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Purification and Regulatory Properties of Phosphoribulokinase from *Hydrogenomonas eutropha* H 16

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1. Phosphoribulokinase was purified 286-fold from extracts of autotrophically grown cells. 2. The enzyme had a molecular weight of 237000 and showed a pH optimum of 9.0 in both crude extracts and purified preparation. $MgCl_2$ was required for activity; full activation was obtained at 5mM-$MgCl_2$ and the K_m was approx. 0.5mM. 3. The ATP-saturation curve was sigmoidal and the degree of positive co-operativity increased at higher $MgCl_2$ concentrations. The ATP-binding sites appeared to be non-interacting at low ribulose 5-phosphate concentrations. 4. Lineweaver–Burk plots for ribulose 5-phosphate showed abrupt transitions between apparently linear sections. The apparent K_m and $V_{max.}$ values increased with increasing concentrations of ribulose phosphate. The transitions may be explained by a sequence of negative and positive co-operativity in the catalytic rate constants. 5. Phosphoribulokinase activity was inhibited by AMP and phosphoenolpyruvate and was activated by NADH. The presence of AMP or phosphoenolpyruvate increased $s_{0.5}$ (substrate concentration required for half-maximal velocity) for both ribulose 5-phosphate and ATP but $V_{max.}$ was not changed. The sigmoidicity of the ATP-saturation curve increased in the presence of AMP but was not affected by phosphoenolpyruvate. The transitions in the ribulose 5-phosphate-saturation curves were more abrupt in the presence of either inhibitor. NADH lowered the $s_{0.5}$ for both ribulose 5-phosphate and ATP. The activator did not affect the degree of positive co-operativity between ATP-binding sites, but the ribulose 5-phosphate-binding sites appeared to be non-interacting in its presence. 6. A sequence of positive and negative co-operativity in the interactions of AMP-binding sites was suggested by the Hill plots. In the presence of NADH (and phosphoenolpyruvate) the sensitivity to inhibition by AMP was less below a certain AMP concentration and increased above that concentration. 7. Examination of the interactions between ligands indicated that phosphoribulokinase can be regulated effectively by changes in effector concentrations similar to those reported to occur *in vivo*.

Phosphoribulokinase [ribulose 5-phosphate kinase; ATP–D-ribulose 5-phosphate 1-phosphotransferase (EC 2.7.1.19)] is a key enzyme in the carbon metabolism of autotrophic organisms. The regulation of phosphoribulokinase activity is an effective way of controlling CO_2 fixation. Studies with extracts of several autotrophic bacteria showed that the site of inhibition of CO_2 fixation by AMP is phosphoribulokinase (Johnson & Peck, 1965; Gale & Beck, 1966; Johnson, 1966; McFadden & Tu, 1967; Rindt & Ohmann, 1969). The regulation of phosphoribulokinase, however, varied with the organism. Phosphoribulokinase from *Pseudomonas facilis* (MacElroy *et al.*, 1969) and *Rhodospirillum rubrum* (Joint *et al.*, 1972*a*) is inhibited by AMP and is activated by NADH. The enzyme from *Thiobacillus neapolitanus* is inhibited by phosphoenolpyruvate (MacElroy *et al.*, 1972) and that from *Chromatium* by both AMP and phosphoenolpyruvate (Hart & Gibson, 1971), but in neither organism is NADH an activator.

Joint *et al.* (1972*b*) isolated and purified phosphoribulokinase as a complex with fructose diphosphatase (EC 3.1.3.11) from *R. rubrum*. The enzyme was also reported to be in a complex with ribulose diphosphate carboxylase (EC 4.1.1.39) in *T. neapolitanus* and *Thiobacillus thioparus* (MacElroy *et al.*, 1968*b*).

We have presented evidence elsewhere that phosphoribulokinase is not associated with fructose diphosphatase or ribulose diphosphate carboxylase in *Hydrogenomonas eutropha* H 16 (Abdelal & Schlegel, 1973). The present paper reports the purification and properties of phosphoribulokinase from *H. eutropha* H 16. Phosphoribulokinase is shown to be inhibited by both AMP and phosphoenolpyruvate and activated by NADH. An attempt is made to correlate the kinetic properties of phosphoribulokinase with the regulation of the enzyme *in vivo*.

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Experimental

Materials

ATP, ADP, AMP, GMP, IMP, CMP, UMP, NADH (free of AMP), $NADP^+$, NADPH, phosphoenolpyruvate, lactate dehydrogenase, gluconate 6-phosphate dehydrogenase and standard proteins for molecular-weight determination were all obtained from C. F. Boehringer, Mannheim, Germany. Protamine sulphate, phenylmethylsulphonyl fluoride, dithiothreitol, ribulose diphosphate and spinach ribulose diphosphate carboxylase were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Whatman DE-52 preswollen DEAE-cellulose was purchased from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. $H_2{}^{14}CO_3$ was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. Ribulose 5-phosphate was prepared by the method described by Pontremoli & Mangiarotti (1962).

All other reagents were obtained at the highest purity possible from commercial sources.

