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THIOL GROUPS OF *ESCHERICHIA COLI* CITRATE SYNTHASE AND THEIR INFLUENCE ON ACTIVITY AND REGULATION

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Summary

The modification of *Escherichia coli* citrate synthase (citrate oxaloacetate-lyase(*pro*-3*S*-$CH_2 \cdot COO^- \rightarrow$ acetyl-CoA, EC 4.1.3.7) with 5,5′-dithiobis-(2-nitrobenzoic acid) has been investigated.

(1) In low ionic strength (20 mM Tris · HCl, pH 8.0): (a) Eight thiol groups per tetramer of the native enzyme reacted with Nbs_2. (b) Two of the eight accessible thiols were modified rapidly with the loss of 26% enzyme activity but with no change in the NADH inhibition. The remaining six were modified more slowly, resulting in a further 60% loss of activity and complete desensitization to NADH. (c) The 2nd-order rate constant for the modification of the rapidly reacting thiols is $2.5 \cdot 10^4\ M^{-1} \cdot min^{-1}$. At the reagent concentrations used (0.1 to 0.2 mM) the modification of the six thiols in the slow kinetic set appeared to be 1st-order; at 0.1 mM dithionitrobenzoic acid their rate of modification was approximately 30 times slower than the thiols in the fast kinetic set.

(2) In high ionic strength (20 mM Tris · HCl, pH 8.0, 0.1 M KCl): (a) Four thiol groups were modified in a single kinetic set and it appeared that these thiols are four of the six slowly modified in the absence of KCl. (b) The modification resulted in 70% loss of enzyme activity and complete loss of NADH inhibition.

(3) From the kinetic analysis it is proposed that the four thiol groups accessible to dithionitrobenzoic acid in the absence and presence of 0.1 M KCl are those involved in the response to NADH. Modification of any one of these four groups produced no reduction in the inhibition; instead, loss of NADH sensitivity was coincident with the appearance of tetrameric protein possessing three substituted thiols, whereas enzyme with one or two modified groups was still fully inhibited by NADH.

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Abbreviation: Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

Introduction

Citrate synthase (citrate oxaloacetate-lyase(*pro*-3*S*-$CH_2 \cdot COO^- \rightarrow$ acetyl-CoA, EC 4.1.3.7) catalyses the condensation of acetyl-CoA with oxaloacetate and thus occupies a key position within the citric acid cycle. The activity of the enzyme from *Escherichia coli* is inhibited by NADH [1—3] and by α-oxoglutarate [4,5], and these two control mechanisms may contribute to the regulation of the production by the cycle of both energy and biosynthetic intermediates.

Several investigations into the nature of these allosteric controls of citrate synthase have centered around chemical modification studies of the enzyme and, in particular, the modification of the protein thiol groups. Weitzman [2] first reported the inactivation of *E. coli* citrate synthase by the thiol-blocking reagents 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2), *N*-ethylmaleimide, and mercuric chloride. Recently, we have demonstrated that sulphydryl modification by photo-oxidation or with Nbs_2 also desensitizes the enzyme to NADH inhibition although there was no loss of response to α-oxoglutarate inhibition [6]. Wright and Sanwal [7] have confirmed that thiol groups are essential for full activity of the enzyme by treatment with 4,4′-dithiodipyridine and Nbs_2.

In the present work we have analysed the kinetics of the modification of *E. coli* citrate synthase with Nbs_2 in an attempt to investigate further the functional relationship between those thiol groups participating in catalytic activity and those involved in the response to NADH. In addition we have examined the influence of ionic strength on the modification process as an extension of our interest in the effects of salt on the enzyme.

Materials and Methods

All chemicals used were analytical grade or the finest grade commercially available. NADH, α-oxoglutarate and Nbs_2 were obtained from Sigma London Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; coenzyme A and oxaloacetate were from Boehringer Corporation (London) Ltd., London, U.K. and Sephadex G-200 was from Pharmacia (Great Britain) Ltd., London, U.K.

The organism used in this work was *Escherichia coli* K12, strain CA244. Glycerol-grown cells were obtained as a frozen paste from the Microbiological Research Establishment, Porton Down, Wilts., U.K.

Assay of citrate synthase

Citrate synthase was assayed spectrophotometrically at 412 nm and 25° C by the method of Srere et al. [8]. Assay mixtures contained 20 mM Tris · HCl (pH 8.0), 1 mM EDTA, 0.1 mM oxaloacetate, 0.15 mM acetyl-CoA and 0.1 mM Nbs_2.

Purification of citrate synthase

The enzyme was purified as described by Danson and Weitzman [6]; its protein concentration was calculated from the absorbance at 280 nm and the value of $2.4 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ for the extinction coefficient (Sanwal, B.D., personal communication).

