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PHOSPHOENOLPYRUVATE CARBOXYKINASE: EFFECTS OF THE HYPERGLYCAEMIC AGENT 3-AMINOPICOLINIC ACID

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Abstract—Chelation by 3-aminopicolinic acid of Fe^{2+}, Co^{2+} and Mn^{2+} has been measured spectrophotometrically. With Fe^{2+} or Co^{2+}, 3-aminopicolinic acid inhibited phosphoenolpyruvate carboxykinase at low metal-ion concentrations. Activation was not observed. The enzyme appears not to bind 3-aminopicolinic acid. 3-Aminopicolinic acid protects phosphoenolpyruvate carboxykinase from inactivation by ferrous ions. Two models are suggested, which could account for activation of gluconeogenesis due to chelation of metal-ions by 3-aminopicolinic acid: (a) phosphoenolpyruvate carboxykinase may be in dynamic equilibrium between inactivation by ferrous ions and reactivation by thiol compounds; and (b) metal-ions may promote product-inhibition by phosphoenolpyruvate, which chelation could alleviate.

Phosphoenolpyruvate (PEP)* carboxykinase [GTP: oxaloacetate carboxy-lyase (transphosphorylating), EC.4.1.1.32] is an important enzyme of gluconeogenesis [1]. 3-Aminopicolinic acid (3AP) raises blood-glucose concentrations [2] and appears to activate gluconeogenesis at or near the PEP carboxykinase step [3]. When PEP carboxykinase, 3AP and a divalent metal ion (Fe^{2+} or Mn^{2+}) are preincubated before assay, 3AP can activate the enzyme [4]; this activation imitates the property of a protein, PEP carboxykinase ferroactivator [5, 6] which occurs in many tissues including liver and kidney [7].

I have shown recently that ferroactivator functions by protecting PEP carboxykinase from inactivation by ferrous ions during the preincubation [8], and has little or no influence on the catalytic properties of the enzyme. Ferrous ions, without preincubation, also activate the pure enzyme. Therefore, 3AP could be protecting the enzyme or changing its kinetic properties, or both.

MATERIALS AND METHODS

Substrates, nucleotides, dithiothreitol and coupling enzymes were supplied by Sigma (London) Ltd. or the Boehringer Corporation (London) Ltd. 3AP was synthesised and made available by the Wellcome Research Laboratories, Beckenham. Quinolinic acid was from Sigma, and 3-mercaptopicolinic acid was a gift from Dr. J. P. Larkin (Wellcome Research Laboratories, Berkhamsted).

Rat-liver PEP carboxykinase was prepared and assayed as described previously [8, 9]. Briefly, the enzyme was either from a crude 45–65% ammonium sulphate precipitate, or purified (to 1.5 or 6.7 units/mg protein) to remove ferroactivator [10, 11].

For routine assays at 25°, enzyme was added last to 1 ml 0.1M imidazole–HCl buffer containing 1.5 mM PEP, 1.25 mM IDP, 50 mM $NaHCO_3$, 5 units of malate dehydrogenase, 0.15 mM NADH, 1 mM dithiothreitol and 2 mM $MnCl_2$ (final pH 6.9–7.1); disappearance of NADH was measured spectrophotometrically at 340 nm. When testing metal-ions and 3AP, dithiothreitol was omitted; 3 mM $MgCl_2$ and other metal-ions as specified were added. PEP-synthesis assays at 25° [8, 12] used 1 ml of 50 mM Hepes/NaOH, pH 7.5, containing 2 mM ITP, 1.5 mM oxaloacetate, 3 mM $MgCl_2$, 15 mM NaF, and other additions as specified. Preincubations, when performed, were at 0° and for 10 min unless otherwise specified; 0.1 ml of preincubation mixture was added to 0.9 ml assay reagents (PEP-synthesis direction) or 50 μl to 0.95 ml (carboxylation direction).

Binding of 3AP to Mn^{2+}, Co^{2+} or Fe^{2+} increased the extinction at 340 nm. Metal salts were added to 1 ml of 200 μM 3AP in 50 mM Hepes/NaOH buffer, pH 7.5, and the extinction recorded.