Growth of cells and preparation of cell extracts

Hydrogenomonas eutropha (=*Alcaligenes eutrophus*) H 16 (Wilde, 1962) (ATCC 17699) was grown in the medium described by Schlegel *et al.* (1961). Cultures were grown at 30°C in a 10 litre fermenter (Braun–Biostat, Melsungen, Germany) and supplied with a gas mixture ($H_2+O_2+CO_2$; 80:10:10) at a rate of 5 litres/min. Cells were harvested in mid-exponential phase, washed once with water, suspended at 0.33g wet wt./ml in 0.1M-triethanolamine–HCl buffer (pH7.6) containing 10mM-$MgCl_2$, and sonically disrupted by a Schoeller ultrasonic probe (Frankfurt, Germany; 20kHz, 600W). The sonication was for 20s/ml of cell suspension at an intensity setting of 7. The crude cell extract was centrifuged at 20 000*g* for 20min and the supernatant was re-centrifuged at 100 000*g* for 1h. Protein was determined by the method of Lowry *et al.* (1951) with crystallized bovine serum albumin as standard.

Enzyme assay

Phosphoribulokinase activity was measured by following the fixation of $^{14}CO_2$ into acid-stable products when the phosphoribulokinase reaction was coupled to spinach ribulose diphosphate carboxylase. The assay was carried out in two stages. The primary reaction mixture (final vol. 0.5ml) contained enzyme, 100mM-glycylglycine–KOH buffer (pH9.0), 10mM-$MgCl_2$, 5mM-ATP and 3mM-ribulose 5-phosphate. The reaction was started by addition of the enzyme or ribulose 5-phosphate and terminated by placing the mixture in a boiling-water bath for 1min. After cooling, the mixture was incubated at 25°C with 0.1ml of 0.1M-Tris–HCl buffer containing excess of ribulose diphosphate carboxylase (enough to convert ribulose diphosphate into 3-phosphoglycerate in 2min), $KH^{14}CO_3$ (25μmol, 0.2μCi), $MgCl_2$ (2μmol), EDTA (dipotassium salt) (0.03μmol) and dithiothreitol (1μmol). After 10min the reaction was stopped by the addition of 0.4vol. of 60% (w/v) trichloroacetic acid and shaken slowly at room temperature for 1h to permit the liberation of excess of $^{14}CO_2$. A 0.4ml sample was placed in a vial containing 20ml of Bray's (1960) solution and counted for radioactivity in a Packard Tri-Carb model 3375 liquid-scintillation spectrometer. With each experiment, reaction mixtures containing no enzyme and others containing no ribulose 5-phosphate were incubated. Controls containing no ribulose 5-phosphate gave counts similar to background values. Controls containing no enzyme gave counts between 1 and 2% of those obtained with the respective reaction mixtures. One enzyme unit catalyses the formation of 1μmol of ribulose diphosphate/min at 30°C. All experiments were done in the range in which reaction rates were constant and proportional to enzyme concentration.

Before assay of crude extracts, samples were equilibrated with 0.02M-triethanolamine–HCl buffer (pH7.6), containing 0.5mM-dithiothreitol and 0.5mM-EDTA (dipotassium salt) by passage through Sephadex G-25 columns. Portions (1ml) were usually passed through 8cm×0.9cm columns.

Purification of phosphoribulokinase from H. eutropha H 16

Step 1. *Preparation of extracts.* Cells were grown autotrophically and extracts were prepared by sonic disruption as described above. Preliminary experiments showed that the stability of phosphoribulokinase in extracts and during the initial purification steps could be increased by the presence of 10mM-$MgCl_2$. The addition of the proteolytic-enzyme inhibitor phenylmethylsulphonyl fluoride at a final concentration of 1mM prevented the loss of phosphoribulokinase activity, which otherwise took place during $(NH_4)_2SO_4$ fractionation. Accordingly, immediately after centrifugation of crude extracts at 100 000*g* for 1h, $MgCl_2$ and phenylmethylsulphonyl fluoride were added to final concentrations of 10 and 1mM respectively. EDTA (dipotassium salt) and dithiothreitol were also added to final concentrations of 0.5mM each. All solutions coming in contact with the enzyme in the following steps contained 0.5mM-EDTA, 0.5mM-dithiothreitol and 10mM-$MgCl_2$.

Step 2. *Protamine sulphate treatment.* Protamine sulphate (1%, w/v, solution, pH7.0) was added with stirring at 0°C at a concentration of 0.025mg/mg of protein in the extract. This concentration was determined from preliminary experiments which

showed no loss of enzyme activity by this concentration and no further decrease in E_{260}/E_{280} on addition of more protamine sulphate. After 20min the precipitate was removed by centrifugation at 20000*g* for 20min.

Step 3. *$(NH_4)_2SO_4$ treatment.* The protein concentration in the supernatant was adjusted to 13mg/ml by dilution with 0.02M-triethanolamine–HCl buffer, pH7.6. The solution was then brought to 35% $(NH_4)_2SO_4$ saturation by the addition of 0.54vol. of $(NH_4)_2SO_4$ solution that had been saturated at 0°C and the pH of which had been adjusted with concentrated KOH to read 7.6 on a glass electrode. Stirring was continued at 0°C for 15min, and after another 15min the precipitate was removed by centrifugation at 20000*g* for 30min. More $(NH_4)_2SO_4$ solution (0.18vol.) was added to the supernatant to bring the saturation to 45%, and the solution was again centrifuged. The precipitate, which contained most of the enzyme activity, was dissolved in a minimal volume of 0.1M-triethanolamine–HCl buffer, pH7.6, containing 0.5mM-EDTA, 0.5mM-dithiothreitol, 10mM-$MgCl_2$ and 0.2mM-NADH. Recovery of the enzyme was less when solid $(NH_4)_2SO_4$ was used for precipitation.

