NADH AND AMP AS ALLOSTERIC EFFECTORS OF RIBULOSE-5-PHOSPHATE KINASE IN *RHODOPSEUDOMONAS SPHEROIDES*

K.-P. Rindt and E. Ohmann

Sektion Biowissenschaften der Universität Halle und
Institut für Biochemie der Pflanzen, Halle
Halle/Saale (DDR), Am Kirchtor 1

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It can be generally accepted that integrated reaction sequences are regulated by control of a strategically placed enzyme (Atkinson, 1966). In many cases these enzymes catalyze reactions that are physiologically irreversible. In view of the central position of the reductive pentose phosphate cycle in autotrophic metabolism we expect this cycle to be regulated in an economical manner. This should be especially the case in organisms which are capable of changing from autotrophic to heterotrophic metabolism, e.g. *Rhodopseudomonas sheroides* (*Rps. sheroides*) or *Rhodospirillum rubrum* (*Rsp. rubrum*). As a key enzyme of reductive pentose phosphate cycle, ribulose-5-phosphate kinase (Ru-5-P kinase) has to be regarded as being of considerable interest. This paper presents evidence that the formation of ribulose-1,5-diphosphate (RuDP) from ribulose-5-phosphate (Ru-5-P) and ATP in *Rps. spheroides* is completely dependent on an allosteric activation of Ru-5-P kinase. This activation may form the molecular basis for a regulatory relationship between the light-driven electron transport system and the "dark" process of CO_2 fixation. Together with inhibition by AMP this most striking feature of Ru-5-P kinase may provide a basic control mechanism in autotrophic metabolism of *Rps. spheroides* and *Rsp. rubrum*.

Methods - Cultures of Rps. spheroides (strain 1760 - 1, Algensammlung Göttingen) were grown photosynthetically in malate glutamate medium. Crude extracts were prepared by grinding of light-grown cells with Alcoa and extracting with 0.05 M Tris-sulfate pH 8.0 containing 50 mM $MgSO_4$ and 0.5 mM mercaptoethanol. After centrifugation at 20,000 x g for 30 min the supernatant was passed through a column of Sephadex G 100. Fractions with highest Ru-5-P kinase activity which appeared in the first protein peak were pooled and the enzyme was precipitated adding ammonium sulfate to 50 % saturation. After centrifugation the precipitate was dissolved in a small amount of Tris buffer pH 8.0. The preparation was free of glyceraldehyde phosphate dehydrogenase but contained excess of ribose phosphate isomerase and ribulose diphosphate carboxylase (RuDPC).

The assay system for Ru-5-P kinase contained in a total volume of 140 µl: 10 µmoles Tris-HCl, pH 8.0, 1 µmole $MgCl_2$, 1 µmole ATP, 0.5 µmoles ribose-5-phosphate (R-5-P), 2 µmoles $NaH^{14}CO_3$ (4.2×10^5 cpm), and additions as indicated. The reaction was started by addition of enzyme solution (about 0.15 mg protein). The reaction mixtures were incubated at 30°. After 15 min the reaction was terminated by addition of 0.1 ml 10 % trichloroacetic acid and acid-stable ^{14}C activity was determined in a 0.1 ml aliquot. The incorporation of $H^{14}CO_3^-$ into acid-stable material followed zero order kinetics during the 15 min assay period and was proportional to enzyme concentration. RuDP carboxylase free of Ru-5-P kinase activity was prepared from Nicotiana alata according to a procedure developed by Steer et al. (1968) for Avena.

RESULTS AND DISCUSSION - As shown in Table I, CO_2 fixation without addition of a substrate in extracts of Rps. spheroides is very poor.

Upon addition of R-5-P and ATP in various amounts there is no significant stimulation of CO_2 incorporation in acid-stable form. Under the same assay conditions extracts of Euglena gracilis show very high activitiy of enzymes involved in CO_2 fixation upon addition of R-5-P and ATP. Since no apparent change of the amount of fixed $^{14}CO_2$ was observed by addition of R-5-P, not even at very

TABLE I

Influence of R-5-P and ATP on the rate of $^{14}CO_2$ incorporation in a cell-free extract of Rps. sheroides in absence of an effector

R-5-P	ATP	cpm	remarks
----------	1 µmole	250	
0.5 µmoles	1 µmole	270	
2.0 µmoles	1 µmole	232	
4.0 µmoles	1 µmole	227	
0.5 µmoles	-------	210	
0.5 µmoles	0.25 µmoles	182	
0.5 µmoles	4.0 µmoles	219	
4.0 µmoles	4.0 µmoles	322	
0.5 µmoles	1.0 µmole	130	enzyme omitted
0.5 µmoles	1.0 µmole	25,680	Euglena extract

Enzyme assay as described, but R-5-P and ATP as indicated.

