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Steady-state kinetic analysis of soluble methane mono-oxygenase from *Methylococcus capsulatus* (Bath)

Jeffrey GREEN and Howard DALTON*
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.

A steady-state kinetic analysis of purified soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath) was performed. The enzyme was found to follow a concerted-substitution mechanism. Methane binds to the enzyme followed by NADH, which reacts to yield reduced enzyme and NAD^+. The reduced enzyme–methane complex binds O_2 to give a second ternary complex, which breaks down to release water and methanol. In this way the enzyme can control the supply of electrons to the active site to coincide with the arrival of methane. Product-inhibition studies (with propylene as substrate) supported the reaction mechanism proposed. K_i values for NAD^+ and propylene oxide are reported. The K_m for NADH varied from 25 μM to 300 μM, depending on the nature of the hydrocarbon substrate, and thus supports the proposed reaction sequence. With methane as substrate the K_m values for methane, NADH and O_2 were shown to be 3 μM, 55.8 μM and 16.8 μM respectively. With propylene as substrate the K_m values for propylene, NADH and O_2 were 0.94 μM, 25.2 μM and 12.7–15.9 μM respectively. Methane mono-oxygenase was shown to be well adapted to the oxidation of methane compared with other straight-chain alkanes.

INTRODUCTION

Methylococcus capsulatus (Bath) is a methanotrophic bacterium that activates its growth substrate methane by oxidizing it to methanol (eqn. 1):

$$CH_4 + NADH + H^+ + O_2 \rightarrow CH_3OH + NAD^+ + H_2O \quad (1)$$

The enzyme responsible for catalysing this initial reaction is methane mono-oxygenase (Colby & Dalton, 1976, 1978). Methane mono-oxygenase is found as either a particulate (membrane-bound) or a soluble (cytoplasmic) protein, depending on the amount of copper available to the cells during growth (Stanley *et al.*, 1983).

Soluble methane mono-oxygenase consists of three proteins, all of which are necessary for mono-oxygenase activity (Dalton, 1980). Protein A is a non-haem iron protein of M_r 210000 that interacts with methane (Dalton, 1980; Woodland & Dalton, 1984*a*). Protein B is a regulatory protein of M_r 15700 lacking prosthetic groups (Green & Dalton, 1985), and protein C is an iron–sulphur flavoprotein of M_r 42000 (Colby & Dalton, 1978, 1979). Protein C is the reductase component of the enzyme and is responsible for the transfer of electrons from NADH to protein A (Dalton, 1980; Lund *et al.*, 1985). Protein A may use the reducing equivalents to activate O_2, allowing an activated oxygen species to be inserted into methane (Dalton, 1980).

Earlier stopped-flow kinetic work in this laboratory was undertaken to study electron transfer through the soluble methane mono-oxygenase complex (Lund *et al.*, 1985). This work, performed aerobically in the absence of the substrate methane, revealed that neither the reduction of protein C by NADH nor electron transfer from protein C to protein A is the rate-determining step in the methane mono-oxygenase reaction, but that the final hydroxylation of methane is probably the rate-limiting step. In any reaction catalysed by a multi-substrate enzyme the rate constants and Michaelis constants observed for one substrate will depend on the concentrations of the other substrates. Thus results obtained in the absence of one substrate may be different from those obtained with all the substrates present. Hence to obtain a complete kinetic picture all the substrates should be present and their concentrations varied in turn.

Joergensen (1985) has published a kinetic study *in vivo* of particulate methane mono-oxygenase in a methanotroph, strain OU-4-1. Random bi-reactant kinetics were observed when methane and O_2 concentrations were varied at constant NADH concentrations. The measured K_m values for methane and O_2 were 1 μM and 0.14 μM respectively, these values being much lower than previous reports (Colby *et al.*, 1977; Joergensen & Degn, 1983).

A steady-state kinetic analysis of the oxidation of methane by soluble methane mono-oxygenase has been prevented by the lack of sufficiently purified enzyme to allow accurate kinetic measurements to be made. However, the recent purification of protein B to near homogeneity (Green & Dalton, 1985) along with the well-established purification protocols for proteins A and C (Woodland & Dalton, 1984*b*; Colby & Dalton, 1979) now permits a basic steady-state kinetic study of this enzyme to be performed.

In this paper we present the results of a three-substrate steady-state kinetic analysis of soluble methane mono-oxygenase and discuss the possible physiological implications of a reaction mechanism for this enzyme.

EXPERIMENTAL

Materials

All chemicals and biochemicals were the best grade available and were obtained from sources reported previously (Colby & Dalton, 1978, 1979). Ethanol-free NAD^+ was obtained from P-L Biochemicals (Milton Keynes, Bucks., U.K.).

* To whom correspondence should be addressed.

Growth of *M. capsulatus* (Bath) and preparation of soluble extracts

Bacterial growth conditions and the preparation of soluble cell extracts were as previously described (Colby & Dalton, 1978).

Purification of methane mono-oxygenase

Proteins A, B and C were purified from soluble cell extracts as previously described (Colby & Dalton, 1979; Woodland & Dalton, 1984*b*; Green & Dalton, 1985).