Step 4. *DEAE-cellulose chromatography.* The solution from step 3 was applied to a column (35cm×1.6cm) of Sephadex G-25 and eluted with 0.02M-triethanolamine–HCl buffer, pH7.6. The protein solution was then pumped on to a column (52cm×1.6cm) of DEAE-cellulose (Whatman DE-52), equilibrated with the same buffer, at a rate of 60ml/h. The column was washed with 100ml of the buffer and proteins were eluted with a linear gradient (0.02–0.4M) of triethanolamine–HCl buffer, pH7.6. The eluate was collected in 5ml fractions. Phosphoribulokinase was eluted between 0.19M- and 0.27M-triethanolamine with the peak of activity at 0.21M. Only fractions with approximately similar specific activity were combined.

Step 5. *$(NH_4)_2SO_4$ treatment.* $(NH_4)_2SO_4$ fractionation was performed on the eluate from DE-52 cellulose. Phosphoribulokinase precipitated between 30 and 40% saturation. The precipitate was dissolved in a minimal volume of 0.1M-triethanolamine–HCl buffer, pH7.6.

Step 6. *Sephadex G-200 chromatography.* The concentrated protein solution (3ml) from step 5 was applied to two columns (90cm×2.6cm and 85cm×2.6cm) of Sephadex G-200 connected in series and eluted with 0.05M-triethanolamine–HCl buffer, pH7.6, at a flow rate of 17ml/h. The elution volume for phosphoribulokinase was 448ml. Only fractions containing peak activity were pooled and mixed. NADH was added, at a concentration of 0.2mM, to the Sephadex G-200 eluate, which already contained 20mM-$MgCl_2$, 0.5mM-EDTA and 0.5mM-dithiothreitol. Before use in kinetic studies, the enzyme solution was dialysed against 0.02M-triethanolamine–HCl buffer, pH7.6, containing 0.5mM-EDTA and 0.5mM-dithiothreitol. Enzyme solutions were kept at 4°C for over a month with no loss in activity or change in sensitivity to effectors. Table 1 summarizes the various steps in the purification, which resulted in a 286-fold overall purification.

Results

Properties of purified phosphoribulokinase

pH optimum. The pH optimum for purified phosphoribulokinase was at pH9.0 in glycylglycine buffer, and the enzyme was 50% inhibited at about pH8.2. Experiments with crude extracts showed a pH optimum also at 9.0. Enzyme assayed in glycylglycine buffer yielded 40% more activity than that assayed in Tris–HCl buffer. Tris–sulphate and triethanolamine–sulphate buffers gave lower enzyme activities than the respective chloride buffers.

Molecular weight. The molecular weight was estimated by filtration on Sephadex G-200 with ferritin (540000), ox liver catalase (240000), rabbit muscle aldolase (147000), bovine serum albumin (67000) and cytochrome *c* (13500) as markers. A value of 230000 was obtained, in agreement with that determined by sucrose-density-gradient centrifugation of the enzyme in crude extracts (Abdelal & Schlegel, 1974).

Table 1. *Purification of phosphoribulokinase*

Enzyme activity was assayed by $^{14}CO_2$ incorporation. Details of the purification procedure are described in the text.

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg)	Total activity (units)
Supernatant	150	39.9	0.31	1855
Protamine sulphate	140	34.5	0.39	1883
$(NH_4)_2SO_4$ (35–45% satd.)	15	70.8	1.33	1412
DEAE-cellulose	115	0.4	17.7	820
$(NH_4)_2SO_4$ (30–40% satd.)	3	8.9	26.6	710
Sephadex G-200	24	0.1	88.9	213

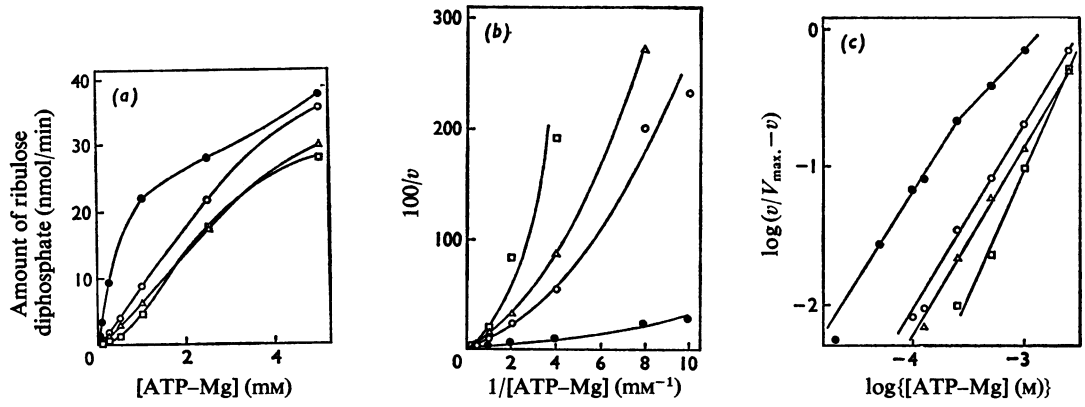

Fig. 1. *ATP-saturation curves for phosphoribulokinase in the presence and absence of effectors*

The initial velocities (v in units/mg of protein) are plotted against ATP concentrations in (*a*) and as double-reciprocal plots in (*b*). Hill plots of the data are shown in (*c*). The enzyme was assayed in glycylglycine buffer at pH9.0 as described in the Experimental section, except that the concentration of ATP was varied as indicated and the concentration of free $MgCl_2$ was kept constant at 5mM. Ribulose 5-phosphate concentration was 3mM. ○, No additions to reaction mixtures ($n = 1.4$); ●, +1mM-NADH ($n = 1.3$; 0.8); □, 0.1mM-AMP ($n = 1.8$); △, +0.1mM-phosphoenolpyruvate ($n = 1.4$).

