Inhibition of Plant Acetyl-CoA Synthetase by Alkyl-adenylates

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Acetyl-CoA Synthetase, Alkyl-adenylates as Inhibitors, I_{50}-values, *Raphanus sativus*

The plant acetyl-CoA synthetase (ACS) is bound to the plastids and provides acetyl-CoA, the starting substrate for *de novo* fatty acid biosynthesis in plastids. This enzymic reaction, which consumes ATP and releases AMP, can be inhibited by different alkyladenylates such as ethyl-, isopropyl-, propyl- or allyl-adenylates as is shown here. The inhibition mechanism is competitive with respect to ATP and non-competitive with respect to acetate. I_{50}-values and the inhibition constants K_i (ATP), K_i (acetate) and K_{ii} (acetate) are given. The results suggest that, also in plants, acetyl-adenylate is the endogenous intermediate in the enzymic formation of acetyl-CoA from acetate by acetyl-CoA synthetase.

Introduction

In higher plants the acetyl-CoA synthetase plays a central role in several biosynthetic pathways of plastids. Besides *de novo* fatty acid biosynthesis, acetyl-CoA is needed for the synthesis of mevalonic acid and isoprenoid lipids as well as branched-chain amino acids. Isolated chloroplasts are capable of incorporating [^{14}C]acetate into fatty acids [1–4]. The enzyme acetyl-CoA synthetase (ACS) activates acetate to acetyl-CoA. ACS forms acetyl-CoA from acetate, ATP and CoA by releasing acetyl-CoA, AMP and pyrophosphate:

$$\text{acetate} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{2+}} \text{acetyl-CoA} + \text{AMP} + PP_i.$$

In higher plants it had been shown that the enzyme acetyl-CoA synthetase is exclusively located in the plastids [5]. This allowed the establishment, with isolated chloroplasts and etioplasts, of a test-system for *de novo* fatty acid biosynthesis starting from [^{14}C]acetate [6, 7]. Besides ACS there is another important acetyl-CoA producing enzyme system present in plastids, the pyruvate dehydrogenase complex [8, 9] which provides acetyl-CoA from pyruvate. In the case of intact spinach chloroplasts the incorporation of [^{14}C]-acetate was, however, preferred to [^{14}C]pyruvate [2, 3, 10]. Today there is still discussion about the enzyme which supplies the major part of acetyl-CoA for the different acetyl-CoA consuming pathways of the chloroplast. There are several observations, which indicate that the acetyl-CoA source depends on plant species [11, 12] on development of the plastids [13] and on the plastidic acetate and pyruvate levels [5, 14, 15]. It seems, however, generally accepted that in developing leaves the major source of acetyl-CoA for *de novo* fatty acid biosynthesis comes from the plastidic ACS activity. The ACS enzyme has been purified and characterized from a number of animal tissues [16] and from baker's yeast [17] and more recently also from spinach leaves [18, 19] and etiolated radish seedlings [20].

In our studies on the characterization of the plant ACS we looked for specific inhibitors of this enzyme. Finding these may help in a further purification of the ACS and also in the investigation of the regulation of the two main acetyl-CoA providing enzyme systems in the chloroplasts of higher plants: the ACS and the pyruvate dehydrogenase. Concerning the mechanism of the ACS reaction, it is known, in animals and yeast, that acetyl-adenylates are formed as tightly bound intermediates in the enzyme reaction [21]. The formation of acyl-adenylates apparently occurs in the case of all AMP-forming ATP-dependent carboxylate ligases [22]. For the ACS from yeast this was proved in detail by isotope exchange, initial rate and product inhibition studies [17]. It was also shown that the yeast ACS reaction is an ordered Bi Uni Uni Bi ping-pong system [17]. Similar results were obtained with the ACS of mammalian sources [21, 23]. With ACS preparations from potato tubers 5 isoenzymes were detected and kinetic studies pointed to

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a similar mechanism as in the yeast system [24]. For the purified plant ACS from spinach [18, 19] or radish [20] the mechanism of the ACS reaction has not yet been clarified, but it can be anticipated that also in these cases acetyl-adenylates may function as transitional intermediates.