Protein concentration was measured spectrophotometrically by the method of Warburg and Christian, as described in [13].

RESULTS

Stabilisation of PEP carboxykinase. When PEP carboxykinase was preincubated with Fe^{2+} and inorganic phosphate, it was rapidly inactivated [8]. When 3AP was present (Fig. 1), it prevented inactivation very effectively. Without Fe^{2+} or phosphate, the enzyme lost a little activity [8]; with 3AP the enzyme was stable. Once inactivated by Fe^{2+} and phosphate (Fig. 1), 3AP could not reactivate.

Preincubation mixtures which contained dithiothreitol and ferrous ions were pink, due to metal–thiol complex-formation [6]; however, when 3AP was present, this colour was not formed. Therefore, complex-formation between 3AP and Fe^{2+} was suspected as a possible factor in protecting the enzyme *in vitro*.

* Abbreviations used: PEP, phosphoenolpyruvate; 3AP, 3-aminopicolinic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid.

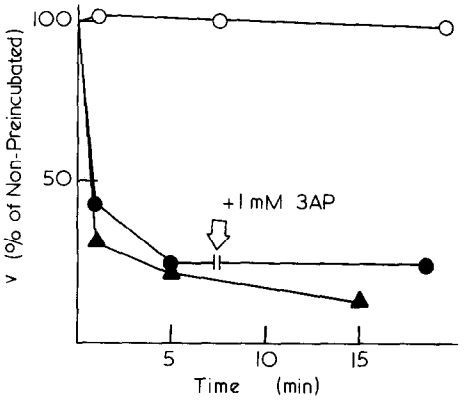

Fig. 1. Effect of 3AP on inactivation of purified PEP carboxykinase by Fe^{2+} and phosphate. All preincubations were at 0° in 0.2 ml of 5 mM Hepes/0.8 mM dithiothreitol buffer pH 7.5, containing 0.054 units of enzyme (1.5 u/mg), 50 μM $FeCl_2$ and 1 mM sodium phosphate. ▲ = no 3AP; ● = + 1 mM 3AP added after 7½ min; ○ = + 1 mM 3AP, present throughout.

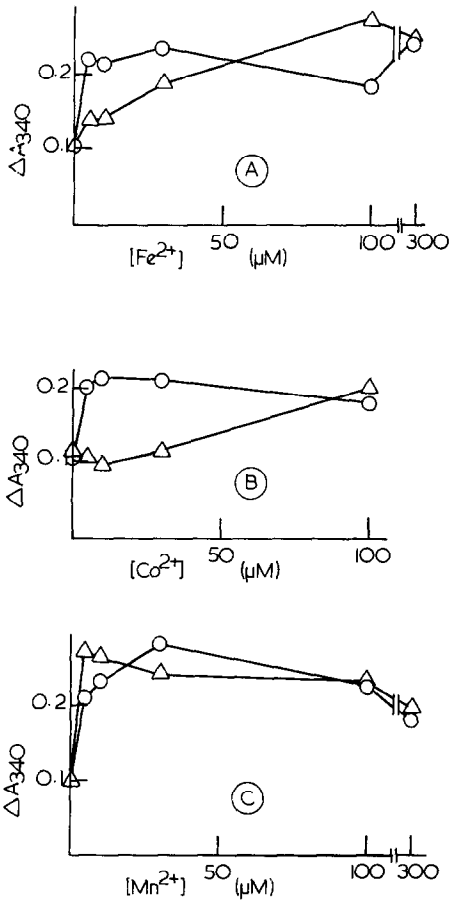

Fig. 2. Effects of 3AP on PEP-synthesis activity of PEP carboxykinase. A, with Fe^{2+}; B, with Co^{2+}; C, with Mn^{2+}. With purified enzyme (6.7 umg) and transition-metals as indicated. ○ without 3AP; △ + 0.2 mM 3AP.

In the presence of Mn^{2+}, or Mn^{2+} + Mg^{2+}, 3AP did not protect the enzyme against heat-inactivation at 48° (see below and Fig. 6).

