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Influence of pN_2 and pD_2 on HD Formation by Various Nitrogenases†

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ABSTRACT: Formation of HD from D_2 has been demonstrated with nitrogenase preparations from *Azotobacter vinelandii*, *Clostridium pasteurianum*, *Klebsiella pneumoniae*, and *Azospirillum* sp. We conclude that the formation of HD from D_2 is a general property of nitrogenases. However, the nitrogenases differ in their K_i values for D_2 (N_2 fixation) and in their rates of catalyzing HD formation; among the nitrogenases tested, *C. pasteurianum* nitrogenase had the lowest activity for formation of HD. When contaminating N_2 was removed from the atmospheres above reaction mixtures, less than 1% of the total electron flux in the system was directed to HD formation; hence, we doubt that N_2-independent HD formation is significant. A working hypothesis is suggested that operates without invoking an N_2-independent reaction for forming HD.

The formation of HD from D_2 by N_2-fixing organisms was discovered in soybean nodules by Hoch et al. (1960). They found this reaction was inhibited by CO and N_2O but enhanced by N_2. Their suggestion that HD was formed from an enzyme-bound diimide moiety may have constituted the first experimental evidence for diimide as an intermediate in biological N_2 fixation.

These observations were expanded by other investigators. Formation of HD from D_2 was observed by Bergersen (1963) in soybean nodules, Dixon (1967) in pea nodules, and Kelly (1968) in nodules of *Medicago lupulina* and *Alnus glutinosa*. Jackson et al. (1968) studied HD formation by crude nitrogenase preparations from *Azotobacter vinelandii* (Av)[1] and confirmed the requirement for N_2 and the inhibition by CO. The facts that MgATP (McNary & Burris, 1962) and a reductant such as ferredoxin (Mortenson, 1964) were required for N_2 fixation had been established, and Jackson et al. (1968) reported that they also were required for formation of HD. Turner & Bergersen (1969), with cell-free extracts from soybean nodule bacteroids, confirmed the work of Jackson et al. (1968). They also reported that the apparent $K_m(N_2)$ for HD formation was much smaller than that for NH_3 production. Kelly (1968, 1969), using crude extracts from *A. vinelandii* and partially purified nitrogenases from *Azotobacter chroococcum* (Ac) and other free-living nitrogen fixers, contrary to others found no enhancement of HD formation by N_2. Vandecasteele & Burris (1970) reported that a small amount of HD was formed by partially purified nitrogenase preparations from *Clostridium pasteurianum* (Cp).

The Kettering research group has studied HD formation in more detail with the Av nitrogenase complex (Bulen, 1976) and later with purified Av nitrogenase (Newton et al., 1976, 1977; Stiefel, 1977; Stiefel et al., 1980; Burgess et al., 1980, 1981; Wherland et al., 1981). They pointed out that HD formation via diimide, as formulated by Hoch et al. (1960), implies electron transfer rather than simple exchange and that one electron is used for each molecule of HD formed. They also suggested that H_2 inhibition and HD formation are two manifestations of the same molecular process. The group also took the position that HD formation has two pathways: an N_2-dependent pathway that is inhibited completely by 1% CO, and an N_2-independent pathway that is inhibited only partially by 1% CO. They reported that in the absence of added N_2 the percentage of total electrons flowing through the N_2-independent pathway is independent of the Av2/Av1 (Av2 = dinitrogenase reductase and Av1 = dinitrogenase) ratio

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[1] Abbreviations: Av, *Azotobacter vinelandii*; Ac, *Azotobacter chroococcum*; Cp, *Clostridium pasteurianum*; Av1, *Azotobacter vinelandii* dinitrogenase; Av2, *Azotobacter vinelandii* dinitrogenase reductase (the same convention is applied to the other nitrogenase components, i.e., Cp1, Kp1, Al1, Cp2, Kp2, Al2, etc.); Kp, *Klebsiella pneumoniae*; Al, *Azospirillum* sp. (apparently *A. lipoferum*); AE, activating enzyme; Tris, tris(hydroxymethyl)aminomethane; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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(Burgess et al., 1980, 1981; Wherland et al., 1981).

Burris & Orme-Johnson (1976) reported the work of Falkner, who studied the effects of pN_2 and pD_2 on HD formation by Av and Cp nitrogenases. The enhancement of HD formation by increasing the pN_2 and pD_2 was more pronounced for Av nitrogenase than for Cp nitrogenase; i.e., HD formation by Cp nitrogenase was relatively weak. They also observed that the apparent $K_m(N_2)$ for HD formation by Av nitrogenase was about one-fourth that for N_2 fixation. Burris et al. (1981) found that HD also was formed by nitrogenase from *Klebsiella pneumoniae* (Kp). Cp2 (which binds tightly to Av1 and thus inhibits Av nitrogenase; Emerich & Burris, 1976) strongly inhibited HD formation by Av nitrogenase.

The present work reports a comparative study of HD formation by highly purified nitrogenases from four different types of N_2-fixing organisms, including the anaerobe *C. pasteurianum*, the facultative anaerobe *K. pneumoniae*, the aerobe *A. vinelandii*, and the microaerobe *Azospirillum* sp. The results provide evidence to support the conclusion that HD formation is a general property of nitrogenases (Burris et al., 1981). The data again show that HD formation by Cp nitrogenase is relatively weak. Our data also indicate that N_2-independent HD formation by nitrogenase is minimal, if it exists at all.

Materials and Methods

Cp Nitrogenase. This was purified from frozen cells of *C. pasteurianum* W5. Cells were disrupted by lysis with lysozyme (Ljones & Burris, 1978); 200 g of frozen cell paste was mixed anaerobically with 400 mL of 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM dithionite. After this cell paste was thawed, 50 mL of the same anaerobic buffer containing 200 mg of crystalline egg white lysozyme, 10 mg of DNase, and 20 mg of RNase was added to the cell suspension, and the mixture was incubated under H_2 with stirring at room temperature for 30 min. The lysed cell suspension was centrifuged at 27000*g* for 20 min. The clear dark brown supernatant was introduced onto an anaerobic DE-52 cellulose column (50 × 200 mm) preequilibrated with 20 mM Tris buffer, pH 7.4, that contained 1 mM dithionite and had been sparged with H_2. The loaded column was washed quickly with 0.15 M NaCl in the same buffer to elute the hydrogenase and other impurities. It then was washed with 0.25 M NaCl in the same buffer to elute the dark brown Cp1, and following this, it was washed with 0.4 M NaCl in the same buffer to elute the yellowish brown Cp2 (Tso et al., 1972). Both Cp1 and Cp2 fractions were concentrated separately on small DE-52 columns. The concentrated Cp1 was purified further on a Sephacryl S-300 column (50 × 900 mm) preequilibrated with 50 mM Tris buffer, pH 8.0, containing 1 mM dithionite. The Cp2 was purified on a Sephadex G-100 column (50 × 900 mm) preequilibrated with the same buffer. All the eluting buffers used for the first DE-52 column were sparged with H_2.