In order to verify whether the plant ACS can be blocked by alkyl-adenylates we synthesized several alkyl-adenylates (alkyl-AMP esters). Some of these (methyl-, ethyl-, propyl- and butyl-adenylate) have been shown to be specific inhibitors of ACS of yeast [25]. These alkyl-adenylates represent structural analogues to the supposed substrate intermediate acetyl-adenylate, whereby the acetyl residue is replaced by alkyl chains. Here we report on the inhibitory potency of several alkyl-adenylates on the purified ACS from radish seedlings and describe the inhibition type and constants.

Materials and Methods

Etiolated radish (*Raphanus sativus* var. Saxa Treib) seedlings were cultivated on water for 6 d in the darkness. Maize plants were cultivated on a mineral containing peat (TKS II, Floratorf) in a 14/10 h day/night cycle. Chloroplasts of spinach and maize seedlings were isolated and their incubation with [^{14}C]acetate was carried out as described by Kobek *et al.* [6].

ACS enzyme assay

The assay of the acetyl-CoA synthetase is based on the non-enzymatic acylation of dithioerythritol (DTE) [26]. The assay was performed as described in [27] with some modifications [28]. The standard assay contained 0.1 Tricine (N-tris(hydroxymethyl)methyl glycine) buffer pH 8, 5 mM $MgCl_2$, 0.25 mM acetate (0.027 μCi ^{14}C), 0.5 mM CoA, 2 mM ATP in a final volume of 50 μl. After 10 min at 30 °C, the enzymic reaction was stopped with an equal volume of a stop-solution which contained 3 M NaCl, 0.2 M Tricine pH 8, 1 M sodium acetate and 20 mM dithioerythritol. The non-enzymic acylation proceeded for 1 h at 30 °C. Thereafter the resulting acylation product was extracted twice with diethylether, the latter evaporated and the radioactivity counted in a liquid scintillation counter using Quicksafe N (Zinsser, Frankfurt) as scintillation medium.

Isolation of the ACS enzyme

The ACS enzyme of etiolated radish cotyledons and of green maize leaves were isolated by a homogenization step in an isolation medium which contained 330 mM sorbitol, 100 mM Tris (tris(hydroxymethyl)-amino methane) pH 9, 2 mM $MgCl_2$, 2 mM DTE and as protease inhibitors, 2 mM benzamidine, 2 mM ε-amino-caproic acid and 0.2 mM phenylmethylsulfonyl fluoride. From this homogenate, plastids were isolated. The ACS of the broken etioplasts was precipitated between 40 to 70% ammonium sulfate solution [20]. The 40–70% pellet was resuspended in buffer pH 8 (100 mM Tricine, pH 8, 2 mM $MgCl_2$ 2 mM DTE and protease inhibitors) and desalted by passing the suspension through a small gel-filtration column (PD 10, Sephadex B-25 M, Pharmacia). Further steps in the isolation of the radish ACS were the application of an FPLC anion exchange column (Fractogel EMD TMAE-650 (M), a dye-ligand affinity column (TSK AF-orange, Merck) and a final gel-filtration column (Merck HW-TSK 50) [20]. After addition of 2 mM DTE the active fractions were stable at −20 °C for a few weeks [20]. The inhibition experiments with alkyl-adenylates were carried out with post gel-filtration ACS preparations, *i.e.* the purest available enzyme fractions. Protein concentrations were determined after Lowry [29] as modified by Bach *et al.* [30].

All given values are means of at least 9 determinations from three independent isolations; maximum deviations were ± 5%.

