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A KINETIC STUDY OF HOMOCITRATE SYNTHETASE ACTIVITY IN THE YEAST *SACCHAROMYCOPSIS LIPOLYTICA*

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Summary

1 A rapid method for estimating the activity of the first enzyme of lysine biosynthesis in yeasts (acetyl-coenzyme A 2-ketoglutarate *C*-acetyl transferase, EC 4 1 3 21) is described

2 In the wild type strain, the fixation of one substrate, *S*-acetyl coenzyme A, shows sigmoidal saturation kinetics The initial rate experiments indicate that the reaction obeys an ordered mechanism, 2-ketoglutaric acid binding before *S*-acetyl coenzyme A

3 The activity is completely inhibited in vitro by lysine and by some lysine analogs, which all show cooperative binding and have an heterotropic effect on 2-ketoglutaric binding sites A second class of effectors is found, including 2-aminoadipic acid, pipecolic acid and dipicolinic acid, which all affect the cooperativity of *S*-acetyl coenzyme A binding sites

4 Two types of mutations which modify these inhibition patterns without affecting the catalytic activity are described One results in a desensitization towards lysine and lysine analogs only The other entirely abolishes the susceptibility towards the second type of inhibitors, without affecting the susceptibility to lysine

5 No variations of the specific activity could be detected in the wild type strain at all, mutants showing an increased or a reduced activity were isolated

6 Our results do not support the existence of isoenzymes at the level of homocitrate synthetase in this yeast

Introduction

The first step of the lysine biosynthetic pathway in yeasts and fungi involves the condensation of 2-ketoglutaric acid with acetyl-coenzyme A (Ac-CoA), yielding homocitric acid and coeznyme A (CoA) [1,2,3,4] The homocitrate

synthetase activity (acetyl-coenzyme A 2-ketoglutarate *C*-acetyl transferase, EC 4.1.3.21), was demonstrated in vitro by isolating the radioactive homocitrate formed during incubation of [^{14}C]acetate, CoA, an Ac-CoA generating system and 2-ketoglutaric acid in the presence of a cell-free extract Feedback inhibition of this activity by lysine was shown to occur both in vivo and in vitro in yeasts and in *Penicillium spirillum* [5,6] The physiological significance of this inhibition remained nevertheless questionable, since high levels of lysine were necessary in order to obtain a significant inhibition.

Probably due to the presence of isoenzyme at this step [7], no mutants for this activity have been isolated up to now in yeasts On the other hand, all methods used in order to measure the homocitrate synthetase activity were very long and tedious, and prevented accurate kinetic study This communication reports that in the yeast *Saccharomycopsis lipolytica*, which is now manageable genetically [8], it is possible both to obtain mutants affected in this step and to conduct kinetic studies easily

Materials and Methods

Strains and media

Cultures were prepared in liquid mineral medium (glucose 1%, ammonium sulfate 0 5%, thiamine 10 ppm and mineral salts, [8]) at 28° C Auxotrophic requirements were compensated by adding 100 mg/l aminoacids

From the wild type strain W29 (ATCC number 20460), lysine-less strains were derived as described elsewhere [8] Lysine can be replaced by 2-aminoadipic acid (an intermediate of the pathway) for lys16 and lys2 strains, but not for lys12 and lys9 strains, which are presumably blocked after this intermediate Lys12 strain was found to be devoid of aminoadipate reductase activity (determined by Sagisaka and Shimura's method [9], to be published), The obtention of the mg-5 strain (ATCC number 20462) as resistant to the toxic analog of lysine, 4-5 transdehydrolysine, has been reported earlier [10], it has been shown that the mutations in lys16 and mg-5 are allelic (homocitrate synthetase structural gene)

Preparation of homocitrate synthetase extracts

Unless otherwise stated, 1 l liquid cultures in mineral medium where shaken for one night at 28° C in 3 l flasks The cells were then harvested at the end of the exponential phase by centrifugation and washed twice with distilled water at 4° C. Subsequent operations were all carried out at 4° C The cells were disrupted in a Braun shaker cell homogeneizer with glass beds in 0 05 M Tris HCl buffer pH 7.4, containing 20% (w/v) glycerol and 1 mM phenylmethylsulfonylfluoride The suspension was then centrifuged 45 min at 70 000 × *g* and the supernatant (containing about 20 mg/ml of proteins in the case of W29 strain) was dialysed for 4 h against 0.02 M Tris · HCl buffer pH 7 4 (containing glycerol and phenylmethylsulfonylfluoride, same as above). This procedure was found to be necessary in order to increase both specific activity and sensitivity to allosteric inhibitors, longer dialysis (8 and 18 h) did not improve the results We verified that 2-aminoadipic acid and lysine accumulated by the strains under certain conditions were indeed eliminated by this procedure (chromato-

graphic tests). The omission of phenylmethylsulfonylfluoride in the buffers resulted in a partial and non-reproducible desensitization of the activity towards lysine the inhibition was then never complete and its maximum ranged between 50 and 80% Lysine or 2-ketoglutaric acid had no significant effect on the susceptibility of the activity towards lysine nor on the specific activity The addition of phenylmethylsulfonylfluoride did not alter the specific activity which was found to be stable for one week at least at 4° C.

We made some attempts in order to purify this activity, but with little success. Consequently, the crude dialysed extract was used routinely

Enzymatic assay for homocitrate synthetase activity

It was usually based on the determination of the CoA liberated during the reaction, although preliminary experiments according to Tucci and Ceci's method [7] were conducted in order to establish that radioactive homocitric acid was indeed formed under these conditions The standard incubation mixture contained 150 nmol phosphate buffer, pH 7 8, 50 nmol 2-ketoglutarate pH 7 0, 0 5 nmol $MgCl_2$, 2 nmol Ac-CoA and crude extract (equivalent to 0 3 mg of proteins), in a total volume of 1 ml The incubations were carried out at 25° C for 10 min for all extracts, except those of lys12 (7 min) The reaction was stopped by adding 50 µl of concentrated sulfuric acid. After removal of the precipitate, the supernatant was diluted 10-fold in 0 1 mM dithio-5,5′-bisnitrobenzoic acid, following the method of Srere et al for citrate synthetase [11]

