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Reduction of cyclopropene by NifV⁻ and wild-type nitrogenases from *Klebsiella pneumoniae*

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The nitrogenase from wild-type *Klebsiella pneumoniae* reduces cyclopropene to cyclopropane and propene in the ratio 1:2 at pH 7.5. We show in this paper that the nitrogenase from a *nifV* mutant of *K. pneumoniae* also reduces cyclopropene to cyclopropane and propene, but the ratio of products is now 1:1.4. However, both nitrogenases exhibit the same K_m for cyclopropene ($2.1 \times 10^4 \pm 0.2 \times 10^4$ Pa), considerably more than the K_m for the analogous reaction with *Azotobacter vinelandii* nitrogenase under the same conditions (5.1×10^3 Pa). Analysis of the data shows that the different product ratio arises from the slower production of propene compared with cyclopropane by the mutant nitrogenase. During turnover, both nitrogenases use a large proportion of the electron flux for H_2 production. CO inhibits the reduction of cyclopropene by both *K. pneumoniae* proteins, but the mutant nitrogenase exhibits 50% inhibition at approx. 10 Pa, whereas the corresponding value for the wild-type nitrogenase is approx. 110 Pa. However, H_2 evolution by the mutant enzyme is much less affected than is cyclopropene reduction. CO inhibition of cyclopropene reduction by the nitrogenases coincides with a relative increase in H_2 evolution, so that in the wild-type (but not the mutant) the electron flux is approximately maintained. The cyclopropane/propene production ratios are little affected by the presence of CO within the pressure ranges studied at least up to 50% inhibition.

INTRODUCTION

The nitrogenase from *Klebsiella pneumoniae* consists of two O_2-sensitive iron–sulphur proteins, a tetrameric MoFe-protein and a dimeric Fe-protein. An iron–molybdenum cofactor, FeMoco, which apparently is, contains, or is part of, the N_2-reducing site, can be extracted from the MoFe-protein. In addition to the fixation of N_2, nitrogenase catalyses the reduction of C_2H_2 and several other small molecules with triple bonds, cyclopropene (the only alkene known to be reduced with facility by nitrogenase) and H^+. All these reductions require both proteins, ATP, a low-potential electron donor (normally dithionite *in vitro*), a bivalent cation (usually Mg^{2+}) and anaerobic conditions. Substrates compete with protons for reduction, but electron flux normally remains constant. CO inhibits the reduction of all substrates by wild-type nitrogenases, but it does not inhibit the evolution of H_2 [1,2].

nifV mutants of *K. pneumoniae* contain a nitrogenase with a normal Fe-protein but a defective MoFe-protein [3]. The enzyme reduces C_2H_2 *in vivo* but not N_2, although N_2 is reduced *in vitro* in conditions of high electron flux. NifV⁻ nitrogenase also reduces HCN with a lower K_m than does the wild-type, and its H_2-evolution activity, unlike that of wild-type nitrogenase, is partially inhibited by CO [3,4]. FeMoco extracted from the *nifV*-mutant MoFe-protein can be combined with the FeMoco-less polypeptides from a *nifB* mutant. The resultant nitrogenase exhibits the NifV phenotype, which provided evidence that FeMoco is the site for binding of N_2 and CO [5]. The metal ion compositions and e.p.r. properties of FeMoco from NifV⁻ Kp1 and from wild-type Kp1 are very similar, indicating considerable structural similarities between the wild-type and NifV⁻ cofactors [5,6], and e.x.a.f.s. studies have not detected significant differences between the local environments of molybdenum in either enzyme [7,8]. However, ENDOR (electron–nuclear-double-resonance) spectroscopy revealed that the molybdenum site is perturbed in the cofactor from the mutant, and it was concluded that it differs from the wild-type cluster probably by addition, subtraction or replacement of a ligating atom at or near the molybdenum. It is believed that the processing factor formed by the *nifV*-gene product, recently proposed to be homocitrate [9], modifies the FeMoco, enhancing N_2 reduction. In so doing it renders H_2 evolution insensitive to inhibition by CO [5].

