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Rapid report

Effect of phosphate residue of NADPH on the interaction between catalytic domains of a multifunctional polyketide synthetic enzyme 6-hydroxymellein synthase

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Abstract

Association of NADPH with the ketoreducing domain of 6-hydroxymellein synthase, a multifunctional polyketide synthetic enzyme of carrot, evoked the alternation of microstructure around the primary binding site of the co-substrates acetyl- and malonyl-CoAs, and this resulted in the marked decrease in K_m value of the enzyme protein for acetyl-CoA. In contrast, the enzyme did not show the increase in the affinity to the substrate when NADPH was replaced by NADH. These results suggest that the phosphate residue attached to 2′-position of adenosyl moiety of NADPH molecule plays an important role in the co-operative interaction between these functional domains of the synthase. © 1998 Elsevier Science B.V.

Keywords: Polyketide synthase; Catalytic domain; Substrate entry

6-Hydroxymellein (6HM) synthase is an inducible multifunctional polyketide biosynthetic enzyme in carrot cells [1,2], and catalyzes the condensation of 1 mol of acetyl-CoA and 4 mol of malonyl-CoA. An NADPH-dependent ketoreduction of the carbonyl group of the top C_2 unit takes place at the triketide intermediate stage to form a dihydroisocoumarin skeleton (Fig. 1(a)). I reported previously [3,4] that the active form of 6HM synthase is organized as a homodimer composed of multifunctional subunits. It is very likely that the two subunits (approximately 130 kDa each) are aligned in an antiparallel direction, and the functional domain for the ketoreduction associates with that for the ketomethylene chain elongation to form two reaction centers in each molecule of the enzyme (Fig. 1(b)). Therefore, the overall organization of 6HM synthase is similar to type IA fatty acid synthase (FAS) in animal cells [5]. However, it appears that both of the two SH groups (cysteine–SH and 4′-phosphopantetheine–SH attached to acyl carrier protein) at the reaction center of 6HM synthase belong to the same subunit (Fig. 1(b)) [3,4] while, in type IA FAS, it has been widely accepted that one of the two SH groups is contributed from the other subunit of the homodimer [6,7]. Recently, I have shown [8] that the association of NADPH molecule with the ketoreducing domain of 6HM synthase

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evokes a certain perturbation of the transacylase structure, the common primary binding site of the co-substrates of the enzyme acetyl-CoA and malonyl-CoA [9]. This alternation of the microstructure of the synthase results in the increase in the affinity of the enzyme protein for acetyl- but not for malonyl-CoA (Fig. 1(c)) [8]. Therefore, it has been demonstrated that the NADPH-associated ketoreducing domain of 6HM synthase contributes in the biosynthetic processes of 6HM not only at the middle stage of the catalytic cycle to reduce the triketide intermediate, but also at the earliest step, the entry of the starter unit into the enzyme protein (Fig. 1(a)). This unique co-operative interaction between the catalytic domain observed in 6HM synthase is a novel class of 'subunit communication' of multifunctional proteins [8]. The primary aim of the present experiments was testing whether or not the functions of NADPH in the series of 6HM synthase-catalyzing reactions can be replaced by NADH.

6HM synthase activity was induced in carrot cells by the treatment of the root tissues with 2-chloroethylphosphonic acid [2], and the synthase was highly purified according to the methods described previously [2,3]. Protein concentrations were determined by the method of Bradford [10]. The purity of the enzyme preparation was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8% gel) according to the method of Laemmli [11]. A densitometric scan on a dual-wavelength chromatoscanner was performed after the proteins were stained with Coomassie brilliant blue, and the results were reported previously [2,8,9]. The standard assay mixture of the synthase activity consisted of 10 mM K-phosphate (pH 7.5), 100 μM of acetyl-CoA, 100 μM of [2-^{14}C]malonyl-CoA (3.7 kBq), approximately 5 pkat of the enzyme preparation, 1% (v/v) of mercaptoethanol and 1 mM NADPH in a total volume of 200 μl. The mixtures were incubated at 37°C for 30 min, and the reaction was terminated by the addi-