The specific activity of Cp1 was 1100–1200 nmol of C_2H_4 formed min^{-1} (mg of Cp1)$^{-1}$, and that of Cp2 was 1950–2100 nmol of C_2H_4 formed min^{-1} (mg of Cp2)$^{-1}$. Both Cp1 and Cp2 contained trace amounts of hydrogenase (about $^1/_{100\,000}$th of the nitrogenase by weight), which gave a specific activity of 5.2 nmol of H_2 evolved min^{-1} (mg of Cp1)$^{-1}$ and 3.2 nmol of H_2 evolved min^{-1} (mg of Cp2)$^{-1}$, respectively, as analyzed with an H_2 electrode (Sweet et al., 1980). The contaminating hydrogenase could not be eliminated completely even by passing the fractions through a hydroxyapatite column, so the minus ATP plus enzyme treatments were used as controls in studying HD formation by Cp nitrogenase.

Kp Nitrogenase. This was prepared from cells of *K. pneumoniae* M5aL; 200 g of frozen cell paste was thawed anaerobically with 400 mL of 50 mM Tris buffer, pH 8.5, containing 1 mM dithionite. The cell suspension was sonicated in 50-mL portions at 50% duty cycle and output setting 5 for 2 min under N_2 with a Heat Systems-Ultrasonics Inc. 350-W, 20-KHz sonicator. Forty milliliters of anaerobic buffer containing 10 mg of DNase and 20 mg of RNase was added to the pooled sonicated cell suspension, and it was incubated under N_2 with stirring at room temperature for 30 min to digest the nucleic acids. After digestion, the cell suspension was heated quickly to 55 °C for 5 min in a hot water bath and cooled immediately to room temperature in an ice bath. The suspension then was centrifuged at 27000*g* for 20 min. The supernatant was introduced onto a DE-52 cellulose column (50 × 200 mm) preequilibrated with 20 mM Tris buffer, pH 7.4, containing 1 mM dithionite, and the column was washed with 0.12 M NaCl in the same buffer until the eluted solution was colorless. The dark brown Kp1 was eluted with 0.22 M NaCl in the same buffer, and this was followed by washing with 0.4 M NaCl to elute the yellowish brown Kp2. After the fractions were concentrated on small DE-52 columns, both Kp1 and Kp2 were purified further by preparative gel electrophoresis (Ludden, 1977). The eluting buffer for collecting Kp2 during electrophoresis should contain 5 mM magnesium acetate; otherwise, Kp2 that is concentrated on a small DE-52 cellulose column sticks to the column and is not eluted even with 1 M NaCl.

The specific activity of Kp1 was 1900–2100 nmol of C_2H_4 formed min^{-1} (mg of Kp1)$^{-1}$. The specific activity of Kp2 was 1000–1100 nmol of C_2H_4 formed min^{-1} (mg of Kp2)$^{-1}$. Neither Kp1 nor Kp2 showed any hydrogenase activity when tested with an H_2 electrode.

Av Nitrogenase. This was purified from the cell paste of *A. vinelandii* OP. The purification procedure was the same as that used for Kp nitrogenase, except that Av1 was purified by crystallization (Shah & Brill, 1973) rather than by preparative gel electrophoresis.

The specific activity of Av1 was 1500–1950 nmol of C_2H_4 formed min^{-1} (mg of Av1)$^{-1}$, and the specific activity of Av2 was 1400–1800 nmol of C_2H_4 formed min^{-1} (mg of Av2)$^{-1}$. Both Av1 and Av2 showed no hydrogenase activity as tested with the H_2 electrode.

Al Nitrogenase. Preparations were purified from frozen cell paste of *Azospirillum* sp. (apparently *Azospirillum lipoferum*, so the abbreviation Al has been used) with some modifications of the procedure reported by Ludden et al. (1978). The cells were disrupted by sonication as described above. Both Al1 and Al2 were purified by preparative gel electrophoresis. The activating enzyme (AE) was purified from the membrane fraction by the method used for purification of AE from *Rhodospirillum rubrum* (Ludden & Burris, 1978). The specific activity of Al1 was 930 nmol of C_2H_4 formed min^{-1} (mg of protein)$^{-1}$, and that of Al2 after activation was 620 nmol of C_2H_4 formed min^{-1} (mg of protein)$^{-1}$. No hydrogenase activity was detected in Al1, in Al2, or in AE.

Assays for Activity. The protein content of the above nitrogenase preparations was determined by the Coomassie blue dye-binding method (Bradford, 1976). The reaction mixture used for Cp, Kp, and Av nitrogenase activity assays contained, in 1 mL, 40 mM Mops–KOH buffer (pH 7.0), 5 mM ATP, 10 mM $MgCl_2$, 20 mM dithionite, 40 mM creatine phosphate (utilization of creatine phosphate always was less than 45% of that supplied), and 0.2 mg of creatine phosphokinase. The reaction mixture used for the Al nitrogenase

system differed only in that the concentration of $MgCl_2$ was increased to 15 mM, the dithionite concentration was decreased to 10 mM, and 0.5 mM $MnCl_2$ was added.

C_2H_2 reduction was followed in rubber-stoppered vaccine bottles (22-mL volume). After the bottle was evacuated and filled with argon 3 times, anaerobic dithionite solution was added with an air-tight syringe, and then dinitrogenase reductase and 10% C_2H_2 were added. AE was added at the same time as Al nitrogenase when the activity of this nitrogenase was assayed. The reaction bottles were preincubated in a 30 °C shaking water bath for 5 min for Cp, Kp, and Av nitrogenase assays, or for 30 min for the Al nitrogenase assay to allow time for activation of Al_2. The reaction was started by the addition of dinitrogenase. After incubation for 15 min (Cp, Kp, and Av nitrogenases), or 20 min (Al nitrogenase), 0.5-mL gas samples were withdrawn with air-tight syringes, and these were analyzed with a Varian Aerograph 600-D gas chromatograph unit equipped with a Porapak R column as described by Stewart et al. (1967).

H_2 evolution was measured with an H_2 electrode (Sweet et al., 1980) to detect any hydrogenase contaminating the nitrogenase preparations. In the study of the pN_2 effects on N_2 fixation in the absence of D_2, H_2 evolution was measured with a gas chromatographic unit equipped with a column of molecular sieve 5A and a thermal conductivity detector.

