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Different Stabilities of Two AMP-forming Acetyl-CoA Synthetases from *Phycomyces blakesleeanus* Expressed under Different Environmental Conditions

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The stability of acetyl-CoA synthetases (ACS1 and ACS2) from *P. blakesleeanus* against temperature, urea and trypsin was studied and compared. Thermal inactivation of ACS1 was biphasic, while that of ACS2 was monophasic. The thermodynamic parameters calculated from the inactivation profiles show ACS2 to be a more thermostable enzyme than ACS1. The presence of ATP and Mg^{2+} exerted a protective effect on both enzymes, and led to a marked increase in the E_a, $\Delta H^{\neq}$, $\Delta S^{\neq}$ and $\Delta G^{\neq}$ values. ACS2 is also much more stable against denaturation with urea; the estimates of ΔG_w (free energy change for protein unfolding at zero denaturant concentration) were 9.4 kJ mol^{-1} and 18.1 kJ mol^{-1} for ACS1 and ACS2, respectively. Finally, a half-life of 44.5 min for ACS2 versus the 21 min for ACS1 indicates that ACS2 is more stable than ACS1 against digestion by trypsin. These results seem to show that ACS2 is more rigid overall than ACS1, which may be essential for preserving its catalytic activity in the stress situation in which it is expressed.

Key words: Acetyl-CoA synthetases, filamentous fungi, stability, thermal inactivation, urea denaturation.

Abbreviations: ACS, acetyl-CoA synthetase.

In some fungi and yeasts, the AMP-forming acetyl-CoA synthetase (AMP-ACS) (EC 6.2.1.1), the enzyme which catalyses the conversion of acetate to acetyl-CoA, has been reported to exist in two isoforms, ACS1 and ACS2. In *S. cerevisiae*, studies have been made of the transcriptional regulation of both structural *ACS* genes, with *ACS1* strongly repressed by glucose (*1*) and *ACS2* expressed in its presence (*1–3*). In *Kluyveromyces lactis*, the *KlACS1* and *KlACS2* genes show similar transcriptional regulation to their homologues in *S. cerevisiae* (*4*). For the enzymes of *P. blakesleeanus*, we found that ACS1 (encoded by the *facA* gene) was induced by acetate and repressed by glucose, while ACS2 (not encoded by the *facA* gene) was detected as a stress response to carbon starvation (*5*). Both purified enzymes can use acetate and propionate as substrates, a substrate specificity similar to that reported for ACS from other microorganisms. *P. blakesleeanus* ACS1 and ACS2 differ in their optimum temperatures (30 and 50°C, respectively), previous experiments indicating furthermore that ACS2 is more stable to temperature than ACS1 (*5*). An optimum temperature of 50°C has been found for *B. japonicum* (*6*) and between 40–50°C for *P. putida* and the halophilic archaea *H. marismortui* enzymes (*7*, *8*), while for hyperthermophilic crenarchaeon ACS, it is reported to be over 90°C (*9*).

P. blakesleeanus cannot grow at temperatures over 30°C and we did not find that the presence of ACS2 allowed it to survive above this temperature. ACS2 may be a stress protein, expressed in an adverse environment for the fungus, and we think that its higher temperature stability may be a reflection of a higher stability to denaturing conditions in general. Proteins, and particularly enzymes, are believed to be quite vulnerable structures, sensitive to environmental changes. They can be denatured by changing their physical or chemical environments, the most common methods being heating, adding a chemical denaturant such as urea or guanidinium chloride, changing the pH or applying high pressure. The denatured (unfolded) state has considerable conformational freedom and thus inherently high configurational entropy. Conversely, the native (folded) state has considerable conformational restrictions and low entropy for, as a protein folds, it loses much of it, which must be balanced by a gain in enthalpy for the free energy to favour folding. Thus, whether a protein folds depends crucially on a balance between diverse stabilizing and destabilizing interactions exhibiting marginal stabilities that are equivalent to only a small number of weak intermolecular interactions (*10*).

The aim of this study was to analyse the stability of ACS1 and ACS2 from *P. blakesleeanus* under several denaturant conditions such us heating, presence of urea and trypsin. The thermodynamic quantities obtained for the two enzymes are compared and analysed in the light

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of overall structural characteristics. Our results show that the higher stability of ACS2 can be advantageous for the maintenance of its activity under adverse conditions in which it is expressed in *P. blakesleeanus*. This experimental approach can be of interest in the study of other enzymes expressed under different stress conditions.

MATERIALS AND METHODS

Materials—*P. blakesleeanus* NRRL 1555 (−) wild type and the *facA*$^-$ mutant, strain MU138 were kindly provided respectively by Dr A.P. Eslava (Dept. of Microbiology and Genetics, University of Salamanca, Spain) and Dr S. Torres-Martínez (Dept. of Genetics and Microbiology, University of Murcia, Spain). Coenzyme A was purchased from Pharmacia Biotech (Uppsala, Sweden). Bradford reactive was from Bio-Rad (Richmond, California, USA). ATP (disodium salt) was from Fluka Chemie A. G. (Switzerland). Sodium acetate was from Merck (Darmstadt, Germany). All other products used were of analytical quality.

