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Activation of acyl condensation reaction of monomeric 6-hydroxymellein synthase, a multifunctional polyketide biosynthetic enzyme, by free coenzyme A

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Abstract

6-Hydroxymellein (6HM) synthase is a multifunctional polyketide enzyme induced in carrot cells, whose fully active homodimer catalyzes condensation of acyl-CoAs and the NADPH-dependent ketoreduction of the enzyme-bound intermediate. 6HM-forming activity of the synthase was markedly decreased when the reaction mixture pH was adjusted from 7.5 to 6.0. However, under these slightly acidic conditions, the acyl condensation catalyzed by the dissociated monomer enzyme was appreciably stimulated by addition of free coenzyme A (CoA). In contrast, the condensation reaction at pH 6.0 was significantly inhibited in the presence of CoA when the reaction was carried out with the NADPH-omitted dimer synthase. Among the kinetic parameters of the acyl condensation, velocity of the monomer-catalyzing reaction at the acidic pH was appreciably increased upon addition of CoA while K_ms did not show any significant change in the presence and absence of the compound. These results suggest that CoA associates with a specific site in the dissociated monomeric form of 6HM synthase, and the velocity of the acyl condensation reaction catalyzed by the CoA-synthase complex appreciably increases in acidic conditions.

Keywords: *Daucus carota*; Umbelliferae; Carrot; CoA; Multifunctional polyketide biosynthetic enzymes; Acyl condensation reaction; Acidic condition; Dissociation of homodimer; Enzyme regulation

1. Introduction

6-Hydroxymellein **1**, (6HM) is a direct precursor of the carrot phytoalexin, 6-methoxymellein (Coxon et al., 1973), and the enzyme catalyzing the biosynthesis of this compound, 6HM synthase, is a multifunctional polyketide synthetic enzyme induced in the cells (Kurosaki et al., 1991). The enzyme catalyzes the condensation of acetyl- and malonyl-CoAs to form the pentaketomethylene chain, and, as shown in Fig. 1a, an NADPH-dependent ketoreduction of the carbonyl group takes place at the triketide intermediate stage (Kurosaki et al., 1989).

The active form of 6HM synthase is organized as a homodimer (Kurosaki, 1995), and two subunits (approximately 130 kDa each) containing the functional domains of the ketoreduction and the acyl condensation are aligned in the head-to-tail direction to form two complete reaction centers (Fig. 1b). The reduction reaction of the carbonyl group in the synthase-bound intermediate is the rate-limiting step in the series of partial reactions which determines the overall rate of 6HM biosynthesis (Kurosaki et al., 1999). It has been shown (Kurosaki et al., 1989; Kurosaki, 1995) that if the reducing co-factor (NADPH) is either omitted or the active homodimer is dissociated into the monomer subunits, the ketoreducing reaction is disabled and triacetic acid lactone **2** (TAL) is liberated as a derailment product instead of 6HM (**1**) (Fig. 1a). We previously demonstrated (Kurosaki et al., 1989, 2000) that free coenzyme A (CoA) is an important allosteric factor stimulating 6HM-biosynthetic activity of the enzyme. It was also shown that the CoA associated with the dimeric enzyme reduced the energy barrier of the ketoreducing reaction by altering the microstructure of the reaction center of the protein and enhanced 6HM (**1**) formation and accumulation. However, the factor(s) affecting the acyl condensation reaction of 6HM synthase, if any, are not yet known.

Since several catalytic reactions and the structural organization of 6HM synthase are similar to those of animal fatty acid synthase (FAS), it has been assumed that these two enzymes share many common properties

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(Wakil and Stoops, 1989; Wakil et al., 1983; Wakil, 1989; Smith, 1994). In animal FAS, it has been shown (Stoops and Wakil, 1981; Wakil, 1989) that Cys-SH of the condensation enzyme is unusually more active at slightly acidic conditions, such as at pH 6.0 and 6.5, indicating that the thiol most likely exists as a thiolate ion and therefore is highly susceptible to alkylation with appropriate SH reagents (McCarthy and Hardie, 1982; Anderson and Kumar, 1987).

In the present study, we first attempted to characterize the possible pH-dependency of 6HM synthase-catalyzing reactions, and special attention was focused on the role of CoA in the acyl condensation of 6HM synthase.