The homogeneity of the enzyme was demonstrated by ultracentrifugation,

gel electrophoresis and gel electrophoresis with sodium dodecyl sulphate. The fact that all 32 cysteine residues per enzyme molecule [6] reacted with Nbs_2 in urea or sodium dodecyl sulphate demonstrated that all these residues were in the reduced (SH) form.

Treatment of the enzyme with Nbs_2

To facilitate the kinetic analysis of the reaction between Nbs_2 and the thiol groups of the protein, pseudo-first-order conditions were established by maintaining at least a 40-fold excess of reagent to available cysteine residues.

Purified citrate synthase (0.1 mg) was incubated in 1.0 ml of 20 mM Tris · HCl (pH 8.0), 1 mM EDTA with 0.1 mM Nbs_2 at 20° C in the dark. At intervals, samples were removed and measurements were made of enzyme activity and the inhibitions produced by NADH (0.2 mM) and by α-oxoglutarate (2.0 mM). The reaction between Nbs_2 and the protein thiol groups was followed by measurement of the increase in absorbance at 412 nm.

The modification procedures were repeated in the presence of 0.1 M KCl.

In the presence of substrates, the initial activity of citrate synthase measured by the above assay method with Nbs_2 was essentially identical with that measured in the absence of Nbs_2 (spectrophotometrically at 232 nm or polarographically). This absence of inactivation by Nbs_2 during the assay made it unnecessary to terminate the Nbs_2 modification of the enzyme by other means.

Analysis of the data

Kinetic analysis of the reaction between Nbs_2 and the protein thiol groups was carried out according to the method of Freedman and Radda [9]. Thus under the pseudo-first-order conditions employed, the modification of a single residue, or more than one residue reacting at identical rates, is described by the following equation:

$$\Delta E_t = \Delta E_\infty (1 - e^{-kt}) \quad (1)$$

where ΔE_t and ΔE_∞ are the changes in absorbance at 412 nm at times t and ∞ respectively, and k is the pseudo-first-order rate constant. The equation can be rearranged to

$$\ln[\Delta E_\infty / (\Delta E_\infty - \Delta E_t)] = kt \quad (2)$$

Thus k is given by the gradient of a plot of $\ln [\Delta E_\infty / (\Delta E_\infty - \Delta E_t)]$ vs. t.

Results and Discussion

In view of the marked effects of high salt concentrations on the physicochemical properties of *E. coli* citrate synthase, especially with respect to the modification of the enzyme's thiol groups [2], the reaction with Nbs_2 has been studied in buffers of both low and high ionic strengths, i.e. 20 mM Tris · HCl (pH 8.0), 1 mM EDTA, in the absence and presence of 0.1 M KCl, respectively.

Treatment of citrate synthase with Nbs_2 in low ionic strength

We have reported previously [6] that the reaction of Nbs_2 with *E. coli* citrate synthase results in the modification of approximately eight thiol groups per

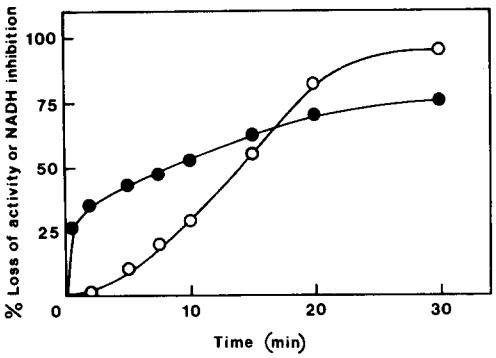

Fig. 1. Effects of Nbs_2 on the activity and NADH inhibition of citrate synthase. Treatment with 0.1 mM Nbs_2 in 20 mM Tris · HCl (pH 8.0), 1 mM EDTA was carried out as described in Materials and Methods. ●, loss of enzyme activity; ○, loss of inhibition by 0.2 mM NADH.

native tetramer (M_r = 230 000). This modification produced a very rapid inactivation of approximately 25%, followed by a slower inactivation until >80% of the initial enzyme activity was destroyed. Whereas this slower loss of activity was exponential, the loss of NADH sensitivity showed a definite lag (Fig. 1). No change in the response to α-oxoglutarate was observed.

A more detailed analysis of this modification process has now been carried out.

(i) Reaction of Nbs_2 *with the thiol groups.* Modification of the protein thiol groups was followed continuously at 412 nm (Fig. 2). Semi-log plots of the data were non-linear indicating that the curve is made up of more than one exponential — a very rapid thiol modification complete within the first few minutes (hereafter referred to as the fast kinetic set(s)) and slower modification resulting in the remaining observed increase in absorbance up to 40 min or longer.