HD formation was assayed with a Varian MAT 250 isotope ratio mass spectrometer. H_2, HD, and D_2 were monitored by the appearance of masses 2, 3, and 4, respectively. The percentages of H_2 and HD were calculated from the total H_2 species after subtracting the corresponding background values. (Small background peaks appear in the mass spectrometer when no gas has been introduced, and these background values commonly are subtracted from observed peaks of the same mass in the sample analyzed. However, when a gas such as Ar is introduced, it may decrease the ionization of other gases, so that a gas in low concentration, e.g., N_2, when corrected for background N_2 observed in the absence of Ar may actually give a negative value for N_2. This response obviously confuses analyses and may support a false assumption that N_2 is absent when it actually is present in a gas mixture. As accurate values for N_2 concentration at low levels are crucial for interpretation of N_2-dependent vs. N_2-independent HD formation, this analytical aberration of mass spectrometry must be kept in mind.) Values were converted to nanomoles by multiplying the percentage value by the volume in milliliters of D_2 gas introduced into the reaction botle and by a constant value of 40370 (1 mL of gas equals 40370 nmol under 100-kPa pressure at 25 °C). Before each sample was analyzed, the background values were measured. The molar ratio of dinitrogenase reductase/dinitrogenase proteins used for the study of HD formation was near 4.5/1 on the basis of molecular weights of 212000 for Cp1, 57647 for Cp2, 219000 for Kp1, 66800 for Kp2, 234000 for Av1, and 64000 for Av2 (Eady, 1980). There are no available data for the molecular weights of components of the Al nitrogenase system, so we have assumed 220000 for Al1 and 60000 for Al2.

Two kinds of reaction vessels were used for studying HD formation: a 22-mL vaccine bottle and a single side-armed Warburg reaction vessel equipped with a capillary adaptor carrying a stopcock. All these containers were calibrated for volume by weighing them empty and full of water. Vaccine bottles were used for time course studies, CO inhibition experiments, and some high pN_2 and pD_2 experiments. The gas mixtures were prepared by injecting measured amounts of gases. Before the reaction was started by addition of dinitrogenase, 0.5 mL of the gas mixture was withdrawn from each reaction bottle as the zero-time sample for background analysis. Minus ATP plus enzyme treatments were used as controls. The reaction was terminated by dipping the bottles into a dry ice bath. Gas samples were injected into the inlet system of the mass spectrometer through a silicone rubber septum mounted in a septum holder with an attached stainless-steel U tube immersed in liquid nitrogen to trap vapors. To terminate activity and to support microdiffusion of NH_3 for analysis, 1 mL of saturated K_2CO_3 was added to the reaction mixture.

The Warburg vessels were used in studying the effects of low pN_2 and pD_2 on HD formation and investigating N_2-independent HD formation. ATP (or H_2O in controls) was injected into the side arm with a long curved needle; other components of the reaction mixture were placed in the main compartment of the vessel. The reaction vessel was connected with the capillary manifold through the stopcock-carrying adaptor. After the flasks were degassed with a vacuum pump, anaerobic dithionite solution was added into the flasks while argon flowed through the flasks; this was followed by the addition of premixed nitrogenase components. The flasks were evacuated again, and then gases were added through a manifold to give the desired gas pressures as measured on an attached Hg manometer. Small gas cylinders containing "highest purity" D_2, Ar, or Ne were connected with the manifold. After the vessels were filled with gases, the stopcocks of the adaptors were closed, and the vessels were immersed in an ice bath until the reaction was started. The reaction was started, 10 min after removing a vessel from the ice bath, by mixing ATP from the side arm with the other components of the reaction mixture; the reaction vessel was placed immediately into a 30 °C shaking water bath for 15, 20, or 30 min as desired. The reaction was terminated by placing the reaction flask in a dry ice bath. The reaction vessel was connected with the inlet system of the mass spectrometer through the stopcock-carrying adaptor, and a gas sample was admitted to the mass spectrometer while the reaction mixture remained in the dry ice bath. After mass spectrometric gas analysis, the Warburg flask was removed from the adaptor, and 1 mL of saturated K_2CO_3 solution was added into the frozen reaction mixture.

Ammonia was assayed by the indophenol method (Chaykin, 1969) after microdiffusion of NH_3 (Burris, 1972). After K_2CO_3 solution was added, the reaction flask was plugged immediately with a rubber stopper equipped with a rough-ended glass rod that had been dipped in 1 N H_2SO_4 solution. NH_3 was diffused overnight in all samples from the alkaline solution to the H_2SO_4; samples were shaken to hasten diffusion. Each rod was immersed in 4.0 mL of phenol reagent (1.4 g of phenol plus 2 mg of nitroprusside in 100 mL of deionized, distilled water), and immediately afterward, 1 mL of freshly prepared alkaline hypochlorite was added (3 g of NaOH plus 2 mL of hypochlorite household bleach in 100 mL of deionized water). After 1 h, the absorbance at 625 nm was measured with a Hitachi spectrophotometer (Model 100-40). A standard curve was prepared in the range of 0–140 nmol of ammonia as $(NH_4)_2SO_4$. If the concentration of ammonia in the sample was greater than 140 nmol, the sample and blank control were diluted with the same reagents. The quantity of NH_3 in any diluted sample was determined from a standard curve prepared by measuring standard samples diluted to the same degree and in the same manner as the experimental samples analyzed.

For determination of ammonia production by the Al nitrogenase system, 0.1 M EDTA was added to the saturated

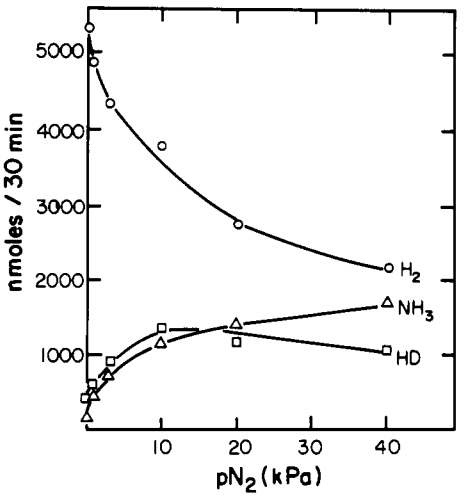

FIGURE 1: Effect of pN_2 on HD formation, H_2 evolution, and NH_3 production by nitrogenase from *Clostridium pasteurianum*. Cp1, 160 μg; Cp2, 180 μg; Cp1 specific activity, 1200 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$; Cp2 specific activity, 2000 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$. pD_2 = 50 kPa.

K_2CO_3 solution to chelate the Mn ions of the reaction mixture. Otherwise, the Mn^{2+} ions appeared to enhance the degradation of protein under alkaline conditions and thus increased the background value for ammonia in the minus ATP plus enzyme control vessels.

