Lysine¹⁶⁴ α of protein farnesyltransferase is important for both CaaX substrate binding and catalysis

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Protein farnesyltransferase (FTase) catalyses the formation of a thioether linkage between proteins containing a C-terminal CaaX motif and a 15-carbon isoprenoid. The involvement of substrates such as oncogenic Ras proteins in tumour formation has led to intense efforts in targeting this enzyme for development of therapeutics. In an ongoing programme to elucidate the mechanism of catalysis by FTase, specific residues of the enzyme identified in structural studies as potentially important in substrate binding and catalysis are being targeted for mutagenesis. In the present study, the role of the positive charge of Lys¹⁶⁴ of the α subunit of FTase in substrate binding and catalysis was investigated. Comparison of the wild-type enzyme with enzymes that have either an arginine or alanine residue substituted at this position revealed unexpected roles for this residue in both

substrate binding and catalysis. Removal of the positive charge had a significant effect on the association rate constant and the binding affinity of a CaaX peptide substrate, indicating that the positive charge of Lys¹⁶⁴ α is involved in formation of the enzyme (E)·farnesyl diphosphate (FPP)·peptide ternary complex. Furthermore, mutation of Lys¹⁶⁴ α resulted in a substantial decrease in the observed rate constant for product formation without alteration of the chemical mechanism. These and additional studies provide compelling evidence that both the charge on Lys¹⁶⁴ α , as well as the positioning of the charge, are important for overall catalysis by FTase.

Key words: enzyme kinetics, farnesylation, prenyltransferase.

INTRODUCTION

A number of key regulatory proteins are post-translationally modified with a 15-carbon farnesyl isoprenoid that is critical for the proper membrane localization of the protein and its cellular function. Protein farnesyltransferase (FTase) catalyses the transfer of the farnesyl group from farnesyl diphosphate (FPP) to proteins containing a C-terminal CaaX motif, where 'C' is a conserved cysteine that is the site of farnesyl modification, 'a' is usually an aliphatic amino acid, and 'X' is methionine, serine, glutamine or alanine. Substrates for FTase include several nuclear lamins, a number of proteins involved in visual signal transduction, and at least six members of the Ras family of proteins [1,2]. The role of farnesylated, activated Ras in tumour formation has led to interest in developing inhibitors of FTase as cancer chemotherapeutics [3,4]. To aid in the design of FTase inhibitors, studies have been undertaken to provide an insight into the catalytic mechanism of FTase, and the roles of specific active site residues in catalysis.

The catalytic mechanism of FTase has been studied extensively. In spite of the fact that FTase can bind either the isoprenoid or the CaaX substrate independently, the general reaction is functionally ordered, with formation of an enzyme (E)·FPP binary complex being an obligate step in the pathway to product formation [5,6]. For steady-state catalysis, product release is rate-limiting under conditions of saturating substrate concentrations; the chemical step of product formation is two to three orders of magnitude faster than the steady-state rate [6–10]. FTase is a zinc metalloenzyme [11,12], and the zinc is required for binding of the protein/peptide substrate, as well as for the chemistry aspects [9,11]. The interaction of the sulphur of the CaaX cysteine thiol with zinc in the active site lowers the pK_a of

the thiol so that a partially charged zinc-thiolate can contribute nucleophilic character to the transition state [9,13–15]. Studies with fluoromethyl-FPP analogues have revealed that the transition state also has electrophilic character, with a partial positive charge on C-1 of the farnesyl group [9,16]. Millimolar concentrations of magnesium ions are required for efficient catalysis, even though magnesium is not necessary for formation of the farnesylated product [9,12,15,17]. The magnesium ions may play an important role in catalysis by co-ordinating the non-bridging oxygen atoms of the diphosphate moiety of FPP to stabilize the leaving group [15,18].