The total change in absorbance at 412 nm (ΔE_∞) was determined by a mathematical extrapolation of the final exponential curve as described by Freedman and Radda [9] rather than by direct observation which would have involved a long scanning time. From the value of ΔE_∞ thus calculated a plot of ln $[\Delta E_\infty/(\Delta E_\infty - \Delta E_t)]$ versus t was constructed and the pseudo-first-order rate constant determined.

Such treatment of the data gave a close fit to a straight line over t = 4 to 35 min and the rate constant (k_2) calculated for this slow kinetic set was 0.0716 min^{-1}. The intercept on the ordinate of this plot, determined by extrapolation of the linear portion, is a measure of the total change in extinction ($\Delta E_{\infty\,1}$) resulting from the modification of the fast kinetic set(s) and subtraction of this from the total ΔE_∞ gives $\Delta E_{\infty\,2}$ (i.e. ΔE_∞ for the slower set). The values obtained from the data were $\Delta E_{\infty\,1}$ = 0.0137 and $\Delta E_{\infty\,2}$ = 0.0343.

The values of ΔE_t for the slow kinetic set were calculated from the estimates of k_2 and $\Delta E_{\infty\,2}$ using Eqn. 1 and subtraction of these from the observed ΔE_t values gave the changes in absorbance with time of modification of the fast set(s). These data were then plotted in the semi-log form described previously, the straight line plot obtained indicating a single fast kinetic set of susceptible thiol groups with a calculated rate constant (k_1) of 2.01 min^{-1}.

Freedman and Radda [9] have pointed out that this sequential method of analysis can lead to a cumulative error in the estimated rate constant of the fast kinetic set. Moreover, a small error in the estimation of the total change in extinction will produce large errors in the calculated values of each rate constant. Therefore, using the estimates of k_1, k_2, $\Delta E_{\infty 1}$ and $\Delta E_{\infty 2}$ calculated above, the values of these parameters giving the best fit to the observed data were obtained by the method described below.

If the modification of the available thiols by Nbs_2 does indeed consist of two pseudo-first-order reactions then the total change in extinction (ΔE_t) at any time t is given by:

$$\Delta E_t = \Delta E_{\infty 1}(1 - e^{-k_1 t}) + \Delta E_{\infty 2}(1 - e^{-k_2 t})$$

Thus it was possible to calculate values of ΔE_t and to compare them with the observed data, and such calculations and comparisons were performed using a FORTRAN program processed on an ICL 4130 computer. This program tested a chosen range of parameter values around the estimated values in order to converge on a unique set that yielded the minimum sum of squares (ss) as defined:

$$ss = \sum(\Delta E_t \text{ calculated} - \Delta E_t \text{ observed})$$

The parameter values yielding the lowest sum of squares were:

$k_1 = 2.418 \text{ min}^{-1}$ $\quad \Delta E_{\infty 1} = 0.0131$

$k_2 = 0.076 \text{ min}^{-1}$ $\quad \Delta E_{\infty 2} = 0.0343$

Comparison between this best fit and the experimental data is shown in Fig. 2. The total changes in absorbance correspond to 2.29 and 5.96 thiol groups modified per enzyme (M_r = 230 000) for the fast and slow kinetic sets respectively.

Modification of citrate synthase was also carried out with 0.2 mM Nbs_2. Identical treatment of the data gave rate constants of $k_1 = 5.025 \text{ min}^{-1}$ and k_2 =

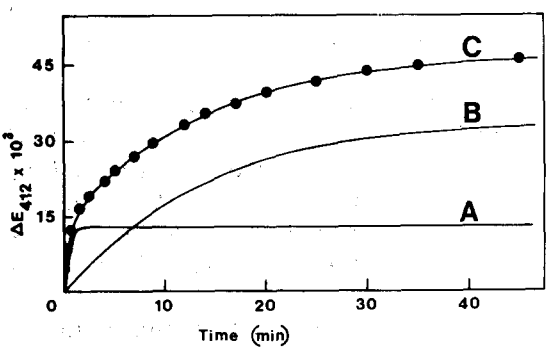

Fig. 2. Modification of citrate synthase with Nbs_2: Reconstruction curves describing the time course of the increase in E_{412}. Citrate synthase (0.1 mg/ml) was treated with 0.1 mM Nbs_2 in 20 mM Tris · HCl (pH 8.0), 1 mM EDTA as described in Materials and Methods. The points (•) are the experimentally observed values of ΔE_{412}. Lines (A) and (B) are the theoretical changes in E_{412} resulting from the modification of the thiol groups in the fast and slow kinetic sets respectively, and were constructed as described in the text. Line (C) is the sum of lines (A) and (B).

0.0691 min^{-1}, the number of thiol groups reacted being 1.89 and 6.00 respectively. Thus, over the range of Nbs_2 concentrations used, the modification of the rapidly reacting thiols is indeed second order (rate constant = $2.5 \cdot 10^4$ $M^{-1} \cdot min^{-1}$). In contrast, the modification of the thiols in the slow kinetic set appears to be first-order.

