Activation and Inactivation of Phosphoenolpyruvate Carboxykinase by Ferrous Ions

C. Hugh REYNOLDS

Biochemical Research Department, The Wellcome Foundation Ltd., Temple Hill, Dartford, Kent DA1 5AH, U.K.

(Received 29 May 1979)

Phosphoenolpyruvate carboxykinase from rat liver cytosol is activated by Fe^{2+} ions in either direction of catalysis. Preincubation of the purified enzyme with Fe^{2+} ions causes a time-dependent irreversible loss of activity; this is not seen with unpurified enzyme. Purified enzyme can be protected from inactivation by Fe^{2+} ions by partially purified protein fractions from liver (ferroactivator fractions). The possible role of ferroactivator and Fe^{2+} ions in regulating phosphoenolpyruvate carboxykinase is discussed.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) catalyses one of the first reactions in the gluconeogenic pathway. Different isoenzymes occur in mitochondria and cytoplasm (Utter & Kolenbrander, 1972), and in rat liver the cytosolic isoenzyme predominates. The crude enzyme from rat liver cytosol is activated by millimolar concentrations of Mg^{2+} ions and is further activated by low concentrations of bivalent transition-metal ions, of which Fe^{2+} is the most effective (Snoko *et al.*, 1971). Purification of the enzyme causes loss of most of the activation by Fe^{2+} ions, and a cytoplasmic protein factor, phosphoenolpyruvate carboxykinase ferroactivator, was shown to restore this response (Bentle *et al.*, 1976). Evidence is presented here that ferroactivator acts by protecting the enzyme from inactivation when the enzyme is preincubated with Fe^{2+} ions, rather than by altering the kinetic properties of the enzyme.

Materials and Methods

Substrates, nucleotides, dithiothreitol, malate dehydrogenase, lactate dehydrogenase and pyruvate kinase were supplied by Sigma (London) Ltd. or Boehringer Corp. (London) Ltd.

Phosphoenolpyruvate carboxykinase was from the 100000g supernatant of 24h-starved rats; 'crude' enzyme was a 45–65% satd. $(\text{NH}_4)_2\text{SO}_4$ fraction of specific activity 0.032 unit/mg of protein (Ballard & Hanson, 1969). Purified enzyme was prepared either by the method of Ballard & Hanson (1969) up to the DEAE-cellulose-chromatography step (specific activity 1.5 units/mg), or was carried on to near-homogeneity (specific activity 6.7 units/

mg) (Philippidis *et al.*, 1972), and was dialysed before use against 50 mM-Tris/HCl/0.1 mM-EDTA/0.5 mM-dithiothreitol buffer, pH 7.5 at 2–4°C. Both purified preparations appeared devoid of ferroactivator (Bentle *et al.*, 1976; Bentle & Lardy, 1977) and behaved identically in metal-activation experiments with or without preincubation.

Enzyme activity was routinely measured at 25°C by the reduction of NADH (0.15 mM) in 1 ml of 0.1 M-imidazole/HCl buffer (final pH 6.9–7.1) (Ballard & Hanson, 1967), containing 1.5 mM-phosphoenolpyruvate, 1.25 mM-IDP, 50 mM- NaHCO_3 , 5 units of malate dehydrogenase, 2 mM- MnCl_2 and 1 mM-dithiothreitol; the activity of crude enzyme was also determined by fixation of [^{14}C]bicarbonate (2 μCi) (Ballard & Hanson, 1967). For testing transition metals, MnCl_2 was replaced by 3 mM- MgCl_2 and dithiothreitol was omitted.

Phosphoenolpyruvate-synthesis assays were as described by Bentle & Lardy (1976), with the addition of 15 mM- NaF (Pogson & Smith, 1975) at 25°C. Preincubations, when performed, were at 0°C and 10 min unless otherwise specified; 0.1 ml of preincubation mixture was added to 0.9 ml of assay reagents (gluconeogenic direction), or 50 μl to 0.95 ml (carboxylation direction). The amount of enzyme used was 0.005–0.02 unit/assay, unless otherwise stated.

Phosphoenolpyruvate carboxykinase ferroactivator (from non-perfused rat livers) was partially purified from 100000g supernatant by heat treatment and Sephadex G-100 gel filtration (Bentle & Lardy, 1977). A major fraction (A), and a minor fraction (B), of lower molecular weight, were isolated.

Protein was measured by the u.v.-absorption method of Warburg & Christian (1941), as described by Dawson *et al.* (1969).

Results

Fe^{2+} ions (and also Mn^{2+} and Co^{2+}) activate purified phosphoenolpyruvate carboxykinase when enzyme is added to mixtures of substrates containing MgCl_2 (Fig. 1), in either direction of catalysis. Crude enzyme gave similar results, and so did purified enzyme to which partially purified ferroactivator was added. Table 1 compares activity after preincubation with Fe^{2+} ions and direct addition to the assay mixture; preincubation leads to some loss of

activity in Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, and to further loss of activity in the presence of Fe^{2+} ions. In other experiments, assays in the carboxylation direction with 2 mM- MnCl_2 or with 3 mM- MgCl_2 + 50 μM - FeCl_2 showed inactivation to have taken place on preincubation of purified enzyme (but not of crude enzyme) with Fe^{2+} ions.