Effect of $MgCl_2$ concentration. Since the stability constant of Mg^{2+}–ribulose 5-phosphate was not determined, exact correlation of phosphoribulokinase activity with free $MgCl_2$ concentration was not possible. However, preliminary studies showed that maximal activity was obtained when the concentration of free $MgCl_2$ was 5mM (on the basis of a value of $73000 M^{-1}$ for the stability constant of $MgATP^{2-}$; O'Sullivan & Perrin, 1964). The K_m for $MgCl_2$ was estimated to be 0.5mM. Concentrations of $MgCl_2$ higher than 10mM inhibited phosphoribulokinase activity.

Dependence of phosphoribulokinase activity on ATP concentration. The ATP-saturation curves in the presence and absence of effectors are shown in Fig. 1(*a*). The ATP-saturation curve in the absence of effectors was sigmoidal. When $V_{max.}$ was determined from the double-reciprocal plot (Fig. 1*b*) and $\log[v/(V_{max.}-v)]$ was plotted against log[ATP], as shown in Fig. 1(*c*), a straight line was obtained with a Hill coefficient (n) of 1.4, indicating positive co-operativity (for the significance of the Hill coefficient, see e.g. Atkinson, 1966). NADH at 1mM activated the enzyme, whereas AMP and phosphoenolpyruvate (each at 0.1mM) inhibited its activity. The inhibition by AMP and phosphoenolpyruvate appeared competitive and the double-reciprocal plots showed that $V_{max.}$ was not affected. $V_{max.}$ was also not changed in the presence of 1mM-NADH. Values for $s_{0.5}$ and $s_{0.1}$ (substrate concentrations required for one-half and one-tenth maximal velocity respectively) were determined from the Hill plots. In the presence of 1mM-NADH, $s_{0.5}$ for ATP decreased from 3.2 to 1.6mM and $s_{0.1}$ from 0.63 to 0.15mM. AMP and phosphoenolpyruvate were used at low concentrations (0.1mM) to determine accurately the shape of the saturation curves in their presence. Hence $s_{0.5}$ did not change significantly in their presence but $s_{0.1}$ increased from 0.63 to 1.1 and 0.83mM in the presence of AMP and phosphoenolpyruvate respectively.

The Hill coefficient, n, was the same in the presence of phosphoenolpyruvate as in its absence, indicating that phosphoenolpyruvate does not influence the degree of co-operative interactions between ATP sites. AMP, however, increased these interactions and the n value increased to 1.9 (Fig. 1*c*). In the presence of 1mM-NADH, a break in the Hill plot is observed approximately at 17% saturation [i.e. 0.5mM-ATP, $v/(V_{max.}-v) = 0.2$]. Below this point an n-value of 1.3 is obtained, whereas above this point the n value decreases to 0.8. Thus it appears that in the presence of the activator NADH the binding of ATP is subject to a sequence of positive and negative co-operativity.

ATP-saturation curves were also obtained in the presence of several fixed concentrations of ribulose 5-phosphate. In the presence of 1 and 3mM-ribulose phosphate, the ATP-saturation curves were sigmoidal whereas in the presence of 0.5mM-ribulose phosphate, the double-reciprocal plot appeared linear (Fig. 2). Hill plots of the data (not shown) yielded an interaction coefficient of 1.0 at 0.5mM-ribulose phosphate concentration. The hyperbolic response in the presence of low concentration of ribulose 5-phosphate may have a physiological significance. It would

ensure the presence of phosphoribulokinase activity when both substrates are present in low concentrations. The effect of $MgCl_2$ concentration on the shape of ATP-saturation curves was also investigated. The curves became more sigmoidal at higher $MgCl_2$ concentrations and the interaction coefficient increased from 1.3 at 1mM to 1.6 at 20mM.

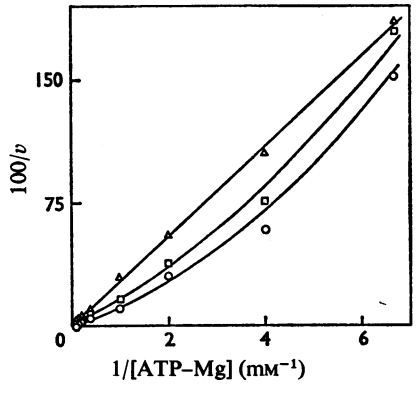

Fig. 2. *ATP-saturation curves for phosphoribulokinase in the presence of several fixed concentrations of ribulose 5-phosphate*

Double-reciprocal plots are shown. The enzyme was assayed in glycylglycine buffer, at pH 9.0 as described in the Experimental section, except that the concentrations of ATP and ribulose 5-phosphate were varied as indicated and the concentration of free $MgCl_2$ was kept at 5mM. Ribulose 5-phosphate concentrations: ○, 3mM; □, 1mM; △, 0.5mM. *v* is in units/mg of protein.

