BBA 66159

THE HYDROGENASE OF *CLOSTRIDIUM PASTEURIANUM*

KINETIC STUDIES AND THE ROLE OF MOLYBDENUM

D. KLEINER AND R. H. BURRIS
Department of Biochemistry, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

(Received May 1st, 1970)

SUMMARY

Partially purified hydrogenase from *Clostridium pasteurianum* was used for kinetic studies and investigations of its activation by molybdenum. Methyl viologen reduction, H_2 evolution and $H_2O/^2H_2$ exchange were employed as assays, and all three reactions were activated at low and inhibited at high concentrations of molybdenum added as permolybdic acid. The inhibition was either uncompetitive or non-competitive. A detailed study of the activation indicated that hydrogenase has separate sites for the activation of H_2 and for activation of the electron carrier, and that molybdenum is loosely bound to the carrier site. It is suggested that molybdenum acts as an electron sink which indirectly takes up an electron from the activated H_2, thus enabling the dissociation of a proton. Double reciprocal plots for methyl viologen reduction showed an intersecting pattern which indicates a reversible sequence for H_2 activation. Activation energies were measured for H_2 and $^2H_2$ evolution, with ferredoxin and methyl viologen as electron carriers, and were found to be 14.6 kcal/mole independent of gas and carrier. Thus, either internal electron transfer or a conformational change within the enzyme is rate limiting. Possible mechanisms of hydrogenase action proceeding by homolytic and heterolytic H_2 cleavage are discussed.

INTRODUCTION

Hydrogenases (EC 1.12.1.1), enzymes occurring most commonly in bacteria, catalyze the reaction:

$$H_2 + \text{electron carrier} \rightleftharpoons \text{reduced electron carrier} + 2\ H^+$$

The carriers take up either one (bacterial ferrodoxin) or two (methyl viologen, spinach ferredoxin) electrons.

TAMIYA AND MILLER[1] investigated the mechanism of hydrogen activation and supported the previously proposed heterolytic cleavage[2].

$$E + H_2 \rightleftharpoons EH^- + H^+$$

Their evidence for this mechanism was: (a) in the $^2H_2O/H_2$ exchange at low hydrogenase concentrations the initial product in the gas phase was mainly H^2H. (b) The *para ortho* H_2 conversion in this system was much slower than the exchange reaction.

As complete purification of hydrogenase has not been reported, only indirect evidence for the metal content and the nature of the active site is obtainable. KIDMAN *et al.*[3] showed that increasing the iron content of the growth medium increased the hydrogenase activity, thus suggesting that hydrogenase contains iron. They also found that the clostridial hydrogenase occurs in the form of at least six isozymes, all with a molecular weight of about 55 000 as determined by gel filtration. SHUG *et al.*[4] reported that hydrogenase loses molybdenum during purification; they could restore activity by addition of solid MoO_3. Our investigations give further evidence for the mechanism of hydrogenase action and the role of molybdenum.

MATERIALS AND METHODS

Growth of C. pasteurianum

C. pasteurianum (Strain W5) was grown in nitrogen-free medium of WESTLAKE AND WILSON[5]. The cells, harvested in the exponential phase of growth, were dried and stored under vacuum at −20°.

Purification of hydrogenase

The dry cells were shaken with 10-fold their weight of 0.02 M Tris–HCl (pH 7.4) under 1 atm of H_2 at 30° for 1 h. After centrifugation (20 min at 37 000 × *g*) the brown supernatant was put on a DEAE-cellulose column (Whatman DE52), and the proteins were eluted stepwise under strictly anaerobic conditions with 0.0, 0.065, 0.09 and 0.4 M $MgCl_2$ in 0.02 M Tris–HCl (pH 7.4). The fraction containing the hydrogenase was eluted together with the Fe–Mo protein of the nitrogenase system with 0.065 M $MgCl_2$ and subsequently was subjected to a heat treatment under H_2 at 60° for 10 min. After centrifugation, the supernatant was concentrated by ultrafiltration (Amicon Diaflo membrane UM10) under H_2 and chromatographed on a Sephadex G-100 or G-200 column. The fractions containing the hydrogenase activity were colorless or slightly greenish. This procedure effected a purification up to 40-fold.