Acetyl-CoA Synthetases—ACS1 from the mycelium of *P. blakesleeanus* NRRL 1555(−) grown on acetate and ACS2 from MU138 [(*P. blakesleeanus facA*$^-$ mutant)] mycelia were purified according to the method of de Cima *et al*. (*5*). Fractions containing homogeneous enzyme, as assessed by SDS-PAGE, were stored at 4°C in 20 mM sodium phosphate buffer pH 7.5, containing 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT and 20% (v/v) glycerol.

Enzyme Assay—Acetyl-CoA synthetase was routinely assayed by measuring the formation of acetylhydroxamate (*11*), with the modifications introduced by Martínez-Blanco *et al*. (*12*), as described previously (*5*). One unit of enzyme activity is defined as the amount of enzyme required for the formation of 1 nmol of acetylhydroxamate in 1 min. Acetyl-CoA synthetase activity was also assayed by a modification of the coupled assay described by Fujino *et al*. (*13*), from which lactate dehydrogenase was omitted, and the pyruvate formed determined by reaction with 2,4-dinitrophenylhidrazone (*14*).

Thermal Inactivation and Thermodynamic Parameter Calculation—Aliquots of purified ACS1 and ACS2 from *P. blakesleeanus* were previously desalted through an Amersham Pharmacia Biotech PD-10 desalting column packed with Sephadex G-25 equilibrated with 20 mM Tris-HCl buffer pH 8.0 containing 20% (v/v) glycerol and eluted with the same buffer. The samples were incubated at different temperatures and the time course of enzymes inactivation were monitored by assaying the enzyme activity in small aliquots withdrawn at the time intervals shown. When the inactivation data did not follow pseudo-first-order kinetics, they were fitted to the following equation, assuming a double exponential inactivation process (*15*):

$$\frac{E_t}{E_o} = (1-A)e^{-(k_1+k_2)t} + Ae^{-k_2 t} \quad (1)$$

where E_o is the original enzyme activity and E_t is the enzyme activity at the time indicated; A is the fraction of activity which inactivates following slow inactivation process, and k_1 and k_2 are the inactivation rate constants for the fast and slow processes, respectively. Weighted non linear least-squares Marquardt regression fitting of the data was performed using the Enzfitter program (*16*).

Activation energy (E_a) was determined from an Arrhenius plot of inactivation rate constants versus 1/T. From the slope, we calculated the Ea value [slope $= -E_a/2.303R$, where R is the universal gas constant ($8.314\,JK^{-1}\,mol^{-1}$)]. Activation enthalpy ($\Delta H^{\neq}$) and activation entropy ($\Delta S^{\neq}$) of the activate state were calculated from a modification of the linear *Arrhenius* equation:

$$\mathrm{Log}\frac{k}{T} = -\frac{\Delta H^{\neq}}{2.303RT} + \log\frac{k_B}{h} + \frac{\Delta S^{\neq}}{2.303R} \quad (2)$$

where k_B is Boltzmann's constant ($1.3805 \times 10^{-23}\,JK^{-1}$) and h is Planck's constant ($6.6256 \times 10^{-34}\,Js$).

Urea Denaturation and Analysis—Aliquots of purified ACS1 and ACS2 from *P. blakesleeanus* were previously desalted through an Amersham Pharmacia Biotech PD-10 desalting column packed with Sephadex G-25 and equilibrated with 50 mM Tris-HCl buffer pH 8.0 and eluted with the same buffer. Denaturing samples at different urea concentrations and incubation times were prepared in 50 mM Tris-HCl buffer pH 8.0 at 30°C. Unfolding of ACS1 and ACS2 from *P. blakesleeanus* as a function of urea concentration was monitored from the residual enzymatic activity of the samples. The data were analyzed assuming a two-state equilibrium and a linear relationship between free energy of unfolding and urea concentration (*17*) using the equations:

$$K_U = \frac{(y_F - y)}{(y - y_U)} = e^{-\Delta G/RT} \quad (3)$$

$$\Delta G = \Delta G_w - mD \quad (4)$$

K_U is the equilibrium constant for unfolding in the presence of urea calculated from Equation 3, where y is the residual activity of the enzyme observed at some point in the transition zone, y_F the residual activity of the folded (native) enzyme, taken as 100% (in the absence of denaturant), and y_U the residual activity of the unfolded (denatured) enzyme, taken as 0. ΔG_w is the Gibbs energy difference between the folded and unfolded states of the enzyme at zero denaturant concentration, D the concentration of denaturant (urea) and m the slope of the linear plot of ΔG versus D.

In-gel Digestion of Proteins—Protein spots were excised from the gels and digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremer, Germany). The digestion protocol used was Schevchenko *et al*.'s (*18*), with minor variations: gel plugs were subjected to reduction with 10 mM dithiothreitol (Amersham Biosciences, Uppsala, Sweden) in 50 mM ammonium bicarbonate (99.5% purity; Sigma Chemical, St. Louis, Missouri, USA), and alkylation was carried out with 55 mM iodoacetamide (Sigma Chemical) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega,

Madison, Wisconsin, USA) at a final concentration of 13 ng/μl in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion allowed to proceed at 37°C for 6 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Sigma Chemical) was added for peptide extraction.

