Isolation and Characterization of the Acetyl-CoA Synthetase from Penicillium chrysogenum

INvolvement of this enzyme in the biosynthesis of penicillins* 

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Acetyl-CoA synthetase (ACS) of Penicillium chrysogenum was purified to homogeneity (745-fold) from fungal cultures grown in a chemically defined medium containing acetate as the main carbon source. The enzyme showed maximal rate of catalysis when incubated in 50 mM HCl-Tris buffer, pH 8.0, at 37°C. Under these conditions, ACS showed hyperbolic behavior against acetate, CoA, and ATP; the K_m values calculated for these substrates were 6.8, 0.18, and 17 mM, respectively. ACS recognized as substrates not only acetate but also several fatty acids ranging between C_2 and C_9 and some aromatic molecules (phenylactic, 2-thiophenacetic, and 3-thiophenacetic acids). ATP can be replaced by ADP although, in this case, a lower activity was observed (37%). ACS is inhibited by some thiol reagents ([5,5'-dithiobis(nitrobenzoic acid), N-ethylmaleimide, p-chloromercuribenzoate], whereas it was stimulated when the reaction mixtures contained 1 mM dithiothreitol, reduced glutathione, or 2-mercaptoethanol. The calculated molecular mass of ACS was 139 ± 1 kDa, and the native enzyme is composed of two apparent identical subunits (70 kDa) in an a_2 oligomeric structure. ACS activity was regulated "in vivo" by carbon catabolite inactivation when glucose was taken up by cells in which the enzyme had been previously induced. This enzyme can be coupled "in vitro" to acyl-CoA:6-aminopenicillanic acid acyltransferase from P. chrysogenum, thus allowing the reconstitution of the functional enzymatic system which catalyzes the two latter reactions responsible for the biosynthesis of different penicillins. The ACS from Aspergillus nidulans can also be coupled to 6-aminopenicillanic acid acyltransferase to synthesize penicillins. These results strongly indicate that this enzyme can catalyze the activation (to their CoA thioesters) of some of the side-chain precursors required in these two fungi for the production of several penicillins. All these data are reported here for the first time.

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The biosynthetic pathway of L-lysine and penicillins in Penicillium chrysogenum and in Aspergillus nidulans is a branched route which starts with the condensation of an acetyl-CoA molecule with α-ketoglutaric acid, leading to the formation of homocitric acid which is the first biosynthetic intermediate (1-4). Later, through a series of reactions similar to those reported for the tricarboxylic acid cycle, L-α-aminoadipic acid is produced. In the specific branch of penicillins L-α-aminoadipic acid is linked with another two amino acids (L-cysteine and L-valine) generating a tripeptide molecule [6-(L-α-aminoadipyl)-L-cysteinyl-D-valine], without antibacterial activity and commonly named ACV (5). In a second step, this compound is cyclized to isopenicillin N (IPN) by the enzyme IPN synthase (6-9). The low antibacterial activity of IPN is greatly increased by replacing the L-α-aminoadipic acid moiety by another acyl-chain (Δ^2-hexenoic, hexanoic, octanoic phenylacetic, and phenoxycetic acids), thus generating different penicillins (10, 11). These transference reactions are catalyzed by a single enzyme, acyl-CoA:6-APA acyltransferase (AT), which requires the previous activation of the acyl-chains to their CoA derivatives (12-14). It has been reported (15-16) that P. chrysogenum catalyzes the activation of phenylactic acid (PAA) (the side-chain precursor of benzylicillin G), to phenylacetyl-CoA (PA-CoA) by a phenylacetyl-CoA ligase (PCL) in the presence of Mg^{2+}, ATP, CoA, and PAA according to the following reaction:

\[
PAA + CoA + ATP \rightarrow \text{PCL} \rightarrow \text{PA-CoA} + AMP + PP_i \quad (1)
\]