Mutagenesis studies and crystal structures have provided an insight into the role of specific residues in the catalytic mechanism of FTase. FTase is a heterodimer consisting of a 48 kDa α subunit and a 46 kDa β subunit [1]. The β subunit structure is an α - α barrel, with a deep central cleft that forms the active site of the enzyme. The active site is near the α/β subunit interface and, while the majority of the active-site residues are derived from the β subunit, both subunits are important for substrate binding and catalysis [1,19]. Early mutagenesis studies suggested that Arg²⁰² β may play a role in the binding of, and specificity for, the CaaX substrate [20]. Recent crystal structures of FTase·isoprenoid·CaaX peptide ternary complexes have shown that Arg²⁰² β and Gln¹⁶⁷ α form hydrogen bonds with the a_2 and X residues, respectively, of the peptide Ca₁a₂X motif [18,21]. The mutagenesis studies also implicated several residues, Arg²⁹¹ β and Lys²⁹⁴ β in particular, in association of the enzyme with FPP [20]. In crystal structures of binary and ternary complexes [18,21–23], several residues were observed to interact with the diphosphate moiety of FPP, including Lys¹⁶⁴ α and Arg²⁹¹ β with the α -phosphate oxygens, and His²⁴⁸ β , Arg²⁹¹ β , Lys²⁹⁴ β and Tyr³⁰⁰ β with the β -phosphate oxygens (Figure 1). Kinetic studies have suggested

Abbreviations used: FTase, protein farnesyltransferase; FPP, farnesyl diphosphate; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; I_{ana}, (E,E)-2-(2-oxo-2-[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]amino)ethyl)phosphonic acid; dansyl-GCVLS, dansyl-Gly-Cys-Val-Leu-Ser.

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Figure 1 Interaction of active-site residues with the diphosphate of FPP

This Figure is derived from an E·FPP binary structure [22] and shows residues Lys¹⁶⁴ α , His²⁴⁸ β , Arg²⁹¹ β , Lys²⁹⁴ β and Tyr³⁰⁰ β that have been observed to hydrogen-bond (dashed lines) with the non-bridging α - and β -phosphate oxygen atoms of FPP.

that Lys¹⁶⁴ α and Tyr³⁰⁰ β are directly involved in catalysis and could act as general acids by donating protons to the diphosphate oxygens to facilitate formation of the positive charge at C-1 [24,25]. Another residue that plays an important role in catalysis is Tyr³⁶¹ β . Mutation of this residue to leucine results in an enzyme that retains efficient substrate binding and chemistry, but does not release the farnesylated product [10].

In the present study, we have investigated the role of a positive charge at position 164 of the α subunit of FTase in substrate binding and catalysis. Previous studies assessing the consequences of altering this residue employed a mutant form of the enzyme in which the lysine was replaced with asparagine and, primarily, steady-state measurements [24,25]. Therefore the precise contributions of interactions of Lys¹⁶⁴ α were difficult to interpret in terms of potential effects on the elementary steps in the catalytic mechanism. In the present study, Lys¹⁶⁴ α was changed to arginine and alanine, and the resulting enzymes were characterized kinetically. Consistent with the aforementioned reports, Lys¹⁶⁴ α is important for catalysis. However, elimination of the positive charge also had a significant effect on CaaX substrate binding, suggesting that this residue plays an important role in formation of the E·FPP·peptide ternary complex. These results indicate that the charge of Lys¹⁶⁴ α and its position in the active site are important for several key steps in the kinetic mechanism of FTase, and expand upon our knowledge of the role of individual residues in FTase catalysis.

MATERIALS AND METHODS

Site-directed mutagenesis, protein expression and protein purification

The pAlter-Ex2-H₆-FT α vector containing the FTase α subunit with an N-terminal hexa-histidine tag, which has been described previously [26], was used for the site-directed mutagenesis. Lys¹⁶⁴ α was changed to arginine (K164R) and alanine (K164A) using the Promega Altered Sites protocol and the primers 5'-AG-GAACAGCCCAGAACTATCAAGT-3' (K164R) and 5'-T-TGAGGAACAGCCCGCAAACCTATCAAGTTT-3' (K164A) (with the altered codons underlined). For the mutants, the pAlter-Ex2-H₆ plasmid containing the mutated FTase α subunit and a pET28a plasmid containing the FTase β subunit [26] were co-transformed into BL21(DE3) *Escherichia coli* cells. The

Figure 2 Structures of FTase substrates and the substrate analogue (I_{ana})

mutant enzymes were purified by Ni²⁺-nitrilotriacetate affinity chromatography, followed by a Q-HP anion-exchange FPLC column [27]. Wild-type FTase was expressed in Sf9 cells, and purified as described previously [28]. Following purification, the enzymes were concentrated to > 1 mg/ml, flash-frozen and stored at -80 °C.