Gas Analysis. The content of N_2 in gas mixtures used in the investigation of N_2-Independent HD Formation (see Results) was analyzed with a MAT 250 isotope ratio mass spectrometer. Masses 28, 29, and 30 were monitored. Samples of gas mixtures for each treatment were collected in the empty all-glass reaction vessels with stopcock closures.

Absorption of Contaminating N_2. "High-purity" commercial gases were contaminated with N_2. The contaminating N_2 was absorbed by molecular sieve 13X at liquid N_2 temperature (Gersberg et al., 1976; Tiedje, 1979). A small glass trap filled with about 10 g of molecular sieve 13X was used for absorbing the contaminating N_2 from D_2 or neon. The inlet tube of the trap was connected to the gas cylinder, and the outlet tube was connected to the capillary manifold. The trap was immersed in liquid nitrogen; a separate trap was used for each kind of gas. Because D_2 also is absorbed by the molecular sieve at liquid N_2 temperature, it must be passed through the trap under pressure (about 70 kPa) at a low flow rate (10 cmHg, or 1.3-kPa decrease in pressure/min). It turns out that less D_2 is absorbed at dry ice temperature than at liquid N_2 temperature, although the efficiency for absorbing the contaminating N_2 is almost the same.

Results

Effect of pN_2 on HD Formation by Cp, Kp, and Av Nitrogenases. Figures 1, 2, and 3 show the effect of pN_2 on HD formation, H_2 evolution, and NH_3 production by Cp, Kp, and Av nitrogenases, respectively. Under atmospheres of 50% D_2 (about 50 kPa) and variable pN_2 and pAr, the rate of HD formation is enhanced by relatively low pN_2's but inhibited somewhat at high pN_2's. The maximal rate of HD formation usually occurs near 0.2 atm N_2, and the percentages of the total electrons that are used for HD formation by Cp, Kp, and Av nitrogenases are ~12, ~24, and ~24%, respectively. As the pN_2 increases to 0.4 atm, the percentages decrease to ~10, ~23, and ~23%, respectively. The rate of HD formation by Cp nitrogenase is about 2.3 times slower than that by Kp or by Av nitrogenase under an atmosphere of 50% D_2/40% N_2/10% Ar (about 50, 40, and 10 kPa).

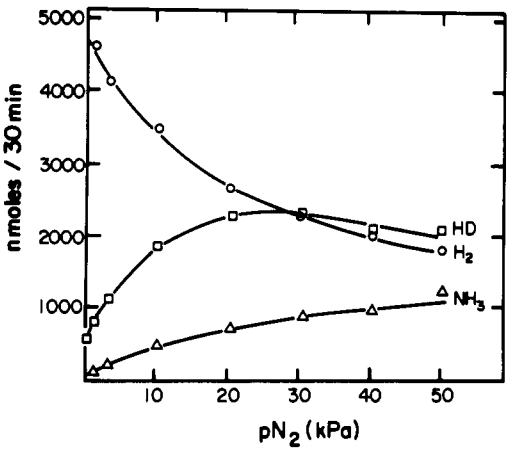

FIGURE 2: Effect of pN_2 on HD formation, H_2 evolution, and NH_3 production by nitrogenase from *Klebsiella pneumoniae*. Kp1, 180 μg; Kp2, 270 μg; Kp1 specific activity, 1900 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$; Kp2 specific activity, 1100 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$. pD_2 = 50 kPa.

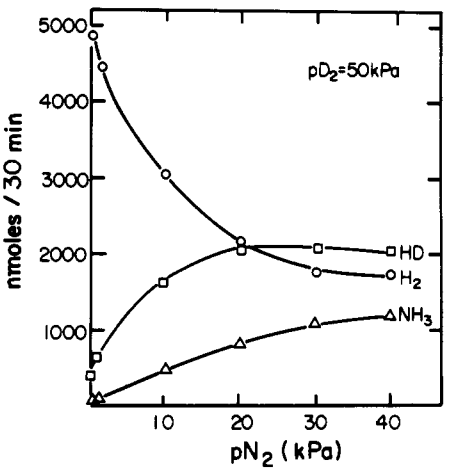

FIGURE 3: Effect of pN_2 on HD formation, H_2 evolution, and NH_3 production by nitrogenase from *Azotobacter vinelandii*. Av1, 200 μg; Av2, 300 μg; Av1 specific activity, 1700 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$; Av2 specific activity, 1600 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$.

H_2 evolution decreases, and NH_3 production increases, with increasing pN_2 above 0.2 atm (about 20 kPa) under our experimental conditions. This is consistent with all published results.

N_2 not only influences the electron allocation but it also influences the total electron flow under D_2-inhibited, N_2-fixing conditions (assuming HD formation requires one electron). A decrease of about 10% occurs in the total electron flow at 40 kPa N_2 relative to 20 kPa N_2 (not shown); this is consistent with the recently published results of Burgess et al. (1981).

Effect of pD_2 on HD Formation by Cp, Kp, and Av Nitrogenases. Figures 4 and 5 show the effect of pD_2 on HD formation, H_2 evolution, and NH_3 production by Kp and Av nitrogenases, respectively. The assumption is made in our calculations of total electron flow that production of one HD requires one electron (per molecule: HD, one electron; NH_3, three electrons; H_2, two electrons); this is logical, and evidence has been presented in its support. Under an atmosphere of 40 kPa N_2 and variable pD_2 and pAr, HD formation increases and NH_3 decreases with increasing pD_2. These results are consistent with all published results. D_2 at 50 kPa inhibits NH_3 production by Kp and Av nitrogenases by 50% (Figures 4 and 5, respectively), but it only inhibits NH_3 production by Cp nitrogenase by 20% (Table I), an inhibition that is only 40%

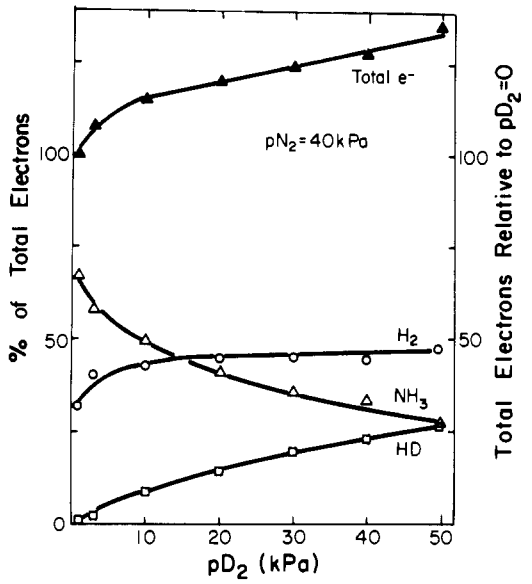

FIGURE 4: Effect of pD_2 on HD formation, H_2 evolution, and NH_3 production by nitrogenase from *Klebsiella pneumoniae*. Kp1, 180 μg; Kp2, 270 μg; Kp1 specific activity, 1900 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$; Kp2 specific activity, 1050 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$. Total electron flow of 100 = 7532 nmol (30 min × total protein concentration).