Methane mono-oxygenase assays

Spectrophotometric assay. Each assay mixture contained, in a total volume of 1 ml: 2 nmol each of proteins A, B and C of soluble methane mono-oxygenase; 12 μmol of sodium phosphate buffer, pH 7.0; various amounts of propylene-saturated 20 mM-sodium phosphate buffer, pH 7.0 (standardized by g.l.c.); various amounts of NADH; water to a total volume of 1 ml. The reactions were performed in sealed 1 ml cuvettes and initiated by the addition of propylene. The reactions were monitored at 340 nm, at 45 °C with a Pye–Unicam SP. 8-200 recording spectrophotometer (Pye–Unicam, Cambridge, U.K.). The reference cuvette contained all the assay components except NADH. Under these conditions the measured oxidation of NADH was proportional to methane mono-oxygenase concentration, and good agreement between the spectrophotometric, polarographic and g.l.c. assays was obtained.

Polarographic assay. Each assay mixture contained, in 3 ml: 5 nmol each of proteins A, B and C; 24 μmol of sodium phosphate buffer, pH 7.0; various amounts of NADH; various amounts of propylene- or methane-saturated 20 mM-sodium phosphate buffer, pH 7.0; water to a total volume of 3 ml. The reaction was initiated by addition of propylene or methane and monitored at 45 °C by means of a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). The oxygen electrode was calibrated by the method of Robinson & Cooper (1970).

G.l.c. assay. Each assay mixture contained, in 1 ml: 2 nmol each of proteins A, B and C; 12 μmol of sodium phosphate buffer, pH 7.0; various amounts of propylene- or methane-saturated 20 mM-sodium phosphate buffer, pH 7.0; various amounts of NADH; water to a total volume of 1 ml. The incubations were performed in sealed 7 ml conical flasks in shaking water baths at 45 °C (Colby & Dalton, 1978). After 30 s equilibration time the reactions were initiated by the addition of NADH to the

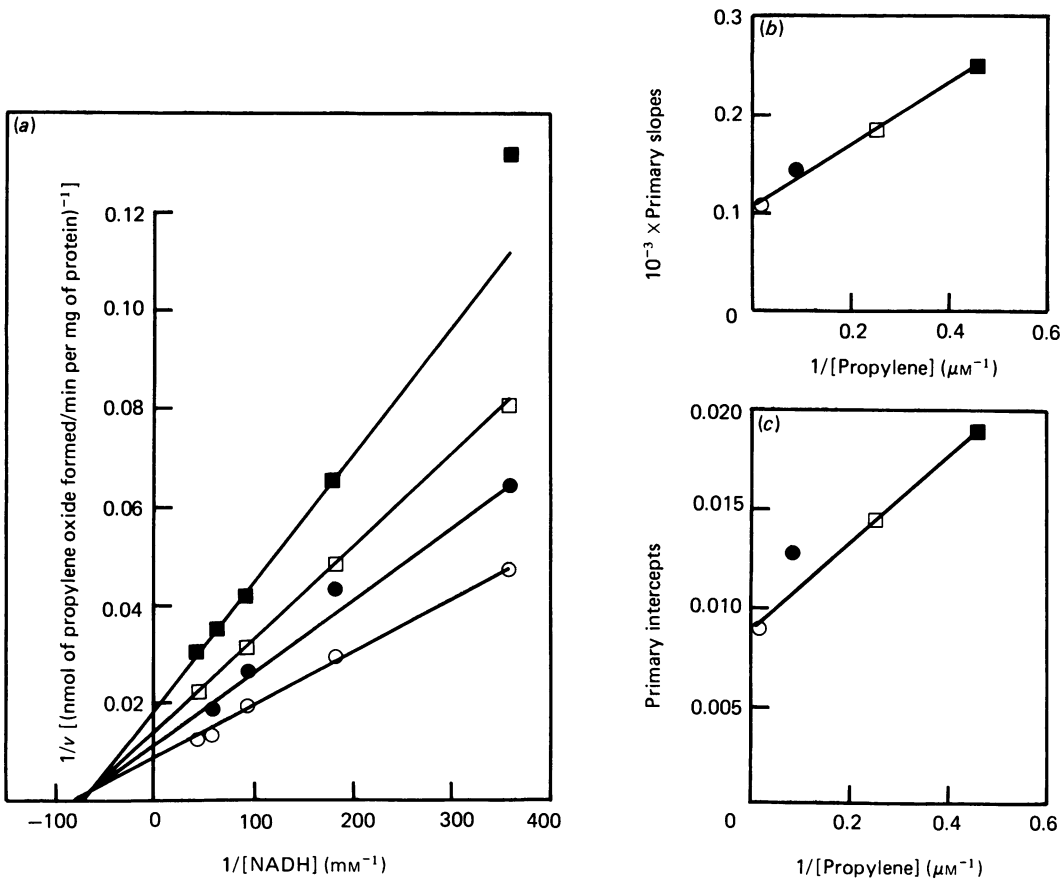


Fig. 1. Steady-state analysis of methane mono-oxygenase at various propylene and NADH concentrations

The g.l.c. assay was used in these experiments (see the Experimental section). The propylene concentrations were: ○, 119 μM; ●, 12 μM; □, 3.9 μM; ■, 2.2 μM. (*a*) shows Lineweaver–Burk plots of primary data. (*b*) and (*c*) illustrate the secondary plots of the same data. The O_2 concentration was fixed at 125 μM for all these experiments. Linear-regression analysis for the secondary plots gave correlations better than 0.914.

