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Phosphoribulokinase from *Nitrobacter winogradskyi*: Activation by Reduced Nicotinamide Adenine Dinucleotide and Inhibition by Pyridoxal Phosphate

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CO_2 fixation by particle-free extracts from *Nitrobacter winogradskyi* increased by addition of reduced nicotinamide adenine dinucleotide (NADH). Ribulose-1,5-diphosphate, however, increased CO_2 fixation, even in the absence of NADH. Phosphoribulokinase (EC 2.7.1.19) was the enzyme of *Nitrobacter* extracts that was activated specifically by NADH. Pyridoxal-5-phosphate inhibited both CO_2 fixation and NADH-activated phosphoribulokinase from *Nitrobacter*. However, it did not affect phosphoribulokinase from spinach leaves. Since the spinach enzyme had also no requirement for reduced pyridine nucleotides, it appears that pyridoxal phosphate interferes only with the binding of NADH and not with the binding of ribulose-5-phosphate and adenosine-5'-triphosphate. The regulation of phosphoribulokinase activity by NADH provided *Nitrobacter* with an energy-dependent control mechanism of CO_2 assimilation.

Reduced nicotinamide adenine dinucleotide (NADH) fulfills one of the identified molecular requirements of the cell-free fixation of carbon dioxide by extracts from the chemoautotrophic *Nitrobacter winogradskyi* (7). In intact cells, NADH is generated by energy-linked reactions that are coupled to the oxidation of nitrite (1, 5, 6) and is consumed in the reductive steps of CO_2 assimilations. NADH also assumes this function when added together with adenosine-5'-triphosphate (ATP), ribose-5-phosphate (ribose-5-P), or ribulose-5-phosphate (ribulose-5-P) and sodium bicarbonate to particle-free extracts from the same microorganism, leading to the fixation of CO_2. However, preliminary experimental evidence from our laboratory indicated that NADH may meet yet another specific requirement of the CO_2 fixation by *Nitrobacter* extracts which was not associated with the reductive steps of carbon dioxide assimilation. More specifically, it appeared that NADH is also essential in the formation of the CO_2 acceptor from ribose-5-P and ATP. This observation was supported somewhat indirectly by a report that identified NADH as an activating effector of phosphoribulokinase (EC 2.7.1.19) activity in extracts from *Rhodopseudomonas spheroides* (12). Consequently, such a function for NADH in the enzymatic pathways of chemoautotrophic CO_2 fixation was further explored, and the results of this study are reported in this paper.

MATERIALS AND METHODS

The strain of *N. winogradskyi* used was originally isolated by H. Engel (Botanical Garden, Hamburg, Germany), from whom it was also obtained. Growth of *N. winogradskyi* and preparation of cell-free extracts were as reported earlier (8). For determinations of phosphoribulokinase activity, these extracts were partially purified by fractionation with $(NH_4)_2SO_4$ (between 35 and 45%, wt/vol) and subsequent molecular sieve filtration of the collected precipitate, which was redissolved in 0.05 M triethanolamine-hydrochloride buffer, pH 8.2, and chromatographed on a Sephadex G-100 (Pharmacia) column (2.5 by 20 cm). The excluded fraction contained the enzyme protein.

CO_2 fixation was measured at 30°C in 3.1 ml, which contained, in addition to cell-free extract equivalent to 1 mg of protein, 50 μmol of triethanolamine-hydrochloride buffer, pH 8.2; 50 μmol of $MgCl_2$; 10 μmol of tetrasodium ATP; 10 μmol of tetrasodium NADH; 118 /mol of $NaH^{14}CO_3$; and 10 μmol of ribulose-1,5-diphosphate (ribulose-1,5-P_2). Incubations were performed in closed plastic tubes containing only the liquid phase, thereby eliminating the necessity for establishing a $NaHCO_3$-CO_2 equilibrium across a gas-liquid interface. CO_2 fixation proceeded linearly for at least 40 min. The reactions were terminated by the addition of 100 μl of glacial acetic acid. $^{14}CO_2$ fixation rates were determined in 100-μl samples of the incubation mixture, which were shaken mechanically for 20 min after the further addition of 100 μl of glacial acetic acid. Fixed ^{14}C was measured in a liquid scintillation spectrometer (Packard Instruments), using Aquasol (New England Nuclear Corp.). Calculations were based on

the specific activity of $NaH^{14}CO_3$ and expressed in nanomoles of CO_2 per minute per milligram of protein.

