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Further kinetic and molecular characterization of an extremely heat-stable carboxylesterase from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*

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The carboxylesterase (serine esterase, EC 3.1.1.1) from *Sulfolobus acidocaldarius* was purified 940-fold to homogeneity by an improved purification procedure with a yield of 57%. In the presence of alcohols the enzyme catalyses the transfer of the substrate acyl group to alcohols in parallel to hydrolysis. The results show the existence of an alcohol-binding site and a competitive partitioning of the acyl-enzyme intermediate between water and alcohols. Aniline acts also as a nucleophilic acceptor for the acyl group. On the basis of titration with diethyl *p*-nitrophenyl phosphate, a number of four active centres is determined for the tetrameric carboxylesterase. The sequence of 20 amino acid residues at the esterase *N*-terminus and the amino acid composition are reported.

INTRODUCTION

Previously we reported on the purification and initial characterization of a heat-stable esterase from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* (Sobek & Görisch, 1988). On the basis of the inhibition pattern and the substrate specificity the esterase was classified as a carboxylesterase (serine esterase, EC 3.1.1.1). For several mammalian and eubacterial carboxylesterases it has been shown that the hydrolysis of esters proceeds via an acyl-enzyme intermediate (Krisch, 1971; Alt *et al.*, 1975). Carboxylesterases have also been shown to catalyse the transfer of the acyl group to alcohols (Greenzaid & Jencks, 1971; Wynne & Shalitin, 1972; Lombardo & Guy, 1981) or amines (Franz & Krisch, 1968; Goldberg & Fruton, 1970; Alt *et al.*, 1975). To obtain information about the catalytic mechanism of the archaebacterial carboxylesterase we studied acyl-group transfer reactions to alcohols and amines as nucleophilic acceptors.

EXPERIMENTAL

Materials

Sulfolobus acidocaldarius (DSM 639) was grown at 70 °C and pH 2.0–2.2 in 50-litre batches as described previously (Grossebüter & Görisch, 1985). The medium described by Brock *et al.* (1972) was used, containing 0.2% (w/v) yeast extract. *p*-Nitrophenyl acetate, *p*-nitroacetanilide, aniline hydrochloride, diethyl *p*-nitrophenyl phosphate and Tris were purchased from Sigma (Taufkirchen, Germany).

5,5′-Dithiobis-(2-nitrobenzoic acid), Coomassie Brilliant Blue R-250, guanidinium chloride and triacetin were from Serva (Heidelberg, Germany). Bovine serum albumin and Azokoll were obtained from Calbiochem (Frankfurt, Germany). Centricon 10 micro-concentrators were from Amicon (Witten, Germany). Q-Sepharose and benzamidine–Sepharose 6B were purchased from Pharmacia (Uppsala, Sweden). Hydroxyapatite was prepared as described by Atkinson *et al.* (1973). Acetanilide was from Riedel-de-Haen (Hannover, Germany). The low-M_r calibration kit was from Boehringer (Mannheim, Germany). Cresol Red was obtained from Merck (Darmstadt, Germany). Yeast extract was from Hartge (Hamburg, Germany). All other organic and inorganic chemicals were of analytical-reagent grade.

Protein determination

Protein was determined by the method of Groves *et al.* (1968); 224 nm and 235 nm were used as isoabsorbance wavelengths, with bovine serum albumin as standard.

Enzyme assay

The standard test for esterase activity was performed with *p*-nitrophenyl acetate at pH 8.0 and 25 °C as described previously (Sobek & Görisch, 1988) in a total volume of 1 ml.

Amidase activity was determined by measuring the hydrolysis of *p*-nitroacetanilide at 25 °C and 50 °C. The reaction mixture contained 0.5 mM-*p*-nitroacetanilide in 0.1 M-Tris/HCl buffer, pH 8.6, and the release of *p*-nitroaniline was monitored at 405 nm (Heymann *et al.*, 1981).

Proteolytic activity was measured by incubating esterase with 50 mg of Azokoll in 5 ml of 10 mM-potassium phosphate buffer, pH 7.0, at 25 °C and 56 °C, followed by centrifugation and measurement of the absorption of the supernatant at 580 nm.