Ferredoxin and other chemicals

In determining the K_m value for ferredoxin, a sample of doubly crystallized pure *C. pasteurianum* ferredoxin obtained from Dr. W. H. Orme-Johnson was employed. The activation energy for H_2 evolution was determined with ferredoxin which was partially purified in the following way: ferredoxin from the crude extract passed through the DE52 column remained adsorbed after elution of most of the proteins with 0.09 M $MgCl_2$. The ferredoxin was eluted with 0.4 M $MgCl_2$, treated for 10 min at 60° and passed through a Sephadex G-25 column after centrifugation.

Cylinder gases were freed from O_2 by passing them through a Deoxo unit for H_2 or through BTS catalyst (BASF) at 100° for N_2. Pure $^2H_2$ and ^2H_2O were obtained from Bio-Rad. All other materials used were of A.R. grade.

Assays

In all assays Tris–HCl was used as buffer at pH 7.4 and 8.1, and phosphate buffer at pH 7.0.

H_2 evolution was measured with a Gilson Volumometer employing respirometer vessels of 10 ml volume carrying 1 atm of N_2. The main compartment contained the enzyme (20–100 μl), the electron carrier and buffer; the side arm always contained 0.2 ml 0.2 M dithionite, which was added at zero time and made up a total liquid volume of 1 ml.

Methyl viologen reduction was measured in anaerobic cuvettes (V = 3.1 ml, 10-mm light path) capped with a rubber stopper and provided with the indicated H_2 pressures without any inert gas. 2 ml of a methyl viologen solution (concentration designated on specific experiments) was present during preincubation at 26°. The enzyme was injected through the stopper and the reduction was followed spectrophotometrically at 700 nm (ε = 2.12 $mM^{-1} \cdot cm^{-1}$ at pH 7.0, 2.36 at pH 8.1 as measured by complete reduction with dithionite). $H_2O/^2H_2$ exchange was carried out in 31-ml reaction vessels containing 2–3 ml buffer and $^2H_2$ at indicated pressures. The vessels were shaken at 100 reciprocal cycles/min, and the reaction was followed mass spectrometrically at 30°.

Disc gel and preparative electrophoresis

For the determination of isoenzymes, gels (0.6 cm × 6 cm) with 7.5% acrylamide (pH 8.9) were used with spacer gels. Preparative gel electrophoresis was performed with a slab apparatus under the same conditions. All samples were run with dithionite to maintain anaerobic conditions. The gels were stained for proteins with Coomassie blue and for activity by inserting them into a solution of methyl viologen saturated with H_2 (ref. 3).

Calculations

All rates are expressed in nmoles H_2 evolved or used per min per mg of protein. For H_2 evolution and methyl viologen reduction the initial rates were recorded, whereas rates were calculated for the $H_2O/^2H_2$ exchange from first order rate constants, which were obtained from the disappearance of $^2H_2$ according to:

$$k = \frac{2.303}{t} \log \frac{[^2H_2]_0}{[^2H_2]_0 - [^2H_2]}$$

in which subscript o denotes initial concentrations and t is the reaction time in min. The rate then was calculated from:

$$v = k \frac{p^2H_2}{760} \cdot \frac{273}{T} \cdot \frac{V_g}{22\,414} \cdot \frac{10^9}{m}$$

in which $p^2H_2 = {}^2H_2$, pressure in torr; T, ambient temperature in °K; V_g, gas volume in cm^3; m, protein content in mg. For all assays we estimated an average deviation of ± 5% per single measurement.

