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Biochemistry

Low stereoselectivity in methylacetylene and cyclopropene reductions by nitrogenase*

(nitrogen fixation/reduction mechanism/isotopic labeling)

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ABSTRACT **The stereochemistry of reductions catalyzed by nitrogenase in ^2H_2O has been investigated by using allene, methylacetylene, and cyclopropene as substrates. Deuterium labeling patterns in the reduction products were determined by mass spectroscopy, infrared spectroscopy, ^2H-decoupled 220-MHz ^1H NMR, and ^1H-decoupled 30.7-MHz ^2H NMR. Reduction of allene gave pure [2,3-$^2H_2$]propene. Reduction of methyl acetylene gave a 1.8:1.0 mixture of [*cis*- and [*trans*-1,2-$^2H_2$]propene. (Similar reduction of acetylene reportedly gave virtually all [*cis*-1,2-$^2H_2$]ethylene.) Reduction of cyclopropene gave [*cis*-1,2-$^2H_2$]cyclopropane and a mixture of [$^2H_2$]propenes. The major propene $^2H_2$ isomers formed were [*trans*-1,3-$^2H_2$]-propene (≈2), [*cis*-1,3-$^2H_2$]propene (≈1) and [2,3-$^2H_2$]propene (≈1). Cyclopropene appears to be unique as a nitrogenase substrate in that it simultaneously undergoes parallel reductions, one of which proceeds with high stereoselectivity while the other proceeds with low stereoselectivity. The weakly selective stereochemistry observed in these reductions is not consistent with a completely concerted dual proton–dual electron transfer mechanism. The results provide a basis to probe stereochemical effects in nitrogenase and in biomimetic model systems.**

Stereochemistry is a characteristic feature of many enzyme-mediated reactions that often provides mechanistically valuable clues concerning active site–substrate interactions. Nitrogenase catalyzes the ATP-dependent reduction of N_2 to NH_3, possibly via enzyme-bound N_2H_2 and N_2H_4 intermediates (1); stereochemistry arising from geometrical isomerism is possible only with the first postulated intermediate, diazene. The instability of free diazene (*cis* or *trans*) has forestalled a direct demonstration of substrate behavior, and it has not proven detectable during nitrogenase-catalyzed N_2 fixation. Among the various adventitious nitrogenase substrates (1), C_2H_2 has been used to explore reduction stereochemistry. Exposure of C_2H_2 to active nitrogenase/^2H_2O assay mixtures reportedly has resulted predominantly or exclusively in formation of [*cis*-1,2-$^2H_2$]C_2H_4 (2–4). A primary kinetic isotope effect has not been observed in these ^2H_2O reductions. This finding has been cited in support of a concerted transfer of two protons and two electrons to substrate, possibly involving a specifically positioned pair of acids derived from the enzyme protein residues (5). Because of its high symmetry ($D_{\infty h}$), C_2H_2 is a comparatively insensitive stereochemical probe: a nonspecific addition of exterior solvent protons to C_2H_2 bound to an active site metal center, or other mechanisms, arguably could account for the observed preferential formation of the *cis*-alkene isomer. Important advantages of C_2H_2 as a substrate are that it interacts efficiently with nitrogenase [$K_m(C_2H_2) \approx K_m(N_2)$] and that it gives a conveniently analyzed product. There are apparent differences in the kinetic behavior of C_2H_2 and N_2, suggestive of incomplete equivalence between the two substrates (6).

We have demonstrated (7) that cyclopropene is a substrate of nitrogenase, being reduced to a mixture of propene and cyclopropane whose proportions are remarkably insensitive (8) to *in vivo* vs. *in vitro* assay. Cyclopropene is a reversible inhibitor of nitrogenase N_2 reduction and is reduced with a K_m value as low as that of N_2 or C_2H_2 (8). In contrast, the acyclic C_3H_4 isomers allene and methylacetylene are poor substrates, judging from their large K_m values (9). Acyclic alkenes such as C_2H_4 or propene do not show detectable substrate activity with nitrogenase (1). The lower symmetry (C_{2v}), rigid ring structure, and structurally differing products available with cyclopropene suggest that it might be useful as a stereochemical probe for reduction by nitrogenase in ^2H_2O. We note that only three [$^2H_2$]C_2H_4 isomers are possible in C_2H_2/^2H_2O reductions, but nine [$^2H_2$]C_3H_6 isomers are possible from cyclopropene. Some are as yet undescribed in the literature.

We report here product characterization studies comparing ^2H_2O/nitrogenase reductions of all three C_3H_4 isomers: cyclopropene, methylacetylene, and allene.

EXPERIMENTAL PART

Nitrogenase. Nitrogenase components were purified from continuously cultured *Azotobacter vinelandii* OP (9). The Fe-Mo protein (Av1) had a specific activity of 2000 (specific activity is given in nmol of C_2H_2 reduced per mg of protein per min) and the Fe protein (Av2) had a specific activity of 1900. Both components were essentially homogeneous by anaerobic gel electrophoresis (10). Experiments were also done with less highly purified fractions.

