Acetyl-CoA : 4-Hydroxybutinylbithiophene O-Acetyltransferase Isoenzymes from *Tagetes patula* Seedlings

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Naturally-occurring hydroxybutinylbithiophene derivatives were acylated by enzyme preparations of *Tagetes patula* seedlings in the presence of distinct acyl-Coenzyme A esters. The O-acyltransferase activity could only be detected after almost complete separation of the enzyme from counter-currently acting esterases which were present in the same extracts. This was achieved by affinity chromatography on Cibachron Blue A. During this procedure, the O-acyltransferase was split, yielding two active fractions. Both had a M_r of 37,000 (±5,000), equal isoelectric properties, a pH optimum of pH 7.0, and were considerably inhibited in the presence of free Coenzyme A. Small differences existed in their affinities for their thiophenic substrates (3,4-dihydroxybutinylbithiophene and 4-hydroxybutinylbithiophene, respectively), as well as for various acyl-CoA esters as cosubstrates. With the preferred cosubstrate, acetyl-CoA, acylation took place at the 4-position of the butinyl side chain of the molecules, forming the naturally-occurring 4-acetoxybutinylbithiophene and 3-OH,4-OAc-butinylbithiophene, respectively. From the other acyl-CoA esters employed, only propionyl-CoA was likewise converted, forming the corresponding O-propionyl esters. The reactions observed are suggested to be catalyzed by two acetyl-CoA: 4-hydroxybutinylbithiophene O-acetyltransferase isoenzymes which exhibit different affinities for particular substrates and cosubstrates. The activities of both the isoenzymes changed drastically if plant material from different developmental stages was used as enzyme source. Therefore, it may be suggested that these isoenzymes play an important regulatory role in the metabolism of naturally-occurring hydroxy- and acetoxybutinylbithiophenes and their derivatives.

Introduction

Thiophene derivatives occur in considerable amounts in some tribes of the *Asteraceae* family. Within the tribe Helenieae, almost all species investigated until now, are reported to contain a high level of several thiophenic compounds [1]. Particularly, the entire genus *Tagetes* exhibits an uniform, characteristic pattern of bi- and terthiophenes; these compounds have been shown to be the major secondary products of adult *Tagetes* plants [2, 3] as well as of *Tagetes* seedlings [4]. The established, characteristic accumulation kinetics of *Tagetes* seedlings render this system attractive to elucidate the metabolism and enzymology of these compounds.

The usefulness of this concept has already been proven. From *Tagetes* plants and hypocotyls, two highly specific acetoxybutinylbithiophene : acetate esterases could be isolated, partly purified and biochemically characterized [5, 6]. The occurrence in nature of the substrates (acetoxybutinylbithiophenes) and the products (hydroxybutinylbithiophenes) of these esterases suggests that these compounds are metabolic intermediates or end products in the thiophene metabolism. The enzymes may therefore be involved in either a biosynthetic or in a degradative pathway. Therefore, the question arises whether or not the plant system has the capability to realize the reverse way, namely, the acetylation of hydroxylated thiophenic compounds. This paper describes the isolation, partial purification and some biochemical properties of a novel enzyme, acetyl-CoA : 4-hydroxybutinylbithiophene O-acetyltransferase from hypocotyls of *Tagetes patula* seedlings.

Materials and Methods

Plant material

Seedlings of *Tagetes patula* L. cv. "Zitronenzwerg" were grown as previously described [4, 6]. Roots and hypocotyls of seedlings, stage '6D2L', were used for substrate production, and hypocotyls, stages '2D2L' and '6D2L', as enzyme source. For definition of stages, see [6].

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Reagents and solvents

All reagents used were of analytical grade, solvents used for HPLC of HPLC-grade. 3,4-Diacetoxybutinylbithiophene (**3**) as well as 4-acetoxybutinylbithiophene (**6**) were prepared from *Tagetes* seedlings by deep temperature extraction, followed by semi-preparative HPLC [7]. 3,4-Dihydroxybutinylbithiophene (**0**) and 4-hydroxybutinylbithiophene (**1**) were prepared by alkaline hydrolysis of **3** and **6**, respectively, and purified as described [6]. The products were stored at −20 °C in ethylene glycol monomethyl ether. The concentration of substrate solutions was determined by UV spectrophotometry and quantitative peak integration on HPLC was done with the extinction coefficients given by [8, 9]. For all HPLC purposes, a bi-distilled CH_3CN/H_2O azeotrop [7] was used as solvent. Free Coenzyme A as well as various Coenzyme A esters were purchased from SIGMA (München, FRG) and Cibachron Blue A from AMICON Corp. (Lexington, MA, USA).