MALDI-MS(/MS) and Database Searching—An aliquot of the above digestion solution was mixed with an aliquot of α-cyano-4-hydroxycinnamic acid (Bruker-Daltonics, Bremer, Germany) in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid. This mixture was deposited onto a 600 μm AnchorChipMALDI probe (Bruker-Daltonics) and allowed to dry at room temperature. MALDI-MS (/MS) data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonics) equipped with a LIFT-MS/MS device (*19*). Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100–1,500 individual spectra averaged. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses analysed in the ion reflector. Measurements were made in part using post-LIFT metastable suppression, which allowed removal of the precursor and metastable ion signals produced after second ion source extraction. Detailed analysis of peptide mass mapping data was performed using flex analysis software (Bruker-Daltonics). Internal calibration of MALDI-TOF mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2,211.105. MALDI-MS/MS calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800–3,200 m/z region. MALDI-MS and MS/MS data were combined through the MS BioTools program (Bruker-Daltonics) to search the NCBInr database using Mascot software (Matrix Science, London, UK) (*20*).

RESULTS AND DISCUSSION

ACS1 and ACS2 from P. blakesleeanus—*Phycomyces blakesleeanus* NRRL 1555 (−) wild type expressed two acetyl–CoA synthetases, ACS1 and ACS2, under different nutritional conditions. ACS1, encoded by the *facA* gene, was found to be induced by acetate and repressed by glucose at the transcriptional level, whereas ACS2, not encoded by the *facA* gene, was detected as a response to carbon starvation (*5*). ACS1 was purified from mycelia of the wild type grown on 2.73% (w/v) acetate for 24 h and ACS2 was from mycelia of a *P. blakesleeanus facA*$^-$ mutant grown for 14 h on 2% (w/v) glucose and then transferred to a medium lacking a carbon source, and maintained for a further 24 h. We have previously reported the purification procedure, made up of ultracentrifugation (100,000 g), ammonium sulphate precipitation, and the following chromatographic steps: Phenyl-Sepharose 6 fast-flow, Hydroxyapatite HT and Green A/Red A cross-linked agarose columns (*5*). The enzyme solutions were stored at 4°C in 20 mM sodium phosphate buffer, pH 7.5, containing 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT and 20% (v/v) glycerol. When adenylate kinase was omitted from the reaction mixture for the coupled activity assay, as described in Materials and Methods, no pyruvate-dinitrophenylhydrazone was formed, indicating that AMP was a reaction product (data not shown), and that therefore both *P. blakesleeanus* enzymes were AMP-forming acetyl-CoA synthetases. We have previously also reported other properties of ACS1 and ACS2 from *P. blakesleeanus* (*5*).

A subunit molecular mass of 74.7 kDa has been estimated for *P. blakesleeanus* ACS1 from the *facA* gene sequence (*21*), which agrees with the native molecular mass determined by us from gel filtration Superose 12HR 10/30 chromatography (data not shown), indicating that ACS1 is a monomeric enzyme. A sample of purified ACS2 eluted from the Red A-agarose chromatography (the last step of the purification procedure) was analysed using native gel electrophoresis (ND-PAGE) on a polyacrylamide gradient (4–20% (w/v) and using 12% SDS-PAGE gels, and in both cases a single band with a molecular mass of 76 ± 1 kDa was obtained, which also suggests a monomeric structure for *P. blakesleeanus* ACS2.

ACS2 was visualized on a 12% SDS-PAGE gel by Mann silver stain, excised from the gel, digested with trypsin and analyzed by tandem mass spectrometry [MALDI –MS(/MS)] of peptides as described in Material and Methods. An ion peptidic fragment from MALDI-TOF spectrum, with a mass of 1366.7608 and with the greatest intensity of all peaks obtained, was selected and analyzed in the tandem time-of-flight (TOF/TOF) mode. MALDI-MS and MS/MS data were combined by means of the MS BioTools program (Bruker-Daltonics) to search the NCBInr database using MASCOT software. The peptide fragment sequence obtained was VTKFYTAPTAIR and the database search revealed a 100% homology (score = 100) with the sequences of, among others, cytoplasmic ACS from *Mus musculus, Gallus gallus, Bos Taurus, Homo sapiens* and *Canis familiaris*, which allowed us to identify ACS2 from *P. blakesleeanus* as an acetyl-CoA synthetase.

Effect of Temperature on Activity and Enzyme Stability—ACS1 and ACS2 from *P. blakesleeanus* have different optimum temperatures, as we have previously reported (*5*): 30°C and 50°C, respectively, preliminary studies indicating that ACS2 is a much more stable enzyme than ACS1. We therefore analysed the effect of temperature on enzymatic activity in detail, as well as on the stability of both *P. blakesleeanus* ACSs enzymes.

Arrhenius plots of log Vmax versus the reciprocal temperature gave straight lines (Fig. 1), from whose slopes we calculated a value for the activation energy (E_a) for the catalytic step of 16.56 ± 0.1 kJ mol^{-1} for ACS1 (in the range from 20 to 30°C) and of 33.2 ± 4.0 kJ mol^{-1} for ASC2 (in the range from 20 to 50°C), indicating that the catalytic process was controlled by a single rate constant in both cases. Similar E_a values to those determined for *P. blakesleeanus* ACS2 have been reported for the *B. japonicum* bacteroid (*6*) and for spinach leaf ACS enzymes (*22*), whereas higher E_a values have been observed for the halophilic archaea *Haloarcula marismortui* ACS (*8*) and for the