Substrates. Allene and methylacetylene were CP (chemically pure) products from Matheson and were purified by low-pressure distillation before use. Cyclopropene was prepared and purified by methods described elsewhere (7). Authentic samples of [*cis*-1,2-$^2H_2$]propene, [*trans*-1,2-$^2H_2$]propene, and [1,1,2-$^2H_3$]propene were purchased from Merck, Sharp & Dohme.

Assay Reagents. (*i*) *ATP stock solution.* A solution of 2.5 g of disodium creatine phosphate tetrahydrate (Pierce) in 20 ml of 99.8% ^2H_2O (Merck, Sharp & Dohme) was lyophilized at −78°C to remove H_2O. To a small beaker was added 1.58 g of the resulting powder, together with 0.11 g of disodium ATP dihydrate (Sigma, grade II); 3.75 ml of 0.25 M Hepes (^2H_2O) buffer (freed of H_2O by lyophilization as above) containing 0.05 M $MgCl_2$ and 7.5 ml of ^2H_2O. After stirring, the mixture was titrated to p^{2}H 7.3 with 1 M NaO^{2}H and added to 3.75 mg of creatine kinase (Sigma, rabbit muscle, type I, 155 units/mg) in a 25-ml graduated cylinder. The solution was made up to 15 ml with ^2H_2O and mixed by several reciprocal transfers, then

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Abbreviations: GC, gas chromatography; IR, infrared; *m/e*, mass-to-charge ratio.

* Preliminary accounts of a portion of this work were given at the Symposium on Bio-Organic Mechanisms, American Chemical Society–Society for Applied Spectroscopy Pacific Conference, Anaheim, CA, Oct. 12–14, 1977, and at the Seventh Harry Steenbock–Third C. F. Kettering International Symposium on N_2 Fixation, University of Wisconsin, Madison, WI, June 12–16, 1978.

transferred in 1-ml aliquots to dry 3-ml ampoules, which were flushed with prepurified N_2, cooled to −78°C, and sealed. The reagent could be stored up to 1 year in liquid N_2 and was thawed as needed. (*ii*) ***Dithionite stock solution.*** Sodium dithionite (Baker), 0.352 g, was placed in a septum-stoppered 25-ml erlenmeyer flask, then pumped and filled with argon repeatedly. An argon-flushed solution of 0.4 ml of 1 M NaO^2H in 10 ml of ^2H_2O was added to the dithionite powder with mixing. The strength of the dithionite stock solution was verified by titration with a degassed solution of methylene blue.

Reduction Assays. Several 21.5-ml septum-stoppered vaccine bottles were evacuated to <20 μm Hg (3 Pa), then filled to 1 atm (101 kPa) with a cyclopropene (0.06 atm)/argon gas mixture by using a vacuum line manifold and vented. C_2H_6 (50 μl) (Matheson, CP grade) was injected into each bottle as an internal gas chromatography (GC) standard. Aliquots (10 μl) were removed from each bottle (and replaced immediately by equivalent volumes of argon) for background GC analysis on a Porapak N column (Analabs) [1 foot (30.5 cm) × 3/16 inch (1 inch = 2.54 cm) glass column, 55°C, helium flow rate 10 ml/min] and on a $AgNO_3$/glycerol on firebrick column (4 feet × 3/16 inch glass column, containing a 1-inch section of Porapak N, 55°C, helium flow rate 30 ml/min). To each bottle mounted in a 30°C shaker bath was added in rapid sequence 0.8 ml of degassed (argon) ATP stock solution (300 μmol of creatine phosphate/10 μmol ATP/31.0 units of creatine kinase/50 μmol of Hepes/10 μmol of $MgCl_2$), 0.5 ml of dithionite stock solution (100 μmol of dithionite), 0.4 ml of degassed (argon) ^2H_2O, and 0.3 ml of nitrogenase [≈3000 units (2 Fe protein:1 Fe-Mo protein)]. The bottles were vented and 10-μl gas aliquots were removed for GC analysis to follow the progress of the reductions. Methylacetylene and allene reductions were done similarly, except that a partial pressure of 0.4 atm was used.

GC-Mass Spectroscopy. Unfractionated gas samples (≈2 μmol) were separated on a stainless steel column (4 feet × 1/4 inch, Porapak N, 100°C, helium flow rate 30 ml/min) and spectra were obtained at 70 eV on an LKB 9000 mass spectrometer.

Product Isolation. The gas portion of each assay bottle was transferred into a 30-ml plastic syringe by gas–liquid displacement using a saturated Na_2SO_4 solution, then separated by preparative GC. The GC preparative column consisted of a 10-foot × 1/4-inch stainless steel column packed with 80–100 mesh Porapak N (70°C, helium flow rate 100 ml/min). Product in the gas effluent (1:40 splitter) was recovered at −196°C in collection tubes equipped with a capillary inlet. No isomerization was detectable in a sample of 98% [*cis*-1,2-$^2H_2$]propene submitted to the complete isolation procedure.

