The kinetic characteristics of the hydrogen uptake reaction of hydrogenase, obtained by conventional activity measurements, led to the proposal of an autocatalytic reaction step in the hydrogenase cycle or during the activation process. The autocatalytic behavior of an enzyme reaction may result in oscillating concentrations of enzyme intermediates and/or products contributing to the autocatalytic step. This behavior has been investigated in the early phase of the hydrogenase-methyl viologen reaction. To measure fast hydrogenase kinetics, flash-reduced methyl viologen has been used as a light-induced trigger in transient kinetic phenomena associated with intermolecular electron transfer to hydrogenase. Here we report fast kinetic measurements of the hydrogenase-methyl viologen reaction by use of the excimer laser flash-reduced redox dye. The results are evaluated on the assumption of an autocatalytic reaction in the hydrogenase kinetic cycle. The kinetic constants of the autocatalytic reaction, i.e. the methyl viologen binding to and release from hydrogenase, were determined, and limits of the kinetic constants relating to the intramolecular (intraenzyme) reactions were set.

Hydrogenases are metalloenzymes that catalyze the reaction $H_2 \rightarrow 2 H^+ + 2 e^-$. Three distinct groups of hydrogenases can be defined, depending on the metal content of the protein. The iron-only enzymes can be found in strict anaerobic bacteria and usually display high sensitivity toward oxygen. [Ni-Fe] and [Ni-Fe-Se] hydrogenases are mostly membrane-bound enzymes found in different kinds of bacteria. They are relatively heat and oxygen stable. A vast majority of known (characterized) hydrogenases belong in the [Ni-Fe] class (1). The [Ni-Fe] enzymes are usually heterodimers involving a small ($\sim$30 kDa) and a large ($\sim$60 kDa) subunit with an overall molecular mass of $\sim$90–100 kDa. The metals are organized into two or three FeS clusters and a [Ni-Fe] binuclear center. The [Ni-Fe] binuclear center or its ligand environment is believed to be the hydrogen-binding site, though the exact binding location has not yet been determined. It is also generally accepted that the FeS clusters transfer electrons from the [Ni-Fe] binuclear center to the terminal electron acceptor, as evidenced from the crystal structure (2, 3).

It is known that hydrogenase needs to be activated under anaerobic conditions (4–6). During activation and the enzyme cycle, several stable intermediates have been determined by electron paramagnetic resonance (EPR) and Fourier transform infrared (FTIR) spectroscopy (7, 8), ranging from the fully oxidized Form A through oxygen-free Form $S_{	ext{lab}}$ and from Form B to the active enzyme (Form C and Form R). These forms have not yet been satisfactorily assigned to the corresponding theoretical enzyme forms of hydrogen splitting.

Although the enzymatic activity of hydrogenase is determined routinely, a number of contradictory results have been published. Despite the many features described in the hydrogenase reaction, the activity of this class of enzymes has not yet been thoroughly explained (5, 9–12). An autocatalytic kinetic step was recently proposed in hydrogenase activation or during the hydrogenase kinetic cycle.2,3

The ability of nitrogen laser flash-reduced methyl or benzyl viologen to initiate a redox transition of a redox protein has been demonstrated previously (14). Here we report fast kinetic measurements of the hydrogenase enzyme reaction involving the use of an excimer laser flash-reduced redox dye. The results are evaluated on the assumption of an autocatalytic reaction in the hydrogenase kinetic cycle.

**EXPERIMENTAL PROCEDURES**

**Strain and Cultivation—Thioacapsa roseopersicina** BBS was a kind gift from Prof. E. N. Kondratieva (Moscow State University, Moscow). The bacteria were grown under standard photosynthetic conditions as described earlier (15). Cells were harvested in the logarithmic phase of growth and were stored at $-20^\circ$C.

**Protein Purification**—The wet cell paste of *T. roseopersicina* was treated with 90% cold ($-20^\circ$C) acetone, and the pellet was dried and stored at $-4^\circ$C. For purification, 15 g of pellet was dissolved in distilled water at 50 °C. The solution was centrifuged at 12,500 $\times$ g for 1 h, and the supernatant was used. The first purification step comprised batch chromatography with Whatman DEAE DE-52 in 20 mM Tris-HCl, pH 7.5. The hydrogenase was washed off with 450 mM NaCl. The next step was butyl-Sepharose column chromatography in 1 mM Tris-HCl at pH 7.5 with 10% ammonium sulfate, using the Amersham Biosciences FPLC system. Hydrogenase eluted at $\approx$0% ammonium sulfate. Two Q-Sepharose Fast Flow columns were used for further purification steps using different buffer systems (20 mM Tris-HCl, pH 7.5, and 50 mM MES, pH 5.5). In both cases, the hydrogenase eluted in the interval 350–500 mM NaCl. Final purification of the hydrogenase was achieved by 9% native preparative polyacrylamide gel electrophoresis. The hydrogenase band was excised, and the electrophoretically pure enzyme was collected on a small Q-Sepharose column.