FPP binding affinity

FPP binding affinity was determined using an equilibrium dialysis assay similar to procedures published previously [29]. A 1 ml sample containing 10 nM tritium-labelled FPP ([1-³H]FPP; 21 Ci/mmol) in 50 mM Tris/HCl, pH 7.7 at 4 °C, 2 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 5 mM MgCl₂, 20 mM KCl, 5 μ M ZnCl₂ and 0.2% (w/v) octyl glucoside was separated by a (1.2–1.4) $\times 10^4$ molecular-mass cut-off membrane from a 1 ml sample containing 0–200 nM FTase and 10 nM [1-³H]FPP in the same buffer. The solutions were allowed to equilibrate at 4 °C overnight, and then aliquots of 20 μ l were removed, before the radioactivity was quantified by scintillation counting. The FPP dissociation constant, K_s^{FPP} , was calculated using eqn (1):

$$([E \cdot FPP]/[FPP]_{tot}) = EP/(1 + K_s^{FPP}/[E]_{free}) \quad (1)$$

where [E·FPP] is the concentration of enzyme-bound FPP, [FPP]_{tot} is the concentration of total FPP in the enzyme chamber, EP is the maximal ratio of bound to total FPP, and [E]_{free} is the concentration of free enzyme.

Peptide binding affinity

The binding affinity of E·isoprenoid complexes for the peptide dansyl-Gly-Cys-Val-Leu-Ser (dansyl-GCVLS) was determined using a fluorescence resonance energy transfer assay [13]. The enzyme (0.02 μ M) was incubated with 0.2 μ M (*E,E*)-2-(2-oxo-2-[[[3,7,11-trimethyl-2,6,10-dodecatrienyl]oxy]amino]ethyl)-phosphonic acid (I_{ana}; Calbiochem, San Diego, CA, U.S.A. (see Figure 2), a non-hydrolysable FPP analogue, at 25 °C in 50 mM Hepes/NaOH, pH 7.7, 5 mM TCEP, 5 mM MgCl₂ and 0.01 μ M

EDTA (in a total volume of 3 ml). The dansyl-GCVLS peptide was titrated into this solution to a concentration of 0.01–1 μM, and the fluorescence (excitation wavelength 280 nm, emission wavelength 496 nm; Perkin-Elmer LS50B) was determined after a 5 min incubation. The peptide dansyl-GAVLS was used to correct for background fluorescence. The peptide dissociation constant, K_s^{pep} , was calculated using eqn (2) for K164A, or eqn (3) for wild-type FTase and K164R [30]:

$$\Delta\text{FL} = \{(\text{EP} - \text{IF}) / (1 + K_s^{\text{pep}} / [\text{dns}])\} + \text{IF} \quad (2)$$

$$\Delta\text{FL} = \text{IF} + \{(\text{EP} - \text{IF}) / 2[\text{E}]\} \{([\text{E}] + [\text{dns}] + K_s^{\text{pep}}) - ([\text{E}] + [\text{dns}] + K_s^{\text{pep}})^2 - 4[\text{E}][\text{dns}]^{1/2}\} \quad (3)$$

where ΔFL is the observed fluorescence corrected for dansyl-GAVLS background, EP is the fluorescence endpoint, IF is the initial fluorescence, and [dns] is the concentration of dansyl-GCVLS.