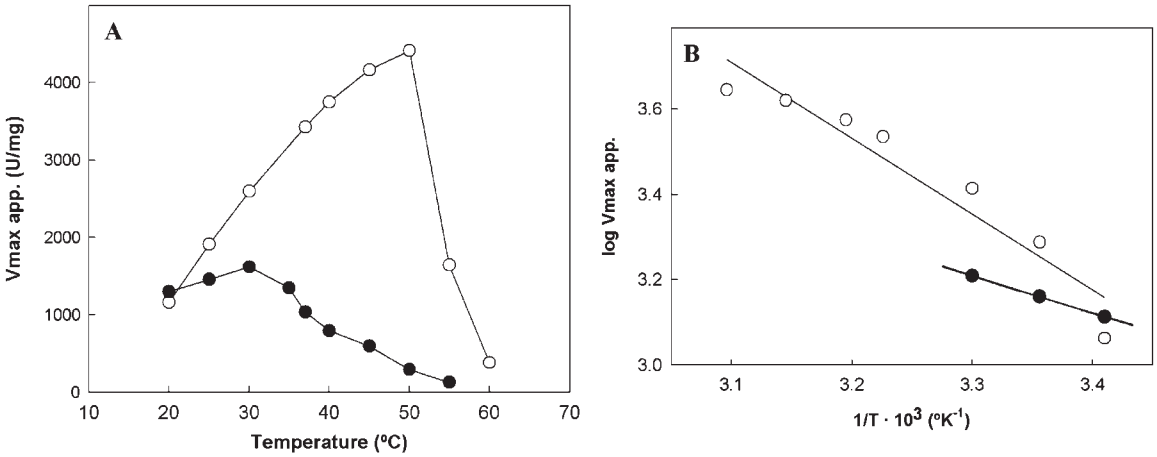

Fig. 1. **Effect of temperature on the enzymatic activity of ACS1 (filled circle) and ACS2 (open circle) from *P. blakesleeanus*.** (**A**) The activities were determined at the temperatures shown by means of the assay described in Materials and Methods in 50 mM Tris-HCl buffer, pH 8.0, with a protein concentration of 22 μg/ml for ACS1 and 6 μg/ml for ACS2. (**B**) Arrhenius plot for the determination of the energies of activation from the data shown in A).

hyperthermophilic crenarchaeon *Pyrobaculum aerophilum* (*9*). E_a values lower than that described for *P. blakesleeanus* ACS1 have not been reported.

Apparent activation-free energy ($\Delta G_{30^\circ C}^{\neq}$), enthalpy ($\Delta H^{\neq}$) and entropy ($\Delta S^{\neq}$) values, *i.e.* 72.2 kJ mol^{-1}, 14.1 kJ mol^{-1} and –192.3 J mol^{-1} K^{-1} for ACS1 and 71.4 kJ mol^{-1}, 31.0 kJ mol^{-1} and –133.6 J mol^{-1} K^{-1} for ACS2, were calculated with a modification of the linear Arrhenius equation as described in Materials and Methods and using 75 kDa as the molecular mass of the enzyme subunit to express the maximum velocity per mol of the catalytic site. ACS1 showed a $\Delta H^{\neq}$ value half of that of ACS2, indicating that, to reach the transition state, catalysis by ACS1 required a lower energy consumption to tense or distort bonds than catalysis by ACS2. The negative value for entropy ($\Delta S^{\neq}$) seems to indicate that the substrates adopt more ordered conformation in the transition state than in the Michaelis-Menten complexes. However the decrease in entropy was lower for ACS2; and this could indicate that, in the transition state, the substrates bind less "rigidly" to ACS2, suggesting that ACS2 has a greater local flexibility at its active site than ACS1. The higher entropic loss for the reaction catalysed by ACS1, which resulted in an increase in $\Delta G^{\neq}$, is compensated for by a lower enthalpic increase and thus, ACS2 showed a slightly lower $\Delta G^{\neq}$ value than ACS1.

ACS2 showed better thermal stability than ACS1. In the absence of added substrates, an incubation of the enzymes for 2 min at 50°C and pH 8.0, in the presence of 20% (v/v) glycerol, caused ACS1 to lose 80% of its activity, whereas ACS2 retained 100%. For this reason, the thermal stability of ACS1 and ACS2 was analysed in depth, by incubating the enzymes in 20 mM Tris-HCl buffer, pH 8.0, containing 20% (v/v) glycerol at the temperatures shown in Figs 2 and 3, either in the absence or in the presence of 10 mM ATP plus 10 mM $MgCl_2$. The reaction rates were determined at 30°C for ACS1 and at 50°C for ACS2.

ACS1 and ACS2 showed different thermal stabilities and inactivation kinetics. As Fig. 2A shows, in the absence of both ATP and Mg^{2+}, and in the temperature range 30–60°C ACS1 thermal inactivation was biphasic: after a rapid initial decrease in activity, a second slow decrease was observed, indicating that the inactivation kinetics responded to two processes with different inactivation rate constants. Analysis of the data shown in Fig. 2A according to Equation (1) allowed us to determine the inactivation rate constant for the fast ($k^1{}_1$) and slow ($k^1{}_2$) processes. In the temperature range 30–40°C, ACS1 is mainly inactivated by the slow process with a half-life of between 400 and 30 min, whereas the rapid process, with a half-life of about 2 min, accounted for a low percentage of enzymatic activity loss. At temperatures over 40°C, ACS1 inactivates mainly by the rapid process: approximately 85% of the enzyme inactivates at 50°C with a half-life of 0.7 min, whereas the remaining 15% inactivates with a half-life ten-fold longer. The presence of ATP and Mg^{2+} has not affected the thermal biphasic inactivation pattern; however, now ACS1 is mainly inactivated by the slow process with a half-life of between 220 and 30 min in the temperature range 42–50°C, whereas the rapid process, with a half-life of about 2 min, accounted for a low percentage of activity. At temperatures over 50°C likewise ACS1 inactivates mainly by the rapid process: approximately 90% of the enzyme inactivates at 62°C with a half-life of 0.8 min, whereas the remaining 10% inactivates with a half-life of 13 min. Thus, the temperature at which the enzyme retains 50% of its activity for a 15 min incubation shifted from 40 to 52°C when ATP and Mg^{2+} were added to the incubation (Fig. 2B).