RESULTS

Isozymes

Isozymes were separated by disc gel electrophoresis[3]; 50 μl (approx. 1 mg) of crude extract was used for separation at 4 mA per tube for 3 h. Fig. 1 shows the activity stain after different times of staining. At least four distinct bands were obtained with

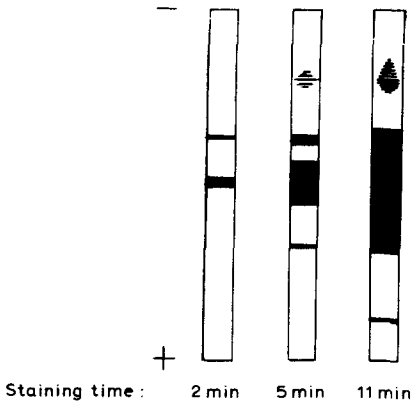
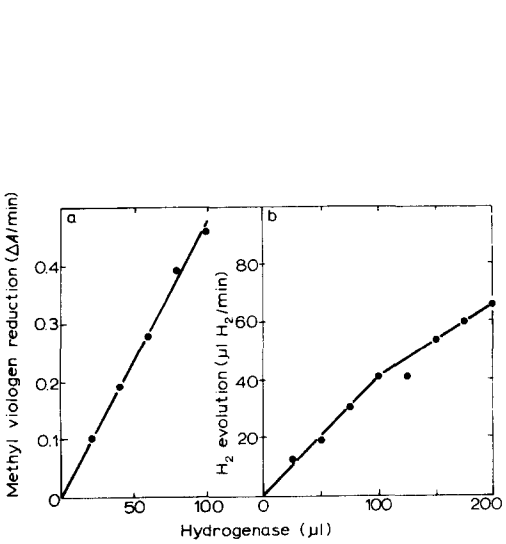


Fig. 1. Isozymes of hydrogenase as indicated by "activity staining" with methyl viologen and H_2.

R_F values of 0.37, 0.50, 0.67 and 0.88. After further purification, only a single activity-stain band was found. However, the R_F value differed; it was 0.66 or 0.50 after the purification described above and 0.50 or 0.40 after purification by preparative electrophoresis. These data confirm the results of KIDMAN *et al.*[3] that hydrogenase exists in various isozyme forms which are interconvertible.

The influence of diffusion

The dependence of the activity on the hydrogenase concentration was determined for all three assays. Fig. 2 shows that in the range tested, the activity increased linearly with enzyme concentration for methyl viologen reduction. H_2 evolution



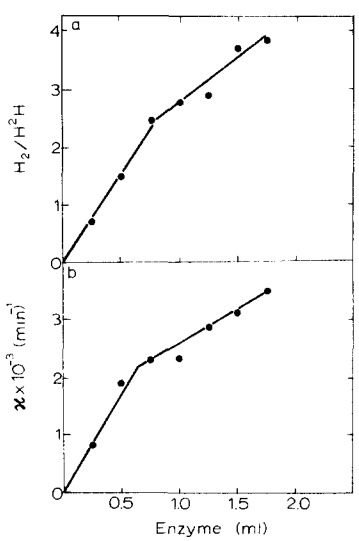


Fig. 2. Dependence of methyl viologen reduction (pH 7.0, 10 mM methyl viologen, pH_2 = 740 torr) and H_2 evolution (pH 7.0, 12 mM methyl viologen) on the concentration of hydrogenase.

Fig. 3. Effect of hydrogenase concentration on initial H_2/H^2H ratio and rate of $H_2O/^2H_2$ exchange (30°, pH 7.4, p^2H_2 = 740 torr); see *Calculations* for description of k.

however seemed to be diffusion limited for activities greater than 40 μl H_2 evolved per min. A limitation also can be seen for $H_2O/^2H_2$ exchange with samples of k greater than $2 \cdot 10^{-3}$ min^{-1} (Fig. 3b). This curve is roughly paralleled by the initial H_2/H^2H ratio in the gas phase (Fig. 3a). At O concentration the H_2/H^2H ratio extrapolates to O, a fact confirmed by using systems of very low activity. A similar dependence of the initial $^2H_2/H^2H$ ratio for the $^2H_2O/H_2$ exchange had been observed previously by TAMIYA AND MILLER[1]. However their extrapolation led to $^2H_2/H^2H = 0.19$ at O hydrogenase concentration. All further experiments were performed in ranges not limited by diffusion.

