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Communication

Kinetic Characterization of Caffeoyl-Coenzyme A-Specific 3-*O*-Methyltransferase from Elicited Parsley Cell Suspensions[1]

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ABSTRACT

The activity of caffeoyl-coenzyme A (CoA) 3-*O*-methyltransferase, an enzyme widely distributed in plants and involved in cell wall reinforcement in a disease resistance response, appears to be subject to a complex type of regulation *in vivo*. In cultured parsley (*Petroselinum crispum*) cells treated with an elicitor from *Phytophthora megasperma* f.sp. *glycinea*, the enzyme activity is rapidly induced by a transient increase in the rate of *de novo* transcription. Parsley caffeoyl-CoA-specific methyltransferase differs in several aspects from other plant *O*-methyltransferases but shows limited homology to bacterial adenine-specific DNA methyltransferases. Kinetic analysis revealed an Ordered Bi Bi mechanism for catalysis, with caffeoyl-CoA bound prior to *S*-adenosyl-L-methionine and feruloyl-CoA released last from the enzyme. The small inhibitory constant determined *in vitro* for feruloyl-CoA suggests that, *in vivo*, the enzyme activity is also under tight control by the steady-state product concentration in addition to the rate of transcription that becomes affected upon elicitor challenge.

Challenge of parsley cell suspension cultures with fungal elicitors induces a concomitant and rapid accumulation of coumarin phytoalexins and the formation of ferulic cell wall esters (14). Both these reactions contribute to the overall disease resistance response, which is commonly observed in incompatible interactions of plants with phytopathogenic fungi. Whereas taxonomically different plants accumulate phytoalexins of different chemical nature (1), the incorporation of ferulic and related acids into cell walls appears to be a widespread phenomenon and invariably requires the activation of the general phenylpropanoid pathway (13). CCoAMT,[2] an enzyme responsible for the formation of feruloyl-CoA, is induced under these conditions (14). CCoAMT was characterized recently from elicitor-treated parsley cells (14, 15), and some homology to bacterial AMTs was noticed (15). This methyltransferase was shown to possess a remarkably narrow substrate specificity for caffeoyl-CoA, and the adenine moiety of CoA was assumed as a cause of homology with adenine-specific DNA-methyltransferases from bacterial sources, which had been proposed earlier to have evolved by gene duplication (7, 12, 15).

In the context of phenylpropanoid biosynthesis, in particular flavonoids and ferulic acid, various OMTs have been studied (6, 16). However, only few have been purified to homogeneity and there is little information on their kinetic mechanisms. None of these OMTs depend on a CoA-ester substrate, and parsley CCoAMT distinctly differs from, for example, caffeic acid OMTs. The unusual features of parsley CCoAMT and the general importance of such enzyme activity for resistance to fungal pathogens at the stage of entry led us to investigate the kinetic mechanism of the elicitor-induced enzyme, with particular attention to control of enzyme activity by product inhibition.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were of analytical grade. AdoMet and AdoHcy were purchased from Sigma, Deisenhofen, and [*methyl*-^{14}C]AdoMet from Amersham-Buchler, Braunschweig. Caffeoyl-CoA and feruloyl-CoA were synthesized according to Stöckigt and Zenk (19).

Cell Culture and Elicitor

The propagation and induction of parsley (*Petroselinum crispum*) cell cultures with crude elicitor from *Phytophthora megasperma* f.sp. *glycinea* (5 mg/40 mL suspension culture) was as described elsewhere (14).

CCoAMT

The purification of CCoAMT from elicited parsley cells including protein determination was reported previously (15). The enzyme fraction eluted from Q-Sepharose chromatography was used throughout these kinetic studies. Catalytic activ-

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[2] Abbreviations: CCoAMT, *S*-Adenosyl-L-methionine:*trans*-caffeoyl-CoA 3-*O*-methyltransferase; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; OMT, *O*-methyltransferase; AMT, adenine-specific methyltransferase.

ity decreased rapidly during purification and inadequately correlated with the high degree of enzyme purity.