Analysis of inactivation rate constants of the fast ($k^1{}_1$) and slow ($k^1{}_2$) processes with the Arrhenius equation allowed us to calculate the E_a values for both processes in the presence and absence of substrates. The other thermodynamic parameters, enthalpy ($\Delta H^{\neq}$), entropy ($\Delta S^{\neq}$) and activation-free energy ($\Delta G^{\neq}$), were calculated

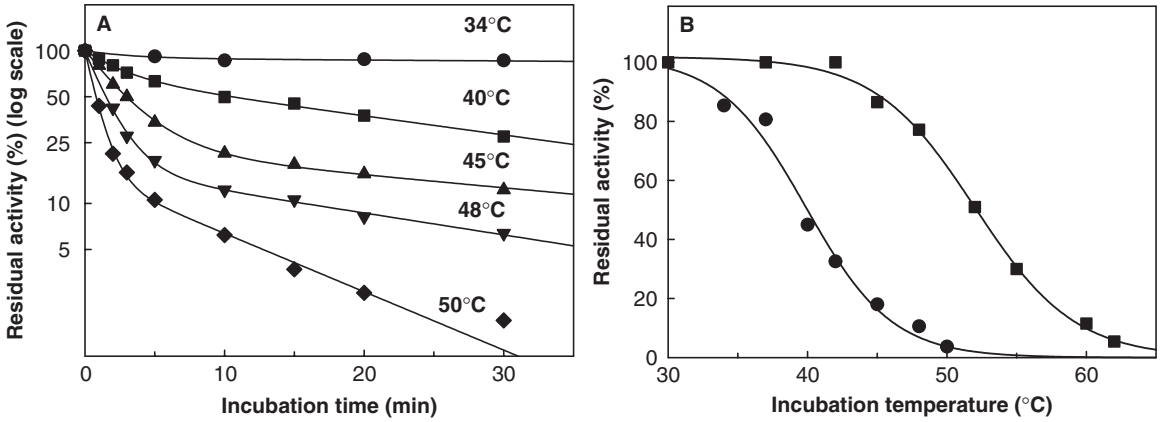

Fig. 2. (**A**) **Thermal inactivation kinetics of *P. blakesleeanus* ACS1 at pH 8.0.** ACS1 (250 μg/ml) was incubated in 20 mM Tris-HCl buffer, pH 8.0 containing 20% (v/v) glycerol. At predetermined times, aliquots were removed and residual activity was determined as described in the text and expressed as relative to zero-time incubation. (**B**) Protective effect of ATP and Mg^{2+} on the thermal inactivation of ACS1 from *P. blakesleeanus* at pH 8.0. ACS1 (25 μg/ml) was incubated in 20 mM Tris-HCl buffer, pH 8.0 containing 20% (v/v) glycerol, in the absence (filled circle) and in the presence of 10 mM ATP and 10 mM $MgCl_2$ (filled squre). After 15 min of incubation, aliquots were withdrawn, and residual activity was determined as indicated in the text and expressed relative to zero-time incubation.