Steady-state and single-turnover kinetics

All steady-state parameters for wild-type FTase and the mutants, except the apparent K_m^{FPP} , were determined using the peptide GCVLS. The assays were performed at 25 °C in 50 mM Hepes/NaOH, pH 7.7, 5 mM MgCl₂, 5 μM ZnCl₂, 2 mM TCEP and 0.2% (w/v) octyl glucoside with 2 μM [1-³H]FPP (10–21 Ci/mmol), and 0.1–30 μM GCVLS. The reactions were initiated by the addition of enzyme (1–5 nM), and aliquots (7.5 μl) were quenched at various times (1–40 min) by the addition of an equal volume of 2-propanol. Products and reactants were separated by TLC on silica-gel plates using a 2-propanol/NH₄OH/water mobile phase (8:1:1, by vol.). Tritium-labelled species were visualized by autoradiography using an intensification screen (TranScreen LE; Kodak, Rochester, NY, U.S.A.) or following fluorescence enhancement (En³Hance; NEN LifeSciences, Boston, MA, U.S.A.). Regions corresponding to substrates and products were excised from the plates and the radioactivity was quantified by scintillation counting. Initial velocities were calculated from a linear fit of the data to 10% product formation on the basis of limiting substrate concentration. To determine the apparent K_m for FPP, the assays contained 5 μM H-Ras, 3–60 μM [1-³H]FPP and 0.1 nM enzyme in the above buffer. Product formation was determined using the filter-binding method described previously [31]. The steady-state rate constant, k_{cat} , and the apparent K_m values for FPP and peptide were calculated using eqn (4):

$$v/[\text{E}]_{\text{tot}} = k_{\text{cat}}[\text{S}]/([\text{S}] + K_m) \quad (4)$$

Single-turnover assays were conducted at 25 °C in 50 mM Hepes/NaOH, pH 7.7, 2 mM TCEP and 5 mM MgCl₂. The E·FPP binary complex was pre-formed by incubation of FTase with [1-³H]FPP (21 Ci/mmol), and the reaction was initiated by the addition of the peptide GCVLS so that the final concentrations were 0.2 μM FTase, 0.1 μM FPP and 0.5–300 μM GCVLS. The reactions (20 μl) were stopped at various times (from 3 s to 5 min) by the addition of an equal volume of 2-propanol, and the reaction components were separated by TLC using plastic-backed silica-gel plates and an 8:1:1 (by vol.) 2-propanol/NH₄OH/water mobile phase. The maximal rate constant for product formation at saturating peptide concentrations (100 μM GCVLS) was determined for both the wild type and the mutant enzymes using a KinTek (Austin, TX, U.S.A.) rapid quench apparatus. The reactions (30 μl) were quenched at various times (0.01–60 s) by the addition of 100 μl of 80% (v/v) 2-propanol/1 M HCl. The solutions were loaded on to glass-backed, laned silica-gel plates, and the components were separated using an 8:1:1 (by vol.) 2-propanol/NH₄OH/water mobile

phase. Following autoradiography as described above, substrate ([1-³H]FPP if the 'quencher' was 2-propanol or [1-³H]farnesol if the quencher was 2-propanol, HCl) and product ([1-³H]farnesyl-GCVLS) were quantified by scintillation counting. The percentage product formed is the ratio of the radioactivity in the product to the total radioactivity. The observed rate constant for product formation, k_{obs} , was calculated using eqn (5):

$$P_t/P_{\text{EP}} = (1 - e^{-k_{\text{obs}}t}) \quad (5)$$

where P_t is the percentage product at time t and P_{EP} is the percentage product at the endpoint of the reaction.

Miscellaneous

The peptides GCVLS and dansyl-GAVLS (> 95% pure) were obtained from Applied Analytical Industries, Inc. (Chapel Hill, NC, U.S.A.) or were synthesized using manual solid-phase techniques. The peptide dansyl-GCVLS was obtained from Commonwealth Biotechnologies, Inc. (Richmond, VA, U.S.A.) and was purified by HPLC before use. Peptide concentrations were determined either by mass or by the reaction of cysteine with 5,5'-dithiobis-(2-nitrobenzoic acid) [32]. H-Ras was expressed in *E. coli* cells, and purified as described previously [31]. Protein concentrations were determined by the Bradford assay [33] using a commercially available dye (BioRad). FPP was obtained from BioMol (Plymouth Meeting, PA, U.S.A.) and [1-³H]FPP was obtained from NEN LifeSciences. Curve fitting was performed with KaleidaGraph (Synergy Software, Reading, PA, U.S.A.). Structures of GCVLS, FPP and the I_{ana} FPP analogue are given in Figure 2.