CCoAMT Assay

The standard assay mixture (50 μL total) consisted of varying amounts of caffeoyl-CoA (added in 2 mM solution in diluted formic acid pH 2–3) and [*methyl*-^{14}C]AdoMet (2.2 GBq/mmol) in 100 mM Tris-HCl buffer (pH 7.5), containing 200 μM $MgCl_2$, 2 mM DTE, and 10% (v/v) glycerol, and employing 1.05 μg partially purified CCoAMT protein. Incubations were started by centrifugation of substrates from the lid to the bottom of Eppendorf tubes and continued for 10 min at 30°C. The reaction was stopped by alkaline hydrolysis (addition of 10 μL 2.8 M NaOH, followed by incubation at 40°C for 15 min and termination by the addition of 6.2 μL 6 M HCl) and the aqueous phase was extracted with ethyl acetate (200 μL). Radioactivity in the extracts was determined by liquid scintillation counting (LKB 121 Rackbeta liquid scintillation counter) of aliquots of the organic phase (150 μL) in a toluene-based cocktail (Rotiszint 22, Roth, Karlsruhe). The reaction velocity was confirmed to be linear with respect to time and protein for at least 30 min at all substrate concentrations employed.

Kinetic data are represented as double-reciprocal plots, which were fitted by linear regression analysis (method of least squares) (20) as well as nonlinear regression analysis (20), and the appropriate model was chosen. Nonlinear regression analysis was performed with a computer program provided by H. Bisswanger, Tübingen. In all instances, the model resulting from nonlinear regression was identical to that generated by fitting the data by the method of least squares.

Table I. *Kinetic Parameters of Parsley CCoAMT*

Parameter	Concentration[a]
	μM
$K_{Caffeoyl\text{-}CoA}$	1.1
K_{AdoMet}	8.2
$K_{iCaffeoyl\text{-}CoA}$	5.2
$K_{iAdoHcy}$	3.5
$K_{iFeruloyl\text{-}CoA}$	11.0

[a] Data were determined from nonlinear regression analysis.

RESULTS

Substrate interaction kinetics with AdoMet as the variable substrate and several fixed concentrations of caffeoyl-CoA gave intersecting lines (Fig. 1). Intercept and slope replots *versus* reciprocal caffeoyl-CoA concentrations (inset, Fig. 1) generated straight lines for usual enzyme binding characteristics, from which catalytic constants can be determined. The initial velocity data were consistent with a sequential binding mechanism where both substrates must bind to the enzyme before any product release, thus excluding a ping-pong mechanism (17).

The order of substrate binding and product release was determined from product inhibition studies. AdoHcy was found to be a noncompetitive inhibitor with respect to both AdoMet and caffeoyl-CoA. Feruloyl-CoA acted as a competitive inhibitor of caffeoyl-CoA and as a noncompetitive inhibitor with respect to AdoMet. These data indicate that feruloyl-CoA is likely to be released last from the parsley CCoAMT in the course of catalysis, provided that an Ordered Bi Bi rather