2. Results and discussion

2.1. Stability of 6HM synthase in acidic buffer

As reported previously, 6HM synthase is very unstable even in neutral buffers, like other multifunctional polyketide enzymes of microbial origin (Beck et al., 1990; Spencer and Jordan, 1992; Kurosaki et al., 1993). The activity of 6HM synthase was completely lost after freezing overnight, and more than 80% decrease in the activity was observed when the preparation was stored in pH 7.5 buffer at 4 °C for 5 days. The addition of polyalcohols and sucrose into the buffers did not appear to stabilize the enzyme. It is reasonable to assume, therefore, that this very unstable protein would irreversibly lose its catalytic activities by transfer to, and storage in pH 6.0 buffer. Thus, we first tested whether or not the synthase activity is restored after treatment of the enzyme protein with slightly acidic buffer for several hours. Accordingly, highly purified 6HM synthase (approximately 10 μg) in 15 ml of pH 7.5 K-Pi buffer was divided equally into two portions, and one of the enzyme solutions was transferred into an Amicon cell (YM-10 membrane). The buffer was replaced by pH 6.0 buffer in an ice bath, and after washing with acidic buffer (2×2 ml), the volume of the enzyme solution was adjusted to 5 ml. The enzyme in the pH 6.0 buffer was then incubated at 37 °C for 4 h, and the acidic buffer was again replaced by 5 ml of the pH 7.5 buffer by ultrafiltration. The other half of the enzyme solution at pH 7.5 was similarly concentrated to 5 ml by ultrafiltration, and

