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Chemical Modification Studies of Yeast Farnesyl Protein Transferase

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Abstract : Phenylglyoxal, diethyl pyrocarbonate (DEPC), and 1-cyclohexyl-3-[2-morpholinoethyl]-carbodiimide metho-*p*-toluenesulfonate (CMC) are modifying reagents specific for arginine, histidine, and aspartate or glutamate, respectively. They were found to inactivate *S. cerevisiae* farnesyl protein transferase (FPTase). The peptide substrate protected the enzyme against inactivation by CMC, and the other substrate farnesyl pyrophosphate showed protection against inactivation by phenylglyoxal, while neither of the two substrates protected the enzyme against DEPC inactivation. These results suggest the presence of aspartate/glutamate, arginine and histidine residues at the active site of this enzyme.
Key words : active site, chemical modification, farnesyl protein transferase,

Farnesyl protein transferase (FPTase) catalyzes the addition of the isoprenoid farnesyl pyrophosphate (FPP) to a cysteine residue of a protein substrate such as Ras (Reiss *et al.*, 1990; Schaber *et al.*, 1990; Manne *et al.*, 1990). Farnesylation of Ras facilitates its membrane anchoring, which is essential for efficient cell transformation by oncogenic forms of Ras (Willumsen *et al.*, 1984). The protein substrates of FPTase, which include nuclear lamin B (Farnsworth *et al.*, 1989) and the γ subunit of transducin (Lai *et al.*, 1990; Fukuda *et al.*, 1990) as well as Ras (Casey *et al.*, 1989), have a carboxyl terminal motif known as CA_1A_2X box, where C is the cysteine that is to be farnesylated, A_1 and A_2 are usually aliphatic amino acids, and X can be Ser, Met, Gln, Cys, or—in yeast—Ala (Reiss *et al.*, 1991; Moores *et al.*, 1991).

The active yeast FPTase is a Zn^{2+}-containing, α/β heterodimer that also requires Mg^{2+} for catalytic action. The 43 kDa β subunit is encoded by the RAM1/DPR1 gene (Goodman *et al.*, 1988), while the 38 kDa α subunit, which also seems to act in another heterodimeric enzyme, geranylgeranyl protein transferase (GGPTase), is encoded by the RAM2 gene (He *et al.*, 1991). Although little has been proven about the structure of the FPTase active site, some suggestions are given. According to biochemical cross-linking studies (Reiss *et al.*, 1992) and photoaffinity-labeling studies (Ying *et al.*, 1994), the β subunit possesses the binding domain for the protein substrate, i.e. the CA_1A_2X box. Also, there is evidence from photo-crosslinking studies that the FPP binding site is located on the β subunit (Omer *et al.*, 1993). Individually, neither α nor β subunit alone is active in catalysis (Chen *et al.*, 1991; Reiss *et al.*, 1992;). Photo-affinity labeling studies indicate that the protein substrate binding locus of the FPTase catalytic site lies in a cleft formed between the two subunits (Ying *et al.*, 1994). Mutagenesis studies of human FPTase indicate that mutant human FPTases, —αN 199K, βD200N, βG249V, and βG349S—corresponding to mutant *S. cerevisiae* alleles—*ram2-1* (N 143K), *ram1-1* (D209N), *ram1-2* (G259V), and *cal1-1*(G328S)—exhibited kinetic defects, particularly in their catalytic efficiency (Omer *et al.*, 1993).

In order to obtain information about what kinds of amino acid residues are involved in the active site(s) and/or the substrate binding site(s), chemical modification studies were performed using phenylglyoxal, diethyl pyrocarbonate (DEPC), and 1-cyclohexyl-3-[2-morpholinoethyl]-carbodiimide metho-*p*-toluenesulfonate (CMC), which are specific modifiers of arginine, histidine, and glutamate or aspartate, respectively. Also protection of the enzyme against these modifiers was tested on FPTase pre-incubated with either FPP or the peptide substrate.

