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Received February 9, 1976

Chemical Probes of Nitrogenase. 1. Cyclopropene. Nitrogenase-Catalyzed Reduction to Propene and Cyclopropane[1]

Sir:

One of the more remarkable properties of nitrogenase consists in its ability to catalyze the reduction of diverse small unsaturated molecules besides N_2, the biological substrate.[2] Prominent among these "adventitious"[3] substrates is C_2H_2, which the enzyme readily reduces to C_2H_4 (but not to C_2H_6).[4] In contrast to C_2H_2, C_2H_4 is completely unreactive to nitrogenase;[5] the origin of this selectivity, an important clue towards the understanding of nitrogenase function, is unknown primarily because an appropriate investigative tool has not been available to enzymologists. It appeared to us reasonable to

$$HC \equiv CH \xrightarrow[ATP,\,2e^-]{N_2ase} H_2C = CH_2$$

$$H_2C = CH_2 \xrightarrow[ATP,\,2e^-]{N_2ase} \text{No reaction}$$

surmise that *a molecule possessing electronic properties intermediate between those of C_2H_2 and C_2H_4 might display partial reactivity with nitrogenase,* and therefore could be of use as a chemical probe to elucidate the molecular basis for such phenomena as C_2H_2 vs. C_2H_4 selectivity and other interesting aspects of nitrogenase catalysis. *Cyclopropene* as a candidate molecule of this type[6] combines a number of attractive features: (1) it clearly satisfies our fundamental criterion; i.e., it is recognized to possess properties in between those regarded as typical for unstrained alkenes and alkynes;[7] (2) it has the most compact steric profile of the three C_3H_4 isomers; and (3) its reduction conceivably could lead to any of several stable products—cyclopropane or propene via a two-electron reduction, propane via a four-electron reduction—which themselves have little or no tendency to interact further with nitrogenase.[8] In this communication we report evidence that nitrogenase prepared from *Azotobacter vinelandii* OP catalyzes the formation of *both propene* and *cyclopropane*

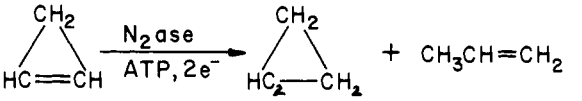

Table I. Nitrogenase-Catalyzed Reduction of Cyclopropene

Expt No.	Assay mixture	Pinit, cyclopropene (atm)[a]	Reaction time (min)	Product formed (nmol)[b] Propene	Cyclopropane	GC column
1	Complete[c]	0.025	10	452	—	*d*
			30	1.26×10^3	—	*d*
			100	1.30×10^3	6×10^2	*e*
2	None	0.02	10	<5[f]	—	*d*
	ATP, DT[g]		10	<5[f]	—	*d*
	Complete[c]		10	347	—	*d*
			57	$\sim 7 \times 10^2$	$\sim 3 \times 10^2$	*h*
			93	7.3×10^2	3.4×10^2	*e*
3	None	0.005	20	$<1 \times 10$[f]	<2[f]	*d, h*
	DT[i]		20	$<2 \times 10$[f]	<2[f]	*h*
	DT, N_2ase[j]		20	$<2 \times 10$[f]	<2[f]	*h*
	Complete[j]		20	155	79	*h*
7	None	0.04	30	<2[f]	<10[f]	*d, k*
	ATP, N_2ase[l]		30	26[f]	<10[f]	*d, k*
	Complete[m]		21.5	367	—	*d*
			105	790	375	*k*
8	None	0.03–0.015	40	<2[f]	<2[f]	*k*
	ATP, DT[n]		13	6[f]	<2[f]	*k*
	complete[o]		13	212	109	*k*
			29	466	262	*k*
10	None	0.035–0.02	20	<8[f]	<5[f]	*k*
	DT, N_2ase[p]		11	<9[f]	<8[f]	*k*
	Complete[q]		10	380	198	*k*