Phosphoribulokinase (EC 2.7.1.19) activity was determined at 30°C and at a 555-nm wavelength in a recording spectrophotometer (Zeiss PMQ II) with an automatic sample changer by following the H^+ formation in the reaction: ribulose-5-P^{2-} + ATP^{4-} → ribulose-1,5-P_2^{4-} + ADP^{3-} + H^+, with phenol red as the indicator dye. Microcuvettes with a 1-cm path length contained, in 380 μl, the following: 2.3 mM ATP, 4.5 mM $MgCl_2$, 0.016 mM phenol red and, unless stated otherwise, 30 μg of protein, 0.3 mM NADH, and 2.6 mM ribulose-5-P. All solutions were adjusted to pH 8.2 with dilute NaOH. The reactions were started by the addition of ribulose-5-P. To convert absorbance changes into changes of H^+ ion concentrations, the buffering capacity of each reaction mixture was always determined by direct, optical titration with dilute, normalized HCl. Only the initial, linear parts of the curves, where changes in total pH were negligible, were used.

Difference spectra of pyridoxal-5-phosphate (PLP)-protein complexes were recorded in a dual-wavelength, split-beam spectrophotometer (model DW-2, Aminco), using microcuvettes with a 2-cm path length.

Protein was determined according to Lowry et al. (9), using crystalline bovine albumin (Calbiochem) as a reference standard.

All chemicals were analytical grades obtained from Mallinckrodt Chemical Works. The biochemicals were purchased from the following sources: β-NAD, disodium β-NADH, tetrasodium NADPH, and disodium ribose-5-P from Boehringer Mannheim; PLP, pyridoxamine phosphate, pyridoxal-hydrochloride, pyridoxamine-dihydrochloride, disodium ribulose-1,5-P_2, and D-penicillamine from Calbiochem; disodium α-NADH, 3-acetylpyridine-NADH, deamino-disodium NADH, disodium ribulose-5-P, and phosphoribulokinase from spinach leaves from Sigma Chemical Co. All other reagents were of the highest purity commercially available.

RESULTS

The effects of ATP or NADH additions or both on the rates of CO_2 fixation in cell-free extracts from *Nitrobacter* in the presence of a CO_2 acceptor or its different precursors are summarized in Table 1. The CO_2 fixation rates with ribulose-1,5-P_2, ribulose-5-P + ATP + NADH, and ribose-5-P + ATP + NADH were virtually identical. This indicated that the requirement for NADH is in the conversion of the precursor to the actual CO_2 acceptor, ribulose-1,5-P_2. Conversely, the omission of ATP or NADH from the incubation mixture led to significant reductions in CO_2 fixation only when ribulose-5-P or ribose-5-P served as substrates, but not with ribulose-1,5-P_2 itself.

Table 2 shows the specificity and relative effectiveness of different forms of pyridine nucleotides and of analogues as they are introduced to replace NADH in an otherwise complete system for cell-free chemoautotrophic CO_2 fixation. Besides β-NADH, only 3-acetylpyridine-NADH was capable of enhancing the rate of CO_2 fixation. Nevertheless, even at equimolar concentrations, it accomplished this less effectively than NADH. Neither β-NADPH nor α-NADH was able to substitute for β-NADH in this particular function, thus indicating a high degree of specificity of the reaction in which NADH participates.

A comparison of the effects of various reduced pyridine nucleotides on the rates of CO_2 fixation by cell-free *Nitrobacter* extracts with their effects on the rates of ribulose-5-P phosphorylation by phosphoribulokinase revealed a high degree of similarities between the two effects

TABLE 1. *Cell-free CO_2 fixation by Nitrobacter extracts*

Additions[a]	CO_2 fixation (nmol of CO_2 per min per mg of protein)
None	0.8
Ribose-5-P	1.3
Ribose-5-P + ATP	3.8
Ribose-5-P + NADH	1.7
Ribose-5-P + ATP + NADH	46.6
Ribulose-5-P	1.2
Ribulose-5-P + ATP	3.9
Ribulose-5-P + NADH	1.8
Ribulose-5-P + ATP + NADH	47.2
Ribulose-1,5-P_2	47.0
Ribulose-1,5-P_2 + ATP	43.8
Ribulose-1,5-P_2 + NADH	44.6
Ribulose-1,5-P_2 + ATP + NADH	44.3

[a] Experimental conditions are outlined in Materials and Methods.

