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Chemical modification of rat liver cytosolic NADP$^+$-linked isocitrate dehydrogenase by *N*-ethylmaleimide

Evidence for essential sulphydryl groups

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Incubation of rat liver cytosolic isocitrate dehydrogenase with *N*-ethylmaleimide (NEM) resulted in the inactivation of the enzyme following pseudo-first order kinetics. Isocitrate affords considerable protection against inactivation whereas NADP$^+$ enhances modification of the enzyme, suggesting localization of the modified group at the active site. Correlation of loss of activity with incorporation of [^{14}C]NEM indicated that two sulphydryl residues/sub-unit are modified of which only one is shown to be involved in catalysis. pH dependence of the inactivation process implicates a reactive group of pK_a 8.1 in catalysis. We conclude that a unique cysteine residue is essential for maximal catalytic activity of isocitrate dehydrogenase.

Cytosolic isocitrate dehydrogenase; Chemical modification; Sulphydryl residue; *N*-Ethylmaleimide

1. INTRODUCTION

NADP$^+$-linked isocitrate dehydrogenase (IDH) (threo-D_s-isocitrate-NADP$^+$ oxidoreductase (decarboxylating) EC 1.1.1.42) catalyses reversible oxidative decarboxylation of isocitrate to α-ketoglutarate. The enzyme is present in both the cytosol and mitochondria of mammalian tissues. In heart and kidney, the enzyme is predominantly localized in mitochondria, whilst in liver it is largely present in the cytosol [1,2]. The two isoenzymes are under different genetic control [3]. The mitochondrial enzyme, which has been implicated to play a key role in the oxidation of isocitrate in the tricarboxylic acid cycle [4], is relatively more studied and has been purified from several tissues, including pig heart [5] and bovine heart [6]. The enzyme from bovine heart has especially been subjected to detailed kinetic and mechanistic studies [7–11]. Chemical modification studies of the pig heart mitochondrial enzyme by Colman and her collaborators have identified critical sulphydryl groups [12,13], glutamyl [14], methionyl [15], arginyl [16] and histidine [17] residues in the binding and catalytic functions of the enzyme. In contrast, very little is known of essential residues involved in the catalytic action of the cytosolic isocitrate dehydrogenase. The enzyme has been isolated from sheep [18], bovine [19] and rat [20] liver. The rat liver enzyme, which we have recently purified, has an apparent M_r of 94,000 by gel filtration and is composed of two identical subunits of M_r 45,000 as judged by SDS-PAGE. The subunit and native enzyme molecular weights are similiar to those reported for the cytosolic enzyme from bovine liver [19] and sheep liver [18], the latter enzyme being a homodimer, with a subunit M_r of 36,000. Polyclonal antibodies raised against the purified rat liver cytosolic enzyme do not cross-react with the rat liver mitochondrial enzyme showing that the two isoenzymes are immunologically distinct (unpublished data). We have noticed that if the enzyme is stored in a buffer without dithiothreitol, it loses activity over a period of time, which suggests that certain sulphydryl groups may have an essential role in the catalytic action of the enzyme. In order to gain an insight into the structure of the active site and catalytic mechanism of the enzyme, we have recently initiated studies to elucidate the roles of critical residues in the catalytic action of the enzyme. In this communication, we report the results of modification studies elucidating the role of reactive cysteine residues. For the enzyme modification, we have employed the sulphydryl reagent, *N*-ethymaleimide (NEM) [21], which has previously been successfully utilized for modification of cysteine residues [13,22,23].

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2. MATERIALS AND METHODS

2.1. *Materials*

NEM was from Sigma Chemical Co. (Poole, Dorset, England). [^{14}C]NEM was purchased from Amersham International plc (Amersham, England). Fast desalting column HR 10/10 was from Pharmacia

(Uppsala, Sweden). Ready Gel scintillation cocktail was from Beckman Instruments Inc. (Fullerton, CA, USA). All other reagents were obtained as described previously [20].

2.2. *Purification of IDH*

$NADP^+$-linked IDH from rat liver cytosol was purified and stored as described previously [20]. The enzyme is apparently homogeneous as judged by SDS-PAGE. On the day of the experiment, the enzyme was desalted into an appropriate buffer using a Pharmacia fast desalting column, HR 10/10.

2.3. *Enzyme assay*

The enzyme was assayed spectrophotometrically at 25°C by monitoring the production of NADPH at 340 nm. The reaction medium, in a volume of 1.4 ml, contained 33 mM Tris-HCl, pH 7.4, 0.33 mM EDTA, 0.1 mM $NADP^+$, 1.33 mM $MnCl_2$ and 1.3 mM DL-isocitrate. Reactions were initiated by the addition of enzyme. One unit of enzyme forms 1 mmol of NADPH/min at 25°C.