Synthesis of alkyl-adenylates

The alkyl-adenylates were synthesized by a combination of the methods given in [25, 31]. 1 mM adenosine-5′-phosphate·H_2O (AMP) was dissolved in 250 ml of the desired alcohol which contained 2 mmol tri-*n*-butyl-amine and 5 mmol dicyclohexylcarbodiimide. This reaction mixture was kept for several days at room temperature. The applied solvent was evaporated (vacuum) and the residue redissolved in about 20 ml H_2O containing 2.2 mmol sodium hydroxide. After filtration the alkaline solution was twice extracted with ether and the alkyl-adenylates in the water-phase further purified using a cation-exchange resin (Amberlite IR-120-(NH_4)). After washing the resin with water, the aqueous alkyl-adenylate solution was evaporated

(vaccum) completely, the dry residue dissolved in a small volume of methanol and the product finally precipitated by addition of about 100 ml acetone. The alkyl-adenylate was filtered and washed with acetone and ether and then dried in vacuum over P_2O_5 at 100 °C for several hours. The yield was between 50 and 75%. The purity of the alkyl adenylates was checked using paper chromatography (with *n*-BuOH/AcOH/H_2O (5/2/3) as solvent) as described in [32]. The R_f-values were 0.34 (methyl-adenylate), 0.44 (ethyl-adenylate), 0.56 (propyl-adenylate) 0.66 (butyl-adenylate) and in good agreement with the values given in [32]. The products did neither contain the applied starting substance AMP nor the reactants tri-*n*-butyl-amine and dicyclohexyl-carbodiimide. The latter was quantitatively transferred to urea during the alkaline hydrolysis step and the tri-*n*-butyl-amine was fully removed in the indicated purification steps. The synthesis of the methyl-, ethyl-, propyl- and butyl-adenylates has been described before [25, 32]. ^{1}H NMR spectra were recorded relative to an internal TMS (tetra-methyl-silane) standard in DMSO-d_6. The relative ppm values of the proton signals were identical to the values given by [25]. The allyl-, isopropyl- and 2-butyl-adenylate were synthesized using the same procedure and the corresponding alcohol.

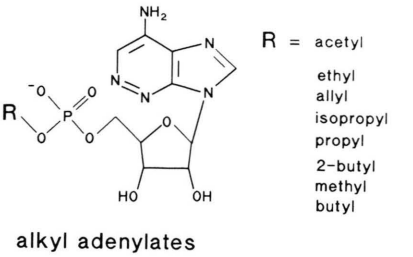

Fig. 1. Chemical structures of the alkyl-adenylates as analogues of the putative endogenous intermediate acetyl-adenylate in the enzymic formation of acetyl-CoA from acetate.

Results and Discussion

The radish ACS has a high substrate specifity for acetate, with very low affinity for propionate and acrylate, whereas other carboxylic acids such as formate, malonate, butyrate or glycine are not used as substrates [20].

In order to obtain more information about the substrate specifity of the ACS and the reaction mechanism of the catalyzed reaction, we investigated various alkyl-AMP esters, which might mimic the tightly enzyme-bound, presumed acetyl-AMP intermediate. The chemical structures of the synthetic compounds are shown in Fig. 1 together with that of acetyl-AMP. The I_{50}-values of the compounds were determined with purified enzyme from radish cotyledons and compared with those obtained with a maize ACS preparation (40–70% $(NH_4)_2SO_4$ pellet desalted). Significant differences between the ACS preparations of the two plants were not obtained, which demonstrates, that the inhibition of ACS by alkyl-adenylates is not dependent on the plant source (monocotyledonous or dicotyledonous plants) or the purification degree of the enzyme (Table I). The I_{50}-value for butyl-adenylate is about 400 times higher than that found for ethyl-adenylate, which as a "C_2"-derivative, is closest to the presumed endogenous acetyl-adenylate and also the strongest inhibitor. Propyl-adenylate and 2-butyl-adenylate were also strong inhibitors. The value for iso-propyl-adenylate is only about 5 times higher than that for ethyl-adenylate. Allyl-adenylate is as effective as propyl-adenylate, whereas methyl-adenylate is far less effective, although the only structural difference in both cases relative to ethyl adenylate is the presence/absence of one methyl-group. This again reflects the substrate specifity of the ACS for acetate and acetyl-adenylate as intermediate. It has been shown that formate is practically not recognized as substrate for radish ACS, whereas propionate competes with acetate [20].