HPLC

Semi-preparative HPLC as well as analytical HPLC was performed using a Spectra-Physics (Santa Clara, CA, USA) SP 8700 ternary solvent delivery system, equipped with a KNAUER (Bad Homburg, FRG) Nr. 731.87 Digital-Spectrophotometer (0.4 mm or 10 mm pathlength). For separation conditions, see [6, 7]. Peaks were integrated on a SP 4100 computing integrator.

Enzyme preparation

All steps were carried out at 0−4 °C under exclusion of UV- or of daylight. The plant material was homogenized in a mortar in 0.1 M KPi, pH 6.5, containing 50 mM K-ascorbate and 0.25 M sucrose under addition of an aliquot of insoluble PVP. After continuous stirring for 15 min, PVP and insoluble residues were removed by filtration on Whatman GFA glass fibre filters, followed by centrifugation (15 min, 28,000 × *g*). The supernatant was fractionated by addition of solid $(NH_4)_2SO_4$ according to [10]. Protein precipitating in a range from 40% to 60% $(NH_4)_2SO_4$ saturation was centrifuged (30 min, 45,000 × *g*), and the pellet was dissolved in 10 mM KPi, pH 6.5, containing 1 mM DTT and 3 mM $MgCl_2$. Low molecular weight compounds were removed by gel chromatography on a Sephadex G-25 column (2.5 × 16 cm, equilibrated with the same buffer). The protein fraction was precipitated again with solid $(NH_4)_2SO_4$ (80% saturation) and, after centrifugation, the pellet was dissolved in 5 mM KPi, pH 6.5, containing 1 mM DTT and 3 mM $MgCl_2$. The sample was applied to a Cibachron Blue A column (2.5 × 20 cm), equilibrated with the same buffer, at room temperature. Protein, bound under these conditions, was eluted by a linear KCl gradient (0−1.5 M). The fractions with highest enzyme activity were collected and concentrated by precipitation as indicated above. The pellets were dissolved in 0.1 M KPi, pH 6.5, and stored below 0 °C. These samples served as standard enzyme source for further experiments.

Enzyme assay

Reaction mixtures were incubated at 30 °C under exclusion of UV- or daylight. The standard mixture contained 5 nmol of the substrate, up to 300 nmol of the cosubstrate and the enzyme preparation under investigation. If 4-hydroxybutinylbithiophene (**1**) was used as substrate, 0.5 mg of bovine serum albumin was added as product stabilizer (*cf.* [5]). A total volume of 100 µl was adjusted with KPi, pH 7.0. Qualitative and quantitative estimation of product formation as well as of enzyme activity was computed after HPLC peak integration [6, 7]. Control assays consisted of blanks in which the cosubstrate was omitted or of blanks with boiled (100 °C, 5 min) enzyme preparations.

Protein assay

A modified Bradford [11] method was used with the PIERCE (Rockford, IL, USA) protein assay reagent.

Results

As shown in Fig. 1A and 1B, the O-acyltransferase (**A1**) catalyzes the acylation of 4-hydroxybutinylbithiophene (**1**) and of 3,4-dihydroxybutinylbithiophene (**0**) in the presence of an acyl donor ('Ac-CoA').

Preliminary experiments showed that **A1** activity is only detectable in the absence of the counter-currently acting 4-acetoxybutinylbithiophene:acetate esterase (**E6**). This enzyme, however, has a considerable activity in *Tagetes* hypocotyls [6]. Certain amounts of **E6** activity can be removed by fractiona-