However, this protein was never purified, nor has the activity been characterized in detail. We have attempted to assay PCL in different low producing or industrial strains of P. chrysogenum but we failed to find it by the reported procedures (15, 16). However, we found a similar enzyme in a strain of Pseudomonas putida (U) able to grow in a chemically defined medium containing PAA as the sole carbon source (17). Recently, Smith et al. (18) have shown that the PCL gene is not linked to the other genes which code for ACV synthase, IPN synthase, and AT. They suggested that PCL could be a nonspecific penicillin biosynthetic enzyme, i.e. a general one involved in the primary metabolism of the fungus. To test this hypothesis we have purified and studied, as an obvious candidate, the acetyl-CoA synthetase (ACS) of P.
**Acetyl-CoA Synthetase and Acyl-CoA: 6-APA Acyl Transferase (ACS) and Acyl-CoA: 6-APA Acyl Transferase (AT) Coupled Assay**—To establish whether some of the substrates activated by ACS to CoA thioesters can be transformed into penicillins, ACS and AT were incubated "in vitro." The assay mixture contained, in a total volume of 126.5 μl: 50 mM HCl-Tris buffer, pH 8.0; 10 mM MgCl₂; 20 mM ATP; 2.4 mM CoA; 12 mM sodium acetate (or the corresponding penicillin side-chain precursor); 2 mM DT; 30 μM 6-APA (free acid); pure ACS (5 μg); and pure AT (10 μg). The reactions were incubated at 30 °C for 60 min and stopped by the addition of a similar volume of methanol (126.5 μl). The antibiotics generated were measured by bioassay against *M. luteus* (19). Control reactions were carried out under the same conditions without ATP, CoA, Mg₂⁺, 6-APA, or the side-chain precursor.

**Determination of Penicillin Synthesis**—Penicillin synthesis was followed in *P. chrysogenum* by measuring the incorporation of [U-¹⁴C]leucine into trichloroacetic acid-insoluble material, as previously reported (4). The effect of cycloheximide on protein synthesis was studied by adding this antibiotic (125 μg/ml) to the flasks at different times (39 and 43 h).

**Carbon Compound Determinations**—Residual D-glucose was measured by the glucose oxidase enzymatic test (22). The quantity of acetate remaining in the broths was measured by the method of Holz (23).

**Gel Electrophoresis**—Isoelectric focusing was carried out as reported by Wrigley (24).

Electrophoresis—Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (SDS-PAGE) was performed in 7% slab gels (25) using phosphorylase b (M, 94,000), bovine serum albumin (M, 67,000), ovalbumin (M, 45,000), and carbonic anhydrase (M, 29,000) as molecular weight standards.

**Starvation Medium**—In some experiments (regulation by glucose, see "Results and Discussion") cultures of *P. chrysogenum* grown in a medium containing acetate and glucose were harvested at different times, washed with sterile saline solution, and resuspended in the same chemically defined medium without glucose and deprived of the nitrogen sources. Under these conditions cellular growth was halted. The *in vitro* synthesis of 6-APA was evaluated by extracting 5 ml of culture (without cells) with 5 ml of n-hexane (twenty times) and washing five times with sterile distilled water. A aliquots of 35 mg (wet weight) were mixed and dried in an oven for 24 h at 40 °C.

**PA Transport System**—Mycelia of *P. chrysogenum* grown in the above medium and conditions were harvested at different times and washed five times with sterile distilled water. Aliquots of 35 mg (wet weight; about 10 mg of dry weight) were suspended in 25 ml Erlenmeyer flask containing 1.4 ml of 0.06 μmol phosphate buffer, pH 6.5, and preincubated at 25 °C for 5 min in a thermostatically controlled shaker. Preincubated (15−17 °C) 0.4 ml of labeled PA (10−20 °Ci) was added to each flask. Incubations were carried out for 30 s, or the required time, halted by addition of 10 volumes of water, rapidly filtered through Millipore filters (0.45-μm pore size), and washed with 3 x 10 ml of sterile distilled water. The filters were dissolved in 10 ml of scintillation fluid and counted as reported. [14C]PA uptake is given as picomoles/min.