The absorbance of the yellow product formed was read at 412 nm at 25° C in a temperature-controlled spectrophotometer (Acta III, Beckman Inst), against a blank without 2-ketoglutaric acid, treated in the same way The reaction velocities measured under these conditions were repeatedly checked, to see that they corresponded indeed to initial velocities and that they were proportional to both time of incubation and enzyme concentration

The pH optimum of the reaction lies between 7 5 and 8 2 There is no absolute need for Mg^{2+} ions in the crude dialysed extract, although there was sometimes a slight stimulation by Mg^{2+} (of about 10%) However, the reaction is sensitive to ethylene-diaminotetraacetate (EDTA) half-inhibition is obtained with 15 mM EDTA

The specific activity was always expressed in laboratory units (absorbance increase/min/mg of proteins) The proteins were determined according to Lowry et al [12]

All chemicals are commercially available, except for 4,5-transdehydrolysine and (amino-3) cyclohexylalanine, which were synthetized on request by the Ecole de Chimie of Mulhouse (France)

Results

Substrate binding and effect of CoA on the activity

Only the results obtained with the strain W29 will be recorded here in detail, but the fact that the binding of the substrates was not modified by lys12 and mg-5 mutations was checked

Substrate fixation was studied by plotting the variations of initial velocities versus various concentrations of one substrate at three different but fixed

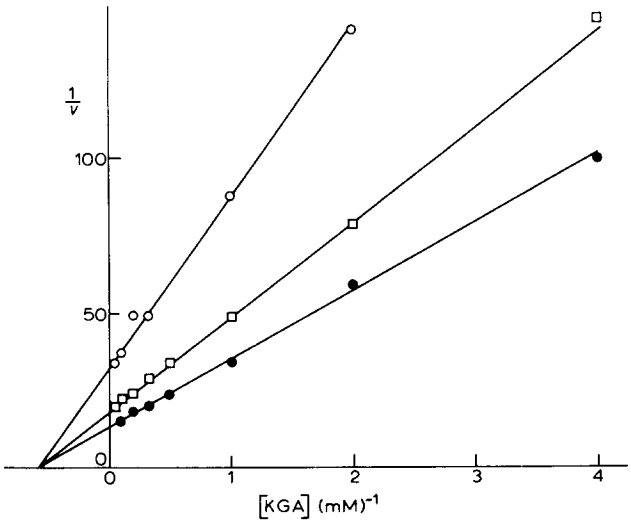


Fig 1 Effect of increasing 2-ketoglutarate concentrations on the initial reaction rates of the wild type homocitrate synthetase at different Ac-CoA levels ○———○, 0 1 mM, □———□, 0 25 mM, ●———●, 1 mM For assay conditions, see Materials and Methods. Initial rates are expressed in laboratory units (absorbance increase at 412 nm after 10 min incubation

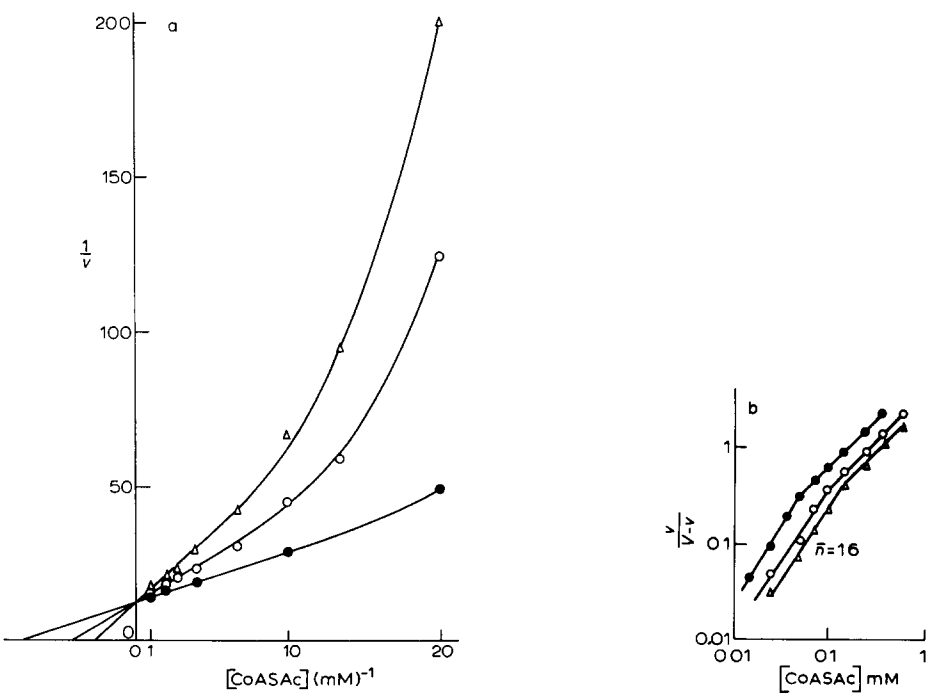


Fig 2 Fixation of Ac-CoA at different 2-ketoglutarate levels (wild type homocitrate synthetase) (a) Reciprocal plot of the initial velocities (expressed as absorbance increase at 412 nm after 10 min incubation) versus Ac-CoA concentrations at different 2-ketoglutarate concentrations ●———●, 50 mM, ○———○, 1 5 mM, △———△, 5 mM (b) Hill's representation [13] of Ac-CoA binding under these conditions For assay conditions, see Materials and Methods.

concentrations of the other The increase of Ac-CoA concentrations affects the V value of the 2-ketoglutarate saturation curves only, but has no effect on the apparent K_m value of 2-ketoglutarate which was found to be in the range of 1 7 mM (see Fig 1) The binding of 2-ketoglutarate always appeared to obey to a Michaelis-Menten equation under these conditions On the contrary, the apparent K_m values for Ac-CoA varied considerably with 2-ketoglutarate concentrations, whereas the V value of the saturation curves was not affected (see Fig. 2) an increase of 2-ketoglutarate concentrations from 5 mM to 50 mM decreased the K_m values for Ac-CoA from 0.40 mM to 0 13 mM. Moreover, an homotropic interaction between Ac-CoA binding sites was observed, and this interaction was not affected by 2-ketoglutaric acid (see insert Fig 2, $\bar{n}$ value [13] for Ac-CoA 1 7)

