A stopped-flow kinetic study of soluble methane mono-oxygenase from *Methylococcus capsulatus* (Bath)

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1. The roles of the three protein components of soluble methane mono-oxygenase were investigated by the use of rapid-reaction techniques. The transfer of electrons through the enzyme complex from NADH to methane/O₂ was also investigated. 2. Electron transfer from protein C, the reductase component, to protein A, the hydroxylase component, was demonstrated. Protein C was shown to undergo a three-electron–one-electron catalytic cycle. The interaction of protein C with NADH was investigated. Reduction of protein C was shown to be rapid, and a charge-transfer interaction between reduced FAD and NAD⁺ was observed; this intermediate was also found in static titration experiments. Thus the binding of NADH, the reduction of protein C and the intramolecular transfer of electrons through protein C were shown to be much more rapid than the turnover rate of methane mono-oxygenase. 3. The rate of transfer of electrons from protein C to protein A was shown to be lower than the reduction of protein C but higher than the turnover rate of methane mono-oxygenase. Association of the proteins was not rate-limiting. The amount of protein A present in the system had a small effect on the rate of reduction of protein C, indicating some co-operativity between the two proteins. 4. Protein B was shown to prevent electron transfer between protein C and protein A in the absence of methane. On addition of saturating concentrations of methane electron transfer was restored. With saturating concentrations of methane and O₂ the observed rate constant for the conversion of methane into methanol was 0.26 s⁻¹ at 18 °C. 5. By the use of [³H₄]methane it was demonstrated that C–H-bond breakage is likely to be the rate-limiting step in the conversion of methane into methanol.

INTRODUCTION

Soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath) is a multicomponent enzyme consisting of three proteins (Colby & Dalton, 1978; Dalton, 1980). The functions of each of the three proteins have been characterized and their chemical and physical properties have been described. Protein A is a non-haem iron protein of *M_r* 210 000 and is the hydroxylase component of the enzyme, interacting with both methane and O₂ (Woodland & Dalton, 1984; Green & Dalton, 1985). It consists of three types of subunit, α (*M_r* 54 000), β (*M_r* 42 000) and γ (*M_r* 17 000), with the site of methane activation located on the α -subunit (Prior & Dalton, 1985). Protein C is an iron–sulphur flavoprotein of *M_r* 42 000 that is responsible for the transfer of reducing equivalents from NADH to protein A (Dalton, 1980; Lund *et al.*, 1985; Lund & Dalton, 1985). Protein B is a low-*M_r* (15 700) single-subunit protein devoid of prosthetic groups, which acts as a powerful regulator of soluble methane mono-oxygenase, possessing the capacity to convert the enzyme from an oxidase into an oxygenase (Green & Dalton, 1985).

A steady-state kinetic study of soluble methane mono-oxygenase was performed earlier and indicated the enzyme operated by Dalziel's concerted-substitution IIB mechanism (Green & Dalton, 1986). Some preliminary stopped-flow data have also been reported for the reduction of protein C by NADH and the transfer of electrons from protein C to protein A (Lund *et al.*, 1985). In the present paper we report a more detailed study of the transfer of electrons through the soluble methane

mono-oxygenase complex and an investigation of the role of protein B in intermolecular transfer.

EXPERIMENTAL

Stopped-flow experiments

All stopped-flow experiments were performed at 18 °C as well as at 25 °C. These temperatures were chosen to maximize the stability of the soluble methane mono-oxygenase proteins during the course of the experiments performed with a Hi-Tech stopped-flow apparatus (Hi-Tech Scientific Ltd., Salisbury, Wilts., U.K.). Fluorescence measurements were made with a 150 W xenon lamp powered by a Hi-Tech LPS 200X power supply.