Effect of alcohols on activity

The effect of alcohols on the release of *p*-nitrophenol from *p*-nitrophenyl acetate was determined in the standard test with 0.13–0.85 unit of esterase. Tris/HCl buffer was replaced by 10 mM-potassium phosphate buffer, pH 8.0. The effect of alcohols on the formation of acetate was measured by utilizing the decolorization of a pH indicator solution caused by the release of acid

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from ester. Because of the lower sensitivity of this test 1.5–8.5 units of esterase were used. The test system contained 0.1 mM-Cresol Red and 0.9 mM-*p*-nitrophenyl acetate in 10 mM-potassium phosphate buffer, pH 8.0. The decrease of absorbance was measured at 580 nm. A quantification of the decrease of absorbance was achieved by a standardization of the test with *p*-nitrophenyl acetate and known amounts of esterase. The rate of acid release was corrected for the proton release from *p*-nitrophenol, which is ionized to the extent of 87.5% at pH 8.0. Within the range of enzyme concentrations used the reaction rate is a linear function of the protein concentration. The rates of hydrolysis and release of *p*-nitrophenol were calculated by reference to equal amounts of esterase. The formation of esters by alcoholysis was demonstrated after complete cleavage of *p*-nitrophenyl acetate by a hydroxamate test described by Hestrin (1949).

Acyl-group transfer to aniline

The acetylation of aniline was determined by a modification of the method described by Franz & Krisch (1968) with triacetin as acyl-group donor. The disappearance of aniline was measured at 286 nm (ϵ 1246 $M^{-1} \cdot cm^{-1}$) and 25 °C in 0.1 M-Tris/HCl buffer, pH 8.6, containing 2 mM-aniline and 29.4 mM-triacetin in a total volume of 1 ml. Within the range of enzyme concentrations used (0.5–3 units) the reaction rate is a linear function of the protein concentration. Initial rates of the acyl-group transfer reaction were determined as a function of the triacetin concentration in the presence of various fixed concentrations of aniline and vice versa. Michaelis constants and maximal velocities were obtained by plotting the data as described by Hanes (1932).

The formation of acetanilide was demonstrated in a preparative reaction mixture: 50 ml of 0.1 M-Tris/HCl buffer, pH 8.6, containing 3.83 mM-aniline and 147 mM-triacetin was incubated at 25 °C with 120 μg of purified esterase. The disappearance of aniline was monitored at 286 nm. At 80% conversion the reaction mixture was extracted twice with 50 ml of diethyl ether. The pooled ether fractions were washed three times with 50 ml of 0.1 M-HCl, dried over anhydrous $MgSO_4$ and evaporated to dryness. The residue was redissolved in ethanol and applied to a thin-layer plate of silica gel UV_{254}. The plate was developed in chloroform/toluene/acetic acid (17:6:17, by vol.). Aniline and acetanilide were detected at 254 nm and identified by comparison with authentic standards.

Amino acid analysis

Purified esterase was dialysed against 20 mM-NH_4HCO_3 and afterwards freeze-dried. Samples containing 0.1 mg of protein were hydrolysed in 6 M-HCl for 24 h, 48 h and 72 h at 110 °C in sealed tubes. Amino acid analysis was carried out on a Biotronic LC 6 000 analyser essentially as described by Spackman (1967). Serine and threonine were determined by extrapolation to zero time. Valine, leucine and isoleucine contents were extrapolated to infinite time of hydrolysis. Cysteine was determined by oxidation with performic acid as described by Moore (1963) and by titration with Ellman's reagent in the presence of 6 M-guanidinium chloride according to the method described by Morino & Snell (1967). Tryptophan was measured in the presence of 6 M-guanidinium chloride (Edelhoch, 1967). The calculation of the number of amino acid residues was based on an M_r for the subunit of 32000 (Sobek & Görisch, 1988).

N-Terminal sequencing

The partial sequence of the *N*-terminal end was determined by using an Applied Biosystems model 470 A gas-phase protein sequencer connected to a 120 A on-line h.p.l.c. system.