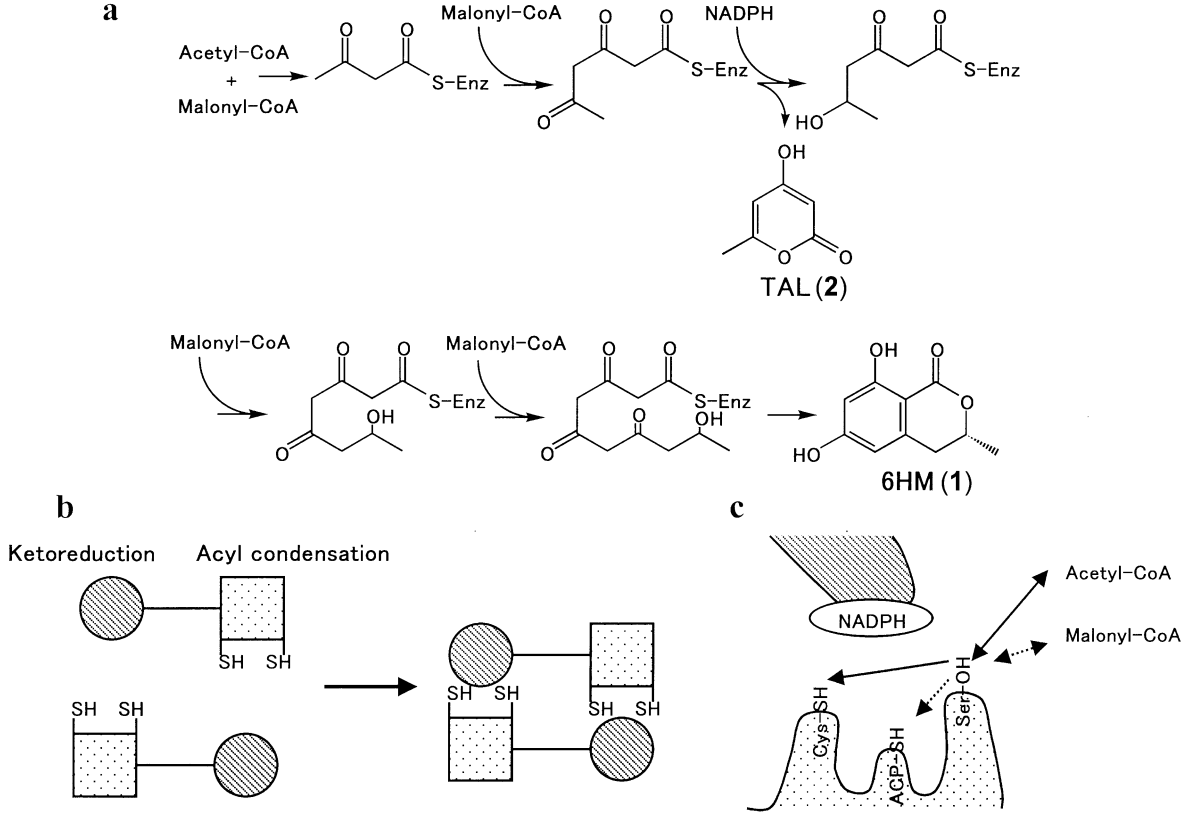

Fig. 1. Various aspects of 6HM synthase (a) catalytic reaction. 6HM synthase catalyzes the condensation of acetyl- and malonyl-CoAs, and an NADPH-dependent ketoreduction takes place at the triketide intermediate stage. Further condensation of malonyl-CoA results in the production of 6HM (**1**). (b) Schematic presentation of the arrangement of the functional domains of homodimeric 6HM synthase. The catalytic domain for ketomethylene chain elongation by acyl condensation is associated with that of ketoreduction belonging to another subunit, and two equivalent reaction centers are organized in each molecule of the active synthase. (c) Schematic presentation of the reaction center of 6HM synthase. The acyl groups transfer from the CoA esters to Ser-OH of transacylase, and they are properly channeled to two SH groups, Cys-SH and ACP-SH, prior to the initiation of the condensation reactions.

incubated for 4 h at 37 °C. The 6HM biosynthetic activities were of these two enzyme preparations were then determined. In repeated experiments, the activity of the acidic buffer-treated enzyme was slightly decreased to 83–91% of the controls in the pH 7.5 buffer (data not shown). These results strongly suggest that the loss of 6HM synthase activities, i.e. for both acyl condensation and ketoreduction, is essentially negligible even after treatment with the pH 6.0 buffer for 4 h. This also implies that the possible difference in the catalytic activities of 6HM synthase between pH 7.5 and 6.0 conditions were not due to the irreversible and/or nonspecific alterations of the protein structures caused by treatment with the acidic buffer, but rather resulted from the pH-dependent changes in the catalytic properties of the enzyme. Therefore, all of the following experiments were done within 4 h after the transfer of 6HM synthase protein to the pH 6.0 buffer using freshly prepared enzyme.

2.2. Effect of CoA on 6HM synthase activities under neutral and acidic condition

The activities of 6HM synthase for 6HM (**1**) and TAL biosyntheses were determined at pH 7.5 and 6.0 under various conditions. As shown in Table 1, transfer of the enzyme from pH 7.5 to 6.0 significantly decreased 6HM (**1**) formation to 24 and 37% of the controls kept in neutral buffer (from 4.21 to 1.01 pkat, and from 3.66 to 1.35 pkat, respectively). The TAL-producing activities were also inhibited under acidic condition either when NADPH is omitted from the reaction mixture or when the enzyme is dissociated into monomers by addition of NaCl (see Experimental). The activities decreased to 31–44% of the controls in the dimer enzyme without the co-factor, and from 30–38% in the dissociated monomer subunits, respectively.

As reported previously (Kurosaki et al., 1999, 2000), addition of submillimolar concentrations of CoA into the 6HM-producing system under the neutral condition resulted in appreciable enhancement of biosynthetic activity. The association of CoA to the enzyme suggests an allosteric interaction that activates the ketoreducing reaction which determines the rate of 6HM (**1**) biosynthesis. In contrast, CoA exhibited a marked inhibitory effect on TAL (**2**) formation by the acyl condensation reaction, catalyzed by either the dimeric synthase without reducing co-factor or the dissociated monomer enzyme by product inhibition mechanism under neutral conditions (Kurosaki, 1998). A set of these experiments was repeated in the present study, and the same results were obtained reproducibly (Table 1). Under the slightly acidic conditions (pH 6.0), CoA also functioned as an activator of 6HM (**1**) production, and a 1.3–1.4-fold increase in the activity (from 1.35 to 1.79 pkat and from 1.01 to 1.45 pkat, respectively) was observed by addition of 0.3 mM CoA as well as in the neutral pH (Table 1). However, in sharp contrast to 6HM (**1**) production, CoA showed quite different effects on the TAL (**2**)-synthetic reactions at pH 6.0. Although, as observed at neutral pH, TAL (**2**) formation by NADPH-free dimer was appreciably inhibited in the presence of CoA (from 0.11 to 0.05 pkat and from 0.10 to 0.06 pkat, respectively), the production of 6 HM (**1**) catalyzed by the dissociated monomer subunits was significantly enhanced upon CoA addition. In repeated experiments, the relative activity of the acyl condensation of the monomer was increased from 0.11 to 0.19 pkat and from 0.11 to 0.22 pkat, respectively, and therefore, the TAL (**2**)-forming ability was elevated 1.7–2.0-fold higher levels in the presence of CoA. The enhanced activity of TAL (**2**) production again decreased to the control level when CoA was removed from the mixture by dialysis (data not shown). It appeared, therefore, that the CoA-induced activation of the acyl condensation reaction by the monomer synthase is a reversible process, and it is very likely that CoA molecule non-covalently associates with the monomer subunits under acidic condition.