The enzyme concentrations are stated in terms of active-site centres. These were calculated from the specific activity of the pure enzyme (73 μmol NADPH formed/min/mg protein) and the sub-unit M_r of 45,000.

2.4. *Radioactivity measurement*

Radioactivity was counted (Auto DPM programme) in 5 ml Ready Gel scintillation cocktail using a Beckman LS 6000TA liquid scintillation counter. All samples were counted at least in duplicate.

2.5. *Modification of IDH with NEM*

Purified IDH (2.5 U/ml) was incubated at 25°C in 100 mM Tris-HCl (pH 7.5), 1 mM EDTA with NEM (0.1–0.25 mM). At appropriate times during the inactivation, aliquots were withdrawn and assayed for the enzyme activity. The rate constant (K_{obs}) for each concentration of NEM was calculated from the slope of semi-logarithmic plots of the respective residual activities against time. In protection studies, the enzyme was incubated with substrates (as indicated in the figure legend) prior to initiating the modification with NEM. Control experiments were performed in the absence of NEM.

In order to investigate the pH dependence of the inactivation process, incubations were carried out with 80 mm NEM in the above mentioned buffer over a pH range of 7.5–9.

2.6. *Stoichiometry of NEM inhibition*

The enzyme (2 μm sub-unit concentration) was allowed to react with [^{14}C]NEM (0.15 mM, 9 mCi/mmol) as described above. At different times, samples were spotted on Whatman 3 MM filter paper squares and washed (3 × 30 min) in 200 ml of 10% (w/v) trichloroacetic acid followed by rinsing in acetone. The paper squares were then dried and counted. The control experiments, without the enzyme, showed that very little (< 2% of enzyme incorporated) radioactivity remained bound to the filter papers following the above washing procedure.

A similar experiment as above was also performed in the presence of 1.4 mM DL-isocitrate in order to assess the effect of this protecting ligand on the pattern of incorporation of [^{14}C]NEM into the enzyme.

3. RESULTS

3.1. *Kinetics of inactivation of IDH by NEM*

Incubation of IDH with an excess of NEM resulted in a time- and concentration-dependent inactivation of the enzyme (see Fig. 1A). The inactivation process followed pseudo-first order kinetics with K_{obs} of 0.95 ± 0.07 min^{-1} (mean ± S.D., $n = 6$) at 0.15 mM of NEM and 25°C at pH 7.5. The relative rapidity of inactivation ($K_{obs} = 0.95$ min^{-1}) at low NEM concentration (0.15 mM) is highly indicative of specific modification of sulphydryl groups. Non-specific modification would proceed much more slowly even at higher NEM concentrations. The rate of inactivation of the enzyme as a function of NEM concentration was linear (Fig. 1B), suggesting that no reversible complex between enzyme and NEM is formed. From the slope, the second order rate constant of 6.6×10^3 $M^{-1} \cdot min^{-1}$ was estimated.

The inactivation process can be represented as:

$$IDH + n\text{NEM} \rightarrow \text{IDH-NEM}_n \quad (1)$$

where n equals the average order of the reaction with respect to NEM, and IDH-NEM_n is the modified enzyme.