Dependence of phosphoribulokinase activity on ribulose 5-phosphate concentration

The double-reciprocal plots for ribulose 5-phosphate curves in the absence and presence of effectors are shown in Fig. 3. In the absence of effectors the Lineweaver–Burk plot (Fig. 3*a*) was not linear. The exact fit of the data required two straight lines to be drawn, one through the points at the high 1/[S] values and one with a greater slope through the points at low 1/[S] values. The apparent K_m value was 0.2mM between 0.01 and 0.06mM-ribulose 5-phosphate, and increased to 1.25mM (approx. six-fold) between 0.06 and 3mM-ribulose 5-phosphate. At low (non-saturating) concentrations of ATP or $MgCl_2$, the double-reciprocal plots for ribulose 5-phosphate (Fig. 3*b*) showed two transitions, in contrast with one transition at saturating concentrations of $MgCl_2$ or ATP. At low ATP or $MgCl_2$ concentrations, the transitions were also less abrupt. The abrupt transition(s) in the double-reciprocal plots for ribulose 5-phosphate were a reproducible observation.

Abrupt transitions in Lineweaver–Burk plots have been reported for several enzymes (for a compilation see Engel & Ferdinand, 1973). Two well-studied examples are glutamate dehydrogenases from ox liver (Engel & Dalziel, 1969) and from *Blastocladiella* (LeJohn & Jackson, 1968). In both cases, similar to

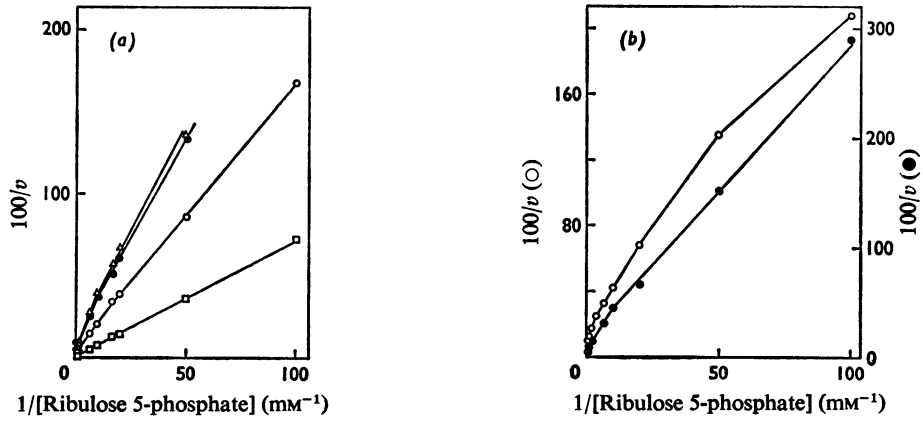

Fig. 3. *Double-reciprocal plots for ribulose 5-phosphate*

(*a*) Plots in the absence and presence of effectors. The enzyme was assayed in glycylglycine buffer at pH 9.0. The concentrations of ATP and free $MgCl_2$ were 5mM each. Ribulose 5-phosphate concentration was varied as indicated. ○, No effectors; □, +1mM-NADH; △, +0.1mM-AMP; ●, +0.1mM-phosphoenolpyruvate. (*b*) Plots at non-saturating concentrations of ATP and $MgCl_2$. ○, ATP and free $MgCl_2$ concentrations were 1 and 5mM respectively. ●, ATP and free $MgCl_2$ concentrations were 5 and 1mM respectively. *v* is in units/mg of protein.

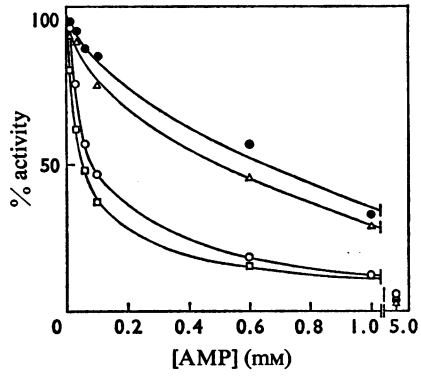

Fig. 4. *Modulation of AMP inhibition by phosphoenolpyruvate and NADH at 1 mM-ATP*

Enzyme was assayed in glycylglycine buffer, pH9.0, as described in the Experimental section, except that the ATP-Mg^{2+} concentration was kept at 1mM. Concentrations of AMP, phosphoenolpyruvate and NADH were varied as indicated. The concentrations of ribulose 5-phosphate and free $MgCl_2$ were 3 and 5mM respectively. Activities, relative to that in the absence of effectors, were 84, 252 and 229% in the presence of 0.1mM-phosphoenolpyruvate, 1mM-NADH and 0.1mM-phosphoenolpyruvate + 1mM-NADH respectively. ○, Inhibition by AMP in the absence of phosphoenolpyruvate and NADH; □, in the presence of 0.1mM-phosphoenolpyruvate; ●, in the presence of 1mM-NADH; △, in the presence of 0.1mM-phosphoenolpyruvate and 1mM-NADH.

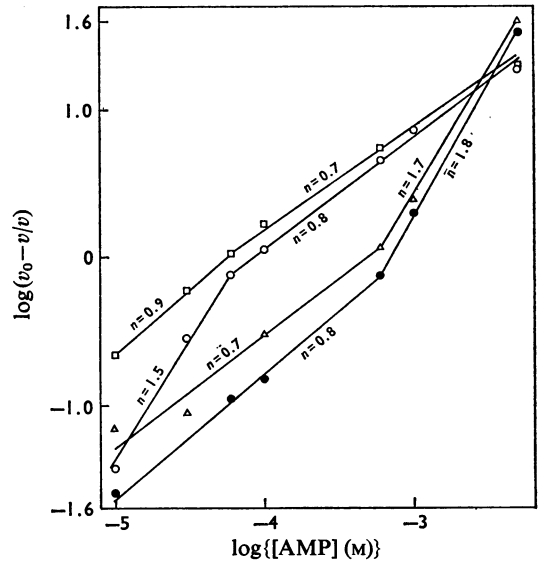

Fig. 5. *Hill plots of the data in Fig. 4*

v_0 is the velocity in the absence of AMP and v is that in the presence of various AMP concentrations as indicated, in units/mg of protein. For definition of symbols see Fig. 4.

results reported above, the differences in slope between the successive linear sections in the double-reciprocal plots for one substrate varied with the concentration of the other substrate. The transitions in Lineweaver–Burk plots are usually explained as the result of negative co-operativity (Dalziel & Engel, 1968; Levitzki & Koshland, 1969). Engel & Ferdinand (1973) suggested that the mathematical requirements to explain these conditions could be better met on the basis of a model involving both negative and positive co-operativity.

