Evidence for an arginine residue at the coenzyme-binding site of *Escherichia coli* isocitrate dehydrogenase

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The arginine-specific reagent phenylglyoxal inactivated the active, dephosphorylated, form of *Escherichia coli* isocitrate dehydrogenase rapidly in a pseudo-first-order process. Both NADP⁺ and NADPH protected the enzyme against inactivation. Phenylglyoxal appeared to react with one arginine residue per subunit, and the extent of the reaction was proportional to the extent of the inactivation. In contrast, the phosphorylated form of isocitrate dehydrogenase did not react detectably with phenylglyoxal. The data indicate that the coenzyme-binding site of isocitrate dehydrogenase contains a reactive arginine residue that is protected by phosphorylation, and are consistent with the hypothesis that phosphorylation of the enzyme occurs close to or at its active site.

INTRODUCTION

The isocitrate dehydrogenase (NADP-linked, EC 1.1.1.42) of *Escherichia coli* is regulated by a reversible phosphorylation system whose biological role is to apportion carbon flux between the tricarboxylic acid cycle and the glyoxylate by-pass during growth on acetate [1–4]. Many of the regulatory properties of the system are now well understood (reviewed in [5]).

One feature of particular interest is that phosphorylation of a single serine residue per subunit inactivates isocitrate dehydrogenase essentially completely [6,7]. This is unusual; in many eukaryotic systems, phosphorylation of an enzyme affects K_m , K_a or K_i values rather than its maximum catalytic capacity. Phosphorylated isocitrate dehydrogenase is unable to bind the coenzyme NADP⁺ [8]. Phosphorylation of, and binding of NADP⁺ to, the active dephosphorylated form of the enzyme seem to give rise to similar conformational changes [8]. These results led to the proposal that the phosphorylation site in isocitrate dehydrogenase is close to its active site and that the introduced phosphate group can interact directly with a positively charged group at the coenzyme-binding site [8,9]. This hypothesis has been supported by recent site-directed mutagenesis studies [10]. Replacement of the phosphorylatable serine residue (Ser-113) by an aspartate residue resulted in complete loss of isocitrate dehydrogenase activity, suggesting that the incorporation of a negative charge plays an important role in the loss of activity caused by phosphorylation [10].

We have therefore attempted to identify groups at the active site of isocitrate dehydrogenase that may play a role in the inactivation of the enzyme by phosphorylation. Here we use chemical modification to show that the coenzyme-binding site contains a uniquely reactive arginine residue that is completely protected in phosphorylated enzyme.

EXPERIMENTAL

Materials

Phenylglyoxal was from Sigma Chemical Co., Poole,

Dorset, U.K., and [7-¹⁴C]phenylglyoxal was from Amersham International, Aylesbury, Bucks., U.K. Other chemicals and biochemicals were obtained as described previously [7,11].

Purification of enzymes

The active form of isocitrate dehydrogenase from *E. coli* ML308 grown on glycerol was prepared as described in ref. [11]. The final step of this purification involves elution of the enzyme from Procion Red-Sepharose with NADP⁺. The phosphorylated form of isocitrate dehydrogenase was prepared by using isocitrate dehydrogenase kinase/phosphatase as described in ref. [7].

To isolate isocitrate dehydrogenase without bound coenzyme, *E. coli* TG1/pCK505 was used. Plasmid pCK505 (kindly given by Professor D. E. Koshland, Jr.) bears the *icd* gene encoding isocitrate dehydrogenase [12], and this strain over-expresses this enzyme some 10-fold relative to the wild-type (results not shown). Cells were grown on L broth containing 100 µg of ampicillin/ml at 30 °C until late exponential phase, then harvested and washed as described in ref. [11]. Preparation of a 60–75%-(NH₄)₂SO₄ precipitate was as described in ref. [11]. This pellet was dissolved in 20 mM-potassium phosphate buffer, pH 6.5, containing 5 mM-potassium citrate and 2 mM-MgCl₂ and dialysed extensively against this buffer. It was filtered and loaded on to a Pharmacia HR10/10 Mono Q column equilibrated in this buffer. The enzyme was eluted with a gradient of 20–200 mM-potassium phosphate buffer, pH 6.5, containing 5 mM-potassium citrate and 2 mM-MgCl₂, run over 25 min at 4 ml/min. The isocitrate dehydrogenase eluted from this column was homogeneous as judged by SDS/polyacrylamide-gel electrophoresis (results not shown).

Assay procedures

Isocitrate dehydrogenase activity [11] and protein concentration [13] were assayed as described previously.

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Inactivation of isocitrate dehydrogenase with phenylglyoxal

Isocitrate dehydrogenase (0.2 mg/ml) was incubated in 50 mM-Mops/NaOH buffer, pH 7.3, containing 1 mM-EDTA at 0 °C with phenylglyoxal (0–30 mM) and other additions as specified in the text. Samples were assayed for isocitrate dehydrogenase activity at various times after the addition of phenylglyoxal. Half-lives and apparent pseudo-first-order rate constants were calculated from semi-logarithmic plots of activity remaining against time. Unless stated otherwise, the enzyme used was isolated from *E. coli* ML303.