TABLE II

Activation of Ru-5-P kinase by NADH

Reaction conditions	cpm
without additions	10
+ 0.15 µmoles NADH	10,100
+ 0.15 µmoles NAD	0
+ 0.15 µmoles NADP	0
+ 0.15 µmoles NADPH	10
+ 0.15 µmoles NADH, R-5-P omitted	0
+ 0.2 µmoles RuDP, R-5-P omitted	5,250
+ 0.2 µmoles RuDP + 0.15 µmoles NADH, R-5-P omitted	5,160
+ 0.15 µmoles NADH + RuDP carboxylase from tobacco	10,040
+ 0.2 µmoles RuDP + RuDP carboxylase from tobacco, R-5-P omitted	5,200

Experimental conditions as described. The results were corrected for background activity (without R-5-P).

high concentrations, it can be concluded that the fixed counts were due to unspecific effects and had nothing to do with the action of Ru-5-P kinase.

Data in Table II show the most striking activation of R-5-P and ATP dependent carbon dioxide fixation by NADH. In the absence of NADH it was found that no carboxylation occurred. The activation is most specific, for other nucleotides such as NAD, NADP, NADPH exhibit no effect. Data of Table 2 also show no effect of NADH on the carboxylation with RuDP as acceptor. Therefore the activation of the ATP and R-5-P dependent CO_2 fixation must occur at some point other than the RuDP carboxylase enzyme site of the reaction sequence. As ribose phosphate isomerase activity of Rsp. rubrum can be measured in an assay system without NADH (Anderson and Fuller, 1967) the site of activation seems to be the Ru-5-P kinase. But the effect of NADH on purified ribose phosphate isomerase has to be determined exactly.

Addition of purified tobacco RuDP carboxylase to the assay system did not increase the CO_2 fixation rate with R-5-P as substrate. In order to facilitate the isomerase equilibrium prior to starting the kinase reaction, the enzyme preparation was preincubated with R-5-P in the absence of ATP. This treatment exhibited no effect on the subsequent ATP dependent CO_2 incorporation. Therefore the Ru-5-P kinase reaction seems to be the rate limiting step.

Although the possibility cannot be excluded that Ru-5-P kinase from Rps. spheroides might show significant activity in the absence of NADH under certain conditions there is no evidence to support this possibility. The essentially absolute requirement of this enzyme for NADH could not be detected until now, because the commonly used estimation of Ru-5-P kinase activity is based on determination of produced ADP in a coupled reaction sequence with pyruvate kinase and lactate dehydrogenase. In this assay system NADH is always present

in large amounts.

An analysis of the initial rate of CO_2 fixation as a function of NADH concentration shows that this relationship is markedly sigmoid in character (Fig. 1). Half-maximum activation is achieved at 0.14 mM NADH. The relationship of reciprocal rate to reciprocal NADH concentration is non-linear and points to deviations from classical Michaelis-Menten kinetics, suggesting that more than one molecule of NADH is bound to the enzyme and that these NADH sites are interacting. If initial rate data for activation of NADH concentration are plotted according to Hill's equation (Changeux, 1963) a straight line is achieved, the slope of which is about 2.5 (Fig. 2). In terms of current theories of allosteric proteins these results seem to indicate that Ru-5-P kinase is an allosteric enzyme the specific activating effector of which is NADH.