The effect of various concentrations of CoA on the acyl condensation reaction of 6HM synthase was also examined. As shown in Fig. 2, TAL (**2**) production

Table 1
Catalytic activity of 6HM synthase under neutral and acidic conditions[a]

		Experimental 1		Experimental 2	
		−CoA	+CoA	−CoA	+CoA
6HM (**1**) formation (pkat)					
	pH 7.5	4.21	6.95	3.66	6.55
	pH 6.0	1.01	1.47	1.35	1.79
TAL (**2**) formation by dimers without NADPH (pkat)					
	pH 7.5	0.25	0.13	0.32	0.13
	pH 6.0	0.11	0.05	0.10	0.06
TAL (**2**) formation by monomers (pkat)					
	pH 7.5	0.29	0.13	0.37	0.21
	pH 6.0	0.11	0.19	0.11	0.22

[a] 6HM (**1**)-forming and TAL (**2**)-forming activities of 6HM synthase at pH 7.5 and 6.0 were determined in the absence or presence of 0.3 mM CoA.

increased with an increase in CoA concentration when the reaction was catalyzed by dissociated monomers of 6HM synthase, and the synthetic ability elevated to approximately 1.7–2.0-fold higher level than that of the control in the presence of 0.3–1 mM of CoA. Addition of 1 mM NADPH or NADH to a monomer-catalyzed reaction did not show any significant change in the CoA-induced activation (data not shown), suggesting that the association of NADPH or NADH with the ketoreducing domain of the monomers did not affect enhancement of the condensation. In contrast, if the reaction was catalyzed by the NADPH-free dimer synthase, CoA exhibited significant inhibitory activity on the condensation processes in a dose-dependent manner (Fig. 2) as was the case at the neutral pH (Kurosaki et al., 1999). As in FAS (Smith, 1994), pantetheine functions as an acyl acceptor in 6HM synthase-catalyzed reaction (Kurosaki and Arisawa, 1999). However, when CoA was replaced by pantetheine, TAL (**2**) biosynthesis was appreciably inhibited in the reactions catalyzed by either NADPH-deprived homodimer or the monomeric form of 6HM synthase (Fig. 3). This result implies that the biochemical properties of CoA as an acyl acceptor is not responsible for stimulation of the condensation reaction.

2.3. Effect of CoA on substrate entry into 6HM synthase under acidic condition

Association of NADPH with the ketoreducing domain of 6HM synthase enhances the substrate entry into the enzyme protein to elevate the activity of 6HM (**1**) biosynthesis (Kurosaki, 1998). Therefore, the possibility was examined that association of CoA under acidic condition alters the process of substrate entry into the reaction center of the monomeric 6HM synthase. Accordingly, two SH groups at the reaction center of 6HM synthase, Cys–SH of ketoacyl synthase and cysteamine-SH attached to the acyl carrier protein (ACP), were specifically alkylated with iodoacetoamide (IoAA) and chloroacetyl–CoA (ClAc–CoA) to simplify estimation of the binding ability of the enzyme toward its co-substrates (Kurosaki, 1996a). In this partially masked protein, it is expected that only Ser-OH of the transacylase structure, the primary binding site of the co-substrates, should function (Fig. 1c). It has been confirmed (Kurosaki, 1996a) that the enzyme does not lose either substrate-binding or substrate-channeling activity even after modification with these reagents. This chemically modified protein was incubated with radiolabeled acetyl- and malonyl-CoAs, and the ratio of the synthase-bound forms of the acyl groups was determined. Since purity of the synthase in each batch of enzyme preparations varied, besides the enzyme being very unstable (Kurosaki, 1996a,b), estimation of the chemical stoichiometry of the synthase-bound acyl groups toward the enzyme was difficult. Results were, therefore, expressed as relative values in which the amount of acetyl group bound to the NADPH-free dimer enzyme without CoA was taken as 1 in each set of experiments as reported previously (Kurosaki, 1996a). As shown in Table 2, addition of 0.3 mM CoA resulted in a marked decrease in the ratio of enzyme-bound acetyl and malonyl groups when these two substrates were added independently. The enzyme-bound forms of the

