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THE KINETICS OF METHYL VIOLOGEN OXIDATION AND REDUCTION BY THE HYDROGENASE FROM *CLOSTRIDIUM PASTEURIANUM*

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Summary

A mechanism for the reduction and oxidation of methyl viologen by *Clostridium pasteurianum* hydrogenase (hydrogen:ferredoxin oxidoreductase, EC 1.12.7.1) is proposed. Double reciprocal plots for methyl viologen reduction and oxidation at pH values 7.0—9.85 are linear, and the plots for reduction and oxidation are intersecting. Such data are consistent with a mechanism in which the H_2 and one methyl viologen bind (either in order or randomly) with subsequent reduction and release of the methyl viologen. A second methyl viologen then is bound, reduced and released. Comparison of the calculated K_{eq}' with the Haldane expression in which both methyl viologens react at the same rate show a large difference. This difference indicates that the two methyl viologens react at different rates. Addition of oxidized electron carriers inhibits the hydrogen-deuterium exchange reaction (i.e., the exchange of protons between H_2 and ^2H_2O). CO reversibly inhibits methyl viologen reduction and is competitive vs. H_2. O_2 acts as an irreversible inhibitor.

Introduction

The iron-sulfur enzyme hydrogenase (Hydrogen:ferredoxin oxidoreductase, EC 1.12.7.1) can catalyze the reversible reduction of various electron acceptors with H_2 [1—3]. The enzyme also will catalyze a reversible hydrogen-deuterium exchange between H_2 and ^2H_2O [4]. The mechanism proposed for the activation of H_2 in the exchange reaction involves a heterolytic split yielding a proton and a hydride · enzyme complex [4,5]. In the reduction of electron acceptors

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Abbreviations: MV^{+2}, oxidized methyl viologen; $MV^{+\cdot}$, reduced methyl viologen; DCPIP, 2,6-dichlorophenol-indophenol.

the enzyme displays properties like those of the H_2 electrode [6]. For methyl viologen reduction, intersecting double reciprocal plots have indicated that H_2 activation is reversible [7]. We investigated the mechanism for the reduction and oxidation of methyl viologen.

Experimental

Materials

Methyl viologen obtained from Koch-Light Laboratories, Ltd., Colnbrook, U.K., was recrystallized twice from methanol/acetone. Elemental analysis showed: C, 56.04%; H, 5.50%; N, 10.88%; Cl, 27.81% (calculated values: C, 56.25%; H, 5.47%; N, 10.94%; Cl, 27.34%.) Buffers were obtained from Sigma Chemical Co. and sodium dithionite from Matheson Coleman and Bell. Deuterium gas was from Bio-Rad Laboratories and CO from Matheson Gas Products.

Any traces of O_2 in H_2, N_2 or Ar were removed by passage of the gas through a 4-ft. long column of BASF catalyst R3-11 (Chemical Dynamics Corp., South Plainfield, N.J.) heated to 160°C. CO was shaken with alkaline pyrogallol before use, and reduced methyl viologen was used to remove O_2 from deuterium gas.

Preparation of hydrogenase

Hydrogenase from *Clostridium pasteurianum* W5 was purified by polyethylene glycol precipitation (PEG 6000, Union Carbide, Corp.), chromatography on two successive DEAE cellulose columns followed by gel filtration on Sephadex G-100 and chromatography on hydroxyapatite. Procedures followed in these steps were similar to those already published [1,8]. Kinetic studies of methyl viologen oxidation and reduction at pH values 7.0, 8.0, 9.0 and 9.85 and comparison of reaction rates with various substrates were run with the same highly purified preparation. In other studies, the hydrogenase used was purified through at least the DEAE cellulose steps. These preparations contained no ferredoxin or Fe-protein of nitrogenase and little or no MoFe-protein of nitrogenase.

Methods

The method of Lowry et al. [9] was used for protein assays.

H_2 evolution from reduced methyl viologen was followed manometrically. All-glass Gilson volumometers equipped with 15-ml Warburg flasks (sidearms capped with serum stoppers) were evacuated and flushed three times with N_2. 1.8 ml of buffered methyl viologen and 0.2 ml of sodium dithionite solution (prepared under N_2 in H_2O at concentrations specified in Results section) were injected through the stoppered sidearm into the flask. The flasks were equilibrated by shaking at 150 reciprocal cycles per min (3—4 cm stroke) at 25°C for 15 min. Without removing the flasks from the bath the enzyme was injected to initiate the reaction. Microliter volumes of enzyme solution were used in all experiments except for the experiment represented in Fig. 1. In this case the final volume was adjusted to equal 2.0 ml. The pH was rechecked after the assay. The assay for reduction of Methylene Blue was prepared similarly.

