The role of isocitrate in control of the phosphorylation of isocitrate dehydrogenase in *Escherichia coli* ML308

E.M.T. El-Mansi, H.G. Nimmo+ and W.H. Holms*

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

Received 22 February 1985

Isocitrate dehydrogenase from *Escherichia coli* is regulated by a reversible phosphorylation mechanism. We confirm here that this permits intracellular isocitrate to rise to a level that can sustain growth on acetate. Isocitrate inhibits isocitrate dehydrogenase kinase and activates isocitrate dehydrogenase phosphatase in vitro. Addition of pyruvate to cultures growing on acetate causes reversible dephosphorylation and activation of isocitrate dehydrogenase, and we show here that this is accompanied by a transient two-fold increase in the intracellular concentration of isocitrate. The data support our suggestion that isocitrate can play a key role in controlling the phosphorylation state of isocitrate dehydrogenase in vivo.

Isocitrate dehydrogenase *Isocitrate concentration* *Glyoxylate bypass* *Protein phosphorylation*

1. INTRODUCTION

The isocitrate dehydrogenase (EC 1.1.1.42) of *Escherichia coli* becomes partially inactivated during growth on acetate in order to allow carbon flux through the glyoxylate bypass [1]. The inactivation of isocitrate dehydrogenase is reversible [1,2] and is mediated by phosphorylation/dephosphorylation [2,3] catalysed by a bifunctional kinase/phosphatase [4,5].

We have made a careful study of the regulatory properties of isocitrate dehydrogenase kinase/phosphatase [6]. Our results, and the observation that the K_m of the glyoxylate bypass enzyme isocitrate lyase for isocitrate is much higher than that of isocitrate dehydrogenase [6–8], led us to propose that phosphorylation of the dehydrogenase facilitates flux through the glyoxylate bypass by rendering the dehydrogenase rate-limiting in the tricarboxylic acid cycle and thus increasing the intracellular concentration of isocitrate [6]. We found that high concentrations of isocitrate favour the dephosphorylation and activation of isocitrate dehydrogenase in vitro and we suggested that in some circumstances the intracellular concentration of isocitrate plays a key role in controlling the phosphorylation state of isocitrate dehydrogenase [6].

Here, we show that the intracellular concentration of isocitrate varies in a manner that is consistent with these proposals.

2. EXPERIMENTAL

2.1. *Materials*

The active form of isocitrate dehydrogenase was prepared as in [9]. Perchloric acid was from BDH Chemicals and *threo*-D_s-(+)-isocitrate was from Sigma. The sources of other chemicals were as in [9].

2.2. *Growth and manipulation of micro-organisms*

E. coli ML308 (ATCC 15224) was grown at 37°C in a mineral salts solution [10] supplemented with either 40 mM sodium acetate, 20 mM glycerol or 10 mM glucose. Inocula were prepared by 3 passages through homologous media (100 ml, 37°C) on an orbital shaker [10]. Turbidities of

* To whom reprint requests should be addressed
+ To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

cultures were measured at 420 nm in a Unicam SP30 spectrophotometer. A turbidity of 1.0 corresponds to a cell density of 160 μg dry wt/ml.

Cultures growing on acetate (800 ml) were aerated by vigorous stirring and the gas phase was supplied with air at 200 ml/min. Pyruvate was added to cultures growing exponentially at a cell density in the range 150–165 μg dry wt/ml. Cultures on glucose or glycerol (100 ml) were grown on an orbital shaker.

2.3. *Preparation of extracts and filtrates*

For cultures growing on acetate, aliquots (4 ml) were mixed rapidly with 30% (w/v) perchloric acid (1 ml) at 0°C and immediately frozen at −70°C. After all the samples had been collected, they were allowed to thaw, kept at 0°C for 10 min and then centrifuged at 4°C and 40000 × *g* for 20 min. Control experiments showed that re-extraction of the resulting pellet released no further isocitrate. The supernatants were carefully decanted into chilled tubes, adjusted to pH 7 by the addition of ice-cold 5 N KOH and centrifuged at 4°C and 20000 × *g* for 20 min to remove precipitated potassium perchlorate. The neutralised supernatants were lyophilised, redissolved in distilled water and assayed for isocitrate either immediately or after storage at −20°C.

In order to allow for any extracellular isocitrate, aliquots of cultures were filtered rapidly through 0.45 μm Millipore filters and the filtrates were treated with perchloric acid exactly as described above.

