The phosphorylation of *Escherichia coli* isocitrate dehydrogenase in intact cells

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The isocitrate dehydrogenase of *Escherichia coli* ML308 can be reversibly activated by addition of pyruvate to cells growing on acetate [Bennett & Holms (1975) *J. Gen. Microbiol.* **87**, 37–51]. By using cells pulse-labelled with [^{32}P]P_i we showed that the activation and inactivation of the enzyme in these conditions correlate with its dephosphorylation and rephosphorylation respectively. Incubation of cell extracts prepared during an activation/inactivation cycle with purified isocitrate dehydrogenase phosphatase confirmed that the pyruvate-induced activation of the dehydrogenase goes essentially to completion. The results show that the reversible changes in the activity of the dehydrogenase in cells grown on acetate are solely due to phosphorylation/dephosphorylation. Inactive ^{32}P -labelled isocitrate dehydrogenase was isolated from cells incubated with [^{32}P]P_i in the presence of acetate. Both this material and purified enzyme phosphorylated *in vitro* were digested with chymotrypsin, and the phosphopeptides were isolated and analysed. Only one phosphopeptide was observed in each case; the results show that the residue phosphorylated *in vivo* is identical with that phosphorylated by purified isocitrate dehydrogenase kinase *in vitro*.

The NADP-linked isocitrate dehydrogenase of *Escherichia coli* (EC 1.1.1.42) can be regulated by phosphorylation (Garnak & Reeves, 1979*a,b*); this is the first example of control of a key metabolic enzyme by phosphorylation in a prokaryote (reviewed by Nimmo, 1984). During growth on acetate, micro-organisms use the glyoxylate bypass to generate the precursors necessary for biosynthesis; the resulting competition between isocitrate lyase and isocitrate dehydrogenase is resolved in *E. coli* by phosphorylation and inactivation of the latter enzyme. The phosphorylation and dephosphorylation of isocitrate dehydrogenase are mediated by a single bifunctional enzyme, isocitrate dehydrogenase kinase/phosphatase (Laporte & Koshland, 1982; Nimmo *et al.*, 1984). Phosphorylation and dephosphorylation of isocitrate dehydrogenase by the kinase/phosphatase *in vitro* cause inactivation and activation respectively (Nimmo *et al.*, 1984).

Reeves' group have demonstrated that, in several K12 strains of *E. coli*, isocitrate dehydrogenase becomes phosphorylated and its activity declines after the addition of acetate to cultures

grown to stationary phase on glucose, glycerol or succinate (Garnak & Reeves, 1979*a,b*; Malloy & Reeves, 1983; Reeves & Malloy, 1983). However, they have not investigated whether there is a quantitative correlation between phosphorylation and inactivation of the enzyme *in vivo*, nor have they shown that either phosphorylation or inactivation can be reversed *in vivo*. Wang & Koshland (1982) showed that the isocitrate dehydrogenase of *Salmonella typhimurium* could be phosphorylated reversibly *in vivo*, but they did not monitor the activity of the enzyme. Thus it has not yet been rigorously demonstrated that the inactivation of isocitrate dehydrogenase *in vivo* is solely due to its phosphorylation.

The activity of the isocitrate dehydrogenase of *E. coli* ML308 can be manipulated readily by altering the available carbon source; for example, the addition of pyruvate to cells growing on acetate causes a rapid 3–4-fold increase in the activity of the enzyme that is reversed on exhaustion of the pyruvate (Bennett & Holms, 1975). In the present paper we use this elegant experimental system to show that the phosphorylation of isocitrate dehydrogenase *in vivo* correlates with inactivation of the enzyme and that the phosphorylation can be reversed *in vivo*. We also show that the serine resi-

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due phosphorylated *in vivo* is identical with that phosphorylated by the purified kinase/phosphatase *in vitro*.

Experimental

Materials

Radiochemicals were obtained from Amersham International, Amersham, Bucks., U.K. Trifluoroacetic acid and propan-2-ol were from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K. The sources of other chemicals and biochemicals were as described previously (Borthwick *et al.*, 1984; Garland & Nimmo, 1984; Nimmo *et al.*, 1984).