In the presence of AMP or phosphoenolpyruvate, the transitions in the double-reciprocal plots are more abrupt than in their absence (Fig. 3*a*). Accordingly, the apparent K_m increases approx. tenfold between the two segments in the presence of AMP or phosphoenolpyruvate in contrast with a sixfold increase in their absence. In the presence of NADH, the Lineweaver–Burk plot for ribulose 5-phosphate becomes linear and the slope of the Hill plot (not shown) is about 1. Thus in the presence of the activator, the ribulose 5-phosphate sites appear to be non-interacting.

Examination of the double-reciprocal plots (Fig. 3*a*) shows that $V_{max.}$ is not changed in the presence of AMP and phosphoenolpyruvate. $V_{max.}$ is increased, however, in the presence of NADH. Values of $s_{0.1}$ and $s_{0.5}$ were estimated from Hill plots: $s_{0.1}$ decreased in the presence of 1mM-NADH (from 0.15mM- to 0.1mM-ribulose 5-phosphate), but increased in the presence of 0.1mM-AMP and 0.1mM-phosphoenolpyruvate to 0.28 and 0.25mM respectively. $s_{0.5}$ also decreased in the presence of 1mM-NADH (from 1.3 to 0.87mM). In the presence of 0.1mM-AMP and 0.1mM-phosphoenolpyruvate, $s_{0.5}$ increased to 2.6 and 2.2mM respectively. Comparison of the changes in $s_{0.5}$ for ribulose phosphate that occur in the presence of effectors with those observed in $s_{0.5}$ for ATP show that NADH had a more pronounced effect on the affinity for ATP, whereas AMP and phosphoenolpyruvate had more pronounced effects on the affinity for ribulose phosphate.

Modulation of AMP inhibition by phosphoenolpyruvate and NADH

To correlate the kinetic data with the regulation of phosphoribulokinase *in vivo* the modulation of AMP inhibition by phosphoenolpyruvate and NADH was examined. The data (Fig. 4) show that at 1mM-ATP and 3mM-ribulose 5-phosphate, the AMP concentration that caused 50% inhibition ($I_{0.5}$) was 0.08mM. In the presence of 0.1mM-phosphoenolpyruvate, $I_{0.5}$ for AMP decreased to 0.05mM. Thus the inhibi-

tory effects of AMP and phosphoenolpyruvate were more than additive. In reaction mixtures containing the activator, NADH (at 1mM), $I_{0.5}$ for AMP increased to 0.64mM. Thus NADH had a profound effect on the sensitivity of the enzyme to AMP, decreasing it by eightfold. When both inhibitors (AMP and phosphoenolpyruvate, each at 0.1mM) were present together with 1mM-NADH, $I_{0.5}$ for AMP was 0.5mM. This is a decrease of tenfold relative to the value when only the two inhibitors are present.

Hill plots of the data (Fig. 5) show both positive and negative co-operativity in the interactions of AMP-binding sites. A break in the plot is observed at approx. 0.06mM-AMP, and interaction coefficients (n) of 1.5 and 0.8 are obtained below and above this concentration respectively. In the presence of 0.1mM-phosphoenolpyruvate, the break in the plot is less apparent, but the slope is decreased in both sections and only negative co-operativity is indicated. The presence of 1mM-NADH reversed the situation; i.e. negative co-operativity ($n = 0.8$) below 0.6mM-AMP and positive co-operativity ($n = 1.8$) above that AMP concentration were indicated. This was also the case when phosphoenolpyruvate was present, together with AMP and NADH. In the absence of other effectors 1mM-AMP caused 90% inhibition and 5mM-AMP only 95% inhibition. The failure to obtain complete inhibition with high AMP concentrations could be explained by the negative interactions among AMP-binding sites at concentrations higher than 0.6mM.

It is noteworthy that in the presence of NADH (or NADH and phosphoenolpyruvate), the response to AMP at concentrations higher than 0.6mM was sigmoidal. Thus although NADH decreased the sensitivity to AMP inhibition below 0.6mM, it actually increased the sensitivity to the inhibitor at high AMP concentrations. The response of phosphoribulokinase to AMP in the presence of phosphoenolpyruvate and NADH is probably the most similar to that prevalent under physiological conditions. The change in the type of modulation of AMP inhibition by NADH at high AMP concentrations would appear to provide the cell with a means of rapidly curtailing phosphoribulokinase activity when the AMP/ATP ratio becomes high.

The same pattern of modulation of AMP inhibition by phosphoenolpyruvate is observed at 3mM-ATP (results not shown). NADH decreased the sensitivity to AMP inhibition in the lower concentration range and increased it in the higher range. $I_{0.5}$ for AMP (estimated from Hill plots) decreased from 0.28mM in the absence of phosphoenolpyruvate to 0.14mM in the presence of 0.1mM-phosphoenolpyruvate. $I_{0.5}$ increased approximately eightfold in the presence of 1mM-NADH (from 0.14mM in the presence of phosphoenolpyruvate alone to 1.1mM in the presence of phosphoenolpyruvate and NADH).