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Materials and Methods

S. cerevisiae FPTase was cloned and expressed in *E. coli* to fuse its β subunit to glutathione S-transferase (GST), and was purified by glutathione-agarose affinity column chromatography (Yi, 1995). GST-PEP, the undecapeptide fused to GST ([GST]-GCVKIKKCVIM), which has the peptide sequence homologous to the C-terminus of $p21^{Ki\text{-}Ras}$, was used as the substrate for FPTase. Fifty millimolar *tris*-[hydroxymethyl]aminomethane (TRIS) buffer (pH 7.5) was used. Enzyme activity was assayed using the simple "acidic ethanol precipitation method" (Moores *et al.*, 1991; Baik, 1994). Approximately 230 μM enzyme was assayed with two substrates 1.9 μM GST-PEP and 0.22 μM FPP. The apparent K_m values for GST-PEP and FPP are 1.12 μM and 0.13 μM respectively (Baik, 1994). FPTase showed no loss of activity over an extended time period in the absence of chemical modification reagents. During the time course of the inactivation reactions using 0-4 mM phenylglyoxal, 0-10 mM DEPC or 0-10 mM CMC, aliquots were withdrawn at 10 minute intervals, quenched and assayed for remaining enzyme activity. In parallel experiments, the enzyme was pre-incubated with 0 μM, 1.9 μM, 3.8 μM concentrations of the substrate GST-PEP at 25°C for 10 min, and reactions were performed separately with 4 mM phenylglyoxal, 5 mM DEPC and 10 mM CMC. Enzyme sets pre-treated with 0 μM, 0.11 μM, 0.22 μM concentrations of FPP were likewise tested. The amount of alcohol added as the solvent for phenylglyoxal and DEPC was adjusted so that it did not affect the enzymatic activity, and the quenching methods used for each modification reaction were proven to be successful without affecting enzymatic activity.

Results

According to the results of inactivation by specific chemical modifiers, it is concluded that aspartate/ glutamate, histidine, and arginine residues, specifically modified by CMC, DEPC and phenylglyoxal, respectively, are critical for enzymatic activity.

Aspartate or glutamate

Carbodiimides exhibit high selectivity for carboxylated residues in proteins. Most of the current work utilizes water-soluble carbodiimides, among which CMC was chosen for the modification of yeast FPTase. Four-fold dilution with 20 mM potassium acetate was successful as a quenching method, and did not disturb enzymatic activity. Increased concentration of CMC accelerated inactivation as a function of incubation time (Fig. 1a). Accordingly, it may be inferred that there is at least one aspartate or glutamate residue at the active site or the substrate binding site of the enzyme.

Inactivation by CMC in the presence of either of the two substrates was studied to determine if substrates would protect the enzyme from inactivation. Protection studies with 0 μM, 1.9 μM, and 3.8 μM concentrations of GST-PEP substrate showed that a higher concentration of the peptide substrate protected the enzyme to a greater extent against inactivation by 10 μM CMC (Fig. 1b). It is not likely that modification of one glutamate residue in the substrate GST-PEP can affect the facility of the catalysis, first because only four of the C-terminal residues CVIM are known to be involved in the enzymatic specificity (Casey *et al.*, 1989), and because the enzyme pre-incubated with GST-PEP showed activity, in the assay where the glutamate residue in GSP-PEP might have been modified. Therefore, the modification of aspartate or glutamate residues seems to influence enzymatic activity by disturbing the peptide sub-

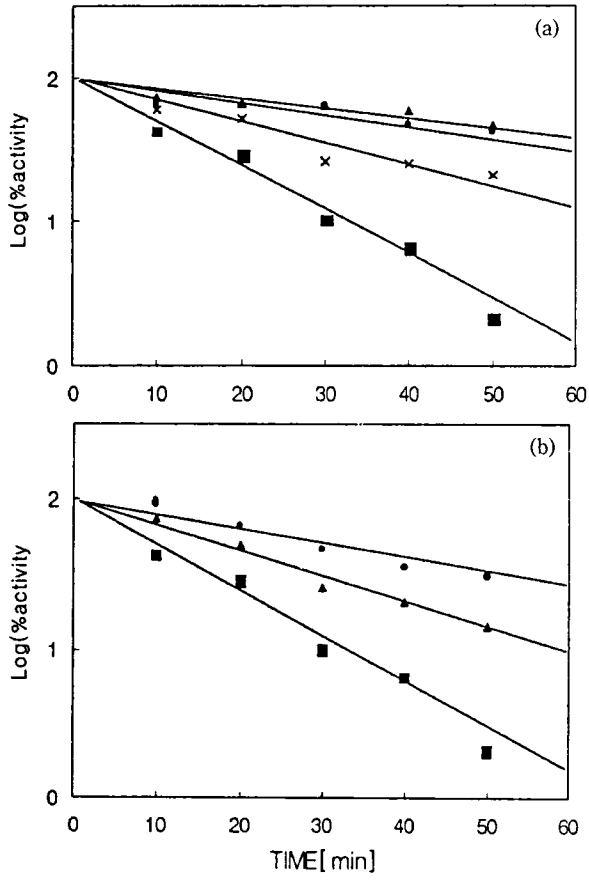

Fig. 1. Inactivation of *S. cerevisiae* FPTase by 1-cyclohexyl-3-[2-morpholinoethyl]-carbodiimide (CMC) metho-*p*-toluenesulfonate as a function of time. (a) Enzyme (230 μM) was treated with 1 mM (▲), 3 mM (●), 8 mM (×), and 10 mM (■) CMC. (b) Protection of the enzyme from inactivation by 10 mM CMC using 0 μM (■), 1.9 μM (▲), 3.8 μM (●) peptide substrate.

strate binding.