Infrared (IR) Spectra. Isolated product was transferred on a vacuum line to an 8-ml gas IR cell equipped with KBr windows. The IR spectra were obtained on a Perkin–Elmer 281 spectrometer. A scan time of 60 min was used to obtain exact band frequencies.

NMR Spectra. Product was transferred under reduced pressure from the IR cell into a 5-mm NMR tube containing 0.4 ml of degassed 100% C^2HCl_3 (Stohler Isotopes) at −196°C. ^2H-decoupled 220-MHz ^1H NMR spectra were obtained at 25°C on a Varian HR-220 spectrometer equipped with a superconducting magnet. The small $CHCl_3$ impurity peak was used as an internal chemical shift reference. This peak and one at chemical shift δ = 1.5 ppm also due to a trace impurity in the C^2HCl_3 solvent were subtracted from the replotted spectra. ^1H-decoupled ^2H 30.7-MHz Fourier transform NMR spectra were determined on a Varian XL-200 spectrometer at −50°C. NMR spectra simulations were generated by using a Varian XL-100 spectrometer computer with the CDOS SPINS subroutine.

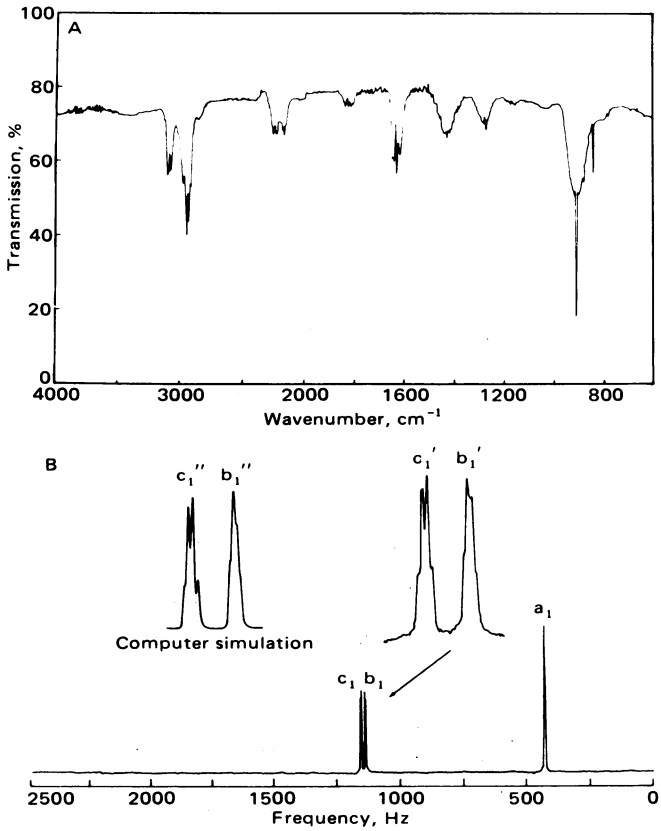

FIG. 1. IR (*A*) and ^2H-decoupled 220-MHz Fourier transform ^1H NMR (*B*) spectra of propene isolated from nitrogenase/^2H_2O reduction of allene. The IR sample (20 μmol) was run against air as blank, using the recommended standard Perkin–Elmer 281 settings for a 12-min scan. For the full NMR spectrum (a_1, b_1, c_1) 64 transients (pulse width 38 μsec, acquisition time 1.64 sec, pulse delay 14 sec) were obtained on the sample in 0.4 ml of C^2HCl_3 over a 2500-Hz sweep width with the abscissa scale zero set 1643 Hz upfield from $CHCl_3$. To obtain the spectrum (b_1', c_1'), 10× abscissa and 2× ordinate scale expansions were used. The simulated vinyl proton spectrum (b_1'', c_1'') was generated for a 250-Hz sweep width and a 1.3-Hz linewidth.