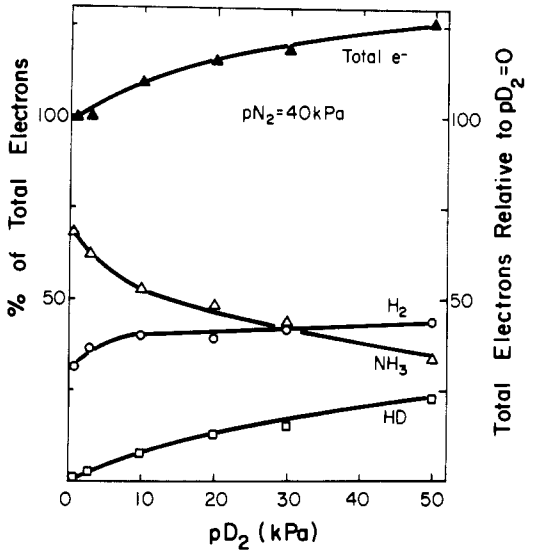

FIGURE 5: Effect of pD_2 on HD formation, H_2 evolution, and NH_3 production by nitrogenase from *Azotobacter vinelandii*. Av1, 160 μg; Av2, 300 μg; Av1 specific activity, 1420 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$; Av2 specific activity, 1380 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$. Total electron flow of 100 = 7756 nmol (30 min × total protein concentration).

the inhibition of Kp or Av nitrogenase. The result is consistent with the observed difference of $K_i(H_2)$ values between Av and Cp nitrogenases. The $K_i(H_2)$ for Av nitrogenase is 10–20 kPa (Hadfield & Bulen, 1969; Hwang et al., 1973; Jackson et al., 1968), while that for Cp is 2.5 times larger (32–55 kPa; Lockshin & Burris, 1965; Dilworth et al., 1965). In contrast to some other reports (Newton et al., 1976, 1977), we observed that H_2 evolution is not constant under different partial pressures of D_2; rather, it increases with increasing pD_2. Under 50 kPa D_2/40 kPa N_2/10 kPa Ar, a 25–30% increase of the total electron flow over that with 1 kPa D_2 and about a 10% increase over that with 10 kPa D_2 occur in the reactions of Kp or Av nitrogenase (Figures 4 and 5). We have no comparable data for Cp nitrogenase but have observed that the total electron flow associated with H_2 evolution by Cp nitrogenase increases about 10% with an increase of pD_2 from 10 to 30 kPa. Total electron flow then decreases to the same level as at pD_2 = 10 kPa when the pD_2 increases to 50 kPa (Table I). It is disturbing that the total electrons used (Figures 4 and 5) increased so markedly with increasing pD_2. As such responses usually have not been reported by others, we are reluctant to attribute any special significance to the observed increases in electrons used.

Table I: Effect of pD_2 on HD Formation, H_2 Evolution, and NH_3 Production by Cp Nitrogenase

pD_2 (kPa)[a]	products						total electron flow (two H_2, one HD, three NH_3)
	H_2		HD		NH_3		
	b	*c*	*b*	*c*	*b*	*c*	
0					212		
10	201	39.1	28	2.7	200	58.2	1030
30	248	43.4	71	6.2	192	50.4	1143
50	208	40.9	98	9.6	168	49.5	1018

[a] We assume the barometric pressure was 750 mmHg or 100 kPa. [b] Nanomoles per minute per milligram of protein. [c] Percent of total electron flow; pN_2 = 40 kPa; [Cp1], 160 μg; [Cp2], 180 μg.

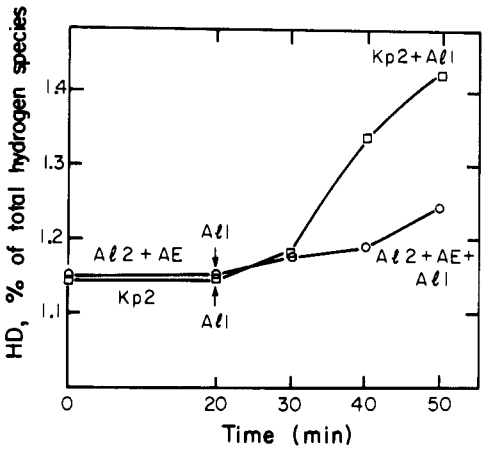

FIGURE 6: Time course of HD formation by the nitrogenase system from *Azospirillum sp.* Al1, 150 μg; Al2, 225 μg; AE, 110 μg. Specific activities (nanomoles of C_2H_2 reduced per minute per milligram of protein): Al1, 930; Al2, 620. In the reaction represented by the upper curve, 150 μg of Al1 was added to the 225 μg of Kp2 at the point indicated. The atmosphere was 50 kPa D_2, 40 kPa N_2, and 10 kPa argon.

Time Course of HD Formation by Al Nitrogenase. There is no report about HD formation by purified nitrogenase from *Azospirillum* sp., although HD exchange by living cells of *A. lipoferum* has been observed (Becking, 1981). The Al nitrogenase system is somewhat more complicated than Cp, Kp, or Av nitrogenases, as the dinitrogenase reductase (Al2) requires an activating enzyme (AE) for its action as does dinitrogenase reductase from *Rhodospirillum rubrum* (Okon et al., 1977; Ludden et al., 1978). Figure 6 shows a time course of HD formation by the Al1 nitrogenase system and by Al1 crossed with Kp2 under 50 kPa D_2/40 kPa N_2/10 kPa Ar. The activity was rather low, but the percentage of total electrons used for HD formation by the Al nitrogenase system was a high as that for Kp or Av nitrogenase (Table II). More HD was formed by Al1 plus Kp2 than that by the Al nitrogenase system itself.

Comparison of HD Formation by Nitrogenases. Table II records a comparison of HD formation by Cp, Kp, Av, and Al nitrogenases under an atmosphere of 50 kPa D_2/40 kPa N_2/10 kPa Ar at similar molar ratios (about 4.5/1) of di-

Table II: Comparison of HD Formation by Cp, Kp, Av, and Al Nitrogenases under 50 kPa D_2/40 kPa N_2/10 kPa Ar Atmosphere[a]

	products					
	H_2		HD		NH_3	
nitrogenase	*b*	*c*	*b*	*c*	*b*	*c*
Cp	204 ± 36	41 ± 4	89 ± 10	9 ± 1	166 ± 2	48 ± 1
Kp	167 ± 14	46 ± 2	177 ± 21	24 ± 2	72 ± 5	30 ± 3
Av	126 ± 20	41 ± 3	136 ± 17	22 ± 1	76 ± 1	37 ± 3
Al	31 ± 1	43 ± 2	35 ± 1	24 ± 1	17 ± 1	33 ± 2

[a] Average values of three duplicate experiments for Cp, Kp, and Av; one duplicate experiment for Al. [b] Nanomoles per minute per milligram of protein. [c] Percent of total electron flow.