**Determination of Penicillins by HPLC**—The different penicillins produced by *P. chrysogenum* grown in the minimal medium reported above for 84 h were analyzed by HPLC. Samples of culture broth (10 ml) were either filtered and lyophilized (for the determination of the total β-lactam antibiotics, including 6-APA) or adjusted to pH 2.0, extracted with isobutylacetate and the antibiotic-rich fraction (organic phase) transferred to 50 μmol phosphate buffer (1/10, v/v) pH 7.0 (11). The organic and the aqueous phases were separated and the latter was lyophilized. The powder obtained in both cases was resuspended in 1 ml of distilled water, and 20 μl was injected in a high performance liquid chromatograph (Spectra-Physics SP 8460).
P. chrysogenum Acetyl-CoA Synthetase

RESULTS AND DISCUSSION

Time Course of the Appearance of Acetyl-CoA Synthetase in P. chrysogenum—Acetyl-CoA synthetase from P. chrysogenum (acetate:CoA ligase (AMP forming), EC 6.2.1.1) is an enzyme that catalyzes the activation of acetic acid to acetyl-CoA in the presence of Mg\(^{2+}\), CoA, ATP, and acetate, according to the following equation

\[
\text{Acetate + CoA + ATP} \rightarrow \text{Acetyl-CoA + AMP + PP}\]  

We have studied this enzyme in mycelia of P. chrysogenum grown in a chemically defined medium containing acetate as the main carbon source. ACS began to be synthesized at the early logarithmic phase of growth (30 h) and its activity increased linearly during the exponential phase, reaching a maximal level at 54 h. Between 54 and 96 h ACS activity decreased rapidly, probably because acetate exhaustion occurred (Fig. 1a). Extracellular penicillin accumulation started to be detectable at 48 h, being maximal at 84 h. From this time to 108 h penicillin titers remained constant (Fig. 1a). A similar curve was found when the penicylactic acid transport system was measured (Fig. 2). The fact that PAA transport system induction and penicillin production started when the acetate was almost exhausted (Figs. 1a and 2) suggests that they are two directly related processes both regulated by acetate. The analysis of the penicillins accumulated in the broths by HPLC showed that about 90% of the total \(\beta\)-lactams produced was penicillin G, the rest being 6-APA. It is worth noting that omission of PAA from the broths drastically changes the nature of the penicillin accumulated since, under these conditions, \(\Delta^2\)-pentenyl penicillin (F) is the only penicillin produced (data not shown).

In view of the above data we decided to take fungal cells grown for 54 h as the source of ACS.

Physicochemical Properties of ACS—Purified ACS (see “Materials and Methods”) runs in 7% SDS-PAGE slab gels as a single band with a Rf of 0.358. The calculated molecular mass for the denatured protein was 70 kDa (Fig. 3). However, by filtration through a Sephadex G-200 (Pharmacia) column (2.8 \(\times\) 34.5 cm) the molecular mass of the enzyme was 139 \(\pm\) 1 kDa suggesting that ACS, in the native form, is a dimer composed of two apparently identical subunits in an \(\alpha_2\) oligomeric structure. Similar molecular weights for both the native enzyme and subunits have been reported in Methanothrix soehnii (26) and in Saccharomyces cerevisiae (27). Contrariwise, the ACS purified from rat liver seems to be composed of 62 kDa monomers (28). Additionally, it has been shown that in A. nidulans and Neurospora crassa the ACS gene codes for a polypeptide chain of about 670 and 660–626 residues of amino acids, respectively (29). Similar results have been reported for the long-chain acyl-CoA synthetase from rat liver (30).

ACS activity was maximal at 37 °C and at a pH value of 8.0. Under these conditions the reaction was linear over 30 min (Fig. 4). The enzyme showed hyperbolic behavior for ATP, CoA, and acetate, the \(K_m\) calculated for each being 17, 0.18, and 6.8 mM, respectively. All these experiments were carried out at saturating concentrations of Mg\(^{2+}\). This ion can be replaced by Mn\(^{2+}\), but in this case a lower rate of catalysis is observed (about 64%).