The inactivation was time-dependent when measured in the direction of phosphoenolpyruvate synthesis (Fig. 2a) or of carboxylation (Fig. 2b). Phosphate potentiated the inactivation by Fe^{2+} ions, in accordance with previous studies (Bentle & Lardy, 1976, 1977). In contrast, crude enzyme, which would also contain ferroactivator, was almost completely stable (Fig. 2c).

Fig. 1. Activation of phosphoenolpyruvate carboxykinase by transition metals

Reactions were initiated by adding purified (6.7 units/mg) enzyme to a mixture of substrates, 3 mM- MgCl_2 , and the indicated concentration of transition-metal ion: O, MnCl_2 ; ●, FeCl_2 ; △, CoCl_2 . (a) Assays of phosphoenolpyruvate synthesis (0.5 μM -EDTA in 50 mM-Hepes buffer, pH 7.5); (b) assays of phosphoenolpyruvate carboxylation [1.5 μg of enzyme 1 μM -EDTA, in 100 mM-imidazole/HCl buffer, pH 7.0; activity ($v_{rel.}$) expressed as fraction of that in the standard assay with 2 mM- MnCl_2].

Fig. 2. Inactivation of phosphoenolpyruvate carboxykinase on incubation with Fe^{2+} ions, and effect of P_i (a), Purified (1.5 units/mg) enzyme and phosphoenolpyruvate synthesis assays; (b), purified (1.5 units/mg) enzyme and carboxylation assays (with 2 mM- MnCl_2); (c), crude enzyme and carboxylation assays. Symbols indicate preincubation conditions: O, in 5 mM-Hepes/0.8 mM-dithiothreitol alone; ●, also with FeCl_2 (99 μM for b, 50 μM for c); △, also with FeCl_2 and Na_2HPO_4 (30 μM and 0.75 mM respectively for a, and 50 μM and 1 mM respectively for both b and c). Preincubation media also contained 5 μM -EDTA (a and b) or 20 μM -EDTA (c).

Table 1. *Effect of preincubation on metal activation of purified (1.5 units/mg) phosphoenolpyruvate carboxykinase*

Preincubations were in 5 mM-Hepes/0.8 mM-dithiothreitol buffer, pH 7.5, at 0°C for 10 min, and 0.1 ml of preincubation mixture was added to 0.9 ml of substrate (+ 3 mM-MgCl₂) etc. to initiate assays. Identical amounts of enzyme protein were present in each assay. Preincubations contained 10 μM-EDTA, and assays 1 μM-EDTA (total).

Preincubation conditions	Transition metal in assay	<i>v</i> (ΔA ₃₄₀ in 5 min)
Buffer only	(None)	0.103
+ 30 μM-FeCl ₂	3 μM-FeCl ₂ *	0.064
+ 100 μM-MnCl ₂	10 μM-MnCl ₂ *	0.215
No preincubation	(None)	0.221
	6 μM-FeCl ₂	0.292
	20 μM-MnCl ₂	0.548

* Carried with enzyme into assay from preincubation; not added separately.

Fig. 3. *Protection by partially purified ferroactivator of phosphoenolpyruvate carboxykinase from inactivation by Fe²⁺ ions*

Preincubations were at 0°C in 60 μl total volume and contained 5 mM-Hepes buffer, pH 7.6, 0.8 mM-dithiothreitol, 100 μM-FeCl₂, 0.83 mM-sodium phosphate, 17 μM-EDTA, ferroactivator solution as indicated, and 0.048 unit of purified (1.4 units/mg) phosphoenolpyruvate carboxykinase. After 10 min, 50 μl was used to initiate the carboxylation enzyme assay. O, Ferroactivator fraction A (147 mg/ml); Δ, ferroactivator fraction B (3.75 mg/ml). Both ferroactivator solutions contained 0.1 mM-EDTA. Assay rates are corrected for traces of phosphoenolpyruvate carboxykinase activity present in the ferroactivator preparations.

Progress curves in the carboxylation assay were usually linear for at least 2 min even in the presence of Fe²⁺, and so substrates probably protect the enzyme from inactivation.

Purified enzyme could be protected from inactivation by Fe²⁺ ions and P_i when partially purified ferroactivator was present (Fig. 3). The effects are dose-dependent, but the protection is not complete under these conditions.