Isocitrate dehydrogenase kinase/phosphatase and the active form of isocitrate dehydrogenase were isolated from *E. coli* ML308 (Borthwick *et al.*, 1984; Nimmo *et al.*, 1984). Phosphorylated isocitrate dehydrogenase was prepared by using kinase/phosphatase and [γ - ^{32}P]ATP as described by Nimmo *et al.* (1984).

Enzyme assays

To measure the activity of isocitrate dehydrogenase in bacterial cultures, samples were diluted, sonicated and assayed as described by Holms & Bennett (1971). During the re-activation of isocitrate dehydrogenase in cell extracts by dephosphorylation, the enzyme was assayed as described by Borthwick *et al.* (1984). One unit of enzyme activity is defined as the amount required to catalyse the formation of 1 μmol of product/min.

Turbidity measurements

Turbidities of cultures were measured at 420 nm in a Unicam SP30 spectrophotometer. A turbidity of 1 corresponds to a cell density of 175 μg dry wt./ml.

Analytical labelling of cells with [^{32}P]P_i

E. coli ML308 (A.T.C.C. 15224) was grown at 37°C on 40 mM-acetate in a low-phosphate medium comprising 0.75 mM-KH₂PO₄, 10 mM-(NH₄)₂SO₄, 2 mM-MgSO₄ and 10 μM -FeSO₄ buffered to pH 7.1 with 62.5 mM-2-[bis-(2-hydroxyethyl)-amino]ethanesulphonic acid/NaOH. Cultures (500 ml) were aerated by vigorous stirring, and the air-space was supplied with air at 400 ml/min. When the cell density reached 440 μg dry wt./ml, pyruvate was added to a final concentration of 4 mM.

At 10 min intervals, samples (5 ml) of the culture were withdrawn, mixed with carrier-free [^{32}P]P_i (final sp. radioactivity 95 Ci/mol) and incubated at 37°C with aeration for 10 min. Samples were then processed for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and for measurement of

the specific radioactivity of cellular ATP. Control experiments showed that the isocitrate dehydrogenase activity in such samples after the 10 min incubation was equal to that in samples taken from the parent culture at the same time.

Polyacrylamide-gel electrophoresis

Samples of the incubation mixtures with [^{32}P]P_i (1 ml) were centrifuged in an Eppendorf 5412 micro-centrifuge for 60 s. The cells were washed twice by resuspension in 1 ml portions of ice-cold 10 mM-Tris/HCl, pH 7.4, and re-centrifugation. The washed cell pellets were resuspended in 0.2 ml of sample buffer [50 mM-Tris/HCl, pH 6.8, 1% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue and 1% (v/v) 2-mercaptoethanol] and heated to 100°C for 2 min. Insoluble material was removed by centrifugation in a microcentrifuge. Samples of the supernatants were subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate on 10% acrylamide slab gels (Laemmli, 1970). The gels were dried and autoradiographed by using Kodak X-OMAT AR film with an intensifying screen. Gel slices were excised, solubilized in 30% (w/v) H₂O₂ and counted for radioactivity in the scintillant described by Borthwick *et al.* (1984).

Measurement of the specific radioactivity of the γ -phosphate group of ATP

A modification of the method of England & Walsh (1976) was used. Samples (1 ml) of the incubations with [^{32}P]P_i were added to 0.2 ml portions of 3 M-HClO₄ and kept at 0°C for 1 h. Precipitated material was removed by centrifugation (20000 g-min) and the supernatants were adjusted to pH 7 with KHCO₃. Precipitated KClO₄ was removed by centrifugation, and the supernatants were removed, freeze-dried, redissolved in water (0.05 ml) and re-centrifuged. At this stage the ATP concentration in each supernatant was about 0.04 mM. Samples (0.04 ml) were incubated with a limiting amount of active isocitrate dehydrogenase (120 pmol of subunits) and isocitrate dehydrogenase kinase (0.01 munit) in 20 mM-NN-bis-(2-hydroxyethyl)glycine/NaOH, pH 9.0, containing 10 mM-MgCl₂ (final volume 0.06 ml) for 3 h. The isocitrate dehydrogenase was fully inactivated by this time. The incorporation of ^{32}P into isocitrate dehydrogenase was measured as described by Nimmo *et al.* (1984) for the assay of isocitrate dehydrogenase kinase. The method was standardized by using solutions of [γ - ^{32}P]ATP of known specific radioactivity. Isocitrate dehydrogenase is a good substitute for the glycogen phosphorylase used by England & Walsh (1976), because only one residue on the dehydrogenase can