Titration with diethyl *p*-nitrophenyl phosphate

Titration of esterase with diethyl *p*-nitrophenyl phosphate was performed as described by Krisch (1966). Purified esterase (22 nmol in 1 ml) was titrated by the addition of diethyl *p*-nitrophenyl phosphate in 10 μl portions containing 4.5 nmol of diethyl *p*-nitrophenyl phosphate. The release of *p*-nitrophenol was monitored at 405 nm. Samples (10 μl) were removed from the reaction mixture to determine the remaining activity in the standard test. The stoichiometry of the reaction with diethyl *p*-nitrophenyl phosphate was calculated assuming an M_r of 128000 for the esterase.

SDS/polyacrylamide-gel electrophoresis

Analytical SDS/polyacrylamide-gel electrophoresis was carried out as described previously (Sobek & Görisch, 1988).

Enzyme purification

With the exception of the heat precipitation all purification steps were performed at 4 °C.

I: Crude extract. A 250 g batch of wet frozen cells (−20 °C) was thawed in 375 ml of 0.1 M-Tris/HCl buffer, pH 7.5. Cells were disrupted by passing the suspension through a French pressure cell at a pressure of 156.6 MPa. Cell debris was collected by centrifugation at 48400 ***g*** for 30 min and resuspended in 150 ml of 0.1 M-Tris/HCl buffer, pH 7.5. The suspension was again passed through the French pressure cell, followed by centrifugation. The combined supernatants of the centrifugation steps were regarded as crude extract.

II: Heat precipitation. The crude extract was heated at 80 °C for 15 min under gentle stirring. After cooling in ice the resulting precipitate was removed by centrifugation at 30000 ***g*** for 30 min. The supernatant was dialysed overnight against 2 × 5 litres of 20 mM-Tris/HCl buffer, pH 7.5.

III: Q-Sepharose chromatography. This step was performed as described previously (Sobek & Görisch, 1988). Precipitation by $(NH_4)_2SO_4$ was omitted. The active fractions were adjusted to pH 6.8 by the addition of diluted acetic acid and applied on to the hydroxyapatite column.

IV/V: Chromatography on hydroxyapatite and benzamidine–Sepharose. These steps were performed as described previously (Sobek & Görisch, 1988).

VI: Concentration of the homogeneous esterase. The active fractions from the benzamidine–Sepharose 6B column were pooled and dialysed against 2 × 5 litres of 20 mM-Tris/HCl buffer, pH 8.0. The dialysed solution was loaded on to a Q-Sepharose column (1 cm × 5 cm) equilibrated with 20 mM-Tris/HCl buffer, pH 8.0.

Table 1. Purification of esterase from *S. acidocaldarius*

For details see the Experimental section.

Step	Total protein (mg)	Total acitvity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	27200	5000	0.18	1	100
Heat precipitation	7100	4900	0.69	3.8	98
Q-Sepharose chromatography	680	4170	6	34	83
Hydroxyapatite chromatography	228	4010	17.6	98	80
Benzamidine–Sepharose 6B chromatography	16.7	2850	170	940	57

Esterase was eluted with the same buffer containing 0.5 mM-NaCl. Fractions (1 ml) were collected. Further concentration steps were performed with Centricon 10 micro-concentrators.

RESULTS

Purification and homogeneity

The purification procedure of the esterase from *S. acidocaldarius* as reported previously (Sobek & Görisch, 1988) is modified by including a heat-precipitation step. By this modification the enzyme is obtained in homogeneous form without chromatography on Mono Q and the yield increases from 20% to 57%. The purification procedure for the esterase from *S. acidocaldarius* is summarized in Table 1. The enzyme is purified 940-fold and shows a specific activity of 170 units/mg when catalysing the hydrolysis of *p*-nitrophenyl acetate. This specific activity is slightly higher than the specific activity reported previously for the homogeneous enzyme (Sobek & Görisch, 1988). The esterase obtained by the modified procedure is homogeneous, as demonstrated by SDS/polyacrylamide-gel electrophoresis.