**Sample Preparation**—Control samples containing 10 mM oxidized methyl viologen in 20 mM Tris-HCl, pH 7.5, were incubated overnight under anaerobic conditions. Before measurement, the sample was poured into a 0.1-mm quartz cell under anaerobic conditions.
Samples containing 5 mg/ml hydrogenase (50 μM) in 20 mM Tris-Cl (pH 7.5) buffer were incubated overnight in an anaerobe box (Coy Laboratories) containing ~10% of hydrogen and 90% of nitrogen. After incubation, the hydrogenase was in activated form (Form C) as revealed by the EPR spectrum. The sample was poured into a 0.1-mm quartz cell and oxidized with Form A by adding a negligible volume of concentrated methyl viologen to a final concentration of 10 mM under anaerobic conditions. The cell was sealed with a quartz cover plate, which maintained anaerobic conditions for several hours.

Kinetic Measurements — The nanosecond flash photolysis setup involved an excimer laser, a controlled-temperature sample holder, a Xenon lamp, a detector, and a DSA sampling oscilloscope connected to an IBM personal computer.

The data collection, evaluation, and model fitting were performed with a specifically modified SPSERV program.5 The program was modified so it would be able to supervise the DSA sampling oscilloscope and collect and process the data.

The geometry of excitation was pseudo front face, and the analyzed volume was completely irradiated by the excitation beam from the excimer laser at 307 nm. The frequency of laser flashes was 10 Hz. The samples were slowly rotated so that each laser flash came into contact with a fresh portion of the sample. After each measurement, a new cylinder of the cell was selected to avoid saturation of the sample (for an explanation of the saturation, see “Results and Discussion”). In every experiment, the temperature of the cell was maintained at 25 °C.

The bandwidth of the measuring light beam for the methyl viologen (control) sample was varied in the range 360–680 nm in 20-nm steps. The bandwidth was 10 nm. 100 flashes were averaged and collected for each wavelength.

The wavelength of the measuring light beam for hydrogenase samples varied in the range 350–600 nm, and kinetic measurements were made at indicated wavelengths. The bandwidth was 30 nm. Data collected from 1000 individual flashes were averaged at each wavelength.

RESULTS AND DISCUSSION

The ability of nitrogen laser flash-reduced methyl viologen to initiate a fast redox transition in redox proteins was demonstrated earlier with flavocytochrome c552 as a model (14). The methyl viologen was reduced by the nitrogen laser flash in less than 100 ns and donated electrons to the protein. Via monitoring the absorption changes at different wavelengths, the rate constant of reduction and the difference spectrum of reduced and oxidized flavocytochrome c552 could be calculated. The disadvantage of using a nitrogen laser is that the excitation wavelength is close to the absorption minimum of oxidized methyl viologen. Accordingly, a high oxidized methyl viologen concentration had to be maintained to furnish a suitable amount of reduced methyl viologen after the laser flash. In this experiment, we preferred excitation with an excimer laser, which permitted a dramatic reduction of the methyl viologen concentration (from 160 mM, in the case of the nitrogen laser, to 10 mM in this case). On the use of a Nd-YAG laser with frequency quadrupling, the laser flash would excite the oxidized methyl viologen at almost exactly the absorption maximum (260 nm), allowing a further reduction of the methyl viologen concentration to 1 mM or even lower.5 This concentration is about the same as the concentration of the methyl viologen used in conventional activity measurements (16).

In the control samples (containing no hydrogenase) the concentration of reduced methyl viologen produced after one excimer laser flash was 1.5 μM. The activation spectrum reconstructed from the signal amplitude of the samples containing only methyl viologen is in excellent agreement with the absorption spectrum of reduced methyl viologen (Fig. 1).

The correspondence of the activation spectrum reconstructed from the signal amplitude demonstrates that methyl viologen reduction did occur. This confirms our previous findings with the nitrogen laser (14).