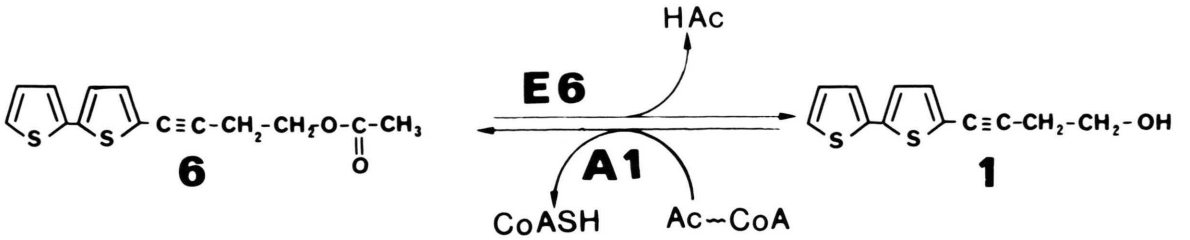

Fig. 1A. Enzymatic deacylation and acylation of naturally-occurring dithiophene derivatives. **6**, 4-Acetoxybutinylbithiophene; **1**, 4-hydroxybutinylbithiophene; **E6**, 4-acetoxybutinylbithiophene:acetate esterase; **A1**, acetyl-CoA:4-hydroxybutinylbithiophene O-acetyltransferase.

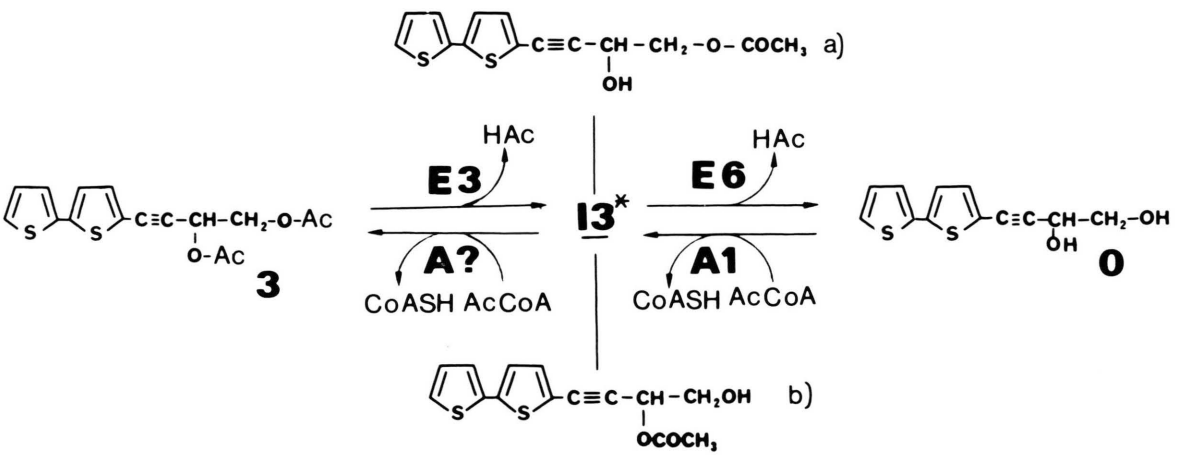

Fig. 1B. Enzymatic deacylation and acylation of naturally-occurring dithiophene derivatives. **3**, 3,4-Diacetoxybutinylbithiophene; **I3*** (alternatively) a) 3-OH, 4-OAc-butinylbithiophene, b) 3-OAc, 4-OH-butinylbithiophene; **0**, 3,4-dihydroxybutinylbithiophene; **E3**, **E6**, acetoxybutinylbithiophene:acetate esterases; **A1**, acetyl-CoA:4-hydroxybutinylbithiophene O-acetyltransferase; **A?**, proposed enzymatic acylation.

tion of crude enzyme extracts with $(NH_4)_2SO_4$ (40%=60% saturation). An almost complete separation of **A1** and **E6** succeeds with affinity chromatography on Cibachron Blue A (Fig. 2 and 3). In this way, the major part of the esterase is eluted under starting buffer conditions whereas O-acyltransferase is eluted with a KCl gradient (Fig. 3). Fractionation of extracts from *Tagetes* hypocotyls, stage '2D2L', yields an apparently homogeneous **A1** activity peak, eluted at about 0.7 M KCl (Fig. 2); using plant material from stage '6D2L', however, two active fractions ('Peak I' and 'Peak II') elute, one at 0.7 M and the other at 1.0 M KCl (Fig. 3). The 'Peak I' and the 'Peak II' enzymes are equally active in catalyzing the acylation of 4-hydroxybutinylbithiophene (**1**). 3,4-Dihydroxybutinylbithiophene (**0**), however, is preferably converted by the 'Peak I' enzyme. Fractions of 'Peak I' and of 'Peak II' showing highest **A1** activity were collected and used for further biochemical characterization of the enzymes.