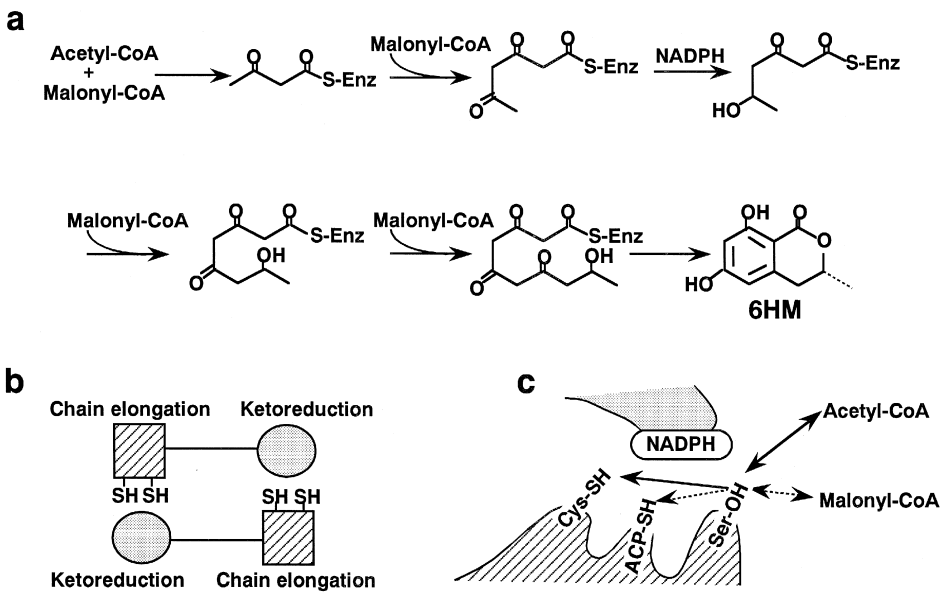

Fig. 1. (a) Catalytic reaction of 6HM synthase. 6HM synthase catalyzes the condensation of acetyl-CoA-and malonyl-CoA, and an NADPH-dependent ketoreduction takes place at the triketide intermediate stage to form a reduced ketomethylene chain. Further condensation of malonyl-CoA results in the production of 6HM [1]. (b) Schematic presentation of the arrangement of the functional domains of homodimeric 6HM synthase. It is assumed that the catalytic domain for ketomethylene chain elongation is associated with that of ketoreduction belonging to another subunit. Two reaction centers are organized in each molecule of the active synthase, and it is likely that two SH-groups at the reaction center are contributed from the same subunit in the homodimer [3]. (c) Schematic presentation of the reaction center of 6HM synthase. NADPH-associated ketoreducing domain interacts with transacylase structure, a common primary binding site of the co-substrates, acetyl- and malonyl-CoAs, to enhance the affinity of the enzyme protein to acetyl-CoA [8]. 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 [9].

tion 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 the development, the radioactivities co-migrated with the authentic 6HM were determined as reported previously in detail [1,8].

To examine the possible difference between NADPH- and NADH-mediated reactions, the activity of 6HM synthase was determined in the presence of various concentrations of these co-factors. As shown in Fig. 2, 6HM synthetic activity of the enzyme increased with the increase in NADPH concentration in a dose-dependent manner. 6HM production by the synthase was also observed when NADH was employed instead of NADPH. However, it appeared that the effect of NADPH on the enzyme activity could not be fully replaced by NADH, and the relative activity of the NADH-dependent 6HM production of the synthase was usually 50–70% of that of the NADPH-mediated reaction within the concentration range tested.