ACS shows low activity in the presence of the amphotiles contained in the buffers required for chromatofocusing. The enzyme migrates in isoelectric focusing gels as a diffuse band between pH 5.6 and 6.0. Similar results have been reported.
TABLE I
Purification of ACS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume</th>
<th>Protein</th>
<th>Enzyme activity</th>
<th>Specific activity</th>
<th>Yield of recovery</th>
<th>Purification</th>
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<tbody>
<tr>
<td>Crude extract</td>
<td>162</td>
<td>1,944</td>
<td>62,641</td>
<td>32.20</td>
<td>100</td>
<td>1.00</td>
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<tr>
<td>Ammonium sulfate precipitation (35–65%)</td>
<td>159</td>
<td>1,240</td>
<td>41,896</td>
<td>33.79</td>
<td>66.88</td>
<td>1.05</td>
</tr>
<tr>
<td>Ultracentrifugation (250,000 × g, 45 min)</td>
<td>25</td>
<td>615</td>
<td>39,040</td>
<td>63.48</td>
<td>62.32</td>
<td>1.97</td>
</tr>
<tr>
<td>Phenyl-Sepharose CL-4B (fractions 9–16)</td>
<td>18.08</td>
<td>97.17</td>
<td>27,542</td>
<td>283.44</td>
<td>43.97</td>
<td>8.80</td>
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<tr>
<td>Concanavaline A (fractions 3–13)</td>
<td>24.75</td>
<td>80.2</td>
<td>24,327</td>
<td>303.32</td>
<td>38.83</td>
<td>9.4</td>
</tr>
<tr>
<td>Sephacryl S-300 (fractions 46–56)</td>
<td>23.65</td>
<td>15.0</td>
<td>18,523</td>
<td>1,204.86</td>
<td>29.57</td>
<td>38.35</td>
</tr>
<tr>
<td>Blue-Sepharose CL-6B eluate (gradient 0.18–0.35 M KCl) (fractions 5–10)</td>
<td>13.2</td>
<td>1.49</td>
<td>11,338</td>
<td>7,608.39</td>
<td>18.09</td>
<td>236.0</td>
</tr>
<tr>
<td>DEAE-eluate (FPLC) (0.20–0.26 M KCl) (fractions No. 20)</td>
<td>0.6</td>
<td>0.050</td>
<td>1,200</td>
<td>24,000</td>
<td>1.92</td>
<td>745.3</td>
</tr>
</tbody>
</table>

FIG. 1. Time course of the appearance of ACS in P. chrysogenum grown in a chemically defined medium. a, cellular growth (O), pH variation (△), penicillin production (○), acetate consumption (△), and ACS activity (●). b, idem when P. chrysogenum was grown in the same chemically defined medium supplemented with glucose (1.5%) (■).

for other acyl-CoA activating enzymes in Pseudomonas putida (17).

Enzyme Stability: Effect of Temperature—Whereas crude extracts are quite stable under prolonged storage at −20 °C in the presence of glycerol (3–4 weeks), the absence of this molecule from the buffers results in a rapid deactivation of the enzyme (more than 70% over 12 h at 2 °C). Purified ACS is even less stable and is particularly sensitive to repeated freezing. Thus, pure enzyme cannot be stored at −20 °C for more than 3 days without a considerable loss of activity (more
than 50%). ACS is also very sensitive to temperatures above 0 degrees, showing very rapid deactivation kinetics when incubated for 30 min at temperatures higher than 30 °C (Fig. 5). Similar results have been reported for other related enzymes (17).

Effect of Cations and Other Molecules—The effect of several cations on ACS activity was studied by adding them to the assays at a final concentration of 1 mM. Whereas monovalent cations (K+, Na+, Li+) did not cause any significant effect, certain divalent cations (Zn²⁺, Cu²⁺, and Hg²⁺) strongly inhibited the enzyme (97, 98, and 100%, respectively). ACS was also inhibited by several agents that react with thiol groups [5,6'-dithiobis(2-nitrobenzoic acid), p-chloromercuribenzoate, and N-ethylmaleimide] when added to the reaction mixtures at a concentration of 1 mM (93, 97, and 100%, respectively). However, DTT, reduced glutathione, and 2-mercaptoethanol (1 mM) increased ACS activity (114, 122, and 140%, respectively). These results suggest that, as reported for phenylacetyl-CoA ligase from P. putida (17), some SH groups of ACS are essential for catalysis to occur.