TABLE 2. *Effects of pyridine nucleotides and their analogues on the cell-free CO_2 fixation by Nitrobacter extracts*

Pyridine nucleotide[a]	CO_2 fixation (nmol of CO_2 per min per mg of protein)
None	4.2
β-NADH	55.3
β-NAD^+	3.8
α-NADH	2.3
β-NADPH	4.0
Deamino-NADH	3.9
3-Acetylpyridine-NADH	36.7

[a] All pyridine nucleotides were added at 1.7 mM final concentrations to an incubation mixture that is described in Materials and Methods.

(Table 3). Just as in CO_2 fixation itself, only β-NADH and, to a lesser extent, its analogue 3-acetylpyridine-NADH were capable of increasing the rate of ribulose-5-P phosphorylation. Even more significantly, this common requirement of an enzymatic pathway and of a particular enzyme (Table 3) identified the enzymatic site of β-NADH activation to be that of phosphoribulokinase.

The effects of increasing concentrations of β-NADH on *Nitrobacter* phosphoribulokinase are shown in Fig. 1. From V_{max} and V_0 values obtained by extrapolation, an activation or binding constant for NADH of $K_a = 3.2 \times 10^{-5}$ M was calculated, which is in good agreement with an earlier value (7). Moreover, the effects of low concentrations of NADH on the V values of enzyme activity (insert, Fig. 1) revealed a slightly sigmoid shape of the total activation curve, a shape that is considered typical for an allosteric interaction between an effector molecule and an enzyme protein (10, 11). However, a Hill coefficient of only 1.8 can be calculated from Hill's plot.

Low concentrations of PLP inhibit both cell-free CO_2 fixation and phosphoribulokinase activity in *Nitrobacter* extracts to an almost identical degree (Fig. 2). The calculated inhibition constant, K_i, for PLP is 2.8×10^{-5} M. In addition, the inhibition of phosphoribulokinase activity by PLP is noncompetitive with respect to NADH (insert, Fig. 2).

The inhibition of phosphoribulokinase activity by PLP is fully reversible by an addition of excess concentrations of D-penicillamine (Fig. 3) or tris(hydroxymethyl)aminomethane. Schiff base formation from D-penicillamine and PLP (3, 14) could lead to dissociation of the PLP-enzyme complex.

The difference spectrum of PLP and the PLP-protein complex is shown in Fig. 4. It is evident that the addition of the enzymatically active protein fraction to 0.2 mM PLP in 0.05 M triethanolamine-hydrochloride buffer, pH 8.2, results in a difference spectrum with absorbance maxima and minima typical for PLP Schiff bases.

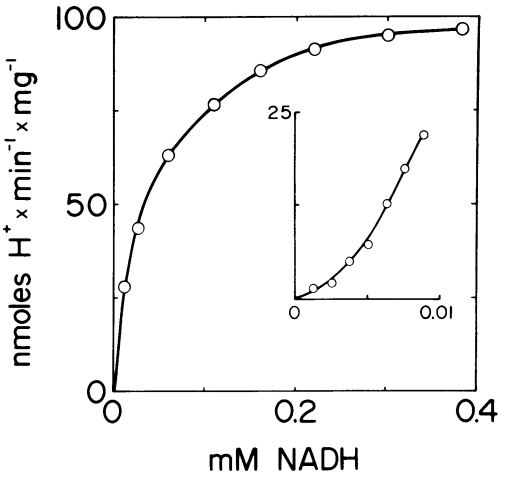


FIG. 1. *Effects of NADH on the initial velocities of the phosphorylation of ribulose-5-P by phosphoribulokinase from Nitrobacter. The rates were determined by the spectrophotometric test described under Materials and Methods. An approximate K_a value of 3.2×10^{-5} M NADH was obtained. The insert shows the shape of the activation curve at low NADH concentration.*