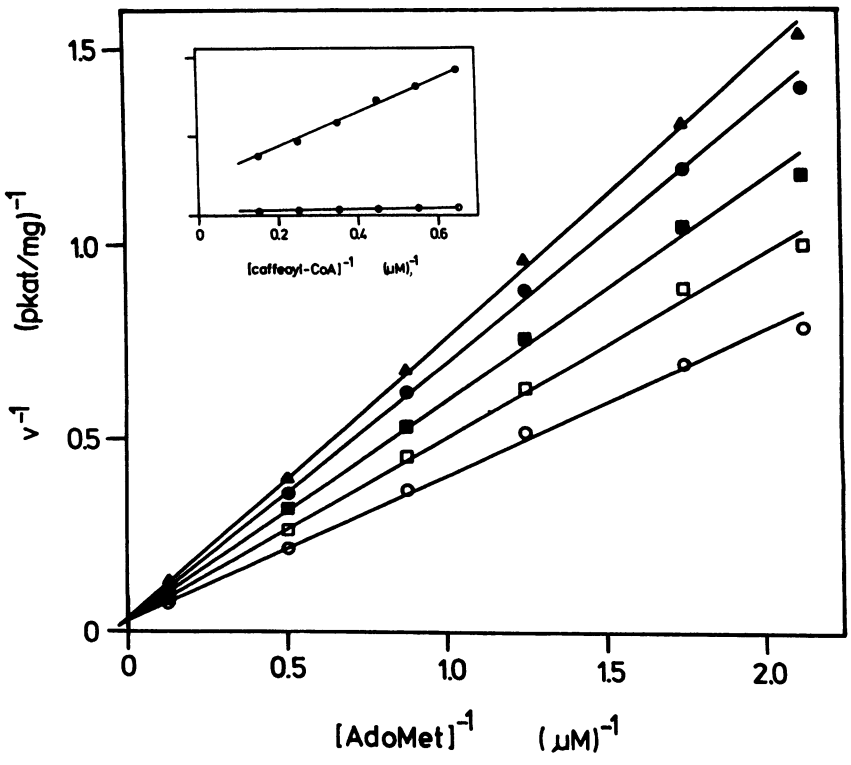

Figure 1. Double-reciprocal plots of initial velocities of CCoAMT with caffeoyl-CoA as the fixed substrate at 1.5 (▲), 1.8 (●), 2.2 (■), 2.9 (□), and 4.0 μM (○) and AdoMet as the variable substrate. Inset, slope (●) and intercept (○) replots *versus* caffeoyl-CoA concentrations.

than a mono-iso Theorell-Chance mechanism (2, 17) is applicable.

The catalytic and inhibitory kinetic constants $K_{Caffeoyl\text{-}CoA}$, K_{AdoMet}, and $K_{iCaffeoyl\text{-}CoA}$, $K_{iAdoHcy}$, and $K_{iFeruloyl\text{-}CoA}$ were calculated from the intercept and slope replots of the generated data (Table I). All values were within one order of magnitude, suggesting that the concentration of feruloyl-CoA *in vivo* may directly modulate the turnover rate of CCoAMT.

DISCUSSION

The product inhibition pattern observed for the parsley CCoAMT is compatible with a steady-state ordered Bi Bi mechanism (2, 17) where caffeoyl-CoA binds prior to *S*-adenosyl-L-methionine, which is followed by sequential release of *S*-adenosyl-L-homocysteine and feruloyl-CoA. An alternative mechanism which may also fit the data is the mono-iso Theorell-Chance mechanism (2, 17) with an inverse sequence of binding and isomerization of the free enzyme. Because in the latter case AdoMet and AdoHcy would bind to different enzyme forms, the distinction between these two mechanisms would require additional binding studies. A mono-iso Theorell-Chance mechanism was proposed previously, for example, for a flavonoid OMT from oat leaves (11) with kinetic patterns of substrate interaction and product inhibition identical to those of parsley CCoAMT. The oat OMT bound efficiently to an AdoHcy-Sepharose column and was eluted with AdoMet. Unlike the oat enzyme, however, parsley CCoAMT did not bind to such an affinity matrix (15). The Ordered Bi Bi mechanism of catalysis is therefore proposed for parsley CCoAMT (Fig. 2). This result is consistent with the ordered Bi Bi mechanism observed with other phenylpropanoid (18) and flavonoid OMTs (9, 10). It differs, for example, from the kinetics of rat liver catechol *O*-methyltransferase, exhibiting a random Bi Bi mechanism (5), or the magnesium-protoporphyrin methyltransferase of etiolated wheat (21), which follows a ping pong mechanism.

CCoAMT is known to be induced *de novo* in cultured parsley cells upon the addition of elicitors, although noninduced control cultures already contain fairly high background activity (approximately one-fifth of maximum), and maximal activity is reached within about 10 to 15 h (14). The small inhibitory constant for feruloyl-CoA suggests that the transcriptional activation of CCoAMT can only be required and reasonably effective under the condition of rapid and high consumption of feruloyl-CoA. This contrasts with, for example, OMTs from cabbage (3) and *Chrysosplenium* (4) accepting caffeic and 5-hydroxyferulic acids or various flavonols as substrates. It remains to be seen whether such enzyme activity is also involved in the lignification of tissues, a process which generally appears to be more sluggish. In this regard, and due to the fact that lignins of low methoxylation are increasingly required, for ecological reasons, in the pulp mill industry (8), the distribution and regulation of CCoAMT activity deserve further attention.

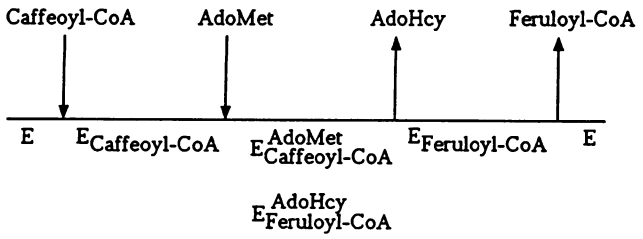

Figure 2. Kinetic mechanism proposed for CCoAMT from elicited parsley cell cultures.

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