Reduction of methyl viologen, FAD, FMN, NAD^+, $NADP^+$ or 2,6-dichloro-

phenolindophenol (DCPIP) was followed continuously on a Gilford spectrophotometer. 2.00 ml of assay medium was pipetted into a 3.1 ml (1-cm light path) anaerobic cuvette that then was capped with a rubber vaccine stopper. The cuvettes were attached to a manifold via 20-gauge hypodermic needles and evacuated 1 min with shaking; this was followed by a flush with H_2. The process was repeated twice more. The cuvettes were equilibrated to the desired H_2 pressure at 25°C. The reaction initiated by injection of the enzyme was followed spectrophotometrically at 560 nm for methyl viologen, 450 nm for FAD and FMN, 340 nm for NAD^+ and $NADP^+$, and 600 nm for DCPIP. The pH was measured before and after the reaction.

The extinction coefficient at 560 nm for methyl viologen was determined experimentally by reduction with excess sodium dithionite. Extinction coefficients for NADH and NADPH (6.2 $mM^{-1} \cdot cm^{-1}$ at 339 nm), for FMN and FAD (12.2 and 11.3 $mM^{-1} \cdot cm^{-1}$ at 450 nm, respectively) and for oxidized DCPIP (20.6 $mM^{-1} \cdot cm^{-1}$ at 600 nm) were taken from the literature [10].

A mixture of *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES), tricine, serine and glycine (50 mM each) was used in the study of variation with pH of kinetic parameters for H_2 evolution and methyl viologen reduction. A 2(*N*-morpholino)ethane sulfonic acid (MES), tricine and glycine mixture (25 mM each) also was used in study of the methyl viologen reduction. Similar values for kinetic parameters of methyl viologen reduction were obtained using either the three or four buffer system. Data from the three buffer system were statistically better and are presented in this paper. When other buffer systems were used they are specified in the Results section.

Hydrogen-deuterium exchange was followed by analysis of the gas phase over the reaction mixture for masses 2, 3, and 4 with a Consolidated-Nier isotope ratio mass spectrometer. The reaction was run in 9.5-ml stoppered serum bottles with 1.0 ml of 20 mM Tris/Cl (pH 8.0) and 101 kilo Pascals (i.e., 760 mm Hg) $^2H_2$. Assay vessels were shaken at 350 reciprocal cycles per min (1-cm stroke at 25°C). Gas samples were removed with a syringe for analysis.

Determination of H_2 and $^2H_2$ solubility. The solubilities of $^2H_2$ and H_2 in water were determined manometrically. To compensate for pressure changes from water vapor, the solubility was determined by difference in gas uptake between a reference flask containing 1.0 ml and a sample flask with 3.0—4.0 ml H_2O. Identical 15-ml flasks with the indicated amounts of H_2O were attached to an all-glass Gilson volumometer and evacuated with shaking at 25°C. Without shaking, the gas was admitted and allowed to remain 60 s before the first reading was taken and shaking restarted. Readings were taken every 2—3 min until equilibration was achieved.

Analysis of data. Kinetic data were analyzed with the computer programs of Cleland [11,12]. In studying the effect of pH on kinetic responses, two or more values were obtained for each kinetic parameter and their weighted means and their standard errors were calculated according to Morrison and James [13].

Results

The extinction coefficient for reduced methyl viologen

Reduction of 0.1 mM methyl viologen with excess sodium dithionite yielded

TABLE I

OXIDATION AND REDUCTION OF VARIOUS SUBSTRATES BY HYDROGENASE AT pH 8, 25°C

	Concentration (mM)	pH_2 (kilopascals)	Rate (mmol reduced/(min · mg))
Reducible substrate			
Methyl viologen	2.5	93.3	1.66
NAD^+	2.0	101.3	0.00
$NADP^+$	2.0	101.3	0.00
FMN	0.2	101.3	1.64
FAD	0.2	101.3	1.30
Methylene blue	2.0	98.6	0.96
DCPIP	0.1	101.3	0.28
Oxidizable substrate		(mmol H_2 evolved/(min · mg))	
Sodium dithionite	20.0	0.00	
Reduced methyl viologen	1.4	0.20	
Reduced Methylene Blue	2.0	0.00	

