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Probing the allosteric activation of pyruvate carboxylase using 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate as a fluorescent mimic of the allosteric activator acetyl CoA

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ABSTRACT

2′,3′-O-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) is a fluorescent analogue of ATP. MgTNP-ATP was found to be an allosteric activator of pyruvate carboxylase that exhibits competition with acetyl CoA in activating the enzyme. There is no evidence that MgTNP-ATP binds to the MgATP substrate binding site of the enzyme. At concentrations above saturating, MgATP activates bicarbonate-dependent ATP cleavage, but inhibits the overall reaction. The fluorescence of MgTNP-ATP increases by about 2.5-fold upon binding to the enzyme and decreases on addition of saturating acetyl CoA. However, not all the MgTNP-ATP is displaced by acetyl CoA, or with a combination of saturating concentrations of MgATP and acetyl CoA. The kinetics of the binding of MgTNP-ATP to pyruvate carboxylase have been measured and shown to be triphasic, with the two fastest phases having pseudo first-order rate constants that are dependent on the concentration of MgTNP-ATP. The kinetics of displacement from the enzyme by acetyl CoA have been measured and also shown to be triphasic. A model of the binding process is proposed that links the kinetics of MgTNP-ATP binding to the allosteric activation of the enzyme.

Introduction

Pyruvate carboxylase is a biotin-dependent enzyme that catalyses the formation of oxaloacetate for the purposes of gluconeogenesis in the liver and replenishment of tricarboxylic acid cycle intermediates in certain tissues (for reviews see [1,2]). The overall reaction occurs in two partial reactions (see Fig. 1). In reaction (1), which occurs in the biotin carboxylation (BC[1]) domain, bicarbonate is activated by phosphorylation to form a putative carboxyphosphate intermediate with concomitant cleavage of MgATP. The 1′N of biotin is then carboxylated, presumably in a reaction with carbon dioxide formed from the reversible decarboxylation of the carboxyphosphate. Carboxybiotin then moves to the carboxyl transfer (CT) domain where reaction (2) occurs, in which there is carboxyl transfer from carboxybiotin to pyruvate and proton transfer from pyruvate to biotin (for reviews on the mechanism of pyruvate carboxylase see [2–4]).

The activity of pyruvate carboxylases from many organisms, including vertebrates, some fungi and some bacteria is allosterically regulated by acyl CoAs, principally acetyl CoA. Many pyruvate carboxylases from these organisms show only low levels of activity in the absence of acetyl CoA. Acetyl CoA has been shown to stabilize the tetrameric structure of the enzyme (α_4 in the majority of cases) [5–9]. The locus of the effects of acetyl CoA on the mechanism of the reaction appears to be primarily on reaction (1) [10–15].

Until the recent publication of the structures of pyruvate carboxylases from *Rhizobium etli* [16] (RePC) and *Staphylococcus aureus* [17], it had been assumed that the subunits comprising the enzymic tetramer operated independently. However, it is apparent that the subunits operate in pairs, with inter-subunit catalysis occurring within these pairs [16,17]. The CT domain of one subunit catalyses the transfer of the carboxyl group to pyruvate from the carboxybiotin of its partner subunit [16,17]. The structure of RePC was determined in the presence of the stable analogue of acetyl CoA, ethyl CoA, with only two of the four

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[1] *Abbreviations used:* PC, pyruvate carboxylase; RePC, *Rhizobium etli* pyruvate carboxylase; BC, biotin carboxylase; CT, carboxyl transferase; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate.

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$$MgATP + HCO_3^- + \text{E-biotin} \rightleftharpoons MgADP + {}^{-2}O_3POCO_2^-.\text{E.biotin} \rightleftharpoons Pi + \text{E-biotin-}CO_2^- \quad \text{- (1)}$$

$$\text{E-biotin-}CO_2^- + \text{pyruvate} \rightleftharpoons \text{E-biotin} + \text{oxaloacetate} \quad \text{- (2)}$$

Fig. 1. In pyruvate carboxylase, reaction (1) occurs at the biotin carboxylation (BC) domain and involves the ATP-dependent activation of bicarbonate to form a putative carboxyphosphate intermediate, from which the enzymic biotin is then carboxylated to form the enzyme–carboxybiotin complex. The carboxybiotin on the biotin carboxyl carrier protein (BCCP) domain then moves to the carboxyl transfer (CT) domain of its partner subunit where the carboxy group is transferred to pyruvate to form oxaloacetate in reaction (2).

subunits having ethyl CoA bound in the allosteric effector site. More importantly, only the pair of subunits with ethyl CoA bound were in a conformation that appeared more conducive for inter-subunit catalysis [16], raising the possibility that pyruvate carboxylase exhibits half-of-the-sites reactivity. There is evidence that another biotin–dependent carboxylase, *Escherichia coli* acetyl CoA carboxylase, operates in this way. The homodimeric biotin carboxylase subunits of this enzyme appear to show half-of-the-sites reactivity, with obligatory switching of activity between the two subunits [18–20]. However, a recent structure of the *S. aureus* enzyme complexed with a single CoA molecule in each of the allosteric sites was determined [21].