Amino acid composition

Table 2 shows the amino acid composition of the esterase from *S. acidocaldarius*. A tryptophan content of two residues per subunit was determined by spectrophotometric measurements of the enzyme in the presence of 6 M-guanidinium chloride. From thiol-group titration experiments with Ellman's reagent a cysteine content of one residue per subunit was calculated. By performic acid oxidation two cysteine residues per subunit were found.

N-Terminal sequence

Fig. 1 lists the sequence of 20 amino acid residues at the esterase *N*-terminus. The first degradation step resulted in three major amino acid derivatives: those of proline, alanine and serine. Proline was the predominant amino acid and is assumed to be the *N*-terminal residue, with alanine and serine probably being contaminants. Residues 2–20 of the esterase could be identified unambiguously.

Titration with diethyl *p*-nitrophenyl phosphate

The esterase from *S. acidocaldarius* was titrated with diethyl *p*-nitrophenyl phosphate. As shown in Fig. 2, the decrease in enzymic activity and the concentration of *p*-nitrophenol released correspond to the amount of diethyl *p*-nitrophenyl phosphate added. The esterase is completely inactivated. From the increase of absorbance at 405 nm, the release of 3.8 mol of *p*-nitrophenol is calculated per mol of enzyme. On the basis of a number of four catalytically active subunits a ratio of 0.95 mol of diethyl *p*-nitrophenyl phosphate per mol of subunit is determined for complete inactivation of the esterase.

Table 2. Amino acid composition of the esterase from *S. acidocaldarius*

The amino acid composition was calculated assuming an M_r of 32000 for the subunit.

Amino acid	Composition (mol of residue/mol)
Asx	33
Thr	13
Ser	19
Glx	28
Pro	16
Gly	29
Ala	20
Val	23
Met	5
Ile	25
Leu	26
Tyr	15
Phe	12
Lys	17
His	3
Arg	12
Trp	2*
Cys	2†
	1‡

* Determined by the method of Edelhoch (1967).
† Determined by the method of Moore (1963).
‡ Determined by the method of Morino & Snell (1967).

Amidase/proteinase activity

Under the conditions described in the Experimental section the esterase from *S. acidocaldarius* shows no amidase or proteinase activity.

Effect of alcohols on esterase activity

With *p*-nitrophenyl acetate as substrate the rate of hydrolysis, measured by the rate of acetic acid release, is

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                5                  10                  15                  20
Pro-Leu-Asp-Pro-Thr-Ile-Lys-Cys-Leu-Leu-Glu-Ser-Gly-Phe-Val-Ile-Pro-Ile-Gly-Lys
(Ala)
(Ser)
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Fig. 1. *N*-Terminal amino acid sequence of the esterase from *S. acidocaldarius*

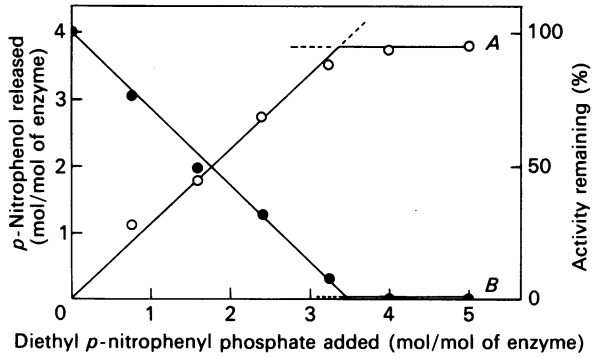

Fig. 2. Titration of the esterase from *S. acidocaldarius* with diethyl *p*-nitrophenyl phosphate

A 0.72 mg portion of homogeneous esterase was used. The mol of diethyl *p*-nitrophenyl phosphate added per mol of esterase are plotted against the remaining activity (curve *B*) and against the mol of *p*-nitrophenol released/mol of enzyme (curve *A*).

strongly decreased in the presence of alcohols. The rate of hydrolysis decreases with increasing concentrations of alcohols and increasing chain length of the alcohols. Propan-2-ol causes a weaker effect than propan-1-ol. With 27 mM-butan-1-ol no acid formation is observed. The total rate of the esterase-catalysed cleavage of *p*-nitrophenyl acetate, measured by the rate of *p*-nitrophenol release, however, increases in the presence of alcohols. The rate of *p*-nitrophenol formation increases with increasing concentrations of alcohols and increasing chain length of the alcohols.

