THE UNIVERSITY OF MANITOBA

A REACTIVE SULFHYDRYL GROUP IN *ESCHERICHIA COLI* CITRATE SYNTHASE

BY

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ABSTRACT

Citrate synthase from acetate grown E. coli (strain K12 3000) has been subjected to sulfhydryl modification using reagents DTNB, 4,4'PDS and IAA.

DTNB reacts with the enzyme to modify 1 sulfhydryl group per subunit quickly and one or more groups more slowly. The rate of reaction for the first group is saturable with respect to DTNB, and exhibits a pH dependence with a pKa of 8.3 $\pm$ 0.2. NADH, an allosteric inhibitor, and ACoA, a substrate, inhibit the reaction, while OAA, the other substrate, has little or no effect. KCl increases the rate of reaction of the initially modified group by increasing the V_{max} for the DTNB-enzyme reaction, and eliminates reaction of secondary sulfhydryls. The kinetic parameters for citrate synthase are altered upon modification by DTNB, such that the Hill coefficients for KCl and ACoA saturation are lower. As initially reported by Danson and Weitzman (Biochem. J., 135, 513 (1973)), DTNB modification causes the enzyme to lose its sensitivity to NADH and some of its activity. I have found that modification by DTNB wipes out the ability of the enzyme to bind NADH and that treatment with DTT regenerates those functions to a considerable degree.

Modification of citrate synthase by 4,4'PDS leads to rapid inactivation as 2.0 groups per subunit are modified. The reaction is inhibited by NADH, and modification of 1 group per subunit eliminates NADH binding by the enzyme. KCl enhances the reactivity of the first group while diminishing that of the second group. Modification with one reagent, followed by exposure to the other, indicates that DTNB and 4,4'PDS react with the same enzyme sulfhydryl group.

Modification of citrate synthase by IAA leads to incorporation of more than 2 carboxymethyl groups per subunit, with a concomitant loss of NADH binding capacity and 4,4'PDS and DTNB reactivity. The overall rate of incorporation was very similar to the rate of loss of NADH binding sites, indicating that the sulfhydryl associated with NADH binding is not selectively modified. The rate of loss of DTNB and 4,4'PDS reactivities were very similar; this observation supports the finding that they react with the same sulfhydryl group.

A model is presented to interpret all these data, in which it is suggested that the susceptible sulfhydryl group exists in or near the NADH binding site, so that modification prevents NADH binding because of steric hindrance. The partial inactivation seen with DTNB and complete inactivation with 4,4'PDS are then seen as a result of heterotropic interactions with the active site. Differing orientations within the site could explain the differing effects of these two reagents on activity.

Several adenylate analogues of NADH bind to the NADH site of citrate synthase, as indicated by their ability to inhibit NADH binding and to protect the enzyme from DTNB and 4,4'PDS modification. These analogues include ADP-ribose, 2'5' and 3'5' ADP, 5'AMP, 3'AMP and NADPH. At low ionic strength, all except 3'AMP activate the enzyme to different extents. The finding that adenylates bind to the allosteric site of E. coli citrate synthase suggests a closer similarity than has been suspected between this enzyme and the citrate synthases of strictly aerobic Gram-negative bacteria, whose NADH inhibition is readily reversed by 5'AMP (Weitzman and Jones; Nature; 219; 270 (1968)).

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACoA	Acetyl Co-enzyme A
ADP	Adenosine diphosphate
ADPR	Adenosine diphosphate ribose
AMP	Adenosine monophosphate
CoA	Co-enzyme A
DEAE-cellulose	Diethylaminoethylcellulose
DTNB	5,5'dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylene diamine tetraacetate
g, mg, μg	gram, milligram, microgram
IAA	Iodoacetic acid
IAM	Iodoacetamide
Kd	dissociation constant
M, mM, μM	molar, millimolar, micromolar
NADH	nicotinamide adenine dinucleotide, reduced
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
nm	nanometer
OAA	oxaloacetate
OD	optical density
4,4'PDS	4,4'dithiodipyridine
POPOP	1,4-bis 2-(5-phenyloxazolyl) -Benzene
PPO	2,5-Diphenyloxazole

SDS	sodium dodecylsulfate
TNB-CN	2-nitro-5-thiocyanatobenzoic acid
tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

