

## Effects of the Calvin Cycle on Nicotinamide Adenine Dinucleotide Concentrations and Redox Balances of *Xanthobacter flavus*

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**The levels of reduced and oxidized nicotinamide adenine dinucleotides were determined in *Xanthobacter flavus* during a transition from heterotrophic to autotrophic growth. Excess reducing equivalents are rapidly dissipated following induction of the Calvin cycle, indicating that the Calvin cycle serves as a sink for excess reducing equivalents. The physiological data support the conclusion previously derived from molecular studies in that expression of the Calvin cycle genes is controlled by the intracellular concentration of NADPH.**

*Xanthobacter flavus* assimilates CO<sub>2</sub> via the Calvin cycle during autotrophic growth. The energy required to operate the Calvin cycle is provided by the oxidation of methanol, formate, thiosulfate, or hydrogen. In addition, heterotrophic growth is supported by a wide range of organic substrates, e.g., gluconate or succinate (15). In this case, CO<sub>2</sub> fixation is not necessary and the Calvin cycle is not induced. To date, three unlinked transcriptional units encoding Calvin cycle enzymes have been identified in *X. flavus*: the *cbb* (14, 21) and *gap-pgk* (13, 17) operons and the *tpi* gene (16). The key enzymes of the Calvin cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and phosphoribulokinase, are encoded by the *cbb* operon.

The LysR-type transcriptional regulator CbbR has been identified in several chemo- and photoautotrophic bacteria (10). This protein controls expression of the *cbb* operon and, in *X. flavus*, also the *gap-pgk* operon (17, 22). We previously showed that purified CbbR protects nucleotides –75 to –29 relative to the transcriptional start of the *cbb* operon in a DNase I footprinting assay. In addition, it was shown that purified CbbR responds to NADPH but not NADH in vitro: DNA binding of CbbR increases threefold and CbbR-induced DNA bending is relaxed by 9° in the presence of NADPH. The apparent  $K_{d[NADPH]}$  was determined to be 75 μM; saturation occurs at approximately 200 μM (23).

The results from these in vitro experiments strongly suggest that the in vivo expression of the *cbb* and *gap-pgk* operons is mediated by CbbR in response to the intracellular concentration of NADPH. To examine this in greater detail, the levels of reduced and oxidized nicotinamide adenine dinucleotides were determined during a transition from heterotrophic to autotrophic growth. *X. flavus* was grown on a mixture of gluconate (5 mM) and formate (20 mM) with pH control by automatic titration with formic acid (25% [vol/vol]) as described previously (15). RuBisCO (5), phosphoglycerate kinase (13), and NAD-dependent formate dehydrogenase (4) enzyme activities were subsequently determined in cell extracts as described previously. Protein was determined by the Bradford method, using bovine serum albumin as a standard (3). Significant for-

mate dehydrogenase activity was already present 1 h following addition of formate to heterotrophically growing *X. flavus*. In contrast, induction of the *cbb* and *gap-pgk* operons, as indicated by the appearance of RuBisCO and increase of phosphoglycerate kinase activity, became apparent 2 h after formate addition (Fig. 1A). During the transition from autotrophic to heterotrophic growth, samples withdrawn from the fermenter were immediately frozen in liquid nitrogen and subsequently freeze-dried. Nicotinamide adenine dinucleotides were extracted from freeze-dried samples (12) and then quantified using a sensitive spectrophotometric cycling assay (2). Following addition of formate to the medium, the concentrations of NAD(H) and NADP(H) increased four- and twofold, respectively, over a period of 5 h (Fig. 2). Prior to addition of formate to the medium, 15 to 25% of the nicotinamide adenine dinucleotide pools were in the reduced form. This percentage increased rapidly following addition of formate to culture and paralleled the increasing activity of formate dehydrogenase (Fig. 1). The rapid increase in redox balance, defined as the ratio of reduced to total nicotinamide adenine dinucleotide, is therefore most likely due to oxidation of formate and the concomitant production of NADH. Similar observations were made when the chemoautotrophic bacterium *Pseudomonas oxalaticus* was transferred from oxalate to formate medium (9). The redox balance reached a maximum 2 h after addition of formate to the medium and subsequently decreased rapidly, even though the activity of formate dehydrogenase and the total concentration of nicotinamide adenine dinucleotide continued to increase (Fig. 1 and 2). The sharp decrease in redox balance coincided with the appearance of RuBisCO activity and the increase in phosphoglycerate kinase activity, which is indicative of operation of the Calvin cycle. This pathway consumes 6 mol of NADH and 9 of ATP for every mole of triosephosphate produced. It is therefore likely that the high demand of autotrophic CO<sub>2</sub> fixation for NADH accounts for the observed decrease in redox balance. The most obvious function of the Calvin cycle is to supply the cell with a source of carbon during autotrophic growth. A second, equally important function is to act as an electron sink in order to dissipate excess reducing power (11, 20). For example, purple nonsulfur bacteria fail to grow photoheterotrophically in the absence of a functional Calvin cycle unless an alternative electron acceptor such as dimethyl sulfoxide is present (6, 19, 24). Interestingly, secondary mutants of RuBisCO-deficient *Rhodobacter sphaeroides*

