Reversible effects of cross-linking on the regulatory cooperativity of *Acinetobacter* citrate synthase

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Citrate synthase was purified from *Acinetobacter calcoaceticus* and treated with the cleavable cross-linking reagent dithiobis(succinimidyl propionate). Cross-linking of the enzyme resulted in the abolition of the sigmoidal responses to inhibition by NADH and re-activation by AMP displayed by the native enzyme. Inhibition and re-activation were still observed but without any cooperativity. Cleavage of the disulphide bonds in the cross-links by treatment with dithiothreitol restored the sigmoidal characteristics of both inhibition and re-activation.

Citrate synthase Acinetobacter calcoaceticus *Cross-linking* *Cooperativity* *Allosteric regulation* *Dithiobis(succinimidyl propionate)*

1. INTRODUCTION

Citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) exhibits a diversity of regulatory behaviour depending on the source organism [1]. The enzyme isolated from Gram-negative aerobic bacteria is allosterically sensitive to inhibition by NADH and re-activation by AMP. Specifically, the citrate synthease of *Acinetobacter calcoaceticus* shows a sigmoidal dependence on each of these effectors and electron microscopic evidence has been presented in support of the view that conformational rearrangements in quaternary structure underlie the changes in enzymic activity [2].

The availability of bifunctional, cross-linking, reagents offers the possibility of 'freezing' a particular conformation of an enzyme and examining the resulting effects on its functional behaviour. If the cross-linking reagent is itself chemically cleavable, then the reversibility of its conformation-freezing action on the enzyme may also be examined. Such investigations may shed some light on the molecular mechanisms underlying the catalytic and/or regulatory activities of the enzyme.

Here, we report some results obtained on treatment of the citrate synthase of *A. calcoaceticus* with the cleavable cross-linking reagent dithiobis(succinimidyl propionate) (DTSP) [3]. It is shown that the normal cooperative homotropic interactions between regulatory effector sites are lost on cross-linking the enzyme but are regained on cleavage of the cross-links.

2. EXPERIMENTAL

2.1. *Materials*

Dithiobis(succinimidyl propionate) (DTSP), was from Pierce and Warriner (Chester); Matrex Gel Red A was from Amicon (Woking). *A. calcoaceticus*, strain 4B, was isolated from water by P.D.J.W.

2.2. *Methods*

Citrate synthase was purified from nutrient-grown *A. calcoaceticus* by a modification of the procedure previously described for the enzyme from *Escherichia coli* [4]. Two additional steps were introduced, both employing affinity chromatography on Matrex Gel Red A. In the first case

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elution was achieved with a salt gradient and in the second case with a mixture of oxaloacetate and coenzyme A, a combination found to be effective in eluting animal citrate synthases from Sepharose-ATP [5].

Assays of citrate synthase activity were performed spectrophotometrically at 412 nm and 25°C as in [6], but in a buffer solution containing 10 mM Tris adjusted to pH 8 with Na_2-EDTA.

Before conducting cross-linking experiments the enzyme was first dialysed exhaustively against 20 mM triethanolamine-HCl (pH 8) to remove all traces of the Tris buffer. The enzyme solution was then diluted with this ethanolamine buffer to a protein concentration of 10–20 μg/ml. A solution of DTSP (25 mM or 125 mM) in acetone was then added to the enzyme to give the desired final concentration (up to 5 mM) and the mixture allowed to stand at room temperature. After 30 min the reaction was terminated by adding an equal volume of 100 mM Tris–HCl (pH 8) and incubating at 4°C for 30 min. For some functional studies the modified enzyme thus prepared was used without further treatment; otherwise it was freed of excess blocked reagent by dialysis against 20 mM triethanolamine-HCl (pH 8).

