

Photoaffinity labelling shows that *Escherichia coli* isocitrate dehydrogenase kinase/phosphatase contains a single ATP-binding site

Isabel Varela* and Hugh G. Nimmo

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland

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Ultraviolet irradiation of *E. coli* isocitrate dehydrogenase kinase/phosphatase in the presence of 8-azidoATP resulted in parallel losses of its kinase and phosphatase activities, and in covalent attachment of the reagent to the protein at a single site. ATP and ADP protected the two activities to similar extents. The data suggest that the activation of the phosphatase by adenine nucleotides results from binding of the nucleotides to the active site of the kinase.

Photoaffinity labeling; 8-AzidoATP; Bifunctional enzyme; Isocitrate dehydrogenase kinase/phosphatase

1. INTRODUCTION

During growth of *Escherichia coli* on acetate, the competition between the tricarboxylic acid cycle and the glyoxylate bypass for the available isocitrate is resolved by partial inactivation and phosphorylation of isocitrate dehydrogenase (ICDH) [1–4]. It has been shown that ICDH kinase and ICDH phosphatase activities can be co-purified to homogeneity [5] and that a single gene, termed *aceK*, encodes both activities [6]. Thus ICDH kinase/phosphatase is a member of the growing family of bifunctional regulatory en-

zymes, examples of which have been found in animal, plant and bacterial systems (e.g. [7–10]). There is considerable interest in the structural organisation, evolution and metabolic role of these enzymes.

ICDH kinase/phosphatase provides a favourable model system for an investigation of these questions. The *aceK* gene has been cloned and over-expressed [6,11] and much is known about the regulation of the kinase/phosphatase and its role in the control of metabolic flux [12–14]. One unusual feature of the ICDH phosphatase activity of the bifunctional enzyme is that it shows an absolute requirement for an adenine nucleotide, either ADP or ATP [5,15]. Since ATP is a substrate and ADP an inhibitor of the kinase, this highlights the question of whether the kinase and phosphatase activities are catalysed at a single site, overlapping sites or spatially distinct sites. The nucleotide sequence of the *aceK* gene has recently been reported [16], but it throws little light on the structural organization of the enzyme. In this paper we report the effects of the photoaffinity labelling reagent 8-azidoATP on the activities of ICDH kinase/phosphatase.

Correspondence address: H.G. Nimmo, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland

* *Present address:* Metabolismo, Nutricion y Hormonas, Instituto de Investigaciones Biomedicas del CSIC, Fundacion Jimenez Diaz, Avenida Reyes Catolicos 2, 28040 Madrid, Spain

Abbreviations: ICDH, isocitrate dehydrogenase; FPLC, fast protein liquid chromatography; Mops, 4-morpholinepropane-sulphonic acid

2. EXPERIMENTAL

2.1. Materials

8-AzidoATP was from Sigma and 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (5.1 Ci/mmol) was from ICN Radiochemicals. Other materials were obtained or prepared as described in [15,17].

2.2. Purification of ICDH kinase/phosphatase

E. coli strain KAT-1/pEM9, which over-expresses the glyoxylate bypass operon of *E. coli* ML308 [11], was grown on acetate, harvested and stored as in [17]. Cell breakage and protamine sulphate treatment were as in [17] except that the extraction buffer was buffer A (50 mM Mops/NaOH, pH 7.3, 1 mM EDTA, 1 mM benzamidine) containing 0.05% 2-mercaptoethanol, 1.2 mM phenylmethylsulphonyl fluoride and 125 mM NaCl. The protamine sulphate supernatant was diluted with buffer A containing 400 mM NaCl so that its conductivity was equal to that of buffer A containing 150 mM NaCl. The material was then loaded onto a column of DEAE-cellulose equilibrated in this buffer and eluted with buffer A containing 0.05% 2-mercaptoethanol and 250 mM NaCl [15]. The active fractions were diluted with buffer A to reduce the NaCl concentration to 100 mM and NAD^+ was added to a concentration of 1 mM. The material was then chromatographed on blue dextran-Sepharose and concentrated as in [15].