For cultures growing on glucose or glycerol, 30% (w/v) chilled perchloric acid (25 ml) was added to the culture (100 ml); the flasks were cooled to 0°C and then treated as above. Culture filtrates (10 ml) were prepared from control flasks and treated as above.

2.4. *Assay of isocitrate*

Isocitrate was assayed spectrofluorimetrically [11] using a Hitachi Perkin-Elmer MPF2A or a Baird Fluoripoint instrument. The amount of extract used was adjusted so that the assay mixture (2 ml) contained 0.1–1.0 nmol isocitrate. The reaction was initiated by the addition of 2 μg purified isocitrate dehydrogenase. The resulting increase in fluorescence was corrected for the increase owing to the dehydrogenase itself. At the end of each assay, 0.5 nmol *threo*-D_s-(+)-isocitrate was added as an internal standard. Intracellular isocitrate was estimated as the difference between the amounts found in the whole culture and the culture filtrate. The isocitrate dehydrogenase used in these experiments contained no detectable aconitase activity, so the presence of citrate (see below) did not interfere with this assay.

2.5. *Assay of isocitrate dehydrogenase*

Culture samples were diluted, sonicated and assayed as described previously [10].

2.6. *High pressure liquid chromatography (HPLC)*

This was carried out at room temperature on an Aminex HPX-87H organic acids column (Bio-Rad). Elution was with 0.01 N H_2SO_4 at a flow rate of 1 ml/min and acids were detected by measurement of A_{215}. Experiments with authentic standards showed that citrate and isocitrate were not resolved by this procedure.

3. RESULTS AND DISCUSSION

3.1. *Measurement of intracellular and extracellular isocitrate*

Previous methods for the measurement of metabolite levels in *E. coli* have involved rapid filtration of culture samples and either immediate extraction [8] or freezing of the collected cells followed by extraction (e.g., [7,11]). The filtration takes several seconds and during this time the air supply to the culture is limited. In *E. coli* the rates of the central metabolic pathways are such that the ATP pool turns over several times per sec [12] and the rate of turnover of the isocitrate pool is also likely to be high. Moreover, anaerobiosis can cause activation of isocitrate dehydrogenase in cells grown on acetate ([13] and our unpublished results).

Any procedure that changes the environment of the cells before metabolic turnover is stopped (e.g., filtration) increases the risk that intracellular metabolite levels change. We therefore decided to measure isocitrate levels by rapid quenching of cultures with perchloric acid. It is generally accepted that this procedure kills cells instantly and prevents further metabolism, and is therefore

superior to other methods. It is necessary in our procedure to correct the total isocitrate present in the culture for any extracellular isocitrate present in the culture medium. While doing these experiments we noticed an accumulation of isocitrate, citrate and 2-oxoglutarate in the growth medium during growth on acetate but not on glucose or glycerol. This is illustrated in fig.1, which shows HPLC traces of culture filtrates taken immediately after inoculation or during exponential growth on acetate. The latter sample contained 2-oxoglutarate and material that comigrated with authentic citrate and isocitrate. Enzymatic analysis showed that 8% of this material was isocitrate, in good agreement with the equilibrium constant of aconitase [14]. During growth on acetate, extracellular isocitrate accumulated and represented

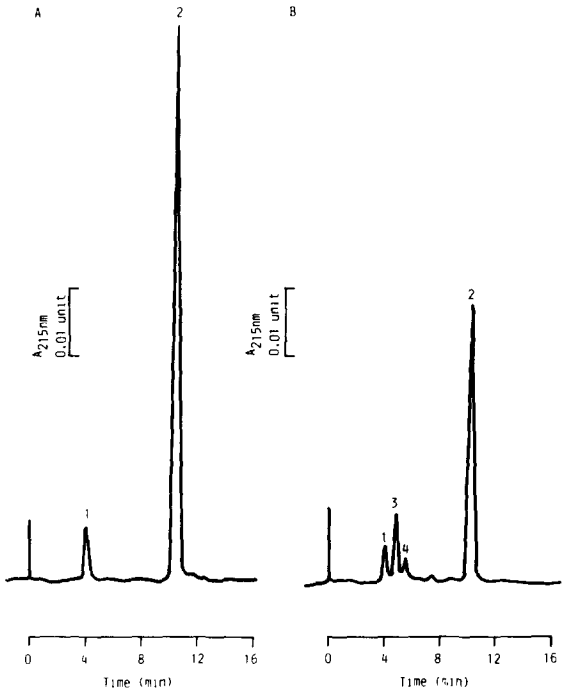

Fig.1. HPLC of culture filtrates. Columns were loaded with 25 μl culture filtrates. The absorbance scale was the same for both traces; the bar represents 0.01 A_{215} units. (A) Immediately after inoculation of acetate growth medium. (B) During exponential growth on acetate; the cell density was 221 μg dry wt/ml. Numbered peaks: 1, solvent front; 2, acetate; 3, 2-oxoglutarate; 4, citrate and isocitrate.