RESULTS AND DISCUSSION

Nitrogenase-Catalyzed Reduction of Allene in ^2H_2O. Allene is a fairly poor substrate (K_m = 0.5 atm) (9, 11). The unique product isolated contained exactly two atoms of deuterium (mass-to-charge ratio m/e = 44). Its IR spectrum (Fig. 1) shows a characteristic $=CH_2$ ν_{CH} triplet (3102, 3090, and 3078 cm^{-1}), as well as $-CH_2-$ ν_{CH} bands at 2977, 2945, 2934, and 2919 cm^{-1}; vinyl ν_{C^2H} $^2H_1$ bands[†] at 2256, 2233, and 2230 cm^{-1} and an alkane ν_{C^2H} $^2H_1$ triplet at 2178, 2172, and 2164 cm^{-1}. Weak overtone $\delta_{=CH_2}$ bands can be seen in the 1850–1800 cm^{-1} region. A $\nu_{C=C}$ group of bands is present, centered at 1637 cm^{-1}. Passing to the especially diagnostic 1100–600 cm^{-1} region, a very strong band at 913 cm^{-1} can be assigned to the Q branch of the out-of-plane $=CH_2$ bending vibration, while a weak band at 845 cm^{-1} is assigned to a 2-^2H C^2H bending mode. No spectral features indicative of *cis*- or *trans*-1-$^2H_1$ groups are evident. Because a reference spectrum of [2,3-$^2H_2$]propene was not available, NMR analysis was employed to verify this assigned structure. An uncoupled 100-MHz ^1H NMR spectrum (not shown) displayed broadened peaks, apparently due to ^2H broadening. The 220-MHz ^2H-decoupled ^1H spectrum of the product has a narrow multiplet at δ 1.68 (a_1) and two quartets

[†] IR spectra of moderate resolution have been published for *cis* and *trans* [1-^2H]- (12), [2-^2H]- (12, 13), and [1,1-$^2H_2$]propene (12, 13); ref. 13 includes a calculational analysis. See also ref. 14.

at δ 4.93 (b_1) and δ 5.00 (c_1). A C2 methine resonance at δ 5.7 is totally absent. The peaks integrate as c_1:b_1:a_1 = 1:1:2. Assuming the [2,3-$^2H_2$]propene structure, generation of a simulated spectrum results in an excellent match of the actual spectrum (Fig. 1). The corresponding ^{1}H-decoupled ^{2}H NMR spectrum (Fig. 2) completely confirms the assignment: singlets of equal intensity at δ 1.78 (3-$^2H_1$-) and δ 5.87 (2-^{2}H-) are observed.

We thus find that highly active *A. vinelandii* components at optimal stoichiometry convert allene uniquely to [2,3-$^2H_2$]propene in ^2H_2O assay mixtures. This product was observed earlier with a less purified preparation (11). The 2,3-$^2H_2$ isomer has been prepared by chemical means, but has not been characterized previously (15). Nitrogenase-catalyzed allene reduction would seem to be an interesting source of reference amounts of this compound in a state of very high isomeric purity.

Nitrogenase-Catalyzed Reduction of Methylacetylene in ^2H_2O. Like allene, methylacetylene is a rather poor substrate of nitrogenase (9) and inhibits C_2H_2 or N_2 reduction only weakly (9). Reduction in ^2H_2O/nitrogenase yielded one major product by GC, m/e = 44. Its IR spectrum (Fig. 3) is quite different from that of the allene-derived product: a $\nu_{=CH_2}$ band near 3030 cm^{-1} is conspicuously lacking, but weak bands assignable to vinyl ν_{CH} are apparent at 3078, 3063, and 3050 cm^{-1}; only the latter occurs in the spectrum of authentic [*cis*-1,2-$^2H_2$]propene. The alkyl ν_{CH} bands centered on 2951 cm^{-1} closely approximate those in the standard spectrum just referred to. In the ν_{C^2H} region weak bands at 2296, 2247, and possibly 2228 cm^{-1} can be assigned to [*cis*-1,2-$^2H_2$]propene, but a band at 2269 cm^{-1} indicates the presence of a *trans*-1-$^2H_1$ isomer. No bands at 2130–2160 cm^{-1} are found, ruling out the presence of a 3-$^2H_1$ isomer. Bands at about 1638, 1627, and 1614 cm^{-1} appear to correspond to $\nu_{C=C}$ modes for *trans*-1-$^2H_1$-, *cis*-1-$^2H_1$-, and 2-^{2}H-containing isomers ([1,1-$^2H_2$]propene also gives the latter two bands). In the spectral area below 1000 cm^{-1}, a strong, sharp band at 876 cm^{-1} affirms the presence of the *cis*-1,2-$^2H_2$ isomer (reference spectrum); however, several bands not seen in the *cis*-1,2-$^2H_2$ reference spectrum are also present. The most prominent among these are two bands of about equal intensity at 882 and 713 cm^{-1}; very weak bands, possibly due to *trans* impurity, are present in the *cis*-1,2-$^2H_2$ reference spectrum at these frequencies. In addition, unassigned weak bands occur at 912 and 721 cm^{-1}. Once again, an NMR spectrum is indispensable for unequivocal characterization of the product. Its ^{2}H-decoupled, 220-MHz ^{1}H NMR spectrum (Fig. 3) shows: a narrow multiplet (a_3) at δ 1.69; a quartet (b_3, J = 1.2 Hz) at δ 4.90; and a quartet (c_3, J = 1.8 Hz) at δ 4.99. There is no detectable methine (δ 5.7) peak. The areas integrate as: c_3:b_3:a_3 = 1.8:1.0:3.3 (insufficient instrument time forced use of a slightly shorter than optimal pulse delay, causing a small artificial enhancement of the methyl integration). The observed spectrum can be fitted to a simulated spectrum (Fig. 3) generated for two major products, consisting of 64% [*cis*-1,2-$^2H_2$]propene and 36% [*trans*-1,2-$^2H_2$]propene. From this, we assign the 882 and 713 cm^{-1} IR bands to the *trans* isomer (they have about half the intensity of the *cis* band at 876 cm^{-1}, but without knowledge of the extinction coefficients a quantitative correlation can only be speculative). Possibly, the very minor band at 912 cm^{-1} is due to a small amount of 2,3-$^2H_2$ isomer; this is beyond the accuracy of the ^{1}H NMR integration to confirm, but might be verifiable by ^{2}H NMR with a larger sample and more transients.