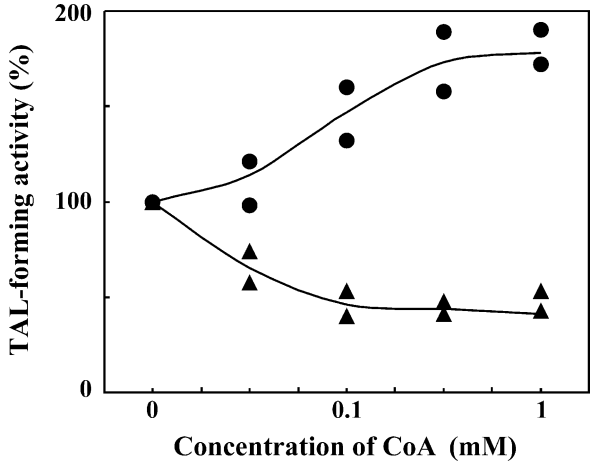

Fig. 2. Effect of CoA on TAL (**2**)-forming reaction catalyzed by 6HM synthase. The acyl condensation reaction catalyzed by 6HM synthase was determined either in the dissociated monomer subunits (•) or in the NADPH-free homodimeric form (▲) of the enzyme in the presence of various concentrations of CoA. The results were expressed as percentages in which the acyl condensation activities determined without CoA (0.19 and 0.38 pkat for the dissociated monomer, and 0.22 and 0.43 pkat for NADPH-omitted dimer, respectively) were taken as 100%.

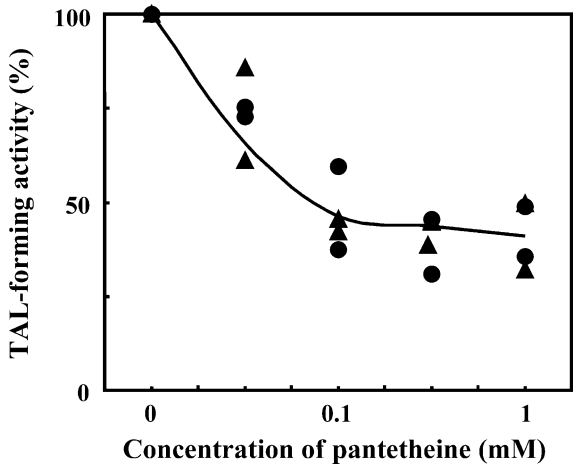

Fig. 3. Effect of pantetheine on TAL (**2**)-forming reaction catalyzed by 6HM synthase. The condensation reaction catalyzed by 6HM synthase was determined either in the dissociated monomers (•) or in the NADPH-omitted dimeric form (▲) in the presence of various concentrations of pantetheine. The results were expressed as percentages in which the activities without pantetheine (0.24 and 0.36 pkat for the monomer, and 0.29 and 0.40 pkat for the NADPH-omitted dimer, respectively) were taken as 100%.

acyl groups decreased to roughly 30–60% of the controls, and similar results were also obtained if the two substrates were added in a 1:1 mixture (Table 2) indicating that CoA functions as an inhibitor of substrate entry into the dissociated monomer. It is concluded, therefore, that this early step of acyl condensation is not the process responsible for CoA-induced stimulation of the reaction under acidic condition.