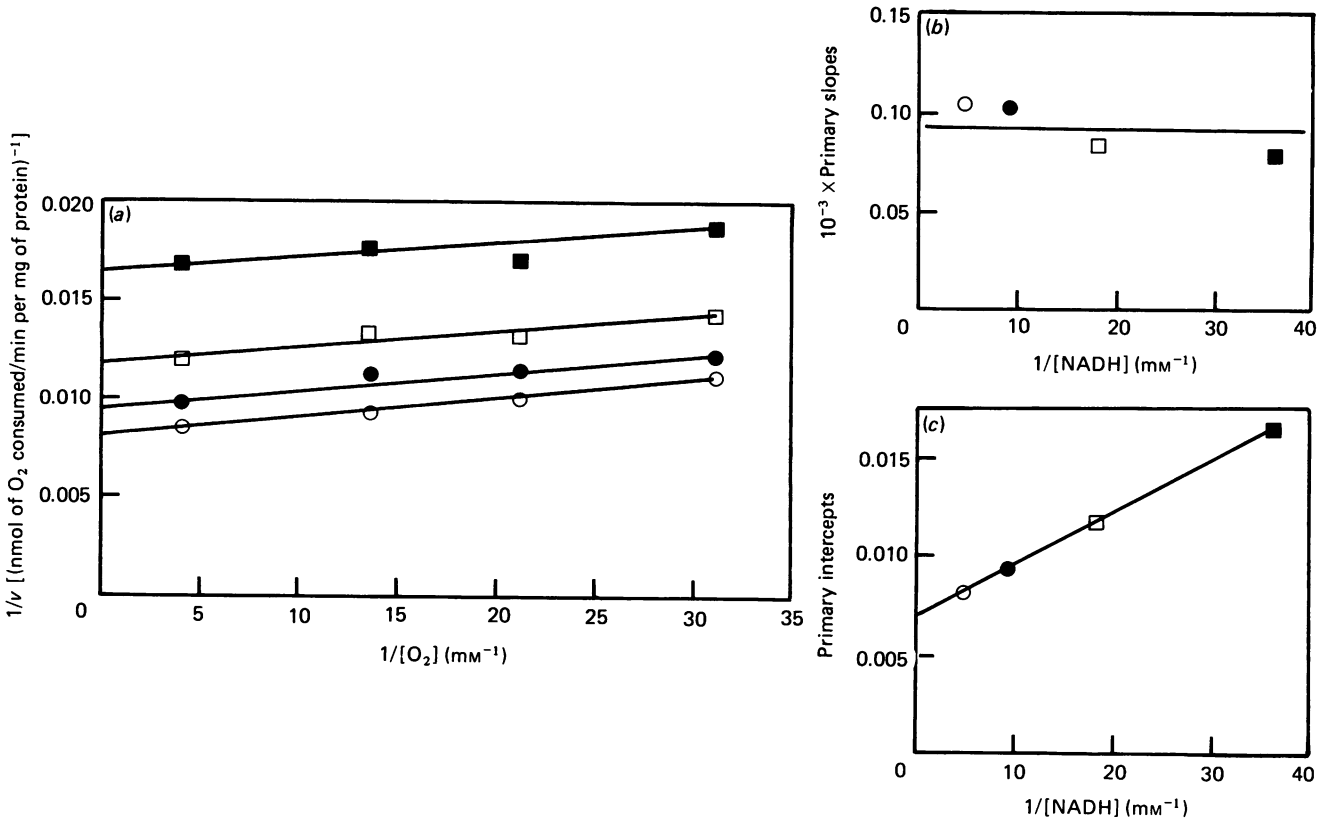


Fig. 2. Steady-state analysis of methane mono-oxygenase at various O_2 and NADH concentration

The polarographic assay was used in these experiments (see the Experimental section). The NADH concentrations were: ○, 0.224 mM; ●, 0.112 mM; □, 0.056 μM; ■, 0.028 mM. (*a*) shows Lineweaver–Burk plots of the primary data. (*b*) and (*c*) illustrate the secondary plots of the same data. The propylene concentration was fixed at 81 μM for all these experiments. Linear-regression analysis for the secondary plots gave correlations better than 0.718 (0.947 for *c*).

assay mixture. After 1 min a sample was removed and the amount of propylene oxide (1,2-epoxypropane) or methanol formed was determined by g.l.c. on a column of Porapak Q (Phase Separations, Queensferry, Clwyd, U.K.) in a Pye–Unicam series 104 chromatograph. The column temperature was 150 °C and the N_2 carrier gas flow rate was 25 ml/min. The detector of the chromatograph was connected to a Hewlett–Packard 3390A integrator (Hewlett–Packard, Geneva, Switzerland). The reactions were linear for at least 3 min under these conditions. Control assays were performed with boiled proteins in order to determine the amount of propylene or methane present in the liquid phase at time zero. Also, samples were removed from the reaction vessels themselves to check the solubilized gas concentrations.

