1. SUMMARY

Increasing the capability of biological systems for hydrogen production requires an understanding of the fundamental level of potential limiting factors. Degradation of various wastes with the concomitant evolution of hydrogen by photosynthetic bacteria has long been proposed as a possible biohydrogen source. Here we present evidence, using growth studies with different nitrogen sources, which indicates that the maximal in vivo activity of the nitrogenase (N_2ase) system of the photosynthetic bacterium, *Rhodobacter capsulatus*, is probably restricted at the level of electron flow. Thus, an increase in catalytic efficiency of at least threefold is possible. We are presently investigating the physiological mechanisms responsible for this metabolic gating. Although very little is presently known in general about the determinants for reaction specificity and efficiency between low-potential redox carriers and enzymes functioning in biological hydrogen production, these results suggest that a consideration of these factors may be extremely important. As a model system, we are undertaking studies of the role of electron carriers in controlling reductant flux through the pyruvate oxidoreductase (POR), N_2ase system. The methods we are using and results obtained should be applicable to other hydrogen-producing systems, for example, POR and hydrogenase.
2. INTRODUCTION

Biological hydrogen production is based on the coupling of the generation of reducing power, either by the cellular machinery (Benemann and Weare, 1974) or in vitro systems (Benemann et al., 1973), to a terminal enzyme capable of reducing protons to hydrogen (either hydrogenase or the hydrogenase reaction of nitrogenase). These terminal enzymes are in general not reduced by specific reductases and require an electron transport factor to be able to draw off the low-potential electrons that have been generated. In many organisms, the suitable electron transport factors are low molecular weight soluble proteins, flavodoxin (e.g., NifF) or ferredoxin (e.g., FdI). Despite the isolation and extensive characterization of many of these electron carriers and their respective electron acceptors, relatively little is known about what factors control their efficiency and reaction specificity. These factors may play important roles in regulating electron flux through the in vivo systems and certainly need to be considered when genetic modifications of pre-existing systems are contemplated with a view towards increasing hydrogen production capability. We present results that indicate that under some circumstances, electron flux to the nitrogenase system limits in vivo nitrogenase activity. The results of stopped-flow spectrophotometric kinetic analysis show that NifF forms a tight complex with Fe-protein whereas FdI does not, suggesting that electron flow is from FdI ($E_m = -510$ mV) $\rightarrow$ NifF ($E_m = -474$ mV) $\rightarrow$ N$_2$ase ($E_m = -470$ mV, [Hallenbeck, 1983]). We have initiated studies on the interaction of NifF and POR (both from K. pneumoniae) and devised schemes for selection of NifF mutants impaired in their interaction with either POR or N$_2$ase.

3. EXPERIMENTAL

3.1. Measurement of in Vivo Nitrogenase Activity and Content

For experiments in which in vivo nitrogenase activity and Fe-protein modification state were analyzed, batch cultures of R. capsulatus SB1003 were grown with -N RCV medium in 1.6 x 20.5 cm tubes sealed with rubber stoppers with needles inserted for gas sparging (argon or N$_2$; 5–10 mL/min). Seven mM glutamate, N$_2$, or limiting amounts of NH$_4^+$ were used as nitrogen sources. In vivo nitrogenase activity was determined by the acetylene reduction method and the amounts of modified and unmodified N$_2$ase proteins were determined by chemiluminescent detection of immunoblots (Yakunin and Hallenbeck, 1997).

3.2. Stopped-Flow Kinetic Analysis

NifF was purified from an over-producing strain of E. coli (Hallenbeck and Gennaro, 1998). FdI and nitrogenase were purified as previously described by Hallenbeck et al. (1982a, b). Anaerobic conditions were maintained by using either an anaerobic chamber or purified nitrogen lines. Carriers were reduced with sodium dithionite. Oxidized species were exchanged into 25 mM HEPES, 10 mM MgCl$_2$ (pH 7.4) by gel filtration (PDG-8) in the anaerobic chamber. NifF$_{SQ}$ (NifF-semiquinone) was conveniently generated by mixing equimolar amounts of NifF$_{ox}$ and NifF$_{HQ}$ (NifF-hydroquinone). All kinetic experiments were carried out at 25 ± 0.1 °C in 25mM HEPES, 10 mM MgCl$_2$ in the anaerobic chamber using a Hi-Tech SF-61 Stopped-Flow Spectrophotometer, and data were analyzed using the associated software. Kinetic analysis followed standard methods for treating scheme 1 under pre-equilibrium and non-steady state conditions.

$$A_{ox} + B_{red} \xrightleftharpoons[k_{+2}]{k_{-1}} AB \xrightarrow{k_{+1}} A_{red} + B_{ox}$$