Comparison of velocities

The rates of methyl viologen reduction (10 mM methyl viologen; $pH_2 = 740$ torr), H_2 evolution (12 mM methyl viologen) and $H_2O/^2H_2$ exchange ($pH_2 = 740$ torr) were compared for various samples of hydrogenase. The ratio of rates differed somewhat with the samples and was on the average 0.8:1:0.5. Thus, all three reactions gave similar specific activities.

Activation by molybdenum

No activation of H_2 evolution was effected by addition of Na_2MoO_4. As SHUG *et al.*[4] had found activation of hydrogenase by solid MoO_3, we tried the addition of MoO_3, solubilized in the following way: MoO_3 was dissolved at 100° in 1% H_2O_2 yielding a yellow solution of permolybdic acid, which then was titrated with solid dithionite to remove excess H_2O_2 until the solution showed a slightly green color. Refluxing for several hours under aerobic conditions destroyed the excess dithionite. If small amounts of this solution (to give a total molybdenum concentration of 1 mM) were added to the enzyme, the color changed from yellow → blue → green → brown during 1 h. The same color changes occurred when the permolybdic acid was treated with an excess of dithionite; apparently the molybdenum is reduced by hydrogenase, possibly to the +5 or +4 state. After addition of MoO_3 in this form, the enzyme samples often showed increased activities, the increases depending upon the stage of enzyme purification (Table I).

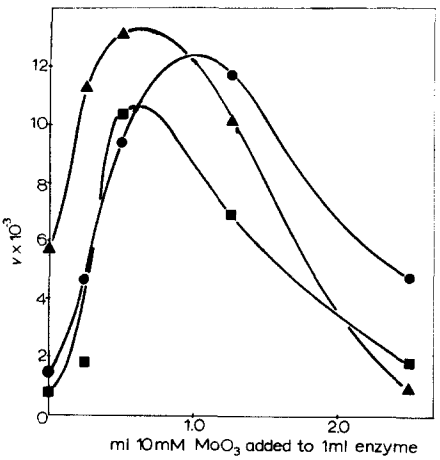
No further increase in molybdenum activation was obtained after passing the heated extract through Bio-Gel P-100 instead of through Sephadex as the last purification step. If the enzyme was passed through a Chelex column, it immediately precipitated and lost all activity; it left a brown band on the column which could not be eluted with strong acids. Activity could not be restored by adding MoO_3 and/or $FeSO_4$.

TABLE I

ENHANCEMENT OF METHYL VIOLOGEN REDUCTION BY ADDED MoO_3

0.1 ml 10 mM molybdenum was added to 1 ml of enzyme.

Purification stage	*% Increase in methyl viologen reduction*
Crude extract	0
DE52	0–20
Heated extract	0–40
Sephadex G-100	40–200



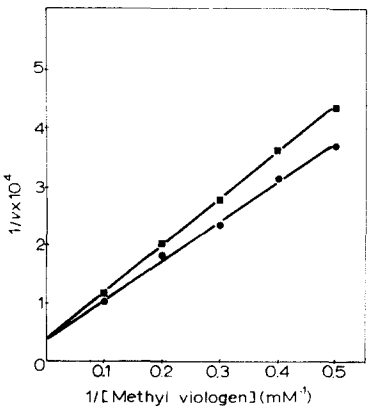
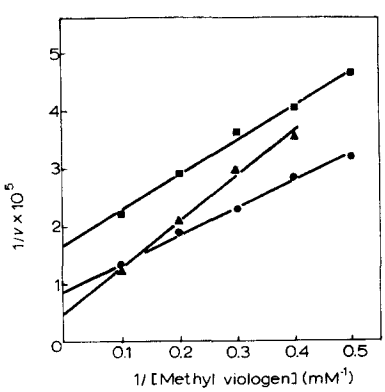


Fig. 4. Effect of varying MoO_3 concentration on H_2 evolution (▲, pH 7.0, 12 mM methyl viologen, 30°), methyl viologen reduction (●, pH 7.4, 10 mM methyl viologen, pH_2 = 740 torr, 26°), and $H_2O/^2H_2$ exchange (■, pH 7.0, p^2H_2 = 740 torr, 30°). Where v appears in this and subsequent figures it is expressed as nmoles H_2 evolved or used/min per mg of protein (see *Calculations*).