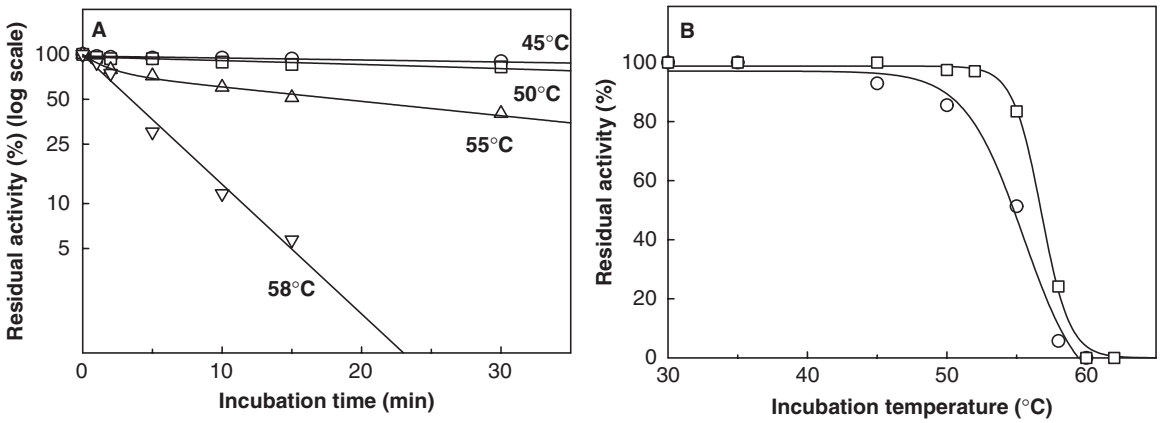

Fig. 3. (**A**) **Thermal inactivation kinetics of *P. blakesleeanus* ACS2 at pH 8.0.** ACS2 (70 μg/ml) was incubated in 20 mM Tris-HCl buffer, pH 8.0 containing 20% (v/v) glycerol. At predetermined times aliquots were removed and residual activity was determined as described in the text and expressed as relative to zero-time incubation. (**B**) Protective effect of ATP and Mg^{2+} on the thermal inactivation of ACS2 from *P. blakesleeanus* at pH 8.0. ACS2 (70 μg/ml) was incubated in 20 mM Tris-HCl buffer, pH 8.0 containing 20% (v/v) glycerol, in the absence (open circle) and in the presence of 10 mM ATP and 10 mM $MgCl_2$ (open square). After 15 min of incubation, aliquots were withdrawn, and residual activity was determined as indicated in the text and expressed relative to zero-time incubation.

by a modification of the linear Arrhenius equation as described in Materials and Methods. Table 1 summarizes the thermodynamic parameter values obtained for thermal inactivation of *P. blakesleeanus* ACS1, and the fast inactivation process is seen not to be affected by the presence of both substrates, ATP and Mg^{2+}. However, the thermodynamic parameter values for the slow inactivation process varied slightly with the presence of both substrates, suggesting that their binding at the corresponding binding sites on the enzyme could stabilize the local conformation of the active site of ACS1. Thus, the slow process could be responsible for the inactivation caused by thermal conformational change affecting the local environment of the ACS1 active centre, while the presence of substrates shifts the fast inactivation process to higher temperatures.

In the absence of substrates, ACS2 was inactivated following a first-order kinetics in the temperature range studied (45–60°C). Analysis of the data shown in Fig. 3A by means of a single exponential decay allowed us to determine the inactivation rate constant for each temperature. The values of this inactivation rate constant were of the same order of magnitude as those obtained for the slow inactivation process of ACS1. The presence of ATP and Mg^{2+} had no effect on the inactivation kinetic pattern, but exerted a protective effect on ACS2 thermal inactivation: the temperature at which the enzyme retains 50% of its activity for a 15-min incubation shifted from 55°C without added substrates to

Table 1. **Thermodynamic parameters for the thermal inactivation at pH 8.0 of ACS1 and ACS2 from *P. blakesleeanus*.**

Parameter	ACS1				ACS2	
	Fast inactivation step		Slow inactivation step			
	In absence of ATP and Mg^{2+}	In presence of ATP (10 mM) and Mg^{2+} (10 mM)	In absence of ATP and Mg^{2+}	In presence of ATP (10 mM) and Mg^{2+} (10 mM)	In absence of ATP and Mg^{2+}	In presence of ATP (10 mM) and Mg^{2+} (10 mM)
E_a (kJ/mol)	100.4 ± 20	108.5 ± 25	161.3 ± 23	196.6 ± 41	276.6 ± 13	537.2 ± 28
$\Delta H^{\neq}$ (kJ/mol)	103.1 ± 17	112.9 ± 30	163.90 ± 23	199.4 ± 41	273.9 ± 58	534.5 ± 29
$\Delta S^{\neq}$ (kJ/mol K)	0.133 ± 0.03	0.153 ± 0.03	0.302 ± 0.04	0.387 ± 0.07	0.53 ± 0.2	1.31 ± 0.11
$\Delta G^{/}_{(50^{\circ}C)}$ = (kJ/mol)	60.0 ± 15	63.4 ± 9	66.4 ± 11	74.4 ± 16	102.7 ± 25	109.75 ± 7

Thermal enzyme inactivation of purified ACS1 and ACS2 from *P. blakesleeanus* was carried out in 20 mM Tris-HCl buffer pH 8.0 containing 20% (v/v) glycerol, as described in the MATERIALS AND METHODS section. The inactivation data were fitted to pseudo-first-order kinetics or double-exponential-inactivation kinetics. Thermodynamic parameters were determined from an Arrhenius plot and a modification of the linear Arrhenius equation, as described in the MATERIALS AND METHODS section.

56.5°C in their presence (Fig. 3B). Analysis of inactivation rate constants according to the Arrhenius equation and a modification of the linear Arrhenius equation as described in Materials and Methods allowed us to calculate the thermodynamic parameter values obtained for thermal inactivation of *P. blakesleeanus* ACS2, which are shown in Table 1. The absence of ATP and Mg^{2+} led to a marked reduction in the E_a value and consequently of $\Delta H^{\neq}$, suggesting that the intramolecular stabilizing forces are associated with the binding of both substrates at the active centre of ACS2. In addition, a lower $\Delta S^{\neq}$ value for the inactivation process in the absence of substrates compared with that observed in their presence suggests that ACS2 molecule adopt a more relaxed conformation as result of the dissociation of substrates. In general, the comparison of the thermodynamic inactivation parameters for *P. blakesleeanus* ACS1 and ACS2 enzymes, in the absence as well as in presence of ATP and Mg^{2+}, led us to conclude that ACS2 has a higher thermal stability than ACS1. From the thermodynamics parameters values for ACS1 and ACS2 showed in Table 1, we can suggest that ACS1 has a more relaxed conformation than ACS2, in the absence or in the presence of ATP and Mg^{2+}, which would bind with more laxity to the active site, contributing with less stabilizing forces to maintain the native conformation of ACS1. This more relaxed conformation of ACS1 could be related with the existence of the fast inactivation process detected in the thermal inactivation of this enzyme. However, in ACS2, the no existence of this rapid inactivation process beside the higher thermodynamic parameter values in absence and in presence of ATP and Mg^{2+} would indicate a more rigid conformation, in which the ATP and Mg^{2+} bind tightly to the active site, contributing, thus, with more intramolecular stabilizing forces to maintain the native conformation. In this regard, rigidity as a prerequisite for high protein thermostability has been proposed as a working hypothesis from the study of hyperthermophilic enzymes (*23*). Our results suggest that ACS2 could combine local flexibility at its active site with a high overall rigidity contributing to its thermostability.