Cyclopropene is reduced by *Azotobacter vinelandii* OP to propene and cyclopropane *in vivo* [10] and *in vitro* [11] in the ratio of 2:1. This ratio is insensitive to enzyme purity over a specific activity range of 40–1200 nmol/min per mg under standard assay conditions [11,12]. The K_m for formation of propene and cyclopropane is 1.0×10^3 Pa (*in vitro* and *in vivo*), corresponding to a molar K_m as low as that of N_2 and C_2H_2 [13], whereas the $k_{cat.}$ for cyclopropene is about half that for acetylene. The ratio of the reaction products may be a useful chemical probe for the nature of the active site of nitrogenase [11–14] and for chemical models of the

Abbreviations used: Kp1 and Kp2, MoFe-protein and Fe-protein components of *Klebsiella pneumoniae* nitrogenase respectively; Av1 and Av2, MoFe-protein and Fe-protein components of *Azotobacter vinelandii* nitrogenase respectively.

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enzyme, and therefore we determined how the NifV$^-$ nitrogenase reacts with cyclopropene.

MATERIALS AND METHODS

Preparation of proteins

Wild-type *Klebsiella pneumoniae* M5a1 and the *nifV* mutant, *Klebsiella pneumoniae* strain 1603, were grown, and the nitrogenase proteins were isolated, as previously reported [4,15,16].

The MoFe-protein component of *K. pneumoniae* nitrogenase (Kp1) had a specific activity of 800 nmol of C_2H_4/min per mg at a Kp2/Kp1 component ratio of 15:1 and of 930 nmol of C_2H_4/min per mg at a Kp2/Kp1 of 30:1. The NifV$^-$ MoFe-protein had a specific activity of 500 nmol of C_2H_4/min per mg at a Kp2/NifV$^-$ Kp1 ratio of 15:1 and of 650 nmol of C_2H_4/min per mg at a Kp2/NifV$^-$ Kp1 ratio of 30:1. Kp2 had a specific activity of 1200 nmol of C_2H_4/min per mg.

Preparation of cyclopropene

Cyclopropene was prepared and purified ($\geqslant 99.7\%$) as described elsewhere [14].

Reagents

All biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. $Na_2S_2O_4$ was recrystallized from the commercial product (Sigma) by a method described elsewhere [17].

Reduction assays

For the assay of nitrogenase activity, C_2H_2 and cyclopropene reductions were performed in 8.0 ml glass vials with rubber serum stoppers. The reaction mixture at pH 7.5 contained, per ml, 12.5 μmol of ATP, 12.5 μmol of $MgCl_2$, 16 μmol of phosphocreatine, 8 units of creatine kinase, 30 μmol of $Na_2S_2O_4$ and 25 μmol of Hepes. For assays of C_2H_2 reduction, $Na_2S_2O_4$ and C_2H_2 were injected through the stopper after the bottle had been evacuated and filled with Ar three times. The pressure in the bottle was lowered to atmospheric by piercing the rubber stopper with a hypodermic needle just before the reaction was initiated by addition of nitrogenase components. After shaking at 30 °C for 10 min, the reaction was terminated with 0.15 ml of 25% (w/v) trichloroacetic acid. Gas samples (100 μl) were analysed with a Varian 2400 gas chromatograph with a Porapak N column and fitted with an FID detector (He as carrier gas).

For assays of cyclopropene reduction, specific amounts of cyclopropene in Ar (prepared on a gas manifold, with the use of a Toepler pump as necessary [14]) were added to a septum-stoppered assay bottle full of Ar at 0.1 MPa (1 atm). The assay bottles were then vented and ethane (20 μl) was added with a syringe to serve as internal standard. The amounts of C_3 hydrocarbon impurities were determined by g.l.c. analysis of 25 μl or 50 μl samples. These samples were immediately replaced by the same volume of argon (0.1 MPa). An anaerobic mixture of water, ATP and $Na_2S_2O_4$ was added, and after 5 min preincubation at 30 °C the pressure in the bottle was adjusted to atmospheric.