Basic stopped-flow experiments consisted of mixing various combinations of proteins A, B and C of soluble methane mono-oxygenase (5 μ M) with NADH (5 μ M). Methane was included in the assays by saturating the protein A-free syringe mixture with methane. All reactions were performed in 50 mM-sodium phosphate buffer, pH 7.0. Reactions were monitored by following the reduction or reoxidation of protein C at 465 nm (both FAD and Fe₂S₂ contribute), 550 nm (Fe₂S₂ reduction) and 750 nm (charge-transfer intermediate). Measurement of methanol production was achieved by coupling the methanol produced to 5 units *Candida boidinii* alcohol oxidase (Sigma Chemical Co., Poole, Dorset, U.K.)/ml. The H₂O₂ thus produced was linked to the reduction of the dye 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (10 mM) by horseradish peroxidase (10 units/ml) (obtained from Sigma Chemical

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Co.), the reaction being monitored by following the absorbance at 405 nm. NADH binding to protein C was monitored fluorimetrically at an excitation wavelength of 360 nm, with emitted light being filtered through a 360 nm filter to exclude contributions from the exciting light. Cytochrome *c* reduction was measured at 550 nm.

Solutions were made anaerobic, if necessary by repeated evacuation and gassing with O₂-free N₂. To ensure that the mixtures remained anaerobic, glucose oxidase (Sigma Chemical Co.) (10 units/ml) and glucose (10 mM) were included in the solutions to remove any remaining O₂. All concentrations given in parentheses are post-mixing.

NADH was standardized spectrophotometrically. Protein was determined by using the Bio-Rad protein reagent (Bio-Rad Laboratories, Watford, Herts., U.K.).

Purification of proteins

Proteins A, B and C were purified from cell extracts of methane-grown *Methylococcus capsulatus* (Bath) as previously described (Woodland & Dalton, 1984; Green & Dalton, 1985; Colby & Dalton, 1978).

Materials

All materials were of the best grade available and were obtained from sources previously described (Colby & Dalton, 1978). [³H₄]Methane was obtained from MSD Isotopes, Montreal, Quebec, Canada.

Steady-state assays

Activity of methane mono-oxygenase was determined by g.l.c. with propene, methane or [³H₄]methane as substrate at 45 °C as previously described for ethene oxidation (Colby & Dalton, 1978). The accumulation of 1,2-epoxypropane or methanol respectively was measured.

RESULTS

Role of protein C

The role of protein C in the soluble methane mono-oxygenase complex is to transfer electrons from NADH

to protein A. This is achieved by passing electrons via its FAD and iron-sulphur centres to the iron centre of protein A (Lund *et al.*, 1985). In this sense protein C acts as a two-electron-one-electron transformase, supplying constant-potential single electrons to protein A from the two-electron packets provided by NADH (Lund *et al.*, 1985). This may be done in two ways, with protein C going through either a two-electron-zero-electron catalytic cycle or a three-electron-one-electron catalytic cycle (Lund *et al.*, 1985). Evidence is now presented for the latter scheme. Stopped-flow kinetic experiments were performed as outlined in Table 1. This experiment takes advantage of the ability of protein C to transfer electrons to various electron acceptors, such as cytochrome *c* at the level of FAD (Lund *et al.*, 1985). Cytochrome *c* reduction was observed at 550 nm after stopped-flow mixing of protein C with various ratios of NADH and cytochrome *c*. Reduction of protein C took place more rapidly than did reoxidation by cytochrome *c*, and therefore apparent first-order rate constants for cytochrome *c* reduction by protein C could be calculated.

When the components were mixed in the proportions 1:1:1 (Table 1, Expt. 1), a single phase of cytochrome *c* reduction was observed, with an apparent first-order rate constant of 19.02 s⁻¹. When a second cytochrome *c* molecule was included in the reaction (Table 1, Expt. 2), a second very slow phase of cytochrome *c* reduction was observed. The amounts of cytochrome *c* reduced were approximately equal. Reduction of the second cytochrome *c* molecule by the one-electron-containing form of protein C is far too slow to account for the observed rate of turnover in catalytic experiments (24 s⁻¹ at 20 °C). Therefore a mechanism in which protein C is first reduced by NADH and is then reoxidized by two successive reductions of cytochrome *c* (i.e. cycling of protein C between fully oxidized and two-electron-reduced) is ruled out.