Product inhibition was only studied with CoA, since the second product of the reaction, homocitric acid, was not available As shown in Fig 3, CoA is a strong competitive inhibitor of Ac-CoA fixation and increased the value of the interaction coefficient of Ac-CoA binding sites to a maximum $\bar{n}$ value of 3 3 These results can be interpreted in terms of a competition for Ac-CoA binding site(s), and consequently the fixation of 2-ketoglutarate is not affected by CoA concentrations up to 0 5 mM (see Fig. 4) At higher CoA concentrations, the initial absorbance of CoA at 412 nm prevented accurate measurements of liberated CoA The mechanism of the reaction thus seems to be of the ordered type 2-ketoglutaric acid binds before Ac-CoA and CoA is probably the first product to be released

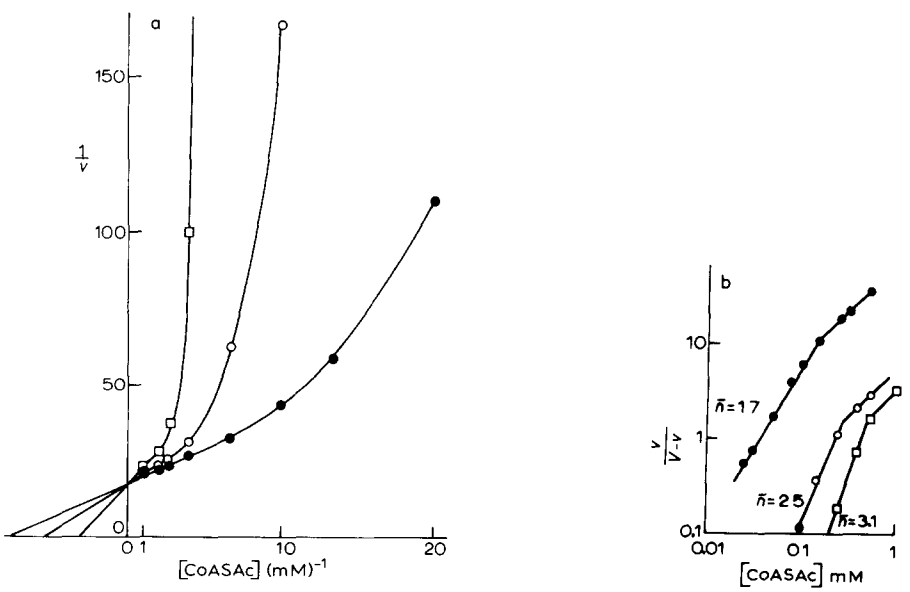


Fig 3 Effect of CoA on the initial reaction velocities at 25 mM 2-ketoglutarate with increasing concentrations of Ac-CoA Initial rates are expressed in laboratory units (absorbance increase at 412 nm after 10 min incubation (a) Reciprocal plot of the variations of initial velocities at different CoA concentrations ●——●, 0, ○——○, 0 02 mM, □——□, 0 1 mM (b) Hill's representation of Ac-CoA binding

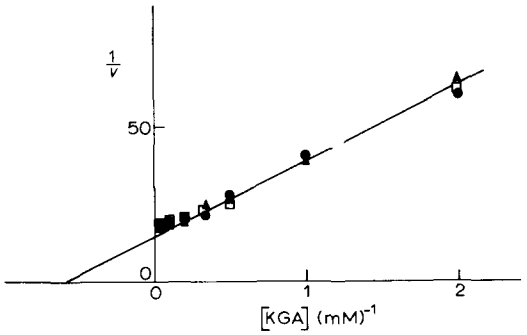


Fig 4 Effect of CoA on the initial rate of the reaction at 1 mM Ac-CoA with increasing 2-ketoglutarate concentrations (wild-type strain extract) Initial velocities are expressed as absorbance increase at 412 nm after 10 min incubation CoA concentrations used are ●——●, 0 mM, □——□, 0 1 mM, ▲——▲, 0 5 mM

Inhibitors of the activity in the wild type strain

Table I shows the effect of various amino acids and lysine analogs at 5 mM concentration on the activity of homocitrate synthetase at saturating substrate concentrations (standard conditions). L-lysine, DL-4,5-transdehydrolysine, DL-(amino-3) cyclohexylalanine, DL-allo-5-hydroxylysine, LL-diaminopimelic

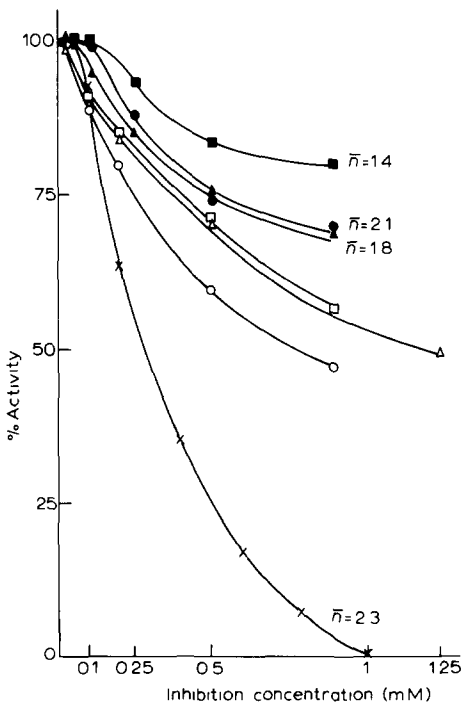
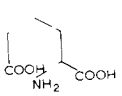
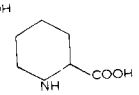
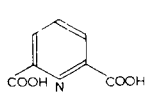


Fig 5 Inhibition of the wild-type homocitrate synthetase Substrate concentrations were following 50 mM 2-ketoglutarate and 2 mM Ac-CoA (full symbols), 25 mM 2-ketoglutarate and 1 mM Ac-CoA (open symbols) The following compounds were tested at increasing concentrations lysine (stars), pipecolic acid (circles), aminoadipic acid (squares), dipicolinic acid (triangles) The residual activities are expressed as the percentage of the activity in the absence of inhibitors. $\bar{n}$' values [15] are given in regard of each curve

TABLE I

EFFECTORS OF HOMOCITRATE SYNTHETASE ACTIVITY

The inhibition of the activity by the compounds listed below was tested at 5 mM concentration (under standard assay conditions (saturating substrates levels, see Materials and Methods)