It is reasonable to assume three possible mechanisms accounting for the low efficiency of the NADH-mediated reaction; (i) low affinity of NADH to the enzyme protein; (ii) low reaction rate of the NADH-mediated reaction; (iii) lack or low intensity of the enhancement of acetyl-CoA entry into the

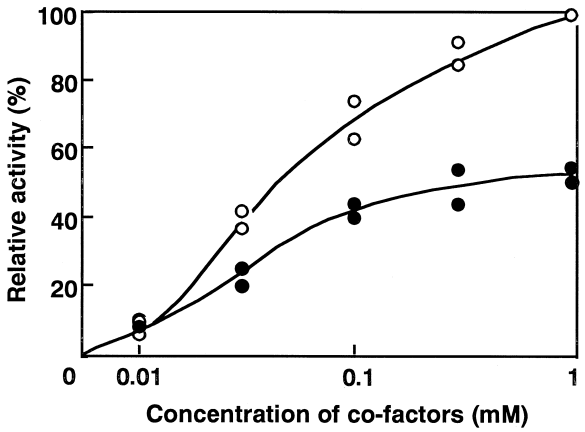

Fig. 2. Effect of various concentrations of NAD(P)H on the catalytic reaction of 6HM synthase. 6HM synthase was induced in and highly purified from carrot root tissues, and the activity of the enzyme was determined in the presence of various concentrations of NADPH (○) or NADH (●). The synthetic activities were expressed as percentages in which the activities observed in the presence of 1 mM NADPH were taken as 100%.

Table 1
Summary of kinetic parameters of NADPH- and NADH-dependent reactions catalyzed by 6HM synthase

Co-factors	Kinetic parameters [a]		
		K_m	Relative V
NADPH	NADPH	70 ± 17 μM	1.0
	Acetyl-CoA	22 ± 4 μM [b]	
	Malonyl-CoA	44 ± 11 μM	
NADH	NADH	10 ± 2 μM	1.1 ± 0.03
	Acetyl-CoA	224 ± 25 μM	
	Malonyl-CoA	48 ± 8 μM	

[a] The results were presented as the means and SDs obtained from three independent experiments.
[b] Previously reported values [8] were presented as K_m for acetyl-CoAs in the NADPH-mediated reaction.

NADH-associated enzyme. In order to elucidate the biochemical basis of the relatively low yield of the NADH-mediated 6HM production, the synthetic reactions were run under the various conditions employing highly purified 6HM synthase, and the kinetic parameters of these reactions were determined. First, possible difference in the affinity of 6HM synthase toward the two reducing co-factors was examined. The assay of 6HM synthase activity was carried out with 20–50 μM of NADPH or NADH, and K_m values for these co-factors were determined by the double reciprocal plot analyses with the method of least squares. K_m of 6HM synthase for NADPH was estimated to be 70 μM while for NADH was 10 μM (Table 1) indicating that the affinity of the enzyme protein for NADPH is appreciably lower than that for NADH. This observation strongly suggests that the difference in the affinity between NADPH and NADH to the enzyme protein is not responsible for the low efficiency of the NADH-mediated product formation.

As reported previously [2], 6HM synthase preparation employed in the present experiments did not consist of a homogenous protein though highly purified, and therefore, the purity and the specific activities of the synthase were varied in each batch of the enzyme preparation. In addition, due to the instability of the enzyme protein, a part of the synthase activity was lost during the purification [2,3]. Therefore, V value for 6HM synthase was appreciably varied in repeated experiments (9–34 nkat/g protein), and the direct comparison of Vs obtained from independent

experiments was impossible. However, the relative ratio of the V values for the NADPH- and NADH-dependent reactions was found to be almost constant irrespective of the specific activity of the enzyme preparations. Therefore, in order to compare the velocities of the NADPH- and NADH-mediated processes, Vs of these two reactions were expressed as the relative values in which V in the NADPH-mediated reaction was taken as 1.0 in each set of the experiments. As shown in Table 1, relative Vs of the NADPH- and NADH-mediated reactions were essentially identical (1.0 and 1.1, respectively) suggesting that the different efficiency of the reducing co-factor-dependent 6HM formation is not caused by the rates of these two reactions.