Fig. 5. Double reciprocal plots for methyl viologen reduction without (■) and with (●) 0.05 ml, 10 mM MoO_3 added to 1 ml enzyme (pH_2 = 740 torr, pH 7.0, 26°).

These observations suggest that molybdenum is an easily dissociable part of the enzyme.

Added MoO_3 affected the rate of all three reactions in a similar way (Fig. 4): at low concentrations it acted as an activator, whereas at high concentrations it inhibited. Na_2MoO_4 also proved inhibitory. The concentration of MoO_3 optimal for



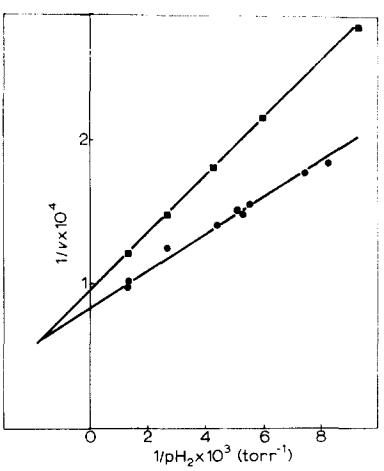
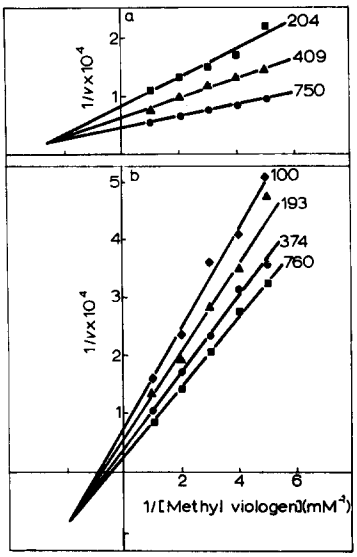


Fig. 6. Double reciprocal plots for methyl viologen reduction (pH 7.0, pH_2 = 740 torr, 26°) showing the effects of various concentrations of MoO_3 added to 1 ml of enzyme: ▲, 0.4 ml 10 mM MoO_3; ●, 0.8 ml 10 mM MoO_3; ■, 1.2 ml 10 mM MoO_3. The enzyme without added MoO_3 showed no detectable activity.

Fig. 7. Double reciprocal plots for methyl viologen reduction as influenced by the pH_2 without (■) and with (●) 0.05 ml 10 mM MoO_3 added to 1 ml enzyme (pH 7.0, 10 mM methyl viologen, 26°).



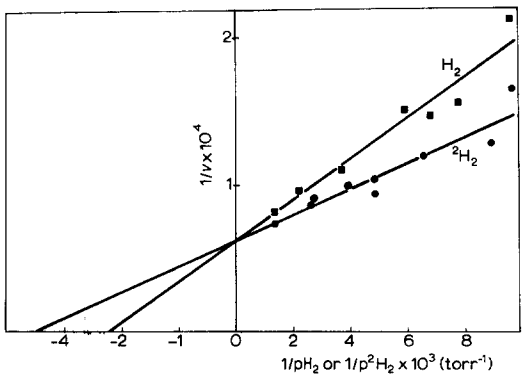


Fig. 8. Double reciprocal plots for methyl viologen reduction at pH 7.0 (a) and 8.1 (b) with different pH_2's (the numbers at the lines give the pH_2 in torr).