Compound tested	Formula	Residual activity (%)
None		100
L-lysine	NH_2-CH_2-CH_2-CH_2-CH_2-CH(-NH_2)-COOH	0
D-lysine	NH_2-CH_2-CH_2-CH_2-CH_2-CH(-COOH)-NH_2	100
DL-allo-5-hydroxylysine	NH_2-CH_2-CH(-OH)-CH_2-CH_2-CH(-NH_2)-COOH	25
DL-4,5-transdehydrolysine	NH_2-CH_2-CH=CH-CH_2-CH(-NH_2)-COOH	10
DL(amino-3)cyclohexylalanine	NH_2-CH(-CH_2-CH_2-CH_2)-CH_2-CH(-NH_2)-COOH	15
LL-diaminopimelic acid	NH_2-CH(-NH_2)-CH_2-CH_2-CH_2-CH(-NH_2)-COOH	10
DL-*S* amino ethylcysteine	NH_2-CH_2-CH_2-S-CH_2-CH(-NH_2)-COOH	100
6-aminocaproic acid	NH_2-CH_2-CH_2-CH_2-CH_2-CH_2-COOH	100
DL-*N*,6-acetyllysine	CH_3-CO-NH-CH_2-CH_2-CH_2-CH_2-CH(-NH_2)-COOH	100
DL-*N*,2-acetyllysine	NH_2-CH_2-CH_2-CH_2-CH_2-CH(-NH-CO-CH_3)-COOH	100
L-Ornithine	NH_2-CH_2-CH_2-CH_2-CH(-NH_2)-COOH	100
Cadaverine	NH_2-CH_2-CH_2-CH_2-CH_2-CH_2($-NH_2$)	100
L-lysine methylester	NH_2-CH_2-CH_2-CH_2-CH_2-CH(-NH_2)-$COOCH_3$	100
Proline		100
Methionine		100
Arginine		100
Glutamate		100
Leucine		100
Isoleucine		100
Valine		100
DL-2-aminoadipate		≤80[1]
L-pipecolate		≤70[1]
Dipicolinate		≤70[1]

[1] Average value of 5 determinations (non-reproducible inhibitions, see text)

acid are the most potent inhibitors, giving an almost complete inhibition at 10 mM concentrations. This type of inhibition seems to be characteristic of compounds sharing following characters· (1) a 6-carbon backbone (*S*-aminoethylcysteine or ornithine are not inhibitors), (2) presence of a free amino group in position 2 (see 6-aminocaproic acid and *N*-2-acetyllysine) and in position 6 (see *N*-6-acetyllysine); (3) a free carboxyl group in position 2 in the L configuration (see cadaverine, D-lysine and L-lysine methylester). On the other hand, the inhibition curves obtained by equimolar mixtures of lysine and of any of these inhibitors are very close to the theoretical curves expected for a strictly additive effect [14] (not shown) it is therefore believed that these compounds act as true lysine analogs, binding to the same site(s) as lysine

Complete inhibition of the activity is achieved at millimolar concentration ranges (Fig. 5) with lysine, which is in marked contrast with the results reported with *Saccharomyces cerevisiae* [5] A cooperative homotropic effect between lysine binding sites was observed, giving a $\bar{n}$'value [15] of 2 3 These effects are independent from the growth conditions

On the contrary, the results ontained with 2-aminoadipic acid were most conflicting. with some preparations no inhibition at all could be found, whereas a maximum of 30% inhibition at 1 mM inhibitor concentration (under standard conditions) has been found in some cases These observations suggested that the enzyme might be desensitized in some preparations, and reminded of earlier results on lysine inhibition, but no stabilizing procedure could be found up to now This holds also for pipecolic acid, although to a lesser extent, and for dipicolinic acid As will be shown below, storage of the cells at −20° C for one night before carrying extraction resulted in a complete desensitization of the activity towards these compounds, but not towards lysine. A cooperative homotropic effect between inhibitors molecules was again observed, giving a $\bar{n}$' value in the range of 2 Only the results obtained with the most sensitive extracts have been reported below.

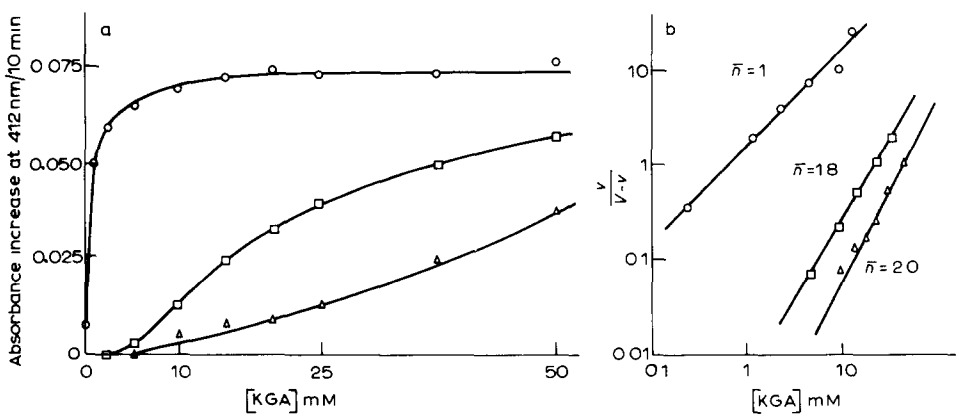


Fig 6 (a) Effect of lysine on the 2-ketoglutarate binding to the wild type enzyme The assay conditions were as given in Fig 1 (with Ac-CoA concentration fixed at 1 mM), except that lysine was added at the following concentrations ○———○, 0 mM, □———□, 0 02 mM, △———△, 0 5 mM (b) Influence of lysine on the cooperativity of KGA binding as shown by the empirical Hill plots [13] Symbols are the same as in (a)