the following extinction coefficients at 560 nm: pH 5.0, 1.08 $mM^{-1} \cdot cm^{-1}$; pH 6.0, 3.86 $mM^{-1} \cdot cm^{-1}$; pH 7.0, 7.71 $mM^{-1} \cdot cm^{-1}$; pH 8.0, 8.05 $mM^{-1} \cdot cm^{-1}$; pH 9.0, 8.03 $mM^{-1} \cdot cm^{-1}$; pH 10.0, 7.89 $mM^{-1} \cdot cm^{-1}$.

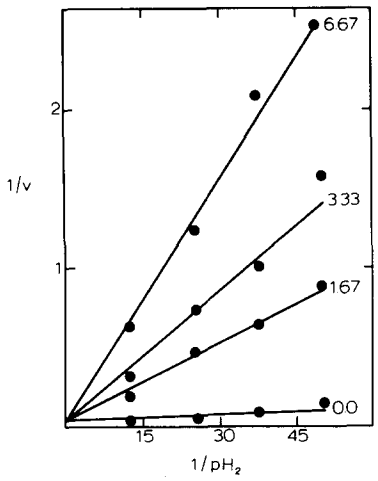
The observed reduction in extinction coefficient below pH 7 may be attributed to production of a colorless dihydropyridyl derivative [14]. Transfer of aliquots of methyl viologen reduced at pH 7 to pH 5 or 6 was accompanied by losses of absorbance in excess of that expected from dilution.

Reaction with various substrates

The rates of oxidation and reduction of various substrates by hydrogenase are indicated in Table I. Neither NAD^+ nor $NADP^+$ were reduced. This contrasts with observations of reduction catalyzed by hydrogenase from *Hydrogenomonas ruhlandii* [15]. Although it is a powerful reducing agent, dithionite failed to promote H_2 evolution without the addition of electron carriers.

Kinetics of oxidation and reduction of methyl viologen

The rate of evolution of H_2 from reduced methyl viologen increased linearly with increasing enzyme concentration to 40 or 50 μl H_2 evolved per min, as observed by Kleiner and Burris [7]. At a shaking rate of less than 120 cycles per min (3—4-cm reciprocating strokes) H_2 evolution was diffusion-limited; assays were run routinely at 150 cycles per min. The relationship of enzyme concentration to activity was checked at all substrate concentrations and pH values. Only those levels of enzyme which gave rates in the linear non-diffusion-limited range were used. Dithionite concentrations of 5, 25 and 75 mM gave the same rates of H_2 evolution in an assay medium containing 0.5 mM methyl viologen in 150 mM Tris/Cl (pH 8.2). Activity was reduced by only 8% when H_2 (101 kilopascals) was used in place or Ar or N_2. Maximal activity occurred between 10 and 20 mM methyl viologen and there appeared to be some substrate inhibition at 30 mM methyl viologen.



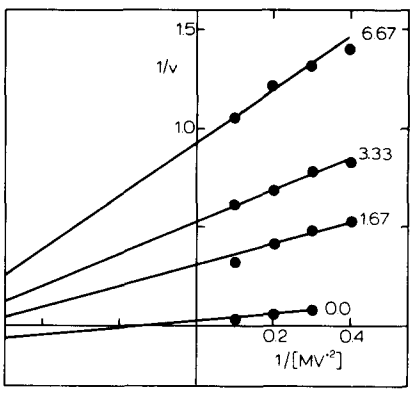


Fig. 1. Double reciprocal plot for methyl viologen reduction at pH 8.1 in the presence of fixed levels of CO (indicated on right-hand margin in kilopascals) at varied levels of H_2 (in kilopascals). Assayed in 75 mM methyl viologen, 20 mM Tris/Cl; v expressed in μmol methyl viologen reduced/(min · mg protein).

Fig. 2. Same as in Fig. 1 except methyl viologen is variable substrate (in mM) and pH_2 is fixed at 73.3 kilopascals.