2.4. Kinetic parameters of monomeric 6HM synthase under acidic condition

The CoA-induced activation of acyl condensation by 6HM synthase was further characterized by determining kinetic parameters of the reactions at pH 6.0 in the absence and presence of 0.3 mM CoA (Table 3). Several kinetic values of the synthase have already been determined at pH 7.5 (Kurosaki, 1998, Kurosaki et al., 2000). In the present study, almost identical values were obtained. In the monomer-catalyzed TAL (**2**) formation at pH 6.0, K_m for acetyl-CoA was slightly increased by addition of CoA (from 383 to 423 μM), and the values for malonyl-CoA were almost comparable in the absence and presence of CoA (51 and 48 μM, respectively). As shown in Table 3, K_ms obtained for the monomeric synthase were essentially similar to those estimated for the NADPH-free dimer synthase in acidic pH. In addition, these values in the TAL (**2**)-forming reactions at pH 6.0, either in the presence or absence of CoA, were essentially comparable to the figures obtained for the reactions at neutral pH (Table 3). Therefore, it is likely that alteration of affinity of the monomeric 6HM synthase to co-substrates upon presumed association of the enzyme to CoA is not responsible for activation of the condensation reaction of the enzyme at acidic pH.

In the next experiment, velocities of the acyl condensation were determined under various conditions. As described above, 6HM synthase preparation in the present experiments did not consist of a homogenous protein though highly purified, and the purity and specific activities of the synthase varied in each batch of enzyme preparation. In addition, due to the instability of the enzyme, part of the synthase activity was lost during purification (Kurosaki, 1998). Consequently, V values for 6HM synthase in TAL (**2**) production varied considerably in repeated experiments (0.7–4.6 nkat/g protein at pH 7.0), and hence direct comparison of Vs obtained from independent experiments was not possible. However, the relative ratio of the V values for the TAL (**2**)-producing reactions catalyzed by 6HM synthase was found to be almost constant irrespective of the specific activity of the enzyme preparations. Therefore, in order to compare the velocities of TAL (**2**) -forming reaction, the results were expressed as relative values in each set of the experiments in which V_{max} of TAL (**2**) production catalyzed by the NADPH-free dimer enzyme without CoA at pH 7.5 was taken as 1. As shown in Table 3, the two TAL (**2**) -producing reactions under neutral conditions appreciably decreased in the presence of CoA (from 1 to 0.75 and 1.12 to 0.71). At pH 6.0, the relative V value of the TAL (**2**)-forming reaction by NADPH-free synthase markedly decreased (from 0.42 to 0.22) as well as in the reactions at the neutral pH. However, in sharp contrast, the V of TAL (**2**) formation catalyzed by dissociated monomers at pH 6.0 appreciably increased upon association with CoA, and the value was elevated to an approximately 2.3-fold higher level than that without CoA (from 0.39 to 0.90). These results strongly suggest that elevation of the velocity of the acyl condensation reaction should be the main reason for the CoA-induced activation of the TAL

Table 2
Substrate-binding activity of 6HM synthase under acidic conditions[a]

			Experiment 1		Experiment 2	
			−CoA	+CoA	−CoA	+CoA
Acetyl-CoA	Dimers without NADPH		1[b]	0.45	1[b]	0.61
	Dissociated monomers		0.84	0.51	1.20	0.39
Malonyl-CoA	Dimers without NADPH		1.12	0.60	0.81	0.55
	Dissociated monomers		0.89	0.41	1.09	0.33
Acetyl-CoA plus malonyl-CoA						
	Dimers without NADPH	Acetyl	0.64	0.31	0.68	0.27
		Malonyl	0.51	0.22	0.44	0.31
	Dissociated monomers	Acetyl	0.47	0.24	0.57	0.24
		Malonyl	0.59	0.32	0.41	0.23

[a] Two SH groups at the reaction center of 6HM synthase were blocked by alkylation, and binding ability of the modified enzyme against acyl-CoAs was examined in the absence or presence of 300 μM of CoA.

[b] Results were expressed as relative values in which the amounts of acetyl group bound to the dimeric form of the modified enzyme without the reducing co-factor were taken as 1 in each set of the experiments.