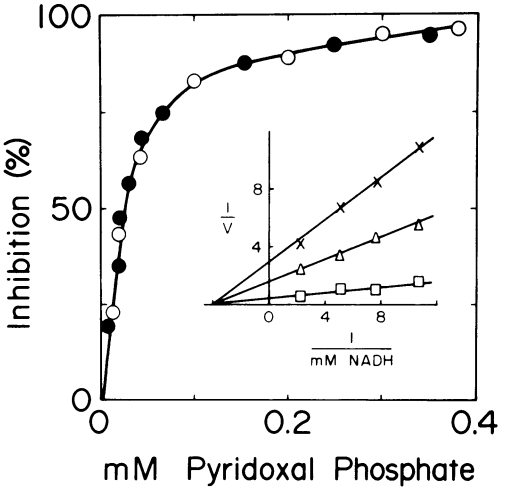


FIG. 2. *Inhibition of NADH-activated CO_2 fixation and phosphoribulokinase activity from Nitrobacter by PLP. The effects of PLP on cell-free CO_2 fixation (●) and phosphoribulokinase activity (○) are shown. (Insert) Lineweaver-Burk plot of the effects of various NADH concentrations at 0.0 (□), 1.8×10^{-5} (△), and 3.6×10^{-5} M PLP (×) indicates the noncompetitive nature of the inhibition. The conditions of the assays are described under Materials and Methods.*

TABLE 3. *Effects of reduced pyridine nucleotides on the phophorylation of ribulose-5-P by phosphoribulokinase from Nitrobacter*

Pyridine nucleotide[a]	Phosphorylation rate (nmol of H^+ per min per mg of protein)
None	5.7
β-NADH	68.4
α-NADH	1.8
β-NADPH	8.1
Deamino-NADH	2.1
3-Acetylpyridine-NADH	44.6
β-NAD^+	3.4

[a] All pyridine nucleotides were added at 1.1 mM final concentrations to an incubation mixture that is described in Materials and Methods.

Various analogues of PLP were compared with regard to their effectiveness as inhibitors of phosphoribulokinase activity from *Nitrobacter* (Table 4). Besides PLP, only pyridoxal shows inhibition of the enzymatic activity. However, as seen from the K_i values, the effectiveness of pyridoxal as enzyme inhibitor is quite reduced, whereas pyridoxamine and pyridoxamine-5-phosphate are ineffective altogether, even at the highest concentrations tested.

Finally, the effects of NADH, NADPH, and PLP on the phosphoribulokinase activity from spinach leaves were studied. It is evident from the data presented in Table 5 that neither NADH nor NADPH increases, or for that matter decreases, the activity of this particular enzyme. Moreover, PLP does not inhibit this enzyme when spinach leaves are its source instead of *Nitrobacter*.

DISCUSSION

The experiments reported in the preceding paragraphs indicate that NADH is essential for the activation of CO_2 fixation by cell-free extracts from *Nitrobacter*. They also show that NADH is necessary for the activation of phosphoribulokinase obtained from this microorganism. For example, the K_a of 3.2×10^{-5} M NADH reported here for the activation of phosphoribulokinase agrees well with an earlier K value of approximately 3×10^{-5} M NADH for the activation of cell-free CO_2 fixation by *Nitrobacter* extracts. Furthermore, PLP shows almost identical inhibition of NADH-activated phosphoribulokinase and NADH-activated CO_2 fixation in cell-free extracts with *Nitrobacter* as the source, since the K values 2.8×10^{-5} M PLP are identical in both cases. Finally, the activation of cell-free CO_2 fixation by NADH can only be observed with ribose-5-P or ribulose-5-P but not with ribulose-1,5-P_2 as substrate.

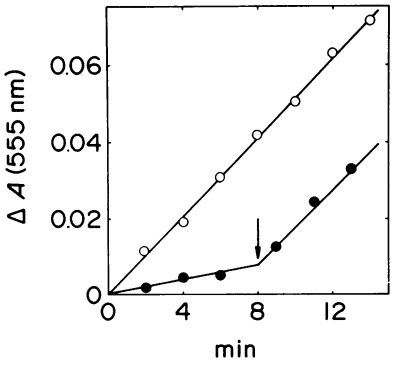


FIG. 3. *Reversal of the PLP inhibition of phosphoribulokinase by* D-*penicillamine. The changes in optical absorbance (A) during ribulose-5-P phosphorylation in the absence* (○) *and in the presence of* 3.6×10^{-5} *M PLP* (●) *are recorded using the phenol red assay (Materials and Methods). At the time indicated by the arrow,* D-*penicillamine was added at a final concentration of 0.02 M. The reversal of PLP inhibition is indicated by an increase in slope to that of the control value.*