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in 5% polyacrylamide gels (or 7.5% gels in the case of the experiments relating to fig.3) as in [7], but in the absence of mercaptoethanol to avoid cleavage of the disulphide bonds in the cross-links. Samples for electrophoresis were first dried under vacuum, dissolved in a solution containing 100 mM sodium phosphate (pH 6.7) 1% (w/v) SDS and 10% (v/v) glycerol, heated at 100°C for 90 s, cooled and loaded into the gel tubes. After electrophoresis, gels were fixed for 1 h in methanol–acetic acid–water (5 : 1 : 5) at 37°C and stained for 30 min in the same mixture containing 0.1% (w/v) Coomassie Brilliant Blue. Destaining was done in methanol–acetic acid–water (1 : 1.5 : 17.5) at 37°C.

3. RESULTS AND DISCUSSION

The enzyme was sensitive to inactivation by DTSP, about 75% of the activity being lost at a reagent concentration of 1.2 mM (fig.1). Reaction with DTSP also resulted in some reduction of the sensitivity of the enzyme to inhibition by NADH,

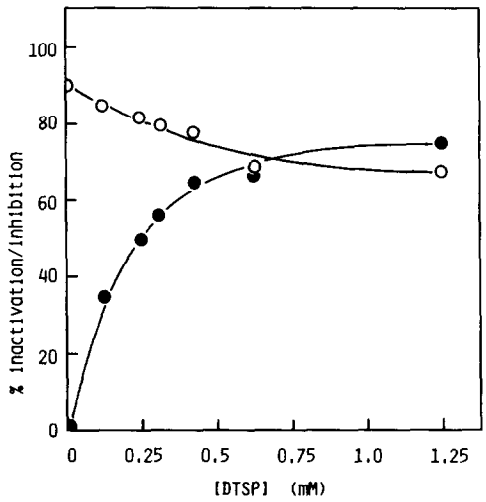

Fig.1. The effect of DTSP on the activity of citrate synthase and on its sensitivity to NADH: (●) inactivation by DTSP; (○) inhibition by 0.2 mM NADH. Excess reagent was not removed by dialysis.

but only to a limited extent (fig.1). Thus even at 1.2 mM DTSP there was still 65% inhibition produced by 0.2 mM NADH. Previous electron microscopic examination of the effect of NADH on *Acinetobacter* citrate synthase [2] revealed a 'swelling' of the enzyme molecule in the presence of NADH, suggesting that the subunits may have moved apart from each other. We therefore anticipated that cross-linking with DTSP might fix the subunits into a structure which could not undergo this swelling and which would therefore render the enzyme desensitized to NADH inhibition. That such desensitization was only slight was initially a disappointment and we therefore examined the enzyme by electrophoresis to confirm that cross-linking had indeed occurred on treatment with DTSP.

The results of SDS–PAGE are shown in fig.2. Whereas the untreated enzyme gave a single band, the enzyme treated with DTSP produced 4 distinct bands and an additional 2 poorly resolved bands. These bands clearly indicate a considerable degree of cross-linking. The band pattern also has implications for the possible hexameric structure of the enzyme which will be discussed in a subsequent paper. Fig.2 also shows that treatment of the cross-linked enzyme with dithiothreitol resulted in a single electrophoretic band which corresponded

Fig.2. SDS–PAGE (5% polyacrylamide) of citrate synthase cross-linked with DTSP: gel 1, native enzyme; gel 2, enzyme cross-linked with 5 mM DTSP; gel 3, enzyme cross-linked with 5 mM DTSP and then treated with 10 mM dithiothreitol.

Fig.3. SDS–PAGE (7.5% polyacrylamide) of citrate synthase cross-linked with various concentrations of DTSP as follows: gel 1, no DTSP; gel 2, 10 mM; gel 3, 5 mM; gel 4, 2.5 mM; gel 5, 1 mM; gel 6, 0.5 mM.

to the monomer of the native enzyme. This indicates that dithiothreitol effectively cleaved the disulphide bonds in the cross-links. Fig.3 shows the effect of various DTSP concentrations on the cross-linking pattern. At the higher concentrations less native monomer was present. However, at 5 mM DTSP the enzyme had lost virtually all its activity and even at 1.25 mM DTSP 75% inactivation resulted. Studies on the functional properties of the modified enzyme were therefore carried out after treatment with 0.6 mM DTSP.