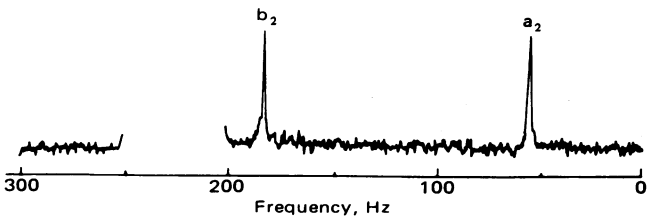

FIG. 2. ^{1}H-decoupled 30.7-MHz ^{2}H Fourier transform NMR spectrum of propene isolated from nitrogenase/^2H_2O reduction of allene (same sample as in Fig. 1). The spectrum (a_2, b_2) was plotted after 100 transients (pulse width 15 μsec, acquisition time 2 sec, no pulse delay) over a 1000-Hz sweep width with the abscissa scale zero set 222 Hz upfield from C^2HCl_3. The spectral region from 200 to 250 Hz is obscured by solvent resonances.

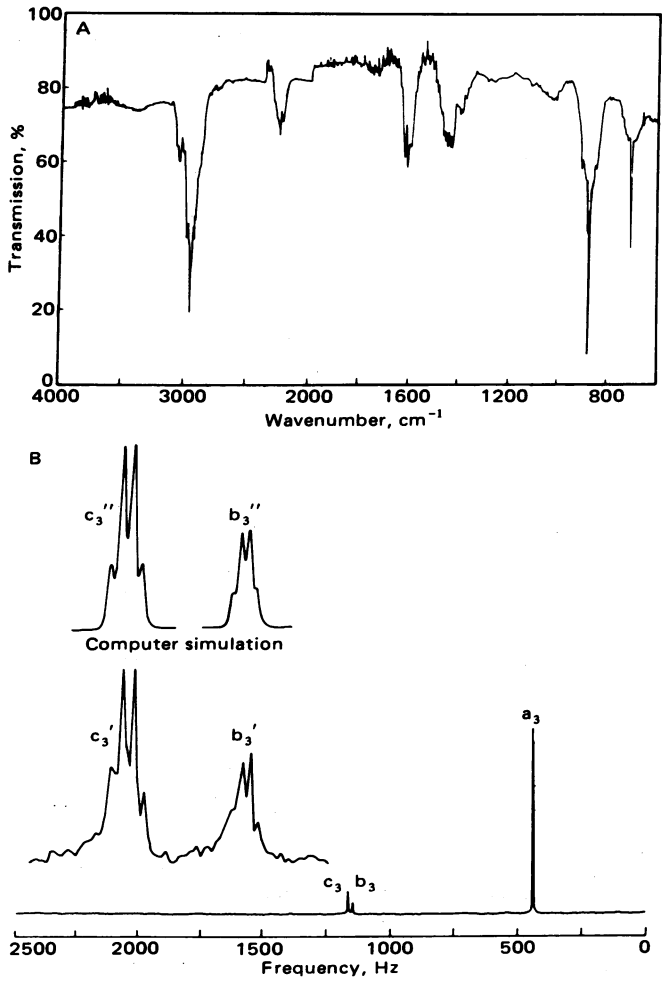

FIG. 3. IR (*A*) and ^{2}H-decoupled 220-MHz Fourier transform ^{1}H NMR (*B*) spectra of propene isolated from nitrogenase/^2H_2O reduction of methylacetylene. The IR sample (≈13 μmol) was run against air as blank with a 2× ordinate expansion (24-min scan). For the full NMR spectrum (a_3, b_3, c_3), 32 transients (pulse width 30 μsec, acquisition time 1.64 sec, pulse delay 24 sec) were obtained on the sample in 0.4 ml of C^2HCl_3 over a 2500-Hz sweep width with the abscissa scale zero set 1658 Hz upfield from $CHCl_3$. To obtain the spectrum (b_2' c_2'), 25× abscissa and 9× ordinate expansions were used. The simulated vinyl proton spectrum (b_2'', c_2'') was generated for a 100-Hz sweep width and a 1.1-Hz linewidth.