be phosphorylated, and the K_m of the kinase for the dehydrogenase is very low (Nimmo *et al.*, 1984; Nimmo & Nimmo, 1984).

Re-activation of isocitrate dehydrogenase in crude cell extracts

Culture samples (3 ml) were centrifuged in an Eppendorf 5412 micro-centrifuge for 60 s. The cells were resuspended in 3 ml of ice-cold Buffer A (10 mM-potassium phosphate, pH 6.5, 0.5 M-KCl, 2 mM-MgCl₂) and sonicated for three bursts (30 s on, 30 s off) at 3 A with a Dawe Soniprobe 1130A; during sonication the extract was chilled in an ice/water bath. Samples (0.07 ml) were then incubated in sealed tubes at 37°C for 4 h in a final volume of 0.1 ml with isocitrate dehydrogenase phosphatase (5 μ units/ml), ATP (2.5 mM), phosphoenolpyruvate (0.5 mM), MgCl₂ (12 mM) and benzamidine hydrochloride (2 mM). The isocitrate dehydrogenase activities of 0.02 ml samples were measured before and after the 4 h incubation. Under these incubation conditions isocitrate dehydrogenase phosphatase is active, but isocitrate dehydrogenase kinase is inactive (Nimmo *et al.*, 1984; Nimmo & Nimmo, 1984). Control experiments showed that the re-activation of isocitrate dehydrogenase went to completion within 4 h.

Purification of ³²P-labelled isocitrate dehydrogenase

E. coli ML308 was grown on 12 litres of basal salts medium (Holms & Bennett, 1971) supplemented with 30 mM-sodium acetate. The cells were harvested in exponential phase at a density of 260 μ g dry wt./ml and resuspended in 500 ml of the low-phosphate medium described above, containing [³²P]P_i (5.33 Ci/mol) but no carbon source. This dense culture (4.7 mg dry wt./ml) was stirred at 37°C and aerated with compressed air (400 ml/min) for 60 min, by which time the specific activity of isocitrate dehydrogenase had increased 3-fold to a constant value. Acetate was then added to a final concentration of 80 mM; this caused a rapid 3-fold decrease in the activity of isocitrate dehydrogenase. No measurable growth occurred during this period. The cells were harvested 30 min after the addition of acetate by centrifugation at 6000 g for 15 min at 4°C, resuspended in 2 vol. of Buffer A and disrupted by 15 bursts of sonication as described above. This extract was then treated as described in the text.

Isolation and analysis of peptides

Samples of phosphorylated isocitrate dehydrogenase were reduced and carboxymethylated (Anfinsen & Haber, 1961) and digested with chymotrypsin (2%, w/w) in 0.5% (w/v) NH₄HCO₃ at 37°C for 48 h. The digests were freeze-dried, dis-

solved in 0.1% (v/v) trifluoroacetic acid and applied to a Waters μ Bondapak C18 column (30 cm $\times$ 3.9 mm). The peptides were eluted with a gradient of 0–40% (v/v) propan-2-ol in 0.1% trifluoroacetic acid (flow rate 1 ml/min, gradient, 0.8% propan-2-ol/min). Peptides were detected by their A_{215} . Isolated peptides were hydrolysed and amino acid analysis was performed as described by Borthwick *et al.* (1984).