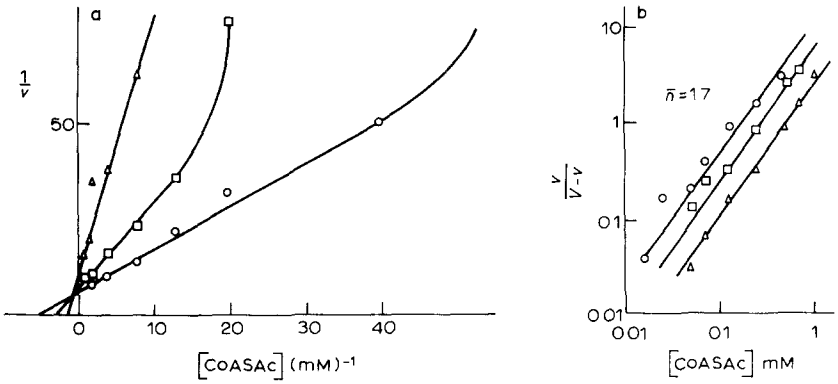


Fig 7 (a) Effect of lysine on the Ac-CoA binding to the wild-type enzyme The assay conditions were as given in Fig 2 (with 2-ketoglutarate concentration fixed at 25 mM), except that lysine was added at the following concentration ○———○, 0 mM, □———□, 0 2 mM, △———△, 0 5 mM Activities (v) are expressed in laboratory units (see Materials and Methods) (b) Hill plots of the same data (same symbols)

Effects of the inhibitors on substrates binding in the wild type strain

The initial velocity patterns for saturation by Ac-CoA and 2-ketoglutarate in the presence of two different but fixed lysine concentrations, showed that lysine acts as a competitive inhibitor of 2-ketoglutarate fixation, whereas it affects both V and K_m values of Ac-CoA saturation curves (Figs. 6a and 7a) An homotropic effect between the binding sites of 2-ketoglutarate was revealed under these conditions, whereas the cooperativity of Ac-CoA binding sites was not modified (Figs 6b and 7b)

When the same experiments were carried out with 2-aminoadipic acid instead of lysine, a competitive inhibition of 2-ketoglutarate fixation and a non-competitive inhibition of Ac-CoA fixation was again observed In this case, however, the cooperativity of 2-ketoglutarate binding sites was not affected ($\bar{n}$ value = 1), whereas an increase of the interaction coefficient of Ac-CoA binding sites from 1 4 to 3 0 was observed in the better case (see above) when 2-amino-

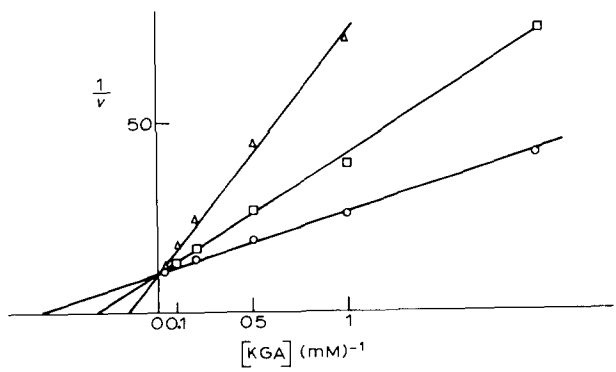


Fig 8 Effect of 2-aminoadipic acid on the initial velocity of the reaction with increasing 2-ketoglutarate concentrations, wild-type strain extract Assay conditions were as described in Fig 6 (Ac-CoA 1 mM), except that lysine was replaced by following concentrations of 2-aminoadipic acid ○———○, 0 mM, □———□, 0 25 mM, △———△, 1 25 mM

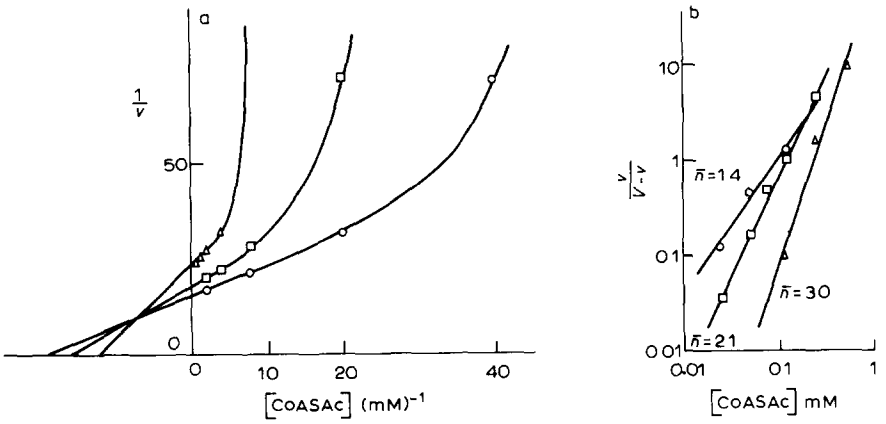


Fig. 9 Effect of 2-aminoadipic acid on the initial velocity of the reaction with increasing Ac-CoA concentrations (25 mM 2-ketoglutarate, wild-type strain enzyme) Conditions are identical to those defined in Fig. 7, except that lysine was replaced by 2-aminoadipic acid at the following concentrations o———o, 0 mM, □———□, 0 25 mM, △———△, 1 25 mM.

adipic concentrations were raised from 0 to 1 25 mM (Figs. 8 and 9).

Dipicolinic acid acted as a non-competitive inhibitor of 2-ketoglutarate fixation and as a competitive inhibitor of Ac-CoA fixation, it affected the cooperativity of both binding sites (Figs 10, 11)

Pipecolic acid was found to exert a non-competitive inhibition on the fixation of both substrates, but to affect the cooperativity of Ac-CoA binding sites only the $\bar{n}$ value was increased from 1 4 to 2 3 when dipicolinic acid concentrations were raised from 0 to 1 mM (Figs. 12 , 13).