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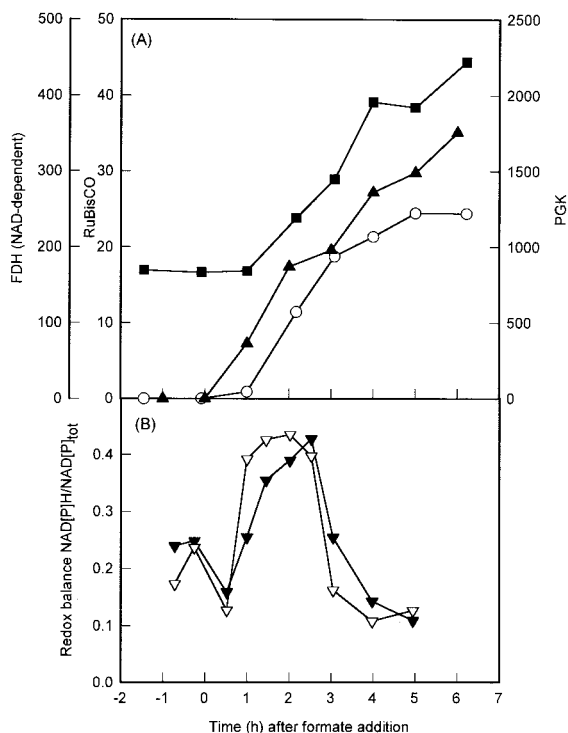


FIG. 1. (A) Activities of RuBisCO (○), phosphoglycerate kinase (PGK; ■), and formate dehydrogenase [FDH (NAD-dependent); ▲] of *X. flavus* growing on 5 mM gluconate. The results following addition of 20 mM formate and automatic titration with formic acid (25% [vol/vol]) at time zero are shown. Enzyme activities are expressed in nanomoles per minute per milligram of protein. (B) NAD(H) (▼) and NADP(H) (▽) redox balance ( $\frac{[NAD(P)H]}{[NAD(P)]} + \frac{[NAD(P)H]}{[NAD(P)]}$ ) of *X. flavus* growing on 5 mM gluconate. The results before and after the addition of 20 mM formate and automatic titration with formic acid (25% [vol/vol]) at time zero are shown.

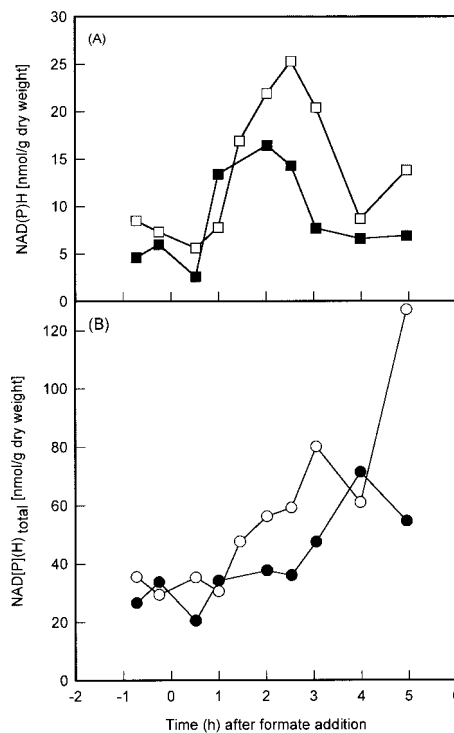


FIG. 2. Concentrations of NADH (A, □) and NADPH (A, ■) and total concentrations of NADH plus NAD<sup>+</sup> (B, ○) and NADPH plus NADP<sup>+</sup> (B, ●) following the addition of 20 mM formate to a culture growing on 5 mM gluconate at time zero.

strains which had regained the ability to grow photoheterotrophically were isolated (24). These mutants induced nitrogenase to reduce protons to H<sub>2</sub>, resulting in dissipation of excess reducing equivalents (8). Induction of the Calvin cycle in *X. flavus* resulted in a rapid decrease of the redox balance to below levels seen before the addition of formate. This suggests that CO<sub>2</sub> fixation via the Calvin cycle is very effective in removing excess reducing power.

The Calvin cycle was induced as the concentration of NADPH approached its maximum (16.4 nmol/g [dry weight]), 1 h following addition of formate to the culture (Fig. 1A and 2A). This corresponds to an intracellular NADPH concentration of 189 to 216 μM, assuming a cellular volume of 3.5 to 4 μl/mg of protein (1, 7, 18). NADPH at this concentration saturates CbbR in vitro, resulting in maximum DNA binding affinity and relaxed DNA bending. The NADPH concentration remained at this level for another 2 h, during which the activity of RuBisCO increased 21-fold. Both the redox balance and the concentration of NADPH subsequently decreased rapidly (Fig. 1B and 2A). However, although the redox balance was reduced to below levels observed before the addition of formate, the NADPH concentration remained twofold higher, at a concentration of 81 to 93 μM. This concentration is slightly above the previously reported  $K_{d[NADPH]}$  of 75 μM. The RuBisCO activity increased only 1.3-fold during this period. The expression levels of the *cbb* operon therefore correspond to the degree of NADPH saturation of CbbR in vitro. This observation supports, but does not prove, our previous conclusion based on

molecular studies that the intracellular NADPH concentration determines the activity of CbbR and hence expression of the *cbb* operon (23). Future research will aim to analyze the interaction between NADPH and CbbR in greater detail.

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