Results and discussion

Phosphorylation and dephosphorylation of isocitrate dehydrogenase in vivo

Preliminary experiments showed that incubation of *E. coli* ML308 cells with [³²P]P_i, followed by gel electrophoresis and autoradiography, gave rise to a background 'ladder' of bands whose intensity increased with incubation time. This 'ladder' probably corresponds to different polyphosphates (see, e.g., Wang & Koshland, 1978); after 20–30 min incubation of cells with [³²P]P_i, the bands are sufficiently intense that interpretation of autoradiographs is very difficult. We wished to monitor the phosphorylation state of isocitrate dehydrogenase over periods of 60 min or more. We therefore labelled cells in small batches by withdrawing samples from a parent culture and incubating these with [³²P]P_i for exactly 10 min before each incubation was terminated and the cells were processed for gel electrophoresis. Control experiments showed that the ATP pool of the cells was equilibrated with the external [³²P]P_i well within this time.

Pyruvate was added to cells growing on acetate. The effects of this on the activity of isocitrate dehydrogenase and the ³²P content of cellular proteins are shown in Figs. 1 and 2. As expected from previous work (Bennett & Holms, 1975), the specific activity of isocitrate dehydrogenase rose some 4-fold over 20–30 min after the addition of pyruvate; it then declined to the basal value by 50 min (Fig. 1). Denaturing gel electrophoresis of samples taken during the incubation gave identical patterns of protein-staining bands (Fig. 2a). The autoradiograph of this gel (Fig. 2b) shows that one of the ³²P-containing bands has a mobility identical with that of pure isocitrate dehydrogenase. This band was destroyed by treatment with proteinase K, but not by ribonuclease (results not shown). Evidence that it is in fact isocitrate dehydrogenase containing ³²P as phosphoserine is given below.

The addition of pyruvate gave rise to a specific loss of ³²P from the isocitrate dehydrogenase band (Fig. 2b, tracks 2–5). The ³²P content of this band increased dramatically 40–60 min after addition of pyruvate (tracks 6–8), and at the same time iso-

Fig. 1. Changes in the activity and the phosphorylation state of isocitrate dehydrogenase after the addition of pyruvate

Pyruvate was added to the parent culture at zero time. The specific activity of isocitrate dehydrogenase (□) is expressed as units of activity divided by the turbidity of the culture at 420 nm. The ³²P content of isocitrate dehydrogenase is expressed as the ratio of the radioactivity in the isocitrate dehydrogenase band to that in band X (see the text) (●).

citrate dehydrogenase was inactivated (Fig. 1). In a further experiment we found that a second addition of pyruvate, after the isocitrate dehydrogenase specific activity had returned to the basal value, gave both re-activation of the enzyme and almost complete removal of ³²P from it (results not shown).

We measured the specific radioactivity of the γ -phosphate group of the cellular ATP pool in samples taken at the same times as those illustrated in Figs. 1 and 2. The values were all very similar and were in the range 1.8×10^5 – 2.4×10^5 c.p.m./nmol. Any changes in the ³²P content of the isocitrate dehydrogenase band therefore cannot be caused solely by changes in the rate of turnover of the phosphate group with no change in the total phosphate content of the enzyme; they must reflect phosphorylation or dephosphorylation of the protein.

Quantification of the ³²P contents of the bands shown in Fig. 2(b) is nevertheless difficult, principally because it is evident that the different tracks were loaded with slightly different amounts of protein. This was caused partly by variable losses during washing and lysis of the cells and partly by growth of the culture during the experiment. We therefore sliced out the isocitrate dehydrogenase band and a reference band (labelled X in Fig. 2b) from the gel shown in Fig. 2, solubilized them and measured their ³²P radioactivity. In a series of

Fig. 2. Changes in the phosphorylation state of isocitrate dehydrogenase after the addition of pyruvate. A denaturing polyacrylamide slab gel was loaded as follows: tracks 1 and 9, 3 μ g of purified active isocitrate dehydrogenase (ICDH); tracks 2–8, 40 μ l of extracts prepared at times 0, 10, 20, 30, 40, 50 and 60 min respectively in the experiment shown in Fig. 1. (a) Gel stained for protein; (b) gel autoradiographed for 24 h.