Table III: Inhibition by CO of HD Formation and NH_3 Production by Cp and Kp Nitrogenases under a 50 kPa D_2/40 kPa N_2/10 kPa Ar Atmosphere

	CO pressure	% inhibition	
nitrogenase	(kPa)	HD	NH_3
Cp	0.3	91	65
	0.5	100	78
	1.0	100	93
	3.0	100	99
	5.0	100	100
Kp	1.0	95	92
	5.0	97	100

nitrogenase reductase/dinitrogenase. The percentage of total electron flow used for HD formation by Cp nitrogenase (~9%) was only about 40% of that used by the others (~23%), and the percentage of total electron flow used for NH_3 production by Cp nitrogenase (~48%) was ~1.5 times more than that used by the others. The percentage of total electron flow used for H_2 evolution by the four nitrogenases showed little difference, as all were around 41–46%.

Effect of CO on HD Formation by Cp and Kp Nitrogenases. CO inhibition of HD formation again was observed. We reexamined the CO effect on HD formation by Cp and Kp nitrogenases, and Table III shows that HD formation by Cp nitrogenase is more sensitive to CO inhibition than that by Kp nitrogenase: 0.5 kPa CO completely inhibited HD formation by Cp nitrogenase, whereas 5 kPa CO gave only 97% inhibition of Kp nitrogenase; 10 kPa CO completely inhibited HD formation by Av nitrogenase as reported by Jackson et al. (1968), Kelly (1968), Bulen (1976), and Newton et al. (1977). HD formation by Cp nitrogenase appeared more sensitive to CO than NH_3 production, as 0.5 kPa CO completely inhibited HD formation but only inhibited NH_3 production by 78%; inhibition of both reactions by 5 kPa CO was complete.

N_2-Independent HD Formation. All published data (Hoch et al., 1960; Bergersen, 1963; Kelly, 1968, 1969; Turner & Bergersen, 1969; Bulen, 1976; Burgess et al., 1980, 1981; Wherland et al., 1981; Burris et al., 1981) showed some HD was formed in the presumed absence of N_2. There are at least three possible explanations for such results: (1) HD formation is supported by a low level of N_2 contaminating the gas phase (Turner & Bergersen, 1969; Bulen, 1976); (2) HD is formed by contaminating hydrogenase in the nitrogenase (Turner & Bergersen, 1969); (3) HD is produced by an N_2-independent HD-formation pathway (Burgess et al., 1980, 1981; Wherland et al., 1981). We also have observed that HD is formed in reaction mixtures nominally free of N_2, but HD formation always is accompanied by formation of a small amount of NH_3, and this implies the presence of N_2. Figure 7 shows that graphical extensions of the HD curve and the NH_3 curve intersect at a negative point on the abscissa. Although we make no claims for quantitative accuracy in the actual point

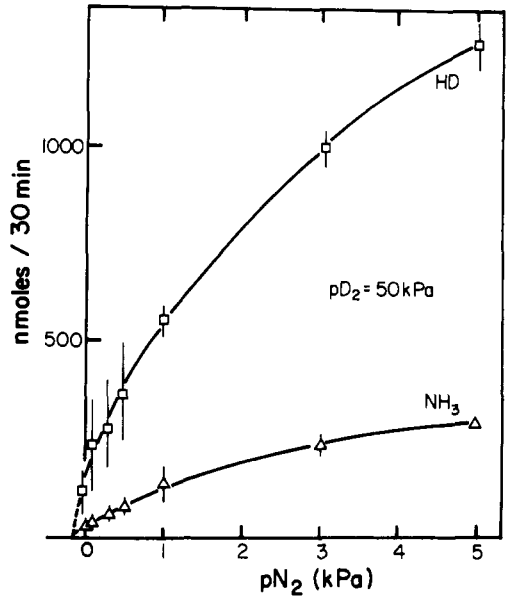

FIGURE 7: Effect of low pN_2's on HD formation and NH_3 production by Kp nitrogenase. Kp1, 175 μg; Kp2, 200 μg. Specific activities (nanomoles of C_2H_2 reduced per minute per milligram of protein): Kp1, 2100; Kp2, 1000. The extensions of the HD and NH_3 curves intersected at a point on the abscissa.

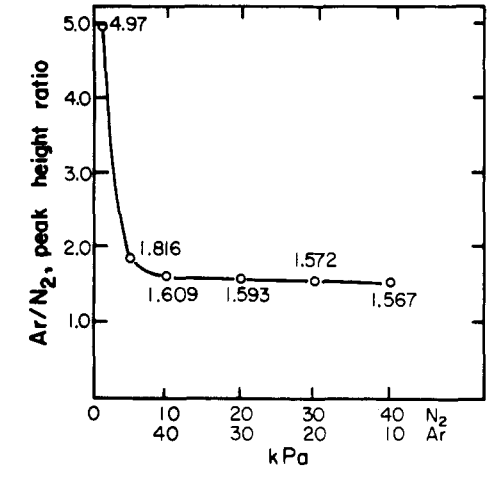

FIGURE 8: Effect of argon on suppressing the peak height of nitrogen in the mass spectrometer. pD_2 = 50 kPa throughout.

of intersection of the two curves, the apparent common point of origin of the curves suggests that the N_2-independent HD formation is caused by contaminating N_2 in the gas mixture nominally free of N_2.

We analyzed all the gases used in these studies and found that gases sold as high-purity products contained N_2, about 0.6% N_2 in D_2 and 0.5% in Ar or Ne. As Ar suppresses the ionization of N_2 (Figure 8) in the mass spectrometer, the real percentage of the contaminating N_2 in Ar is higher than in-

Table IV: HD Formation by Cp, Kp, and Av Nitrogenases under a 50 kPa D_2/50 kPa Ne Atmosphere[a]

nitrogenases	N_2 content in gas phase[b] 28	29	30	HD (% of total electron flow)	NH_3 (nmol in 30 min)
Cp1, 200 μg; Cp2, 275 μg	−36	−0.2	0.60	0	0
Cp1, 200 μg; Cp2, 250 μg	−12	−0.1	0.57	0.85	4
Kp1, 150 μg; Kp2, 180 μg	−15	−0.01	0.63	0	0
Kp1, 150 μg; Kp2, 180 μg	−36	−0.16	0.64	0	0
Kp1, 200 μg; Kp2, 260 μg	−1.8	0.03	1.10	0	0
Kp1, 450 μg; Kp2, 540 μg	−16.8	−0.16	0.65	0.81	2.5
Kp1, 450 μg; Kp2, 540 μg; 100% D_2	−1.5	0.13	4.58	2.7	44
Av1, 230 μg; Av2, 330 μg	−6.9	0.08	1.0	1.86	4
Av1, 230 μg; Av2, 330 μg	−3.0	−0.03	0.9	0.93	7
Av1, 210 μg; Av2, 315 μg	−13.5	−0.06	0.88	2.02	0
Av1, 200 μg; Av2, 300 μg	−6.3	0	1.3	2.25	2