By cochromatography with authentic reference substance, the reaction product formed with **1** as substrate and acetyl-CoA as cosubstrate was identified as 4-acetoxybutinylbithiophene (**6**) (Fig. 1A). With **0** as substrate and acetyl-CoA as cosubstrate, a product is formed which exhibits exactly the same retention time on HPLC as the intermediate product **'I3'** of the 3,4-diacetoxybutinylbithiophene:acetate esterase reaction **E3** [6]. The two possible structures of **I3** are given in Fig. 1B as a) and b).

As far as investigated, with one exception (see below), the properties of the 'Peak I' and of the 'Peak

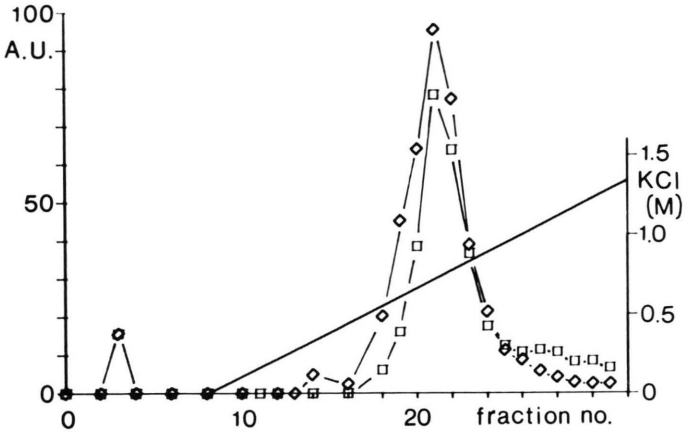

Fig. 2. Fractionation of acetyl-CoA:4-hydroxybutinylbithiophene O-acetyltransferase on Cibachron Blue A. Enzyme source: Hypocotyls of *Tagetes patula*, stage '2D2L'. ◇—◇: Formation of **13** (substrate: **0**); □—□: Formation of **6** (substrate: **1**). Straight line: KCl gradient. Enzyme activity is expressed as arbitrary units (A.U.).

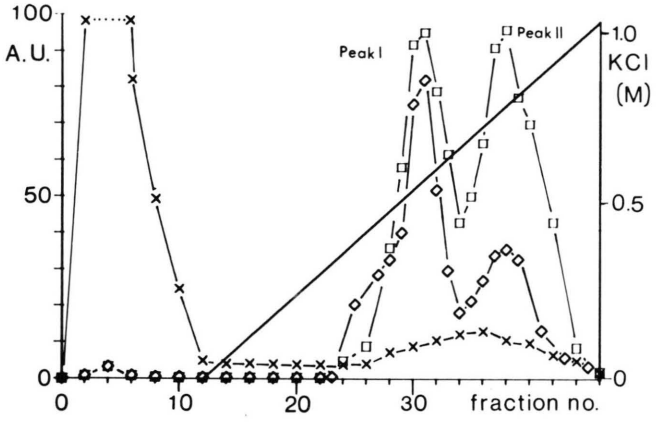

Fig. 3. Fractionation of acetyl-CoA:4-hydroxybutinylbithiophene O-acetyltransferase isoenzymes ('Peak I' and 'Peak II') on Cibachron Blue A. Enzyme source: Hypocotyls of *Tagetes patula*, stage '6D2L'. ×—×: activity of 4-acetoxybutinylbithiophene:acetate esterase. Other symbols used as given in Fig. 2.