Initial rates were proportional to the amount of the three proteins added, provided that they were each present in excess of 0.5 nmol. Calculations based on the relative specific activities of the individual proteins and their respective M_r values suggest the equimolar amounts of proteins A, B and C used in these assays is similar to the situation in the cell. Also, titrimetric studies indicated that maximal specific activities were obtained when equimolar amounts of the three proteins were present in the assays (results not shown).

Standardization of gases

The concentration of propylene and methane in the liquid phase of assays was determined by g.l.c. by using a column of Porapak T (2.1 m × 4 mm internal diam., temperature 65 °C, carrier gas flow rate 25 ml/min), in a Pye–Unicam 104 gas chromatograph. Standard gas mixtures were prepared by flushing a 1-litre vessel with Ar and then sealing the vessel with a Suba-Seal. A measured amount of propylene or methane was then added via a gas-tight syringe closed with a silicone stopper. Fixed amounts of the test gas/argon mix were then injected by forcing the needle through the silicone stopper and septum of the gas chromatograph on to the Porapak T column, to calibrate the column. In this way loss of gas to the atmosphere between sampling and loading on to the column was prevented. Higher alkanes (C_2H_6 to C_5H_{12}) were standardized in the same way except for liquids, which were injected directly on to a Porapak Q column.

Variation of O_2 concentrations

O_2 concentrations were measured with a calibrated oxygen electrode. Where O_2 concentrations were less than saturating all the components of the assays were repeatedly degassed and flushed with O_2-free N_2 (except for the gas-saturated buffers, where the buffers were degassed and then exposed to propylene or methane). The final O_2-depleted solutions were sealed under N_2 for short-term storage. The O_2 concentration in the final assays could be varied in the oxygen-electrode chamber by combining the O_2-depleted components of the assays with untreated buffers. Whenever O_2 was the substrate

whose concentration was varied the polarographic assay was used to measure the activity, thus giving an accurate determination of O_2 at zero time.

Definition of a unit of enzyme activity

Specific activities are expressed as nmol of NADH or O_2 consumed, or propylene oxide or methanol produced, per min per mg of protein A.

Determination of protein

Protein was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Watford, Herts., U.K.), with bovine serum albumin standards.

RESULTS

Good agreement (within 5%) was obtained among the polarographic, spectrophotometric and g.l.c. methane mono-oxygenase assay systems described in the Experimental section. This is important for the comparison of activities from separate experiments, and also to show that the soluble methane mono-oxygenase is fully coupled and the mono-oxygenase reaction is catalysed to the total exclusion of the oxidase reaction catalysed by proteins A and C (Lund *et al.*, 1985; Green & Dalton, 1985).

The general rate equation for a three-substrate enzyme-catalysed reaction is (Dalziel, 1969):

$$[E]/V_0 = \phi_0+\phi_A/[A]+\phi_B/[B] +\phi_C/[C]+\phi_{AB}/[A][B]+\phi_{AC}/[A][C] +\phi_{BC}/[B][C]+\phi_{ABC}/[A][B][C] \quad (2)$$

The initial-rate equations for other mechanisms may be derived as special cases of this general equation. The kinetic parameters ϕ_0, ϕ_A etc. can be determined graphically by plotting the reciprocal of the initial rate against the reciprocal of the concentration of each substrate at fixed concentrations of the other two (Dalziel, 1969).

The physiological substrate for methane mono-oxygenase is methane, which is oxidized to methanol (eqn. 1). Since methanol is also a substrate for methane mono-oxygenase (Colby *et al.*, 1977), product-inhibition studies would prove impossible with methane as a substrate for the enzyme. It is for this reason that our initial studies were performed with propylene as the hydrocarbon substrate, since the product, propylene oxide (Colby *et al.*, 1977), is not further metabolized by the enzyme.

Steady-state analysis with propylene as substrate

Lineweaver–Burk plots in which the concentrations of propylene and NADH were varied at a fixed concentration