15–16% of the total isocitrate content of the culture.

There are at least 3 possible explanations of the appearance of these compounds in the extracellular fluid. Firstly, leakage or passage across the inner cell membrane could be mediated either by carriers or by simple diffusion; for isocitrate there is a large concentration gradient across the membrane that is generated by the high intracellular concentration required to sustain growth on acetate (see below). Secondly, there might be periplasmic enzymes which could generate the compounds and leave them to equilibrate across the outer membrane. Thirdly, cell lysis would release intracellular pools into the medium. It is possible to discount the last explanation because lysis would liberate all the intracellular solutes and there is no evidence for release of other compounds (e.g., other intermediates of the Krebs cycle) into the extracellular fluid. It is clear that the first two explanations are worthy of further study. However, the question of which of these two (if either) is correct is not relevant to the argument of this paper.

3.2. *Changes in the intracellular concentration of isocitrate*

The intracellular content of isocitrate in cells growing exponentially on acetate, corrected for extracellular isocitrate, was found to be 1.54 ± 0.04 μmol/g dry wt (mean ± SD, n = 8). Assuming that 1 g dry wt of cells corresponds to 2.7 ml intracellular water [15], the intracellular concentration of isocitrate is 0.57 mM. No isocitrate was detectable in cultures growing exponentially on glucose or glycerol, or in the corresponding culture filtrates and, from the sensitivity of the fluorimetric assay, the intracellular concentration of isocitrate in these cells must be less than 20 μM.

Our value for the intracellular concentration of isocitrate in cells growing on acetate is rather higher than the previously reported values of 0.39 μmol/g dry wt [11] and 160 μM [8]. This discrepancy could be due to metabolism of isocitrate during the filtration procedure used in other studies [8,11] or perhaps to strain differences. However, the great difference in the intracellular concentration of isocitrate between cells grown on acetate and those grown on glucose or glycerol is in agreement with other workers [8,11]

and is consistent with our suggestion that isocitrate dehydrogenase is rate-limiting in the tricarboxylic acid cycle only during growth on acetate [6].

We have previously shown that addition of pyruvate to *E. coli* ML308 growing on acetate causes activation of isocitrate dehydrogenase, owing to its dephosphorylation, and an increase in growth rate, both of which persist until the pyruvate has been utilised [1,2]. We have measured the intracellular concentration of isocitrate during such experiments and typical results are shown in fig.2. Addition of pyruvate causes a very rapid, transient, 2-fold increase in the intracellular concentration of isocitrate, and a 4-fold activation of isocitrate dehydrogenase. This activation corresponds to essentially complete dephosphorylation of the enzyme [2]. The isocitrate concentration increases at least as fast as the isocitrate dehydrogenase activity and it declines to the control level prior to full deactivation of the dehydrogenase.

We suggested that the activation of isocitrate dehydrogenase observed in these conditions is triggered by an increase in the intracellular concentration of isocitrate, which is an inhibitor of isocitrate dehydrogenase kinase and an activator of isocitrate dehydrogenase phosphatase [6]. To explain the increased concentration of isocitrate we proposed that pyruvate can be converted to acetyl-CoA, and thus fed into the tricarboxylic acid cycle, more rapidly than can acetate itself [6]. The results shown in fig.2 are thus fully consistent with our view of the role of isocitrate in the activation of isocitrate dehydrogenase.