In the case of hydrogenase, it is more difficult to demonstrate a redox transition as compared with flavocytochrome c552. Hydrogenase is not only a redox protein but also a catalyst that produces hydrogen in this reaction. Because the volume of the sample is limited and hydrogen cannot leave it, after a while the back reaction of the hydrogenase should be taken into account. Thus, upon prolonged irradiation the forward and back reactions of the hydrogenase will come into equilibrium, and, therefore, no further net methyl viologen oxidation will be observed. Rotation of the sample holder and irradiation of a fresh portion of the sample can partially resolve this problem; but, after the entire surface of the cell has been irradiated, the whole sample becomes saturated with hydrogen gas, and no further disappearance of the reduced dye can be observed despite the presence of hydrogenase in the sample. Hence, one sample is suitable for only a limited number of experiments.

The results of fast kinetic measurements on the hydrogenase-methyl viologen reaction at different wavelengths are presented in Fig. 2. The rate of change of the absorption of hydrogenase samples on reaction with methyl viologen is very

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Footnotes:
1. Copyrighted in 2000 by Csaba Bagvinka.
2. Cs. Bagvinka and M. J. Maroney, unpublished data.
characteristic. At every wavelength where reduced methyl viologen absorbs, a fast initial increase can be observed, followed by damped oscillatory behavior of the kinetic trace. The spectral dependence of the initial amplitude follows the methyl viologen absorption spectrum (there is no observable initial increase in Fig. 2 at 450- and 500-nm excitation, where the reduced methyl viologen absorption spectrum exhibits a minimum). This is exactly the same phenomenon as observed in hydrogenase-free samples.

The measured rate of change of $\Delta$OD for a hydrogenase/methyl viologen sample can be fitted with the sum of a fast and a slow decay curve ($A_1\exp(-t/\tau_1)$ and $A_2\exp(-t/\tau_2)$) and two damped sinusoidal time functions ($A_3\sin(\omega t - t_01)\exp(-t/\tau_3)$ and $A_4\sin(\omega t - t_02)\exp(-t/\tau_4)$) (Fig. 3).

The amplitude of the fast decay curve ($A_1$) reflects the methyl viologen concentration and the $\tau$ value for this exponential ($\tau_1$) remains constant throughout the wavelength range measured. The reduced methyl viologen concentration decreases as the hydrogenase takes over the electrons from the reduced methyl viologen. We therefore assign this process to the disappearance of reduced methyl viologen by the transfer of electrons to hydrogenase. The rate constant for the electron transfer from the reduced methyl viologen to the hydrogenase (calculated according to Ref. 14) is $k = 1.33(5)\times10^6\text{ M}^{-1}\text{s}^{-1}$. This value is of the same order of magnitude as the $10^9\text{ M}^{-1}\text{s}^{-1}$ found for Chromatium vinosum hydrogenase by cyclic voltammetry (17).

We have not been able to assign the second decay to any process. This decay can be visualized at some wavelengths as a slight decrease in the kinetic trace with time. This is clearly pronounced at 350 nm in Fig. 2, where the damped oscillations are not around the zero value. The dependence of the amplitude ($A_2$) on the wavelength demonstrates no clear-cut tendency. The $\tau$ value for this exponential ($\tau_2$) changes from 15,000 ms at 350 nm to 400 ms at 600 nm, but the fitting error for the high values was high (>50%). This change in $A_2$ and $\tau_2$ indicates that the concentration of a corresponding reaction partner is changing during the measurement. Because we collected 1000 flashes with a frequency of 10 Hz in order to obtain a reasonable signal-to-noise ratio, a slow process can develop during the data collection (100 s), and a hydrogenase component, the substrate (the reduced viologen dye) or the product (the hydrogen gas) can diffuse over the cell and interfere with the following measurement. We therefore assign this process as a methodological artifact.

The kinetic characteristics of the hydrogenase reaction, obtained by conventional activity measurements, led us to the conclusion that there should be an autocatalytic reaction step in the hydrogenase cycle or during the activation process. The autocatalytic interaction between two enzyme forms results in a conformational change of the enzyme, thereby activating it. We know from experimental results that such conformational changes are possible and that different conformations of hydrogenase exist. Following the changes in behavior of the hydrogenase under SDS-PAGE conditions, we were able to distinguish between three different states of the enzyme. The first conformer is present under aerobic conditions. Incubation of the enzyme under anaerobic conditions changes it to a different conformer, whereas incubation under hydrogen and methyl viologen transforms it into a third conformational state (18–20). We do not know, however, if these conformational changes are associated with an autocatalytic reaction. It is very interesting to note, however, that, similarly to prion proteins, the hydrogenases are partially resistant to proteolytic digestion (20).