RESULTS

Structural information on E·FPP binary and E·isoprenoid·peptide ternary complexes have suggested that several residues in the active site could be involved in catalysis. In particular, Lys¹⁶⁴α is in close proximity to C-1, the reactive carbon of the farnesyl group, and has been observed to interact with an oxygen of the α-phosphate of FPP via a hydrogen bond (Figure 1) [18,21,22]. Previous studies in which Lys¹⁶⁴α was changed to asparagine suggested that this residue is important for catalysis, but not for substrate binding [24,25]. However, these studies did not specifically address the role of the positive charge at position 164α on substrate binding and catalysis. To perform a more systematic investigation of the role of Lys¹⁶⁴α in the reaction catalysed by FTase, and to assess directly the importance of the side chain of this residue, we used site-directed mutagenesis to construct two mutants: K164R, in which the lysine was changed to arginine to maintain the positive charge, and K164A, in which the lysine was changed to alanine to eliminate the side chain altogether. An important consideration in the studies detailed below is that for mammalian FTase, the rate-limiting step in steady-state catalysis is substrate binding and product release, respectively, at subsaturating and saturating substrate concentrations [6,7]. Therefore the steady-state parameters K_m , k_{cat} and k_{cat}/K_m do not necessarily reflect the thermodynamics of substrate binding or the chemical step of product formation. To draw conclusions about the role of specific residues, including Lys¹⁶⁴α, in FTase catalysis, it was considered very important to determine the influence of the residue on the individual steps in the mechanism.

Involvement of Lys¹⁶⁴α in substrate binding

In crystal structures of E·FPP binary and E·isoprenoid·peptide ternary complexes, the residues Lys¹⁶⁴α, His²⁴⁸β, Arg²⁹¹β, Lys²⁹⁴β

Table 1 Kinetic constants for wild-type FTase and Lys¹⁶⁴ mutants

All values for kinetic parameters were obtained with GCVLS, except K_S^{FPP} (dansyl-GCVLS) and K_m^{FPP} (H-Ras). The apparent K_m values for GCVLS and FPP were determined at 2 μM FPP and 5 μM H-Ras respectively. ND denotes not determined.

Kinetic parameter	Wild-type	K164R	K164A
K_S^{FPP} (μM)	0.018 ± 0.002	0.012 ± 0.002	0.025 ± 0.007
K_S^{pep} (μM)	0.016 ± 0.002	0.016 ± 0.001	0.153 ± 0.015
K_m^{FPP} (μM)	0.006 ± 0.001	0.004 ± 0.001	0.004 ± 0.001
K_m^{pep} (μM)	0.57 ± 0.11	0.44 ± 0.12	3.16 ± 0.4
k_{cat} (s^{-1})	0.058 ± 0.003	0.014 ± 0.001	0.025 ± 0.001
$k_{\text{obs}}^{\text{max}}$ (s^{-1})	4.6 ± 0.5	0.55 ± 0.07	0.11 ± 0.01
k_1 ($\text{M}^{-1} \cdot \text{s}^{-1}$)	6×10^5 *	ND	1.3×10^4
$K_{1/2}$ (μM)	20*	ND	11

* These values were obtained from [9].

Figure 3 Effect of mutation of Lys¹⁶⁴ on peptide binding affinity

Peptide dissociation constants were determined using 0.02 μM enzyme, 0.2 μM I_{ana} , a non-hydrolysable FPP analogue and increasing concentrations of dansyl-GCVLS. The curves were obtained by fitting eqn (2) or (3) to the data to give K_S^{pep} values of 0.016 ± 0.002 μM (wild-type, $\circ$), 0.016 ± 0.001 μM (K164R, $\bullet$), and 0.153 ± 0.015 μM (K164A, $\square$). The relative fluorescence is the observed fluorescence, with background subtracted, divided by the maximal fluorescence of 66.4 (wild-type), 81 (K164R) or 106 (K164A).