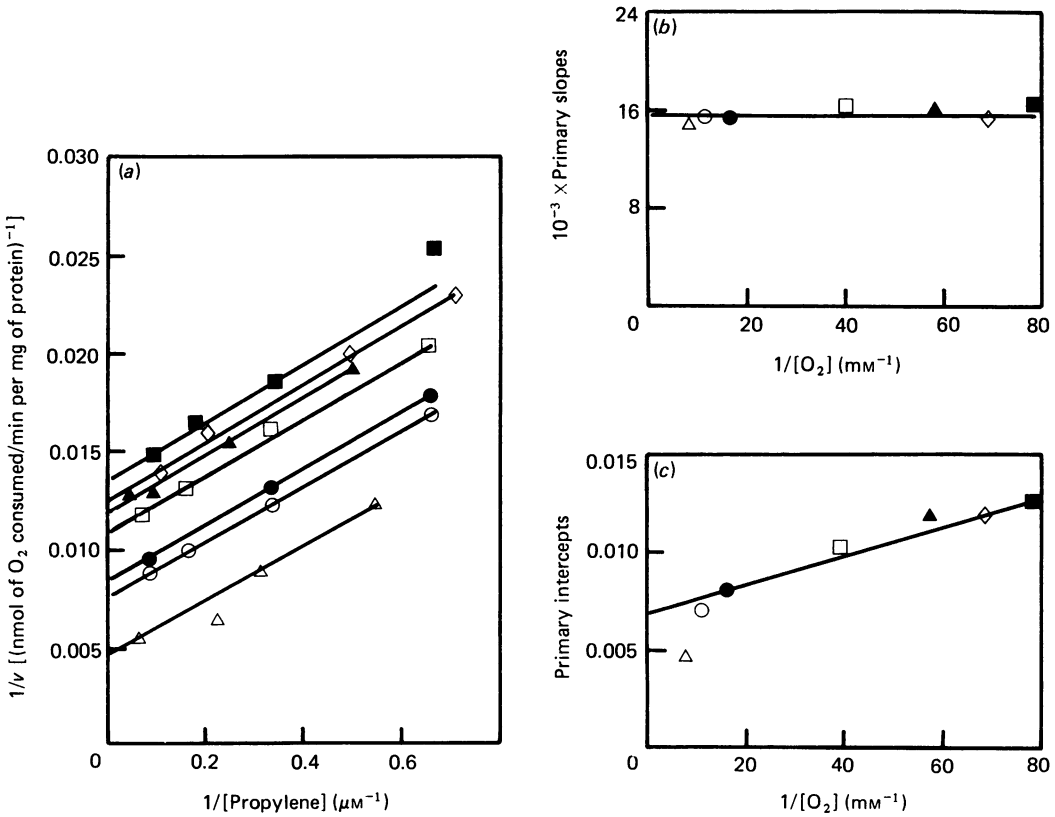


Fig. 3. Steady-state analysis of methane mono-oxygenase at various propylene and O_2 concentrations

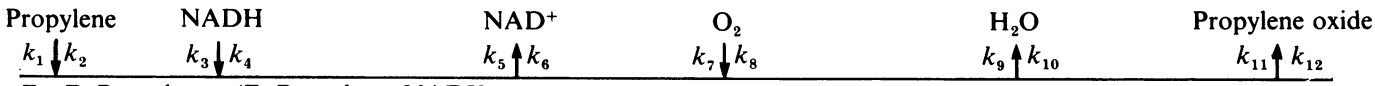
The polarographic assay was used in these experiments (see the Experimental section). The O_2 concentrations were: △, 130 μM; ○, 94 μM; ●, 63 μM; □, 25 μM; ▲, 17.2 μM; ◇, 14.5 μM; ■, 13 μM. (*a*) shows Lineweaver–Burk plots of the primary data. (*b*) and (*c*) illustrate the secondary plots of the same data. The NADH concentration was fixed at 94 μM for all these experiments. Linear-regression analysis for the secondary plots gave correlations better than 0.891 (0.943 for *c*).

of O_2 are shown in Fig. 1. A converging pattern of lines was observed intersecting at a point whose horizontal coordinate is -66 mM^{-1}. Two other sets of Lineweaver–Burk plots illustrate results obtained when the concentrations of O_2 and NADH were varied at a fixed propylene concentration (Fig. 2) and when the concentrations of O_2 and propylene were varied at a fixed NADH concentration (Fig. 3). Both these latter series yielded sets of parallel lines.

The initial-rate equation that fits these results has the form:

$$[E]/V_0 = \phi_0 + \phi_{Propylene}/[Propylene] + \phi_{NADH}/[NADH] + \phi_{O_2}/[O_2] + \phi_{Propylene,NADH}/[Propylene][NADH] \quad (3)$$

Reaction schemes that conform to this initial-rate equation must include a ternary complex of enzyme, propylene and NADH (Dalziel, 1969). One mechanism that is consistent with these constraints is:



(4)

This type of reaction is known as a concerted-substitution type IIb mechanism (Dalziel, 1969) or a Bi Uni Uni Bi Ping Pong mechanism (Cleland, 1963). A concerted reaction of the enzyme with propylene and NADH occurs to form a reduced form of the enzyme, and this reduced enzyme then reacts with the third substrate, O_2, in a separate step.

The values of the kinetic constants for the reaction can be estimated from the secondary plots in Figs. 1, 2 and 3 and are summarized in Table 1. It is clear that methane mono-oxygenase has low K_m values for both propylene and O_2 and therefore a high affinity for both these substrates.

Steady-state analysis of methane mono-oxygenase with methane as substrate

A similar steady-state kinetic analysis of methane mono-oxygenase with the physiological substrate, methane, was performed. The primary data yielded patterns of converging and parallel lines similar to those described for propylene (Figs. 1, 2 and 3). The kinetic constants obtained from secondary plots (Figs. 4*a*, 4*b*, 4*c* and 4*d*) are summarized in Table 2.