[a] Initial partial pressures (balance, Ar to 1 atm), determined manometrically or estimated by GC. [b] All experiments conducted at 30°. C_2H_6 used as internal standard in expt. 7, 8, and 10. Apparent activities in some experiments limited by agitation rate. [c] 4.9 mg CP-S N_2ase protein in 2 ml of assay mixture as described in text. [d] Porapak N, 2 ft × 3/16 in. [e] Porapak N, 10 ft × 1/4 in. [f] Background value. [g] 0.55 ml of H_2O, 0.80 ml of ATP-generator (ATP (12.5 μM), $MgCl_2$ (12.5 μM), CP (62.5 μM), CPK (16 units), HEPES (62.5 μM, pH 7.3)), and 0.5 ml of 0.08 M DT. [h] 4 ft × 3/16 in. $AgNO_3$/ethylene glycol/firebrick, 1.5 in. × 3/16 in. Porapak N. [i] 0.15 ml of H_2O, 0.1 ml of 0.25 M HEPES/0.05 M $MgCl_2$ (pH 7), 0.25 ml of 0.08 M DT. [j] As in *i*, plus 3.3 mg of CP-S N_2ase protein in 0.1 ml of 0.01 M sodium phosphate buffer (pH 7). Complete mixture additionally contained 0.4 ml of ATP-generator. [k] 14 ft × 3/16 in $AgNO_3$/glycerol/firebrick, 2 in. × 3/16 in. Porapak N. [l] 0.6 ml of H_2O, 0.8 ml of ATP-generator (note *g*) and 3.65 mg of CP-S N_2ase protein in 0.1 ml of 0.01 M sodium phosphate buffer (pH 7). [m] As in *l*, plus 0.50 ml of 0.08 M DT. [n] 0.8 ml of ATP-generator (note *g*), 0.65 ml of H_2O, and 0.5 ml of 0.08 M DT. [o] As in *n*, plus 1.84 mg of CP-S N_2ase protein in 0.05 ml of 0.01 M sodium phosphate buffer (pH 7). [p] 0.45 ml of 0.25 M HEPES/0.05 M $MgCl_2$ (pH 7), 0.5 ml of 0.08 M DT and 9.18 mg of CP-S N_2ase protein in 0.25 ml of 0.01 M sodium phosphate buffer, pH 7. [q] As in *p*, plus 0.8 ml of ATP-generator (note *g*).

from cyclopropene; *i.e., cyclopropene is a two-electron substrate of the enzyme and reduction is partly accompanied by cleavage of the three-membered ring.*

Some pertinent data concerning the reduction are presented in Table I. Incubation of nitrogenase[9] in 2 ml of an aqueous assay mixture[11] containing ATP (5 μM), $MgCl_2$ (5 μM), CP (25 μM), CPK (16 units), HEPES (25 μM, pH 7.3), and DT (20 μM) under an atmosphere of dilute cyclopropene[12] in Ar resulted in the formation of substantial quantities of propene[14] and cyclopropane, detected by GC analysis with several column types and directly identified in scaled up runs by ^{1}H NMR spectrometry. Neither gas was generated by control mixtures lacking enzyme, ATP-generator, or DT. For all assays shown, propane formation was undetectable (<1% of propene formed).

In other experiments, it was found that no reduction of cyclopropene to propene or cyclopropane occurred in complete assay mixtures when CP-S nitrogenase was replaced with equivalent amounts (in terms of activity units) of either isolated Fe-Mo or Fe protein; however, the recombined enzyme components strongly catalyzed reduction to both products. Kinetic studies of the reduction revealed a smooth, initially linear evolution of both propene and cyclopropane beginning promptly after initiation by ATP, DT, or nitrogenase. The slopes of the linear portions of rate plots were direct functions of the number of enzyme units present in the assay mixtures when ATP and electron donor were maintained in excess, and the total amount of reduction product was limited by total ATP available with DT in excess. H_2 (0.01 atm) could not be substituted for DT as the in vitro reductant, and added H_2 (0.01 atm) failed to enhance product yields when DT was limiting.