and Tyr³⁰⁰ have been observed to interact with the non-bridging oxygen atoms of the diphosphate moiety of FPP (Figure 1). Mutation of some of these residues to alanine, most notably Arg²⁹¹ and Lys²⁹⁴, has been shown to cause a significant decrease in the affinity of the FTase mutants for FPP [20], suggesting that a loss of these interactions is important for FPP binding. However, these enzymes still retained relatively high-affinity FPP binding ($K_S^{\text{FPP}} < 0.05$ μM), indicating that the majority of the binding energy can be attributed to substantial interactions elsewhere, most probably between the farnesyl carbons and hydrophobic residues in the active site. To determine whether alteration of Lys¹⁶⁴ affects the affinity of the enzyme for FPP, equilibrium-dialysis experiments were performed. The dissociation constants (K_S^{FPP}) determined for K164R (0.012 μM) and K164A (0.025 μM) were comparable with the value obtained for the wild-type enzyme (0.018 μM) (Table 1), indicating that even complete removal of the side chain of Lys¹⁶⁴ does not have a significant effect on the binding affinity of FPP. These results confirm, and significantly extend, the previously published data

Scheme 1 General kinetic scheme for the reaction catalysed by FTase

The CaaX substrate association rate constant and the rate constant for the chemical step are denoted by k_1 and k_2 respectively. The observed rate constant for product formation in the single-turnover assays is represented by k_{obs} . For wild-type FTase, the steady-state rate constant k_{cat} is a measurement of product release. F-CaaX denotes farnesylated CaaX peptide or protein product.

on the mutant, in which asparagine was substituted for lysine at this position [24,25].

To investigate the role of the positive charge at position 164 α in formation of an E·isoprenoid·peptide ternary complex, dissociation constants were measured for a dansyl-labelled peptide, dansyl-GCVLS, which corresponds to the CaaX sequence of H-Ras. We had previously employed these techniques to directly measure peptide substrate binding affinities for FTase, and to show that the presence of bound isoprenoid on the enzyme markedly enhances the affinity for CaaX peptide substrates [13]. Binding studies were performed on both wild-type FTase and the Lys¹⁶⁴ mutants containing the bound FPP analogue I_{ana} (see the Materials and methods section); competition experiments showed that neither mutation compromised I_{ana} binding (results not shown). The binary E· I_{ana} complexes of wild-type FTase and the K164R mutant have high affinity for dansyl-GCVLS, with both exhibiting K_S^{pep} values of 0.016 μM (Figure 3 and Table 1). Surprisingly, the binding affinity of the K164A· I_{ana} complex for dansyl-GCVLS was 10-fold lower, with a K_S^{pep} value of 0.153 μM . Competition assays using the peptide GCVLS resulted in K_S^{pep} values similar to those obtained for dansyl-GCVLS (results not shown), confirming that the presence of the dansyl group did not affect the affinity of the enzyme for the peptide. These results suggest that the positive charge at position 164 α is involved in the formation of the E·isoprenoid·CaaX ternary complex.

Involvement of Lys¹⁶⁴ in catalysis

To determine the effect of removal of the positive charge at position 164 α on the chemical step of product formation, single-turnover reactions were used to directly probe the chemical step. These techniques have been used previously to study the catalytic mechanism of both wild-type FTase and various mutated and metal-substituted forms of the enzyme [9,10,15,26,34]. For the wild-type enzyme, the observed rate constant for product formation at saturating peptide concentrations, $k_{\text{obs}}^{\text{max}}$, represents the chemical step in the reaction (k_2 in Scheme 1). The value of $k_{\text{obs}}^{\text{max}}$ for the wild-type enzyme obtained in this study, 4.6 s^{-1} (Table 1), is consistent with previously reported values [9,15]. Mutation of Lys¹⁶⁴ to arginine caused an 8.5-fold decrease in the maximal observed rate constant for product formation, $k_{\text{obs}}^{\text{max}} = 0.54$ s^{-1} (Figure 4A), even though the positive charge was retained. Removal of the positive charge resulted in an even greater decrease in the maximal observed rate constant for product formation, with a $k_{\text{obs}}^{\text{max}}$ of 0.11 s^{-1} for K164A. These results suggest that both the presence of the positive charge and the positioning of the charge in the active site impact on the ability of the enzyme to form a farnesylated product. This finding is consistent with a previous report that showed that conversion of Lys¹⁶⁴ into asparagine resulted in a significant decrease in the rate constant for product formation [25].