Table 1. Kinetic constants for methane mono-oxygenase with propylene as substrate

The data were obtained from Figs. 1, 2 and 3.

Term	Value	
K_m for propylene	0.94 μM,	—
K_m for NADH	25.2 μM,	25.4 μM
K_m for O_2	15.9 μM,	12.7 μM
$V_{max.}$ (turnover relative to 1 molecule of protein A)	0.67 s^{-1},	0.63 s^{-1}
ϕ_{AB}	0.19 M^2·min,	0.16 M^2·min

Product-inhibition studies

In order to confirm the mechanisms predicted by the initial-rate kinetic analysis, the technique of product inhibition was used with propylene as substrate (for reasons outlined above methane could not be used in these studies). The inhibition patterns obtained were: NAD^+ showed competitive inhibition with respect to O_2, non-competitive inhibition with respect to propylene and mixed competitive/non-competitive with respect to NADH; propylene oxide showed uncompetitive inhibition with respect to O_2, competitive inhibition with respect to propylene and non-competitive inhibition with respect to NADH. K_i values obtained from secondary plots of the data are shown in Table 3. The inhibition patterns are consistent with the mechanism shown in eqn. (4) since propylene oxide was a competitive inhibitor with respect to propylene, NAD^+ was a competitive inhibitor with

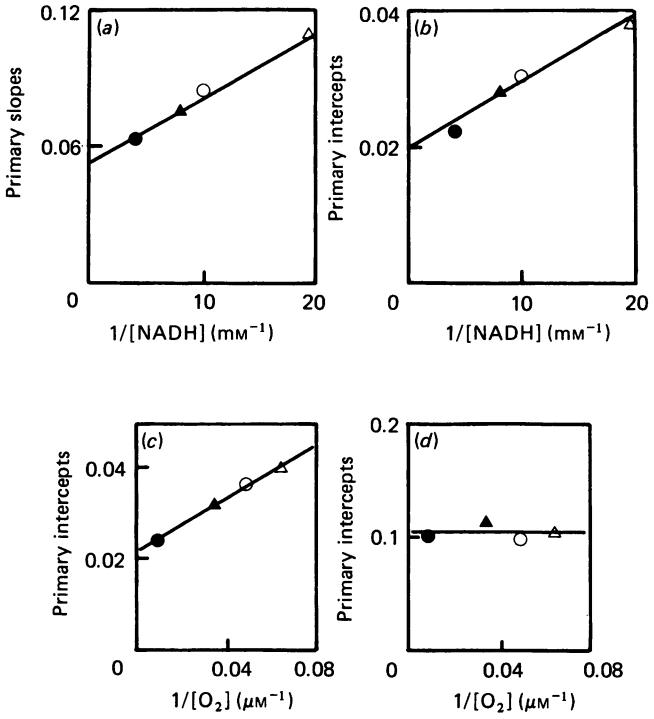


Fig. 4. Steady-state analysis of methane mono-oxygenase with methane as the hydrocarbon substrate

Secondary plots obtained from Lineweaver–Burk plots, in which methane was the substrate whose concentration was varied. For (*a*) and (*b*) the NADH concentration was fixed at 250 μM and the O_2 concentration was set at: ●, 117 μM; ▲, 28.1 μM; ○, 20.4 μM; △, 15.6 μM. For (*c*) and (*d*) the O_2 concentration was fixed at 125 μM and the NADH concentration was set at: ●, 250 μM; ▲, 125 μM; ○, 100 μM; △, 50 μM. Linear-regression analysis showed correlations better than 0.948.

Table 2. Kinetic constants for methane mono-oxygenase with methane as substrate

The data were obtained from secondary plots of initial-rate data expressed as Lineweaver–Burk plots.

Term	Value
K_m for CH_4	3 μM
K_m for NADH	55.8 μM
K_m for O_2	16.8 μM
$V_{max.}$ (turnover relative to 1 molecule of Protein A)	0.19 s^{-1}
ϕ_{AB}	2.65 $M^2 \cdot min$

Table 3. K_i values for product inhibitors of methane mono-oxygenase

K_i values were obtained from the intercept on the horizontal axis of secondary plots of slopes and intercepts of primary Lineweaver–Burk plots (Table 2) plotted against the concentration of the product inhibitor. — indicates the slopes/intercepts of the primary plots were constant, and thus no K_i could be determined from secondary plots.

Inhibitor and varied substrate	$K_{i(slope)}$ (mM)	$K_{i(intercept)}$ (mM)
NAD^+: propylene	2.6	2.9
NAD^+: NADH	3.0	9.7
NAD^+: O_2	2.2	—
Propylene oxide: propylene	0.9	—
Propylene oxide: NADH	0.75	3.8
Propylene oxide: O_2	—	1.5

respect to O_2 and propylene oxide was a non-competitive inhibitor with respect to NADH. These findings support the order of substrate binding illustrated in eqn. (4).