The concentrated enzyme was diluted with buffer A containing 1 mM dithiothreitol and 0.05% Lubrol PX to reduce the NaCl concentration to 100 mM. The material was loaded onto an FPLC MonoQ column and eluted with a gradient of 100–500 mM NaCl in this buffer run over 25 min at 1 ml/min. The active fractions were diluted with buffer B (50 mM Bicine/NaOH, pH 9.0, 1 mM dithiothreitol, 1 mM benzamidine, 0.05% Lubrol PX) to reduce the NaCl concentration to 100 mM, loaded onto a MonoQ column and eluted with a gradient of 100–500 mM NaCl in buffer B run as above. In each case the kinase/phosphatase was eluted at approx. 0.34 M NaCl. The pooled fractions from the second MonoQ column were dialysed overnight into buffer A containing 1 mM dithiothreitol, 100 mM NaCl and 0.05% Lubrol PX, concentrated by centrifugation through a Centricon 30 filter (Amicon) to approx. 0.1 ml and chromatographed on an FPLC Superose 12 column at a flow rate of 0.3 ml/min. The pooled fractions from this column were homogeneous by the criterion of SDS-polyacrylamide gel electrophoresis.

2.3. Enzyme assays

ICDH kinase was assayed by monitoring the inactivation of *E. coli* ICDH and ICDH phosphatase by monitoring the release of ^{32}P from ICDH that had been phosphorylated in vitro using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ [15].

2.4. Photoaffinity labelling of ICDH kinase/phosphatase

Solutions of 8-azidoATP, 50 mM in 50 mM Mops/NaOH, pH 7.3, 1 mM EDTA, were stored at -20°C in darkness. ICDH kinase/phosphatase was dialysed into buffer A containing 0.2 mM dithiothreitol and 0.05% Lubrol PX to minimise the reaction between 8-azidoATP and dithiothreitol [18]. The enzyme (kinase activity 2–5 nmol/min) was incubated in this buffer with 8-azidoATP, 10 mM MgCl_2 and other additions as indicated in a final volume of 0.1 ml in 1 ml quartz cuvettes at

0°C . The enzyme was pre-incubated with the nucleotides in darkness for 20 min prior to irradiation to allow complete equilibration to occur. The samples were irradiated at 254 nm, 5 cm from a UVSL-58 lamp (UV Products Inc.).

At each time point, duplicate samples were removed, kept in darkness at 0°C and then assayed as described above. In some experiments 10 μCi of 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ per incubation was included. Samples (25 μl) were removed, denatured and analysed on 10% polyacrylamide gels in the presence of 0.1% SDS [19]. After electrophoresis the gel was soaked for 30 min in 10% isopropanol, 10% acetic acid and rinsed thrice in water to remove unbound label, stained with Coomassie blue, destained, dried and autoradiographed for 48 h at -70°C with an intensifying screen.

For peptide mapping, enzyme after the second MonoQ column (approx. 15 μg kinase/phosphatase) was labelled with 10 mM 8-azidoATP (20 $\mu\text{Ci}/\mu\text{mol}$), denatured and subjected to

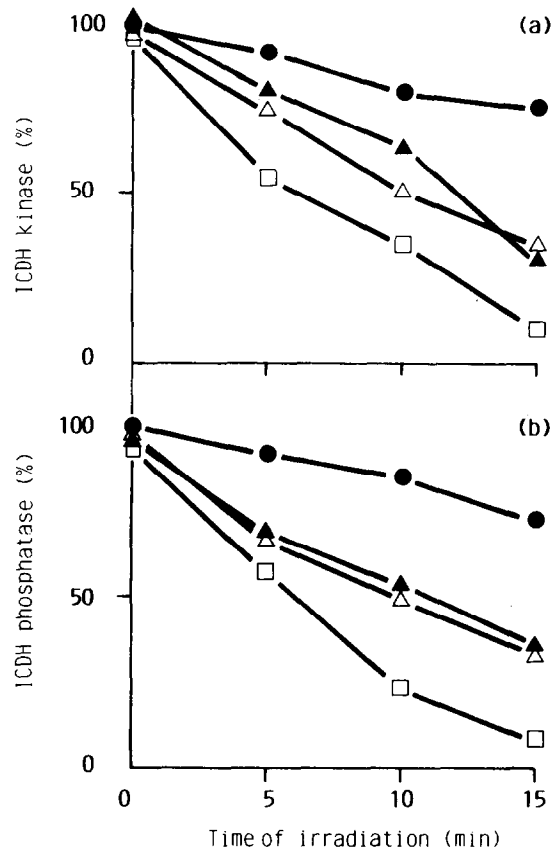


Fig.1. Photoinactivation of ICDH kinase and ICDH phosphatase by 8-azidoATP. ICDH kinase/phosphatase was irradiated as described in section 2 for the indicated times in the absence of 8-azidoATP (●) or in the presence of 5 mM 8-azidoATP with no other additions (□), 5 mM ADP (Δ) or 5 mM ATP (▲). ICDH kinase (a) and ICDH phosphatase (b) activities are expressed as a percentage of the control activity (no 8-azidoATP) at zero time.

gel electrophoresis as above. The band corresponding to the kinase/phosphatase was excised from the gel, dried, rehydrated and digested with trypsin for 24 h as described [20]. The peptide mixture was resolved on a Waters μ Bondapak C18 reverse-phase column using a linear, 0–100% gradient of acetonitrile in 0.1% trifluoroacetic acid over 30 min at 1 ml/min.