Conformational Stability—Stability refers to the maintenance of a defined functional state under extreme conditions. The conformational stability of a protein is defined as the free energy change, ΔG, for the folded $\leftrightarrow$ unfolded reaction under physiological conditions (*24*). We have determined the conformational stability of ACS1 and ACS2 from *P. blakesleeanus* at pH 8.0 and 30°C by analyzing the chemical denaturation with urea as denaturant. Figure 4 shows the transition curves for urea denaturation obtained for ACS1 and ACS2 after 20 min of incubation for different urea concentrations, as well as the linear relationship between the free energy of unfolding and urea concentration in the ranges 0–3.5 M (ACS1) and 0–5.5 M (ACS2), by assuming that the unfolding of both enzymes follows a two-state mechanism in which only the native (folded) state and the denatured (unfolded) state are present at significant concentrations in the transition region. Many soluble globular proteins can be reversibly unfolded by urea and exhibit two-state behaviour, a condition on which analyses of unfolding free energy measurements have been based (*25*, *6*). The linear extrapolation method is the one most commonly used for thermodynamic analyses (*27*, *28*) and assumes that the linear dependence of ΔG on denaturant concentration observed in the transition region continues to zero concentration, and thus it is possible to calculate the free energy change for protein unfolding at zero denaturant concentration (ΔG_w).

A similar denaturation analysis was carried out for both enzymes for another two incubation times, 5 and 10 min, with urea. As no significant deviations in m and urea concentration of mid-denaturation ($U_{1/2}$) values were observed for different times of urea incubation, average values of 1.0 ± 0.07 kcal mol^{-1} M^{-1} and 2.25 M for ACS1 and 1.2 ± 0.06 kcal mol^{-1} M^{-1} and 3.6 M for ACS2 were used to calculate the values of the free energies of unfolding in H_2O (ΔG_w) by multiplying m x $U_{1/2}$. The values of ΔG_w calculated were 2.25 kcal mol^{-1} (9.4 kJ mol^{-1}) for ACS1 and 4.32 kcal mol^{-1} (18.1 kJ mol^{-1}) for ACS2. As can be seen, ACS2 showed a conformational stability value nearly double that of ACS1, so ACS2 is much more stable than ACS1. However, the conformational stability of both enzymes is low under these experimental conditions, namely in the absence of any stabilizer such as glycerol, and it has been reported that almost all naturally occurring globular proteins have conformational stabilities between 5 and 15 kcal mol^{-1} (21–63 kJ mol^{-1}) (*24*).

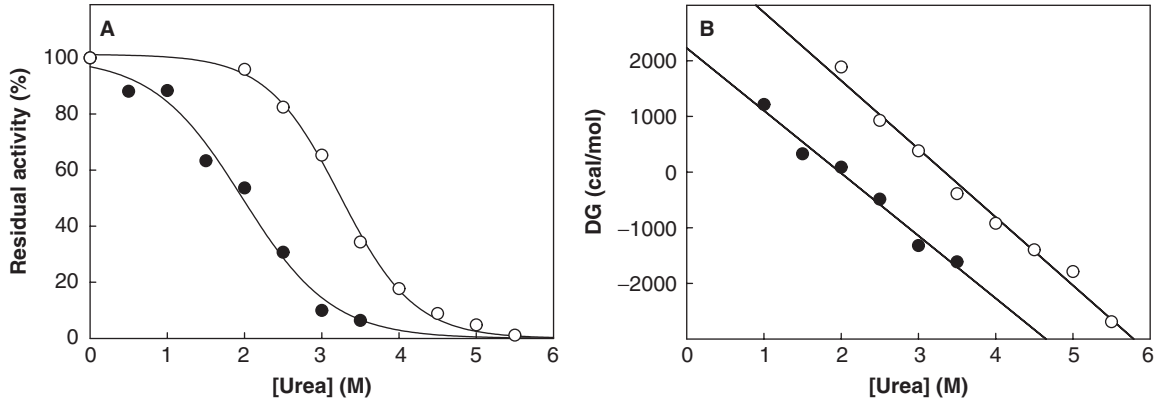

Fig. 4. **(A) Stability curves for denaturation by urea of ACS1 and ACS2 from *P. blakesleeanus* at pH 8.0 and 30°C.** ACS1 (54 μg/ml) (filled circle) and ACS2 (44 μg/ml) (open circle) were incubated in 50 mM Tris-HCl buffer pH 8.0 at 30°C with each of urea concentrations indicated. After 20 min of incubation, aliquots were withdrawn, and residual activity was determined as indicated in the text and expressed relative to zero-time incubation. **(B)** Dependence of free energy of unfolding of ACS1 (filled circle) and ACS2 (open circle) on urea concentration. ΔG values were calculated for the data given in A) according the equations (3) and (4) defined in the text.