With increasing levels of enzyme the reduction activity was linear to 5 μmol methyl viologen reduced per min. Saturation with methyl viologen occurred above 50 mM. At pH 7 or above reduction was linear with time. However, below pH 7 the rate of reduction changed rapidly such that accurate initial velocities could not be obtained. Conversion of reduced methyl viologen to the colorless derivatives may account for the difficulty in obtaining linear initial velocities for reduction by hydrogenase and H_2 below pH 7.

CO reversibly inhibited methyl viologen reduction. Inhibition was competitive vs. H_2 (K_i, 666 pascals CO) at saturating levels of methyl viologen (75 mM) and noncompetitive vs. methyl viologen (K_{is}, 1037 pascals CO; K_{ii}, 113 pasclas CO) at 73.3 kilo pascals H_2 (Figs. 1 and 2).

O_2, in air, inactivated hydrogenase as measured by methyl viologen reduction. Half the activity was lost at 25°C in 2—3 min. No activity remained after 60 min. Evacuation followed by incubation under H_2 restored only a few per cent of the activity, and addition of dithionite did not alleviate the loss.

The double reciprocal plots for methyl viologen reduction with methyl viologen as variable substrate and H_2 as changing fixed substrate, or vice versa, were linear and intersecting. An example is shown in Fig. 3. The double reciprocal plots for H_2 evolution are shown in Fig. 4. Values for K_i and K_m for the substrates of H_2 evolution and methyl viologen reduction at various pH values are listed in Table II. The parameters V/K_m and V are plotted to show any variations with pH (Fig. 5 and 6). V_m/K_{H_2} is essentially pH-independent, whereas V/K_m for oxidized and reduced methyl viologen are highly pH-dependent. A plot of the Haldane expression and calculated K_{eq}' vs. pH presented in the Discussion is shown in Fig. 7.

Isotope studies

The solubility of H_2 at 25°C in H_2O was 16.1 ml gas at STP per l H_2O (rang-

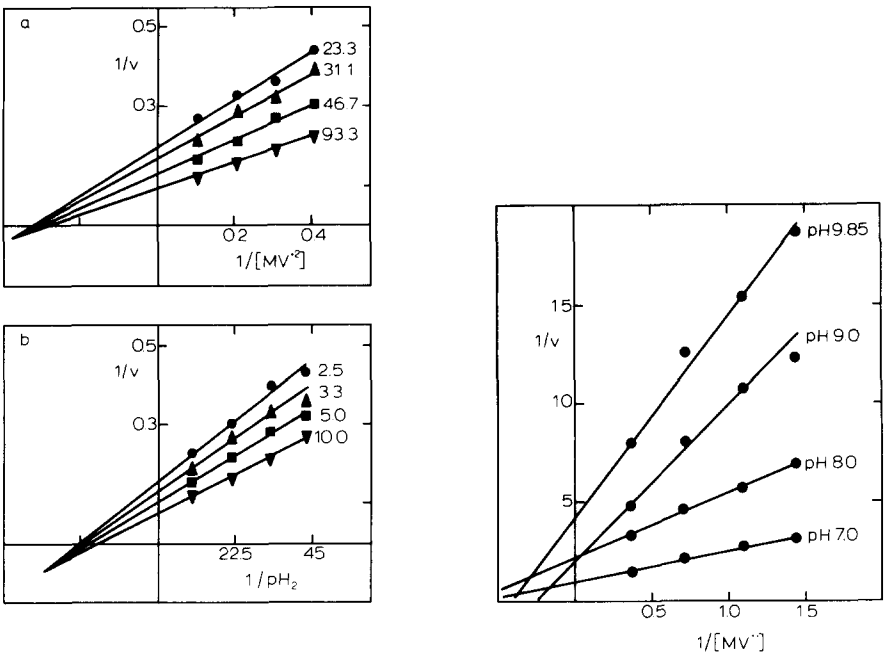


Fig. 3. Double reciprocal plots for methyl viologen reduction at pH 9.0 ($1/v$ in min · mg protein/mmol methyl viologen reduced; $1/[MV^{+2}]$ in mM^{-1}; $1/pH_2$ in megapascals^{-1}). The fixed substrate, H_2, in plot a is noted at the right-hand margin in kilopascals. Similarly MV^{+2} is noted in plot b in mM.