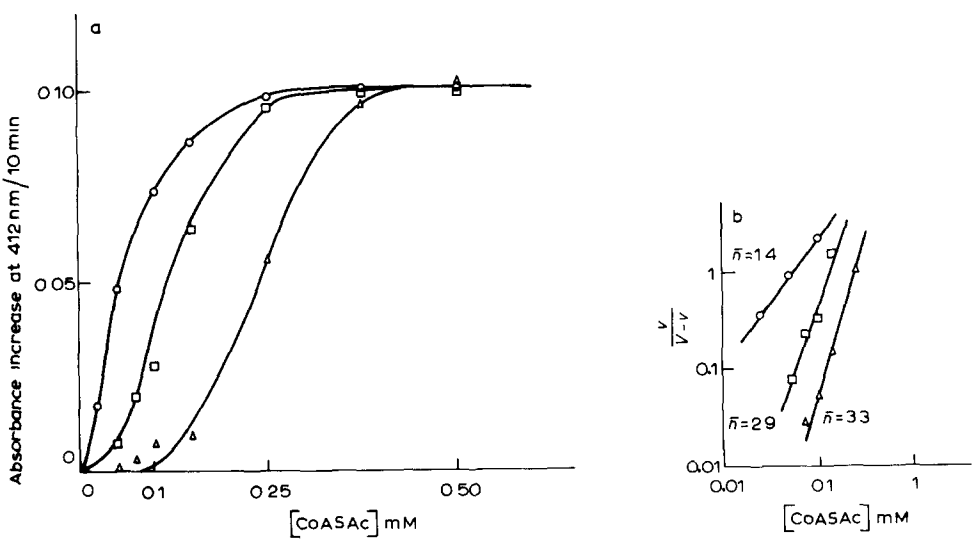


Fig 10 (a) Effect of dipicolinic acid on Ac-CoA binding to the wild-type enzyme The conditions are the same as defined for Fig 7 (25 mM 2-ketoglutarate), except that the following dipicolinate concentrations were used o———o, 0 mM, □———□, 0 1 mM, △———△, 0 5 mM (b) Influence of dipicolinic acid on the cooperativity of Ac-CoA binding as evidenced by the Hill plot

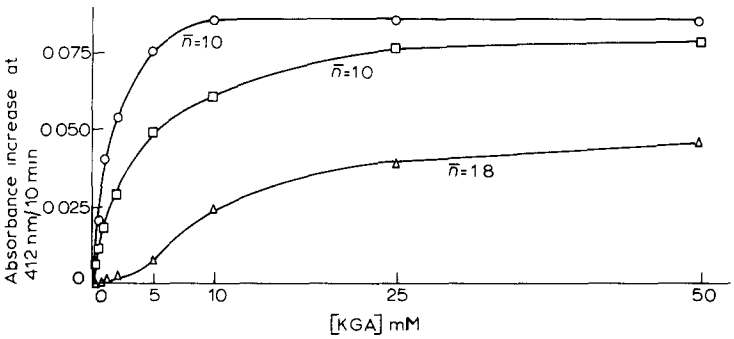


Fig 11 Effect of dipicolinic acid on 2-ketoglutarate binding to the wild-type enzyme Experimental conditions were as described in Fig 6 (1 mM Ac-CoA), except that lysine was replaced by following dipicolinate concentrations o———o, 0 mM, □———□, 0 25 mM, △———△, 1 25 mM

Modification of the inhibition patterns

Although the inhibition by non lysine-like compounds did vary considerably at saturating substrate concentrations from one extract to the other, the effects on substrate binding could always be detected, even with 2-aminoadipic acid, when freshly harvested wild-type cells were used for enzyme preparation On the other hand, storage of the cells for one night at −20° C resulted in a complete loss of these last effects, but did not affect substrate binding nor susceptibility towards lysine

A similar situation was found with the enzyme extracted from lys12 strain, and also if freshly harvested cells were used The saturation curves of each substrate were found to be similar in lys12 and W29 strains As shown on Fig. 14, lysine is a competitive inhibitor of the homocitrate synthetase of lys12, inhibition being complete in the range of millimolar concentrations under

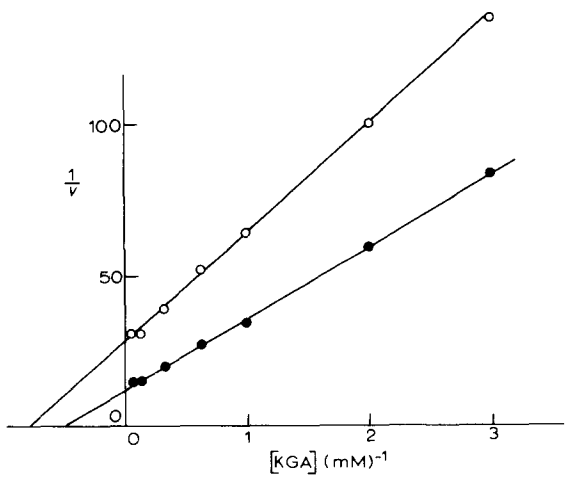


Fig 12 Effect of pipecolic acid on 2-ketoglutarate binding to the wild-type enzyme (reciprocal plot) Experimental conditions were as defined for Fig 6 (1 mM Ac-CoA), except that lysine was replaced by following pipecolate concentrations •———•, 0 mM, o———o, 1 mM

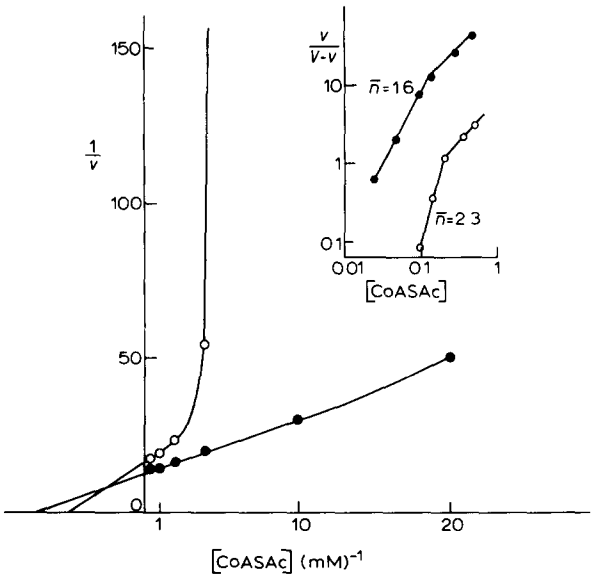


Fig 13 Effect of pipecolic acid on Ac-CoA binding to the wild-type enzyme (reciprocal plot) Experimental conditions were as described in Fig 7 (25 mM 2-ketoglutarate), except that lysine was replaced by following pipecolate concentrations ●——●, 0 mM, ○——○, 1 mM Insert influence of pipecolic acid on the cooperativity of Ac-CoA binding sites, as evidence by the Hill plot Symbols are the same in both representations