The assay was started by addition of a mixture of purified nitrogenase components at a specified formal ratio. Gas samples for time-course investigations were withdrawn at fixed intervals for analysis of propene and cyclopropane and they were replaced by equal volumes of Ar. For K_m and CO-inhibition experiments, the assays were terminated after 15 min with 0.15 ml of 25% trichloroacetic acid (the reaction was linear for at least 15 min), after which the gas was analysed for cyclopropane, propene and H_2. The cyclopropane and propene measurements were corrected for background. Cyclopropane and propene were determined with a Varian gas chromatograph equipped with a glass column (0.9 m × 4.5 mm) packed with $AgNO_3$-impregnated firebrick and a short plug of Porapak N (0.9 m × 25 mm), with He as carrier gas. The column with $AgNO_3$/Porapak N removes cyclopropene, and Porapak N separates ethane, propene and cyclopropane [11]. H_2 was determined on 0.5 ml headspace samples with a Varian 3700 gas chromatograph equipped with a molecular-sieve (0.5 nm pore size; 60–80 mesh) copper column (1.8 m × 3 mm) and a thermal-conductivity dectector (Ar as carrier gas).

RESULTS

Cyclopropene is reduced to propene and cyclopropane by both wild-type and NifV$^-$ *K. pneumoniae* nitrogenases. The time courses for product formation are linear for at least 900 s. The ratio of propene to cyclopropane is approx. 2:1 for the enzyme from wild-type *K. pneumoniae*, similar to the ratio observed for the nitrogenase from *A. vinelandii* OP [11,12] (Fig. 1). However, for the NifV$^-$ nitrogenase the ratio fell to approx. 1.4:1 (Table 1 and Fig. 1).

The wild-type and mutant nitrogenases exhibit a K_m of $2.1 \times 10^4 \pm 0.2 \times 10^4$ Pa cyclopropene, as determined by a range of weighted and non-weighted linear-regression methods [18–20] (Fig. 1). In a parallel experiment with *A. vinelandii* OP proteins, Av2/Av1 ratio 20:1 under identical assay conditions, a K_m of 5.1×10^3 Pa was found for reduction of cyclopropene (J. P. Gemoets & C. E. McKenna, unpublished work). The reported K_m [11,12] of 1.5×10^3 Pa was determined with a 10-fold lower Av2/Av1 ratio (2:1) and different assay conditions. Double-reciprocal plots for the individual reduction products propene and cyclopropane show K_m values not significantly different from the value obtained from total hydrocarbon product calculations. This suggests that the two *Klebsiella* enzymes have similar binding sites for cyclopropene. The nitrogenase from the mutant reduces cyclopropene with a $V_{max.}$ of approx. 490 nmol/min per mg, whereas the wild-type enzyme has a specific activity of 662 nmol/min per mg as derived from Fig. 1. The lower specific activity for the mutant enzyme may be due to a factor such as lower intrinsic activity, but could also arise from incomplete purification.

The different product ratio arises from a slower production of propene compared with cyclopropane by the mutant species. The plots for cyclopropane in Fig. 1 are coincident, which suggests that the mutant modification affects only the reduction pathway to propene.

A large proportion of the total electron flow goes to H_2 formation when cyclopropene is the substrate (Table 1). For NifV$^-$ nitrogenase, 73–75% of the electrons go to H_2 production at 1.0×10^4 Pa cyclopropene (i.e. $K_m/2$), whereas the wild-type enzyme forms slightly more H_2 at comparable pressures, e.g. 80–85% at 1×10^4 Pa cyclopropene. Extrapolation of the H_2-evolution data as a function of cyclopropene pressure and neglecting any possible substrate inhibition indicates that approx. 20%

Table 1. Reduction of cyclopropene by the nitrogenase of wild-type *K. pneumoniae* and of a NifV⁻ mutant

Data for wild-type *A. vinelandii* are included for comparison. For experimental details see the text.

