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Purification and characterization of phosphoenolpyruvate carboxykinase from the anaerobic ruminal bacterium *Ruminococcus flavefaciens*

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Abstract Phosphoenolpyruvate (PEP) carboxykinase was purified 42-fold with a 25% yield from cell extracts of *Ruminococcus flavefaciens* by ammonium sulfate precipitation, preparative isoelectric focusing, and removal of carrier ampholytes by chromatography. The enzyme had a subunit molecular mass of ~66.3 kDa (determined by mass spectrometry), but was retained by a filter having a 100-kDa nominal molecular mass cutoff. Optimal activity required activation of the enzyme by Mn^{2+} and stabilization of the nucleotide substrate by Mg^{2+}. GDP was a more effective phosphoryl acceptor than ADP, while IDP was not utilized. Under optimal conditions the measured activity in the direction of PEP carboxylation was 17.2 μmol min^{-1} (mg enzyme)$^{-1}$. The apparent K_m values for PEP (0.3 mM) and GDP (2.0 mM) were 9- and 14-fold lower than the apparent K_m values for the substrates of the back reaction (oxaloacetate and GTP, respectively). The data are consistent with the involvement of PEP carboxykinase as the primary carboxylation enzyme in the fermentation of cellulose to succinate by this bacterium.

Key words Rumen · Phosphoenolpyruvate · Phosphoenolpyruvate carboxykinase · CO_2 fixation · *Ruminococcus flavefaciens*

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Abbreviations *IEF* Isoelectric focusing · *MALDI* Matrix-assisted laser desorption/ionization · *OAA* Oxaloacetic acid · *PEP* Phosphoenolpyruvate

Introduction

Phosphoenolpyruvate carboxykinase (PEP carboxykinase, EC 4.1.1.32) catalyzes the reversible carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (OAA) according to the reaction:

$$PEP + CO_2 + MgNDP \leftrightarrow OAA + MgNTP$$

where CO_2 is an unspecified reactive carboxyl species (CO_2 or HCO_3^-); the identity of the nucleotide di- and triphosphates varies among different organisms (Utter and Kolenbrander 1972). The enzyme has been purified from a wide variety of organisms and tissues [see summary by Schomburg and Salzmann (1990)]. PEP carboxykinase has been studied intensively in tissues of vertebrate animals, where it functions mainly in the direction of OAA decarboxylation to generate PEP for gluconeogenesis and has only a minor role in converting PEP to OAA for replenishment of tricarboxylic acid cycle intermediates removed in biosynthetic reactions (Utter and Kolenbrander 1972). PEP carboxykinases from bacteria have received relatively little study. A gluconeogenic role has been proposed for the enzyme in certain aerobic bacteria (Bridgeland and Jones 1967; Salverry et al. 1989; Shrago and Shug 1969; Teraoka et al. 1970;) and anaerobic bacteria (Chao and Ng 1986) growing on lactate and other organic acids. In contrast, the enzyme is thought to be a major carboxylation enzyme for the generation of an OAA intermediate in succinate-producing saccharolytic anaerobes (Scardovi 1963; Hopgood and Walker 1969; Miller 1978; Samuelov et al. 1991).

Ruminococcus flavefaciens is an anaerobic cellulolytic ruminal bacterium. In the rumen habitat, this species is both a major fiber-digesting agent and a major producer of acetic and succinic acids, the latter being an important precursor of propionate (Hungate 1966). Acetate and pro-

pionate, along with other short-chain fatty acids produced during ruminal fermentation, are absorbed through the rumen wall for subsequent oxidation to provide energy and biosynthetic precursors for the animal (Hungate 1966). In *R. flavefaciens* FD-1, yields of the primary fermentation end products succinate and acetate do not vary substantially as a function of growth rate (Shi and Weimer 1992). This relative constancy of carbon flux to end products may be exerted through controlling the metabolism of PEP, the branch point of the carbohydrate fermentation pathway. Isotopic studies with whole cells have revealed that the carboxyl group of succinate is derived from CO_2 (Shi et al. 1997); in cell extracts, PEP carboxykinase is the major carboxylation enzyme and the only enzyme among nine catabolic enzymes tested whose activity was related to both bacterial growth rate and succinate production rate in cellulose-limited continuous cultures (Shi 1992). Despite the purportedly central role of PEP carboxykinase in anaerobic succinate production (Scardovi 1963; Hopgood and Walker 1969; Miller 1978; Samuelov et al. 1991), this enzyme has not been purified from any ruminal bacterium, although it has been purified from the nonruminal saccharolytic anaerobe *Anaerobiospirillum succiniciproducens* (Podkovyrov and Zeikus 1993). This paper reports the isolation of PEP carboxykinase from *R. flavefaciens* FD-1 and describes some of the salient characteristics that suggest its major role in catabolic CO_2 fixation.