The autocatalytic behavior of an enzyme reaction leads to an oscillating concentration of enzyme products contributing to the autocatalytic step. On the assumption of a simple autocatalytic reaction for hydrogenase activation, as shown in Reaction 1 (the autocatalytic step is between $E_2$ and $E_3$, as indicated by the curved back arrow),

The changes in concentration of $E_2$ and $E_3$ would be reflected by a damped oscillation with $\omega^2 = k_1k_0E_1$, if $k_1k_0E_1 < k_2^2$ and if we can neglect second-order terms (21, 22). $E_3$ is the active form of the enzyme that is able to catalyze hydrogen evolution or hydrogen splitting. The reaction cycle closes back to $E_2$ (recent model) or $E_0$, the autocatalytic step being included in the enzyme cycle or left only in the activation process.

$k_0$ describes an intraenzyme reaction (probably a conformation change or charge redistribution), while $k_1$ is the autocatalytic rate constant. It should be noted that, at the start of the
experiment, the hydrogenase is in the E₁ form, and this is not
the oxidized form (Form A) of the enzyme (see sample prepa-
rated). The two oscillating concentrations observed in our ex-
periment would correspond to E₂ and E₃ in the above model.
The spectral dependence of the initial amplitudes of both of
the damped sine curves can be described as the difference in
absorption of the spectra of reduced and oxidized hydrogenase,
which has a maximum at around 440 nm, though the fitting
errors are rather large (Fig. 4). The spectral dependence of
the amplitude of the second oscillation is the mirror image of
the first. This implies that the oscillation occurs between an ox-
idized and a reduced form of hydrogenase.

An alternative explanation is that, while the first oscillation
proceeds from a reduced hydrogenase, the second oscillation
originates from the reduced dye. As a consequence, it is possi-
ble that an enzyme form (if any) binding the reduced dye is
responsible for this spectral component, or, alternatively, the
methyl viologen plays the role of the autocatalytic partner. To
be able to select between these two possibilities, the rate of
changes of the spectra of the hydrogenase-methyl viologen
samples should be measured in smaller wavelength steps. Un-
fortunately, the saturation of the hydrogenase samples by hy-
drogen and the limited volume did not allow us to obtain more
precise wavelength information by using the same hydrogenase
sample.

The frequencies (0.9 (1) - 10⁸ s⁻¹ and 1.04 (9) - 10⁹ s⁻¹) of the two
damped oscillations are very close (within the fitting error);
only the phases differ. This is in accordance with the above
autocatalytic model, where the two oscillations should have the
same frequency. The k₁k₂E₁ value calculated from the average
ω of this fit is roughly 6 × 10⁶ s⁻². For the conditions discussed
above, k₀k₁E₁ ≪ k₂, which puts k₂ at > 3 × 10⁶ s⁻¹. In the
knowledge of the overall concentration of the enzyme and on
the assumption that the enzyme is in the E₁ form at the begin-
ing of the experiment, k₀k₁ ~ 10⁻¹¹ M⁻¹ s⁻².

The rate of the autocatalytic process should be higher than or
at least the same order of magnitude as that of the reaction
with the real substrate (reduced methyl viologen in our case);
otherwise, we would not be able to observe the autocatalytic
behavior of the reaction because it would be very fast. A com-
parison of the reaction rate constant of the reduced methyl
viologen binding, calculated from the fast decay of the reduced
dye, puts k₁ at around or higher than 10⁸ M⁻¹ s⁻¹. As a result,
k₀ < 10⁸ s⁻¹.

In summary, the ability to use flash-reduced methyl viologen
as a light-induced trigger in transient kinetic phenomena as-
associated with the intermolecular electron transfer of hydro-
genase has been demonstrated. The possibility of fitting the kinet-
ics with a hydrogenase autocatalytic process has been
demonstrated, and the kinetic constants of the autocatalytic
reaction and the binding of methyl viologen or its release from
hydrogenase have been determined. Limits of the kinetic con-
stants relating to the intramolecular (intrazyme) reactions have also been set.

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Autocatalytic Oscillations in the Early Phase of the Photoreduced Methyl Viologen-initiated Fast Kinetic Reaction of Hydrogenase
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