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Possible regulatory characteristics of the fructose diphosphatase–phosphoribulokinase complex from *Rhodospirillum rubrum*

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SUMMARY

The activity of fructose diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) from *Rhodospirillum rubrum* is inhibited most markedly by $MgATP^{2-}$ and magnesium pyrophosphate ($MgP_2O_7^{2-}$). Other less potent inhibitors include $MgADP^-$, ribulose 5-phosphate, 3-phosphoglyceraldehyde, thiamine pyrophosphate and guanosine triphosphate. The activity of phosphoribulokinase (ATP:D-ribulose-5-phosphate 1-phosphotransferase, EC 2.7.1.19) is enhanced by NADH and inhibited by AMP.

Elsewhere[1] we have described the isolation and purification of a protein complex possessing the activities of both alkaline fructose diphosphatase (fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) and phosphoribulokinase (ATP:D-ribulose-5-phosphate 1-phosphotransferase, EC 2.7.1.19) from *Rhodospirillum rubrum*.

Such a complex seemed to be of some interest since the component enzymes did not catalyse successive reactions in the reductive pentose phosphate cycle. Rather, the feature that linked the enzymes was the fact that both had been implicated as possible control points for the regulation of photosynthetic CO_2 assimilation. Possibly, isolation of this complex reflects the fact that enzymes of the Calvin cycle are organised or associated in some way within the cell, and that one of the functions of such an organization is to facilitate control of this cycle. It seemed possible, therefore, that the mechanism by which the Calvin cycle is regulated might be better elucidated by studying these possible

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regulatory enzymes as part of a complex than after their activities had been separated.

All the results presented in this paper have been obtained from studies with the fructose diphosphatase–phosphoribulokinase complex from autotrophically-grown *R. rubrum*, strain S1. The methods of growth, extraction and purification of the complex, and assay of the two enzymes are described elsewhere[1]. All measurements were made within 24 h of the preparation of a new batch of the pure complex; that is, before it had broken down to its subunits. In attempting to understand the way in which the activities in the complex are regulated, we have emphasized three features: the kinetics of substrate and activator saturation curves, the effect of Mg^{2+} on pH optima, and the identity of inhibitors and activators.

Priess *et al.*[2] and Morris[3] observed that the relationship between reaction rate and fructose 1,6-diphosphate concentration for the fructose diphosphatase from spinach chloroplasts was sigmoidal. However, with our preparation from *R. rubrum* there was no such relationship; the curves being hyperbolic. From double-reciprocal plots the Michaelis constants (K_m) of fructose diphosphatase were $1.88 \cdot 10^{-4}$ M (determined by the phosphate assay) and $1.85 \cdot 10^{-4}$ M (determined by the coupled enzyme assay).

MacElroy *et al.*[4] observed that the relationship between reaction rate and ATP concentration for the phosphoribulokinase from *Hydrogenomonas facilis* was sigmoidal. No data were given for the other substrate, ribulose 5-phosphate. However, in our experiments, the curves for both ATP and ribulose 5-phosphate were hyperbolic. From the double-reciprocal plots the K_m value for ATP was $3.4 \cdot 10^{-4}$ M, and that for ribulose 5-phosphate was $2.5 \cdot 10^{-4}$ M.

Like the alkaline fructose diphosphatase from other photosynthetic organisms, that from *R. rubrum* required Mg^{2+}. The relationship between fructose diphosphatase activity and Mg^{2+} concentration was sigmoidal, and maximum activity was achieved with 5 mM. These results are comparable to those obtained with the fructose diphosphatase from spinach chloroplasts[3]. Activity of phosphoribulokinase also depended on the presence of Mg^{2+}. The precise relationship between reaction rate and Mg^{2+} concentration was difficult to determine since no activity could be detected with concentrations less than 0.25 mM; maximum activity was obtained with Mg^{2+} concentrations of 2.5 mM.

Priess *et al.*[2] observed that the pH optimum of spinach chloroplast fructose diphosphatase shifted to lower pH values with increasing Mg^{2+} concentrations in the reaction mixture. Bassham *et al.*[5] observed the same effect with the ribulose diphosphate carboxylase (EC 4.1.1.39) from spinach chloroplasts. In our experiments, however, increasing the Mg^{2+} concentration from 5 to 40 mM did not affect the pH curves of either fructose diphosphatase or phosphoribulokinase from *R. rubrum*.