Materials and methods

Cell growth and enzyme purification

Ruminococcus flavefaciens FD-1 was grown for 36 h at 39° C in two carboys that contained ten-liter volumes of CO_2-sparged modified Dehority medium (Weimer et al. 1991) supplemented with 4.5 g cellobiose l^{-1}. At the end of the incubation, the culture was amended with 4 mM $MgCl_2$ and 0.02 mM $MnCl_2$, and cells were concentrated using a Pellicon horizontal-flow filtration device (Millipore, Bedford, Mass., USA) before pelleting by centrifugation at 12,000 × *g*. Cells were immediately suspended in buffer [5 mM K phosphate, 2 mM $MgCl_2$, 0.02 mM $MnCl_2$, (pH 8.0)] and broken by a single passage through a cold French pressure cell at 124 MPa. The cell lysate was centrifuged at 35,000 × *g* at 2° C for 60 min to yield a clear supernatant (cell extract). All of the above procedures were conducted under a CO_2 atmosphere.

Purification of phosphonenolpyruvate (PEP) carboxykinase was initiated by subjecting the cell extract to $(NH_4)_2SO_4$ precipitation. The fraction at 50–75% saturation, which contained most of the PEP carboxykinase activity, was resuspended in 5 mM K phosphate buffer (pH 8.0). Portions (1.4 ml) of this solution were applied to each of six columns (6 × 1.4 cm diameter, in 10-cm^3 plastic hypodermic syringe barrels) containing Bio-Gel P6DG (Bio-Rad, Richmond, Calif., USA) that had been previously equilibrated with four volumes of the same buffer. These columns were centrifuged at 150 × *g* for 1 min to recover the desalted enzyme-containing eluate. This solution was applied to a Rotofor preparative isoelectric focusing (IEF) cell (BioRad). The IEF cell was prefocused with 2% (v/v) Bio/Lyte carrier ampholytes (BioRad) having a nominal pH range of 3–5. IEF was carried out at constant power (12 mA) for 4 h. The four active fractions (pH range 4.1–4.5) were pooled and refocused, and the four active fractions from the second focusing (pH range 4.1–4.3) were pooled and desalted as above to remove carrier ampholytes and divalent cations and to exchange the buffer to 50 mM K phosphate (pH 8.0).

Determination of physical properties of the enzyme

Subunit molecular mass was determined by matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry, essentially by the method of Beavis et al. (1992), using ~20 pmol purified enzyme, α-cyano-4-hydroxycinnamic acid as adsorbing matrix, and β-lactoglobulin (18.30 kDa) as an external standard. Spectral resolution of the molecular ion peak was ~0.162 kDa (full width at half maximal peak height). Subunit molecular mass was verified by SDS-PAGE at constant current (27 mA) in 0.75-mm-thick gels having a polyacrylamide content of 12.5% in the resolving gel and 3% in the stacking gel. Molecular masses were determined using standards of 14.4–97.4 kDa (BioRad).