II' enzymes turned out to be exactly the same. Both fractions show a linear reaction up to 20 min, at protein concentrations up to 50 μg per ml reaction mixture. The molecular weight determined on Sephadex G-200 [12] is 37,000 ± 5,000 (Fig. 4). The pH optimum lies around pH 7.0, and the activity is half maximal at pH 6.0. Measurements above pH 8.0 gave incorrect values because of spontaneous hydrolysis of products formed. The reactions are not affected by excess amounts of either **6** or **3** in the reaction mixture. Free Coenzyme A, however, inhibits the acyltransferase activity to some extent. As shown in Fig. 5, the reactions are influenced already in the presence of about 0.3 μM of CoA, and are completely blocked if the concentration of CoA exceeds 3 mM. No enhancement or inhibition of the transferase was observed by adding either SH reagents like

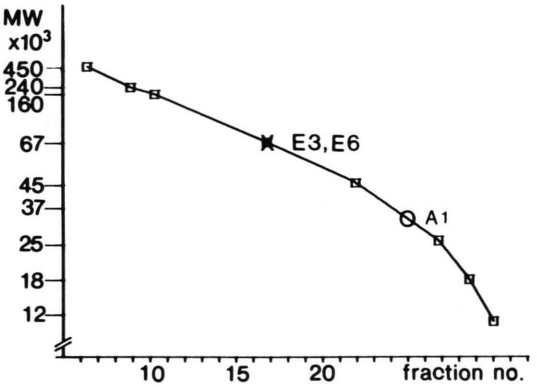

Fig. 4. M_r determination of acetoxybutinylbithiophene:acetate esterases (**E3**) and (**E6**) (×) and of acetyl-CoA:4-hydroxybutinylbithiophene O-acetyltransferase (**A1**) (○) by gel chromatography on Sephadex G-200. Column size: 1.6 × 70 cm; buffer used: 0.1 M KPi, pH 8.0. Calibration of column by standard proteins (□—□).

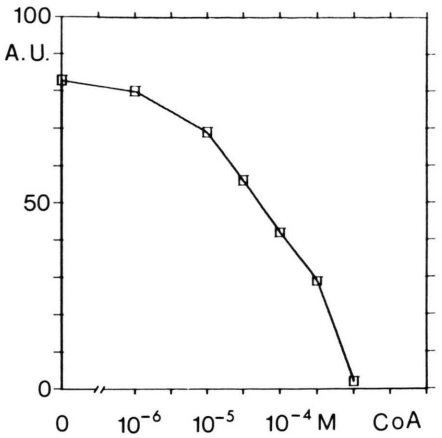

Fig. 5. Effect of increasing concentrations of free Coenzyme A (CoA) on the activity of acetyl-CoA : 4-hydroxybutinylbithiophene O-acetyltransferase (**A1**). Enzyme activity is expressed as arbitrary units (A. U.).

DTT or thiol directed reagents like PCMB. The only difference between 'Peak I' and 'Peak II' activities, however, is the apparent conversion rate of the mono- and the dialcohol derivatives in the presence of various acyl-Coenzyme A esters as cosubstrates (Table I). With acetyl-, propionyl-, butyryl- and with methylmalonyl-CoA, the corresponding substituted butinylbithiophene derivatives are formed. The retention times of these products on HPLC correspond to the expected values based on their polarity. The 'Peak I' enzyme clearly has an higher affinity for 3,4-dihydroxybutinylbithiophene (**0**), then for 4-hydroxybutinylbithiophene (**1**). The reverse situation is observed with the 'Peak II' enzyme. For 'Peak I', acetyl-CoA is the favoured cosubstrate, but propionyl-CoA is also converted at a considerable rate. The 'Peak II' enzyme, however, shows a likewise high affinity for acetyl-CoA as well as for propionyl-CoA. In comparison, conversion rates with butyryl-CoA and methylmalonyl-CoA are low; no reaction could be observed using malonyl-CoA or more complex or longer-chain substituted Coenzyme A esters like S-acetoacetyl-CoA or oleolyl-CoA.

Discussion

This is the first report about the enzymatic acylation of naturally-occurring dithiophene derivatives. From their biochemical properties (see below), the enzymes involved in these reactions are proposed to be two acetyl-CoA : 4-hydroxybutinylbithiophene O-acetyltransferase isoenzymes. The acetyltransferase (**A1**, see Fig. 1A, 1B) from *Tagetes patula* hypocotyls catalyzes the formation of 4-acetoxybutinyl-

Table I. Substrate/cosubstrate specificity of acyl-CoA : 4-hydroxybutinylbithiophene O-acyltransferase isoenzymes.

