ALLOSTERIC INHIBITION OF NADP-LINKED MALIC ENZYME FROM AN EXTREME HALOPHILE BY ACETYL-CoA

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1. Introduction

The enzymes from extremely halophilic bacteria require high concentrations of salts for both activity and stability [1, 2]. Very little is known about their regulation; only two halophilic enzymes, aspartate transcarbamylase [3] and threonine deaminase [4], both from *Halobacterium cutirubrum*, have been reported to be affected by allosteric effectors.

Malic enzyme (L-malate: NADP oxidoreductase (decarboxylating), EC 1.1.1.40) from *H. cutirubrum* requires high concentrations of NH_4^+ or K^+ ions for activity [5]. Since malic enzyme is affected by several allosteric inhibitors in non-halophilic organisms such as *Escherichia coli* [6, 7], it was considered of interest to determine whether the halophilic enzyme was also inhibited by the same metabolites. The results presented in this communication show that acetyl-CoA acts as a powerful inhibitor of the halophilic malic enzyme in the presence of 1 M NH_4Cl or 3 M KCl. This inhibition is remarkably affected by pH. The enzyme shows modulator-dependent cooperativity [8]; the hyperbolic saturation curve for L-malate is converted into a sigmoidal curve by the inhibitor.

2. Materials and methods

L-malate and deoxyribonuclease I were purchased from Sigma Chemical Co., St. Louis, Mo.; NADP and CoA from Boehringer, Mannheim; Sephadex G-200 from Pharmacia, Uppsala; NH_4Cl, KCl and NaCl were analytical reagents obtained from Merck, Darmstadt. Acetyl-CoA was synthesized from CoA and acetic anhydride by the method of Stadtman [9] and assayed with 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB) [10]

H. cutirubrum was grown, harvested and washed as previously described [5]. The cells were suspended in 0.05 M Tris-HCl buffer, pH 7.6, containing 5 M NaCl and 1 mM EDTA (0.45 ml per g, wet wt) and disrupted at 5° by four treatments (15 sec each) in an MSE sonic disintegrator (Measuring and Scientific Equipment, Ltd., London, England) at maximum power. To the homogenate were added $MgCl_2$ (2 mM final conc.) and deoxyribonuclease I (50 μg per ml of suspension), incubated at 30° for 30 min, and centrifuged at 25,000 *g* for 45 min at 4°. 4 ml of the supernatant fluid were percolated through a column of Sephadex G-200 (30 × 2 cm) equilibrated with the Tris-HCl-NaCl-EDTA buffer solution. Malic enzyme was eluted immediately after the void volume. The fractions with high specific activities were pooled and fractionated with acetone at −10°. The active fraction (20–33% acetone, v/v) was dissolved in 2 ml of the Tris-HCl-NaCl-EDTA buffer solution, and dialysed overnight against the same buffer, yielding a yellowish preparation, purified about 10-fold with respect to the crude extract, and free of any NADPH oxidizing activity, either in the absence or in the presence of acetyl-CoA. The purified preparations were also unable to destroy acetyl-CoA, as demonstrated by direct assay of the thioester in the reaction mixtures with DTNB. Malic enzyme was assayed spectrophotometrically as described in the legends to figures. Protein was determined by the method of Lowry et al. [11].

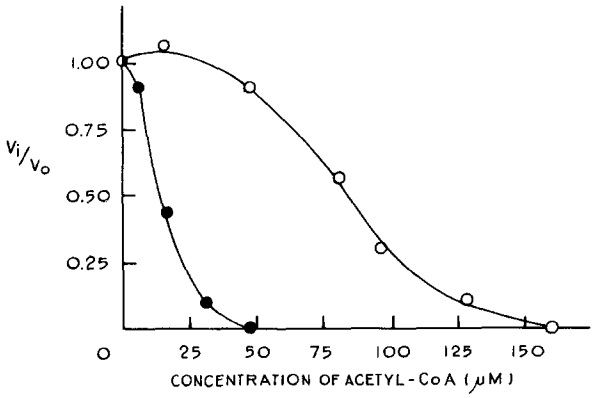

Fig. 1. Inhibition of the halophilic malic enzyme by acetyl-CoA. The reaction mixtures contained (in micromoles) in a final volume of 1 ml: Tris-HCl buffer, pH 7.6, 40; $MnCl_2$, 1; NADP, 0.1; L-malate, 1.5; NH_4Cl, 1000 (o—o—o) or KCl, 3000 (•—•—•); acetyl-CoA as stated on the abcissa. 130 μg of enzyme were used per assay. Reaction velocities are expressed as nanomoles of NADPH formed per min under the conditions of assay. V_o (reaction velocity in the absence of acetyl-CoA) was 15 in the presence of NH_4Cl, and 10 in the presence of KCl. V_i is the reaction velocity in the presence of the inhibitor. The reaction was started by the addition of the enzyme after equilibration of the otherwise complete reaction mixture in the chamber of a Beckman DB-G spectrophotometer at 30° for 1 min, and followed as the increase of absorbance at 340 nm.

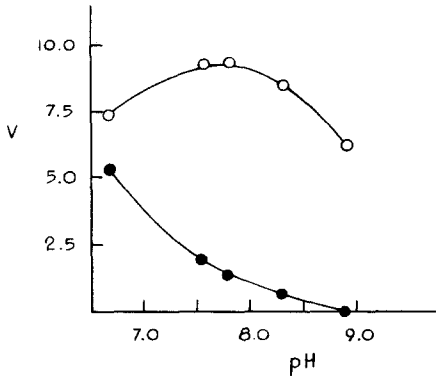

Fig. 2. Effect of pH on the reaction velocity and the inhibition by acetyl-CoA. Experimental conditions as stated in the legend to fig. 1, in the presence of NH_4Cl, except for the amount of enzyme (65 μg), and the pH of the Tris-HCl buffer (162 μmoles), which varied as stated on the abcissa. (o—o—o), no acetyl-CoA; (•—•—•), 96 μM acetyl-CoA.