Kinetic effects on PEP carboxykinase. In the gluconeogenic direction, 3AP alters the response to Fe^{2+} and Co^{2+} (Figs. 2A and B), but not to Mn^{2+} (Fig. 2C). With Fe^{2+} and Co^{2+}, higher concentrations of metal-ions are needed to activate, but the optimal activation is not much affected. Chelation of these ions by 3AP would lower their effective concentrations. The apparent activation by 3AP at low concentrations of Mn^{2+} is repeatable; however, its significance is uncertain because under these conditions the assay-rate increased with time [14] and 3AP may be decreasing this lag without increasing the final rate. Very similar results were given by crude enzyme (not shown).

In the carboxylation direction, 3AP also shifted the responses to Fe^{2+} and Co^{2+} (Figs. 3A and B) towards higher metal concentrations, while that for Mn^{2+} (Fig. 3C) was unaltered.

Binding of 3AP to metal ions. 3-Aminopicolinic acid has a maximum absorbance at approximately 320 nm (Fig. 4A). It was noticed fortuitously, when performing enzyme assays, that addition of Fe^{2+}, Co^{2+} or Mn^{2+} increased the absorbance at 340 nm. Figs. 4A–C show that the increase in absorbance is largest at or near 340 nm, and hence this wavelength was used for spectrophotometric titrations of 3AP with metal ions. Cobalt gave the largest absorbance change with rather tight binding (Fig. 5A); Mn^{2+} showed weaker binding. The titration-curve with Fe^{2+} was anomalous (Fig. 5A): when the concentration of Fe^{2+} exceeded half the concentration of 3AP,

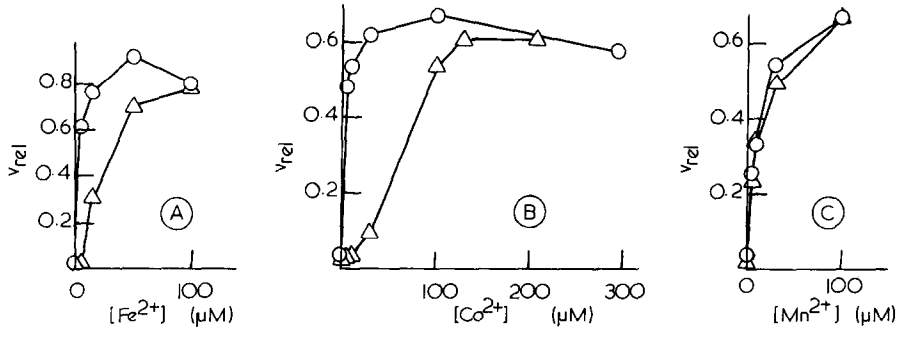

Fig. 3. Effects of 3AP on carboxylation activity of PEP Carboxykinase. A, with Fe^{2+}; B, with Co^{2+}; C, with Mn^{2+}. With purified enzyme (6.7 u/mg) and transition-metals as indicated. ○ = without 3AP; △ + 0.2 mM 3AP.