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Citrate synthase [citrate oxaloacetate - lyase (CoA-acetylating) E. C. 4.1.3.7] catalyzes the reaction through which the acetyl carbons of acetyl coenzyme A enter the Krebs cycle:

$$\text{Acetyl-CoA} + \text{Oxaloacetate} + H_2O = \text{Citrate} + \text{CoA} + H^+$$

The Krebs cycle is a major energy producing pathway in eukaryotic cells, and the citrate produced by the reaction catalysed by citrate synthase is broken down stepwise to give back oxaloacetate, yielding 12 molecules of ATP through coupling with oxidative phosphorylation. In cells which contain glyoxysomes, the enzyme functions in the glyoxylate shunt in which two acetyl units are converted to succinate. In bacteria, especially anaerobes, the series of reactions beginning with the one catalyzed by citrate synthase leads to the synthesis of glutamic acid. Thus citrate synthase occupies a key position in the metabolism of most organisms and the control of its activity has been postulated to be important in various regulatory mechanisms.

Molecular Weight Studies

In recent years, a number of investigations have centered around the question of the molecular weight and state of aggregation of the native *E. coli* citrate synthase.

Weitzman and Dunmore (1969) have studied by means of gel filtration the molecular weights of twelve citrate synthases representing three different regulatory types i) NADH-insensitive enzymes from Gram-positive bacteria and eucaryotic organisms ii) NADH-inhibited enzymes

in which the inhibition is relieved by AMP and iii) NADH-inhibited/AMP-insensitive enzymes. These three categories were distinguished by Weitzman and Jones (1968). E. coli citrate synthase is an example of the last group. The citrate synthases in Weitzman and Dunmore's study fell into two groups - "large" molecules that eluted before bovine liver catalase (MW approximately 250,000) on a Sephadex G-200 column and were sensitive to regulation by NADH and "small" molecules that eluted after rabbit muscle lactate dehydrogenase (MW approx 140,000) and were insensitive to NADH.

Faloona and Srere (1969) have carried out molecular weight determinations on the E. coli enzyme using the gel filtration medium Bio-Gel A. They found that at pH 8.1 in the presence of 0.1 M KCl and 0.1 M mercaptoethanol, the molecular weight was approximately 280,000 grams/mole. Removal of potassium did not result in dissociation or aggregation of the enzyme.

Zonal sedimentation and sedimentation velocity experiments by Wright and Sanwal (1971) led to the conclusion that E. coli citrate synthase exists as an equilibrium mixture of monomers of 60,000 $\pm$ 3000 MW, tetramers of 245,000 $\pm$ 10,000 MW and octamers of 486,000 $\pm$ 20,000 MW. Only the tetramer was reported to have enzymic activity and the association-dissociation phenomenon was found to be pH dependent, with predominantly octamer existing at pH 7.0 and the enzyme irreversibly dissociating to form monomers at pH 11.0. A subunit molecular weight estimate of 60,000 to 65,000 grams/mole was obtained through SDS polyacrylamide gel electrophoresis, although it should be noted that it is my feeling that the citrate synthase sample was not adequately "bracketed" in mobility on

gels by known standards, so that there was considerable uncertainty in this estimate.

Srere (1972) has reported that sedimentation equilibrium experiments indicate that E. coli citrate synthase exists as a tetramer of MW 210,000 grams/mole.

Danson and Weitzman (1973) have used SDS polyacrylamide gel electrophoresis to determine a subunit molecular weight for E. coli citrate synthase of 55,000 ± 4,000. Ultracentrifugation studies at pH 8.0, using the same diffusion coefficients as Wright and Sanwal (1971), gave a MW of 57,000 and 230,000 for the two species present. These authors have confirmed the dissociation at pH 11.0 into monomers, as found by Wright and Sanwal, but disagree with the finding that at pH 7.0 in the presence of 2 mM DTT, the enzyme exists only as the octameric species. They have found that under these conditions the tetrameric form predominated, although the enzyme concentration used was much lower than that of Wright and Sanwal (1971). By use of analytical band centrifugation of the active enzyme-substrate complex, these authors confirm that the tetramer is the active species.