The original aim of this work was to investigate nucleotide binding to RePC by using a fluorescent analogue of ATP, viz 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) (see Fig. 2). However, we found that MgTNP-ATP activates the enzyme in a similar way to acetyl CoA and by measuring the increase in fluorescence associated with MgTNP-ATP binding to the enzyme, we have been able to probe the connection between activator binding and this activation. In this paper we also report the characteristics of the activation of the bicarbonate-dependent ATP cleavage catalysed by RePC at concentrations above those required to saturate the enzyme with MgATP as a substrate, or, more simply, super-catalytic concentrations of MgATP.

Experimental procedures

Expression and purification of RePC in E. coli

BL21(DE3) were transformed with the pET-17b $(His)_9$ RePC plasmid [16] or R472S mutant and the pCY216 plasmid containing *E. coli* biotin protein ligase (BirA) [22] for expression. The R472S mutant was constructed by site-directed mutagenesis using RePC WT sequence as the template as previously described [15]. The mutagenic primers used were K472S-F (5′-aagcgccaggac**tct**gcgacgaagctt-3′, bold indicates the codon changed for serine) and K472S-R (5′-aagcttcgtcgc**agag**tcctggcgctt-3′). The nucleotide sequence of the mutant was verified by DNA sequencing. Wild-type and R472S RePC were overexpressed in 8 L batch cultures of LB media (containing 200 μg ml^{-1} ampicillin, 30 μg ml^{-1} chloramphenicol, 1 mg L^{-1} biotin and 25% w/v arabinose) which were inoculated with an overnight culture of the freshly transformed *E. coli* BL21(DE3)cells. Large-scale fermentations were carried out in a 10 L carboy equipped with an air inlet hose, which was connected to the house air line and fitted with a gas diffusion stone to ensure proper aeration and agitation of the media throughout growth and induction. Batch cultures were grown at 37 °C to an OD_{600} = 0.9–1.2. They were then chilled on ice for 20 min before IPTG was added to a final concentration of 0.1 mM in the carboy, prior to transferring to a 16 °C water bath for approximately 48 h. Cells were harvested by centrifugation, yielding about 80*g* of cell paste. RePC was purified using Co^{2+}-affinity chromatography [16]. The purified enzyme was stored in 0.1 M Tris–Cl, pH 7.8 containing 30% glycerol at −80 °C after being flash frozen in liquid nitrogen.

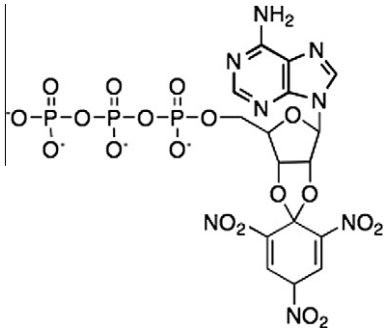

Fig. 2. Structure of 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP).

Estimation of biotin concentration in pyruvate carboxylase

Biotin concentrations of the enzyme solutions were determined by subjecting the enzyme to proteolysis as described previously [23]. The biotin content of the hydrolysates was then assayed according to the method described by Rylatt et al. [24]. Enzyme concentrations used are expressed in terms of the biotin concentration.

Pyruvate carboxylation assays

Assays were performed at 30 °C in 0.1 M Tris–Cl, pH 7.8 in the presence of 20 mM $NaHCO_3$, 6 mM $MgCl_2$, 1 mM ATP and 10 mM pyruvate. Oxaloacetate formation was measured by coupling with malate dehydrogenase (11 units mL^{-1}) and 0.24 mM NADH. Assays were performed in the presence or absence of varying concentrations of acetyl CoA at different fixed concentrations of MgTNP-ATP (Jena Bioscience). To assess the effect of super-catalytic concentrations of MgATP on the reaction, MgATP was the varied substrate and reactions were performed with no acetyl CoA present. Apparent k_{cat} values were calculated by dividing the measured reaction velocity by the biotin concentration of the RePC used in the assay.

Bicarbonate-dependent ATP-cleavage assays

Assays were performed as described previously [25]. Briefly, the reactions contained 0.1 M Tris–HCl, pH 7.8, 2.5 mM MgATP, 2.5 mM $MgCl_2$, 20 mM $NaHCO_3$, 0.33 mM NADP, 0.25 mM acetyl-CoA, 5 μM α-glucose-1-phosphate, 1 mg mL^{-1} glycogen, 2 units mL^{-1} phosphorylase *a*, 3 units mL^{-1} phosphoglucomutase and 3 units mL^{-1} glucose-6-phosphate dehydrogenase. Production of NADPH was determined by measuring the increase in absorbance at 340 nm. Apparent k_{cat} values were calculated by dividing the measured reaction velocity by the biotin concentration of the RePC used in the assay.

Fluorescence emission spectra

Fluorescence emission spectra of MgTNP-ATP were recorded on a Cary spectrofluorometer with an excitation wavelength of 408 nm and emissions recorded between 500 and 600 nm. Experiments were performed at 30 °C in 0.1 M Tris–Cl, pH 7.8 and 20 mM