The conformational stability of any protein depends on a delicate balance between large contributions of diverse stabilizing and destabilizing forces involved in the formation of the folded, native three-dimensional structure, giving rise to a marginal free energy difference of stabilization. Although the establishment of the effective contribution of each of the forces obviously implies an exhaustive study, our results allowed us to deduce interesting data on the conformational stability of ACS1 and ACS2. The slope of the linear plot of ΔG versus D [concentration of denaturant (urea)], m, is related to the nonpolar area of protein exposed to solvent during denaturation (*29*). Since the values obtained for ACS1 and ACS2 are similar, 1.0 and 1.2 respectively, one might expect the hydrophobic effect contribution (a majority force that stabilizes the native structure) to be similar for both enzymes. If this is so, it seems reasonable to assume that the difference in ΔG_w would be due to the different contributions of the other majority forces operating in the opposite direction, namely hydrogen bonding and the conformational entropy, and thus to a higher contribution of hydrogen bonding and/or a lower conformational entropy in ACS2.

Tryptic Digestion Stability—We determined the stability of ACS1 and ACS2 against tryptic digestion at pH 8.0 and 25°C, a loss of time-dependent activity occurring (ratio 5 : 1) of both in both cases. Both ACS1 and ACS2 showed a pseudo-first-order inactivation kinetics (Fig. 5). ACS2 was more stable to inactivation by trypsin than ACS1: the half-life ($t_{1/2}$) for ACS2 was double that for ACS1 (44.5 min as opposed to 21 min). The larger stability of ACS2 against inactivation by trypsin may be due to: (i) a lower amount of arginine and lysine in the aminoacid sequence, (ii) lesser accessibility of some of these aminoacids to trypsin action, although the amounts were the same and/or (iii) the distance of some of these aminoacids from the active centre of ACS2, with a consequently lesser effect of the proteolytic cleavage by trypsin on activity. At present we cannot rule out the possible contribution,

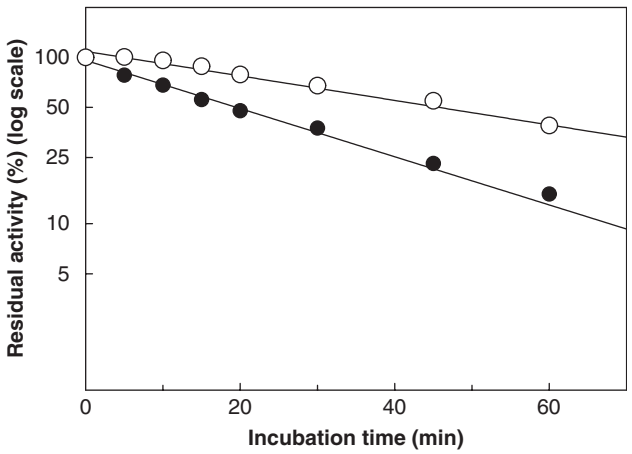

Fig. 5. **Trypsin inactivation kinetics of ACS1 (filled circle) and ACS2 (open circle) from *P. blakesleeanus* at pH 8.0 and 25°C.** ACS1 (90 μg/ml) and ACS2 (70 μg/ml) were incubated, in separate experiments, with trypsin (18 and 14 μg/ml, respectively) in 50 mM Tris-HCl buffer pH 8.0 containing 20% (v/v) glycerol at 25°C. At the times shown on the graph, aliquots were removed and residual activity was determined as relative to zero-time incubation.

to some extent, of any of these factors to the greater stability of ACS2. We have determined the pI value of ACS2 by chromatofocusing by using a PBE 94 Polybuffer exchanger (Amersham Biosciences). This pI value was slightly more alkaline for ACS2 (6.4) than for ACS1 (6.12). From the aminoacid sequences for ACS stored in the Swissprot/TrEMBL data base (http://www.expasy.org/sprot) and using the informatic online program ProtParam we have found the existence of a good relationship between the ratio of the (Arg + Lys) percentage and the (Asp + Glu) percentage and the pI value, as an increase in the ratio correspond to an increase in the pI value. Differences in amino acid sequences or alterations resulting from chemical modification may be responsible for increased conformational

stability, and although no systematic amino acid substitutions produced this effect, statistical method show that thermoenzymes have a higher Arg:Lys ratio than mesozymes (*30*). The higher stability of ACS2 against temperature and urea denaturation could imply a greater rigidity of the enzyme, allowing us to suggest that some of these arginine and lysine residues might be less accessible to trypsin action.

CONCLUSIONS

In this study, we examined the stability under several denaturing conditions of the two acetyl-CoA synthetases (ACS1 and ACS2), which are expressed in *P. blakesleeanus* under different physiological conditions. Our most interesting finding was that ACS2 is a more stable enzyme than ACS1 against temperature, urea and trypsin denaturation. We think that this greater stability may be due to the contribution of several stabilization factors, such as a few additional hydrogen bonds, lower conformational entropy and/or the presence of stabilizing amino acids. Proteins exhibit marginal stabilities that are equivalent to only a small number of weak intermolecular interactions and rigidity is a prerequisite for thermostability (*23*, *30*). Our results support the idea that *P. blakesleeanus* ACS2 is overall more rigid than ACS1 and that this rigidity may be essential for preserving its catalytic active structure in unfavourable conditions, which is expressed as a response to the stress generated by carbon starvation.

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