Like Ru-5-P kinase from some other chemoautotrophs or photoautotrophs (Aleem and Huang, 1965, Johnson, 1966, Johnson and Peck,

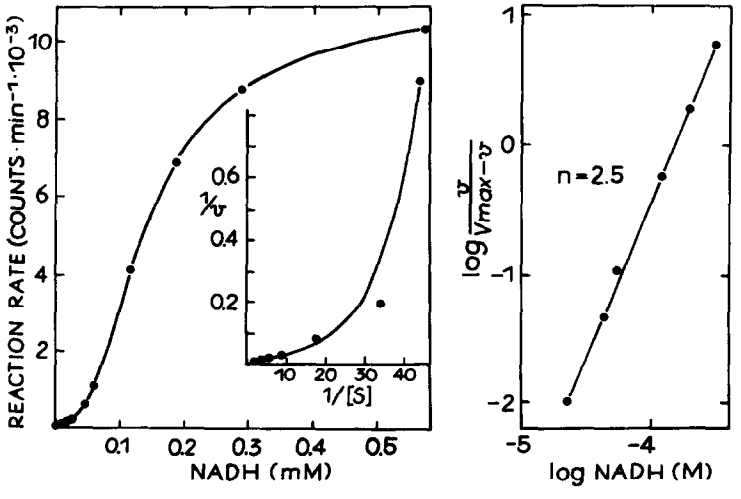

Fig. 1 (left). Kinetic of NADH activation of CO_2 fixation in extracts of Rps. spheroides. Experiments were performed as described.

Fig. 2 (right). Hill plot of the data from Fig. 1.

1965, Mayeux and Johnson, 1967), the enzyme from Rps. spheroides is inhibited by AMP. The degree of AMP inhibition depends on the concentration of NADH. At 0.6 mM NADH about 0.3 mM AMP is necessary to decrease the reaction rate about 50 %. At 0.14 mM NADH (Half maximum saturation of effector) only about 0.08 mM AMP is required for the same degree of inhibition (Table III). NAD seems to have no inhibitory effect because it does not influence the rate of CO_2 fixation even at concentrations five times that of NADH. The exact character of AMP inhibition remains to be determined, but preliminary results indicate an allosteric mechanism too.

TABLE III

Influence of AMP and NAD on NADH activated CO_2 fixation in extracts of Rps. spheroides

NADH	additions	cpm
0.57 mM	----------	7,210
0.57 mM	0.14 mM AMP	6,020
0.57 mM	0.36 mM AMP	3,240
0.14 mM	----------	3,630
0.14 mM	0.07 mM AMP	2,020
0.14 mM	0.14 mM AMP	1,070
0.14 mM	0.70 mM NAD	3,600

Experimental conditions as described.

On the basis of the present data a detailed allosteric characteristic of the enzyme cannot yet be presented. The Hill coefficient points to at least 2.5 binding sites for the modifier NADH. Of course, the exact number of binding sites for NADH cannot be given from the kinetic data presented because of the implication of non-exclusive binding on the degree of cooperativity (Rubin and Chan-

geux, 1966). It must therefore await the result of special binding studies with more purified enzyme preparations.

The physiological significance of the data reported here is that activation of Ru-5-P kinase by NADH and inhibition by AMP might represent a basic control mechanism in autotrophic metabolism of Rps. sheroides. The importance of an efficient control mechanism of autotrophic CO_2 fixation must be stressed for systems which may switch from aerobic dark metabolism to anaerobic light metabolism or viceversa. It appears from the data presented in this paper that both NADH and AMP may be involved in regulating primary events of CO_2 fixation via the pentose phosphate cycle in Rps. spheroides. Upon illumination the process of bacterial photosynthetic electron transport results in the production of NADH. This may be received as a signal by Ru-5-P kinase. The reductive pentose phosphate cycle is inactive if NADH is not present in critical amounts. In Rsp. rubrum, the Ru-5-P kinase of which behaves in an essentially similar manner (unpublished), the level of NADH rapidly drops down (Jackson and Crofts, 1968) during transition from anaerobic light to aerobic dark conditions. At low light intensities the level of AMP should increase as NADH concentration decreases. This control phenomenon has the consequence that the extremely energy-consuming process of CO_2 fixation may only be operating with full capacity if there is no risk in the supply of energy for other important synthetic processes. We propose that both inhibition of Ru-5-P kinase by AMP and activation by NADH may be of equal importance for integrating the cell's economy by regulating the balance between energy-yielding and energy-requiring processes in such a way as to prevent the ATP level from being exhausted.

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