At a concentration of 54 mM-butan-1-ol the rate of release of *p*-nitrophenol is 4.4-fold higher than the rate observed in its absence. Propan-2-ol causes a weaker effect than propan-1-ol. At higher concentrations of butan-1-ol and propan-1-ol again a decrease of the rate is observed (Fig. 3). The difference between the rate of the release of *p*-nitrophenol and the rate of acid formation represents the esterase-catalysed transesterification of *p*-nitrophenyl acetate by reaction with alcohols as nucleophiles. After complete release of *p*-nitrophenol from *p*-nitrophenyl acetate the resultant esters were assayed as hydroxamates in the reaction mixture. Thus the esterase from *S. acidocaldarius* catalyses the acyl-group transfer reaction to alcohols. The transfer of the substrate acyl group to alcohols occurs in parallel to the hydrolysis of substrate. These results indicate a competitive partitioning of an acyl-enzyme intermediate between water and alcohol.

The possible mechanisms of esterase-catalysed hydrolysis/alcoholysis reactions and their kinetic equations were discussed by Wynne & Shalitin (1972). With the archaebacterial esterase a plot of the hydrolysis/alcoholysis ratio against 1/[alcohol] (Fig. 4) indicates the existence of an alcohol-binding site for propan-2-ol (Wynne & Shalitin, 1972). The existence of a hydrophobic

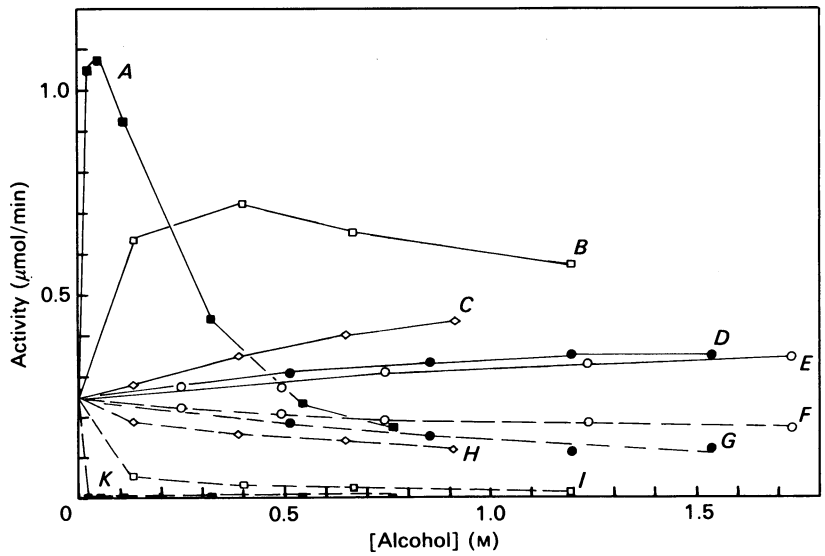

Fig. 3. Effect of alcohols on the release of *p*-nitrophenol (curves *A–E*) and the formation of acetic acid (curves *F–K*) from *p*-nitrophenyl acetate

The activities refer to 2 μg of purified esterase, measured at 0.9 mM-*p*-nitrophenyl acetate at 25 °C in 10 mM-potassium phosphate buffer, pH 8.0. Curves *A* and *K*, butan-1-ol; curves *B* and *I*, propan-1-ol; curves *C* and *H*, propan-2-ol; curves *D* and *G*, ethanol; curves *E* and *F*, methanol.

alcohol-binding site on the enzyme for primary alcohols is also indicated by the increase of alcoholysis with increasing alkyl chain length (Fig. 3).