The above results are consistent with essential known requirements and properties of previously described nitrogenase-substrate reduction systems,[2a-c] and taken together exclude the possibility that cyclopropene is being reduced via an inadvertent, nonspecific process bearing no significant relation to any of the mechanistic events involved in N_2 fixation or the conversion of C_2H_2 to C_2H_4 by the enzyme. A unique feature of the cyclopropene reduction is the formation of two *isomeric* products in substantial amounts. Furthermore, under many conditions the relative quantities of the two products formed appear to correspond to a fixed, perhaps integral ratio; in all our time course experiments to date, including those in which either the Fe-Mo or the Fe protein was at a saturating concentration relative to its complementary component, the propene:cyclopropane initial rate quotient was 2.0 ± 0.2. Present uncertainty in the value of the Henry's law coefficient for cyclopropene in water prevents us from making a reliable estimate for $K_{m(app)}$, but our preliminary measurements indicate that the maximal velocity (V_m) for cyclopropene reduction to the two C_3H_6 products is sensibly smaller than the V_m for C_2H_2 reduction.

The occurrence of protein-bound Fe and Mo in the nitrogenase components has led to much speculation that one or both metals may be intimately involved in the binding and subsequent reduction of N_2, C_2H_2, and analogous substrates,[2a-c,16] thus stimulating efforts to elaborate possibly related nonenzyme chemistry based on Mo, Fe, or other transition metals.[2b,16,17] The feasibility of π-bonded cyclopropene-transition metal complexes has been recognized for some time.[7] Structures invoking mixed π and σ bonding have been proposed for certain compounds derived from Fe and Co carbonyl complexes and triphenylcyclopropenyl bromide,[18] insertion products from reactions of cyclopropenes with various metal salts and complexes have been reported,[19] and more recently reversibly-formed Pt(0) complexes of molecular cyclopropenes have been characterized.[20] However, direct precedent from well-understood model chemistry for the novel enzyme-catalyzed unsubstituted cyclopropene reduction we have described here cannot be cited, and should be sought. It is tempting to consider the abrupt change from inertness to reactivity with nitrogenase that is encountered in passing from C_2H_4 to cyclopropene and C_2H_2 in terms of the presence of *two* orthogonal sets of π/π^* orbitals in the latter molecule;[21] thus the lessened reactivity of cyclopropene relative to C_2H_2 could be ascribed to the less favorable overlap or donor-acceptor properties of the distorted geometry, quasi-π/π^* Walsh orbitals that are coplanar with the three-membered ring. Unequivocal conclusions concerning this and alternative hypotheses must await the results of detailed investigations, currently in progress in our laboratory, of the reduction mechanism and other aspects of interactions between nitrogenase and cyclopropene.

Acknowledgment. We thank Professor R. H. Burris for the gift of a culture of *A. vinelandii OP,* Dr. Richard C. Burns for a preprint of ref 2e, and Professor K. L. Servis for helpful advice concerning FT NMR experiments. Purchase of the Varian XLFT-100 NMR spectrometer used in this work was made possible by an NSF Departmental Equipment Award (GP43411). This research was supported by a grant from the Research Corporation (No. 7292) and in part by a Biomedical Sciences Support Grant RR07012-08 from the National Institutes of Health. M.-C. McKenna acknowledges support from NSF Grant No. BMS 13608.