Incorporation of [7-¹⁴C]phenylglyoxal into isocitrate dehydrogenase

Incubations were carried out as described above except that [7-¹⁴C]phenylglyoxal (10 mM; 5 μ Ci/ μ mol) was used. To quantify protein-bound radioactivity, samples (10 μ l) were spotted on to 2.5 cm-diameter filter-paper discs. Discs were washed for 4 $\times$ 15 min in 10 % (w/v) trichloroacetic acid (10 ml/disc), dried and their radioactivities counted. Control experiments showed that this washing procedure completely removed unused phenylglyoxal and that the protein-bound radioactivity was stable over the duration of the washing.

RESULTS

Inactivation of isocitrate dehydrogenase with phenylglyoxal

Phenylglyoxal inactivated the dephosphorylated form of isocitrate dehydrogenase rapidly in a pseudo-first-order process, and both NADP⁺ and NADPH afforded protection against this inactivation. Typical data are shown in Fig. 1. The half-life of the enzyme in the presence of 20 mM-phenylglyoxal at pH 7.3 and 0 °C, without coenzyme, was 2.9 ± 0.6 min (mean $\pm$ s.d., $n = 15$). A plot of apparent pseudo-first-order rate constant against phenylglyoxal concentration showed biphasic behaviour (Fig. 2). In contrast with the effects of phenylglyoxal, two other arginine-specific reagents, butane-2,3-dione and cyclohexane-1,2-dione, gave little or no inactivation of isocitrate dehydrogenase at concentrations of up to 150 mM and 50 mM respectively.

The protection against inactivation afforded by NADP⁺ was investigated further by measuring the half-life of the enzyme at different NADP⁺ concentrations (Fig. 3). The K_s for NADP⁺ was found to be 60 μ M: this should be compared with a K_m of 36 μ M determined in the presence of 0.03 mM-DL-isocitrate under somewhat different conditions (results not shown).

The presence of DL-isocitrate also protected the isocitrate dehydrogenase isolated from strain ML308 against inactivation by phenylglyoxal (results not shown). This result is in apparent conflict with the fact that the enzyme obeys a compulsory-order mechanism with NADP⁺ binding first [14]. One possible explanation of this discrepancy is that the purified enzyme contains small amounts of NADP⁺. The presence of NADP⁺ in the isocitrate dehydrogenase isolated from *E. coli* ML308 was confirmed fluorimetrically after reduction to NADPH, and was presumably due to incomplete removal by dialysis of the coenzyme used in elution of the Procion Red-Sepharose column. Isocitrate dehydrogenase free of NADP⁺ was therefore isolated from *E. coli*

Fig. 1. Effect of coenzyme on the inactivation of isocitrate dehydrogenase by phenylglyoxal

Isocitrate dehydrogenase from *E. coli* ML308 was inactivated with 20 mM-phenylglyoxal as described in the Experimental section either alone ($\blacktriangle$) or in the presence of 100 μ M-NADPH ($\triangle$) or 200 μ M-NADP⁺ ($\circ$). Enzyme from strain TG1/pCK505 was inactivated with 20 mM-phenylglyoxal in the presence ($\square$) or in the absence ($\blacksquare$) of 100 μ M-DL-isocitrate.

Fig. 2. Effect of phenylglyoxal concentration on the rate of inactivation of isocitrate dehydrogenase

Enzyme was inactivated with phenylglyoxal with no other additions. The apparent pseudo-first-order rate constants (k'_{app}) were estimated as described in the Experimental section.

Fig. 3. Protection of isocitrate dehydrogenase against phenylglyoxal by NADP⁺

Enzyme was inactivated with 20 mM-phenylglyoxal in the presence of the indicated concentration of NADP⁺. Half-lives were estimated as described in the Experimental section.

TG1/pCK505. Inactivation of this enzyme by phenylglyoxal, and protection against inactivation by NADP⁺, were qualitatively similar to those for the enzyme from *E. coli* ML308. However, DL-isocitrate (100 μM) gave no detectable protection of the enzyme from strain TG1/pCK505 (Fig. 1).

Stoichiometry of the reaction of isocitrate dehydrogenase with phenylglyoxal

The incorporation of [7-¹⁴C]phenylglyoxal into both active and phosphorylated isocitrate dehydrogenase was measured as described in the Experimental section. The results of a representative time-course experiment are shown in Fig. 4. Incorporation of ¹⁴C radioactivity into the active form of the enzyme proceeded in parallel with its loss of activity. This is illustrated more clearly in Fig. 5, which shows a plot of the extent of incorporation of phenylglyoxal into the protein against the extent of inactivation for active dehydrogenase, either alone or in the presence of 200 μM-NADP⁺ or 100 μM-DL-isocitrate. These results reveal that incorporation of approx. 1 mol of phenylglyoxal/mol of isocitrate dehydrogenase subunits gave complete inactivation of the enzyme. In replicate experiments in the absence of NADP⁺ or isocitrate, complete inactivation corresponded to incorporation of 0.98 ± 0.06 mol of phenylglyoxal/mol of subunits (mean ± S.D., *n* = 4).

