Role of reducing co-factors in catalytic reactions of 6-hydroxymellein synthase, a multifunctional polyketide biosynthetic enzyme in carrot cells

Fumiya Kurosaki *, Kousuke Togashi, Munehisa Arisawa

Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-0194, Japan

Received 5 June 2000; received in revised form 14 August 2000; accepted 16 August 2000

Abstract

6-Hydroxymellein (6HM) synthase, a multifunctional polyketide biosynthetic enzyme in carrot cells, is capable of catalyzing the acyl-CoA condensation and the ketoreduction in the presence of the nucleotide reducing co-factors. Although free CoA at high concentrations functioned as the activator of the NADPH-dependent 6HM formation, the compound exhibited an appreciable inhibitory activity toward the reaction mediated by NADH. CoA showed a potent inhibitory activity against substrate entry into the reaction center of the NADH-associated enzyme while, in the presence of NADPH, the compound slightly inhibited the formation of the acylated enzyme. The catalytic rate of the synthase was appreciably decreased when NADPH was replaced by the deuterium-labeled compound, however, the \( k_H/k_D \) value was markedly reduced if NADH and [D]NADH were employed as the reducing co-factors. These results suggest that the phosphate group attached to 2'-position of the adenosyl moiety of NADPH associated with the ketoreducing domain of 6HM synthase plays an important role in the regulation of the enzyme activity.

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Keywords: Daucus carota; Polyketide biosynthetic enzyme; Reducing co-factor; Rate-limiting step; Multifunctional enzyme; Deuterium isotope effect

1. Introduction

6-Hydroxymellein (6HM) is a direct precursor of carrot phytoalexin, 6-methoxymellein, and the enzyme catalyzing the biosynthesis of the compound, 6HM synthase, is a multifunctional polyketide synthetic enzyme induced in the cells [1,2]. The enzyme catalyzes the condensation of acyl-CoAs to form the polyketomethylene chain and the ketoreduction of the enzyme-bound triketide intermediate in the presence of NADPH or NADH (Fig. 1a). It was demonstrated [3] that this hydride transfer process is the late-limiting step in the series of partial reactions, and determines the overall rate of 6HM biosynthesis. The active form of 6HM synthase is organized as a homodimer, and two subunits (approximately 130 kDa each) containing the functional domains of the ketoreduction and the ketomethylene chain elongation are aligned in a head-to-tail direction [4]. Therefore, the organization of 6HM synthase is similar to multifunctional fatty acid synthase (FAS) in animal cells [5–7], however, unlike in animal FAS, it appears that both of two SH groups (Cys-SH and 4'-phosphopantetheine-SH attached to acyl carrier protein) at the reaction center of 6HM synthase belong to the same subunit (Fig. 1b and c) [8]. In this class of FAS, it is widely accepted [6,9] that both acetyl and malonyl moieties load onto Ser-OH at the transacylase structure as the common primary binding site during any stage of the chain elongation, and an undesired acyl group attaches to this structure is rapidly exchanged to the proper one by the ester-exchanging reaction with CoA as the acyl acceptor. In contrast, when

* Corresponding author. Fax: +81-76-4345052.
E-mail address: kurosaki@ms.toyama-mpu.ac.jp (F. Kurosaki).
the CoA concentration rises, the equilibrium in the translocation of substrates between CoA- and enzyme-bound forms is shifted to CoA-esters to decrease the concentration of free CoA. Therefore, CoA at low concentrations functions as an activator for FAS [10,11] while it exhibits the inhibitory activity to the enzyme at high concentrations [6,9].

We have reported previously [12] that, in NADPH-associated 6HM synthase, CoA at low concentrations functions as an activator as well as in FAS, however, in sharp contrast to FAS, the synthase activity appreciably increases even in the presence of relatively high concentrations of CoA. On the other hand, we have also reported that biochemical properties of the NADPH- and NADH-associated synthase are quite different in several respects. For example, only NADPH- but not NADH-synthase complex shows the insensitivity against cerulenin [13], and affinity of the synthase toward acetyl-CoA specifically increases only in the NADPH-associated form [14,15]. The primary aim of the present study was elucidation of the roles of the nucleotide reducing co-factors and CoA in the regulation of 6HM synthase activity. Special attention was focused on the possible role of the phosphate group attached to 2’-position of adenosyl moiety of NADPH (2’P), the structural difference between NADPH and NADH, in the enzyme regulation.