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(1) Presented in part at the US-USSR Joint Symposium on Chemical Catalysis, Homogeneous Catalysis Section, Princeton University, Princeton, N.J., June 23-25, 1975.
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(3) We introduce this term to emphasize that aside from the fixation of N_2 itself, which may involve hydrazine as an intermediate,[2f] none of the established nitrogenase-catalyzed substrate reductions have a known physiological role in contemporary organisms.
(4) Thus making possible a sensitive, convenient, and widely-used assay for nitrogenase based upon GC separation and analysis of the two hydrocarbons (ref 2a,c): standard specific activities for nitrogenase used in this work are for C_2H_2 reduction (nm of C_2H_4/(mg of protein min).
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(9) *A. vinelandii OP* was harvested from a continuous culture maintained in log phase; details will be published elsewhere. Harvested cells were disrupted at 10,000-15 000 psi in a French pressure cell, and the nitrogenase complex was purified by a modification of published procedures through cellulose phosphate resolubilization of a protamine sulfate precipitate.[10] This storage-stable preparation (designated CP-S) had a specific activity of 150 ± 5. Fe-Mo and Fe proteins were isolated from gel-filtered CP-S by DEAE-cellulose chromatography, and had limiting specific activities of 1610 and 850, respectively.
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(11) Abbreviations: ATP, adenosine 5'-triphosphate, disodium salt; CP, creatine phosphate, disodium salt; CPK, creatine phosphokinase; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-ethanesulfonic acid; DT, sodium dithionite.
(12) Cyclopropene was prepared by the method of Closs[13] and purified by repeated trap-to-trap vacuum line transfers at −78 to −118° followed by preparative GC, or in some cases by low pressure, low temperature fractional distillation. Cyclopropene, propene, cyclopropane, propane, allene, methyl acetylene, and other possible hydrocarbon impurities were determined by analytical GC and also by ir and ^{1}H NMR spectrometry.
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(14) Contaminating cyclopropene isomers or allylic impurities were excluded as sources of the propene as follows: (1) methyl acetylene, present at 1-5% levels in crude, freshly prepared cyclopropene, was not detectable by FID GC analysis in the purified samples employed in the enzyme ex-

periments; the amounts of propene formed (Table I) exceed this detection limit by factors of 10^2–10^3; (2) the allene content (≤0.5%, ir) of the purified cyclopropene samples corresponded to a maximum partial pressure of 10^{-4} atm in assay gas mixtures; the maximal initial rate of propene production calculated[15] for this value is several orders of magnitude less than observed rates, and in fact the amounts of propene formed exceeded the maximal allene contamination by factors of 10 or more; and (3) traces of allyl chloride and allyl amine carried over in the collection of crude cyclopropene were no longer detectable (FID GC, ir) after purification and can also be disregarded by stoichiometry alone, although there is no evidence that either compound can undergo reduction by nitrogenase. Nonhydrocarbon products of nonreductive decomposition of cyclopropene due to contact with the aqueous assay mixture cannot be an important source of propene formation because this should lead to an increased rate of such formation as a function of preincubation time, whereas experimentally the opposite was observed (C. E. McKenna, unpublished data).

(15) Using a K_m (allene) value of 0.4 atm and assuming V_m (allene) ≤ V_m (C_2H_2) (ref 2e).

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(22) Recipient of 1976 Sigma Xi Student Research Award, U.S.C.

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Received February 9, 1976

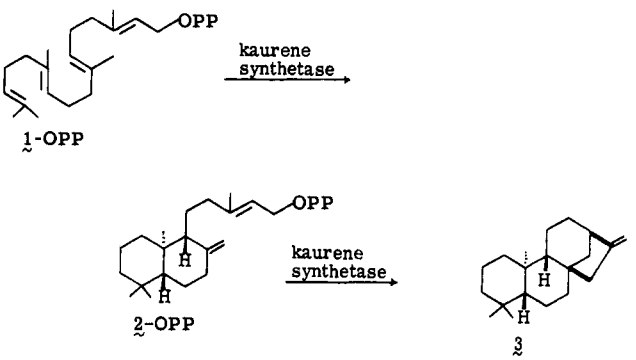
Enzymatic Cyclization of (*R,S*)-14,15-Oxidogeranylgeranyl Pyrophosphate to 3α- and 3β-Hydroxykaurene[1]

Sir:

The first step in the biosynthesis of the majority of the known diterpene natural products from *all-trans*-geranylgeranyl pyrophosphate (**1**-OPP), is a double cyclization to the labdane nucleus, a process apparently initiated by enzymatic protonation of the 14,15-double bond.[2] Thus, the tetracyclic diterpene kaurene (**3**), a biosynthetic precursor of the gibberellin plant growth regulators,[2,3] is formed from **1**-OPP by way of the bicyclic intermediate, copalyl pyrophosphate (**2**-OPP).[4] Although many members of the gibberellin family and certain other diterpenes possess a hydroxyl group at C_3,[5] this functional group is apparently introduced by an oxygenation reaction subsequent to the cyclization stages.[2,3,6] In contrast, the characteristic C_3 hydroxyl group present in most cyclic triterpenes and sterols is a direct outcome of the enzymatic cyclization of (3*S*)-2,3-oxidosqualene.[7] We wish to report that the (*R,S*)-14,15-epoxide (**4**-OPP) of geranylgeranyl pyrophosphate is cyclized to a mixture of 3α- and 3β-hydroxykaurene (**5** + **6**)[1] by soluble enzyme preparations containing the diterpene cyclase, kaurene synthetase.[8,9]

all-trans-Geranylgeraniol (**1**-OH)[10] was rendered radioactive (MnO_2 oxidation; NaB^3H_4 reduction) and converted to the known (*R,S*)-14,15-epoxide (**4**-OH, 47 mCi/mm)[8b] by means of regioselective terminal hypobromination of **1**-OAc.[12] Phosphorylation of **4**-OH was accomplished with the modified Cramer procedure,[13] the pyrophosphate (**4**-OPP) being obtained in 20% yield after purification by ion exchange chromatography on Dowex 1-X8[14] and elution from an Amberlite XAD-2 column to separate ammonium formate.[15] The ammonium pyrophosphate was characterized by its specific activity (46 mCi/mm), typical thin layer chromatographic (TLC) behavior,[16a] and reconversion to **4**-OH (75%, identification by TLC mobility)[16b] by treatment with bacterial alkaline phosphatase.

Scheme I



Soluble preparations of endosperm homogenates from immature *Echinocystis macrocarpa* (wild cucumber) seed are known to contain kaurene synthetase activity.[17] A series of preliminary experiments was performed with these preparations as the source of enzyme. The epoxy pyrophosphate is converted in substantial amounts to an extractable, radioactive fraction (X) which runs ahead of the epoxy alcohol (**4**-OH) on TLC[16c] to the region expected for hydroxy diterpenes. Smaller amounts of extractable radioactivity were associated with the triol (from epoxide and pyrophosphate hydrolysis) and one or two very small radioactive fractions were seen at positions intermediate between reference markers of the triol and epoxy alcohol. No distinct radioactive peak was associated with the epoxy alcohol itself. Heat-inactivated enzyme preparations did not catalyze the formation of X. Treatments with bacterial alkaline phosphatase at the end of the initial incubation period did not increase the amounts of X extracted. The plant growth retardant Amo-1618, which strongly inhibits the enzymatic cyclization of **1**-OPP,[18] greatly reduced incorporation into X. Under appropriate TLC conditions,[16b] X is resolved into two closely running components (4:1 ratio), the more polar and predominant of which migrated with authentic 3 α-hydroxykaurene (**5**).[19]

Preparative incubations were carried out with 7.3 g of lyophilized endosperm from *E. macrocarpa* which was reconstituted in 235 ml of cold pH 7.4 buffer (0.1 M Tris and 0.01 M KH_2PO_4). Magnesium chloride hexahydrate (0.47 mmol) and epoxy pyrophosphate (2.1 mg, 4.2 μmol, 420 × 10^6 dpm) were then added. After 15 h at 30° the incubation was terminated by addition of 230 ml of acetone, and the products were separated by extraction with benzene. Purification by preparative TLC[16b] afforded radioactive product (161 × 10^6 dpm, 38%) with R_f 0.5–0.6. The radioactivity was separated into a slightly more polar major fraction (96.6 × 10^6 dpm, 23%, ~260 μg) and a less polar minor fraction (20.7 × 10^6 dpm, 5%, ~60 μg) by means of high pressure liquid chromatography (Corasil type II column, 0 → 20% ethyl acetate/hexane gradient). The major and minor products were identified as 3α- and 3β-hydroxykaurene (**5** and **6**),[1] respectively, by direct comparisons with authentic specimens prepared from the natural diterpene abbeokutone.[20,21]