Fig. 9. Double reciprocal plots for methyl viologen reduction with H_2 and $^2H_2$ (pH 7.0, 10 mM methyl viologen).

methyl viologen reduction was somewhat higher than for the other reactions, but this may only have reflected the fact that the pH of the reaction was 7.4 instead of 7.0. Double reciprocal plots for methyl viologen reduction showed the following characteristics: At very low noninhibitory concentrations of MoO_3, the plots of $1/v$ *vs.* 1/[methyl viologen] at $pH_2 = 740$ torr gave straight lines that intersected on the $1/v$ axis (Fig. 5). At higher MoO_3 concentrations, the activation by molybdenum was not apparent because of domination by its inhibitory effect, which appeared to be either of the noncompetitive or uncompetitive type (Fig. 6). If the pH_2 was varied at a constant methyl viologen concentration (same mixture employed in Fig. 5), the data yielded double reciprocal plots in which the straight lines intersected to the left of the $1/v$ axis (Fig. 7).

Kinetics of methyl viologen reduction

The kinetics of methyl viologen reduction were measured at pH 7.0 and 8.1 with methyl viologen concentration as a variable and with different levels of H_2 supplied as fixed substrate. Double reciprocal plots (Figs. 8a, 8b) showed intersecting patterns at both pH's. The kinetic parameters varied with pH and pH_2 as shown in Table II.

Methyl viologen reduction by H_2 and $^2H_2$

Kinetic experiments were performed to follow the reduction of 10 mM methyl viologen (pH 7.0) with H_2 and $^2H_2$ at various pressures. A double reciprocal plot (Fig. 9) showed that $^2H_2$ supports faster reduction, because $^2H_2$ has the lower K_m value (143 torr for $^2H_2$, 227 torr for H_2). The solubility of H_2 in water is 15.6 cm^3 H_2/l water at 1 atm and 25° (ref. 6). Assuming linearity of solubility with pressure, 227 torr

TABLE II

KINETIC PARAMETERS FOR METHYL VIOLOGEN REDUCTION

pH	pH_2	K_m (*mM*)	V_{max}* ($\times 10^{-4}$)
8.1	204	2.94	1.21
	409	2.70	1.60
	750	2.23	2.22
7.0	100	10.5	1.25
	193	13.9	1.82
	374	16.1	2.85
	760	21.0	3.90

* All velocities, as indicated in the text, are expressed as nmoles H_2 evolved or used/min per mg protein.

would give a K_m value of 0.2 mM for H_2. No solubility data for $^2H_2$ were available, but the lower K_m value for $^2H_2$ could be attributable to greater solubility of $^2H_2$ than of H_2. However, the large difference in K_m more probably arises from the higher affinity of $^2H_2$ for the enzyme. Assuming that equal orbitals are involved in the bonding, the difference in zero-point energies favors greater stability for the E–$^2H_2$ complex than for the E–H_2 complex.

Activation energies for H_2 evolution

The apparent activation energies were compared at pH 7.0 for the four substrates ferredoxin, methyl viologen, 100% H_2O and 90% ^2H_2O. From Fig. 10 the temperature coefficients recorded in Table III were obtained. Within experimental error, the apparent activation energies were the same for all reactions. It should be noted that no change in activation energy is apparent at 18° unlike the response of many enzymes. The 4 times slower evolution of $^2H_2$ than H_2 was not surprising, as most reactions proceed more slowly in ^2H_2O than in H_2O (ref. 7). The K_m values for H_2 evolution at pH 7.0 were 64 μM for ferredoxin and 4.4 mM for methyl viologen as substrates.

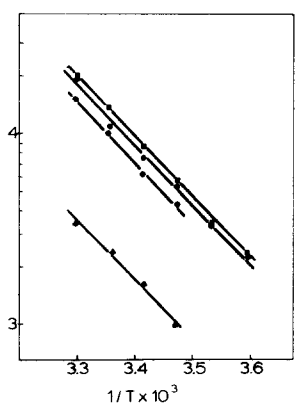


Fig. 10. Activation energies for H_2 and $^2H_2$ evolution with methyl viologen or ferredoxin as carrier (pH 7.0): ■, H_2 evolution, ferredoxin as carrier (0.6 mg); ●, H_2 evolution, methyl viologen as carrier (17 mM); ◆, H_2 evolution, methyl viologen as carrier (12 mM); ▲, $^2H_2$ evolution, methyl viologen as carrier (12 mM, 90% ^2H_2O).