Protein source	$10^5 \times$ Cyclopropene pressure (Pa)	Specific activity (nmol/min per mg)				H_2 activity (as % of total activity)	Ratio propene/cyclopropane
		Cyclopropane	Propene	H_2	Total		
$NifV^-$ mutant of *K. pneumoniae*	0.010	10	14	777	801	97	1.43
	0.012	13	18	756	787	96	1.45
	0.016	18	26	741	785	94	1.43
	0.025	24	35	717	776	92	1.43
	0.041	35	52	671	758	86	1.47
	0.065	50	74	586	710	83	1.48
	0.100	77	100	475	652	73	1.29
	0.110*	65	96	247	408	61	1.47
Wild-type *K. pneumoniae*	0.010	11	24	1445	1479	98	2.18
	0.012	13	29	1446	1488	97	2.28
	0.017	17	38	1382	1437	96	2.24
	0.025	25	56	1343	1424	94	2.20
	0.041	37	84	1288	1409	91	2.28
	0.067	58	125	1208	1391	87	2.16
	0.096†	70	158	1030	1258	82	2.24
	0.099	90	159	1044	1293	81	1.76
Wild-type *A. vinelandii*	0.041	84	167	855	1106	77	1.98

* All experiments used 0.187 mg of Kp1 per assay, except this, which used 0.213 mg and a Kp2/Kp1 ratio of 10:1, not 20:1.
† All experiments used 0.170 mg of Kp1 per assay, except this, which used 0.149 mg and a Kp2/Kp1 ratio of 10:1, not 20:1.

($NifV^-$ Kp1) and 30% (Kp1) of the total electron flow will go to H_2 at K_m.

The total of electron flux recovered in reaction products (propene, propane and H_2) tends to decrease with increasing cyclopropene pressure. At approx. 1×10^4 Pa cyclopropene ($K_m/2$) with $NifV^-$ nitrogenase, 19% of the electrons are not recovered as reaction products (Table 1), and the comparable value for the wild-type nitrogenase at the same pressure of cyclopropene is 17%.

The effects of CO upon cyclopropene reduction and upon cyclopropane, propene and H_2 evolution are shown in Table 2 and Fig. 2. The data show that CO inhibits the reduction for both *K. pneumoniae* proteins. The $NifV^-$ nitrogenase is the more sensitive to CO inhibition, 50% inhibition being observed at approx. 10 Pa, an order of magnitude less than the value of

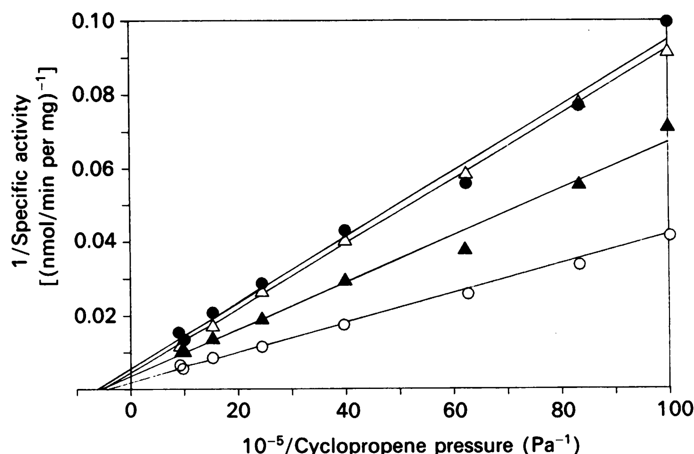

Fig. 1. Cyclopropene reduction by wild-type and NifV⁻ nitrogenases

△, Cyclopropane production by wild-type nitrogenase; ○, propene production by wild-type nitrogenase; ●, cyclopropane production by NifV⁻ nitrogenase; ▲, propene production by NifV⁻ nitrogenase.