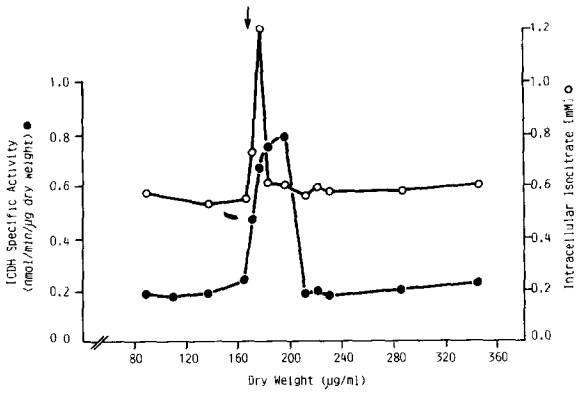

Fig.2. Changes in intracellular isocitrate concentration after addition of pyruvate. Isocitrate dehydrogenase specific activity (●) and the intracellular concentration of isocitrate (○) were measured in a culture growing exponentially on acetate. Pyruvate was added to the culture at the point indicated by the arrow.

Our results show that, in the conditions of fig.2, the intracellular concentration of isocitrate is in the range 0.5–1.0 mM. The K_m of isocitrate lyase for isocitrate is relatively high; values of 3 mM [7] and 0.6 mM [8] have been reported. Thus, the increase in isocitrate concentration caused by addition of pyruvate would increase the rate of flux through isocitrate lyase and the glyoxylate bypass as well as the activity of isocitrate dehydrogenase and the rate of flux through the tricarboxylic acid cycle. In addition, pyruvate can be used directly for biosynthesis and this will reduce the demands made on the glyoxylate bypass. These factors provide an explanation for the increased growth rate seen in these conditions [1].

Isocitrate inhibits isocitrate dehydrogenase kinase sigmoidally, with an apparent K_i (for DL-isocitrate) in the range 15–60 μM [6]. It activates isocitrate dehydrogenase phosphatase at concentrations in the range 0–5 mM DL-isocitrate. However, the apparent K_i of the kinase for isocitrate increases with the concentration of isocitrate dehydrogenase [6]. The concentration of the dehydrogenase in vivo [9] is considerably higher than the concentrations used in the assays of the kinase and phosphatase [6]. It is therefore very difficult to assess whether the effects of isocitrate in vivo (fig.2) are mediated by activation of the phosphatase, inhibition of the kinase or both.

The present results fully support our theory [6] that during growth on acetate flux through the glyoxylate bypass is facilitated by maintenance of a high intracellular concentration of isocitrate. Addition of pyruvate can increase the isocitrate concentration and this effects a redistribution of carbon flux by causing activation of isocitrate dehydrogenase. A similar explanation probably accounts for the activation of the dehydrogenase caused by the addition of glucose, malate or other dicarboxylic acids [1]. Our results represent the first direct evidence that the intracellular concentration of isocitrate can, in some circumstances, control the phosphorylation state of isocitrate dehydrogenase.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Science and Engineering Research Council, London. We thank Margaret Cowan for excellent technical assistance.

REFERENCES

[1] Bennett, P.M. and Holms, W.H. (1975) J. Gen. Microbiol. 87, 37–51.
[2] Borthwick, A.C., Holms, W.H. and Nimmo, H.G. (1984) Biochem. J. 222, 797–804.
[3] Garnak, M. and Reeves, H.C. (1979) Science 203, 1111–1112.
[4] LaPorte, D.C. and Koshland, D.E. (1982) Nature 300, 458–460.
[5] Nimmo, G.A., Borthwick, A.C., Holms, W.H. and Nimmo, H.G. (1984) Eur. J. Biochem. 141, 401–408.
[6] Nimmo, G.A. and Nimmo, H.G. (1984) Eur. J. Biochem. 141, 409–414.
[7] Bautista, J., Satrustegui, J. and Machado, A. (1979) FEBS Lett. 105, 333–336.
[8] Walsh, K. and Koshland, D.E. (1984) J. Biol. Chem. 259, 9646–9654.
[9] Borthwick, A.C., Holms, W.H. and Nimmo, H.G. (1984) Eur. J. Biochem. 141, 393–400.
[10] Holms, W.H. and Bennett, P.M. (1971) J. Gen. Microbiol. 65, 57–68.
[11] Lowry, O.H., Carter, J., Ward, J.B. and Glaser, L. (1971) J. Biol. Chem. 246, 6511–6521.
[12] Holms, W.H., Hamilton, I.D. and Robertson, A.D. (1972) Arch. Mikrobiol. 83, 95–109.
[13] Holms, W.H. and Nimmo, H.G. (1982) Biochem. Soc. Trans. 10, 319–320.
[14] England, P.J., Denton, R.M. and Randle, P.J. (1967) Biochem. J. 105, 32C–33C.
[15] Winkler, H.H. and Wilson, T.H. (1966) J. Biol. Chem. 241, 2200–2211.