As DTSP clearly cross-links the enzyme, we examined the resulting effects on the regulatory behaviour of the enzyme in more detail. Fig.4 shows the inhibition by NADH and re-activation by AMP as a function of the concentrations of NADH and AMP, respectively. For the native enzyme, both these dependence curves are strongly sigmoidal. However, after cross-linking with 0.6 mM DTSP, inhibition and re-activation were still observed, but both these properties showed a hyperbolic, non-sigmoidal, dependence on effector concentration. As mentioned above, some desensitization to NADH accompanied the cross-linking, but the AMP re-activation was undiminished.

It thus appears that the cooperative interactions between effector sites, which give rise to the sigmoidal responses to NADH and AMP, are abolished on cross-linking the enzyme with DTSP. It is also apparent that the loss of cooperative interactions does not require total cross-linking of the enzyme into its full native polymeric state. Thus when the enzyme was treated with 0.6 mM DTSP (resulting in complete loss of sigmoidal regulatory behaviour), SDS–PAGE revealed that about 50% of the enzyme was still in the monomeric form, the other 50% being mainly dimeric with very small amounts of higher polymeric forms. These results suggest that partial cross-linking of the enzyme interferes with subunit interactions sufficiently to destroy fully the effector cooperativity.

Cleavage of the cross-linked enzyme with dithiothreitol resulted in restoration of the sigmoidal dependences of inhibition and re-activation on NADH and AMP concentrations (fig.4). In order to prevent atmospheric reoxidation of the thiol groups (and hence possible reformation of cross-

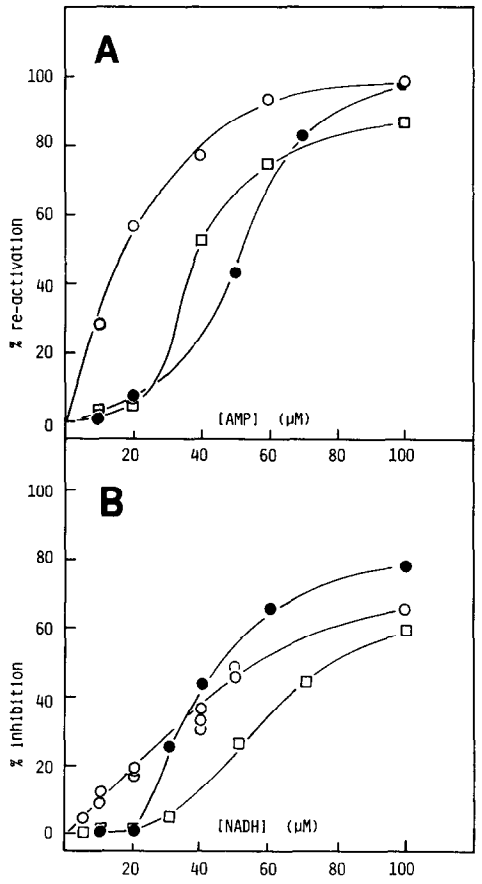

Fig.4. The effect of DTSP on the AMP re-activation (A), and NADH inhibition (B) of citrate synthase: (●) native enzyme; (○) enzyme treated with 0.6 mM DTSP; (□) enzyme treated first with 0.6 mM DTSP and then with 10 mM dithiothreitol. Excess reagents were removed by dialysis against 20 mM triethanolamine–HCl (pH 5.5). AMP re-activation was measured in the presence of 0.2 mM NADH.

links) and also to remove excess dithiothreitol, the enzyme was dialysed at pH 5.5. Under these conditions, the final product gave 90–100% monomeric species on SDS–PAGE. Thus cleavage of the cross-links permits the operation of subunit interactions and the display of regulatory cooperativity.