experiments of this type, we detected no systematic variations in the ^{32}P content of the reference band during the course of the incubation. The ratios of the ^{32}P contents of the isocitrate dehydrogenase band and band X are shown in Fig. 1. These results show that there is an inverse correlation between the ^{32}P content of isocitrate dehydrogenase *in vivo* and its activity. The ^{32}P content of the enzyme immediately before the addition of pyruvate is, however, anomalously low (Fig. 1 and Fig. 2b, tracks 2 and 7). This is probably because the rate of turnover of the phosphate group in isocitrate dehydrogenase at the steady state before the addition of pyruvate is sufficiently low that the group had not equilibrated fully with the cellular ATP pool in the 10 min incubation with $[^{32}\text{P}]\text{P}_i$. Other small deviations from an exact inverse relation between the phosphorylation state of the enzyme and its activity (e.g. Fig. 1, 40 min) could be due to the occurrence of slight dephosphorylation and re-activation of the enzyme during the preparation of the samples for gel electrophoresis; this can take place when cultures become anaerobic (Holms & Nimmo, 1982; E. M. T. El-Mansi, W. H. Holms & H. G. Nimmo, unpublished work), but it does not seem to occur during the rapid sonication used in the measurement of enzyme activity.

The results presented above thus show for the first time that there is a reasonable inverse correlation between the phosphorylation state of *E. coli* isocitrate dehydrogenase *in vivo* and its activity. They also demonstrate that the enzyme can be dephosphorylated by simple manipulation of the available carbon source.

Re-activation of isocitrate dehydrogenase in extracts

In order to investigate whether the activation of isocitrate dehydrogenase after the addition of pyruvate goes to completion, sonicated samples taken during an experiment similar to that shown in Figs. 1 and 2 were treated with isocitrate dehydrogenase kinase/phosphatase under conditions designed to promote dephosphorylation of the dehydrogenase. The samples were assayed for isocitrate dehydrogenase activity before and after 4 h incubation with the kinase/phosphatase; control experiments showed that activation of the dehydrogenase went to completion within this time. The results (Fig. 3) reveal that the isocitrate dehydrogenase activity of the samples taken at the peak of the activation caused by pyruvate was not further increased by the kinase/phosphatase. However, for the other samples the incubation with kinase/phosphatase caused up to a 4-fold increase in the specific activity of the dehydrogenase; the specific activities of the samples after this activation *in vitro* were similar to the value reached at the peak of the pyruvate-induced activation *in vivo*.

Fig. 3. Activation by isocitrate dehydrogenase phosphatase of isocitrate dehydrogenase in extracts of cells harvested during a pyruvate-induced activation/inactivation cycle

The experiment was performed exactly as described for the parent culture in the experiment shown in Fig. 1. Samples were assayed for isocitrate dehydrogenase before (●) or after (□) 4 h incubation with isocitrate dehydrogenase phosphatase. The specific activity of isocitrate dehydrogenase is as given for Fig. 1. Pyruvate was added to the culture at the time indicated by the arrow.

These results show that the activation of isocitrate dehydrogenase *in vivo* caused by the addition of pyruvate goes essentially to completion; they are compatible with our observation (Fig. 2) that ^{32}P is almost completely removed from the enzyme in these conditions. Taken together, the results in Figs. 1–3 comprise much stronger evidence than has been presented hitherto that the reversible activation/deactivation of isocitrate dehydrogenase in *E. coli* grown on acetate is due solely to the dephosphorylation and rephosphorylation of the enzyme.

Isolation of isocitrate dehydrogenase labelled with ^{32}P *in vivo*

In order to show that the ^{32}P -containing band shown in Fig. 2(b) and discussed above is indeed phosphorylated isocitrate dehydrogenase, we isolated the material on a preparative scale. We made use of the fact that, in cells grown on acetate and then starved of carbon, isocitrate dehydrogenase becomes fully re-activated (Bennett & Holms,

1975); subsequent re-addition of acetate results in inactivation of the enzyme. The procedure used for labelling the cells with [^{32}P]P_i is described in the Experimental section. Samples taken during this experiment were subjected to denaturing polyacrylamide-gel electrophoresis and autoradiography as described for Fig. 2; the results (not shown) revealed that, as expected, ^{32}P was incorporated into the isocitrate dehydrogenase band after the addition of acetate.