3. RESULTS

In preliminary experiments, carried out in darkness to prevent possible photoinactivation of the enzyme, we found that 5 mM 8-azidoATP was unable to replace ATP as a substrate for ICDH kinase. However, 8-azidoATP could partially satisfy the adenine nucleotide requirements of ICDH phosphatase; 5 mM 8-azidoATP gave some 15% of the phosphatase activity obtained with an almost saturating concentration, 0.625 mM, of ATP.

Preincubation of ICDH kinase/phosphatase with 8-azidoATP in darkness resulted in no significant loss of either activity (not shown). Irradiation in the absence of 8-azidoATP gave slight losses of activity but irradiation in the presence of

8-azidoATP gave a marked time-dependent inactivation of both ICDH kinase and ICDH phosphatase. The inactivation observed after 7 min irradiation varied with the 8-azidoATP concentration over the range tested (1–10 mM). A concentration of 5 mM 8-azidoATP was chosen for further experiments and typical time-courses of inactivation of ICDH kinase and ICDH phosphatase are shown in fig.1. ATP and ADP could protect both activities against photoinactivation (fig.1), indicating that the interaction between the enzyme and 8-azidoATP was specific. The kinase and phosphatase activities declined at similar rates (fig.1). Moreover, the concentration dependence of the protection against inactivation afforded by ATP was very similar for the two activities (fig.2).

Irradiation of ICDH kinase/phosphatase in the presence of 8-azido- $[\alpha\text{-}^{32}\text{P}]$ ATP resulted, as expected, in a time-dependent incorporation of ^{32}P into the protein that was abolished in the presence of 20 mM ATP (fig.3). This is in agreement with the observation that 20 mM ATP gives essentially

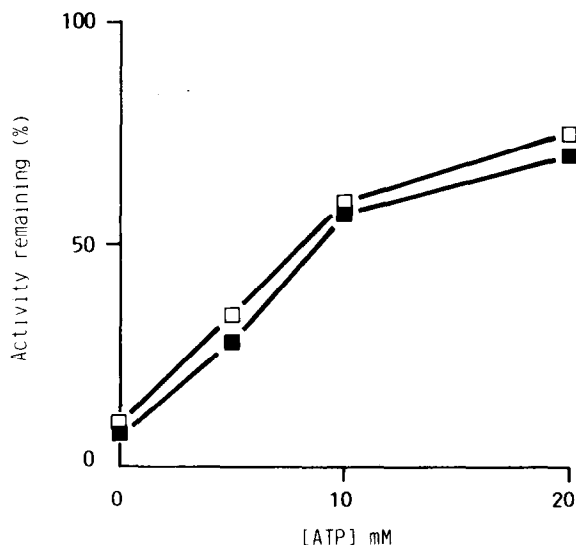


Fig.2. Protection against photoinactivation afforded by ATP. ICDH kinase/phosphatase was irradiated as described in section 2 in the presence of 5 mM 8-azidoATP and the indicated concentrations of ATP for 15 min. ICDH kinase (\square) and ICDH phosphatase (\blacksquare) activities remaining after irradiation are expressed as a percentage of the control activity (no 8-azidoATP) prior to irradiation. Irradiation of the control reduced the kinase and phosphatase activities to 76% and 81%, respectively.