Assays and reagents

Enzyme assays were conducted in glass semimicrocuvettes (1-cm path length) sealed with soft rubber stoppers and having a liquid volume of 1.0 ml and a gas phase volume of ~0.2 ml. PEP carboxykinase was assayed in both directions by coupling the activity to exogenously added dehydrogenase enzymes; the rate of NADH oxidation was measured spectrophotometrically at 340 nm. In the direction of PEP carboxylation, the standard 1-ml reaction mixture contained 50 mM Na borate/succinate buffer (pH 6.9), 4 mM $MgCl_2$, 0.02 mM $MnCl_2$, 25 mM $NaHCO_3$, 1 mM GDP, 0.3 mM NADH, 2 units malate dehydrogenase (from bovine heart), 7.5 µg purified PEP carboxykinase, and a CO_2 gas phase (established by evacuation of gas phase for several minutes, followed by flushing with O_2-free CO_2). The reaction was initiated by addition of PEP to a concentration of 8 mM. For assays conducted to measure the pH profile of the enzyme, the amount of malate dehydrogenase was increased to 6 units to compensate for its reduction in activity at extremes of pH. In the direction of oxaloacetate (OAA) decarboxylation, the standard 1-ml reaction mixture contained 50 mM Hepes (pH 7.1), 4 mM $MgCl_2$, 0.02 mM $MnCl_2$, 25 mM $NaHCO_3$, 5 mM GTP, 5 units each of pyruvate kinase and lactate dehydrogenase (from rabbit muscle), 1 mM ADP, 0.3 mM NADH, 7.5 µg purified PEP carboxykinase, and a CO_2 gas phase; the reaction was initiated by addition of K-OAA to a concentration of 8 mM. All coupling enzymes were obtained from Sigma as suspensions in 50% glycerol or 3.2 M $(NH_4)_2SO_4$ and were used directly without dialysis.

Protein was measured by the method of Bradford (1976) using commercial dye reagents (BioRad), and using egg white lysozyme (Sigma, St. Louis, Mo., USA) as a standard.

Determination of kinetic parameters

Kinetic parameters for the substrates GDP and PEP were determined with the purified enzyme by measuring the reaction rate at varying GDP concentrations with each of four fixed PEP concentrations (0.0625, 0.125, 0.25, and 0.50 mM) or at varying PEP concentrations with each of four fixed GDP concentrations (0.57, 0.667, 0.8, and 1.0 mM). Kinetic parameters for Mn^{2+} were determined by varying Mg^{2+} concentration at four different fixed levels of Mn^{+2} (0.005, 0.0067, 0.01, and 0.02 mM). For each assay, the concentrations of other reaction components were as listed above. Apparent K_m values were determined from Lineweaver-Burk plots with data fitted by least-squares linear regression. Y-intercepts of the Lineweaver-Burk plots were plotted against the inverse of the concentration of secondary substrate to determine V_{max} and true K_m; this method of systematically varying the concentration of secondary substrate permits determination of kinetic parameters without the necessity of using saturating concentrations of either substrate (Phillips 1993). Correlation coefficients (r^2 values) for these primary and secondary plots ranged from 0.976 to 1.000 (mean 0.993).

Table 1 Purification of phosphoenolpyruvate (PEP) carboxykinase from *Ruminococcus flavefaciens* FD-1. Assays were performed in the direction of PEP carboxylation

Purification step	Volume (ml)	Protein (mg)	Activity (μmol min^{-1})	Specific activity [μmol min^{-1} (mg protein)$^{-1}$]	Recovery (%)	Purification (- fold)
Crude extract	195	669	274.2	0.41	100	1
$(NH_4)_2SO_4$ (after desalting)	380	21.9	142.6	6.5	52	16
Isoelectric focusing	8.4	4.16	63.1	15.2	23	37
Bio-Gel P6DG	15.2	3.98	68.5	17.2	25	42

Results

Enzyme purification

Phosphoenolpyruvate (PEP) carboxykinase from *Ruminococcus flavefaciens* FD-1 was purified 42-fold with an overall recovery of 25% by a relatively simple procedure involving $(NH_4)_2SO_4$ precipitation, desalting, preparative-scale isoelectric focusing, and final desalting (Table 1). The pure, desalted enzyme retained activity upon lyophilization and rehydration. The enzyme had a broad pH profile with a pH range of 5.3–8.6, half-maximal activity at 6.0 and 8.0, and maximal activity at pH 6.9. The enzyme was not inactivated or inhibited by O_2.

Physical properties

Matrix-assisted laser desorption/ionization (MALDI) analysis (Fig. 1) revealed that the enzyme had a subunit molecular mass of 66.316 kDa, with an accuracy of approximately one part in 1,000; two minor contaminants (molecular mass 44.25 and 26.62 kDa) were also detected. SDS-PAGE revealed a single band of approximate molecular mass of 68 kDa, indicating a single subunit type. However, enzyme activity was completely retained by a 100-kDa nominal molecular mass cutoff filter.