2. Materials and methods

2.1. Chemicals

6-Hydroxymellein was prepared by demethylating 6-methoxymellein isolated from fungi-infected carrot root tissues with BBr₃ in anhydrous CH₂Cl₂ as described previously in detail [1,8]. Chloroacetyl-CoA (ClAc-CoA) was synthesized according to the method of Kawaguchi et al. [16]. 2-Chloroethylphosphonic acid, acetyl-CoA, malonyl-CoA, CoA, pantetheine, NADPH, NADP, NADH, NAD, ATP and bovine serum albumin

Fig. 1. (a) Catalytic reaction of 6HM synthase. 6HM synthase catalyzes the condensation of acetyl- and malonyl-CoAs, and an NAD(P)H-dependent ketoreduction takes place at the triketide intermediate stage to form a reduced ketomethylene chain. Further condensation of malonyl-CoA results in the production of 6HM. (b) Schematic presentation of the arrangement of the functional domains of homodimeric 6HM synthase. The catalytic domain for ketomethylene chain elongation is associated with that of ketoreduction belonging to another subunit, and two reaction centers are organized in each molecule of the active synthase. (c) Schematic presentation of the reaction center of 6HM synthase. The acyl groups bound to Ser-OH of transacylase are properly channeled to two SH groups, Cys-SH and acyl carrier protein (ACP)-SH, prior to the initiation of the condensation reactions.
were purchased from Sigma. Dithiothreitol (DTT) and iodoacetoamide (IOAA) were obtained from Wako Pure Chemicals while glucose-1-D (98%) was from Aldrich. Glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides) and hexokinase (from yeast) were from Oriental Yeast. [2-14C]Acetyl-CoA (specific activity 2.1 GBq:mmol) and [2-14C]malonyl-CoA (specific activity 2.2 GBq:mmol) were from New England Nuclear. All other chemicals were reagent grade.

2.2. Induction, purification and assay of 6HM synthase

6-Hydroxyellein synthase was induced in carrot root tissues by treatment with 2-chloroethylphosphonic acid [1,2], and the synthase protein was highly purified according to the methods described previously [3]. Protein concentrations were determined by the method of Bradford [17], and the purity of the synthase in the enzyme preparation was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [18]. After staining with Coomassie Brilliant Blue, the concentration of the synthase in the preparation was determined by a densitometric scan on a dual wavelength chromatoscanner [3,13,19]. The standard assay mixture of the synthase activity consisted of 10 mM K-phosphate (pH 7.5), 100 μM of acetyl-CoA, 50 μM of [14C]malonyl-CoA (3.7 kBq), 2–5 pkat of the enzyme preparation, 2 mM of DTT, 1 mM of NADPH or NADH and, if necessary, various concentrations of free CoA or pantetheine in a total volume of 100 μl. The mixtures were incubated at 37°C for 30 min, and the reaction was terminated by the addition of 50 μl of 50% (v/v) acetic acid. The products were extracted with 200 μl of ethyl acetate by blending, and 50 μl-aliquots were applied onto a silica gel TLC plate. After development, the radioactive co-migrated with the authentic 6HM were determined as reported previously in detail [1–3]. In some experiments, the reaction was coupled with a CoA-scavenging system, and 1 mM ATP, 2 mM MgCl2, 2 mM Na-citrate and approximately 0.1 nkat of ATP: citrate lyase, which had been partially purified according to the method of Stern et al. [10], were added in a total volume of 260 μl. In some experiments, 30 μM of CoA or pantetheine was also added into the assay mixture.

2.3. Modification and binding assay of 6HM synthase

Two SH groups at the reaction center of 6HM synthase were blocked by alkylation according to the method described previously in detail [20]. In brief, DTT was removed from the synthase preparation by dialysis, and Cys- and ACP-SHs were alkylated by the incubation of the enzyme protein with 5 mM of IOAA plus 1 mM of ClAc-CoA at 37°C for 15 min. After the alkylation, 7 mM DTT was added to the mixture to quench the excess SH inhibitors, and the sample was dialyzed against 20 mM K-phosphate buffer containing 5 mM DTT (pH 7.0) to remove these reagents. Binding abilities of the partially masked 6HM synthase toward the co-substrates were determined by the incubation with [14C]-labeled acyl-CoAs according to the method described previously [12,20]. The assay mixture consisted of 10 mM K-phosphate (pH 7.5), 10 μM of [14C]acetyl-CoA or [14C]malonyl-CoA (7.4 kBq), 1 mM of NADH, 5 μg proteins from the enzyme preparation (approximately 50 pkat/assay) and 5 mM DTT in a total volume of 100 μl. The reaction was run for 2 min at 37°C, and was terminated by the addition of 500 μl of 2 M trichloroacetic acid. To the reaction mixture was added bovine serum albumin (100 μg) as a carrier, and the enzyme protein was recovered by precipitation. The resultant pellets were denatured and subjected to SDS-PAGE, and, after electrophoresis, gel slices containing the enzyme protein were excised with a blade [20]. They were immersed in 0.5 ml of Solvable (New England Nuclear), and the radioactivities were determined.