Acyl-group transfer to aniline

Esterase from *S. acidocaldarius* catalyses an acyl-group transfer with triacetin as acyl donor and aniline as acyl acceptor. Initial velocities at various concentrations of triacetin in the presence of changing fixed concentrations of aniline follow regular Michaelis–Menten kinetics. Initial rates at variable concentrations of aniline in the presence of changing fixed concentrations of triacetin show inhibition at concentrations of aniline above 2 mM. At low concentrations of aniline, where no substrate inhibition is apparent, the initial velocities follow regular Michaelis–Menten kinetics and Hanes (1932) plots yield straight lines (Figs. 5*a* and 6*a*). In the presence of aniline hydrolysis of triacetin is still occurring. This indicates that, as with alcohols, the acetyl-group transfer to aniline competes with hydrolysis. Under these conditions apparent K_m values were determined from replots of the slopes of Hanes plots from a study of the acyl-group transfer reaction at various concentrations of aniline at different fixed concentrations of triacetin and vice versa (Figs. 5*b* and 6*b*). The apparent K_m values for triacetin and aniline were both determined to be 1.6 mM. From the obtained $V_{max.}$ values a specific activity of 23.7 units/mg was calculated for acetyl-group transfer to aniline.

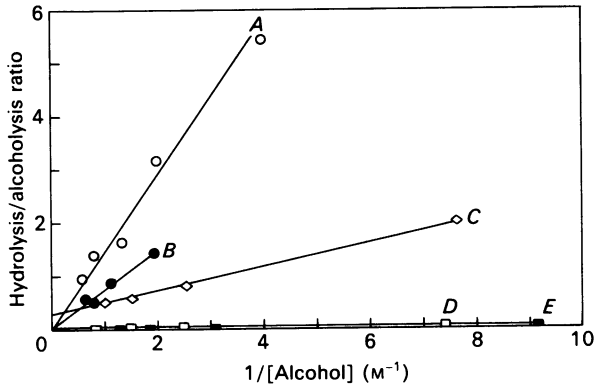

Fig. 4. Effect of the concentration of alcohols on the hydrolysis/alcoholysis ratio

Hydrolysis/alcoholysis ratios are plotted against 1/[alcohol]. The values of the hydrolysis/alcoholysis ratios were calculated from the data in Fig. 3. Curve *A*, methanol; curve *B*, ethanol; curve *C*, propan-2-ol; curve *D*, propan-1-ol; curve *E*, butan-1-ol.

DISCUSSION

The purification procedure described in this paper represents a modification of the procedure reported previously (Sobek & Görisch, 1988). To increase the amount of esterase in the crude extract, the cell suspension was passed twice through a French pressure cell at high pressure. Inclusion of a heat-precipitation step allows omission of the Mono Q chromatography. This procedure results in a substantially higher yield of homogeneous enzyme. The carboxylesterase from *S. acidocaldarius* was purified 940-fold and 57% of the starting activity was recovered. When hydrolysing *p*-nitrophenyl acetate the enzyme shows a specific activity of 170 units/mg, which is slightly higher than the specific activity reported previously for the homogeneous enzyme (Sobek & Görisch, 1988).

Four active centres were found per molecule of tetrameric carboxylesterase by titration with diethyl *p*-nitrophenyl phosphate. Therefore each subunit contains one active centre. With respect to the *N*-terminal amino acid sequence the archaebacterial carboxylesterase from *S. acidocaldarius* shows no similarity to other known sequences of carboxylesterases. No significant similarity is found to either the two carboxylesterases isolated from *Drosophila mojavensis* (Pen *et al.*, 1986) or the carboxylesterase from *Caenorhabditis elegans* (McGhee, 1987).