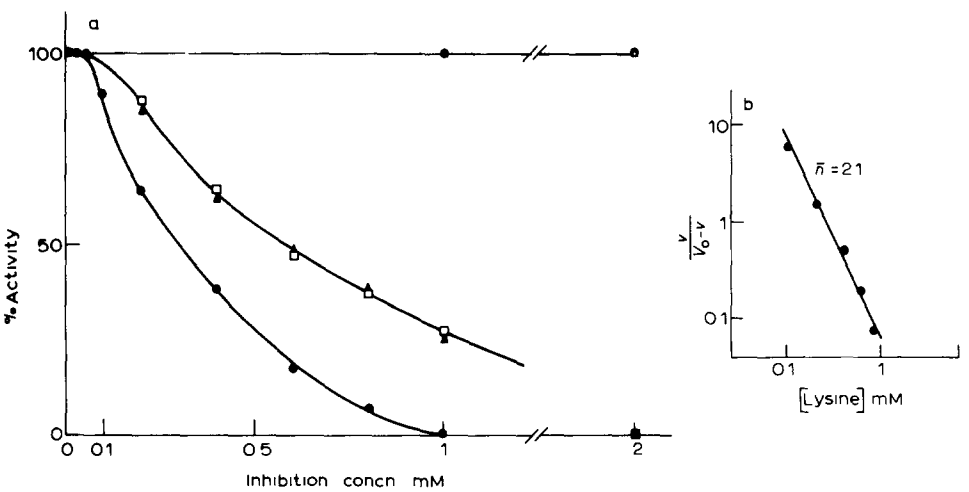


Fig. 14 (a) Inhibition of lys12 homocitrate synthetase The assay conditions were as described for "standard conditions" in Materials and Methods (50 mM 2-ketoglutarate, 2 mM Ac-CoA), except that the following compounds were added ●——●, lysine, ○——○, 2-aminoadipic acid, △——△, dipicolinic acid, □——□, equimolar mixture of lysine and 2-aminoadipic acid, ▲——▲, equimolar mixture of lysine and dipicolinic acid The values are plotted in terms of total inhibitors concentrations, (incubation time 7 min) (b) Cooperativity of the lysine binding sites as evidenced by the empirical plot of Taketa and Pogell [15]

standard conditions. But surprisingly, no effect could ever be detected with 2-aminoadipic acid, dipicolinic acid and pipecolic acid, (see Fig 14) Nor did these inhibitors have any effect at all on the saturation curves of each substrate (not shown)

At present there is no explanation for this phenomenon lys12 is unlinked to known mutations affecting homocitrate synthetase (to be published), the phenomenon is not strain specific, since independently induced mutations allelic to lys12 show the same phenotype. Whatever explanation this lack of inhibition may have in vivo desensitization of the activity due to the accumulation of an inhibitor [16], greater instability of the enzyme extracted from the mutant cells, (in)direct interaction between homocitrate synthetase and the aminoadipate reductase system , it appears that it is possible to affect the binding site(s) of the non lysine-like compounds without affecting the catalytic center nor the lysine site

The binding of both substrates in the absence of effectors was not affected by the presence of the mg-5 mutation (not shown) The activity was found to be sensitive to 2-aminoadipic acid, pipecolic acid and dipicolinic acid These inhibitors showed cooperative binding (see Fig 15) and heterotropic effects on the binding of the substrates like those previously reported for the wild type extract (not shown) The activity was also sensitive to lysine, but with two new

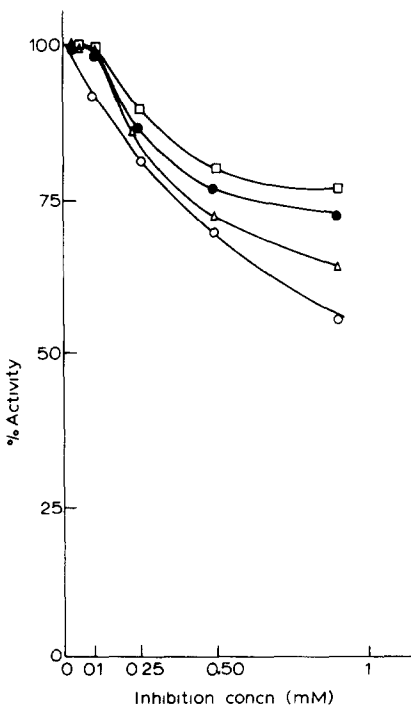


Fig 15 Inhibition of mg-5 homocitrate synthetase The assay conditions were as described under Materials and Methods for "standard conditions" (50 mM 2-ketoglutarate, 2 mM Ac-CoA), except that the following compounds were added ○———○, lysine, ●———●, 2-aminoadipic acid, △———△, dipicolinic acid, □———□, pipecolic acid

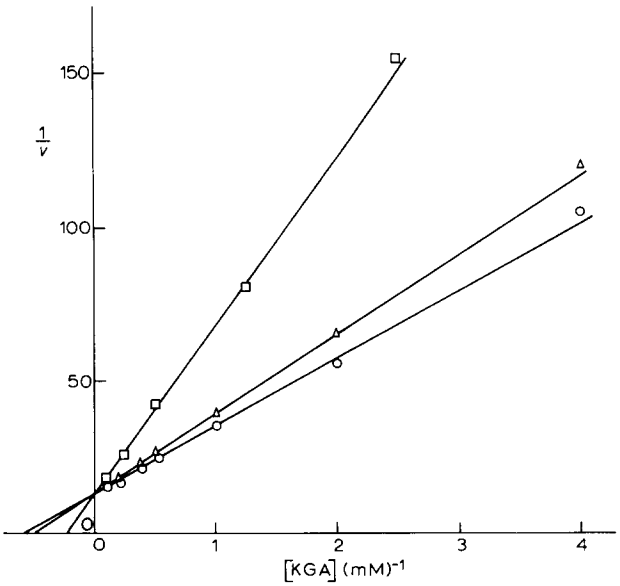


Fig 16 Inhibition of mg-5 homocitrate synthetase by lysine effect of lysine on the 2-ketoglutarate saturation curves (Ac-CoA concentrations 1 mM) Lysine concentrations were o———o, 0 mM, △———△, 0 25 mM, □———□, 1 mM

characteristics (1) at millimolar concentrations, about 50% residual activity was retained, (2) homotropic effects on lysine binding sites were lost (Fig. 15) so as were heterotropic effects on 2-ketoglutarate binding sites (Fig. 16) When mg-5 extracts are used, lysine appears to be competitive michealian inhibitor of 2-ketoglutarate binding.