2.4. Preparation of stereospecifically deuterium-labeled NADH

4-S-[4-D]NADH was prepared essentially according to the method described by Wilken et al. [21]. NAD was reduced with glucose-6-phosphate dehydrogenase in the presence of [1-D]glucose-6-phosphate which was generated from glucose-1-D by the action of hexokinase. The labeled NADH was purified by an ion exchange chromatography on a DEAE-Sephacel column (Pharmacia, 1.6 × 11 cm) with a linear gradient of NaCl (0–0.5 M) in a total 100 ml of 20 mM K-phosphate buffer (pH 7.0).
Table 1
Effect of ATP: citrate lyase on the activity of the NADH-associated 6HM synthase

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>+ Citrate lyase</td>
<td>91</td>
</tr>
<tr>
<td>+ Boiled citrate</td>
<td>78</td>
</tr>
<tr>
<td>+ Citrate lyase + citrate</td>
<td>29</td>
</tr>
<tr>
<td>+ Citrate lyase + citrate</td>
<td>79</td>
</tr>
<tr>
<td>+ Citrate lyase + citrate + CoA (30 μM)</td>
<td>81</td>
</tr>
<tr>
<td>+ Pantetheine (30 μM)</td>
<td></td>
</tr>
</tbody>
</table>

*6HM synthase activities of controls which received only Mg²⁺ and ATP as the additional factors were taken as 100%, and the results were expressed as percentages.

3. Results

3.1. Effect of low concentrations of free CoA on NAD(P)H-dependent 6HM synthase reaction

We have reported [12] that, as well as in animal FAS, free CoA spontaneously generated during the enzyme reaction functions as an activator of 6HM synthase when NADPH is employed as the reducing co-factor. In order to examine the possible effect of 2’P in the catalytic reactions in the presence of low concentrations of CoA, the compound was quenched by coupling the reaction system with ATP: citrate lyase, a CoA-scavenging enzyme [10]. The synthase activity was appreciably inhibited in the presence of the native citrate lyase plus its substrate (Table 1), however, the lyase-induced inhibition was restored to almost the control level by the addition of 30 μM of free CoA. Recovery of the inhibition of the synthase activity was also observed when pantetheine was employed as an acyl acceptor suggesting that, as well as in NADPH-dependent reaction, the acyl acceptor is essential for the catalytic reaction of NADH-associated 6HM synthase. It is very likely, therefore, that the rapid transfer back reaction of undesired acyl groups to appropriate acyl acceptors is essential for the proper entry of the co-substrates irrespective of the chemical species of the nucleotide reducing co-factors associated with the ketoreducing domain of 6HM synthase.

3.2. Effect of high concentrations of free CoA on NAD(P)H-dependent 6HM synthase reaction

In our previous work, it was demonstrated [12] that, unlike in FAS, submillimolar concentrations of CoA added to the assay mixture of NADPH-associated 6HM synthase functioned as an activator, and the enzyme activity increased to about 170% level of the control in the presence of 1 mM of CoA. Several lines of evidence suggested [12,22] that a certain allosteric interaction between CoA molecule and the NADPH-enzyme complex alters the microstructure around the reaction center, and this affects the ketoreducing process, the rate-limiting step of 6HM biosynthesis, to enhance the overall catalytic rate. Therefore, it was tested whether or not this ‘unusual’ property of free CoA in the 6HM-forming reactions is similarly observed in the NADH-associated synthase. CoA was added to the reaction system of 6HM synthase in the presence of NADH, and the effect of relatively higher concentrations of the compound on 6HM formation was examined. As shown in Fig. 2, the activity of 6HM synthase decreased according to the increase in CoA concentrations, and the residual activity of the enzyme was found to be approximately 40% of the control in the presence of 1 mM of CoA. Pantetheine also exhibited a marked inhibitory activity to the reaction catalyzed by NADH-synthase in a dose-dependent manner, and 6HM formation decreased to about 40% level in the presence of 100 μM of the comp-
Table 2
Binding activity of NADH-associated 6HM synthase toward the co-substrates in the presence of acyl acceptors