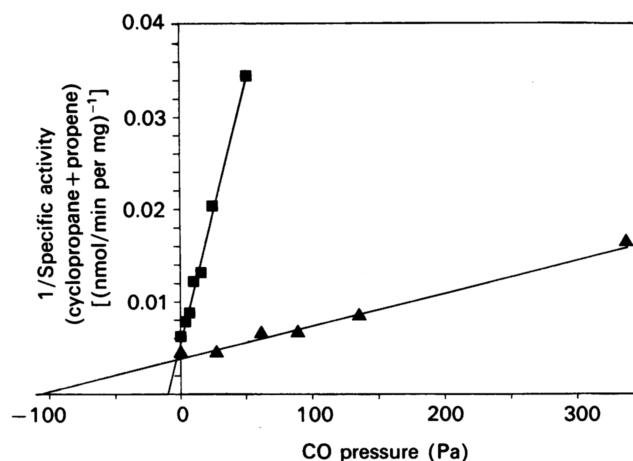

Fig. 2. Effect of CO upon product formation during cyclopropene reduction

▲, Cyclopropane+propene production by wild-type nitrogenase; ■, cyclopropane+propene production by NifV⁻ nitrogenase.

Table 2. Inhibition by CO of the reduction of cyclopropene by the nitrogenases of wild-type *K. pneumoniae* and of a NifV⁻ mutant

For experimental details see the text.

Protein source	CO pressure (Pa)	Specific activity (nmol/min per mg)				H_2 activity (as % of total activity)	Ratio propene/ cyclopropane
		Cyclopropane	Propene	H_2	Total		
NifV⁻ mutant of *K. pneumoniae*	0.0	65	96	247	408	61	1.47
	3.4	52	76	216	344	63	1.46
	6.7	47	66	218	331	66	1.42
	10.1	35	47	195	277	71	1.35
	15.6	31	45	162	238	68	1.46
	24.5	22	27	199	248	81	1.23
	50.5	15	14	195	224	87	0.97
Wild-type *K. pneumoniae*	0.0	70	158	1030	1258	82	2.24
	27.0	71	155	982	1208	81	2.16
	61.0	47	106	1155	1308	88	2.26
	89.0	46	103	1068	1217	88	2.25
	135	37	82	1243	1362	91	2.23
	335	19	42	1189	1250	95	2.18

approx. 110 Pa for the wild-type system. H_2 evolution by the mutant system is much less affected than is cyclopropene reduction, the inhibition being approx. 15% and relatively independent of the CO pressure above 10 Pa (Table 2). CO inhibition of cyclopropene reduction by the nitrogenases coincides with a relative increase in H_2 evolution in both systems, to such an extent that with the wild-type, but not with the mutant, the electron flux is approximately maintained. This is a significant difference between the two systems. Table 2 shows that the ratios of cyclopropane to propene exhibit little sensitivity to the presence of CO within the pressure ranges studied up to at least 50% inhibition.

DISCUSSION

It has been postulated that the reaction chemistry of nitrogenases is consistent with the involvement of a hydridic metal site (for a review see ref. [21]), and this idea is supported by the reduction of difluorocyclopropene by the *A. vinelandii* enzyme, which involves loss of fluoride ([14]; C. E. McKenna, H. Eran & T. Nakajima, unpublished work). The reduction of cyclopropene to cyclopropane seems to follow the classical reduction pathway for an alkene or alkyne at a hydridic metal centre (see, e.g., ref. [22]), even to the extent that there is some stereochemical control, since cyclopropene is hydrogenated by *A. vinelandii* nitrogenase primarily in the *cis* positions [23].