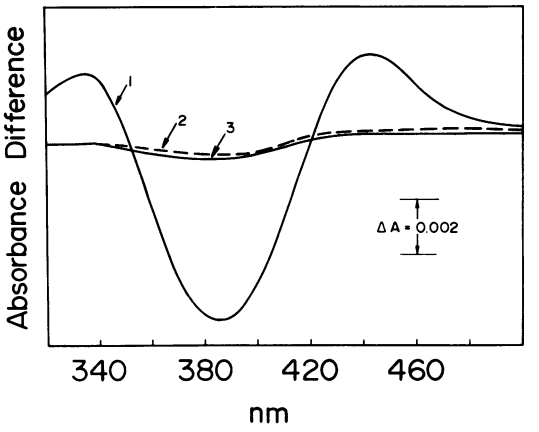
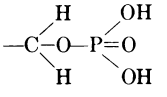
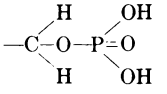
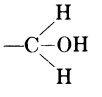
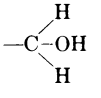


FIG. 4. *Schiff-base difference spectra from PLP and the phosphoribulokinase active protein fraction from Nitrobacter. (1) PLP + protein versus PLP; (2) PLP versus PLP; (3) PLP + protein versus PLP + protein. The conditions are listed under Materials and Methods; the protein concentration was 150 μg/ml.*

Since the activity of phosphoribulokinase from *Nitrobacter* is lost during purification and can only be restored by addition of NADH or 3-acetylpyridine-NADH, it is likely that NADH binds reversibly to the enzyme protein. Moreover, the slightly sigmoid shape of the activation curve of phosphoribulokinase, which becomes apparent only at lower NADH concentrations, could indicate an allosteric mechanism (10, 11). Yet, a Hill coefficient of only 1.8 could be calculated from Hill's plot, which is low for allosteric interactions. In addition, the requirement for NADH is rather specific and does not involve NADH oxidation.

PLP has been shown previously to bind mainly to lysyl residues of proteins, whereby it can affect even the activities of enzymes for which PLP is not a coenzyme (2, 4, 13, 15). It is therefore interesting that PLP inhibits the phosphoribulokinase activity of *Nitrobacter*. In fact, various PLP analogues can serve as convenient probes, revealing some of the molecular requirements of the inhibition. For example, for full inhibitory effectiveness, the aldehyde group of PLP is essential as is the methylphosphate ester group. These requirements, together with the complete reversibility of the

TABLE 4. *Inhibitory effects of PLP and its analogues on the phosphorylation of ribulose-5-P[a] by phosphoribulokinase from Nitrobacter*

Inhibitor	R_1	R_1	K_i (M)
PLP			2.8×10^{-5}
Pyridoxamine phosphate			$>5 \times 10^{-1}$
Pyridoxamine			$>5 \times 10^{-1}$
Pyridoxal			8.0×10^{-2}

[a] Ribolose 5-P: 

TABLE 5. *Effects of reduced pyridine nucleotides and PLP on the phosphorylation of ribulose-5-P by phosphoribulokinase from spinach leaves*

Additions[a]	Phosphorylation rate (nmol of H^+ per min per mg of protein)
None	75.4
β-NADH	76.1
β-NADPH	75.7
PLP	75.3
β-NADH + PLP	76.0

[a] Conditions of the test are identical with those described in Materials and Methods for the same enzyme from *Nitrobacter*. The final concentration of the different pyridine nucleotides is 1.7 mM, and that of PLP is 5 mM.

inhibition by D-penicillamine, which readily forms a Schiff base with PLP, may suggest that PLP also forms a Schiff base with the enzyme protein, an assumption that is also strengthened by the difference spectra obtained with PLP and the protein fraction. One might also speculate that this bond is formed in the vicinity of the NADH-binding site, which is thereby altered. Moreover, such an assumption would be in agreement with the noncompetitive nature of the PLP inhibition with respect to NADH and with the results obtained with phosphoribulokinase from spinach leaves. This particular enzyme does not have a requirement for reduced pyridine nucleotides as activators, nor is it inhibited by PLP.

Finally, it may be of interest to note that the specific activation of phosphoribulokinase by NADH renders this particular *Nitrobacter* enzyme dependent on the intracellular steady-state concentrations of NADH, which in turn are maintained by energy-linked NADH-generating reactions of nitrite oxidation. Thus, NADH becomes a regulator of an early enzymatic step of cellular CO_2 assimilation and can thereby modify the general energetic efficiency of intact *Nitrobacter* cells.

ACKNOWLEDGMENTS

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