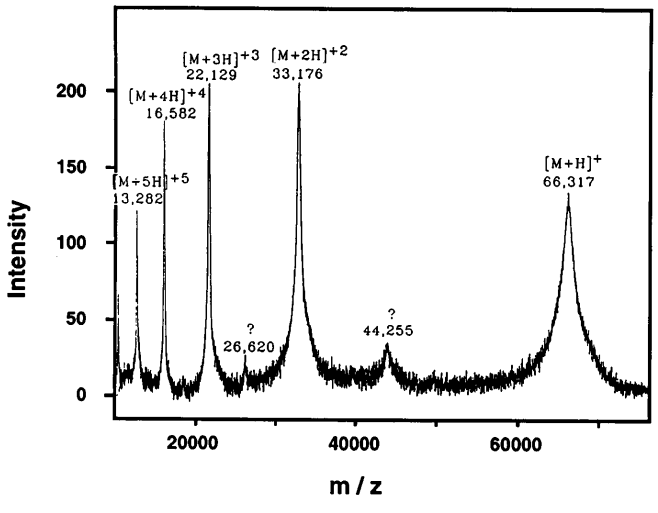

Fig. 1 Matrix-assisted laser desorption/ionization (MALDI) mass spectrometric determination of subunit molecular weight of phosphoenolypyruvate (PEP) carboxykinase. The peak at *m/z* = 66.317 kDa for the singly ionized protein corresponds to a native molecular mass of 66.316 ± ~0.07 kDa. The small peaks at *m/z* = 26.620 and 44.255 kDa correspond to minor contaminants

Kinetic parameters in the direction of PEP carboxylation

Lineweaver-Burk plots of enzyme activity were linear for both GDP and PEP at all four concentrations of secondary substrates. Apparent K_m values, determined at the highest concentrations of secondary substrates tested, were 0.29 mM for PEP and 2.0 mM for GDP. The y-intercept values (apparent $1/V_{max}$ values) from these primary plots were replotted against the inverse of substrate concentration to determine the true K_m and V_{max} values. The true K_m for PEP (0.21 mM) was nearly tenfold lower than the true K_m for GDP (2.0 mM). Assuming that the enzyme has a single catalytic site, the calculated V_{max} of 18.6 μmol min^{-1} (mg enzyme)$^{-1}$ translates to a turnover number for the enzyme of 1,230 min^{-1}. ADP could be used as an alternate nucleotide substrate at 10% of the rate for GDP, but IDP could not serve as a substrate. Lineweaver-Burk plots were linear for ADP, with an apparent K_m of 9.8 mM.

Activation of enzyme by divalent cations

Titration of the fully activated enzyme with EDTA produced a biphasic profile of inhibition (Fig. 2A). The biphasic inhibition of enzyme activity by EDTA in this assay system indicates that both Mn^{2+} and Mg^{2+} (exogenously added to the reaction mix) were required for full activation of the enzyme. Since EDTA has a much higher affinity for Mn^{2+} (log stability constant at pH 7 = 10.7) than for Mg^{2+} (log stability constant at pH 7 = 5.4), the scarcer Mn^{2+} was titrated first upon EDTA addition (Dawson et al. 1979), producing a strong reduction in activity at low EDTA concentration (cf. slopes in Fig. 2B and 2C). The fact that some activity was retained at EDTA concentrations sufficient to complex the Mn^{2+} suggests that Mg^{2+} can also activate the enzyme, albeit less effectively. Based on analogy with other PEP carboxykinases (Schramm et al. 1981; Rohrer et al. 1986), it appears that the low levels of Mn^{2+} directly activated the enzyme, while the high levels of Mg^{2+} stabilized the nucleotide diphosphate substrate. Other divalent cations (Co^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}) at concentrations of up to 4 mM failed to substitute for Mg^{2+}or Mn^{2+}. Lineweaver-Burk plots of enzyme activity