Methylacetylene is thus reduced to propene by the purified enzyme with only limited (28%) stereoselectivity. Acetylene reportedly was reduced with up to 100% stereoselectivity (2–4). It would be of interest to confirm the latter finding by using the highly purified components now available. Our results demonstrate unambiguously that reduction of the two linear C_3H_4 isomers does not pass through a common intermediate. nor can either isomer be a rearrangement intermediate on the reduction pathway for the other. Concerted, exclusively *syn* vs. *anti*,

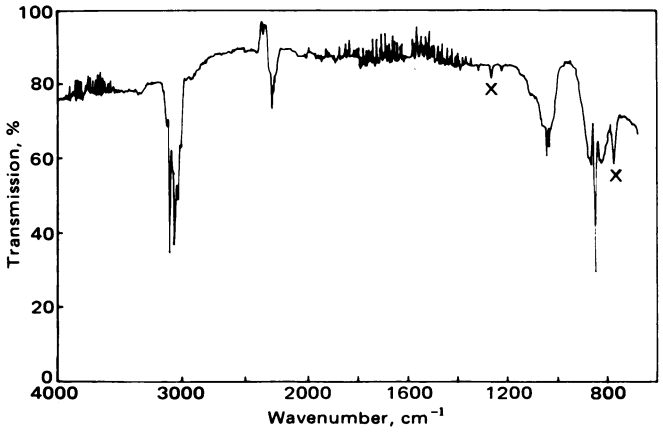

FIG. 4. IR spectrum of cyclopropane isolated from nitrogenase/^2H_2O reduction of cyclopropene. The IR sample (≈9 μmol) was run against air as blank, using a 2× ordinate expansion and a 24-min scan. Peaks marked X are assigned to cell background.

protonation cannot be distinguished in the allene reduction but is ruled out in the methylacetylene experiment, because both *cis*- and *trans*-$^2H_2$ isomers are formed in substantial amounts.

Nitrogenase-Catalyzed Reduction of Cyclopropene in ^2H_2O. We have already noted that cyclopropene rivals N_2 and C_2H_2 in its effectiveness as a substrate (9). For this and other reasons stated in the introduction, the pattern of deuterium labeling in its reduction products holds particular interest. Two major products were in fact isolated, identified as propene and cyclopropane by GC. Mass spectral analysis showed that each product contained two deuterium atoms (m/e = 44). The IR spectrum of the *cyclopropane* product (Fig. 4) could be compared directly to published spectra of [*cis*- and [*trans*-1,2-$^2H_2$]cyclopropane (16). Within the limits of accuracy imposed by the sample size, the product corresponds to the *cis* isomer; *trans* isomer present, if any, amounts to less than 5–10%. The IR spectrum of the *propene* product (Fig. 5) shows that a mixture of $^2H_2$ isomers is present. Lack of a complete set of reference spectra again hinders full interpretation. Nevertheless, a partial analysis of the spectrum can be accomplished and is fairly revealing. The $\nu_{=CH}$ region has a broad complex absorption with peaks at 3133, 3095, 3075, 3053, and 3030 cm^{-1}. There is an alkyl ν_{CH} group centered at about 2944 (main peaks at 2977, 2944, 2931, and 2919 cm^{-1}). In the ν_{C^2H} region absorptions assignable both to (=C^{2}H) (2286, 2264, 2240, and 2220 cm^{-1}) and (—C^{2}H) (2179 and 2166 cm^{-1}) bands are observed; the latter are about twice as intense as the former. The $\nu_{C=C}$ band system is complex, with peaks at 1668, 1657, 1649, 1644, 1640, 1634, 1627, and 1619–1614 cm^{-1}. Below 1100 cm^{-1} numerous strong, medium, and weak bands occur. Weak bands at 882, 876, and 713 are consistent with the presence of small amounts of the two [1,2-$^2H_2$]propenes. Major bands unassignable for lack of reference spectra are seen at 994, 973, 816, 798, and 784 cm^{-1}. Assignment of the strong 912 cm^{-1} band uniquely to a [2,3-$^2H_2$]propene mixture component is prevented by the absence of a proportionally intense band at 845 cm^{-1} (a weak band is present at 843 cm^{-1}). Other weak bands are visible at 955, 854, and 778 cm^{-1}. Preliminary 100-MHz undecoupled ^{1}H NMR spectra of the product (not shown) established that protons experiencing all possible chemical shift environments in propene were present in substantial amounts. The peaks were broadened, obscuring multiplet structure; the broadening was not affected by raising the temperature but was largely removed by broad-band ^{2}H decoupling. A 220-MHz ^{2}H-decoupled ^{1}H NMR spectrum (Fig. 5) confirmed the existence of a complex mixture, with multiplets at δ 1.67–1.70, 4.87–5.04,