Fig. 4. Double reciprocal plots for H_2 evolution at various pH values ($1/v$ in min · mg protein/mmol H_2 evolved; $1/[MV^{+\cdot}]$ in mM^{-1}).

ing from 15.0 to 17.2 ml gas for eight determinations). This value compares favorably with literature values of 15.6 ml [16] and 17.5 ml [17]. $^2H_2$ solubility was 17.4 ml gas at STP per liter H_2O (ranging from 16.1 to 18.7 ml for 7 determinations). Kleiner and Burris [7] reported that V for methyl viologen reduction at pH 7 in phosphate buffer was the same for $^2H_2$ and H_2, whereas the K_m for $^2H_2$ was only 63% of that for H_2. The same effect was observed in this study at pH 7 in 20 mM phosphate but not at pH 8 in 50 mM Tris/Cl. Since H_2 and $^2H_2$ are similar in solubility, the differing K_m values are not due to solubility effects.

Hydrogen-deuterium exchange was inhibited by addition of either Methylene

TABLE II

K_m AND K_i VALUES FOR OXIDIZED OR REDUCED METHYL VIOLOGEN AND H_2

pH	K_m ($MV^{+\cdot}$) (mM)	K_m (MV^{+2}) (mM)	K_i (MV^{+2}) (mM)	K_m (H_2) (kilopascals)	K_i (H_2) (kilopascals)
7.0	1.56 ± 0.10	15.4 ± 10.5	13.0 ± 4.5	29.1 ± 11.1	24.0 ± 4.4
8.0	1.62 ± 0.10	8.77 ± 1.85	5.60 ± 1.37	50.1 ± 12.8	40.0 ± 8.3
9.0	4.03 ± 0.59	6.19 ± 1.05	2.02 ± 0.49	74.9 ± 11.1	22.3 ± 6.1
9.85	2.19 ± 0.28	1.51 ± 0.98	1.43 ± 0.91	50.1 ± 13.8	44.5 ± 24.1

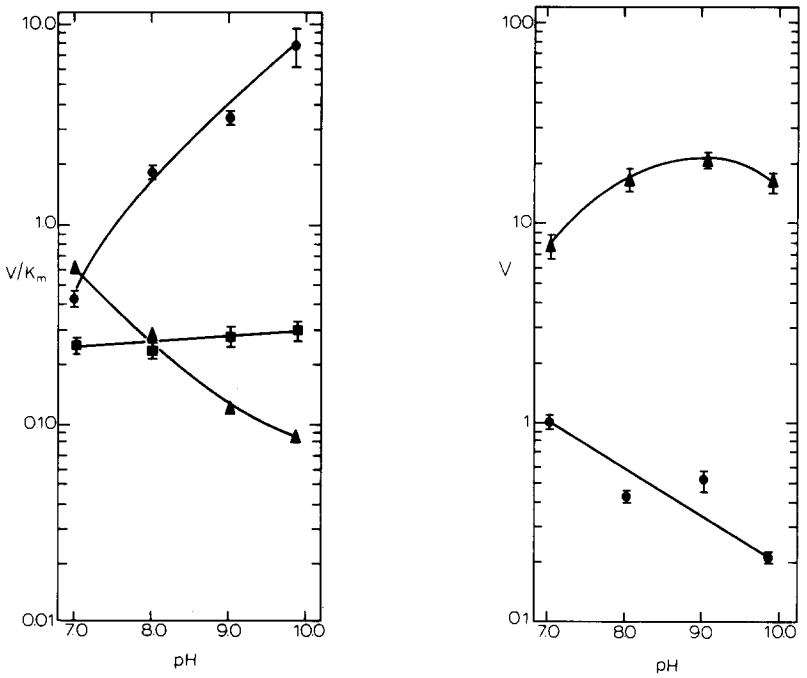


Fig. 5. pH dependence of V/K_m. (V/K ($MV^{+\cdot}$) in mmol H_2 evolved/(min · mg protein · mM methyl viologen), ▲——▲; V/K (MV^{+2}) in mmol methyl viologen reduced/(min · mg protein · mM methyl viologen), ●——●; V/K_m (H_2) in mmol methyl viologen reduced/(min · mg protein · kilopascals H_2), ■——■).

Fig. 6. pH dependence of V for H_2 evolution (V in mmol H_2 evolved/(min · mg protein), ●——●) and methyl viologen reduction (V in mmol methyl viologen reduced/(min · mg protein), ▲——▲).