Figure 4 Product formation in single-turnover reactions

Single-turnover assays were conducted with 0.2 μM enzyme and 0.1 μM [³H]FPP, and the observed rate constants for product formation were calculated using eqn (5). **(A)** Product formation by wild-type (●), K164R (○), and K164A (■) at 100 μM GCVLS. The observed rate constants for product formation at saturating peptide concentration, $k_{\text{obs}}^{\text{max}}$, are $4.6 \pm 0.5 \text{ s}^{-1}$ for wild-type, $0.54 \pm 0.07 \text{ s}^{-1}$ for K164R and $0.11 \pm 0.01 \text{ s}^{-1}$ for K164A. The percentage product endpoint ranged from 21–31.5%. **(B)** Product formation by K164A with 0.5 (●), 1 (○), 5 (■), 20 (□) or 100 (◆) μM GCVLS. The percentage product endpoint ranged from 65–79%.

Figure 5 Dependence on peptide concentration for steady-state catalysis

Assays were performed with 1–5 nM enzyme, 2 μM FPP, and the indicated concentrations of the peptide substrate GCVLS. The dependence of $v/[E]_{\text{tot}}$ on peptide concentration yielded k_{cat} and apparent K_m^{FPP} values [eqn (4)] of $0.058 \pm 0.003 \text{ s}^{-1}$ and $0.57 \pm 0.11 \text{ μM}$ for wild-type (●), $0.014 \pm 0.001 \text{ s}^{-1}$ and $0.44 \pm 0.12 \text{ μM}$ for K164R (○) and $0.025 \pm 0.001 \text{ s}^{-1}$ and $3.16 \pm 0.4 \text{ μM}$ for K164A (■) respectively.

We also investigated the effect of mutation of Lys¹⁶⁴α on the nature of the reaction mechanism. For wild-type FTase, the rate constant for the chemical step, k_2 , is significantly higher than the rate constant for CaaX substrate dissociation, k_{-1} (see Scheme 1) [6,8]. Therefore the reaction mechanism is characterized by two irreversible steps: binding of the CaaX substrate and chemistry. Consequently, $K_{1/2}$, the concentration of peptide that results in a half-maximal rate constant for product formation under single-turnover conditions, calculated from the peptide concentration-dependence does not represent equilibrium binding of the peptide, and $K_{1/2} > K_s^{\text{pep}}$ [9]. To determine whether this mechanism is conserved in the absence of a positive charge at position 164α, the dependence of the observed rate constant for product formation on the peptide substrate concentration was determined for K164A. As shown in Figure 4(B), product formation by K164A is dependent upon the concentration of GCVLS used in the assay. Fitting a hyperbolic equation representing a rapid-equilibrium mechanism $\{k_{\text{obs}} = k_{\text{obs}}^{\text{max}}[\text{pep}]/([\text{pep}] + K_{1/2})\}$ to the observed rate constants for product formation resulted in a $K_{1/2}$ of 11 μM for K164A, which is similar to the value of 20 μM reported for the $K_{1/2}$ of wild-type FTase [9]. The finding that the value of $K_{1/2}$ for K164A is significantly higher than K_s^{pep} (i.e. 11 μM compared with 0.153 μM) indicates that, despite the 40-fold reduction in the observed rate constant for product formation, the mechanism of K164A has not changed to one involving rapid-equilibrium substrate binding. Therefore the reaction mechanism is conserved, despite the loss of the positive charge at position 164α. A linear fit of k_{obs} at low peptide concentrations resulted in a rate constant for peptide association, k_1 , of $1.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for K164A. This k_1 value is approx. 50-fold lower than the previously reported value for wild-type FTase ($6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$; [9]). These data are consistent with the decrease in peptide affinity observed for K164A, and provide additional evidence that the positive charge at position 164α plays a role in the formation of the E·FPP·peptide ternary complex.