Table I. Alkyl-adenylates as inhibitors of the plant ACS, determined with purified radish ACS (post gel-filtration; 193 nmol × min^{-1} × (mg protein)$^{-1}$) or with crude ACS-containing enzyme preparation from maize chloroplasts (40–70% ammonium sulfate pellet, desalted; specific activity 13.1 nmol × min^{-1} × (mg protein)$^{-1}$).

	I_{50} values [μM]	
	ACS (radish)	ACS (maize)
Ethyl-adenylate	0.38	0.3
Isopropyl-adenylate	1.8	1.7
Allyl-adenylate	2.7	2.7
Propyl-adenylate	2.8	2.8
2-Butyl-adenylate	8.2	11.0
Methyl-adenylate	24.2	35.6
Butyl-adenylate	117.9	135.4

The inhibition potency of these alkyl-adenylates as inhibitors of *de novo* fatty acid biosynthesis we also checked in our chloroplast test-system [33, 34]. The results with intact chloroplasts from a monocotyledonous (maize) and a dicotyledonous plant (spinach) are shown in Table II. The alkyl-adenylates had to be applied in higher concentrations as compared to the direct ACS enzyme assay. This may be due to a reduced uptake-rate of the alkyl-adenylate inhibitors by the chloroplasts. Alkyl-adenylates are fairly water-soluble compounds and it is known from the uptake of herbicides that more lipophilic substances permeate the chloroplast better and faster than do their hydrophilic derivatives. We can also assume that the alkyl-AMP-inhibitors are transported into the chloroplast in a way similar to that for ATP, but it has to be considered that the direct uptake of ATP in a chloroplast under light conditions is a very slow process [35]. From this, it appears that the uptake of the alkyl-adenylate may be the rate limiting step in the inhibition of the ACS in intact chloroplasts. Supporting this view is the fact that preincubation of the chloroplast with the inhibitor increases the inhibition (Table II) and that the differences between the compounds tested are relatively small in contrast to the values for the purified enzyme. The percentage inhibition of the ACS by alkyl-adenylates was higher for maize chloroplasts than for spinach chloroplasts. Chloroplasts and etioplasts of other plant sources should be examined because *de novo* fatty acid biosynthesis of the latter strongly depends on the addition of external ATP [7]. Consequently with etioplasts even lower I_{50} values of the alkyl-adenylates can be expected.

Further kinetic inhibition studies with the purified radish ACS enzyme were performed. For ethyl-adenylate, the inhibition was competitive with respect to ATP and non-competitive for acetate (Fig. 2). We determined the inhibition constants K_i (in the case of competitive inhibition) as well as K_i and K_{ii} values (in the case of non-competitive inhibition with respect to acetate) of the inhibitors. The K_i value represents the equilibrium constant for the reaction "enzyme + inhibitor $\rightleftharpoons$ enzyme-inhibitor-complex (EI)" and the K_{ii} value the equi-