Other compounds were also tested for possible regulatory effects. The following compounds had no effect on the activity of phosphoribulokinase (at 1mM): acetyl phosphate, pyruvate, 3-phosphoglycerate, magnesium-pyrophosphate, GMP, IMP, UMP, CMP and NADPH. ADP inhibited phosphoribulokinase but $I_{0.5}$ was very high (4.2mM) under standard assay conditions. $I_{0.5}$ for phosphoenolpyruvate was 0.1mM at 5mM-ATP and 0.5mM-ribulose 5-phosphate and also at 1mM-ATP and 3mM-ribulose 5-phosphate; 1mM-phosphoenolpyruvate caused 90% inhibition in both cases.

Preliminary experiments with crude extracts showed the same pattern of inhibition and activation as was found with the purified phosphoribulokinase preparations.

Discussion

The pH optimum of phosphoribulokinase of *H. eutropha* H 16 is higher than that reported for the enzyme from *Chromatium* (Hart & Gibson, 1971) or *T. neapolitanus* (MacElroy *et al.*, 1972). The enzyme is similar to that in other bacteria in the requirement for bivalent metal ions and in the requirement for 5mM-$MgCl_2$ for full activation (MacElroy *et al.*, 1972).

The data reported in the present work indicate the presence of positive co-operativity in ATP binding. A sigmoidal relationship between reaction rate and ATP concentration was also observed with the enzyme from *Chromatium* (Hart & Gibson, 1971). A hyperbolic response, however, was reported for the enzymes from *R. rubrum*, but no results were given (Joint *et al.*, 1972*a*). *T. neapolitanus* enzyme showed a hyperbolic response at its pH optimum and a sigmoidal one below that pH. The differences in the ATP-saturation curves among these organisms may not be real. Our results showed that although the ATP-saturation curve was sigmoidal at high ribulose 5-phosphate concentrations, the response was different at low concentrations. At 0.5 mM-ribulose 5-phosphate the double-reciprocal plot appeared linear and the Hill plot gave an n value of 1.0 below 1mM-ATP. Both Joint *et al.* (1972*b*) and MacElroy *et al.* (1972) used low ribulose 5-phosphate concentrations; 0.2 and 0.5mM respectively. Thus it is possible that at higher ribulose 5-phosphate concentrations sigmoidal curves would have been obtained for the enzymes of these bacteria too.

The ribulose 5-phosphate-saturation curves were also not hyperbolic. The double-reciprocal plots showed at least one abrupt transition. In contrast with this behaviour, the ribulose 5-phosphate-saturation curves for the enzymes from *R. rubrum* (Joint *et al.*, 1972*a*, no values were given), *Ps. facilis* (Ballard & MacElroy, 1971) and *T. thioparus*

(MacElroy *et al.*, 1972) were reported to be hyperbolic. The relationship for the *Chromatium* enzyme was reported to be sigmoidal (Hart & Gibson, 1971). Although there is no reason to expect similar kinetic behaviour for one enzyme from different sources, some of the differences in the ribulose 5-phosphate curves may be only the result of different assay conditions. The ribulose phosphate curve for *Ps. facilis* was determined in the presence of 2mM-NADH, which is an activator of the enzyme (Ballard & MacElroy, 1971). We have shown (Fig. 3) that the abrupt transitions in the reciprocal plots for *H. eutropha* H 16 enzyme disappear in the presence of 1mM-NADH; the plot appears linear and the Hill plot yielded an n value of about 1.0. In other cases (Hart & Gibson, 1971; MacElroy *et al.*, 1968*a*) only a limited range of ribulose 5-phosphate concentrations were used. Hart & Gibson (1971) used a range of concentrations of 0.2–0.5mM and reported a $s_{0.5}$ value of 0.22mM. MacElroy *et al.* (1968*a*) used a range of concentrations from 0.006 to 0.05mM and reported a K_m value of 0.04mM. Examination of the data shown in Fig. 3 for the *H. eutropha* H 16 enzyme show that in each of these limited ranges the experimental points fit a single straight line. It is possible that only one enzyme species (ES_1, ES_2, ...) was examined in the studies of Hart & Gibson (1971) and MacElroy *et al.* (1968*a*) and thus the double-reciprocal plot appeared linear.

The increase in apparent K_m and $V_{max.}$ values with increasing concentrations of ribulose 5-phosphate might serve (as was suggested for other systems by Conway & Koshland, 1968) to insulate the cell against great changes in phosphoribulokinase activity in response to fluctuations in ribulose 5-phosphate concentrations. The cell would then regulate phosphoribulokinase activity according to the concentrations of the other substrate (ATP) and the effectors. This would be advantageous to *H. eutropha* cells, since ribulose 5-phosphate is involved in reactions other than those of CO_2 fixation.

In addition to the co-operative interactions observed with both ATP and ribulose 5-phosphate, phosphoribulokinase from *H. eutropha* H 16 is inhibited by both AMP and phosphoenolpyruvate and is activated by NADH. It is similar in this respect to the enzyme from *Ps. facilis* (Ballard & MacElroy, 1971). There are differences in the regulation of the enzyme between these two organisms and other bacteria. Phosphoribulokinase from *R. rubrum* is inhibited by AMP, activated by NADH, but not affected by phosphoenolpyruvate (Joint *et al.*, 1972*a*). The enzyme from *Chromatium* (Hart & Gibson, 1971) is inhibited by AMP and phosphoenolpyruvate but not affected by NADH. The *T. neapolitanus* enzyme was reported initially (MacElroy *et al.*, 1968*a*) to be inhibited by AMP, but the purified enzyme was inhibited by phosphoenolpyruvate and only marginally affected by AMP and NADH (MacElroy *et al.*, 1972).