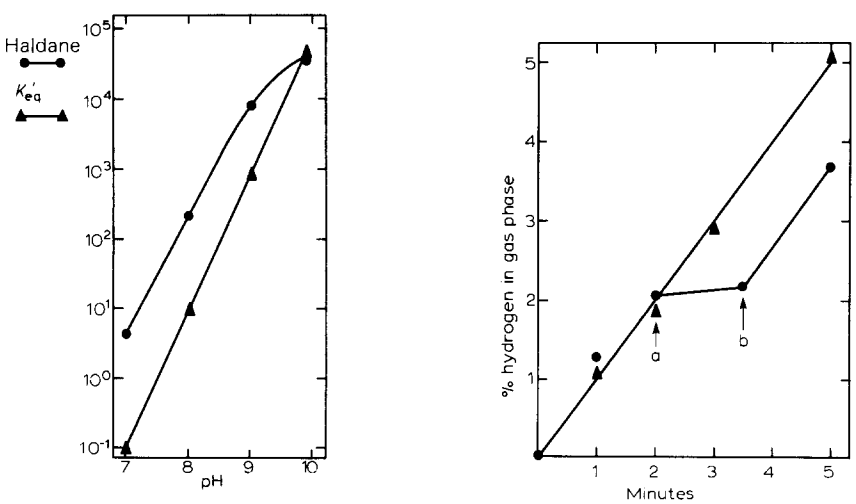


Fig. 7. pH dependence of Haldane expression and calculated K_{eq}' (in atm^{-1}). K_{eq}' calculated using a redox potential of —444 mV for methyl viologen.

Fig. 8. Influence of methylene blue (10 mM) on hydrogen-deuterium exchange. Dye was injected into the reaction vessel at a and was almost fully reduced at b. The upper plot is a control injected only with buffer.

Blue or methyl viologen until the dye was reduced (Fig. 8). Then the exchange reaction resumed its original rate.

Discussion

The overall reaction of hydrogenase-catalyzed reduction of methyl viologen is:

$$H_2 + 2MV^{+2} \rightarrow 2H^+ + 2MV^+.$$

Several features of the kinetic data are notable. The intersecting double reciprocal plots (Fig. 3) indicate that H_2 and MV^{+2} bind sequentially. The order of binding may be ordered or randomly sequential. Absence of curvature in the plots suggests that only one methyl viologen reacts at a time. These data are consistent with the model shown in Fig. 9. This is a Bi Uni Uni Uni Ping Pong mechanism described by Cleland [18]. The concentration C in Cleland's equation has been replaced by B (Fig. 9), because B and C each equals the MV^{+2} concentration. The rate equation is:

$$v = (k_1k_3k_5k_7k_9AB^2 - k_2k_4k_6k_8k_{10}P^2)\, E_t/\text{denominator}$$

in which v is reaction velocity, A, B and P are concentrations of H_2, MV^{+2}, $MV^{+\cdot}$, respectively, and the denominator is that for the Bi Uni Uni Uni Ping Pong mechanism in which C equals B. The expressions for K_m, K_{ia} and V_m are as follows:

$$V_1/K_a = k_1$$

$$V_1/K_b = (k_3k_5k_7k_9)/(k_7k_9(k_4 + k_5) + k_3k_5(k_8 + k_9))$$

$$V_2/K_p = (k_4k_6k_8k_{10})/(k_4k_6(k_8 + k_9) + k_8k_{10}(k_4 + k_5))$$

$$K_{ia} = (k_2k_7k_9(k_4 + k_5))/(k_1(k_7k_9(k_4 + k_5) + k_3k_5(k_8 + k_9)))$$

in which V_1 and V_2 are the maximum velocities in the reduction and oxidation of methyl viologen, respectively; K_b is the K_m for MV^{+2} and K_p is the K_m for $MV^{+\cdot}$ and K_{ia} is the dissociation constant for H_2. The Haldane expression is:

$$K'_{eq} = (k_1k_3k_5k_7k_9)/(k_2k_4k_6k_8k_{10})$$

The Haldane (K'_{eq} is the K_{eq} at a particular pH. In the case in which the rate constants for the reaction of each methyl viologen are the same (i.e., $k_3 = k_7$,

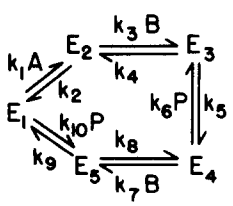


Fig. 9. Model for reduction of methyl viologen by hydrogenase (E_1, E_2, enzyme forms; A, H_2 concentration; B, MV^{+2} concentration; P, $MV^{+\cdot}$ concentration; k_1, k_2, etc.; rate constants).