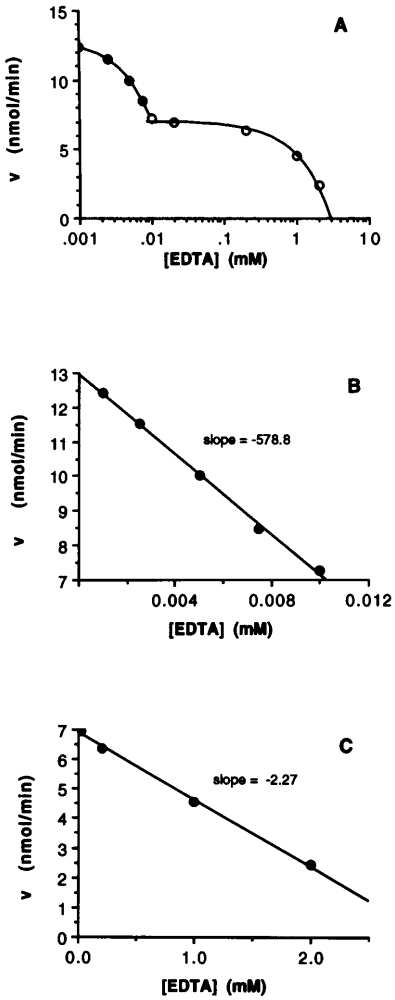

Fig. 2 Inhibition by EDTA of fully activated phosphoenolpyruvate (PEP) carboxykinase, assayed in the direction of PEP carboxylation. **A** Semilogarithmic plot of reduction of activity upon addition of various concentrations of EDTA. **B** Linear plot of the reduction of activity upon addition of low concentrations of EDTA (titration of Mn^{2+}). **C** Linear plot of the reduction of activity upon addition of high concentrations of EDTA (titration of Mg^{2+}). See text for discussion

were linear for Mg^{2+} at four different Mn^{2+} concentrations. A plot of the y-intercepts (apparent $1/V_{max}$ values) from these primary plots versus the inverse of $[Mn^{2+}]$ revealed a true K_m for Mn^{2+} of 0.0087 mM.

Kinetic parameters in the direction of oxaloacetate decarboxylation

In the direction of oxaloacetate (OAA) decarboxylation, the enzyme displayed linear Lineweaver-Burk plots for both OAA (apparent K_m = 2.7 mM) and GTP (apparent K_m = 28.7 mM). While the concentrations of substrate used in these plots were well below the concentration required for saturation of enzyme activity and, thus, yield only approximate apparent K_m values, the data nevertheless indicate that the affinities for substrates in the direction of OAA decarboxylation are well below those in the direction of PEP carboxylation.

Discussion

Although phosphoenolpyruvate (PEP) carboxykinases have been isolated from many different organisms and tissues, this report describes the first purification of this enzyme from a ruminal bacterium. Despite the simplicity of the purification procedure, PEP carboxykinase was purified 42-fold to a measured specific activity [17.2 μmol min^{-1} (mg enzyme)$^{-1}$] similar to those reported for the most active PEP carboxykinases from vertebrate tissues (Ballard and Hanson 1969; Goto et al. 1979, 1980; Gallwitz et al. 1988). PEP carboxykinase accounted for ~2.4% of the protein in cell extracts, a value consistent with its apparently major role in carbohydrate catabolism. The substrates for the *Ruminococcus flavefaciens* PEP carboxykinase reaction used for PEP carboxylation show a 9- to 14-fold lower apparent K_m than do those used for oxaloacetate (OAA) decarboxylation. In comparison, in the nonruminal saccharolytic *Anaerobiospirillum succiniciproducens*, the apparent K_m values are two- to sixfold lower for the substrates in the direction of PEP carboxylation than for those in the direction of OAA decarboxylation (Podkovyrov and Zeikus 1993). This fact, combined with the known production of succinate as a major product of carbohydrate fermentation, suggests that the preferred physiological direction of the reaction in vivo is PEP carboxylation. The use of PEP carboxykinase as a major catabolic enzyme has been suggested previously for several other succinogenic anaerobic bacteria including *R. flavefaciens* strain C (Hopgood and Walker 1969) and *Succinivibrio dextrinosolvens* (Scardovi 1963) based solely on the abundance of this enzyme in cell extracts. In contrast, PEP carboxykinases in a number of bacteria that do not produce succinate (e.g., the PEP carboxykinases from the tissues of vertebrate animals) are considered to operate in the direction of OAA decarboxylation to provide PEP for gluconeogenesis (Bridgeland and Jones 1967; Shrago and Shug 1969; Teraoka et al. 1970; Chao and Ng 1986; Salverry et al. 1989).