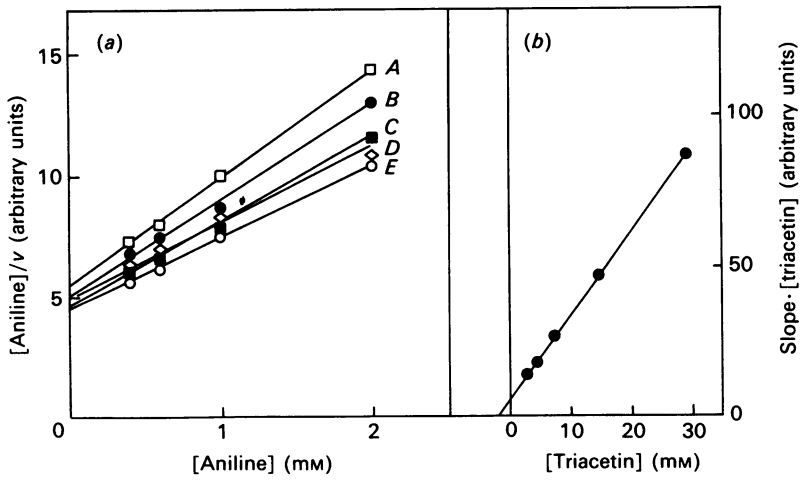

Fig. 5. Determination of the apparent K_m for triacetin

A 12 μg portion of homogeneous esterase was used. (*a*) The concentration of aniline was varied at different fixed concentrations of triacetin: curve *A*, 2.94 mM; curve *B*, 4.2 mM; curve *C*, 7.35 mM; curve *D*, 14.7 mM; curve *E*, 29.4 mM. (*b*) Replot of the slopes of (*a*).

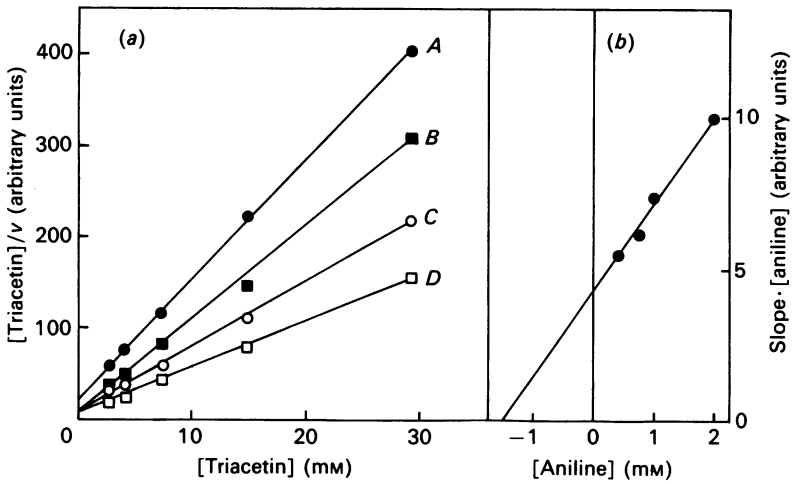

Fig. 6. Determination of the apparent K_m for aniline

A 12 μg portion of homogeneous esterase was used. (*a*) The concentration of triacetin was varied at different fixed concentrations of aniline: curve *A*, 0.4 mM; curve *B*, 0.6 mM; curve *C*, 1 mM; curve *D*, 2 mM. (*b*) Replot of the slopes of (*a*).

With *p*-nitrophenyl acetate as substrate a decreased rate of hydrolysis is observed in the presence of alcohols. Under the same conditions the rate of *p*-nitrophenol release increases, which indicates a two-step reaction mechanism for the carboxylesterase from *S. acidocaldarius*, with the formation of an acyl-enzyme intermediate. In the presence of alcohols a competitive partitioning of the acyl-enzyme intermediate between water and alcohol is observed. Thus the archaebacterial carboxylesterase exhibits the same reaction mechanism as the eukaryotic carboxylesterases from bovine liver (Wynne & Shalitin, 1972), pig liver (Greenzaid & Jencks, 1971) and human pancreas (Lombardo & Guy, 1981). The kinetic data show the presence of an alcohol-binding site on the enzyme for propan-2-ol. The increase of the rate of *p*-nitrophenol release and the strong inhibition of hydrolysis by alcohols suggest that with *p*-nitrophenyl acetate the deacylation of the acyl-enzyme intermediate is the rate-limiting step. Like alcohols, aniline can act as nucleophilic acceptor for the substrate acyl group.

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