Detergents (0.5% w/v) also affected the activity of this enzyme, SDS being the compound that elicited a strongest degree of inhibition (100%). Other molecules such as Tween 20, Tween 80, Tween 100, and Nonidet P40 inhibited activity to a lower extent (24, 30, 36, and 14%, respectively); this effect was similar to that reported for other enzymes or enzymatic systems (17, 31–32).

Substrate Specificity—In an attempt to characterize the specificity of this enzyme, different molecules were tested as substrates of ACS. Using the hydroxylamine procedure as the assay method (see “Materials and Methods”), we found that ACS recognizes as substrates: acetic, propionic, butyric, and valeric acids but with different efficiencies (100, 48, 20, and 11%, respectively). Taking into account the substantial levels of catalysis with substrates other than acetate, we have designed a highly sensitive method to evaluate low levels of conversion of some rare substrates related to penicillin biosynthesis. By coupling in vitro ACS and AT, these substrates can be converted into penicillins and evaluated by bioassay (see “Materials and Methods”). The results presented in Table II and Fig. 6 indicate that the substrate specificity of this enzyme is quite broad and that ACS can activate many other molecules to acyl-CoA derivatives: hexanoic, Δ⁵-hexenoic, heptanoic, octanoic, Δ³-octenoic, phenylactic, 2-thiophene acetic, and 3-thiophenecetic acids. These acyl-CoA derivatives can be used by P. chrysogenum to produce in vivo the corresponding penicillins, implicating that ACS could play an important role in the biosynthetic pathway of such antibiotics. Moreover, the fact that the acetyl-CoA synthetase obtained from the β-lactam producer A. nidulans also activates these kinds of compounds (Table II), strongly reinforces the above conclusion.

It can be observed that ACS recognizes better as substrates Δ⁵-hexenoic acid and Δ³-octenoic acids than their saturated molecules (hexanoic and octanoic acids) (see Table II). These results contrast with the higher titers of saturated side chain-containing penicillins (DF and K) reported by us (11) when P. chrysogenum was cultured in industrial broths in the absence of PAA. We suggest three alternative explanations to justify this observation: (i) the inhibitory effect (owing to their detergent properties) on the enzymatic system (ACS-AT) caused by hexanoic and octanoic acids would lead to a lower rate of catalysis when these acids are used as substrates; (ii) the synthesis of hexanoyl- and octanoyl-CoA (required by AT for the biosynthesis of penicillins DF and K) might be catalyzed in vivo by a different enzymatic system (fatty acid β-oxidation or directly through a reaction catalyzed by a different acyl-CoA synthetase) and (iii) the structure of fatty acids containing double bonds at the C₃ position (Δ³-hexenoic...
and Δ1-octenoic) would permit the acquisition of an appropriate configuration (more rigid) which would facilitate the binding of the substrates at the active site. A similar structural requirement was reported when the final concentration of octanoic acid was reduced (0.6 and 0.2 mM) it was added more efficiently to 6-APA (as measured by the hydroxylamine assay method) when ACS was incubated with acyl-acids whose carbon length ranges between C6 and C8, does not actually mean that this enzyme was fully unable to activate these substrates; it is possible that the sensitivity of the method was too low. The quantities of the acyl-CoA products generated in the reactions were quite sufficient to be detected by bioassay against the raw materials routinely used in industrial fermentations.

In order to shed light on these points, several experiments were performed. We have tested different concentrations of hexanoic and octanoic acids in the ACS-AT reaction mixture to establish whether they are able to act as inhibitors of the enzyme (see Table II). We observed that when the final concentration of octanoic acid was reduced (0.6 and 3 mM) it was added more efficiently to 6-APA (as measured by the generation of the corresponding penicillins). This effect was less pronounced when lower concentrations of hexanoic acid were used (see Table II). These results suggest that, in vivo, P. chrysogenum can utilize ACS to obtain all the acyl-CoA thioesters required to synthesize penicillins G, F, DF, and K. However, we cannot rule out that other acyl-CoA activating enzyme(s), which also could serve to synthesize hexanoyl- and octanoyl-CoA, might be induced in this fungus was cultured in complex industrial broths.