The following compounds (in final concentration up to 5 mM) had no effect on the activity of fructose diphosphatase; AMP, inorganic orthophosphate, NAD^+, NADH, $NADP^+$, NADPH, 3-phosphoglyceric acid, fructose 6-phosphate, fructose 1-phosphate, glucose 6-phosphate, ribose 5-phosphate, phosphoenol pyruvate and citrate.

Of the compounds tested the only ones which had any effect were $MgATP^{2-}$, magnesium pyrophosphate ($MgP_2O_7^{2-}$), $MgADP^-$, ribulose 5-phosphate, 3-phospho-

glyceraldehyde, thiamine pyrophosphate and GTP (Table I). Fig. 1 shows the Lineweaver–Burk plot for fructose diphosphatase in the presence of two concentrations of $MgATP^{2-}$.

TABLE I

INHIBITOR CONSTANTS OF COMPOUNDS WHICH AFFECT FRUCTOSE DIPHOSPHATASE ACTIVITY

For each inhibitor, fructose diphosphatase was assayed in two ways: (a) by coupling with excess glucose phosphate isomerase (EC 5.3.1.9) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and measuring the change in absorbance at 340 nm due to the reduction of $NADP^+$; and (b) by assaying the inorganic phosphate produced in the assay by the method of Lowry and Lopez[14]. The two assays gave identical results for all inhibitors. Two concentrations of each inhibitor were used for the determination of K_i.

Compound	*K_i (mM)*	*Type of inhibition*
$MgATP^{2-}$	2.60	Non-competitive
$MgP_2O_7^{2-}$	3.33	Non-competitive
$MgADP^-$	5.70	Non-competitive
Ribulose 5-phosphate	5.0	Non-competitive
3-Phosphoglyceraldehyde	4.95	Non-competitive
Thiamine pyrophosphate	7.52	Non-competitive
GTP	$v_p < V$ $K_p > K_m$	Mixed

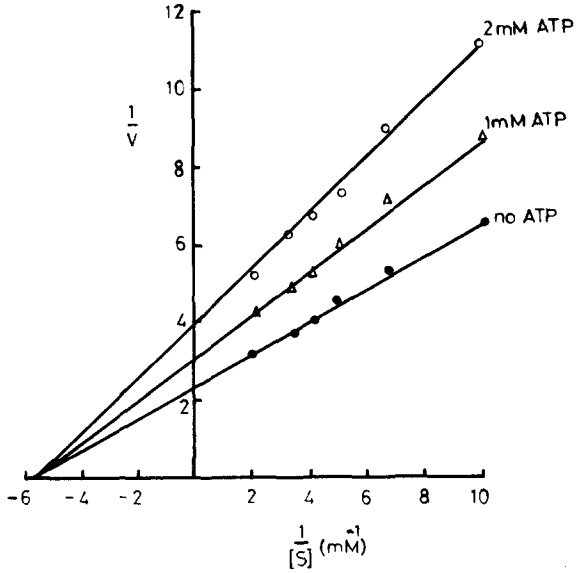

Fig. 1. Effect of ATP on reaction velocity. Lineweaver–Burk plot for fructose diphosphatase in the presence of two concentrations of ATP. The reaction mixture contained: enzyme, 100 mM Tris–HCl buffer (pH 8.5) and 5 mM $MgCl_2$ (increased to 6 mM in the presence of 1 mM ATP and to 7 mM in the presence of 2 mM ATP, to maintain the same free Mg^{2+} concentration). Inorganic phosphate produced in the reaction was assayed by the method of Lowry and Lopez[14]. Velocity is expressed as μmoles phosphate produced per min.

Similar plots were obtained for the other inhibitors. The most potent inhibitors (Table I) were $MgATP^{2-}$ and $MgP_2O_7^{2-}$. Since ATP and pyrophosphate complex with Mg^{2+} (ref. 6), additional Mg^{2+} was added to maintain the free Mg^{2+} concentration the same in the presence of the inhibitors as in their absence. Apart from the mixed inhibition shown by GTP, all inhibition was non-competitive.

