure 5C), indicating that the type of metal in the enzyme can also influence its ability to recognize a peptide substrate in a catalytically competent fashion.

Figure 5 Substrate specificities of Zn-GGTase-I and Cd-GGTase-I

(A) Analysis using prenyldiphosphates and protein substrates. The activities of both Zn-GGTase-I (solid bars) and Cd-GGTase-I (hatched bars) were examined using different combinations of the standard isoprenoid and protein substrates used in GGTase-I and FTase assays (i.e. FPP, GGPP, Ras-CVLS, Ras-CVLL) as indicated. Assays were conducted as described in the Experimental procedures section. (B) Analysis using tetrapeptides. The ability of Zn-GGTase-I and Cd-GGTase-I to use the tetrapeptides CVFL, CVFM and CVLL as substrates was determined using the TLC-based assay described in the Experimental procedures section. The respective enzyme and substrate under the various conditions analysed are indicated. An autoradiogram of the TLC plate is shown. (C) Quantification of the activity of Zn-GGTase-I and Cd-GGTase-I using peptide substrates. The autoradiogram shown in (B) was processed by densitometric quantification. Enzyme activity under the indicated conditions is shown. For (A), (B) and (C) the data shown represent the means of duplicate determinations from a single experiment, which is representative of two such experiments.

DISCUSSION

The results obtained from this study show that although FTase and GGTase-I have similar primary sequences, substrates and enzymic properties, the two enzymes differ in their specific metal requirements. While FTase requires Mg^{2+} for activity, this metal is not required for, nor does it seem to influence, GGTase-I activity. These results are in contrast with previous reports from both this and another laboratory that indicated an Mg^{2+} requirement for activity of both enzymes [8,27]. In the case of [8], we have determined that the discrepancy is due to the assay methods employed in the previous study, in which assays using metal-depleted enzymes were conducted with excess EDTA in the assay that chelated the added Zn^{2+} . The activity restoration seen upon addition of Mg^{2+} was actually due to its ability to tie up the EDTA and thereby allow restoration of the activity of GGTase-I by added Zn^{2+} (F. L. Zhang and P. J. Casey, un-

published observations). The modified assay employed in this study (see the Experimental procedures section) effectively circumvented this problem, and has allowed an unambiguous determination of the Mg^{2+} requirements (i.e. the lack thereof) for GGTase-I activity.

Mg^{2+} is required for many enzymic reactions, and the metal can play both structural and catalytic roles in enzyme action [35]. While we cannot yet ascribe one or the other of these roles to Mg^{2+} with regard to activities of the CaaX prenyltransferases, the metal is certainly important in the functioning of FTase. Moreover, since GGTase-I does not require this metal, even though it catalyses a reaction almost identical with that of FTase, it seems likely that the role of Mg^{2+} in the action of FTase is distinct from one involving an interaction with the diphosphate moiety of the isoprenoid substrate. This assessment is also consistent with the finding that high-affinity binding of FPP by FTase is independent of the presence of Mg^{2+} [36]. Further analysis of the reaction mechanisms of FTase and GGTase-I may lead to a better understanding of the roles of Mg^{2+} in enzymic reactions.

We have confirmed a previous finding [27] that Cd^{2+} can replace Zn^{2+} in restoring activity to metal-depleted GGTase-I. Since the properties of Cd^{2+} are very similar to those of Zn^{2+} , it is likely that Cd^{2+} binds to the same site that is normally occupied by Zn^{2+} in the enzyme. Other metals tested (Co^{2+} , Mn^{2+} , Ni^{2+} , Hg^{2+}) either cannot occupy the Zn^{2+} site in a functional manner, or they may be able to occupy the site but dissociate rapidly in the presence of 50 μM EDTA. Cd^{2+} substitution for Zn^{2+} broadens the substrate specificity of GGTase-I, indicating that the metal ion may play a direct role in substrate binding and/or catalysis by the enzyme; a somewhat similar influence of Cd^{2+} -substitution on the properties of FTase has been observed [37]. The FTase study also revealed that Cd^{2+} -FTase is inactivated by EDTA more slowly than its Zn counterpart, suggesting a higher affinity of Cd^{2+} for the enzyme. The fact that Cd^{2+} has a larger ionic radius than Zn^{2+} may indicate that the former metal can interact with potential ligands more efficiently than Zn^{2+} [38]; such a property could explain both the tighter binding of Cd^{2+} to FTase observed in the previous study and the broadened substrate specificity of the Cd^{2+} enzyme compared with its Zn^{2+} counterpart seen here.

Two potential models for an interaction between substrates and the zinc atom in GGTase-I can be envisaged. The first is that one or both substrates can directly coordinate with the zinc, and the second is that substrates bind to a site distinct from that occupied by zinc. Data in support of the first model come from our finding that binding of GGPP protects GGTase-I from inactivation by metal-chelating reagents; however, this view is blunted somewhat by the data indicating that GGPP binding to the enzyme is neither dependent on, nor influenced by, the presence of Zn^{2+} . The Zn^{2+} requirement for peptide and protein substrate binding initially suggested, both to us and to others [24], that the Cys -SH group of this substrate might interact directly with the bound metal, but the data reported here, indicating that peptide substrate binding does not slow dissociation of the metal from the enzyme, suggest that this is not so, at least in the case of peptide substrate binding to free enzyme. The effect of zinc depletion on peptide substrate binding may be due to some type of conformational change that disrupts the peptide-binding site. Alternatively, there could in fact be a zinc-Cys interaction that is only manifest during the actual catalytic process. What is clear from these studies is that additional evidence is required before the precise role(s) of the zinc in catalysis by the protein prenyltransferases can be assigned. Such evidence will likely come either from detailed biophysical

studies of metal-substituted enzymes, such as those performed on a zinc metalloenzyme termed Ada that catalyses a reaction chemically similar to that of the protein prenyltransferases [39,40], or from direct crystallographic analysis of the enzymes.

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