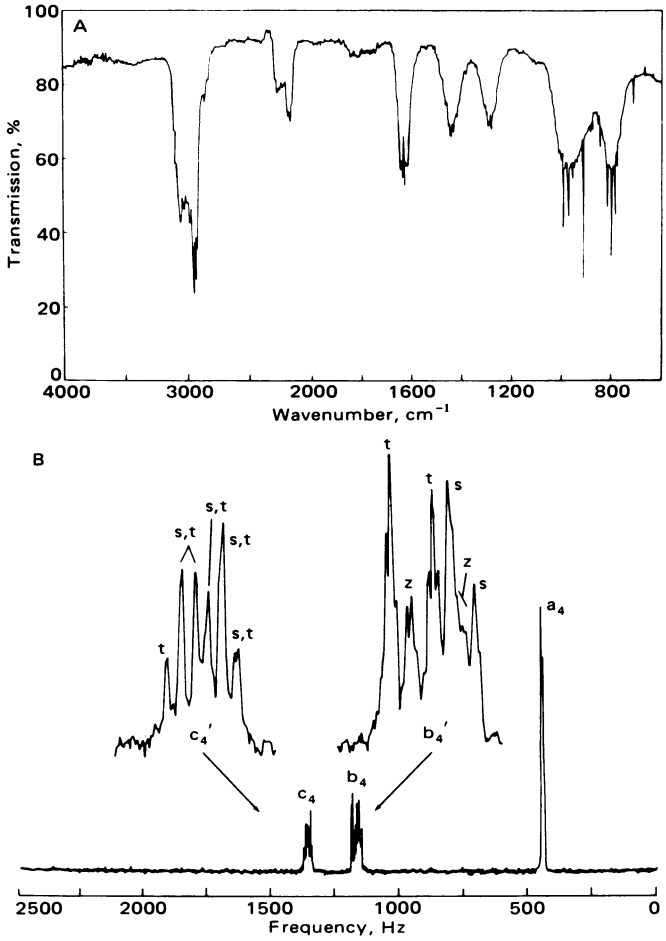

FIG. 5. IR (*A*) and ^{2}H-decoupled 220-MHz Fourier transform ^{1}H NMR (*B*) spectra of propene isolated from nitrogenase/^2H_2O reduction of cyclopropene. The IR sample (≈20 μmol) was run against air as blank with the standard Perkin–Elmer 281 settings for a 24-min scan. For the full NMR spectrum (a_4, b_4, c_4), 16 transients (pulse width 30 μsec, acquisition time 1.64 sec, pulse delay 19 sec) were obtained on the sample in 0.4 ml of C^2HCl_3 over a 2500-Hz sweep width with the abscissa scale zero set 1652 Hz upfield from $CHCl_3$. To obtain the spectrum (b_4', c_4') 10× abscissa and 4× ordinate scale expansions were used. Assignments: s = [*cis*-1,3-$^2H_2$]propene; t = [*trans*-1,3-$^2H_2$]-propene; z = [2,3-$^2H_2$]propene.

and 5.74–5.87. The integrated areas are a_4:b_4:c_4 = 1.0:1.6:2.7. Close examination of the vinyl multiplets suggests major components having *cis*-1,2 and *trans*-1,2 protons. These cannot be due to [1,2-$^2H_2$]propene species (ruled out by IR) and therefore can only be assigned to [*cis*- and [*trans*-1,3-$^2H_2$]-propene. The *trans*-$^2H_2$ isomer would give a doublet of triplets for its =CH resonance, with J = 16, 2 Hz. This is found in the spectrum (Fig. 5) at the downfield end of the =CH multiplet near δ 5.0 (t in b_4'). The corresponding methine signal near δ 5.70 (t in C_4') should then be a doublet of triplets (J = 16, 6 Hz) (Fig. 5). The *cis*-$^2H_2$ isomer would also give doublets of triplets near δ 4.8 (J = 10, 1 Hz) (s in b_4') and near δ 5.7 (J = 10, 6 Hz) (s in c_4'). Simulation (not shown) on the basis of an empirically derived mixture (2 *trans* to 1 *cis*) then accounts reasonably well for the methine multiplet but still leaves important features of the terminal vinyl multiplet unexplained. This suggests an additional major component having a deuterium atom at the methine position. Because 1,2-$^2H_2$ isomers were excluded, the 2,3-$^2H_2$ isomer is left. We have seen that this compound has quartets at δ 5.0 and δ 4.9; assignment (z in b_4') is in fact possible (Fig. 3), thereby accommodating all major peaks in the multiplet. We ascribe the slight differences in δ values for "equivalent"