Chemical precedent for the formation of propene is not so easy to find. Cyclopropane is known to form metallacyclobutanes under certain circumstances ([24], and references cited therein). Cyclopropene might also form a metallacyclobutene in analogous fashion (though this has yet to be demonstrated) and the metallacyclobutene then could produce propene. In any case, previous measurements on *A. vinelandii* have excluded the direct conversion of cyclopropane into propene mediated by that enzyme [11]. However, ring-opening reactions of cyclopropene to yield co-ordinated vinylketen have been reported [25]. The protonation of cyclopropene itself is believed to occur by way of an allylcarbocation $CH_2{=}CH{-}CH_2^+$ (see ref. [26]), and it has been suggested that this also occurs in model molybdothiol systems [27], though with little supporting evidence.

The observation that the ratios of products from *A. vinelandii* nitrogenase both *in vivo* and *in vitro* with Av2 in excess of Av1 are the same (2 mol of propene to 1 mol of cyclopropane) makes more plausible the earlier suggestion [14] that cyclopropene is a chemical probe for the nitrogenase mechanism. FeMoco has been reported to catalyse the reduction of C_2H_2, but not of N_2 [13,28], and it can also catalyse the reduction of cyclopropene to cyclopropane by $NaBH_4$ in aqueous *N*-methylformamide solution [13]. After such a reduction FeMoco is still capable of restoring nitrogenase activity to reconstituted enzyme [29]. Only in the presence of a suitable apoprotein is propene also produced. The molybdenum–thiol nitrogenase model systems [27] have also been used to reduce C_2H_2, N_2 and cyclopropene, the last to give propene and cyclopropane in a ratio that varies with pH, being 2:1 at pH approx. 1 and 1:2 at pH approx. 7, although these ratios can be perturbed by certain complexing agents and it is not known whether the molybdothiol reagent does not change with pH. The product ratio with the molybdothiol reagent is therefore pH-dependent [27], although it is not clear why.

A common factor in these systems may be an iron–molybdenum–sulphur cluster, though it is hardly likely to be exactly the same in every case. In the case of the NifV⁻ enzyme, the difference in the cluster that provokes the difference in propene/cyclopropane ratio presumably arises from the absence of the processing factor, now claimed to be homocitrate [9], formed by the *nifV*-gene product. This lowers the proportion of propene in the product, without perturbing the electron flux devoted to formation of cyclopropane.

It is thus not evident what the structural difference between the wild-type and mutant Kp1 proteins might be. However, *a priori*, the mechanistic consequence of this difference would seem to express itself in a change in the product ratio, which could arise in one of the following ways: a change in the local redox potential or

chemistry of one of two sites, each of which is product-specific; a change in the partitioning of a common intermediate between two pathways, one of which causes ring-opening, and the other of which retains the ring; a change in protonation, perhaps related to local pH, rather than electronation, affecting product distribution as observed in the molybdothiol model systems [27].

CO is an inhibitor of cyclopropene reduction by both *K. pneumoniae* nitrogenases, but the NifV$^-$ enzyme is markedly the more sensitive. The ratio of propene to cyclopropane is not altered by the presence of CO up to a partial pressure of 16 Pa. This suggests that the CO acts by diverting the electron flux towards protons before the electrons reach the binding site, which may be, to judge from the common K_m values, similar in both nitrogenase variants.

Investigation of the variation of product ratio with component ratio, pH, ligand and metal type should enable us better to assess the significance of the observations made with nitrogenases *in vivo* and *in vitro*. We have already shown that $[MoH_4(Ph_2PCH_2CH_2PPh_2)_2]$ reacts with cyclopropene in the presence of HBF_4,Et_2O to yield cyclopropane and propene, in marked contrast with the reduction of cyclopropene by molybdothiol systems under acid conditions, which yields predominantly propene [27]. Extension of these experiments should provide an insight into the components and local conditions necessary to produce a fully competent nitrogenase.

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