The harvested cells (12g) were disrupted by sonication and the resulting ^{32}P -labelled extract was mixed with an extract prepared from unlabelled cells (24g) grown on acetate as described by Borthwick *et al.* (1984). The inactive form of isocitrate dehydrogenase was then purified to homogeneity as described previously (Borthwick *et al.*, 1984). Samples from each stage in the purification were analysed by denaturing-gel electrophoresis (Fig. 4a); the corresponding autoradiograph is shown in Fig. 4(b). No distinct ^{32}P -containing bands are visible for the crude extract (track 1); this is because the labelled extract was diluted with unlabelled extract and less protein was loaded on track 1 than on the tracks of Fig. 2. Nevertheless a comparison of Figs. 2 and 4 shows clearly that the ^{32}P -containing band in Fig. 2 identified as isocitrate dehydrogenase does indeed

correspond exactly to the inactive isocitrate dehydrogenase isolated in this experiment.

The properties of inactive isocitrate dehydrogenase have been described previously (Borthwick *et al.*, 1984). In agreement with that work, the specific activity of the inactive enzyme isolated in this experiment was less than 1 unit/mg, over 200-fold lower than the value for the active form of the enzyme (Borthwick *et al.*, 1984). The ^{32}P content of the enzyme, 41760 c.p.m./mg, corresponded to the presence of about 0.5 phosphate group per subunit. However, this value can only be an approximation; the calculation involves the assumption that the phosphorylation states of isocitrate dehydrogenase in the two extracts were equal. This assumption is questionable, because the isocitrate dehydrogenase in cells grown on acetate can be partially re-activated during harvesting and cell breakage (Borthwick *et al.*, 1984) and the two batches of cells were grown, harvested and broken in slightly different ways. The apparent phosphate content of 0.5 molecule/subunit is therefore not incompatible with our earlier work, showing that, *in vitro*, isocitrate dehydrogenase is fully inactivated by the incorporation of 1 phosphate group per subunit (Nimmo *et al.*, 1984).

In contrast with our results, the ^{32}P -containing isocitrate dehydrogenase that was isolated from *E.*

Fig. 4. Purification of ^{32}P -labelled isocitrate dehydrogenase

The enzyme was labelled and isolated as described in the text. A denaturing polyacrylamide slab gel was loaded as follows: track 1, crude extract (21 μg of protein); track 2, supernatant after protamine sulphate treatment (14 μg); track 3, $(\text{NH}_4)_2\text{SO}_4$ precipitate (25 μg); track 4, after first DEAE-cellulose column (13 μg); track 5, after Procion Red-Sepharose column (8 μg); track 6, after second DEAE-cellulose column (2.5 μg); tracks 7 and 8, after chromatofocusing (3 μg and 6 μg respectively). Details of the purification procedure are given by Borthwick *et al.* (1984). (a) Gel stained for protein; (b) autoradiograph (7 days).

coli K12 cells incubated with [^{32}P]P_i by Garnak & Reeves (1979b) was partially active (specific activity 48 units/mg at 25°C); the ^{32}P content of this species appears to correspond to the presence of at

least 4 phosphate groups per subunit (Nimmo, 1984). On the basis of the purification method used by Garnak & Reeves (1979b), we have suggested that this species might comprise a mixture of phosphorylated and unphosphorylated forms of the enzyme (Borthwick *et al.*, 1984). It is not, however, clear why its ^{32}P content should be so high; this may reflect a difference between *E. coli* ML308 and K12 strains.

Isolation and analysis of the phosphopeptides from isocitrate dehydrogenase labelled in vitro and in vivo

We wished to compare the ^{32}P -phosphorylated residue(s) in isocitrate dehydrogenase that had been labelled *in vitro* by using purified isocitrate dehydrogenase kinase/phosphatase (Nimmo *et al.*, 1984) with those in the enzyme that had been labelled *in vivo* as described above. We therefore subjected purified samples to reduction, carboxymethylation and digestion with chymotrypsin as described in the Experimental section. Each digest was then fractionated by high-pressure liquid chromatography. One section of the chromatogram is shown for each sample in Fig. 5. The two profiles are strikingly similar. Since the sample phosphorylated *in vitro* was prepared with active isocitrate dehydrogenase, this confirms our previous conclusion (Borthwick *et al.*, 1984) that the inactive enzyme isolated from cells grown on acetate is identical with the active form of the enzyme except for the presence of a phosphate group in the former.