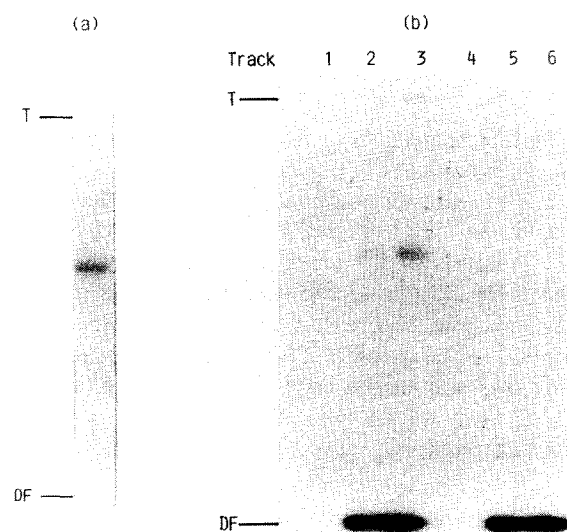


Fig.3. Incorporation of 8-azido- $[\alpha\text{-}^{32}\text{P}]$ ATP into ICDH kinase/phosphatase. Panel a shows an SDS-polyacrylamide gel of purified ICDH kinase/phosphatase. In panel b, the enzyme was incubated with 5 mM 8-azidoATP in the absence (tracks 1–3) or presence (tracks 4–6) of 20 mM ATP. Samples were taken for gel electrophoresis after irradiation times of 0 min (tracks 1 and 4), 10 min (tracks 2 and 5) and 15 min (tracks 3 and 6). T and DF indicate the top of the gel and the dye front, respectively.

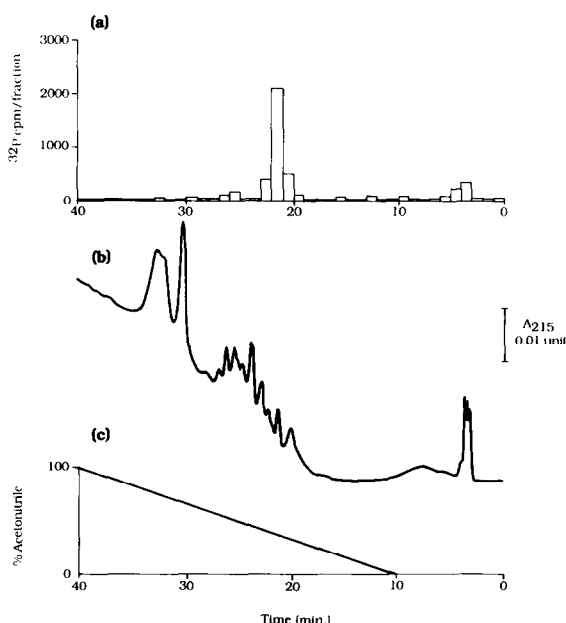


Fig.4. Peptide mapping of ^{32}P -labelled ICDH kinase/phosphatase. The tryptic digest (4000 cpm) was injected onto the column at time 0, and 1 ml fractions were collected. Panel a shows the ^{32}P content of each fraction, panel b the absorbance at 214 nm and panel c the acetonitrile gradient. The background increase in A_{214} is due to the acetonitrile gradient.

complete protection against the photoinactivation due to 8-azidoATP (fig.2).

ICDH kinase/phosphatase was labelled with 8-azido- $[\alpha\text{-}^{32}\text{P}]$ ATP and then digested with trypsin. Analysis of the resulting peptides by reverse-phase HPLC (fig.4) showed that a single peptide peak contained more than 90% of the radioactivity applied to the column. Thus 8-azidoATP reacts at only one site in the enzyme under conditions in which both its kinase and phosphatase activities are destroyed.

4. DISCUSSION

We failed to detect any inactivation of ICDH by ICDH kinase/phosphatase in the presence of 8-azidoATP. Thus ICDH kinase appears unable to transfer the γ -phosphoryl group of 8-azidoATP to ICDH at a significant rate. However the photoinactivation of ICDH kinase by 8-azidoATP, and the protection against this afforded by ATP and ADP (figs 1,2), suggests that the azido

analogue can in fact interact with the active site of the enzyme. It is noteworthy that other nucleotides, including GTP, UTP and CTP, can interact with the enzyme as shown by inhibition studies, yet are not substrates for it [13].

Previous work has shown that ICDH phosphatase is absolutely dependent on the presence of an adenine nucleotide for activity [5,15]. Our data reveal that 8-azidoATP destroys the activity of ICDH phosphatase in parallel with that of ICDH kinase by reacting at a single site in the enzyme. The simplest explanation of these results is that the enzyme contains only a single ATP-binding site, the kinase active site, and that activation of the phosphatase by adenine nucleotides results from binding at this site. While this work was in progress, Stueland et al. [21] reported that ICDH kinase/phosphatase possesses an intrinsic ATPase activity. They suggested that the enzyme contains only one active site and that the phosphatase reaction results from the kinase back reaction tightly coupled to ATP hydrolysis. Our data confirm one prediction of this model, that the enzyme contains only one ATP-binding site.

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