Since both $MgATP^{2-}$ and ribulose 5-phosphate inhibited the activity of fructose diphosphatase, it seemed possible that addition of them together might cause an inhibition greater than would be expected. Both these compounds are substrates of the other enzyme in the complex phosphoribulokinase. Addition of them together might cause some kind of conformational change in the complex and so inhibit fructose diphosphatase activity. However, inhibition by the two compounds added together was additive, no enhanced inhibition being observed. Similarly, fructose 1,6-diphosphate did not affect phosphoribulokinase.

The activity of phosphoribulokinase measured with the ^{14}C-assay technique[1] was always lower than when the continuous coupled enzyme was used. This was due to the activation of phosphoribulokinase by NADH – one of the components of the coupled enzyme assay mixture. The enzyme was fully activated by a NADH concentration of 0.05 mM and this represented a 5-fold stimulation of the activity measured in the absence of NADH. No comparable effect was observed with NADPH, NAD^+ and $NADP^+$. During the assay in the presence of NADH, absorption at 340 nm did not decrease, thus suggesting that, although NADH stimulated phosphoribulokinase activity, it was not utilized during the course of the reaction.

The activity of phosphoribulokinase was inhibited by AMP. The type of inhibition was non-competitive. The K_i value determined from studies with a single concentration of AMP and with changing concentrations of ribulose 5-phosphate was 1.2 mM. The K_i value determined in the presence of changing ATP concentrations was 1.8 mM. $MgADP^-$ also inhibited phosphoribulokinase activity, but the concentrations required were much higher than with AMP: K_i values of 7.1 mM (when ribulose 5-phosphate concentration was varied) and 6.4 mM (when ATP concentration was altered). Compounds which did not affect the activity of phosphoribulokinase were ribulose 1,5-diphosphate, fructose 1,6-diphosphate, fructose 6-phosphate, phosphoenol pyruvate, inorganic pyrophosphate and inorganic orthophosphate.

Fructose diphosphatase is a well-known regulatory enzyme in the process of gluconeogenesis in non-photosynthetic organisms[7], regulation of gluconeogenesis being achieved, in part, through the inhibition of fructose diphosphatase by AMP and citrate. There is no comparable understanding of the role of the alkaline fructose diphosphatase in the regulation of the Calvin cycle. Several workers have tried to find regulatory inhibitors of the photosynthetic fructose diphosphatase. Morris[3] observed that the fructose diphosphatase from spinach chloroplasts was inhibited by $MgATP^{2-}$, $MgADP^-$ and $MgP_2O_7^{2-}$; there was no evidence of inhibition by AMP. The type of inhibition suggested allosteric interactions. Scala *et al.*[8] observed that fructose diphosphatase from Navy Bean was inhibited by ATP, AMP and fructose 1-phosphate; the inhibition was competitive.

However, the fructose diphosphatase from *Rhodopseudomonas palustris* was not inhibited by ATP, AMP, ADP or inorganic pyrophosphate, but it was inhibited by GTP[9]. In a study with a second fructose diphosphatase from spinach leaves (activity did not depend on the presence of Mg^{2+}) the enzyme was activated by reduced ferredoxin[10].

The present results with *R. rubrum* agree, in part, with those of Morris[3] from studies with fructose diphosphatase from spinach chloroplasts. In particular, it is interesting that both fructose diphosphatases are inhibited most strongly by ATP and inorganic pyrophosphate, and do not show inhibition by AMP. Possibly, therefore, this inhibition by ATP and pyrophosphate is of general significance in the control of CO_2 assimilation (there is some evidence that, in addition to ATP, inorganic pyrophosphate can also be a product of photophosphorylation in *R. rubrum*[11]). However, the precise mechanism by which such inhibitions would regulate CO_2 assimilation *in vivo* remain unknown. The fructose diphosphatase of *R. rubrum* differs from that of spinach chloroplasts in two possibly important ways: firstly, that the substrate (and inhibitor) kinetics are not sigmoidal, and secondly that the pH optimum is not affected by increasing Mg^{2+} concentration (*cf.* refs 2 and 3).

The studies of phosphoribulokinase reported here agree with those obtained with other autotrophic bacteria[4,12,13]. That is, the activation of phosphoribulokinase by NADH and its inhibition by AMP appear to be general methods of regulating the reductive pentose phosphate cycle in autotrophic bacteria. This combination of NADH activation and AMP inhibition would appear to be a sensitive means of regulating the activity of the Calvin cycle when light (or, in chemolithotrophic bacteria, the inorganic reductant) is no longer available.

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