peak of a probably pentamer component and a small peak of a trimer at the same pH (Fig. 1c). After changing the pH to 8.5 but still with tris-HCl buffer (Fig. 1d) the oxidized growth hormone shows a different proportion between the two polymers, with a shift to a lower degree of polymerization.

At the present time it is not possible to elucidate the reason for the higher polymerization after oxidation or the shift to a lower degree of polymerization on increasing the pH.

I am grateful to Dr O. Trygstad, Oslo, for the radio-immunological assay, Mr Knud D. Schmidt, Pharmacist, for the amino acid sequence analysis, and Miss Lone Henriksen, Mrs Elise Marnø Jørgensen and Mrs Jeanette Laier Henriksen for their diligent assistance throughout this work.

- Andrews, P. (1964). *Biochem. J.* **91**, 222.
 Andrews, P. (1965). *Biochem. J.* **96**, 595.
 Andrews, P. (1966). *Nature, Lond.*, **209**, 155.
 Andrews, P. & Folley, S. J. (1963). *Biochem. J.* **87**, 3r.
 Craig, L. C. (1956). In *Ciba Found. Colloq.: Endocrinology* vol. 9, p. 107. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. Boston, Mass.: Little, Brown and Co.
 Fønss-Bech, P. & Li, C. H. (1954). *J. biol. Chem.* **207**, 175.
 Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1958). *Meth. biochem. Anal.* **2**, 359.
 Li, C. H., Liu, W.-K. & Dixon, J. S. (1966). *J. Amer. chem. Soc.* **88**, 2050.
 Liu, W.-K., Dixon, J. S. & Li, C. H. (1964). *Biochim. biophys. Acta*, **93**, 428.
 Ornstein, L. (1964). *Ann. N.Y. Acad. Sci.* **121**, 321.
 Wilhelmi, A. E. (1961). *Canad. J. Biochem. Physiol.* **39**, 1659.

Biochem. J. (1966) **101**, 44c

Reduced Nicotinamide-Adenine Dinucleotide as an Allosteric Effector of Citrate-Synthase Activity in *Escherichia coli*

By P. D. J. WEITZMAN

Department of Biochemistry, University of Leicester

(Received 23 September 1966)

It has been shown (Weitzman, 1966a) that the citrate synthase (EC 4.1.3.7) of *Escherichia coli* differs from the enzyme purified from mammals and yeast in being insensitive to inhibition by ATP: instead, the enzyme is powerfully inhibited by NADH, and this is reversible with the concentration of acetyl-SCoA. It is the purpose of this communication to present evidence which indicates that, under certain circumstances, the inhibitory action of NADH may be abolished without loss of the catalytic activity of the enzyme, and that such circumstances may also produce conformational changes in the enzyme protein.

Except where otherwise stated, assays were performed by the polarographic measurement of CoASH production (Weitzman, 1966b) in buffer, of composition 20 mM-tris-10 mM-MgCl₂-1 mM-EDTA, at pH 8.0 in the presence of 0.2 mM-oxaloacetate and 0.16 mM-acetyl-SCoA. Citrate synthase was purified from *E. coli* strain 114-IL⁺ (Kornberg, 1966) to a specific activity of 30 μ moles of CoASH produced/min./mg. of protein.

As shown in Fig. 1 (curve C) the inclusion of 0.2 mM-NADH in the above assay system virtually abolished enzyme activity at pH values of less than 7.5, but had progressively less effect as the pH was raised; at pH 8.5 only 52% inhibition was observed. At pH 9.2 the enzyme was no longer inhibited by

NADH, although (Fig. 1, curve A) the catalytic activity at this pH, in the absence of NADH, was still 50% of that observed at the optimum value of pH 8.0. Titration of NADH confirmed that it has no dissociating group in the pH region 8-9. Thus it is most probable that the enzyme itself is modified by the change of pH and that the regulatory and catalytic sites on the enzyme are not identical.

Since a change in the ionic environment appears to modify the regulatory site, the effect of increased salt concentration was examined at pH values at which the enzyme normally showed strong inhibition by NADH. It was found that, at pH 8.0, the presence of 0.2 M-KCl in the assay medium left the catalytic activity essentially unchanged but completely abolished the sensitivity to NADH (Fig. 1, curve D). Moreover, although the activity of the enzyme at this pH was virtually the same in the absence or presence of 0.2 M-KCl, the presence of this salt markedly affected the activity of the enzyme at other pH values, and particularly in that region (pH 8.0-8.5) at which, in the absence of KCl, NADH ceases to exert its inhibitory effect (Fig. 1, curves A and B). Similar effects were observed with other salts (NaCl, KNO₃, Na₂SO₄) but, at 0.2 M, these were less effective than KCl in overcoming the inhibition; likewise, the inhibitory action of NADH