(ii) Inactivation by Nbs_2. The inactivation of citrate synthase by Nbs_2 (Fig. 1) was subjected to the same method of kinetic analysis as were the thiol modifications. Data from several experiments gave a total loss of activity of 84% (±4%) with a pseudo-first-order rate constant for the slower phase of the inactivation (t = 0.5 to 30 min) of 0.071 min^{-1}. This rate of inactivation was very close to the rate of modification of the slow set of thiols and similarly its value was independent of the Nbs_2 concentration over the range studied (0.1 mM to 0.2 mM Nbs_2). It was not possible to determine the rate constant for the rapid inactivation although it was estimated to be complete in less than 0.5 min, the intercept from the semi-log plot indicating a rapid loss of 26% of the initial enzyme activity.

In agreement with observations of Weitzman [2], addition of 0.1 M KCl to enzyme that had been inactivated by Nbs_2 produced approximately 40% reactivation, suggesting that, at least for some thiols, involvement in enzyme activity may be indirect.

(iii) Desensitization to NADH by treatment with Nbs_2. The relationship between thiol modification and desensitization to NADH is less obvious than that between the loss of thiols and inactivation of the enzyme, for there was a marked lag in the desensitization process whereas the modification of the thiols was exponential. Inhibition studies on partially desensitized enzyme demonstrated that the observed loss of response to NADH was not due to an increase in the K_i for this effector.

Ray and Koshland [10] have put forward two possible models to explain such lag phenomena. Application of the first, that of progressive denaturation, would assume that modification of the residues in the fast kinetic set does not affect NADH inhibition but does affect the protein molecule so that previously unreactive thiols, essential for NADH regulation, become susceptible to attack: such a situation would result in a non-linear desensitization. The model predicts that those residues required for NADH inhibition should be modified non-linearly, i.e. with a lag comparable to that observed for the desensitization.

However, the kinetic analysis demonstrated that both sets of thiols reacted with Nbs_2 in an exponential fashion from zero time. Moreover, modification of the thiol groups in the fast kinetic set was complete within approximately 0.5 min whereas full NADH inhibition was still observed after 2.0 min incubation with Nbs_2.

The second model postulates that more than one residue is involved in the NADH inhibition, such that modification of any one of the thiols does not diminish the regulation, and desensitization only occurs when two or more groups have been modified. In this case the progress of residue modification should be exponential, as was indeed observed, and this model is therefore favoured over that of progressive denaturation as an explanation of the experimental observations. In accordance with such a model, a semi-log plot of NADH inhibition against time described a curve whose slope was initially zero,

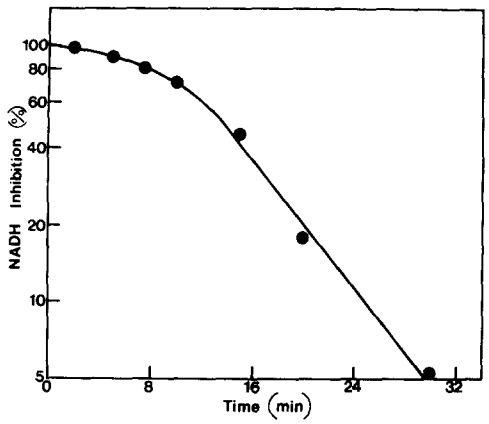

Fig. 3. Determination of the rate of desensitization of citrate synthase to NADH inhibition by Nbs_2. The data for the desensitization to inhibition by 0.2 mM NADH from Fig. 1 were plotted in the semi-log form of % NADH inhibition versus incubation time. The rate constant (k) is determined from the gradient of the linear portion (k = 2.303 × gradient).

increased in a negative sense with time, and eventually approached a constant value (Fig. 3).

From the linear portion of this plot the rate constant for the loss of NADH inhibition was calculated to be 0.147 min^{-1} (k_N). This value is in fact twice that for the modification of the thiols in the slow kinetic set (k_2 = 0.076 min^{-1}) and describes the rate of conversion of the enzyme species possessing full NADH inhibition to a species exhibiting no sensitivity to NADH.

Therefore, the model describing the desensitization process most closely is that modification of the thiols in the fast kinetic set results in no loss of NADH inhibition; rather it is the more slowly reacting thiol groups that are essential for the response to this effector. Moreover, the data suggest that the modification of these thiols in the slower set proceeds to give an enzyme which is fully inhibited by NADH (hence the lag in the desensitization) and which possesses two thiols, the modification of either of the two resulting in a completely desensitized enzyme (hence $k_N = 2k_2$). This proposed model is discussed in more detail later as further relevant information was gained from the modification of the enzyme in the presence of 0.1 M KCl.