Substrate*	Cosubstrate*	Product formation Rt* [min]	'Peak I' (% V_{max})*	'Peak II' (% V_{max})
4-OH*	Acetyl-CoA	7.47	87	100
3,4-OH*		7.72	100	35
4-OH	Propionyl-CoA	9.88	62	100
3,4-OH		10.11	85	25
4-OH	Butyryl-CoA	12.60	15	17
3,4-OH		13.30	15	9
4-OH	Methyl-malonyl-CoA	9.70	12	14
3,4-OH		9.96	12	6

No enzymatic conversion observed with: Malonyl-CoA, S-acetoacetyl-CoA and oleolyl-CoA.

* Separation conditions: Column 4 × 250 mm Spherisorb ODS II, 5 µ. Solvent systems: For 4-hydroxybutinylbithiophene (4-OH), CH_3CN/H_2O 75:25, isocratic; for 3,4-dihydroxybutinylbithiophene (3,4-OH) as substrate: CH_3CN/H_2O 65:35. Substrate concentrations: 5×10^{-6} M each; cosubstrate concentrations: 1×10^{-3} M each. V_{max} values are expressed as relative arbitrary units of HPLC peak integration.

bithiophene (**6**), a major accumulated dithiophene of this species [1–5], and of **I3**, the intermediate product of the 4-acetoxybutinylbithiophene : acetate esterase reaction [6]. Most probably, **I3** itself serves as substrate of another specific O-acetyltransferase **A?** (Fig. 1B) which leads to the synthesis of the likewise naturally-occurring 3,4-diacetoxybutinylbithiophene (**3**) [6]. The acylation of **1** in position 4 of the butinyl side chain by **A1** strongly suggests that **0** is also acylated on this particular position. This is supported by an earlier hypothesis regarding the substitution pattern of **I3** by biochemical and physiological data [6]. Therefore, the structure of **I3** is supposed to be 3-OH,4-OAc-butinylbithiophene (formula a) in (Fig. 1B). The enzyme **A1**, involved in these reactions, occurs apparently in two isoenzymic forms which exhibit different substrate/cosubstrate specificities. In comparison with the biochemical properties of other O-acetyltransferases which have been described to be involved in the secondary metabolism of plants [13–15], the pH optimum, determined for **A1**, seems to be low; a mentholacetyltransferase from *Mentha* [13], an isoamyl alcohol acetyltransferase from *Musa* [14] and an acetyl-CoA : deacetylvindoline O-acetyltransferase from *Catharanthus* [15] have a pH optimum in the range of pH 8.0 to 9.0. However, the M_r of the *Tagetes* enzyme (37,000 ± 5,000) is in agreement with the M_r of the other systems (37,000, 40,000 and 45,000, respectively). Similar to the *Tagetes* acetyltransferase, the *Catharanthus* enzyme shows no end product inhibition and is affected by the co-product, Coenzyme A. Although comprehensive data are not given, the results regarding the cosubstrate specificity of the *Catharanthus* and of the *Mentha* enzymes correspond to the data reported here for *Tagetes*. The former enzymes also accept propionyl-CoA as well as acetyl-CoA as acyl donors. The *Mentha* acetyltransferase, however, also transfers butyryl-CoA to an high extent.

The sequence of metabolic steps in the turnover of thiophene derivatives is still unresolved. From tracer experiments, compounds like **6** and **1** appear to be 'inactive' end products of thiophene metabolism [1, 16]. Previous results, based on enzymological investigations [5, 6] as well as the results reported here, however, suggest that these compounds are rather 'active' intermediates. The stage-dependent presence or absence of one or both of the O-acetyltransferase isoenzymes suggests a certain regulatory role of these enzymes in thiophene metabolism. Hence, the level of the different substituted thiophenic acetoxy-compounds in the plant can be controlled fourfold: by substrate availability, by enzyme synthesis, by coproduct inhibition as well as by the action of the counter-currently working 4-acetoxybutinylbithiophene : acetate esterase (**E6**) [6]. It is conceivable that the function of the acetyl-CoA : 4-hydroxybutinylbithiophene O-acetyltransferase lies in the production of a 'pool' of thiophenic acetoxy-derivatives from which, on demand, equivalents may be provided for the synthesis of other, biosynthetically-related, thiophenic compounds.

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