Variation of $K_{m(app.)}$ for NADH with hydrocarbon substrate

In order to provide further evidence for the proposed mechanism for methane mono-oxygenase, use was made of alternative hydrocarbon substrates of the enzyme (Colby *et al.*, 1977). It can be seen from eqn. (4) that if NADH were the first substrate to bind to the enzyme the K_m for NADH should be independent of the second substrate. Evidence to suggest that this was not the case was obtained from the data already presented (Tables 1 and 2), in which the K_m for NADH was 55.8 μM when methane was the hydrocarbon substrate and 25.2 μM with propylene. The $K_{m(app.)}$ for NADH with benzene as substrate, however, was determined to be 78 μM and with methanol as substrate it was 294 μM. This variation in the K_m for NADH with different hydrocarbon substrates could only occur if the hydrocarbon bound to the enzyme before NADH, different hydrocarbon–enzyme complexes having different affinities for NADH. If NADH bound before the hydrocarbon there would not be any variation in the K_m for NADH with different hydrocarbon substrates, and therefore the findings support the mechanism proposed in eqn. (4).

Table 4. Apparent kinetic constants for soluble methane mono-oxygenase for the series of alkane substrates CH_4–C_5H_{12}

Activities were determined by using the g.l.c. assay method (see the Experimental section). The O_2 and NADH concentrations were held constant at 131 μM and 500 μM respectively, and the concentration of hydrocarbon substrate was varied. Data obtained were plotted on Lineweaver–Burk plots, from which $V_{max.(app.)}$ and $K_{m(app.)}$ were determined. Where more than one product was produced, both were summed to calculate the specific activity.

Alkane substrate	$V_{max.(app.)}$ (nmol/min per mg of protein A)	$K_{m(app.)}$ (μM)
Methane	56.0	3.0
Ethane	83.3	60.6
Propane	34.5	12.5
Butane	90.9	500.0
Pentane	10.3	392.0

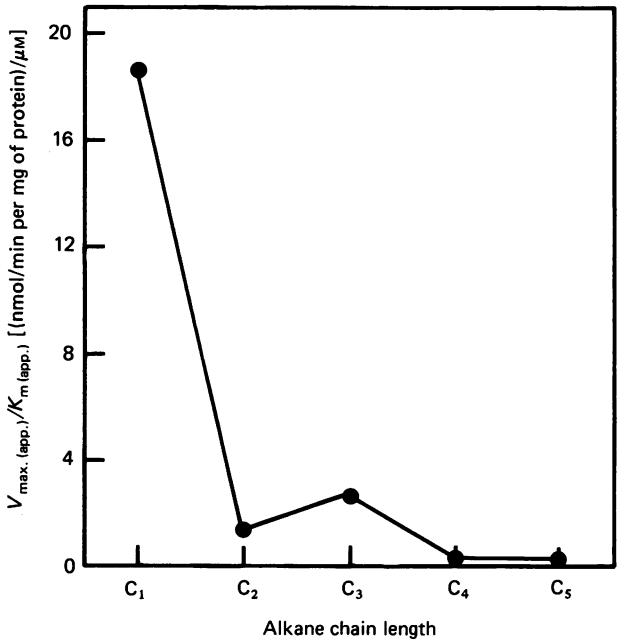


Fig. 5. Relationship between $V_{max(app.)}/K_{m(app.)}$ and carbon chain length of substrate for soluble methane mono-oxygenase

The ratio $V_{max.(app.)}/K_{m(app.)}$ is a measure of the affinity of the enzyme for a particular substrate. Data for this Figure were obtained from Table 4.

Adaptation of methane mono-oxygenase to the oxidation of methane

It is well established that methane mono-oxygenase has the capacity to oxidize a wide range of hydrocarbon substrates (Colby *et al.*, 1977; Stirling & Dalton, 1977); however, only methane is physiologically significant. In order to establish how well methane mono-oxygenase is adapted to oxidizing methane, the $K_{m(app.)}$ and $V_{max.(app.)}$ values for the series of straight-chain alkanes CH_4–C_5H_{12}

were determined. The data presented in Table 4 and Fig. 5 show that the $V_{max.(app.)}/K_{m(app.)}$ ratio for methane is almost 10-fold greater than for the other alkanes considered, indicating that methane mono-oxygenase is a far more efficient enzyme with methane as substrate than with C_2H_6–C_5H_{12} alkanes.

DISCUSSION

Steady-state results reported in this paper suggest that soluble methane mono-oxygenase has the reaction mechanism shown in eqn. (4). This mechanism involves two different ternary complexes but not a quarternary complex of the enzyme and its three substrates. Similar mechanisms have been proposed for a number of hydroxylases (White-Stevens *et al.*, 1972; Nakamura *et al.*, 1970; Spector & Massey, 1972; Strickland & Massey, 1973). An alternative mechanism consistent with the initial-rate data is Dalziel's (1969) concerted-substitution type IIc mechanism or Cleland's (1963) Bi Bi Uni Uni Ping Pong mechanism; however, this mechanism is inconsistent with other facts known about the enzyme, as it would require the release of both products from the first ternary complex before the addition of the third substrate. This clearly cannot be the case here, as methane must be bound in a ternary complex with O_2, since molecular O_2 is the source of the oxygen atom incorporated into methane to yield methanol.