TABLE III

ACTIVATION ENERGIES FOR HYDROGEN EVOLUTION

Gas evolved	*Carrier*	*Carrier concn.*	*Activation energy (kcal/mole)*
H_2	Ferredoxin	0.6 mg/ml	14.6
H_2	Methyl viologen	17 mM	14.5
H_2	Methyl viologen	12 mM	14.6
$H^2H + {}^2H_2$	Methyl viologen	12 mM	14.8

Properties of purified hydrogenase

Small amounts of about 80% pure hydrogenase were obtained by preparative gel electrophoresis. During electrophoresis the enzyme lost most of its activity; H_2 evolution with 12 mM methyl viologen as carrier indicated an activity of $v = 2.9 \cdot 10^{-3}$ nmoles H_2/min per mg protein. Analysis showed 0.0–0.2 molybdenum per molecule, assuming a molecular weight of 60 000 (refs. 3, 8). It is possible that this low molybdenum content resulted from a loss of molybdenum during electrophoresis. This loss could have contributed to the partial inactivation during electrophoresis. These hydrogenase samples did not catalyze any oxidation of benzidine by H_2O_2; this shows that the hydrogenase does not contain iron bound to porphyrin.

DISCUSSION

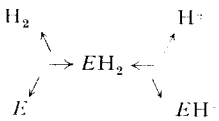
The data in Fig. 3a shows that the initial H_2/H^2H ratio depends strongly on the activity of the system. This agrees with observations on the hydrogenase of *Proteus vulgaris*[1] but disagrees with the results from *Desulfovibrio desulfuricans*[9] and *Scenedesmus obliquus*[10]; in these organisms the initial $^2H_2/H^2H$ ratio was found to be independent of the activity. In contrast to the results of TAMIYA AND MILLER[1], our data indicate that the ratio extrapolates to zero for zero hydrogenase concentration. In terms of the mechanism of RITTENBERG AND KRASNA[2] this supports formation of a hydride which cannot exchange with water.

The similar rates of the three reactions (H_2 evolution, methyl viologen reduction, $H_2O/^2H_2$ exchange), together with their similar patterns of activation and inhibition by MoO_3, indicate that the $H_2O/^2H_2$ exchange is part of the mechanism of H_2 evolution and methyl viologen reduction. A more detailed insight into the nature of the active sites is given by investigations concerned with the influence of MoO_3 on the reduction of methyl viologen. The intersection of the double reciprocal plots on the $1/v$ axis with varying methyl viologen concentrations (Fig. 5) and their intersection to the left of the $1/v$ axis with varying pH_2 (Fig. 7) indicate that two different sites exist for H_2 and methyl viologen activation. Molybdenum is bound to the methyl viologen site and can dissociate only if no methyl viologen is bound. It is not necessary that molybdenum dissociates from the enzyme during each cycle.

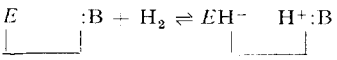
The results showing the activation of the $H_2O/^2H_2$ exchange by MoO_3 indicate that molybdenum is also essential for H_2 activation, although it is not present at the H_2 site. We assume that an electron transfer bridge exists connecting the H_2 site and the molybdenum site. Molybdenum then could act as an electron sink, or as a con-

nection to an electron sink, which takes up one electron from hydrogen and thus makes the cleavage of H_2 possible. Probably each site binds its substrate without influencing the other.

The intersecting patterns (Fig. 8) for methyl viologen reduction are expected because of the reversibility of the sequence of H_2 activation,



and because the product H^+ always is present in the reaction medium. TAMIYA AND MILLER[1] proposed an asymmetrical H_2 site for accepting a proton and a hydride ion arising from the heterolytic cleavage of H_2:



They suggested that site E is a metal, because many metal ions in solution are capable of activating H_2 by the formation of a hydride[11]. In terms of this mechanism, our results concerning the activation by molybdenum lead to the suggestion that the hydride formed might be stabilized by the transfer of an electron to the molybdenum. Thus Mo^{6+} could be reduced to Mo^{5+}, as arbitrarily assumed in Fig. 11a in the mechanism for $^2H_2O/H_2$ exchange. The proton at site X is postulated to undergo rapid exchange with the solvent to form H^2H.