Table 3
Kinetic parameters of condensation reaction catalyzed by 6HM synthase

			−CoA	+CoA
K_m for acetyl-CoA (μM)	pH 7.5[a]	Dimers without NADPH	284±43	468±52
		Dissociated monomers	318±144	524±24
	pH 6.0	Dimers without NADPH	417±81	344±46
		Dissociated monomers	383±83	423±68
K_m for malonyl-CoA (μM)	pH 7.5	Dimers without NADPH	43±16	41±18
		Dissociated monomers	40±13	46±15
	pH 6.0	Dimers without NADPH	57±11	49±18
		Dissociated monomers	51±8	48±7
Relative *V*max[b]	pH 7.5	Dimers without NADPH	1	0.75±0.30
		Dissociated monomers	1.12±0.15	0.71±0.27
	pH 6.0	Dimers without NADPH	0.42±0.11	0.22±0.14
		Dissociated monomers	0.39±0.14	0.90±0.21

[a] K_m values of 6HM synthase at neutral pH have been reported previously, however, the set of the experiments was repeated in the present study. Since almost identical K_ms were reproducibly obtained, the previously reported values are presented for reference (Kurosaki et al., 1999).

[b] *V*s of TAL-producing reactions catalyzed by 6HM synthase were expressed as relative values in which 6HM (**1**) formation by the homodimer enzyme at pH 7.5 without CoA was taken as 1. K_m and V_{max} values were presented as means and S.D. obtained from four independent experiments.

(**2**)-forming reaction catalyzed by the monomer subunits of 6HM synthase under acidic condition.

In the present study, it has been shown that association of CoA molecule with 6HM synthase monomer subunits results in activation of the acyl condensation reaction to liberate TAL (**2**) as product. We reported previously (Kurosaki et al., 1999, 2000) that the ketoreducing process, the rate-limiting step of 6HM (**1**) biosynthesis, is appreciably activated by a certain allosteric interaction when CoA associates with the homodimer form of 6HM synthase. One possible explanation for these results is that there are two CoA-binding sites in 6HM synthase, and the first site would function only in the homodimeric form to activate the reducing reaction upon association with CoA (Fig. 1c). Another CoA-binding site might accept CoA molecule only in the dissociated monomer form to stimulate the acyl condensation reaction. Alternatively, it is also possible that the same site for CoA-binding is involved with the monomer and dimer (plus NADPH) but with different outcomes in the two different reactions. CoA-induced activation of the acyl condensation catalyzed by monomeric 6HM synthase was specifically observed under the acidic condition (Table 2); however, at present, it is not clear why stimulation of the monomer-catalyzed condensation by CoA is observed only at slightly acidic pH. Although no evidence is available to explain this observation, it is possible that a key amino acid residue involved in monomer-specific CoA-binding site exhibits affinity against CoA only in the acidic condition.

In animal FAS, it was demonstrated (Strom et al., 1979; Strom and Kumar, 1979) that the activity of enoyl reductase was markedly stimulated in the presence of submillimolar levels of CoA, and an almost 5-fold increase in the activity was observed. Although the catalytic domain corresponding to enoyl reductase does not exist in 6HM synthase, regulation of the activities of this class of multifunctional proteins that catalyze acyl condensation and ketoreduction by association with CoA might be potentially an important phenomenon. Further studies to elucidate the mechanisms of the CoA-induced stimulation of acyl condensation catalyzed by 6HM synthase are in progress.

3. Experimental

3.1. Chemicals

6HM (**1**) was prepared by demethylating 6-methoxymellein isolated from fungal-infected carrot roots with BBr_3 in anhydrous CH_2Cl_2 as reported in detail (Kurosaki et al., 1989). TAL (**2**) was synthesized from dehydroacetic acid (nacalai tesque) as reported (Kurosaki et al., 1989), and ClAc–CoA was prepared according to the method of Kawaguchi et al. (1981).

2-Chloroethylphosphonic acid, acetyl-CoA, malonyl-CoA, NADPH, NADH, CoA, pantetheine and bovine serum albumin were purchased from Sigma, while dithiothreitol (DTT) and IoAA were from Wako Pure Chemicals. [2-^{14}C]Acetyl-CoA (2.1 GBq/mmol) and [2-^{14}C]malonyl-CoA (2.2 GBq/mmol) were obtained from Perkin Elmer. All other chemicals were reagent grade.