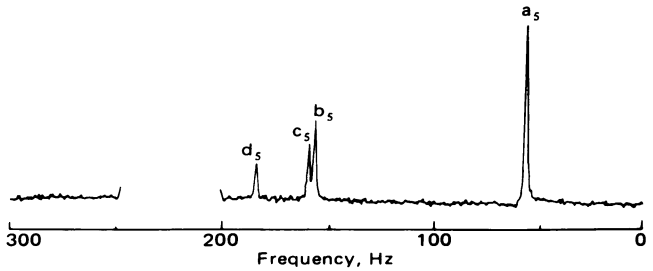

FIG. 6. ^{1}H-decoupled 30.7-MHz ^{2}H NMR spectrum of propene isolated from nitrogenase/^2H_2O reduction of cyclopropene (same sample as in Fig. 5). The spectrum (a_5, b_5, c_5, d_5) was plotted after 400 transients (pulse width 15 μsec, acquisition time 2 sec, no pulse delay) over a 1000-Hz sweep width with the abscissa scale zero set 222 Hz upfield from C^2HCl_3. The spectral region from 200 to 250 Hz is obscured by solvent resonances.

protons to small chemical shift effects from deuterium at different positions in the molecules. Simulation studies suggest an optimal fit for a 3:1 mixture of the 1,3- and 2,3-$^2H_2$ isomers. With these proportions it can be easily calculated that the ratios c_4:b_4:a_4 should be 1.0:1.7:2.7, which fits the actual integration data (see above) within experimental error.

To confirm the assignments made, a ^{1}H-decoupled ^{2}H NMR spectrum of the propene product was also obtained (Fig. 6). The analysis predicts four distinct signals: one each from methine-$^2H_1$ at δ 5.7 (d_5) (2,3-$^2H_2$ isomer); *cis*-vinyl ^{2}H at δ 5.0 (c_5) (*cis*-1,3-$^2H_2$ isomer); *trans*-vinyl ^{2}H at δ 4.8 (b_5) (*trans*-1,3-$^2H_2$ isomer); and methyl ^{2}H at δ 1.7 (a_5) (all isomers). These are observed. The predicted intensities are d_5:c_5:b_5:a_5 = 1:1:2:4. Reliable (calibrated) integrations of the Fourier transform ^{2}H spectrum have not been obtainable, but by inspection (Fig. 6) the experimental signals are approximately in these proportions. Returning now to the IR spectrum (Fig. 4) for this product, it is apparent that at least part of the 912-cm^{-1} band is attributable to the 2,3-$^2H_2$ isomer. Of the remaining major bands, we propose to assign the doublet at 994 and 973 cm^{-1} to the [*trans*-1,3-$^2H_2$]propene and the triplet at 816, 798, and 784 cm^{-1} to [*cis*-1,3-$^2H_2$]propene. These assignments are in fact fairly consistent with published analyses of the corresponding *cis*- and *trans*-$^2H_1$ isomers.

It therefore appears that for cyclopropene reduction to cyclopropane proceeds with rather high stereoselectivity, whereas reduction to propene proceeds with rather low selectivity. With reduction of the cyclopropene double bond, protons, whether donated by specific enzyme acids or by solvent, add to the ring in *syn* fashion. With cleavage of a cyclopropene C—C single bond, substantial addition of two protons across this bond is observed to yield in ^2H_2O reductions a 1,3-labeled product. However, the product equivalent of more *anti* than *syn* addition is obtained, resulting in an excess of *trans*-1,3 label. Because most (75%) of the product is 1,3-labeled, the dominant mechanistic pathway cannot include prereductive isomerization of cyclopropene to either allene or methylacetylene, because as we have proven other products would then be expected. The presence of a minor amount of 2,3-$^2H_2$-labeled propene, with traces of 1,2-$^2H_2$-labeled propenes, nevertheless makes such isomerization possible as a secondary mechanism. Perfectly concerted two-proton and two-electron transfer would require formation of a single 1,3-$^2H_2$ isomer; thus, it cannot be involved in the major pathway to propene,‡ and alternative mechanisms are needed, in which protonation either lags or precedes electron transfer or is concerted with it but occurs in discrete single-proton steps. Examples of the latter in which *syn* or *anti* 1,2-addition of a nucleophilic metal and proton is followed by reductive elimination *concerted* with protonation from a specific side of the opening ring do not predict the observed products, however.

Two final points are worth emphasizing. First, the reductions reported here were conducted under a specific set of assay parameters. Variation of these parameters, and addition of other substrates or inhibitors may effect the reduction stereochemistry in an interpretively useful way. (Systematic reexamination of C_2H_2 reduction stereochemistry may also be informative.) Second, the results already obtained present a relatively complex and subtle new challenge (17) to biomimetic systems. It is hoped that attempts to meet this challenge and others generated by new physical methods (18) may result in more highly evolved nitrogenase model chemistry.

‡ Unless unspecific isomerization of [*cis*-1,2-$^2H_2$]cyclopropane via a 1,2- ring cleavage is involved; however, we have not observed any nitrogenase-catalyzed isomerization of added cyclopropane (up to 0.4 atm) under the experimental conditions described herein. This does rule out prereductive insertion to a vinyl carbene intermediate as an alternative mechanism.

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