On the other hand, if we propose that electron transfer to molybdenum is an essential step in the isotopic exchange, a mechanism involving a homolytic cleavage of H_2 also agrees with the experimental data under certain conditions (Fig. 11b). It must be assumed that the molybdenum can pick up only one electron. Then only one H atom could form a stable X–H bond, whereas the other H atom would exchange with $^3H_3O^+$. This mechanism agrees with experimental results only if the rates for the elementary steps are in the sequence: proton exchange > H^2H release > electron transfer. If the electron transfer were faster than the H^2H release, both H atoms should exchange and produce an initial $^2H_2/H^2H$ ratio > 0. If these rates were about equal,

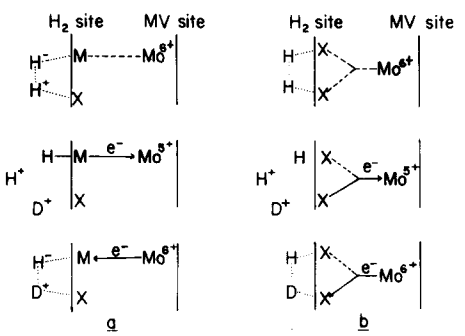


Fig. 11. Possible mechanisms for $^2H_2O/H_2$ exchange: a with heterolytic H_2 cleavage; b with homolytic H_2 cleavage. M is assumed to be a metal site; - - - indicates possible routes of electron transfer. MV is methyl viologen.

some $^2H_2$ in addition to H^2H should be formed. This may occur with hydrogenases from other organisms[1,9,10].

H_2 evolution from different substrates requires the same activation energy (Fig. 10). This indicates that the rate limiting step is monomolecular, either a conformational change of the enzyme, or electron transfer within the hydrogenase,and would agree with mechanism of Fig. 11b.

ACKNOWLEDGMENTS

We thank Dr. W. H. Orme-Johnson for a sample of pure ferredoxin and Dr. W. W. Cleland for helpful advice and discussion of the kinetic problems. This work is published with the approval of the Director of the Wisconsin Agricultural Experiment Station and was supported in part by grant GB-483 from the National Science Foundation and by research grant AI-00848 from the Division of Research Grants, National Institutes of Health.

REFERENCES

1 N. TAMIYA AND S. L. MILLER, *J. Biol. Chem.*, 238 (1963) 2194.
2 D. RITTENBERG AND A. I. KRASNA, *Discussions Faraday Soc.*, 20 (1955) 185.
3 A. D. KIDMAN, B. A. C. ACKRELL AND R. N. ASATO, *Biochim. Biophys. Acta*, 159 (1968) 185.
4 A. L. SHUG, P. W. WILSON, D. E. GREEN AND H. R. MAHLER, *J. Am. Chem. Soc.*, 76 (1954) 3355.
5 D. W. S. WESTLAKE AND P. W. WILSON, *Can. J. Microbiol.*, 5 (1959) 617.
6 J. W. MELLOR, *Comprehensive Treatise on Inorganic and Theoretical Chemistry*, Vol. 1, 1957, p. 302.
7 L. MELANDER, *Isotope Effects on Reaction Rates*, Ronald Press, New York, 1960.
8 D. KLEINER AND R. H. BURRIS, *Federation Proc.*, 29 (1970) 894, abs.
9 A. I. KRASNA, E. RIKLIS AND D. RITTENBERG, *J. Biol. Chem.*, 235 (1960) 2717.
10 H. HARTMAN AND A. I. KRASNA, *J. Biol. Chem.*, 238 (1963) 749.
11 J. HALPERN, *Advan. Catalysis*, 11 (1959) 301.