The nature of the activated oxygen species in the catalytic cycle of methane mono-oxygenase is unknown. However, it has been demonstrated that free peroxide is not involved in hydroxylation and that O_2 reacts with protein A (Green & Dalton, 1985). This effectively rules out the possibility of a peroxyflavin intermediate (Poulsen & Ziegler, 1979). Activation of O_2, in many cases, may be regarded as irreversible (Hayaishi, 1974); however, we have no evidence to suggest whether this is or is not the case for the soluble methane mono-oxygenase system.

The K_m determined for methane was low at 3 μM, a previous estimate for $K_{m(app.)}$ for methane in crude extracts of *M. capsulatus* (Bath) being 160 μM (Colby *et al.*, 1977). The new value may be of great physiological significance, since it would allow *M. capsulatus* (Bath) to oxidize methane even when the substrate was present at low concentrations in Nature. Also, the low K_m determined for O_2 means the organism can continue to oxidize methane, leading to energy generation via methanol, formaldehyde and formate dehydrogenases, when both methane and O_2 concentrations are low. Estimates made *in vivo* of the K_m for methane of particulate methane mono-oxygenase (Joergensen, 1985) of a methanotroph, strain OU-4-1, support the low K_m values reported here. Joergensen (1985) determined the K_m for methane to be 1 μM and the K_m for O_2 to be 0.14 μM. This suggests that earlier estimates of K_m for methane were high (Colby *et al.*, 1977; Joergensen & Degn, 1983).

Product-inhibition studies provided evidence to support the proposed mechanism. Product-inhibition data have to be considered with caution, since the inhibitor may bind to more than one form of the enzyme; this may be the case with NAD^+, resulting in a mixed-type inhibition with respect to NADH.

Further support for the proposed mechanism was obtained with the observation that the K_m for NADH was affected by the nature of the hydrocarbon substrate. Clearly, if NADH were the first substrate to bind to the enzyme, this would not be the case. The use of alternative substrates in this way can be a powerful tool for distinguishing between various mechanisms (Engel, 1977). It is noted that the high K_m for NADH (294 μM) observed with methanol as the substrate indicates that this compound is not oxidized *in vivo* by methane mono-oxygenase, as has been proposed (Cornish *et al.*, 1984), given the high activities of methanol dehydrogenase present in the cells. Also, by decreasing the affinity of methane mono-oxygenase for NADH, the dissociation of the enzyme–methanol complex is favoured rather than the further oxidation of methanol by methane mono-oxygenase. This is important in conserving energy for the cell, since methanol dehydrogenase generates energy whereas methane mono-oxygenase consumes it, in the form of NADH.

A common characteristic of other enzymes sharing a Bi Uni Uni Bi Ping Pong mechanism is that the enzyme–substrate complex is much more readily reduced by NADH than is the enzyme alone (Nakamura *et al.*, 1970; Strickland & Massey, 1973). Experiments to investigate this phenomenon with methane mono-oxygenase have not yet been performed. However, it has been shown that electron transfer can occur aerobically in the absence of a hydrocarbon substrate from protein C to protein A (Lund *et al.*, 1985), and that this occurs at a rate sufficient to support the rate of turnover of protein A catalysing the oxidation of methane. In order to be certain that the results reported by Lund *et al.* (1985) are a full representation of kinetics of electron transfer during methane oxidation the experiments must also be performed anaerobically [since some of the methane mono-oxygenase may be acting in an uncoupled manner under the conditions used (Green & Dalton, 1985)], and aerobically in the presence of methane.

In conclusion, this paper presents evidence to suggest that the reaction mechanism of methane mono-oxygenase takes the following form. Methane binds to the enzyme, followed by NADH, which reacts to yield a reduced form of the enzyme and the release of NAD^+. This means proteins A and C act in a co-operative manner with respect to substrate binding. The reduced form of the enzyme then binds O_2 to form a second ternary complex, which breaks down to release the products water and methanol (eqn. 4). Thus the enzyme can control the supply of electrons to the active site to coincide with the arrival of methane. In this way reducing equivalents, which may be in short supply for cells growing on methane (Anthony, 1982), can be conserved.

The mechanism proposed here is different to that put forward by Joergensen (1985) for particulate methane mono-oxygenase, where a random bi-reactant mechanism was proposed in which the binding of one substrate to the enzyme decreases its affinity for the other substrate. This provides further evidence that the soluble and particulate methane mono-oxygenases are quite separate and distinct enzymes.

Results obtained with the series of alkanes CH_4–C_5H_{12} showed methane mono-oxygenase to be well adapted to the oxidation of methane despite its well-established lack of substrate specificity (Colby *et al.*, 1977). Methane was oxidized more efficiently than any of the other alkanes in this series, suggesting the lack of specificity may be an

evolutionary by-product of the optimization of conditions for methane oxidation.

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