In contrast with the active form of isocitrate dehydrogenase, the phosphorylated form seemed to be completely resistant to reaction with phenylglyoxal (Fig. 4). In four separate experiments, incubation of phosphorylated isocitrate dehydrogenase with 10 mM-[7-¹⁴C]phenylglyoxal for up to 150 min resulted in no detectable incorporation of radioactivity into the protein. To confirm that this change in the susceptibility of the arginine residue(s) of isocitrate dehydrogenase to chemical modification was specifically due to phosphorylation, the phosphorylated enzyme was fully dephosphorylated and re-activated by incubation with isocitrate dehydrogenase kinase/phosphatase as described in ref. [7]. After overnight dialysis of the re-activated enzyme, addition of

Fig. 4. Incorporation of [7-¹⁴C]phenylglyoxal into isocitrate dehydrogenase

Incorporation of ¹⁴C radioactivity into the active (●) and phosphorylated (○) forms of isocitrate dehydrogenase with no other additions was carried out as described in the Experimental section. The activity of the active form of the enzyme was measured in the same experiment (▲).

Fig. 5. Correlation between the incorporation of [7-¹⁴C]phenylglyoxal into isocitrate dehydrogenase and inactivation of the enzyme

Experiments similar to that shown in Fig. 4 were carried out with the isocitrate dehydrogenase of *E. coli* ML308 either alone (○ and □, two independent experiments) or in the presence of 200 μM-NADP⁺ (▲) or 100 μM-DL-isocitrate (●).

phenylglyoxal caused a rapid loss of isocitrate dehydrogenase activity, similar to the results in Figs. 1 and 4 (results not shown).

DISCUSSION

Arginine residues are commonly found at anion-binding sites in proteins (see, e.g., [15] and [16]). The inactivation and protection data reported in Figs. 1 and 3 indicate that *E. coli* isocitrate dehydrogenase contains at least one arginine residue at its coenzyme-binding site. Isocitrate does not protect against inactivation provided that coenzyme is absent; this is consistent with the compulsory-order kinetic mechanism of isocitrate dehydrogenase [14]. It is interesting to note both that the apparent pseudo-first-order rate constant for the inactivation by phenylglyoxal is not linearly related to the reagent concentration (Fig. 2) and that two other arginine-specific reagents have very little effect on the enzyme. The data suggest that phenylglyoxal may have some affinity for the coenzyme-binding site of isocitrate dehydrogenase, although simple saturation kinetics are not obeyed (Fig. 2).

We have consistently observed that the incorporation of one molecule of phenylglyoxal per isocitrate dehydrogenase subunit completely inactivates the enzyme (Fig. 5). The stoichiometry of the reaction between phenylglyoxal and arginine residues in proteins has been quoted as 2:1 (e.g. [17] and [18]) or 1:1 [19]. It has been suggested that the reaction stoichiometry depends on the environment of the arginine residue involved [15,19]. The simplest interpretation of our data is that the coenzyme-binding site of isocitrate dehydrogenase contains one arginine residue that reacts with one molecule of phenylglyoxal. We cannot, however, rule out the possibility that the phenylglyoxal/arginine stoichiometry is 2:1 and that the enzyme displays 'half-of-the-sites' reactivity in this context. Accurate determination of the stoichiometry of the reaction, by comparison of ^{14}C incorporation with the destruction of arginine residues, would be very difficult because of the presence of a total of 17 arginine residues per subunit [10]. However, the results suggest that only one residue is particularly reactive towards phenylglyoxal. Similar observations have been made for glyoxal. We cannot, however, rule out the possibility (e.g. [15] and [16]).

Phosphorylation of isocitrate dehydrogenase has a dramatic effect on the susceptibility of the enzyme to reaction with phenylglyoxal. It completely protects the residue at the coenzyme-binding site that reacts in the dephosphorylated enzyme. This result is consistent with the hypothesis that the phosphorylation site in isocitrate dehydrogenase is close to its active site and that the introduced phosphate group can interact with a positively charged group at the coenzyme-binding site [8,9]. It is interesting to note in this context that the phosphorylatable serine residue is immediately C-terminal to an arginine residue [10,20,21]. However, we cannot rule out the possibility that the phosphorylation site is spatially

remote from the active site but that phosphorylation triggers a conformational change that prevents binding of the coenzyme. Attempts to distinguish between these possibilities by identifying the arginine residue that reacts with phenylglyoxal have been unsuccessful, because the arginine-phenylglyoxal adduct formed in this case is not stable under the conditions required for isolation of peptides (results not shown). Although the 2:1 phenylglyoxal-arginine adduct is stable under acid conditions [17], in several cases the modification of an active-site arginine residue has yielded a product that is not stable enough to allow isolation of labelled peptides [15]. The lack of stability observed here is consistent with the interpretation (see above) that a 1:1 adduct is formed in this case. However, because of this lack of stability, the possibility that the arginine residue at the coenzyme-binding site is residue 112, immediately adjacent to the phosphorylatable serine residue 113 [10], has not been subjected to a direct test.

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