Treatment of citrate synthase with Nbs_2 in high ionic strength

(i) Reaction of Nbs_2 with the thiol groups. Incubation of citrate synthase with 0.1 mM Nbs_2 in the presence of 0.1 M KCl resulted in the modification of 4.0 (±0.5) thiols per enzyme molecule (M_r = 230 000). Analysis of the time course for the reaction demonstrated it to be composed of a single exponential, the semi-log plot of $\Delta E_\infty/(\Delta E_\infty - \Delta E_t)$ against time being linear with an intercept of zero (Fig. 4). The first-order rate constant for the modification of these four thiol groups was 0.53 (±0.03) min^{-1}.

(ii) Inactivation and desensitization to NADH. The reaction of Nbs_2 with the four thiols of citrate synthase resulted in a 70% loss of enzyme activity and complete desensitization to NADH (Fig. 5). As in the modification with Nbs_2

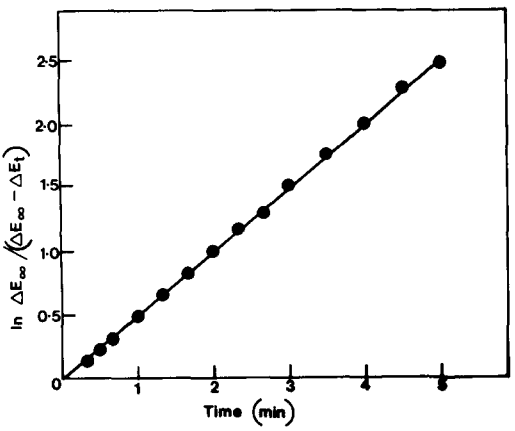

Fig. 4. Modification of citrate synthase with Nbs_2 in KCl. Citrate synthase (0.19 mg/ml) was incubated with 0.1 mM Nbs_2 in buffer of composition 20 mM Tris · HCl (pH 8.0), 1 mM EDTA, containing 0.1 M KCl, and the change in E_{412} (ΔE) followed with time. The data are plotted as ln $[\Delta E_\infty/(\Delta E_\infty - \Delta E_t)]$ versus time. The rate constant is given by the gradient.

in the absence of KCl, there was a lag in the loss of NADH inhibition, whereas the time course for the inactivation was exponential. The inclusion of 0.1 M KCl in the assay again produced considerable reactivation of the modified enzyme, i.e. only about 40% loss of activity was apparent when 0.1 M KCl was present in the assay (Fig. 5). Semi-log plots of the data demonstrated single pseudo-first-order inactivations with rate constants of 0.60 min^{-1} and 0.50 min^{-1} for the assays performed in the absence and presence of KCl respectively. This close agreement between the observed rates of modification and inactivation suggests that the susceptible thiols are necessary for full catalytic activity, although again this involvement may be indirect.

These cysteine residues also appear to be essential for NADH regulation. As with the Nbs_2 modification experiments in the absence of KCl, the exponential modification of the four thiols in the presence of the salt leads to the conclusion that the lag in the desensitization process probably reflects the fact that

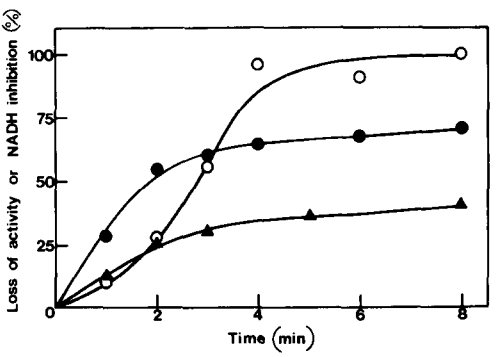

Fig. 5. Inactivation and desensitization to NADH inhibition of citrate synthase by Nbs_2 in KCl. Citrate synthase was incubated with 0.1 mM Nbs_2 in 20 mM Tris · HCl (pH 8.0), 1 mM EDTA, containing 0.1 M KCl. ●, loss of enzyme activity when assayed in the absence of KCl; ▲, loss of activity when assayed in the presence of 0.1 M KCl; ○, loss of inhibition by 0.2 mM NADH (assayed in the absence of KCl).

more than one thiol group must be modified before any loss of NADH inhibition is observed. Indeed, a semi-log plot of inhibition by NADH versus time described a curve whose slope increased negatively from zero to a constant value of −0.5, giving a rate constant of 1.15 min^{-1}. Again, this constant is approximately twice that for the thiol modification (k = 0.53 min^{-1}) and therefore suggests that loss of NADH inhibition results from reaction of Nbs_2 with either of two thiols on a modified enzyme molecule showing full regulation.