Arginine

Arginine residues are usually involved in binding sites rather than catalytic sites, playing an important role in binding anionic substrates, cofactors or effectors (Riordan *et al.*, 1977). The role of arginine residues in enzymatic activity can be studied by using specific arginine modifiers such as phenylglyoxal and 2,3-butanedione. The guanidine moiety capping the side chain of arginine characteristically adds to these 1,2-dicarbonyl compounds (Nishimura and Kitajima, 1979). The use of phenylglyoxal was developed by Takahashi (Takahashi, 1988) and has since been applied to the study of the role of arginine residues in proteins. Phenylglyoxal, like glyoxal, reacts with α-amino groups at a significant rate. Stoichiometrically, 2 moles of phenylglyoxal react with 1 mole of arginine. Enzymes may be protected from inactivation by dicarbonyl reagents by the presence of substrates that bind to arginine residues. Quenching with 200 mM L-arginine-HCl was successful without disturbing the enzymatic action. A control treated with 1% ethanol also showed no disruption of enzymatic action. Increased concentration of phenylglyoxal accelerated inactivation as a function of incubation time (Fig. 2a). The substrate FPP showed almost perfect protection against inactivation by 4 mM phenylglyoxal (Fig. 2b). Thus, it is highly probable that at least one arginine residue is critical for enzymatic action by way of binding the substrate FPP and participating in the catalytic reaction.

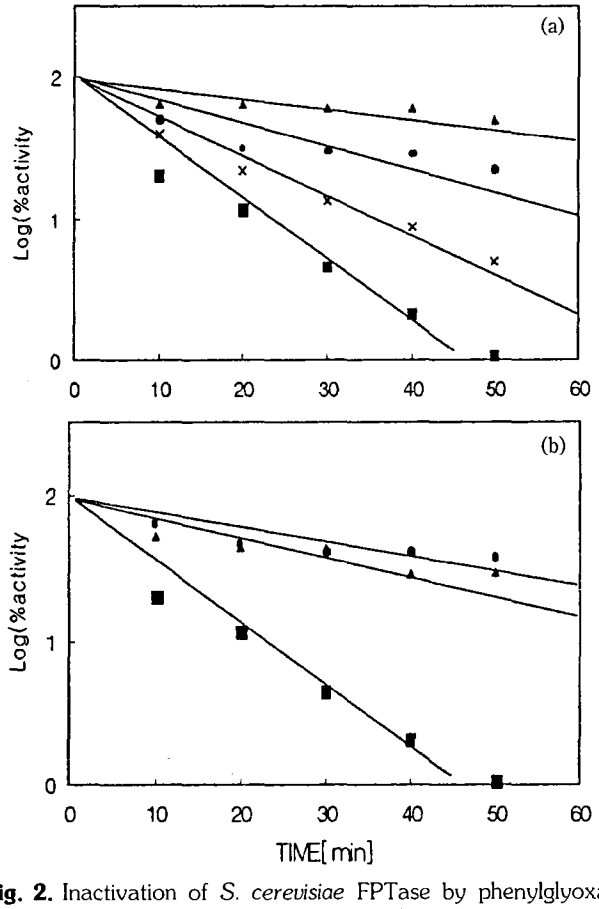

Fig. 2. Inactivation of *S. cerevisiae* FPTase by phenylglyoxal as a function of time. (a) Enzyme (230 μM) was treated with 0.1 mM (▲), 1 mM (●), 3 mM (×), and 4 mM (■) phenylglyoxal. (b) Protection of the enzyme from inactivation by 4 mM phenylglyoxal using 0 μM (■), 0.11 μM (▲), 0.22 μM (●) of substrate farnesyl pyrophosphate.

Histidine

Since many enzymes contain histidine residues which are critical for the catalytic process, site-specific modification of this residue has been the subject of many studies. DEPC, an ethoxy formic anhydride, is currently used for specific modification of histidine in proteins. Because histidine is quite sensitive to photo-oxidation, the reaction mixture was kept in the dark. To reduce the possibility of nonspecific base catalyzed hydrolysis at high pH, the reaction was performed at relatively lower pH of 7.0 (Lundblad, 1995). Four-fold dilution with 50 mM imidazole was used as the quenching method. Increased amount of DEPC accelerated inactivation as a function of incubation time (Fig. 3). It is certain that at least one histidine residue is involved in the catalytic action of the enzyme.

Neither of the two substrates showed protection, suggesting that the substrate binding site and the catalytic site may not be closely adjacent, and that the critical histidine residue may be exposed without significant shielding effects by either of the two substrates.