[a] Both D_2 and neon were passed through molecular sieve 13X at liquid N_2 temperature. [b] Mass spectrometer peak heights corrected for background.

dicated directly by peak heights on the mass spectrometer. In Figures 1–3, formation of HD with no added N_2 required about 5% or greater of the total electrons used, a requirement not unlike the 6% and 9% observed by Wherland et al. (1981) when they employed commercial gases in their experiments. The experiments represented by our Figures 1–3 were performed with commercial cylinder gases, and although the gases were sold as high-purity products, they were contaminated with N_2. To minimize this problem, we have used molecular sieve 13X to absorb the contaminating N_2 in D_2 and in Ne (Ar freezes at liquid N_2 temperature, so we have used Ne instead of Ar as the inert gas). The treatment yields Ne virtually free of N_2, as no mass 28, 29, and 30 peaks above background are detectable mass spectrometrically in the treated Ne. It is more difficult to free D_2 of N_2. After D_2 has passed through a molecular sieve trap at 70 kPa at a low flow rate, a detectable peak remains at mass 30. When we used treated D_2 and Ne in studying N_2-independent HD formation, we found little or no N_2-independent HD formation by nitrogenase. Table IV shows that under an atmosphere of 50 kPa D_2/50 kPa Ne, only 0–0.85% of the total electron flow was used for HD formation by Kp and Cp nitrogenases (in the last experiment listed for Kp nitrogenase, note that "100% D_2" was used rather than 50 kPa D_2 + 50 kPa Ne); 1–2% of the total electron flow was used for HD formation by Av nitrogenase. Usually a small amount of NH_3 production occurred in reaction mixtures nominally free of N_2 (Figure 9).

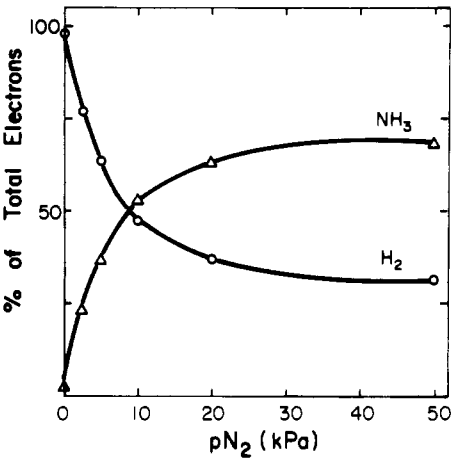

FIGURE 9: Effect of pN_2 on the production of H_2 and NH_3 by Av nitrogenase. Av1, 180 μg; Av2, 225 μg. Specific activity of 666 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$ for protein mixture for H_2 production at $pN_2 = 0$. Total electron flow of 100 = 1168 nmol (30 min × total protein concentration).

Discussion

HD formation from D_2 occurs in all the N_2-fixing agents tested in vivo and in vitro. As there have been no reports of negative results, it seems reasonable to conclude that HD formation is a general property of the nitrogenases. Although most earlier detailed work has been confined to *A. vinelandii*, we also have shown HD formation by purified nitrogenases from *C. pasteurianum*, *K. pneumoniae* (Figures 1–3), and *Azospirillum* sp. (Table II). The rate of HD formation by Cp nitrogenase is lower than that of other nitrogenases tested. Table II shows that under an atmosphere of 50 kPa D_2/40 kPa N_2/10 kPa Ar and at a similar molar ratio of dinitrogenase reductase/dinitrogenase (ratio about 4.5/1), the percentage of total electrons used by Kp, Av, and Al nitrogenases for HD formation was 22–24%, whereas that by Cp was less than 40% as great (9%/23%).

Our data show that under 40 kPa N_2 and 60 kPa Ar, 32% of the total electrons are used for H_2 evolution and 68% for NH_3 production by Av nitrogenase (Figure 9). Under 50 kPa D_2/40 kPa N_2/10 kPa Ar, the corresponding percentages are 44% for H_2 and 33% for NH_3 (Figure 5); i.e., D_2 causes a decrease from 68% to 33% in the total electrons used for NH_3 production. Excess electrons [100% − (44% + 33%) = 23%] must be allocated to other products. In the experiment recorded in Figure 5, about 23% of the total electrons were used in HD formation.

The percentage inhibition by 50 kPa D_2 of NH_3 production by Kp and Av nitrogenases was about 50% (Figures 4 and 5), whereas inhibition of production of NH_3 by Cp was only 21% (Table I). These lower values for Cp are close to the disparity in ratio of $K_i(H_2)$ between Cp and Av nitrogenases. The $K_i(H_2)$ for Cp nitrogenase (Lockshin & Burris, 1965; Dilworth et al., 1965) is 32–35 kPa, whereas that for Av nitrogenase (Hadfield & Bulen, 1969; Jackson et al., 1968; Hwang et al., 1973) is about 45% of that (10–20 kPa). The larger $K_i(H_2)$ for Cp nitrogenase indicates that the Cp nitrogenase is less sensitive to H_2 (or D_2) inhibition than is Av nitrogenase; hence, at a given pD_2, relatively less HD is formed and more NH_3 is produced by Cp nitrogenase.

HD formation by nitrogenase has offered valuable insight into the mechanism of biological N_2 fixation. Several schemes have been proposed for illustrating the mechanism of HD formation by nitrogenase (Hoch et al., 1960; Bergersen, 1963; Jackson et al., 1968; Newton et al., 1976, 1977; Schrauzer, 1976; Chatt, 1980; Wherland et al., 1981; Burgess et al., 1981; Turner & Bergersen, 1969). Although the 1960 scheme of Hoch et al. still presents most of the basic facts of HD for-

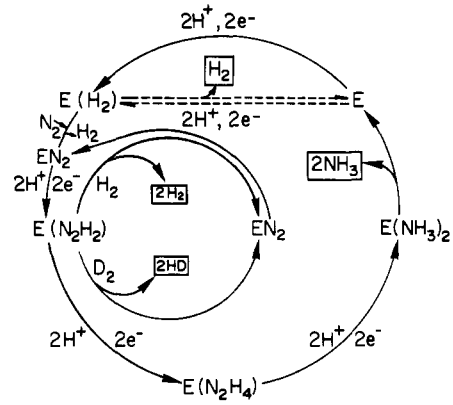

FIGURE 10: Working scheme illustrating the mechanism of H_2 evolution, HD formation, and NH_3 production by nitrogenase. E represents nitrogenase, and the broken line represents N_2-independent H_2 evolution.