PEP carboxykinase from *R. flavefaciens* FD-1 differs in some respects from the well-characterized PEP carboxykinases from other organisms. Its subunit molecular mass (~66.3 kDa) is at the lower end of the range (67–83 kDa) reported for most other organisms (Schomburg and Salzmann 1990), and its pI (~4.2) is substantially lower than the pIs of the PEP carboxykinases of *Anaerobiospirillum succiniciproducens* (pI 4.9; Podkovyrov and Zeikus 1993) and of organisms that do not use the enzyme primarily for CO_2 fixation (pI 5.0–6.5; Goto et al. 1980; Gallwitz et al. 1988). Similar to most other PEP carboxykinases, the *R. flavefaciens* enzyme is activated by divalent metal cations. However, unlike several other PEP carboxykinases (Snoke et al. 1971; Colombo et al. 1978), activation of the enzyme by divalent cations was observed only with Mn^{2+} and Mg^{2+}. The true K_m for PEP of 0.21

mM is similar to the 0.3–0.4 mM reported for several other PEP carboxykinases (Hebda and Nowak 1982; Harlocker et al. 1991).

The *R. flavefaciens* enzyme differs most from other PEP carboxykinases with respect to nucleotide utilization. GDP was the preferred substrate, but its true K_m of 2.0 mM is well above the values reported for the apparent K_m values in rat liver cytosol (8 μM; Colombo et al. 1978) and ribbed gill muscle tissue (24 μM; Harlocker et al. 1991). PEP carboxykinase from *R. flavefaciens* displayed a fivefold lower apparent K_m for GDP than for ADP, the preferred substrate for the enzyme from most plants (Utter and Kolenbrander 1972) and micro-organisms (Cannata 1970; Teraoka et al. 1970; Samuelov et al. 1991). IDP, the preferred cofactor in several mammalian systems (Utter and Kolenbrander 1972; Noce and Utter 1975; Colombo et al. 1978), was not utilized. *R. flavefaciens* produces considerable amounts of succinate despite the low affinity of its PEP carboxykinase for GDP. This may be due to the fact that the alternative catabolic fate of PEP (conversion to pyruvate via pyruvate kinase as the first committed step in acetate production) has an obligatory requirement for ADP rather than GDP as the phosphate acceptor; the utilization of different phosphoryl acceptors effectively reduces competition between the two pathways for PEP. Further characterization of the partitioning of PEP into the succinate and acetate pathways will require in vivo measurement of concentrations of the various reactants.

While PEP carboxykinase has not been previously isolated from other ruminal bacteria, several workers have reported its presence in cell extracts. Even within this group, considerable diversity appears to exist. In *R. flavefaciens* C, the enzyme resembles that from our strain FD-1 in its preference for GDP over ADP, but differs in its ability to use Co^{2+} as an activating cation (Hopgood and Walker 1969). In *Succinovibrio dextrinosolvens*, the enzyme displays a low pH optimum (4.8), a marked preference for Co^{2+} as activating cation, and a requirement for ADP as phosphoryl acceptor (Scardovi 1963). In the nonruminal succinate-producing bacterium *Anaerobiospirillum succiniciproducens*, the enzyme uses Co^{2+} or Mn^{2+}, but requires ADP (Podkovyrov and Zeikus 1993).

In *R. flavefaciens* and other succinate-producing ruminal bacteria, the use of PEP carboxykinase rather than PEP carboxylase and pyruvate carboxylase as a primary carboxylation enzyme has the net effect of both conserving energy (by substrate-level phosphorylation of GDP) and providing OAA, the conversion of which to succinate permits the disposal of two pairs of reducing equivalents generated during conversion of carbohydrates to PEP. The use of PEP carboxykinase as a gluconeogenic enzyme is obviated by the nutritional specialization of this anaerobic bacterium, which can subsist only by fermentation of cellulose and cellodextrins (Hungate 1966), compounds which provide any glucosyl precursors necessary for anabolic reactions.

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