3.2. Induction, purification and assay of 6HM synthase-catalyzing reactions

6HM synthase was induced in carrot cells by treatment of root tissues with 2-chloroethylphosphonic acid (Kurosaki et al., 1989). The synthase was purified according to methods described previously (Kurosaki et al., 1993). Protein concentrations were determined by the method of Bradford (1976). The purity of the

synthase was assessed by a densitometric scan after separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970), and the results were reported previously (Kurosaki et al., 1993). The standard assay mixture for the determination of 6HM-producing activity of the enzyme consisted of 10 mM K-Pi (pH 7.5), acetyl-CoA (100 μM), [2-^{14}C]malonyl-CoA (50 μM) 3.7 kBq), enzyme preparation (2–5 pkat), DTT (2 Mm) and NADPH (1 mM) in a total volume of 100 μl unless otherwise noted. Formation of TAL (**2**) by 6HM synthase was achieved by employing either the dissociated monomer subunits of the synthase or the homodimer without NADPH. For dissociation of the homodimeric to the monomer subunits, the enzyme was incubated with 2 M NaCl at 37 °C for 5 min prior to the assay, and the TAL (**2**)-forming reaction was carried out in the presence of the same NaCl concentration (Kurosaki, 1995). The assay mixtures were incubated at 37 °C for 30 min, and the reactions were terminated by the addition of 50% (v/v) acetic acid (50 μl). The products were extracted with EtoAc (200 μl), and 50 μl-aliquots were applied onto a silica gel TLC plate. After development, the radioactivities co-migrating with authentic 6HM (**1**) or TAL (**2**) were determined as reported previously in detail (Kurosaki et al., 1989, 1993).

3.3. Chemical modification and substrate-binding assay of 6HM synthase

Two SH groups at the reaction center of 6HM synthase, Cys-SH and ACP-SH, were irreversibly blocked by alkylation with IoAA and ClAc–CoA according to the method described previously (Kurosaki, 1996a). In brief, DTT was removed from the synthase preparation by dialysis, and the enzyme was incubated with IoAA (5 Mm) ClAc–CoA (1mM) at 37 °C for 15 min to block Cys- and ACP-SHs, respectively (McCarthy and Hardie, 1982; Anderson and Kumar, 1987). After alkylation, DTT (7mM) was added to the mixture to quench excess SH inhibitors, and the sample was dialyzed against 20 mM K-Pi buffer containing 5 mM DTT (pH 7.5) to remove these reagents. When necessary, the enzyme preparation was dialyzed against the buffer containing 2 M NaCl for the dissociation of the homodimers to the monomer subunits. The partially masked 6HM synthase was then incubated with [^{14}C]-labeled acyl-CoAs to estimate the binding properties toward the latter substrates according to the method described previously (Kurosaki, 1996a). The assay mixture consisted of 10 mM K- Pi (pH 7.5), [^{14}C]acetyl-CoA or [^{14}C]malonyl-CoA (10 μM, 7.4 kBq), NADPH (1 mM), 5 μg proteins of the enzyme preparation (approximately 50 pkat/assay) and DTT (5mM) in a total volume of 100 μl. In some experiments, non-radiolabeled substrates were appropriately added to the assay mixture. The reaction was run for 2 min at 37 °C, and was terminated by the addition of TCA (500 μl, 2 M). Bovine serum albumin (100 μg) was then added to the reaction mixture as a carrier, and the precipitated proteins were recovered by centrifugation (700 X *g*, 5 min). The samples were denatured and subjected to SDS–PAGE, and the position of 6HM synthase subunits was determined with the molecular weight markers (Bio-Rad) after staining with Coomassie Brilliant Blue (Kurosaki et al., 1991). The gels containing the enzyme were excised with a blade, and the radioactivities were determined as described (Kurosaki, 1996a).

3.4. Determination of kinetic parameters of 6HM synthase

In order to determine the kinetic parameters of 6HM synthase under various reaction conditions, the assay was carried out with a series of concentrations of each of the substrates. K_m values for acetyl-CoA were estimated by a set of enzyme reactions with a fixed concentration of malonyl-CoA (50 μM) and 20–100 μM of acetyl-CoA, while, for estimation of malonyl-CoA, the reactions were run with 100 μM of acetyl-CoA and 3–10 μM of malonyl-CoA (Kurosaki, 1996b), respectively. The results were analyzed by the double reciprocal plots with the method of the least squares.

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