Treatment of citrate synthase with Nbs_2 in low ionic strength after modification in the presence of 0.1 M KCl

The extent of inactivation, the kinetics of the desensitization to NADH inhibition and the partial reactivation by KCl all tentatively suggest that the four cysteines modified in the presence of KCl may be four of the six thiols in the slow kinetic set which are susceptible to Nbs_2 when the salt is absent. If this is correct, Nbs_2 modification in the absence of KCl, after modification in the presence of salt, might result in a rapid modification of two thiol groups with two more being attacked at a much slower rate.

After incubation of the enzyme with Nbs_2 in the presence of 0.1 M KCl for 14 min (see Figs. 4 and 5), unreacted Nbs_2 was removed by extensive dialysis against 20 mM Tris · HCl (pH 8.0), 1 mM EDTA containing 0.1 M KCl. The KCl was subsequently removed by further dialysis against the Tris · HCl, EDTA, buffer alone. The enzyme was then incubated with 0.1 mM Nbs_2.

From the increase in absorbance at 412 nm with time it was evident that a very rapid modification occurred, probably accompanied by a slower attack. Kinetic analysis was difficult because it was not possible to calculate the total ΔE_∞, the modification being too slow after 2 min incubation. However, estimates indicated that 1.85 (±0.15) thiols were rapidly modified, the semi-log plot indicating a rate constant of 2.4 (±0.2) min^{-1}. The number of thiols in the slow kinetic set could not be determined although the rate of their modification appears to be approximately 50—100 times slower than that of the rapid set.

The rate constant of 2.4 min^{-1} is in fact identical with the value determined for the modification of the two thiols reacting rapidly with 0.1 mM Nbs_2 in the absence of KCl (k_1 = 2.4 min^{-1}). Moreover, this modification was also accompanied by a partial but rapid loss of the enzyme activity remaining after the Nbs_2 treatment in KCl. In all probability, therefore, the thiols susceptible to Nbs_2 in the presence of 0.1 M KCl are four of the six slowly modified in the absence of the salt.

Desensitization to NADH inhibition

As discussed above, when citrate synthase was treated with Nbs_2 in buffer of low ionic strength modification of the 6 thiols in the slow kinetic set produced complete loss of NADH inhibition. However, studies in the presence of 0.1 M KCl suggest that of these 6 thiol groups, modification of only 4 results in desensitization. The observation that the loss of inhibition on modification with Nbs_2, either in the presence or absence of KCl, proceeds, after a lag, at twice the rate at which individual thiols are modified therefore suggests that loss of any 2 of the 4 thiols produces no desensitization, but the subsequent

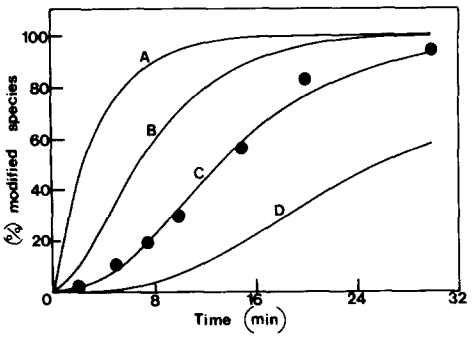

Fig. 6. Estimation of the relative amounts of each species present during the chemical modification of citrate synthase with Nbs_2. As described in the text and Appendix, the percentages of molecules possessing at least 1 (A), 2 (B), 3 (C) and 4 (D) modified thiol groups were calculated. The points (•) are the experimentally observed values for the % losses of NADH inhibition (see Fig. 1).

modification of either of the remaining 2 results in an enzyme insensitive to NADH.

Considering the modification of these 4 thiols implicated in NADH inhibition, the proposed model predicts that the loss of inhibition should be coincident with the appearance of enzyme possessing 3 modified thiol groups. Assuming that the modification of any one thiol group proceeds independently of the modification of the other three and that all 4 thiols react with Nbs_2 at the same rate, it is then possible to determine at any time in the reaction the percentage of tetrameric enzyme molecules with only 1, 2, 3 or 4 thiols modified (see Appendix).

For the modification by Nbs_2 in the absence of KCl the relative amounts of the modified species, together with the losses of NADH inhibition, are shown in Fig. 6. The data clearly fulfil the prediction that 3 out of the 4 thiols in question must be modified to produce desensitization to NADH inhibition. Similar calculations were made for the modification in 0.1 M KCl, but the data showed a poorer correlation between the loss of inhibition and the appearance of a particular modified species. Thus it was unclear from the plots whether 3 or 4 of the thiols must be modified before loss of inhibition is observed. The only deciding factor between the two possibilities is that the rate of desensitization (k = 1.15 min^{-1}) corresponds with the rate of appearance of the enzyme with 3 modified thiols (k = 1.06 min^{-1}) rather than 4 (k = 0.53 min^{-1}), thus favouring the former possibility.