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+ CoA</td>
<td>+ Pantetheine</td>
<td>Control</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>1b</td>
<td>0.38</td>
<td>0.42</td>
<td>1</td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>1.14</td>
<td>0.27</td>
<td>0.32</td>
<td>0.88</td>
</tr>
<tr>
<td>Acetyl-CoA–acetyl</td>
<td>0.41</td>
<td>0.19</td>
<td>0.21</td>
<td>0.44</td>
</tr>
<tr>
<td>+ malonyl-CoA–malonyl</td>
<td>0.57</td>
<td>0.24</td>
<td>0.16</td>
<td>0.51</td>
</tr>
</tbody>
</table>

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 presence of 300 μM of CoA or pantetheine. Controls did not receive these acyl acceptors.

b Results were expressed as relative values in which the amounts of acetyl group bound to the modified enzyme were taken as 1 in each set of the experiments.

Therefore, it has been clearly demonstrated that NADPH- and NADH-associated 6HM synthase proteins show the quite different sensitivities to high concentrations of free CoA, and the compound functions as an activator only toward the enzyme complexed with NADPH.

3.3. Effect of high concentrations of free CoA on substrate entry into 6HM synthase

We reported previously [12] that free CoA showed only slight inhibitory activity against the binding ability of NADPH-6HM synthase complex toward the co-substrates, and the ratio of the enzyme-bound acyl groups slightly decreased to 70–90% of the controls even in the presence of 300 μM CoA. In contrast, it was reported [10] that CoA showed a potent inhibitory activity toward the substrate-binding ability of FAS, and the ratio of the enzyme-bound acyl groups decreased to the level of about 10% of the control. In the next experiments, it was tested whether or not the CoA-induced slight inhibition of substrate entry into 6HM synthase protein was similarly observed when NADPH was replaced by NADH. The ratio of the synthase-bound forms of the acyl groups of the substrates was measured in the absence and presence of the acyl acceptors. In order to simplify the estimation of the binding ability of the enzyme toward its substrates, two SH groups at the reaction center of the synthase, Cys-SH and ACP-SH, were alkylated with IoAA and ClAc-CoA, respectively, according to the methods reported previously [12,20]. In this partially masked enzyme protein, it is expected that only Ser-OH of transacylase structure, the primary binding site of the acyl groups, remains to be free (Fig. 1c). As reported previously [3,20], an attempt to estimate the chemical stoichiometry of the synthase-bound acyl groups against the enzyme protein was unsuccessful because of the varied purity of the synthase in each batch of the enzyme preparations and the unstability of the enzyme protein. Therefore, the results were expressed as relative values in which the amount of acetyl group bound to the chemically modified enzyme protein was taken as 1 in each set of the experiments. As shown in Table 2, addition of 300 μM of CoA resulted in a marked decrease in the enzyme-bound acetyl and malonyl groups when these two substrates were added independently. The enzyme-bound forms of the acyl groups decreased to roughly 30–40% levels of the controls, and the similar results were also obtained when the two substrates were added as 1:1 mixture (Table 2). It appears, therefore, that CoA exhibits the potent inhibitory activity toward substrate entry into the NADH-associated enzyme protein while, in the NADPH-enzyme complex, it functions as a very weak inhibitor. Addition of pantetheine also showed the similar results and a marked decrease in the enzyme-bound forms of the substrates was observed in the presence of this acyl acceptor (Table 2).

3.4. Deuterium isotope effect of NAD(P)H-dependent 6HM synthase reaction

As described above, the NADPH-dependent ketoreduction at the triketide intermediate stage
(Fig. 1a) is the rate-limiting step, and a marked deuterium isotope effect ($k_{\text{H}}/k_{\text{D}} 5.2$) was observed when 4-pro-S-hydrogen of NADPH was replaced by deuterium [3]. In order to examine the possibility that the difference in the chemical species of the reducing co-factor associated with 6HM synthase would affect the rate-limiting process, we determined the deuterium isotope effect in NADH-dependent 6HM formation (Table 3). The assay of the synthase was carried out in the presence of 1 mM of NADH or 4-S-[4-D]NADH, and $1.6 \pm 0.4$ ($n = 4$) was obtained as $k_{\text{H}}/k_{\text{D}}$ value (Table 3). In parallel, a set of the experiments to determine $k_{\text{H}}/k_{\text{D}}$ for NADPH-mediated 6HM formation was also repeated, and the values essentially identical to that reported previously [3] were obtained (4.9–5.6). The present observation strongly suggests that the contribution of NADPH and NADH to the ketoreducing reaction is significantly different, and 2’P of NADPH plays an important role in the determination of synthetic rate of 6HM.