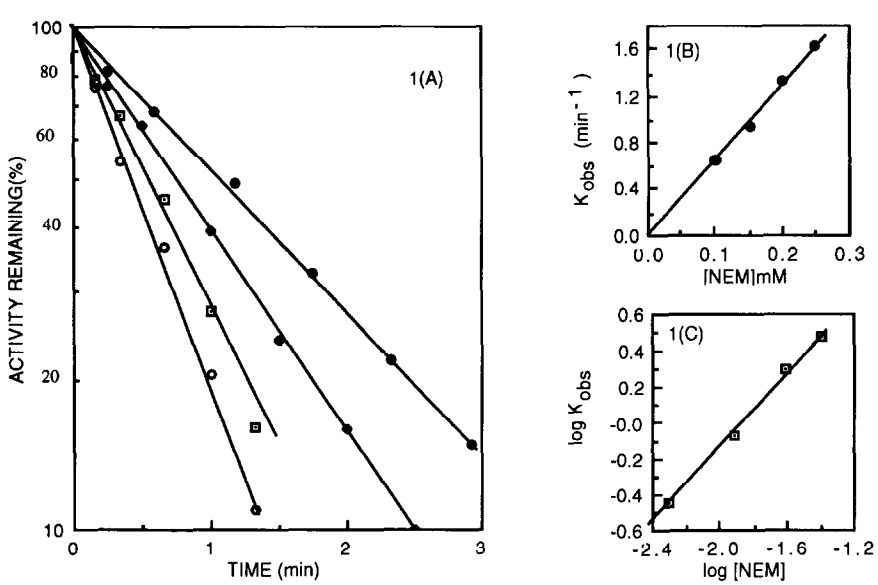

Fig. 1. Time-course of inactivation of IDH by NEM. (A) Purified IDH was treated with NEM as described in section 2. NEM concentrations were 0.1 mM (●), 0.15 mM (■), 0.2 mM (□) and 0.25 mM (○). (B) K_{obs}, the pseudo-first order rate constant (determined from lope of the inactivation lines) was plotted against NEM concentration. (C) A double-logarithmic plot of inactivation rates against concentration of NEM. The slope of the graph, which is approximately 1, indicates that modification of a single sulphydryl group is involved in the inactivation of IDH.

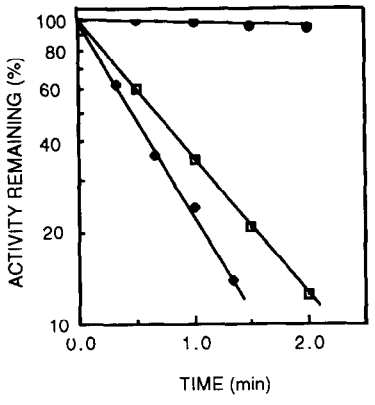

Fig. 2. Effect of substrates on the rate of inactivation of IDH by NEM. IDH was incubated with NEM (0.15 mM, □) in the presence of DL-isocitrate (1.33 mM, ●) and $NADP^+$ (0.1 mM, ■). The residual enzyme activity was measured as described in section 2. The concentration of active isomer D_s-isocitrate is half that of DL-isocitrate.

The inactivation of the enzyme can be described by the differential equation:

$$-d[IDH]/dt = k[IDH]\cdot[NEM]^n \quad (2)$$

Under the experimental conditions of [NEM] >> [IDH], integration yields:

$$\log K_{obs} = \log K + n\log[NEM] \quad (3)$$

A double-log plot of K_{obs} against [NEM] [24] gave a straight line (Fig. 1C) with a slope of ~ 1, which is the reaction order with respect to NEM. This indicates that only 1 mole of NEM reacts with 1 mole enzyme sub-unit to cause the inactivation of the enzyme.

The effect of incubating the enzyme with NEM in the presence of ligands is shown in Fig. 2. Substantial protection against inactivation is afforded by isocitrate. In contrast, $NADP^+$ enhances the rate of modification of the enzyme by 40%.

3.2. *Determination of the stoichiometry of the inactivation reaction with NEM*

The time-dependent incorporation of [^{14}C]NEM into IDH is shown in Fig. 3. The data indicate that the complete loss of enzyme actvity is associated with modification of 2 NEM reactive sulphydryl residues per enzyme sub-unit.

If the modification is carried out in the presence of isocitrate, however, then only 1 mole of NEM is incorporated per mole of enzyme sub-unit without inactivation of the enzyme (data not shown). This indicates that only one of the two sulphydryl residues is essential for the catalytic action of the enzyme. Binding of isocitrate prevents modification of this residue.

3.3. *pH dependence of the rate of inactivation of IDH by NEM*

As shown in Fig. 4A, the pseudo-first order rate constant increases more than 4-fold over the pH range 7.5–9.0. At pH 9.0 the rate of inactivation only approximates to pseudo-first order kinetics. Therefore, studies at higher pH were not attempted. If it is assumed that the protonated form of the reacting group in the enzyme is inactive and the unprotonated residues are modified by NEM, then the following equation can be used to describe the dependence of the rate constant on pH:

$$K_{obs} = \frac{(K_{obs})_{max}}{1+[H^+]/K_a} \quad (4)$$

which yields:

$$K_{obs}[H^+] = K_a(K_{obs})_{max} - K_aK_{obs} \quad (5)$$

where K_a = the dissociation constant of the reacting group, and $(K_{obs})_{max}$ = the pseudo-first order rate constant of the unprotonated reacting group.

A plot of $K_{obs}[H^+]$ vs. K_{obs} (Fig. 4B) gave a straight line from which values of $(K_{obs})_{max} = 2.25$ min^{-1} and $pK_a = 8.13$ were estimated. The theoretical curve obtained using the above values fits the experimental data reasonably well (Fig. 4A). The value of 8.13 obtained in this study is in the right region for a cysteine residue which in the free form has a value in the 8–8.6 range, although the neighbouring micro-environment groups around it may conceivably shift its pK_a.