In summary, we demonstrate that the lack of catalysis observed (according to the hydroxylamine assay method) when ACS was incubated with acyl-acids whose carbon length ranges between C6 and C8, does not actually mean that this enzyme was fully unable to activate these substrates; it is possible that the sensitivity of the method was too low. The quantities of the acyl-CoA products generated in the reactions were quite sufficient to be detected by bioassay against M. luteus after being transformed into penicillins (see Table II).

The high quantity of penicillin F obtained when Δ1-hexenoic acid was tested as a substrate (Table II), indicates that this enzymatic system (ACS-AT) could probably produce in vivo higher amounts of this penicillin if the appropriate side chain precursors (or unsaturated fatty acids) were present in the raw materials routinely used in industrial fermentations. This high capacity to synthesize penicillin F could be the reason why this antibiotic is the only penicillin produced in this fungus when this fungus was cultured in complex industrial broths.
the fact that penicillin F was not accumulated when PAA is added to the chemically defined medium, suggests that Δ⁴
hexenyl-CoA is not synthesized in the presence of phenylacetic acid.

The comparative study of the substrate specificity of ACS from *P. chrysogenum* and *A. nidulans* (Table II) suggests that it is quite different from that reported for phenylacetyl-CoA ligase (PCL) from *P. putida* (17). Although these three enzymes recognize similar substrates, the enzyme of *P. putida* preferentially activated phenylacetic acid and also, but with a lower efficiency, different acyl-acids, indicating that they are two different enzymes. The relevance of these results will be discussed below.

When ATP was replaced by other nucleotides (ADP, UTP, UDP, CTP, CDP, GTP, or GDP) ACS activity was only detected when ADP, UTP, CTP, and GTP were used as substrates. The structural similarity between ATP and ADP, to a certain extent (37%) permitted the synthesis of acetyl-CoA, whereas in the other cases activity was almost negligible (10, 7, and 3%, respectively). Similar results have been reported for phenylacetyl-CoA ligase from *P. putida* (17) and benzoyl-CoA synthetase from *Rhodopseudomonas palustris* (36).

Carbon Catabolite Inactivation of ACS—Fig. 1b shows that ACS was synthesized at a similar rate when *P. chrysogenum* was grown in the basal medium containing acetate or acetate and glucose (1.5% w/v) for 48 h, whereas later, when (due to the exhaustion of acetate) glucose started to be catabolized, ACS activity decreased rapidly. The lack of consumption of glucose seems to be due to the preferential utilization of acetate by this fungus when a mixture of these two carbon sources (acetate and glucose) was present in the medium. The preferential utilization of acetate has been reported both in *P. chrysogenum* and *A. nidulans* (36, 37). Similar results were obtained when soy bean oil (1% w/v) was supplied together with acetate in the chemically defined medium. Under these conditions, *P. chrysogenum* did not start to consume the oil until the acetate had been exhausted (at 24 h 100% of oil remains; at 48 h 80%; whereas at 60 h, due to the consumption of acetate, only 20% of the initial oil was detected). These experiments indicate that in the presence of acetate, fatty acids (oils), like glucose, are not (or are very poorly) taken up by the cells. When glucose consumption started (at about 42 h of growth) a rapid loss on the measurable activity of ACS was observed (Fig. 1b). Although the ACS of *A. nidulans* has been shown to be controlled at the level of mRNA accumulation by carbon catabolite repression (38), the rapid loss of activity of the enzyme from *P. chrysogenum* cannot be explained by this mechanism alone. The *in vivo* half-life of ACS, evaluated by halting protein synthesis in 39- and 43-h-old cultures with cycloheximide (125 μg/ml), was estimated to be 10 h (Fig. 7a), i.e. higher than the time required to inactivate ACS when glucose is catabolized (about 2 h, Fig. 1b). This glucose effect cannot be explained by transcriptional repression (38, 39), and we conclude that a faster posttranscriptional mechanism of control of the enzymatic activity does exist: carbon catabolic inactivation (40–46). To study further this effect, 125 μg/ml cycloheximide was added to cultures grown for 42 h in minimal medium containing acetate and glucose, and the rate of the disappearance of ACS was studied. Fig. 7b shows that in both cases (with and without CH) the rate of disappearance was quite similar, suggesting that protein synthesis is not required for ACS inactivation. Similar results have been reported for a different enzyme (fructose-1,6-diphosphatase) in yeasts (47, 48). To establish whether the effect caused by glucose on ACS activity was or was not reversible, cultures incubated for 42 h in the presence of acetate and glucose were harvested at different times (42.5 h and 43 h), washed, resuspended in starvation medium (see “Materials and Methods”) and incubated in it for different times. ACS activity was measured *in vitro* at short intervals (Fig. 7b). In no case was the restoration of the lost activity observed nor after Sephadex G-25 filtration, dialysis, heat treatment, or by incubating the extracts with different phosphodiesterases. All these results strongly suggest that an irreversible change in the structure of the enzyme had been produced that handicapped the condensation of acetate to acetyl-CoA.