mation, our experimental results and the accumulated knowledge of the mechanism of biological N_2 fixation suggest modifications as incorporated in the scheme in Figure 10. This scheme shows two separate routes for H_2 evolution (*not* HD formation). One major pathway is N_2 independent (upper pathway, Figure 10), and the other pathway is N_2 dependent. In the absence of N_2, or other nitrogenase-reducible substrates, all the electrons provided support H_2 evolution by the N_2-independent pathway. With N_2 present, N_2 occupies the H_2 evolution site and can be reduced to produce NH_3. Although N_2 and H_2 are competitive at the active site, N_2 cannot inhibit H_2 evolution completely, even at a high pN_2 (Hwang et al., 1973; Rivera-Ortiz & Burris, 1975). Thus, H_2 may be evolved although NH_3 is being released from the enzyme, and only one molecule of H_2 in evolved per molecule of N_2 reduced (eq 1). By literal interpretation of eq 1, 25% of the total electrons

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 \quad (1)$$

should be used for H_2 evolution and 75% for NH_3 production at saturating pN_2. We actually observed that under 40 kPa N_2 (nonsaturating with N_2), the percentage of total electrons used by Av nitrogenase for H_2 evolution was 32% and for NH_3 production was 68% (Figure 9).

There may be another N_2-dependent pathway of H_2 production which occurs only in the presence of H_2 and is analogous to HD formation in the presence of D_2. Bulen (1976) postulated that this occurs from the "enzyme-bound diimide" intermediate (eq 2); the position has been supported

$$[E + N_2 + 2H^+ + 2e^-] + H_2 \rightarrow E + N_2 + 2H_2 \quad (2)$$

by the Kettering research group subsequently. When D_2 is inhibiting N_2 fixation, D_2 reacts with the enzyme-bound diimide to produce HD (eq 3). The enzyme-bound diimide

$$[E + N_2 + 2H^+ + 2e^-] + D_2 \rightarrow E + N_2 + 2HD \quad (3)$$

intermediate is a key compound from which the following can occur: NH_3 production, N_2-dependent formation of H_2, or N_2-dependent formation of HD. Although the second type of N_2-dependent H_2 evolution (and the HD formation) depends upon N_2, N_2 only acts as a catalyst in H_2 and HD production, for in these reactions, it is regenerated as shown in eq 2 and 3.

Figures 1–3 show that HD formation is enhanced by rather low levels of N_2, the maximal enhancement being near 10–20 kPa N_2. When the pN_2 increases further, the rate of HD formation is inhibited somewhat. This inhibition may result because high levels of N_2 block the reaction of D_2 with the enzyme-bound diimide intermediate and thus decrease the rate of HD formation.

The influence of the pD_2 and the pN_2 on the total electron flow under D_2-inhibited, N_2-fixing conditions is complicated. The total electron flow decreases somewhat with increasing pN_2 (data not shown), whereas it increases with increasing pD_2 (Figures 4 and 5). These observations require verification, and detailed kinetic studies will be necessary to clarify this complex of reactions.

These investigations have established that HD formation is a general property of nitrogenases, as demonstration of the reaction has been extended from the commonly studied *A. vinelandii* to *C. pasteurianum*, *K. pneumoniae*, and *Azospirillum* sp. Our studies also have raised serious questions about the ability of nitrogenase to catalyze an N_2-independent formation of HD.

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Registry No. HD, 13983-20-5; D_2, 7782-39-0; N_2, 7727-37-9; CO, 630-08-0; nitrogenase, 9013-04-1.

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Evidence Showing That a Proline-Specific Endopeptidase Has an Absolute Requirement for a Trans Peptide Bond Immediately Preceding the Active Bond†

Lung-Nan Lin and John F. Brandts*

ABSTRACT: The proline-specific endopeptidase (EC 3.4.21.26) from *Flavobacterium meningosepticum* is specific for the cleavage of peptide bonds on the C-terminal side of prolyl residues. Such bonds will normally exist in the all-trans configuration. However, the preceding peptide bond in the sequence (i.e., on the N-terminal side of the prolyl residue) will exist as a mixture of cis and trans forms in solution. In this study, the activity of the proline-specific endopeptidase toward the substrates *N*-Cbz-Gly-Pro-MCA (where MCA = 4-methylcoumarinyl-7-amine) and *N*-Cbz-Gly-Pro-Leu-Gly has been examined. At a high ratio of enzyme activity/substrate concentration, the hydrolysis pattern for each substrate shows two well-separated kinetic phases. It is concluded that the fast kinetic phase, whose velocity depends on enzyme concentration, results from the direct hydrolysis of the active substrate bond (i.e., either the Pro–MCA or Pro–Leu bond, respectively) in molecules where the preceding Gly–Pro bond is trans. The slow phase, whose velocity is independent of enzyme concentration, is rate-limited by the cis-to-trans isomerization of those substrate molecules which initially have the preceding Gly–Pro bond in the cis configuration. That is, substrate molecules having the cis form of the Gly–Pro bond which precedes the active bond cannot be hydrolyzed directly but must first isomerize to the trans form before cleavage can occur. The amplitude, relaxation time, and activation energy for the slow phase are consistent with this interpretation. Thus, the proline-specific endopeptidase from *Flavobacterium* has an absolute requirement for a trans peptide bond at the position immediately preceding the active bond.

Previous studies from this laboratory on the isomeric specificity of proteases have demonstrated that proline-specific aminopeptidases such as prolidase and aminopeptidase P exhibit trans specificity toward the active X–Pro bond (where X is any amino acid residue); i.e., only the trans form of the N-terminal X–Pro peptide bond can be cleaved by these two enzymes. The cis form is not a hydrolyzable substrate and must isomerize to the trans form before it can be cleaved. Taking advantage of the isomeric specificity of these enzymes, the kinetic and thermodynamic properties of isomerization for several short peptides with X-Pro at the N terminus and for polyproline have been elucidated (Lin & Brandts, 1979a,b, 1980). Recently, we showed that an endopeptidase, trypsin, also exhibits trans specificity toward a following X–Pro bond; i.e., trypsin can only cleave an active Lys–X bond in a substrate with the Lys-X-Pro sequence when the following X–Pro bond is in the trans form (Lin & Brandts, 1983a). This isomeric specificity of endopeptidases enabled us to study the properties of an X–Pro peptide bond situated inside the polypeptide chain. Trypsin, in combination with aminopeptidase P, was used to examine proline-93 isomerization in oxidized RNase[1] (Lin &

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