The enzymic activity lost on reaction with DTSP was not regained on cleavage with dithiothreitol, suggesting that the inactivation arises from the modification of certain amino groups on the enzyme rather than from constraints imposed by the cross-linking itself. This was confirmed by reaction of the enzyme with TSP; i.e., the material produced by cleavage of DTSP with excess of dithiothreitol. Enzyme so treated suffered a loss of activity similar to that brought about by the equivalent concentration of DTSP itself, but the dependences of inhibition and re-activation on the concentrations of NADH and AMP were still sigmoidal and closely resembled those of native enzyme (fig.5).

To eliminate the possibility that the changes in regulatory cooperativity produced by cross-linking

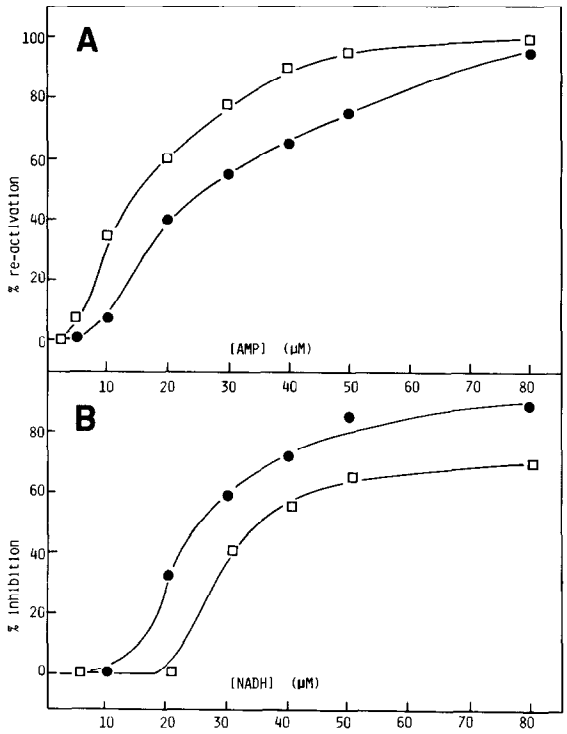

Fig.5. The effect of thiosuccinimidyl propionate (TSP) on the AMP re-activation (A), and NADH inhibition (B) of citrate synthase. DTSP was incubated at 25°C with an excess of dithiothreitol for 30 min. The TSP produced was reacted with citrate synthase as described for DTSP in section 2.2. Excess reagents were removed by dialysis against 20 mM triethanolamine–HCl (pH 5.5): (●) native enzyme; (□) enzyme treated with 0.6 mM TSP. AMP re-activation was measured in the presence of 0.2 mM NADH. Note that the slightly more sensitive responses to both NADH and AMP observed here with the native enzyme compared with the data in fig.4 are attributable to different enzyme preparations used for the two sets of experiments.

with DTSP were due to dissociation or major modification of enzyme structure, several properties of the native and cross-linked enzyme were compared. Similar molecular sizes for the two forms were indicated by the coincidence of their elution peaks on gel filtration through a column of Sephadex G-200. The K_m values were also similar; for the native enzyme these were 3.9 μM and 125 μM for oxaloacetate and acetyl-CoA, respectively, while the corresponding values for the cross-linked enzyme were 3.1 μM and 167 μM. Finally, rabbit antiserum raised against the pure native enzyme exhibited identical inactivation characteristics against native and cross-linked enzyme. The native and cross-linked enzymes are thus closely similar except for the nature of their responses to NADH and AMP.

In a study on *E. coli* aspartate transcarbamylase [8] it was found that cross-linking resulted in complete loss of cooperativity with respect to aspartate and in stabilisation of the 'T' state, thus preventing the allosteric transition to the 'R' state. When cross-linked in the presence of substrate analogues, the K_m for aspartate decreased, indicating that a stabilised 'R' state had been produced. For this enzyme, cross-linking studies showed that subunit interactions are necessary for the $R \rightleftharpoons T$ transitions.

It is not known in what kinetic state the *Acinetobacter* citrate synthase is stabilised by cross-linking. Examination of the kinetic parameters following cross-linking in the presence of substrates and effectors should give some indication of this. It is also proposed to examine by electron microscopy possible size changes of the cross-linked enzyme produced by effector binding and to compare these with previous observations on the native enzyme [2].

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