A summary of the modification by Nbs_2 and the effects on the activity of the enzyme and its regulation by NADH is presented in Table I.

From our present analysis of the reaction of Nbs_2 with the thiol groups of *E. coli* citrate synthase it has been possible to describe more precisely the relationship between the susceptible residues involved in enzyme activity and those that are essential for regulation by NADH.

The incomplete loss of catalytic activity and the partial reactivation by KCl of the Nbs_2-treated enzyme indicate an indirect role for the modified thiols, such as maintenance of the active conformation. It is possible that the introduction of several bulky thionitrobenzoate moieties into the active site

TABLE I

MODIFICATION OF CITRATE SYNTHASE BY Nbs_2

The data were obtained by modification of the enzyme with 0.1 mM Nbs_2 except the values in parentheses which are for modification with 0.2 mM Nbs_2. (—) indicates that no value could be measured.

Buffer	Thiol groups modified		Activity		Inhibition by NADH	
	Number	Rate constant $(min)^{-1}$	% lost	Rate constant $(min)^{-1}$	% lost	Rate constant $(min)^{-1}$
20 mM Tris · HCl (pH 8.0), 1 mM EDTA	2.29 (1.89) 5.96 (6.00)	2.42 (5.03) 0.076 (0.069)	26 58	— 0.071	0 100	0 0.147
20 mM Tris · HCl (pH 8.0), 1 mM EDTA + 0.1 M KCl	4.0	0.53	70	0.55	100	1.15
20 mM Tris · HCl (pH 8.0), 1 mM EDTA (after modification in 20 mM Tris · HCl (pH 8.0), 1 mM EDTA + 0.1 M KCl)	1.85 —	2.4 ≈0.03—0.06	16 —	— —	— —	— —

region of the enzyme causes loss of activity through steric hindrance, although we have observed similar extents of inactivation with 2-nitro-5-thiocyanobenzoate, the cyano group incorporated being small and uncharged and thus less likely to produce large conformational changes in the protein [11].

It will be noticed that the rate of inactivation by Nbs_2 observed here is considerably less than that reported by Weitzman [2]. The difference in the modification procedures is that 10 mM $MgCl_2$ was formerly included in the incubation buffer, and this comparison has led Weitzman and Packman (unpublished) to demonstrate that low concentrations of Mg^{2+} do, indeed, greatly enhance the rate of inactivation by Nbs_2, suggesting the possibility that Mg^{2+} alter the conformation of the enzyme significantly.

In contrast to the partial inactivation produced by Nbs_2, complete loss of inhibition by NADH was observed. However, from the model proposed, it is unlikely that these thiols are directly involved in the binding of NADH since modification of a single thiol would then be accompanied by partial desensitization. Instead, it is more likely that they have an indirect role such as in subunit interactions which may be essential for the response to the inhibitor. Indeed, at any time point during the desensitization by Nbs_2, the concentration of NADH required to produce half maximal inhibition at that point remains constant, suggesting that binding of the effector to the enzyme is not affected by the modification; rather the response to the binding is impaired.

The present data do not give any definite indications as to why the 8 susceptible thiol groups per enzyme tetramer are modified in two kinetic groups of two and six thiols respectively. No dissimilarity between the polypeptide chains of *E. coli* citrate synthase has been detected. Molecular weight studies,

peptide fingerprinting [7] and end-group analyses [12] indicate very similar if not identical subunits, although in the absence of sequence data their precise identity remains to be established.

If, in fact, the polypeptides are identical, each may possess two susceptible thiol groups. The four thiols in one group (one thiol per subunit) appear to react with Nbs_2 in an homologous manner and are accessible to modification in the presence and absence of 0.1 M KCl. The other set of four thiols is only accessible in the absence of high salt concentrations, when two react very rapidly whilst the other two do so much more slowly. This non-identical behaviour of these four thiols may result from subunit interactions within the enzyme; modification of one by Nbs_2 may induce a conformational change in an adjacent subunit reducing the rate of modification of the thiol in that subunit. This would be analogous to the concept of half-site reactivity, as has been observed, for example, in the alkylation of thiols in glyceraldehyde-3-phosphate dehydrogenase [13].

Support for such an hypothesis has come from studies in our laboratory (Danson, Harford and Weitzman, unpublished) on a mutant *E. coli* citrate synthase which is a dimeric molecule and has lost the characteristic subunit interactions of the wild-type enzyme. Interestingly, this mutant enzyme does not show a biphasic inactivation with Nbs_2; rather, the loss of activity is described by a single exponential with a rate constant very similar to that of the two rapidly modified thiols in the wild-type enzyme. Further comparative studies with other thiol-blocking reagents may provide more useful information concerning this problem.