In our previous work, it has been demonstrated [12] that the addition of submillimolar concentrations of free CoA appreciably reduces the deuterium isotope effect in NADPH-associated 6HM synthase, and $k_{\text{H}}/k_{\text{D}}$ value decreases from 5.2 to 2.8 in the CoA-treated enzyme. Therefore, we concluded [12] that the ketoreducing reaction is, at least, one of the partial reactions responsible for the CoA-induced stimulation of NADPH-associated 6HM synthase activity. Considering the several differences in biochemical properties between NADPH- and NADH-associated 6HM synthase, we examined the possible change in the deuterium isotope effect of NADH-synthase complex in the presence of CoA. As shown in Table 3, the CoA-induced reduction of the isotope effect in NADPH-associated 6HM synthase [12] was not observed in the NADH-enzyme complex, and essentially identical $k_{\text{H}}/k_{\text{D}}$ values were obtained in the absence and presence of free CoA (1.6 and 1.7, respectively). This result strongly suggests that the association of CoA molecule with 6HM synthase protein stimulates the ketoreducing reaction only when the enzyme is complexed with NADPH but not with NADH. Addition of pantetheine to the NADH-associated synthase also did not affect the isotope effect. These observations, together with our previous results [12,22], suggest that an allosteric interaction between 2’P of NADPH molecule at the ketoreducing domain with a certain structure around the reaction pocket of 6HM synthase protein associated with CoA plays a central role in the CoA-induced enhancement of the rate-limiting reaction to increase the apparent rate of 6HM biosynthesis.

### Table 3

<table>
<thead>
<tr>
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<th>$k_{\text{H}}/k_{\text{D}}$ values$^a$</th>
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</thead>
<tbody>
<tr>
<td>[D]NADH</td>
<td>1.6 $\pm$ 0.4</td>
</tr>
<tr>
<td>[D]NADH + 300 µM CoA</td>
<td>1.7 $\pm$ 0.3</td>
</tr>
<tr>
<td>[D]NADH + 300 µM pantetheine</td>
<td>1.5 $\pm$ 0.7</td>
</tr>
</tbody>
</table>

$^a$ 6HM synthase activity was determined under the standard assay conditions (100 µM of acetyl-CoA, 50 µM of [14C]malonyl-CoA and 1 mM of [D]NADH) in the presence or absence of the reducing co-factors. The results were presented as the means and standard deviations obtained from four independent experiments.

4. Discussion

In the present study, it has been clearly demonstrated that structural difference between NADPH and NAD, 2’P, plays important roles in the regulation of 6HM synthase activity. The CoA-induced allosteric effect, which results in the stimulation of the synthase activity, is specifically observed in the NADPH-associated synthase but not in the enzyme complexed with NADH. It appears that CoA functions as a potent inhibitor toward substrate entry into the reaction pocket only in the NADH-synthase. It is also likely that 2’P contributes the control of the rate-determining process of 6HM biosynthesis even in the absence of free CoA.

What kind of interaction between 2’P and 6HM synthase protein is responsible for these changes of the catalytic properties of the enzyme? Recently, Suh et al. have reported [23] that the activity of monoxygenase from *Saccharomyces cerevisiae* appreciably depends on the nucleotide reducing co-factors, and the enzyme uses NADPH 12 times more efficiently than NADH. Amino acid sequence analysis suggests that Lys residue(s) of the enzyme protein act as counterions of 2’P of NADPH, and this electrostatic interaction is implicated in the characteristic of the nucleotide-mediated enzyme reaction. Similar results have also been reported from several proteins containing...
NADPH/NADH binding domain from a wide variety of biological sources ranging from bacteria to mammalian cells [24–26], and the specific recognition of 2’P of NADPH with two positively charged residues, such as Lys and Arg, is demonstrated in this class of proteins. It might be expected, therefore, that, as well as these NAD(P)H-dependent proteins, a certain interaction between 2’P of NADPH and basic amino acid residues in 6HM synthase structure alters several catalytic properties of NADPH-associated 6HM synthase. Further elucidation of the interaction between the nucleotide co-factors and 6HM synthase. Further elucidation of the interaction between 2’P of NADPH and basic amino acid residues in 6HM synthase alters several catalytic properties of NADPH-associated 6HM synthase. Further elucidation of the interaction between the nucleotide co-factors and 6HM synthase structure and its role in the regulation of the enzyme activity is in progress in our laboratory.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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F. Kurosaki et al. / Plant Science 160 (2000) 113–120 119