3. Results and discussion

As shown in fig. 1, acetyl-CoA was a powerful inhibitor of the malic enzyme from *H. cutirubrum*. In the presence of 1.5 mM L-malate (concentration near the apparent K_m for this substrate) 50% inhibition was attained at about 80 μM acetyl-CoA in the presence of 1 M NH_4Cl as activating salt, whereas in the presence of 3 M KCl the acetyl-CoA concentration required for the same effect was even lower, 15 μM. The curves were markedly sigmoidal; Hill plots of the same results yielded apparent n values of about 3 (KCl) or 4 (NH_4Cl).

Fig. 2 shows that the inhibition by 96 μM acetyl-CoA in the presence of 1.5 mM L-malate was markedly dependent on the pH of the reaction mixture, being only 30% at pH 6.7, and 100% at pH 8.9. Since the difference in catalytic activity at the same pH values was small, these results suggest that acetyl-CoA is acting at a site different from the active site.

Fig. 3 shows that the plot of reaction velocity against concentration of L-malate was hyperbolic, but it was converted into a sigmoidal curve by 96 μM acetyl-CoA. The inhibition was competitive, the apparent K_m for L-malate being changed from 2 mM to 5.2 mM. The apparent n value changed from 1.04 to 2.4 in the presence of the inhibitor. The results of fig. 3 were obtained with 1 M NH_4Cl as activating salt. Similar results were obtained in the presence of 3 M KCl, although the inhibitory effect of acetyl-CoA was much stronger (see fig. 1).

When the effect of acetyl-CoA on the saturation curve for NADP at 10 mM L-malate was studied (fig. 4) hyperbolic curves were obtained either in the presence or in the absence of the inhibitor. The inhibition was of the mixed type, since the apparent K_m for NADP increased from 39 to 55 μM, and the V_{max} was decreased by 30%, in the presence of 160 μM acetyl-CoA. These results agree very well with those of Sanwal and his co-workers for the *E. coli* enzyme [6, 7] and, although they do not rule out the possibility of binding of acetyl-CoA to the same enzyme form which binds NADP, they suggest that the physiological "target" substrate [8] for the inhibition of the halophilic enzyme by acetyl-CoA is also L-malate.

The concentrations of acetyl-CoA required for half-maximal inhibition were considerably lower than those

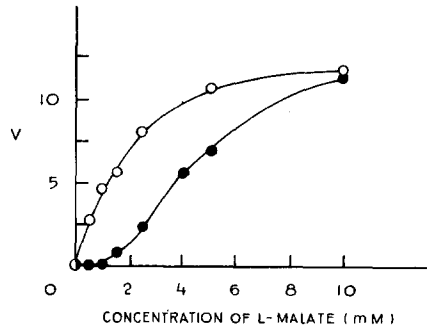

Fig.3. Effect of acetyl-CoA on the saturation curve for L-malate. Experimental conditions as stated in the legend to fig. 1, in the presence of NH_4Cl, except for the amount of enzyme (60 μg). L-malate, as stated on the abcissa. (o—o—o), no acetyl-CoA; (•—•—•), 96 μM acetyl-CoA.

reported for the enzymes from *E. coli* [6, 7] and a marine *Pseudomonas* [12]. Since preliminary experiments showed that the halophilic enzyme was only little affected by oxalacetate, NADH and glyoxylate, acetyl-CoA is probably the major regulatory metabolite for the *H. cutirubrum* enzyme. The significance of the inhibition is probably similar to that suggested for the *E. coli* enzyme [6]; under the conditions for growth of the halophile, with amino acids as main carbon source, the function of the malic enzyme would be essentially degradative.

The pH dependence of the inhibition by acetyl-CoA

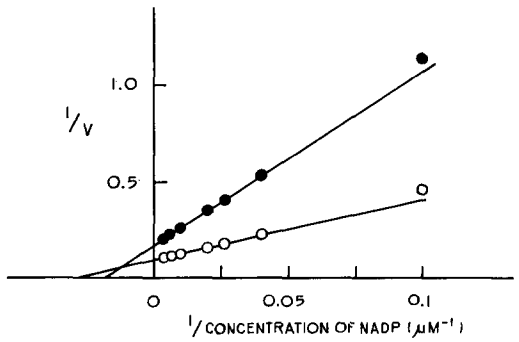

Fig. 4. Effect of acetyl-CoA on the saturation curve for NADP. Experimental conditions as stated in the legend to fig. 1, in the presence of NH_4Cl, except for the amount of enzyme (70 μg), L-malate, 10 μmoles, and NADP, as stated on the abcissa. (o—o—o), no acetyl-CoA; (•—•—•), 160 μM acetyl-CoA.

of the malic enzymes from *E. coli* and *H. cutirubrum* was exactly the opposite. The *E. coli* enzyme [6] was inhibited by 50% by 0.25 mM acetyl-CoA at pH 7.0, and was insensitive to the same concentration of the inhibitor at pH 9.0. Kushner and his co-workers [3] have also reported an opposite behaviour of the halophilic aspartate transcarbamylase, when compared with the same enzyme from yeast, with respect to the effect of pH on the inhibition by CTP.

These differences might be related to the halophilic nature of the enzymes, which it has been proposed depends on an increased acidity of the protein [1, 2]. It is noteworthy that the sigmoidicity of the saturation curve of the halophilic threonine deaminase disappeared at the lower pH values tested [4]. The study of more examples is necessary in order to assess if this pH dependence is a peculiarity of allosteric halophilic enzymes.

Acknowledgements

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