To characterize the effect of changes at position 164 of the α subunit on steady-state catalysis, K_m and k_{cat} were determined for the wild-type enzyme and both mutants. As observed in the binding studies, removal of the positive charge in the K164A mutant primarily affects the peptide substrate. The apparent K_m^{pep} for K164A, 3.16 μM, was approx. 6-fold higher than the apparent K_m^{pep} for wild-type FTase or K164R (Figure 5 and Table 1). The apparent K_m values for FPP, determined with full-length H-Ras protein, were similar to the K_s^{FPP} values and were not significantly different for the three forms of the enzyme. The steady-state rate constant, k_{cat} , is minimally affected by substitution of the lysine at position 164α with arginine or alanine (Table 1). Previous reports suggested that an asparagine-for-lysine substitution had a more significant impact on steady-state catalysis [24,25]. Substantial decreases in k_{cat} for FTase can reflect problems with release of the farnesylated product or a change in the rate-limiting step in the reaction. For wild-type FTase and K164R, the observed rate constant for product formation is 80-fold and 40-fold higher respectively than the steady-state rate constant, indicating that product release is the rate-limiting step in overall catalysis by these enzymes. However, for K164A the observed rate constant for product formation is only 4-fold higher than the steady-state rate constant, suggesting that product formation could be partially rate-limiting in steady-state catalysis.

DISCUSSION

The results presented here increase our understanding of the role of Lys¹⁶⁴α in the catalytic mechanism of FTase. By probing

individual steps in the reaction pathway, we have shown that both the charge and the position of the charge of Lys¹⁶⁴ α are important for catalysis. Mutation of this residue to either arginine or alanine causes a significant decrease in the observed rate constant for product formation. Surprisingly, we found that elimination of the positive charge at position 164 α also had substantial effects on formation of the E·FPP·peptide ternary complex, as determined both by a decrease in the peptide association rate constant and by an increase in the peptide dissociation constant. The decrease in the peptide binding affinity for K164A could be caused by a change in the conformation of the isoprenoid that alters the synergism between binding of the isoprenoid and peptide substrates. Alternatively, Lys¹⁶⁴ α could play a more direct role in formation of the ternary complex by interacting with the peptide substrate.

There have been two proposals for the role of Lys¹⁶⁴ α in catalysis. In one model, Lys¹⁶⁴ α acts as a general acid by donating a proton to an α -phosphate oxygen to stabilize the diphosphate leaving group and facilitate formation of the positive charge at C-1 of the farnesyl group [25]. The position of the charge would be extremely important in this model, since the distance between the proton and the oxygen would directly impact on the ability of this residue to act as a general acid. However, the general acid model was formulated on the basis of an analysis of the pH-dependence of steady-state catalysis that, for FTase, is difficult to interpret. In an alternative model of catalysis, the diphosphate leaving group is stabilized by co-ordination with magnesium [15]. This co-ordination by magnesium would require the displacement of at least some of the residues that are observed to interact with the diphosphate oxygens in the crystal structures. The crystal structure of an E·isoprenoid·peptide ternary complex with bound manganese provides some evidence for a change in the conformation of Lys¹⁶⁴ α due to co-ordination of the metal [18]. In this structure, the manganese is co-ordinated to the region of the I_{ana} FPP analogue that mimics the diphosphate of FPP, and Lys¹⁶⁴ α is shifted away from the analogue. The other residues that interact with the non-bridging diphosphate oxygen atoms, His²⁴⁸ β , Arg²⁹¹ β , Lys²⁹⁴ β and Tyr³⁰⁰ β , do not have altered conformations. Repositioning of Lys¹⁶⁴ α upon magnesium binding could make the positive charge available to act in other aspects of catalysis. In this regard, while our results and those obtained previously [24,25] support the assignment of a catalytic role for Lys¹⁶⁴ α , we cannot rule out the possibility that the rate constant for product formation measured for the Lys¹⁶⁴ α mutants represents a step other than chemistry, such as a conformational change, that is rate-limiting in the reaction.

The availability of structural data and a detailed understanding of the catalytic mechanism of FTase have made it possible to evaluate the role of individual residues in the reaction catalysed by this enzyme. From this information, residues have been identified that are involved in every aspect of the function of this enzyme from substrate binding to product release. Lys¹⁶⁴ α is of particular interest because of its position close to the reactive carbon of the farnesyl group and the diphosphate leaving group. In addition, this residue is strictly conserved both across species and in the related enzyme protein geranylgeranyltransferase type II [35], suggesting that it is an integral part of the prenylation reaction. Further kinetic and structural studies with Lys¹⁶⁴ α mutants will be required to elucidate the exact role of this residue in protein prenylation.

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