Kinetic studies revealed further differences and similarities of phosphoribulokinase in different organisms. The inhibition by AMP and phosphoenolpyruvate of the enzyme appeared competitive with respect to both ATP and ribulose 5-phosphate in *H. eutropha* H 16 (Figs. 1 and 3) and also in *Chromatium* (Hart & Gibson, 1971). However, the inhibition by phosphoenolpyruvate of the enzyme from *Ps. facilis* (Ballard & MacElroy, 1971) and by AMP of that from *R. rubrum* (Joint *et al.*, 1972*a*) was non-competitive. The effects of AMP and phosphoenolpyruvate on substrate-saturation curves also differed from one organism to another. AMP increased the interactions among ATP sites for the *H. eutropha* H 16 enzyme (Fig. 1), but had no effect on these interactions for the *Chromatium* enzyme (Hart & Gibson, 1971). MacElroy *et al.* (1972) reported that the hyperbolic ATP curve became sigmoidal in the presence of phosphoenolpyruvate for the enzyme from *T. neapolitanus*, whereas this compound did not affect the slope of the Hill plot with ATP for phosphoribulokinase from *H. eutropha* H 16. The enzyme from *Chromatium* (Hart & Gibson, 1971) was similar to that from *H. eutropha* in the potentiation of AMP inhibition by phosphoenolpyruvate.

Ballard & MacElroy (1971) reported that although NADH activates phosphoribulokinase of *Ps. facilis*, the inhibition by phosphoenolpyruvate could not be reversed by excess of NADH. Our studies with the enzyme from *H. eutropha* H 16 showed that in the presence of NADH, the sensitivity to AMP inhibition decreased below a certain threshold concentration but increased above that threshold. This result of interaction between the two effectors may have important physiological implications. Therefore for each set of substrates, phosphoenolpyruvate and NADH concentrations, a certain threshold of AMP exists; below that concentration the response to certain increments of AMP concentration is minimal and above that concentration the response is sharp.

Kinetic studies, reported in the present paper, indicate that the activity of phosphoribulokinase can be regulated effectively by changes in effector concentrations similar to those reported to occur *in vivo*. Under conditions of 'Leerlauf oxidation' (hydrogen oxidation in the absence of CO_2 and NH_3 which implies excess of energy; Schlegel & von Bartha, 1961), the concentrations of ATP, AMP, phosphoenolpyruvate and NADH in *H. eutropha* H 16 were estimated to be 2.4, 0.5, 0.1 and 1.0mM respectively (A. M. Cook & H. G. Schlegel, unpublished work). ATP concentration was estimated to be 3mM in growing *H. eutropha* H 16 cells. In glucose-grown *Escherichia coli* cells, Lowry *et al.* (1971) reported similar concentrations of ATP (2.6mM) and phosphoenolpyruvate (0.08mM) but

lower AMP (0.14mM). They also reported that the ATP concentration remained reasonably constant and that even when cells were forced into stationary phase, the ATP concentration fell by only 50%. The ATP/AMP ratio, however, varied considerably under different conditions. It appears reasonable then to assume ATP concentrations of 2–3mM in cells under conditions of unlimited energy supply. This concentration is below $s_{0.5}$ for ATP (3.2mM in the absence of effectors) and phosphoribulokinase activity would change according to the concentrations of phosphoenolpyruvate, AMP and NADH. We have shown that when NADH and phosphoenolpyruvate concentrations are 1 and 0.1mM, $I_{0.5}$ for AMP is 1.1mM. This AMP concentration is much higher than that measured *in vivo* for *H. eutropha* cells, and we would therefore expect full phosphoribulokinase activity under these conditions. Indeed, in the presence of 0.5mM-AMP (measured for *H. eutropha* cells under conditions of unlimited energy supply), phosphoribulokinase activity is 97% of that in the absence of effectors (Fig. 4). Unfortunately, values for the concentrations of substrates and effectors of phosphoribulokinase under conditions of limited energy supply are lacking for *H. eutropha* cells. We might expect, however, the ATP/AMP ratio and NADH concentration to decrease. In the absence of NADH, 1mM-AMP caused 90 and 80% inhibition at 1 and 3mM-ATP respectively. In the intermediate ranges of NADH and AMP, the activity of phosphoribulokinase would vary according to their concentrations.

The role of phosphoenolpyruvate inhibition of phosphoribulokinase activity is somewhat unclear. It may serve, as Hart & Gibson (1971) suggested, as a negative feedback control mechanism to maintain a balance between the synthesis and utilization of metabolic intermediates under autotrophic conditions. With *H. eutropha* H 16 phosphoenolpyruvate inhibition may assume more importance under mixotrophic or heterotrophic conditions. The concentration of phosphoenolpyruvate in *H. eutropha* H 16 cells grown on fructose and malate was 0.25 and 0.6mM respectively (I. Probst & H. G. Schlegel, unpublished work). The synthesis of phosphoribulokinase is repressed under heterotrophic conditions (Abdelal & Schlegel, 1974) and the activity of the remaining enzyme would be further inhibited by the higher concentration of phosphoenolpyruvate. In this way the operation of the reductive pentose phosphate cycle would be decreased.

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