The changes in the reactivity of the thiol groups on increasing the ionic strength of the incubation buffer are indicative of salt-induced conformational changes in the enzyme and further support our recent studies (Danson and Weitzman, unpublished) on the effects of KCl on *E. coli* citrate synthase. As previously mentioned, KCl also produces partial reactivation of the Nbs_2-modified enzyme and this may explain the claim of Faloona and Srere [14] that Nbs_2 reacts with citrate synthase from *E. coli* with little loss of enzyme activity. Their reaction was carried out in 0.1 M Tris · HCl and the enzyme was assayed in 0.1 M Tris · HCl containing 0.1 M KCl; under these conditions we have found only 30% loss in enzyme activity.

Preliminary studies on the action of Nbs_2 on citrate synthase from other Enterobacteriaceae have been made [15]. Similar to the *E. coli* enzyme, citrate synthases from *Klebsiella aerogenes*, *Serratia marcescens* and *Arizona arizonae* were completely desensitized to NADH by Nbs_2. Considerable inactivation of the enzyme from *Klebsiella* and *Arizona* was also observed, although the *Serratia* enzyme retained over 70% of its activity even after prolonged incubation with the reagent. The very similar sensitivities towards Nbs_2 of the NADH inhibition suggest that the citrate synthases from the Enterobacteriaceae may share a common mechanism of regulation. As NADH inhibition is a characteristic feature of citrate synthases from diverse Gram-negative bacteria [15] it will be interesting to examine the extent of similarity and of thiol group involvement in the enzymes from a range of organisms within this taxonomic group.

Appendix

Chemical modification of citrate synthase with Nbs_2

Consider the modification of 4 thiol groups on each enzyme molecule. It is assumed that all the thiols react with Nbs_2 at an identical rate, the Nbs_2 being in at least 40-fold excess over available thiol groups, thus ensuring that the modification is a pseudo-first-order process. If PA represents an enzyme molecule with 4 unmodified thiols, PB with 3, PC with 2, PD with 1 and PE with no unmodified thiols, then during the reaction with Nbs_2:

$$PA \xrightarrow{k_a} PB \xrightarrow{k_b} PC \xrightarrow{k_c} PD \xrightarrow{k_d} PE$$

where k_a, k_b, k_c and k_d are the respective rate constants. Let k represent the rate constant for the modification of each thiol group. Let $[PA]_0$ represent the concentration of PA at zero time and $[PA]_t$ the concentration of PA at time t; similarly with PB, PC, PD and PE.

Assuming first-order modifications, it can be shown that:

$$[PA]_t = [PA]_0 e^{-4kt}$$

$$[PB]_t = 4[PA]_0(e^{-3kt} - e^{-4kt})$$

$$[PC]_t = 6[PA]_0(e^{-2kt} - 2e^{-3kt} + e^{-4kt})$$

$$[PD]_t = 4[PA]_0(e^{-kt} - 3e^{-2kt} + 3e^{-3kt} - e^{-4kt})$$

$$[PE]_t = [PA]_0 - ([PA]_t + [PB]_t + [PC]_t + [PD]_t)$$

The full derivation of these equations can be supplied on request.

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References

1 Weitzman, P.D.J. (1966) Biochim. Biophys. Acta 128, 213–215
2 Weitzman, P.D.J. (1966) Biochem. J. 101, 44c–45c
3 Weitzman, P.D.J. and Jones, D. (1968) Nature 219, 270–272
4 Wright, J.A., Maeba, P. and Sanwal, B.D. (1967) Biochem. Biophys. Res. Commun. 29, 34–38
5 Weitzman, P.D.J. and Dunmore , P. (1969) FEBS Lett. 3, 265–267
6 Danson, M.J. and Weitzman, P.D.J. (1973) Biochem. J. 135, 513-524
7 Wright, J.A. and Sanwal, B.D. (1971) J. Biol. Chem. 246, 1689–1699
8 Srere, P.A., Brazil, H. and Gonen, L. (1963) Acta Chem. Scand. 17, S129–S134
9 Freedman, R.B. and Radda, G.K. (1968) Biochem. J. 108, 383–391
10 Ray, W.J. and Koshland, D.E. (1961) J. Biol. Chem. 236, 1973–1979
11 Degani, Y. and Patchornik, A. (1974) Biochemistry 13, 1–11
12 Tong, E.K. and Duckworth, H.W. (1975) Biochemistry 14, 235–241
13 Levitzki, A. (1974) J. Mol. Biol. 90, 451–458
14 Faloona, G.R. and Srere, P.A. (1969) Biochemistry 8, 4497–4503
15 Weitzman, P.D.J. and Danson, M.J. (1976) Curr. Top. Cell. Regul. 10, 161–204