In summary, the absence or low titers of natural penicillins and penicillin G found in the culture broths of *P. chrysogenum* when glucose was added at the very beginning of the fermentation (see Fig. 1b) could be explained by the following overlapping mechanisms: (i) by carbon catabolite inactivation of ACS when this enzyme has been synthesized during growth on acetate (this report), and also by carbon catabolite repression of the gene encoding this enzyme, as has been reported in *A. nidulans* (38); (ii) by glucose repression of IPN synthase (49); and (iii) by repression of the phenylacetic acid transport system (50–52).

ACS and PTS regulation could be explained by a common mode of control. Thus, repression of PTS could be an indirect effect of the inactivation of ACS, since if PAA is not activated, phenylacetyl-CoA (which seems to be the true inducer of PTS) (52), is not produced and therefore phenylacetic acid cannot be taken up by the cells (inducer exclusion). The fact that PTS was not induced until acetate was exhausted (Fig. 2) fits well with this interpretation.

**Fig. 7. Inactivation of ACS by glucose.** a, half-life of ACS (●) when *P. chrysogenum* was grown in the basic chemically defined medium for 39 and 43 h (V). Arrows indicate the time at which cycloheximide (CH) (125 μg/ml) was added. After antibiotic addition, protein synthesis is completely and immediately halted (as we assured by [14C]leucine incorporation). b, rate of ACS deactivation when *P. chrysogenum* was grown for 42 h in a chemically defined medium containing acetate (●) or acetate and glucose (○). Idem when at 42 h. CH (125 μg/ml) was added (△, ▢). Some cultures grown in the glucose containing medium were harvested at different times (42.5 and 43 h), washed to eliminate the glucose, and resuspended in starvation medium, and ACS was measured at short intervals (V). The arrow indicates the time at which CH was added.
formation of this antibiotic, when the ACS-AT coupled system was incubated in vitro with CoA, ATP, Mg²⁺, 6-APA, DTT, phenylacetate, and acetate, was not observed (see Table II). In this reaction, mainly acetyl-CoA (but no or very little phenylacetyl-CoA) is formed and taking into account that acetyl-CoA is not a substrate of AT (11, 12), no penicillin can be produced. On the contrary, we detected synthesis of penicillin G when the concentration of acetate in the reaction was reduced (0.05 and 1 mM, see Table II). Similar results have been obtained when this enzymatic system was incubated with Δ⁶-hexenoic acid and acetate (Table II), strongly supporting the hypothesis that ACS also catalyzes in vivo the activation (to their CoA derivatives) of other molecules used as penicillin-side chain precursors. However, this situation (an excess of acetate or other acyl-acids with respect to PAA) would be a rare event in industrial fermentations of penicillin side chains.

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