Other residues

Other amino acid residues were also tested for inac-

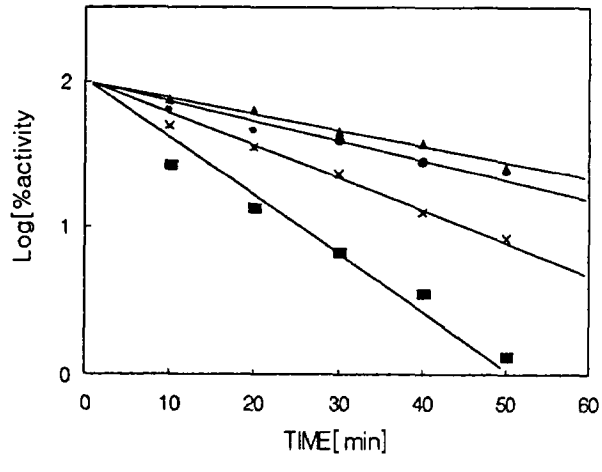

Fig. 3. Inactivation of 230 μM *S. cerevisiae* FPTase by 0.1 mM (▲), 1 mM (●), 5 mM (×), and 10 mM (■) DEPC as a function of time.

tivation by specific modifiers (Lundblad, 1995), but do not seem to be critical for enzymatic activity (Data not shown). Tryptophan modification by N-bromo-succinimide and lysine modification by maleic anhydride or succinic anhydride rarely affected enzyme activity. Modification of serine or threonine by *p*-methylsulfonylfluoride does not seem to inhibit enzymatic activity, which lessens the possibility that a serinyl or threoninyl hydroxyl group might participate in catalysis. Iodoacetate, which modifies cysteine, methionine, histidine, and lysine with relatively low specificity, influenced enzyme activity only slightly. Histidine has already been proven critical; lysine does not clearly affect enzyme activity, as shown by only partial inactivation by pyridoxal-5'-phosphate which usually modifies amino acids having potent nucleophilic side chain functional groups such as cysteine, histidine, lysine and methionine. Hydrogen peroxide must have affected the easily oxidizable amino acids with low specificity, such as histidine, tryptophan, methionine, and cysteine, and/or have induced conformational changes by forming undesirable disulfide linkages to result in inactivation of the enzyme.

Discussion

The studies of the substrates and metal cofactors suggest that FPP binds to the β subunit and the peptide substrate binds into the interface between the two subunits. Zn^{2+} is essential for peptide binding and is attached to the β subunit, which discriminates FPP from GGPP, a substrate of another prenylation enzyme geranylgeranyl protein transferase (GGPTase) (Reiss *et al.*, 1992). FPP does not seem to require any cationic cofactor for binding to the active site, while transfer of the bound prenyl group to the bound peptide acceptor requires Mg^{2+} (Reiss *et al.*, 1992). The proposed kinetic mechanism is a simple, random-ordered, two substrate reaction, where FPP and the peptide substrate can bind to the enzyme in any order (Pompliano *et al.*, 1992; Reiss *et al.*, 1992). Comparison of known genes of human, bovine, murine and *S. cerevisiae* FPTase, in view of sequence homology, led to site-directed mutagenesis studies. The yeast mutant *ram2-1* allele, in which residue 143 of the α subunit was Lys instead of the normal Asn, primarily affected CVIM peptide substrate utilization, and the *ram1-1* allele of yeast, having Asn instead of the normal Asp for residue209 of the β subunit, increased the K_m value for CVIM (Omer *et al.*, 1993). According to the results of similar site-directed mutagenesis, α H145D had an increased K_m value for the CVIM peptide substrate and is proposed to be involved in peptide binding (Baik, 1994). β H258A also showed decreased enzyme activity (Yi, 1995).

In summary, aspartate/glutamate, arginine and histidine residues seem to be involved in the active site of *S. cerevisiae* FPTase. It is possible that the activity drop resulted from changes in the overall enzyme structure, rather than from modification of specific active site amino acid residues. However, it is reasonable to believe the activity drop resulted mostly from modification of specific amino acids, since the enzyme protected by the substrate retained its activity under modifying conditions. It is possible, considering the results of other studies, that α H145 and β D209 may be positioned at the peptide binding domain, and one or two of these residues may be coordinated to Zn^{2+} or act as the enzymatic base extracting the thiolic proton while stabilizing the transition state of the nucleophilic attack by the sulfur atom of the cysteine residue of the peptide substrate. β H258 may also serve one of these roles. It is also inferred that β D 209 is the most probable residue affected by CMC inactivation and participates in binding of the peptide substrate. An arginine residue may directly bind the anionic pyrophosphate leaving group of FPP, which was shown to protect the enzyme against inactivation by phenylglyoxal.

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