Genetically the mg-5 mutation was shown to be situated in the homocitrate synthetase gene [10]

TABLE II

SPECIFIC ACTIVITIES OF HOMOCITRATE SYNTHETASE IN WILD TYPE AND LYSINE-LESS STRAINS

The strains were cultivated on mineral medium supplemented with $5\ 10^{-4}$ M lysine and harvested at the end of the exponential phase ($8\ 10^{7}$ cells/ml) They were then extracted as described under Materials and Methods and dialysed twice against 0 02 M Tris HCl buffer pH 7 4 (each time for 2 h) It was verified that this procedure was sufficient in order to decrease the concentrations of the accumulated products (2-aminoadipate in the case of lys12) to such extent that the concentration of these products in the test assay could be considered as negligible (below 0 01 mM) Enzyme activities were determined as described in the text, and expressed in absorbance increase/min/mg of protein

Strain tested	Position of the block	Homocitrate synthetase activity
W29	no block	0 030
lys12	aminoadipate reductase	0 102
lys9	after aminoadipate	0 032
lys16	homocitrate synthetase	0 001
lys2	before aminoadipate	0 029

Variations of the homocitrate synthetase specific activity

No change of the specific activity of the wild type strain could ever be detected (1) during the growth cycle, (2) on mineral medium supplemented with lysine, aminoadipic acid, dipicolinic acid, pipecolic acid or yeast extract (all 1 g/l), (3) on different carbon sources (glycerol, glucose, lysine, all 20 g/l), (4) on different nitrogen sources (ammonium sulfate, proline, arginine or lysine)

Three types of lysine-less mutants have been obtained in respect to the level of homocitrate synthetase (see Table II). (1) those which are not affected at all in their activity, (2) those which have lost their activity these are all situated in a single locus, show intraallelic complementation [10] and are thus considered to be affected in the structural gene of homocitrate synthetase, (3) those which are affected in the aminoadipate reductase system, are allelic of lys12 and are not linked to previous mutants all these mutants show a higher homocitrate synthetase activity (average increase by a factor of three)

So, if repression of the homocitrate synthetase activity could up to now not be evidenced in this system, an apparent derepression by a factor of three was found in lys12 and allelic strains, but not in other mutants blocked either earlier or later in the pathway

Discussion

The first enzyme of the lysine pathway in the yeast *Saccharomycopsis lipolytica* exhibits non-michaelian characteristics, which are partly consistent with the K system defined by Monod et al. [17] in that (a) total inhibition is achieved at high lysine concentrations, (b) the inhibition is overcome by increasing concentrations of the substrate 2-ketoglutarate, (c) the inhibition causes 2-ketoglutarate saturation kinetics to become cooperative, and increasing inhibitor concentrations increase the cooperativity If the enzyme already exists under two conformations (at least) in the absence of substrates, one can assume that the equilibrium between these two forms is not affected by 2-ketoglutaric acid (i e. it binds equally to the "active" and to the "inactive form), but that the binding of Ac-CoA which then occurs favours the "active" form, thus displacing the equilibrium The presence of lysine would modify this situation in such a way that 2-ketoglutarate would preferentially bind the non-lysine receptive form (thus revealing the cooperativity of 2-ketoglutarate binding sites), whereas Ac-CoA binding, which occurs later, would not be modified From the $\bar{n}$ and $\bar{n}'$ values reported, a minimum of 3—4 sites is required on the protein for the substrates and for lysine.

From the results obtained with mg-5 extracts, it can be assumed that lysine and substrate binding sites are different, since lysine binding can be affected without affecting substrate binding. The michaelian inhibition patterns found in this case for lysine suggest that the modification introduced by the mg-5 mutation leads to an equivalent affinity of lysine for all conformations of the protein, without affecting its oligomeric structure (Ac-CoA shows cooperative binding) The results obtained with the non lysine-like inhibitors are more difficult to explain in the hypothesis of a pure K system, since pipecolic and dipicolinic acids acted as non-competitive inhibitors of the activity A kinetic

interpretation of their mode of action will probably be possible only when the enzyme will have been purified (in order to exclude for example protein-mediated interactions in a complex) and when more suitable conditions for studying these inhibitions will have been set up. Preliminary studies on the inhibition patterns obtained by mixing lysine and any of these compounds have shown complex interactions, resulting in an increase or in a reversal of these inhibitions (to be published)

The regulation of the homocitrate synthetase levels in *Saccharomycopsis lipolytica* was not the main aim of this communication, but it should be noticed that the situation appears to be different from that found in other systems [3,5,6] no repression by lysine alone could be detected at all, even in lys12 strain (where the specific activity appears to be derepressed by a factor 3) Studies with double mutants, carrying lys12 and an early block in the pathway, such as diploid studies, will probably throw some light on these phenomena

There is at present no evidence for the existence of an isoenzymic system in our yeast, at this step, and two observations argue against such an hypothesis (a) the possibility of obtaining monogenic lysine-less mutants which have almost entirely lost the activity, (b) the possibility of obtaining complete inhibition of the activity, at least in vitro, at millimolar lysine concentrations (which would not have been expected if one of the isoenzymes had functions other than ensuring lysine synthesis) On the other hand, and in conflict with the situation reported in *Saccharomyces cerevisiae* [5], the regulation of the intracellular lysine concentrations seems to be mainly achieved at the level of homocitrate synthetase, since mutants desensitized at this step, like mg-5, are lysine accumulating [10].

Finally, a puzzling problem remains in the biosynthesis of lysine by yeasts, i e the existence of a branch in the pathway [5] Such a branch has already been described in some fungal systems, leading to the production of antibiotics [18,19] or of secondary metabolites like dipicolinic acid [20], or to pipecolic acid [21]. Our results show that the first enzyme of the pathway is, in one yeast at least, sensitive to other feedback inhibitors than lysine, and are an indication of the possibility of the existence of such a branch in yeasts as well

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