Fellowship 1-F1-GM-29, 518-01 from the General Medical Sciences Division. B.M.S. is a Public Health Service Predoctoral Fellow.

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Received April 13th, 1967

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Biochim. Biophys. Acta, 139 (1967) 524–526

BBA 61140

Allosteric fine control of citrate synthase in *Escherichia coli*

Citrate synthase (citrate oxaloacetate-lyase (CoA acetylating), EC 4.1.3.7) occupies a key position in the tricarboxylic acid cycle in that it effects the entry of carbon, in the form of acetyl-CoA, into the cycle. The combustion of this carbon in later steps of the cycle provides the energy which is subsequently trapped as ATP. The metabolic regulation of citrate sythase activity would thus also regulate energy production.

Previous studies[1] suggested that citrate synthase activity may be regulated by the level of NADH. Furthermore, the observation that the enzyme may be desensitized towards the inhibitor without loss of catalytic activity indicated that NADH acts as an allosteric effector[2]. Since NADH may be considered both a "product" of the tricarboxylic acid cycle and an intermediate in the formation of ATP, its effect on citrate synthase activity may constitute an essential feedback control mechanism in *Escherichia coli*, especially since (unlike the similar enzyme from mammals[3,4], plants[5] and yeast[6]) the *E. coli* enzyme is essentially insensitive to inhibition by ATP (ref. 1). In order to study further the nature and mechanism of the metabolic control of citrate synthase, the enzyme has been isolated from *E. coli* strain K 12 in a highly purified state. This has permitted a study of the action of possible allosteric effectors both in stimulating and inhibiting enzymic activity.

The organisms were grown up in 15 l of medium[7] in a carboy at 30° with 50 mM acetate as sole carbon source. After washing the cells with water and disrupting them by ultrasonication, the purification procedure[8] consisted of the following steps: (1) treatment of the sonic extract with protamine sulphate (1 mg per

Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

10 mg protein); (2) fractionation with $(NH_4)_2SO_4$ the enzyme precipitating at 55–70% saturation; (3) chromatography on DEAE-cellulose at pH 8, the enzyme being eluted by 0.2 M KCl; (4) gel filtration on Sephadex G-200. The enzyme thus prepared catalyzed the formation of 115 μmoles of CoASH/min per mg protein; it was shown to be approximately 90% homogeneous by disc electrophoresis on acrylamide gels[9]. Throughout the purification, enzyme activity was assayed by the continuous polarographic measurement of CoASH production[10]. However, it was found that the purified enzyme, unlike cruder preparations used earlier[2], was not inhibited by the chromogenic thiol reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), so that assays could conveniently be performed spectrophotometrically at 412 mμ with this reagent[11]. Accordingly, this assay procedure was employed in all the kinetic studies here reported. In view of the effect of salt in overcoming the NADH inhibition[2] the buffer solution used in the assay contained 20 mM Tris (free base) adjusted to pH 8.0 with sodium–EDTA. Assays were performed in the presence of 0.2 mM oxaloacetate, 0.16 mM CoASAc and 0.1 mM DTNB, in a total volume of 1.0 ml.

The specific and powerful inhibitory action of NADH on enzyme activity was confirmed with the purified enzyme. Thus under the above assay conditions 85% inhibition was produced by 0.2 mM NADH. No inhibition was produced by any of the following substances when tested at a concentration of 1 mM: NAD^+, $NADP^+$, NADPH, AMP, ADP, ATP. The selective desensitization of the enzyme to NADH previously reported[2] was confirmed with the purified enzyme; thus in the presence of 0.2 M KCl or at pH 9.2 no inhibition was produced by 0.2 mM NADH. However, the behaviour of the purified enzyme with NADH displays some important differences from these earlier results. In particular, the inhibition by NADH appears to be independent of the concentration of either substrate and is not reversed by higher CoASAc concentrations. Moreover, the extent of inhibition exhibits a sigmoid dependence on the NADH concentration (Fig. 1, Curve A), indicating cooperativity in the action of this inhibitor.

Various metabolites were examined for their ability to reactivate the NADH-inhibited enzyme. No effect was observed with NAD^+ and, as expected, ATP was similarly without effect. However, both AMP and ADP produced considerable reac-

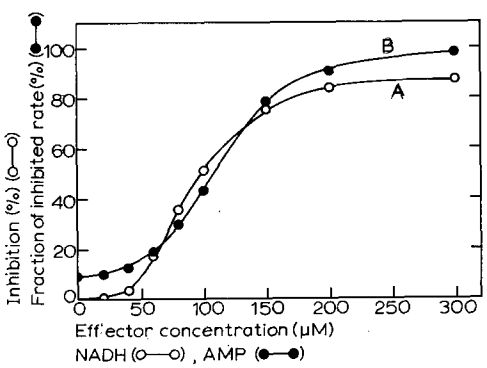

Fig. 1. Dependence of citrate synthase inhibition and reactivation on the effector concentration. Assays were performed as described in the text. Curve A shows the inhibition produced by NADH. Curve B shows the reactivation produced by AMP in the presence of 1 mM NADH.

tivation when present at 2 mM concentration. Further studies with lower concentrations of these substances showed that AMP is a powerful reactivator. Thus the 90% inhibition produced by 1 mM NADH was virtually completely overcome by 0.3 mM AMP (Fig. 1, Curve B). ADP appears to be a very much weaker reactivator with only 5–10% of the potency of AMP. Fig. 1 also shows that the reactivation of NADH-inhibited enzyme by AMP exhibits a sigmoid dependence on the AMP concentration, again implying cooperativity in the action of this effector.

These observations are in accord with the predictions of the model which MONOD, WYMAN AND CHANGEUX[12] have proposed for allosteric regulatory enzymes, in which the equilibrium between two states of the enzyme (active and inactive) will be displaced by the preferential binding of a particular effector to one of the states. Cooperativity is then to be expected in the action of such effectors. The sigmoid curves of Fig. 1 show that both NADH and AMP behave in this way. Further evidence that the behaviour of citrate synthase is in keeping with such a model has also been obtained by electron microscopy of the purified enzyme[13]. In these studies it has been shown that NADH produces a distinct change in the shape of the enzyme, and that in the presence of KCl, which is known to overcome the NADH inhibition, this shape change is reversed.

It is tempting to speculate on the advantages conferred on *E. coli* by the regulation of citrate synthase through both NADH and AMP. In view of the fact that AMP, which is an indicator of energy depletion in the cell, serves as a positive effector it might be expected that the negative effector, or signal of an energy-rich state, would be ATP itself. However, the tricarboxylic acid cycle serves a dual role in the production of both energy and biosynthetic intermediates; since biosynthesis requires the availability of ATP, it might be disadvantageous were just such a condition to inactivate citrate synthase. Regulation through NADH, on the other hand, may permit the build-up of ATP required for biosynthesis without inhibiting citrate synthase.

I thank Professor H. L. KORNBERG, F.R.S., for his continued advice and interest, and Mrs. PATRICIA DUNMORE for technical assistance. This work was aided by the Science Research Council through Grant No. B/SR/652.

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Received April 26th, 1967