Fig. 1. pH-dependence of citrate-synthase activity and inhibition by NADH. Assays were carried out polarographically in the presence of 0.2mM-oxaloacetate and 0.16mM-acetyl-SCoA. Curve A: enzyme activity measured in buffers of composition 20mM-tris-10mM-MgCl₂-1mM-EDTA (20mM-Na₂B₄O₇ was substituted for tris at pH 9.2). Curve B: activities measured as for curve A but with the addition of 0.2M-KCl. Curve C: extent of inhibition produced by 0.2mM-NADH in the above buffers in the absence of KCl. Curve D: inhibition measured as for curve C but in the presence of 0.2M-KCl.

was much less when 0.2M-tris buffer at pH 8.0 was used instead of the customary 0.02M-tris.

These findings suggest that KCl acts by producing a conformational change in the enzyme whose effect may be either to modify the protein at the regulatory site and so to prevent the binding of NADH or, alternatively, to block the effect of bound NADH on the affinity of the enzyme for acetyl-SCoA. In support of this suggestion are the following observations of changes in the properties of the enzyme occasioned by the presence of KCl:

(1) The heat stabilities of the enzyme with and without KCl were different. Thus when the enzyme was heated at 55° in 20mM-tris-1mM-EDTA buffer, pH 8.0, it lost half of its activity in 5min., but retained over 90% of its activity if heated for this time in the presence of 0.2M-KCl.

(2) In the presence of 0.2M-KCl, the enzyme was resistant to thiol reagents that otherwise produced inactivation. The activity of the enzyme, when measured as the rate of chromophore formation from CoASH and 5,5'-dithiobis-(2-nitrobenzoic acid) (Srere, Brazil & Gonen, 1963), was considerably less than that determined polarographically, and preincubation of the enzyme for 5min. with 0.1mM-5,5'-dithiobis-(2-nitrobenzoic acid) in pH 8.0 buffer (20mM-tris-1mM-EDTA) virtually abolished its activity. However, if this treatment with 5,5'-dithiobis-(2-nitrobenzoic acid) was carried out

in the presence of 0.2M-KCl, no inactivation was observed. Further, the activity of the enzyme as determined with 5,5'-dithiobis-(2-nitrobenzoic acid) was identical with that determined polarographically if 0.2M-KCl was also present. Moreover, the addition of KCl (to 0.2M) to the enzyme after it had been inactivated by treatment with 5,5'-dithiobis-(2-nitrobenzoic acid) resulted in considerable reactivation (more than 50%).

Similar results were obtained when the enzyme was treated with two other thiol reagents, *N*-ethylmaleimide and HgCl₂. In these experiments, neither the polarographic nor 5,5'-dithiobis-(2-nitrobenzoic acid) assay procedures could be used because the thiol reagents added would react with CoASH; enzyme activity was therefore measured spectrophotometrically by the method of Dixon & Kornberg (1962). After preincubation of the enzyme for 5min. at pH 8.0 with either 0.1mM-*N*-ethylmaleimide or 1μM-HgCl₂ only 10% or less of the original activity was retained, but no loss of activity was observed when 0.2M-KCl was also present. Moreover, as with 5,5'-dithiobis-(2-nitrobenzoic acid), addition of KCl to enzyme that had been inactivated by *N*-ethylmaleimide or HgCl₂ produced more than 50% reactivation.

Although the nature of the change in the enzyme produced by KCl is still under investigation, the effects produced by thiol reagents suggest that SH groups are involved at the catalytic site, but, since KCl can reverse these effects, that this involvement may be indirect. This is also indicated by the pH profiles of the enzyme observed in the absence of KCl (Fig. 1, curves A and C). The decrease in activity noted at pH values above 8 coincides with that pH region at which SH groups dissociate, and the inhibition exerted by NADH is also half-maximal in this region. It is thus possible, that, in media of high ionic strength, SH groups on the enzyme, which are in some way necessary for enzyme activity at low ionic strength, no longer govern its catalytic properties; these SH groups may also be involved in the regulatory action of NADH.

I thank Professor H. L. Kornberg, F.R.S., for much helpful discussion and encouragement. I am also grateful to Mrs Patricia Dunmore for her excellent technical assistance. This work was aided by the Science Research Council through Grant B/SR/652.

Dixon, G. H. & Kornberg, H. L. (1962). In *Methods in Enzymology*, vol. 5, p. 633. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Kornberg, H. L. (1966). *Biochem. J.* **99**, 1.

Srere, P. A., Brazil, H. & Gonen, L. (1963). *Acta chem. scand.* **17**, S129.

Weitzman, P. D. J. (1966a). *Biochim. biophys. Acta*, **128**, 213.

Weitzman, P. D. J. (1966b). *Biochem. J.* **99**, 18p.