In the next experiments, the possibility was examined that the NADPH-induced enhancement of acetyl-CoA entry into the enzyme protein is observed even if NADPH is replaced by NADH. I reported previously [8] that K_m value of 6HM synthase for acetyl-CoA was 22 μM when the reaction was run in the presence of NADPH. The same set of the experiments was repeated in the present study with a series of the concentrations of acetyl-CoA (20–100 μM), and essentially identical values (19–27 μM) were obtained for the NADPH-dependent reaction (Fig. 3). In contrast, the value for the NADH-mediated reaction was found to be much higher (224 μM) than that for NADPH (Table 1). This figure was almost comparable with that obtained for the synthase from

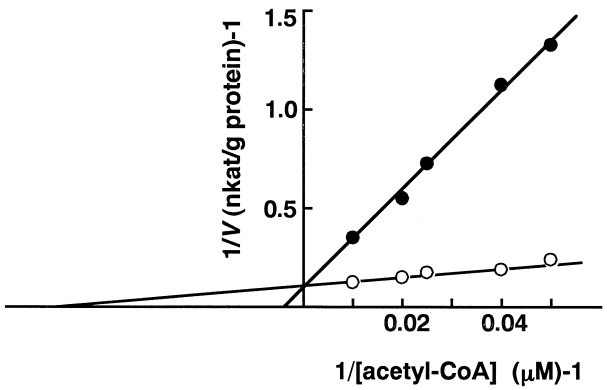

Fig. 3. Double reciprocal plot analyses of the NADPH- and NADH-mediated reactions of 6HM synthase. Primary reciprocal plots were determined at a series of concentrations of acetyl-CoA (20–100 μM) in the presence of 1 mM of NADPH (○) or NADH (●), and a typical set of the results is presented.

which these reducing co-factors were omitted (284 μM) [8]. Therefore, it is assumed that, unlike in NADPH, NADH-associated 6HM synthase protein shows, if any, only a very little effect on the interaction between ketoreductase and transacylase domains which increases in the affinity to acetyl-CoA. In contrast to the marked difference observed in K-acetyl-CoA, K_ms for malonyl-CoA in the NADPH- and NADH-dependent 6HM production were almost identical (44 and 48 μM, respectively) as determined with 3–10 μM of malonyl-CoA (Table 1).

Results obtained in the present study suggest that the phosphate residue attached to the 2′-position of adenosyl moiety of NADPH molecule (2′-P) plays an important role in the co-operative interaction between the NADPH-associated ketoreducing domain and the transacylase structure which results in the enhancement of the affinity of the enzyme for acetyl-CoA. In sharp contrast to acetyl-CoA, it appeared that the affinity of another substrate of 6HM synthase, malonyl-CoA, does not depend on the species of the reducing co-factors associating with the ketoreducing domain. I showed previously [8] that K_m value for malonyl-CoA does not change even if the reducing co-factors were omitted. Therefore, it has been clearly demonstrated that the microenvironmental alternation of the enzyme structure evoked by 2′-P of NADPH solely influences the affinity for acetyl-CoA but not for malonyl-CoA. It has been also suggested that 2′-P is an important partial structure which affects the affinity of NADPH to the enzyme protein. K_m value of the synthase for NADPH is 7-fold higher than that for NADH. Therefore, it is very likely that 2′-P of NADPH molecule exhibits both positive and negative effects on the catalytic reaction of 6HM synthase; increase in the affinity of the enzyme protein to acetyl-CoA and decrease in the affinity of NADPH itself. The apparent low efficiency of NADH in the 6HM-forming reaction would result from the ‘balance’ of these positive and negative effects caused by the phosphate residue. In our previous work, it was demonstrated that the transfer of pro-*S*-hydrogen at the 4-position of NADPH to the carbonyl carbon atom of the triketide intermediate is the rate-limiting step of the series of partial reactions involved in 6HM biosynthesis [2]. However, no obvious difference was observed in the velocities of the NADPH- and NADH-mediated reactions suggesting that 2′-P in

NADPH structure does not affect the hybrid transfer process.

In conclusion, it has been demonstrated that 2′-P of NADPH molecule is an essential partial structure in the novel class of co-factor-induced subunit communication between the catalytic domains of a multifunctional polyketide biosynthetic enzyme, 6HM synthase. This phosphate residue would directly or indirectly evoke a certain alternation of the microenvironmental circumstance around the primary binding site of the substrates to increase the affinity of the synthase protein toward acetyl-CoA. Further characterization of the interaction between NAD(P)H-associated ketoreducing domain and transacylase structure of 6HM synthase is in progress in my laboratory.

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