$k_4 = k_8$, $k_5 = k_9$ and $k_6 = k_{10}$), the Haldane expression is simplified to:

$$K'_{eq} = k_1(k_3k_5)^2/k_2(k_4k_6)^2$$

Expressed in terms of the kinetic parameters the Haldane expression becomes:

$$K'_{eq} = (V_1K_p/V_2K_b)^2/K_{ia}$$

Derivation of K_{ia} for the model shows that it equals twice the experimental value. In calculating the value of the Haldane expression from experimental values for the kinetic parameters this was taken into consideration.

The Haldane expression (K_{eq}') and the $K_{eq}'^*$ (calculated from redox potentials) differ greatly (Fig. 7). Such a difference indicates that the Haldane expression fails to represent the data and that the assumption that rates of reaction of the methyl viologens are equal is incorrect. The $K_{eq}'^*$ was calculated using a potential of −444 mV for methyl viologen at all pH values (Eisenstein and Wang report this value at pH values 7.4, 9 and 11 [19]; Michaelis and Hill [20] and Stombaugh et al. [21] report −446 mV and −445 mV, respectively, at pH 11), and the equation:

$$K'^*_{eq} = \text{antilog}_{10}\left(\frac{nF}{2.3\,RT}\,\Delta E'_0\right)$$

(n equals 2, the number of electrons involved in the complete oxidation of H_2; F, Faraday's constant; R, the universal gas constant; $\Delta E'_o$, the redox potential for methyl viologen). A dependence of the methyl viologen redox potential on concentration has been reported [21]. Extrapolation to infinite methyl viologen concentration from these data gives a value of −443 mV. At lower concentrations the potential is more negative. If the Haldane values are equated to $K_{eq}'^*$ and redox potentials for methyl viologen then are calculated, the values obtained are: pH 7.0, −394 mV; pH 8.0, −403 mV; pH 9.0, −416 mV; pH 9.85, −446 mV. The actual redox potentials would be unlikely to fluctuate through these extremes. The Haldane expression probably fails to equal the calculated $K_{eq}'^*$ at the lower pH values because of differing reaction rates of the two methyl viologens.

The competitive inhibition of CO vs. H_2 during methyl viologen reduction indicates binding of CO to the form of the enzyme which binds H_2. Considering the small size of CO and H_2 molecules, it seems unlikely that binding of CO on a site or sites remote from that of H_2 would cause this type of inhibition. The inhibition constant for CO (666 Pascals, 5.8 μM) is similar to values reported for its inhibition of hydrogenase from *Proteus vulgaris* (18 μM) and *Desulfovibrio desulfuricans* (3 μM). This inhibitor was competitive vs. H_2 in the hydrogen-deuterium exchange reaction [22]. Vs. methyl viologen the inhibition was noncompetitive ($K_{i\,slope}$, 1040 pascals and $K_{i\,intercept}$, 113 pascals). The observations suggest that CO binds near or at the H_2 site.

O_2 also has been shown to inhibit hydrogenase in algae and bacteria [23,24 25]. Fisher et al. [23] observed that in *P. vulgaris* the inhibition was reversed by evacuation or addition of dithionite. Reactivation by evacuation indicated that the inactive enzyme was oxygenated. The inhibition of *C. pasteurianum* hydrogenase by O_2 was not fully reversible in contrast to the inhibition of *P. vulgaris* hydrogenase. Complete inactivation occurred in 60 min (with 50%

loss of activity in the first 2—3 min) and could only be reversed by a few per cent when O_2 was removed by evacuation followed by incubation under H_2. Subsequent addition of dithionite had no effect.

Methylene Blue or methyl viologen will inhibit H-^{2}H exchange at pH 8 until all dye is reduced (Fig. 8). The flux of electrons going to dye reduction at the concentration of dye used is three to four times the flux involved in H-^{2}H exchange. The V_m for H_2 evolution is lower than the V_m for methyl viologen reduction at pH 8, possibly indicating that electron flow to production and release of H_2 may be slower than the oxidation of H_2.

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