4. DISCUSSION

In the present study, rapid inactivation of rat liver cytosolic IDH by chemical modification has been achieved with the sulphydryl reagent, NEM. The modifying reagent inactivates IDH with a second order rate constant of 6.6×10^3 $M^{-1}\cdot min^{-1}$ at pH 7.5 and 25°C. The studies on the stoichiometry of the cysteine modification using [^{14}C]NEM indicate that 2 cysteine residues/sub-unit are modified during the inactivation of the enzyme, however, in the presence of isocitrate, only one mole of NEM is incorporated per mole of enzyme subunit without inactivation. This further supports the re-

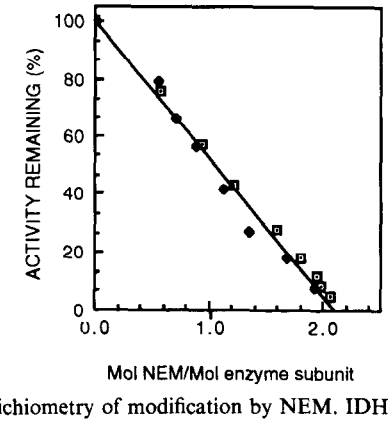

Fig. 3. Stoichiometry of modification by NEM. IDH was incubated with [^{14}H]NEM as described in section 2. The data shown are from two independent experiments.

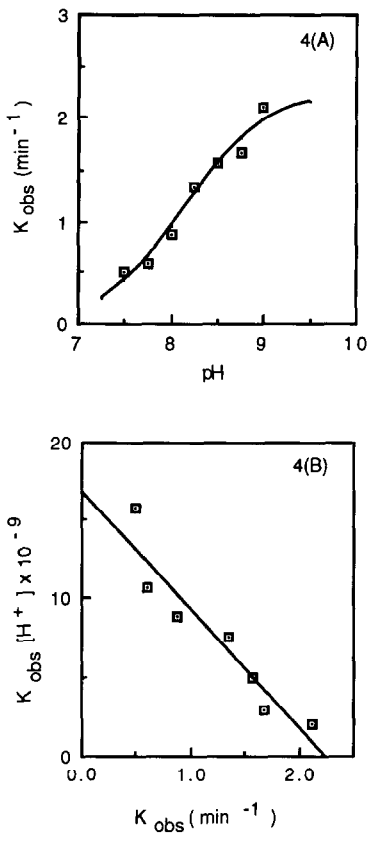

Fig. 4. Effect of pH on the inactivation rates of IDH. (A) The enzyme was incubated with 80 μM NEM between pH 7.5 and 9.0 as described in section 2. Pseudo-first order rate constants were plotted against pH. The points in the figure represent the experimental values, whereas the solid line was calculated with the values of $(K_{obs})_{max}$ = 2.25 min^{-1} and a pK_a of 8.13 deduced from Fig. 4B. (B) The figure represents the plot of $K_{obs}[H^+]$ against K_{obs} according to Eqn. 5.

sults of the kinetic analysis of the inactivation process by the method of Levy et al. [24] which have shown that modification of only one cysteine residue/sub-unit is responsible for the catalytic inactivation of the enzyme. The second cysteine residue is thus apparently not involved in the catalytic action of the enzyme. Significant protection against inactivation was afforded by isocitrate. This observation could be interpreted to mean that the essential cysteine residue is located in the isocitrate binding site, however, the alternative interpretation, that binding of isocitrate causes a conformational change in the enzyme which renders the cysteine residue inaccessible to NEM, can not be ruled out. Enhancement of the inactivation rate in the presence of $NADP^+$ supports a model involving substrate-induced conformational changes. Such conformational changes in the enzyme upon substrate binding are well documented for IDH [8,9]. The above results indicate that the critical residue may be located at the active site of the enzyme. In the absence of the knowledge of the primary sequence of the enzyme and the sequence of amino acids around the modified cysteine, it is not possible to identify the essential residue. Analysis of the pH profile of the inactivation process with NEM suggests that a critical residue with a pK_a of 8.1 is involved. This further supports the view-point that the amino acid residue modified by NEM is an essential cysteine residue.

The results obtained in this study may be compared with those involving the modification of sulphydryl groups with NEM in the catalytic action of the pig heart mitochondrial IDH [13]. Two functional groups have been shown to be modified, of which one was responsible for inactivation of the enzyme, while modification of the second residue caused a 3- to 4-fold increase in the K_m for isocitrate. Isocitrate, in the presence of $MnSO_4$, afforded considerable protection and a specific reaction at the active site of the enzyme was indicated.

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