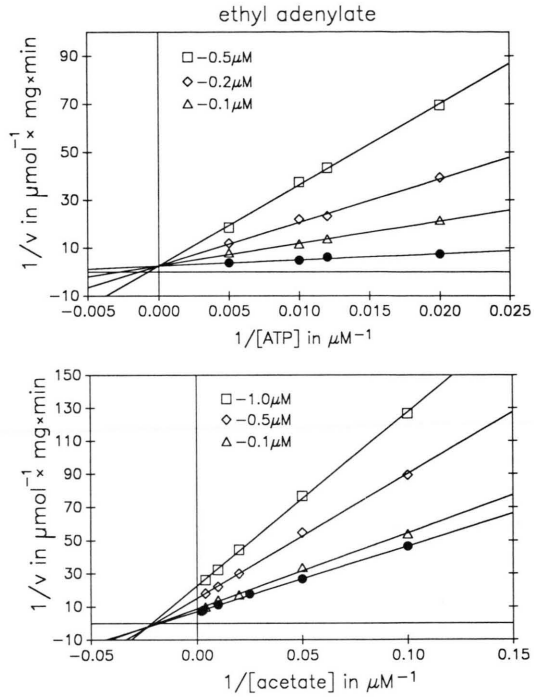

Fig. 2. Inhibition of purified radish ACS by ethyl-adenylate with ATP and acetate as variable substrates. The concentration of the non-variable cofactors were 5 mM $MgCl_2$, 2 mM ATP, 1 mM CoA, 1 mM acetate. Under these conditions specific activity was 193 nmol × min^{-1} × (mg protein)$^{-1}$. Closed circles = controls without inhibitor.

Table II. Percentage inhibition of [^{14}C]acetate incorporation into the total fatty acid fraction of isolated chloroplasts from spinach and maize by different alkyl-adenylates without preincubation or with a preincubation period of 20 min. Mean of 4 independent experiments, maximum deviations ± 5%. The incorporation rates of the controls amounted to 30 (spinach) and 26 (maize) nmol [^{14}C]acetate × h^{-1} × (mg total chlorophyll a + b)$^{-1}$.

	Spinach chloroplasts	Maize chloroplasts	
	no preincubation	no preincubation	20 min preincubation
Ethyl-adenylate (50 μM)	43%	62%	73%
Isopropyl-adenylate (50 μM)	27%	49%	62%
Allyl-adenylate (100 μM)	35%	79%	92%
Propyl-adenylate (100 μM)	37%	69%	87%

Table III. K_i(ATP), K_i(acetate) and K_{ii}(acetate) values of alkyl-adenylates for the radish ACS which are competitive inhibitors with respect to ATP and non-competitive inhibitors with respect to acetate.

Inhibitor	K_i(ATP) [μM]	K_i(acetate [μM]	K_{ii}(acetate) [μM]
Ethyl-adenylate	0.042	0.465	0.543
Isopropyl-adenylate	0.107	1.16	1.34
Allyl-adenylate	0.208	2.01	1.98
Propyl-adenylate	0.229	2.13	1.97
Methyl-adenylate	2.45	–	–
2-Butyl-adenylate	–	8.89	6.3

librium constant for the reaction "enzyme-substrate-complex (ES) + inhibitor $\rightleftharpoons$ enzyme-substrate-inhibitor-complex (ESI)"; for details see [36]. For all other alkyl-adenylates examined, the same results as for ethyl-adenylate were obtained which suggests that ATP is the first substrate to combine with the free ACS enzyme and then is followed by acetate. Variations of the alkyl residue of the inhibitor had no influence on the binding sequence. The K_i and K_{ii} values are given for the tested alkyl-adenylates as shown in Table III. The K_i values were determined by plotting the slopes and intercepts (in the case of non-competitive inhibition) of Lineweaver-Burk plots *versus* the concentration of inhibitor. These secondary plots were again linear and K_i and K_{ii} were calculated from the slopes. The K_i(acetate) values of all tested alkyl-adenylates were 10-fold higher than the K_i(ATP) values and were about the same as the I_{50}-values determined with the enzyme preparations.

From the data presented here we conclude that the K_i and I_{50}-values for the inhibitors parallel the substrate specifity of the ACS. From the inhibition pattern it seems reasonable to assume that the reaction mechanism of radish ACS is similar to that of the enzyme from mammalian sources and yeast. The specificity and efficiency of the alkyl-adenylates as inhibitors of plant acetyl-CoA synthetase provide evidence that they either mimic the transition state and the acetyl adenylate intermediate of the reactions. Alkyl-adenylates can be regarded as transitional state-type inhibitors of the plant ACS.

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