Tong and Duckworth (1975) have reported a subunit molecular weight for E. coli citrate synthase of 46,000 ± 2000 g/mole, using SDS polyacrylamide gel electrophoresis. Cross-linking with dimethyl suberimidate before electrophoresis led to six bands whose apparent molecular weights were almost integral multiples of 47,000 g/mole. Sedimentation equilibrium experiments in guanidine HCl gave a molecular weight average of 43,400 ± 300 g/mole. Various conditions seemed to

influence the aggregation state of the enzyme. At pH 7.0 and 7.8 monomers through greater than decamers were present; at pH 9.0 only dimers were seen and at pH 10.0, large aggregates (up to 20 subunits) were present. At pH 7.8, in the presence of 0.05 M KCl, dimer and hexamer are the only species present and increasing the KCl concentration to 0.10 M converts all of the enzyme to hexamer.

The native molecular weight of E. coli citrate synthase has been reported to be greater than 250,000 (Weitzman and Dunmore, 1969), 280,000 (Faloona and Srere, 1969), 210,000 (Srere, 1972) or 245,000 (Wright and Sanwal, 1971). The finding of Tong and Duckworth (1975) that the enzyme exists as a heterogenous mixture of various oligomeric forms at the pH and concentration ranges used in these studies renders these values meaningless, since they are based on methods which are incapable of distinguishing the continuous MW variation present. Sedimentation coefficients quoted are reflections of a complex association-dissociation equilibrium, whose values fortuitously could be interpreted in terms of certain oligomeric structures. The subunit molecular weight, on the other hand, is reported to be 47,000 (Tong and Duckworth, 1975); 52,500 (Srere, 1972); 55,000 (Danson and Weitzman, 1973) or 60,000 grams/mole (Wright and Sanwal, 1971). All of these measurements derived in part or whole from SDS gel electrophoresis experiments, and reflect valid disagreement over the true value. The subunit molecular weight of Tong and Duckworth (1975) is the one I have most confidence in, since this value has been obtained using a number of different and sensitive methods to determine a true subunit molecular weight, and it has been duplicated a number of times in the hands of other workers. (data not presented)

Allosteric Properties

Extensive functional studies have been carried out on the enzyme from the time Ochoa's group isolated the enzyme from E. coli in 1950. Originally the enzyme was assayed by coupling it to malate dehydrogenase and following the appearance of NADH at 340 nm. Srere, Brazil and Gonen (1963) developed an assay based on the reaction of Ellman's (1959) reagent, DTNB with the free sulfhydryl of CoA, a product of the enzyme's reaction, to produce a highly colored species.

Weitzman (1966a) was the first to show that NADH inhibits citrate synthase from E. coli, as well as other bacterial sources, but was ineffective towards yeast and mammalian citrate synthase. Inhibition of the enzyme was essentially complete at 0.1 mM NADH and dependent on the amount of ACoA present. No inhibition was observed with NAD^+, $NADP^+$ or NADPH (each at 0.3 mM), but high concentrations of ATP (20 mM) produced 75% inhibition. Thus NADH appeared to be the prime feedback inhibitor in the bacterial system of energy production.

In other studies, Weitzman (1966b) showed that changing the pH from 7.5 to 9.2 abolished the NADH inhibition effect on the enzyme, although the catalytic activity was lowered by only 50%. From this finding, the allosteric nature of the enzyme was first postulated. Weitzman also found that addition of 0.2 M KCl completely removed the sensitivity of the enzyme to NADH, although it did not affect the catalytic activity at pH 8.0. However, in the pH range 8.0 - 9.0, addition of KCl would increase the catalytic activity approximately 40%. KCl also seemed to protect the enzyme from the effects of heat and conferredresistance to thiol reagents that otherwise produced inactivation.

Weitzman (1967) reported some significant differences from earlier work (Weitzman, 1966a,b) on a purified preparation of citrate synthase, notionally of E. coli origin. These included lack of inactivation by DTNB, NADH inhibition that was noncompetitive rather than competitive with respect to ACoA, sigmoidal dependence of the degree of inhibition on NADH concentration, rather than hyperbolic, and the fact that AMP and ADP could relieve NADH inhibition of the enzyme. Further investigation revealed that the enzyme under study was not from E. coli, but from Acinetobacter lwoffi, a Gram-negative strict aerobe which had contaminated the culture (Weitzman and Danson, 1976).