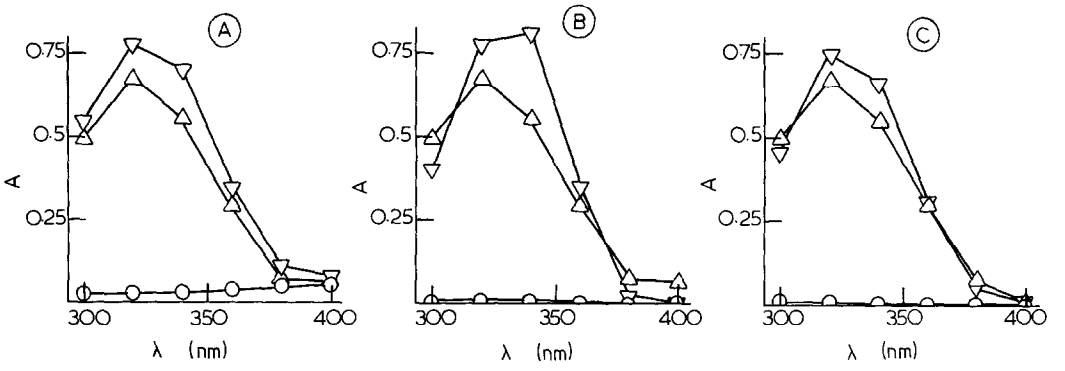

Fig. 4. u.v. absorption spectra of 3AP and effects of metal ions. A, with Fe^{2+}; B, with Co^{2+}; C, with Mn^{2+}. ○ = 50 mM Hepes–NaOH, pH 7.5 + 0.1 mM metal salt; △ = Hepes + 0.2 mM 3AP; ▽ = Hepes + 0.2 mM 3AP + 0.1 mM metal salt ($FeCl_2$, $CoCl_2$ or $MnCl_2$, as appropriate). Hepes alone gave the same absorbance as Hepes + $MnCl_2$.

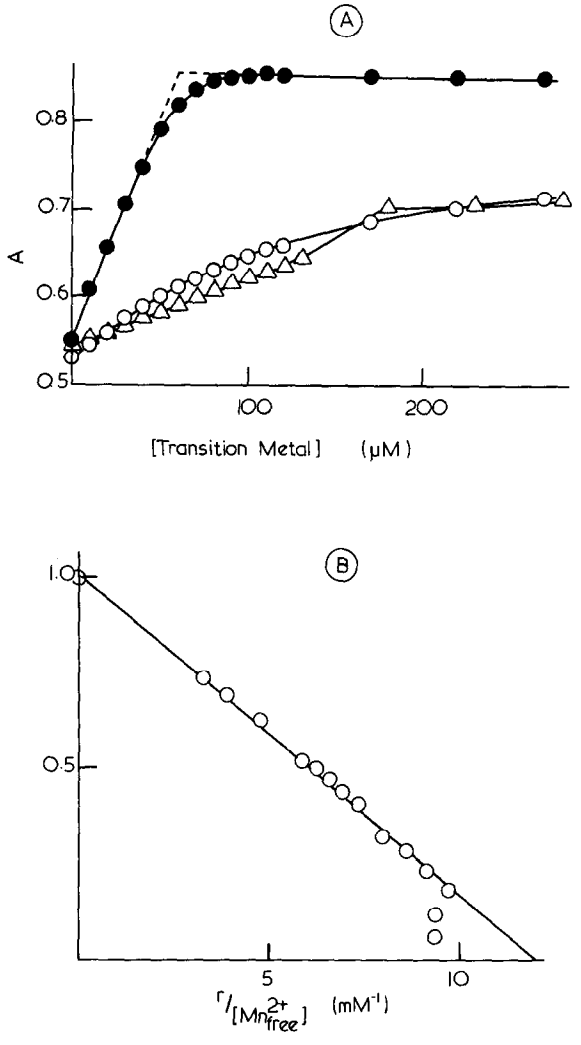

Fig. 5. Titration of 3AP with metal ions. A, absorbance vs metal-ion concentration: △ = + Fe^{2+}; ● = Co^{2+}; ○ = + Mn^{2+}. B, Scatchard plot of apparent binding of Mn^{2+} to 3AP, where r represents the fractional saturation with Mn^{2+}.

a further (time-dependent) increase in absorbance began. The Fe^{II}–(3AP) complex may be readily oxidisable to Fe^{III}–(3AP), while Fe^{II}–$(3AP)_2$ would appear to be stable. If $FeCl_2$ was diluted into the cuvette 5–10 min before adding 3AP, the extra absorbance was only formed after adding the 3AP, and therefore $Fe^{2+}(H_2O)_6$ was oxidised more slowly than Fe^{II}–(3AP).

With Co^{2+} (Fig. 5A) the extrapolated end-point of the titration is at 60 μM $CoCl_2$, which is nearest to three 3AP molecules complexing to each Co^{2+} ion. The linearity up to at least half-saturation suggests that each 3AP molecule in the complex has its u.v. absorbance altered identically. The cumulative dissociation-constant for all three molecules ($K_2 \times K_2 \times K_3$) is roughly 2700 $(\mu M)^3$. If each dissociation-constant was equal, their value would be 14 μM. Alternatively, if the first two bound very much more strongly than the third, the value of K_3 would be approximately 17 μM.