Discussion

Our most highly purified preparation of phosphoenolpyruvate carboxykinase had a specific activity of 6.7 units/mg for carboxylation at 25°C. This compares well with literature values for this purification method [e.g. 5 units/mg for the preparation used by Bentle & Lardy (1976), which they described as nearly homogeneous on polyacrylamide gels], but is well below the value of 18 units/mg obtained by Colombo *et al.* (1978) using a GTP affinity column. Both this and the less highly purified preparation (1.5 units/mg) appeared devoid of ferroactivator protein: they were both inactivated when preincubated with Fe²⁺ ions, with or without phosphate. Each preparation had been chromatographed on both Sephadex G-100 and DEAE-cellulose, and each of these processes should separate the enzyme from ferroactivator (Bentle *et al.*, 1976).

The results described above show that purified phosphoenolpyruvate carboxykinase can be strongly activated by Fe²⁺ ions in the gluconeogenic direction, in the absence of ferroactivator. Activation in the carboxylation direction has been reported by Colombo & Carlson (1978) and Hammond & Balinsky (1978). Earlier studies used preincubation with Fe²⁺ ions and ferroactivator to produce activation (Bentle *et al.*, 1976; Bentle & Lardy, 1977; MacDonald *et al.*, 1978); however, the results presented here show that the enzyme is partially inactivated *in vitro* by incubation with Fe²⁺ ions, but is protected by protein fractions with ferroactivator activity. So the higher activity in the presence of ferroactivator is not due to activation in the presence of ferroactivator; rather, it is due to Fe²⁺-induced inactivation in the absence of ferroactivator. Ferroactivator causes no significant changes in kinetic properties of the enzyme without preincubation (Reynolds, 1979a,b), e.g. affinities for metal ions, *K_m* for phosphoenolpyruvate or *K_i* for the inhibitors quinolinate and 3-mercaptopycolinate. Therefore ferroactivator probably does not regulate the kinetic activity of phosphoenolpyruvate carboxykinase *in vivo*.

However, phosphoenolpyruvate carboxykinase may be in a dynamic equilibrium between inactive enzyme, whose formation is catalysed by Fe²⁺, and re-activated enzyme, which may be formed by reaction with thiols. Hebda & Nowak (1978) reported that thiols re-activated chicken phosphoenolpyruvate carboxykinase, and age-inactivated rat liver enzyme can be slowly re-activated by over-

night dialysis against buffer containing dithiothreitol (C. H. Reynolds, unpublished work). If such a dynamic equilibrium exists, then ferroactivator may affect the equilibrium, favouring active enzyme. Ferroactivator may also have a function unrelated to phosphoenolpyruvate carboxykinase; it is present in tissues where this enzyme is low or absent, such as erythrocytes (MacDonald *et al.*, 1978).

The hyperglycaemic agent 3-aminopicolinic acid, which has been reported to activate pure phosphoenolpyruvate carboxykinase in a manner analogous to the protein ferroactivator (MacDonald & Lardy, 1978), protects the enzyme very effectively from inactivation by Fe^{2+} ions and P_i (Reynolds, 1979a); this is probably attributable in part to chelation of Fe^{2+} ions.

The technical assistance of Mr. P. T. J. Claxton is gratefully acknowledged. I thank Dr. N. R. Lazarus for advice and encouragement.

References

- Ballard, F. J. & Hanson, R. W. (1967) *Biochem. J.* **104**, 866–871
- Ballard, F. J. & Hanson, R. W. (1969) *J. Biol. Chem.* **244**, 5625–5630
- Bentle, L. A. & Lardy, H. A. (1976) *J. Biol. Chem.* **251**, 2916–2921
- Bentle, L. A. & Lardy, H. A. (1977) *J. Biol. Chem.* **252**, 1431–1440
- Bentle, L. A., Snoko, R. E. & Lardy, H. A. (1976) *J. Biol. Chem.* **251**, 2922–2928
- Colombo, G. & Carlson, G. M. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1423
- Colombo, G., Carlson, G. M. & Lardy, H. A. (1978) *Biochemistry* **17**, 5321–5329
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd edn., pp. 625–626, Oxford University Press, London
- Hammond, K. D. & Balinsky, D. (1978) *Int. J. Biochem.* **9**, 199–211
- Hebda, C. & Nowak, T. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 1296
- MacDonald, M. J. & Lardy, H. A. (1978) *J. Biol. Chem.* **253**, 2300–2307
- MacDonald, M. J., Bentle, L. A. & Lardy, H. A. (1978) *J. Biol. Chem.* **253**, 116–124
- Philippidis, H., Hanson, R. W., Reshef, L., Hopgood, M. F. & Ballard, F. J. (1972) *Biochem. J.* **126**, 1127–1134
- Pogson, C. I. & Smith, S. A. (1975) *Biochem. J.* **152**, 401–408
- Reynolds, C. H. (1979a) *Biochem. Pharmacol.* in the press
- Reynolds, C. H. (1979b) *Comp. Biochem. Physiol.* in the press
- Snoko, R. E., Johnston, J. B. & Lardy, H. A. (1971) *Eur. J. Biochem.* **24**, 342–346
- Utter, M. F. & Kolenbrander, H. M. (1972) *Enzymes 3rd Ed.* **6**, 117–168
- Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384–421