These findings prompted Weitzman and Jones (1968) to survey a large number of citrate synthases from bacterial sources in order to investigate the seemingly different kinetic details of NADH inhibition of these two Gram-negative bacteria. The organisms could be divided into two major groups according to whether or not their citrate synthases are sensitive to inhibition by NADH. The NADH-sensitive group consisted of the Gram-negative bacteria whereas the NADH-insensitive group comprises the Gram-positive group. The NADH-sensitive group of citrate synthases could be subdivided further into two groups. The strict aerobes, of which A. lwoffi is an example, showed NADH inhibition which could be relieved by AMP, while the facultative anaerobes (e.g. E. coli) showed no AMP reactivation. Various authors have presented exceptions to these patterns (Taylor, 1970; Swissa and Benziman, 1973; Srere, 1972) and these have been discussed by Danson and Weitzman (1976).

Jangaard, Unkeless, and Atkinson (1968) have presented work on the inhibition of E. coli citrate synthase by ATP. They have found that ATP strongly inhibits the enzyme in pH range of 6.0 to 7.3, then the

inhibition decreases with increasing pH until ATP becomes slightly stimulatory above pH 7.6. AMP and ADP inhibit to a lesser degree than ATP at low pH (below 7.0) and stimulate to a greater extent above pH 7.2. OAA in the reaction mixture prior to initiating the reaction seems to protect the enzyme from ATP inhibition. At pH 6.4, ATP decreases the apparent affinity of the enzyme for both ACoA and OAA, in contrast to the mammalian enzymes where only the Km for ACoA is altered. The authors also reported that enzyme activity is influenced by EDTA, Mg^{2+}, K^{+} and changes in the molarity of the buffer.

Faloona and Srere (1969) have investigated the effect of K^{+} on highly purified E. coli citrate synthase. They found that K^{+}, and to a lesser extent other monovalent cations, markedly increased the stability of the enzyme to urea denaturation, and activated the enzyme up to 20 fold, with a concomitant lowering of the apparent Km's for ACoA and OAA. K^{+} also normalized sigmoidal reciprocal plots of velocity versus ACoA concentration. A shift in the ultraviolet absorption spectrum was observed when potassium was added to the salt free enzyme. Since KCl was said to have no effect on the molecular weight, these authors have concluded that the K^{+} effects are due to conformational changes in the enzyme.

Wright and Sanwal (1971) have shown through equilibrium - binding studies that ACoA binds in a cooperative manner, both in the absence and presence of KCl, although cooperative rate - concentration plots in the absence of KCl become Michaelian hyperbolas in its presence. An unexpected finding was the fact that in the absence of ACoA, the maximum amount of binding for NADH did not exceed 5% of the sites available. Both inhibitors,

αKG and NADH, bind to the enzyme in a negatively cooperative manner in the absence of ACoA, but in a positively cooperative manner in its presence. The binding of both inhibitors, especially that of NADH, is considerably enhanced in the presence of ACoA. These results are in conflict with later studies discussed below, and must be viewed with caution, since the enzyme used in these studies contained large amounts of ammonium sulfate.

Danson and Weitzman (1973) have reported that histidine residues appear to be involved in enzyme activity, as well as the αKG effector site, as judged by the pH dependence of the rates of inactivation by photo-oxidation with Methylene Blue, photo-inactivation with Rose Bengal and destruction of activity by diethyl pyrocarbonate. Also, modification with 2-hydroxy-5-nitrobenzyl bromide indicates the participation of tryptophan in the activity of the enzyme. Photo-oxidation studies suggested the involvement of cysteine at the NADH effector site, and this was confirmed by the desensitization to NADH inhibition produced by treatment of the enzyme with DTNB. The authors suggest that the inactivation of the enzyme after modification with this reagent is due to the additional involvement of cysteine in catalytic activity.

Harford and Weitzman (1975) have conducted multiple-inhibition studies on citrate synthase from Pseudomonas aeruginosa, a Gram-negative bacterium in the same class as E. coli, as well as enzyme from eukaryotic and Gram-positive bacterial sources. They have found that ATP acts in all cases as an isosteric inhibitor at the ACoA site but that NADH acts isosterically with the eukaryotic and Gram-positive bacterial citrate synthases and as a specific allosteric inhibitor in the case of the Gram-