With Mn^{2+}, a Scatchard binding plot is shown in Fig. 5B. It is assumed that three 3AP molecules bind per Mn^{2+}, each giving an equal absorbance change. The linearity of the plot indicates that, if these assumptions are justified, the 3AP molecules each have equal affinity for Mn^{2+}; the slope gives a dissociation-constant of 85 μM.

Binding of 3AP to enzyme. Quinolinate and 3-mercaptopicolinate are inhibitors of PEP carboxykinase, and they compete for PEP in the carboxylation reaction [9]. Their structural similarity with 3AP suggests that 3AP might also alter the affinity of the enzyme for either of these inhibitors, or for PEP itself. Therefore K_m for PEP was determined with 3AP present, and K_i for quinolinate and for 3-mercaptopicolinate (Table 1). There is no clear effect of 3AP on any parameter. Results must be interpreted with caution; the concentrations of free metal-ions, and of their various complexes, are not the same in each experiment, although optimal metal-ion concentrations were used.

The rate of heat-inactivation [15] at 48° was slightly increased by 0.8 mM 3AP (Fig. 6); this can be attributed to chelation of Mn^{2+} (25 μM total) rather than

Table 1. Kinetic constants for PEP carboxykinase: effects of 3AP*

Inhibitor	Metal-ion	K_m (PEP) (μM)		K_m (μM)	
		No 3AP	+ 3AP	No 3AP	+ 3AP
3-Mercaptopicolinate	Mn^{2+}	240	185	22	18
Quinolate	Fe^{2+}	140–385	250	39–78	93

* Determined as described previously [9], with purified rat-liver enzyme (specific activity 6.7 u/mg). Quinolinate, 1 mM; 3-mercaptopicolinate, 50 μM; Mn^{2+}, 100 μM; Fe^{2+}, 50 μM without 3AP and 100 μM with 3AP.

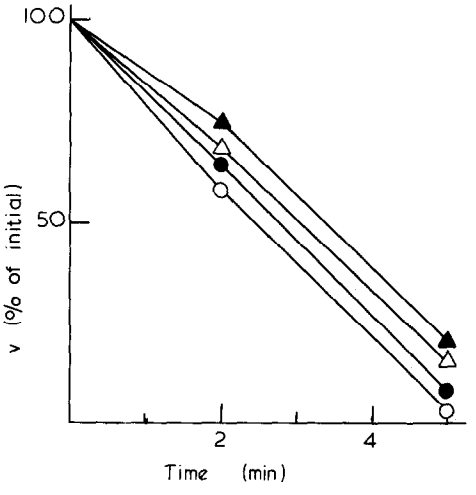

Fig. 6. Effect of 3AP on thermal inactivation of PEP carboxykinase at 48°. Enzyme (0.053 units of dialysed 45–65% ammonium sulphate fraction) was incubated at 48° in 50 mM Hepes–NaOH buffer pH 7.5, and 50 μl samples were withdrawn for normal carboxylation assay. Circles, + 25 μM $MnCl_2$ only; triangles, 25 μM $MnCl_2$ + 3 mM $MgCl_2$. Filled symbols, without 3AP; open symbols, + 0.8 mM 3AP.

to enzyme-binding by 3AP, since Mn^{2+} stabilised the enzyme under these conditions [15].

DISCUSSION

Chelation of metal ions by 3AP. Binding of 3AP by Co^{2+}, Fe^{2+} and Mn^{2+} is sufficiently strong to decrease the concentrations of the free ions *in vivo*. Furthermore, low concentrations of 3AP might catalyse the oxidation of Fe^{2+}. The concentrations *in vivo* of transition-metals are likely to be below optimal for PEP carboxykinase in normal liver: cytosolic Mn is 1.8 μM [5] and total cytosolic Fe is quoted as 185 μM [5], of which only a small proportion is likely to be Fe^{2+}; cobalt, at 0.07 p.p.m. in liver (see [15]) would be 1.2 μM if evenly distributed throughout the tissue.