For each sample one peak (labelled A in Fig. 5) contained over 80% of the total recovered ^{32}P radioactivity. To investigate the purity of the material in these peaks, we subjected 2 nmol of peak-A material from isocitrate dehydrogenase labelled *in vitro* to two-dimensional peptide 'mapping' (Bates *et al.*, 1975); only one ninhydrin-positive peptide was detected, and this contained all the ^{32}P radioactivity (results not shown). A corresponding analysis for the enzyme labelled *in vivo* was not performed owing to the limited amount of material available; however, the similarity between the two profiles (Figs. 5a and 5b) strongly suggests that the peptide in peak A from this sample was also pure.

The amino acid compositions of the purified ^{32}P -containing peptides from the two samples are shown in Table 1. The results confirm that the two peptides are identical. The residue phosphorylated in isocitrate dehydrogenase either *in vitro* (Nimmo *et al.*, 1984) or *in vivo* (Garnak & Reeves, 1979b) is a serine residue. The purified phosphopeptide contains only one such residue (Table 1). Our results therefore show clearly that the residue phosphorylated in isocitrate dehydrogenase *in vivo* is identical with that labelled by our purified prep-

Fig. 5. Separation of chymotryptic peptides from ^{32}P -labelled isocitrate dehydrogenase

Samples were prepared and run as described in the Experimental section; fractions were collected manually. Only the 25–50 min (20–40% propan-2-ol) section of each chromatogram is shown (the 0–25 min sections for the two samples were essentially identical): (a) 10 nmol of isocitrate dehydrogenase labelled *in vitro*; (b) 5 nmol of isocitrate dehydrogenase labelled *in vivo*.

Table 1. *Amino acid compositions of purified phosphopeptides from isocitrate dehydrogenase labelled in vitro or in vivo*

Samples (2 nmol) of each peptide (peaks labelled A in Fig. 5) were hydrolysed for 20 h, with 1 nmol of norleucine as internal standard. Values are expressed as molar ratios relative to Ala and are uncorrected for destruction or partial hydrolysis. The values for enzyme labelled *in vitro* represents the mean of duplicates that agreed to within 10%. The composition for the enzyme labelled *in vivo* was determined only once.

Amino acid	Composition	
	Enzyme labelled <i>in vitro</i>	Enzyme labelled <i>in vivo</i>
Asx	2.5	3.2
Thr	1.2	1.3
Ser	0.9	0.8
Glx	1.6	2.3
Pro	1.8	2.4
Gly	3.0	2.8
Ala	1.0	1.0
Val	1.6	1.8
Ile	1.1	1.3
Leu	2.2	2.3
Tyr	0.5	0.6
Phe	0.2	0.2
His	0.9	0.9
Lys	0.8	0.8
Arg	0.5	0.1

aration of isocitrate dehydrogenase kinase/phosphatase *in vitro*. In addition, the fact that over 80% of the ^{32}P present in phosphorylated isocitrate dehydrogenase is associated with a single peptide containing only one serine residue is fully compatible with the observed phosphorylation stoichiometry of one per subunit (Laporte & Koshland, 1983; Nimmo *et al.*, 1984).

The results reported in the present paper establish rigorously that the activity of isocitrate dehydrogenase in *E. coli in vivo* is controlled by its phosphorylation state and that the bifunctional kinase/phosphatase that we and others have isolated (Laporte & Koshland, 1982; Nimmo *et al.*, 1984) is responsible for the phosphorylation. It is noteworthy that only one residue in isocitrate dehydrogenase can be phosphorylated *in vitro* or *in vivo*; this is unlike many eukaryotic systems, in

which multisite phosphorylation has been observed (see, e.g., Cohen, 1983). This will simplify our future analysis of the molecular details of the phosphorylation.

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