The binding of Mn^{2+} by 3AP was studied by MacDonald and Lardy [4], using electron paramagnetic resonance of Mn^{2+}. Their method complements that used here; they measured a change of the environment of Mn^{2+}, while I measured a change in that of 3AP. Assumptions about stoichiometry were necessary in each case, but both methods yielded dissociation constants for 3AP of roughly 10^{-4}M. The binding of 3AP to Mn^{+} is clearly weaker than to Co^{2+} or Fe^{2+}, and the effect on the enzyme activity is very much weaker.

Since quinolinate and 3-mercaptopicolinate are well-characterised reversible inhibitors of PEP carboxykinase, it was expected that 3AP would also bind to the enzyme. However, the experiments reported here do not support this idea: affinities for PEP, quinolinate and mercaptopicolinate are scarcely affected by 3AP; and 3AP does not protect the enzyme against heat-inactivation, but only against Fe^{2+}–induced inactivation. MacDonald and Lardy also reported that 3AP does not affect the K_m for oxaloacetate [4], and have listed references to other iron-chelating agents which are hyperglycaemic or hypoglycaemic [17]. Iron-chelating agents which do not bind to PEP carboxykinase may be hyperglycaemic, while those that can bind to this enzyme may also inhibit it, neutralising or reversing the hyperglycaemic effect. Two models are suggested which may account for the activation of gluconeogenesis mediated by lowering the concentrations of free transition-metal ions by chelation. One involves inactivation of PEP carboxykinase by ferrous ions, and the other involves product-inhibition by PEP.

Protection of PEP carboxykinase from inactivation. Ferrous ions inactivate PEP carboxykinase at 0°, and 3AP effectively protects the enzyme. When 3AP is added to partly-inactivated enzyme it is not reactivated, in accordance with a previous report [4]; oxidation of thiol groups in the enzyme is catalysed by Fe^{2+} [18]. Protection of existing enzyme, so that the rate of degradation was less than the rate of synthesis, would allow more enzyme to accumulate. However this effect would be slow, as the half-life of the enzyme in rat-liver is normally about 6 hr [15], and yet 3AP begins to raise blood glucose within 10 min [3].

Alternatively, PEP carboxykinase may always be in a partly inactivated state *in vivo* due to a dynamic equilibrium between inactivation by O_2 and Fe^{2+}, and reactivation by thiols such as reduced glutathione or reduced thioredoxin [8]. Reactivation could be allowed to predominate if 3AP effectively lowered the concentration of Fe^{2+}, probably by chelation. Quinolinate, although an inhibitor of PEP carboxykinase and of gluconeogenesis, gives a rapid increase in the assayable activity of the enzyme [19] after liver perfusion and can also chelate Fe^{2}. Dithiothreitol prevents inactivation but does not activate the enzyme in supernatant fractions from rat livers [14];

thiol compounds restore the activity of sub-active purified chicken-liver enzyme [20].

Possible kinetic effects of 3AP on PEP carboxykinase. The only kinetic effect shown here of 3AP on the activity of PEP carboxykinase is inhibition at low concentrations of Fe^2 and Co^{2+}. However, the concentrations of substrates, products and activators in liver cytoplasm are different from those used in assays *in vitro*.

The K_s for PEP has been estimated kinetically to be 40 μM with Mn^{2+} as activator [21], which is lower than K_m when PEP is substrate; magnetic resonance gave evidence that the liver mitochondrial enzymes from pig [22] and the chicken [20], and the sheep kidney mitochondrial enzyme [23] bind PEP through a bridge with Mn^{2+}. The importance of a transition-metal for PEP-binding is also suggested by the lack of activity, with Mg^{2+} alone, in carboxylating PEP; oxaloacetate is readily decarboxylated with Mg^{2+} alone. Since the concentrations of oxaloacetate, GDP and GTP are low *in vivo*, product-inhibition by PEP might be appreciable provided that divalent transition-metal ions were present to enable it to bind. A chelating agent such as 3AP, by reducing the concentration of transition-metal ions, could reduce PEP-binding and hence allow activation in the gluconeogenic direction. This needs to be examined by direct kinetic studies at near-physiological concentrations of metabolites.

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