However, when a second equivalent of NADH is included along with the second cytochrome *c* molecule (Table 1, Expt. 3) both equivalents of cytochrome *c* are reduced at a rate approximately equal to the catalytic turnover rate. No slower phase of reduction was

Table 1. Catalysis of cytochrome *c* reduction by protein C of soluble methane mono-oxygenase

Protein C was mixed, anaerobically, with cytochrome *c* and NADH. Cytochrome *c* reduction was monitored at 550 nm at 18 °C in the stopped-flow apparatus. ΔA values represent the relative change in absorbance observed in each phase of the experiments.

Experiment	First phase		Second phase	
	Rate (s ⁻¹)	Rel. ΔA	Rate (s ⁻¹)	Rel. ΔA
1. Protein C (8.5 μ M) NADH (8.5 μ M) Cytochrome <i>c</i> (8.5 μ M)	19.02	1576		
2. Protein C (8.5 μ M) NADH (8.5 μ M) Cytochrome <i>c</i> (17 μ M)	16.23	2117	1.62	1893
3. Protein C (8.5 μ M) NADH (17 μ M) Cytochrome <i>c</i> (17 μ M)	19.18	3625		
4. Protein C (4.25 μ M) NADH (8.5 μ M) Cytochrome <i>c</i> (17 μ M)	5.87	2656	0.57	853

Fig. 1. Catalytic cycle of protein C

observed. Hence two equivalents of NADH were required to reduce two cytochrome *c* molecules at a rapid rate. The most likely mechanism for this phenomenon is that the second NADH is further reducing the one-electron form of protein C to a three-electron reduced form, which then reacts rapidly with the second cytochrome *c* molecule.

Since the product of the latter reaction would be the two-electron reduced form, such a mechanism predicts that, if four cytochrome *c* molecules and two NADH molecules per protein C molecule are involved in the reaction, three of the four cytochrome molecules will be rapidly reduced and the fourth will be slowly reduced (Table 1, Expt. 4). The results obtained show this prediction to be correct: 75.7% of the absorbance change occurs in the fast phase of the reaction ($k' = 5.87 \text{ s}^{-1}$) and the rest in a slow second phase ($k' = 0.57 \text{ s}^{-1}$). Therefore, during turnover, protein C cycles between a three-electron reduced form and a one-electron reduced form (Fig. 1).

Interaction of protein C with NADH

Some stopped-flow kinetic studies have been performed previously (Lund *et al.*, 1985), which indicated that protein C equilibrated rapidly with NADH followed by a slow reduction of protein C. We have extended those previous studies by investigating in more detail the binding of NADH to protein C, the reduction of the flavin and iron-sulphur centres of protein C, the possible presence of charge-transfer intermediates and the breakdown of the reduced-protein C-NAD⁺ complex.

The binding of NADH to protein C was investigated fluorimetrically as described in the Experimental section. The results indicated that NADH bound rapidly to protein C with a second-order rate constant of $40 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$.

The reduction of protein C by NADH was also rapid (Fig. 2), with a second-order rate constant of

Fig. 2. Interaction of protein C with NADH

- $$\left. \begin{aligned} k_1 &= 40 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \\ k_2 &= \text{approx. } 0 \text{ s}^{-1*} \\ k_3 &= 2.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \\ k_4 &= \text{approx. } 0 \text{ s}^{-1*} \\ k_5 &= 76 \text{ s}^{-1} \\ k_6 &= 1 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1} \\ k_7 &= 3.6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1\dagger} \\ k_8 &= 1 \text{ s}^{-1\dagger} \end{aligned} \right\} k_{\text{diss.}} = 80 \times 10^{-3}$$

* Within the limits of detection of this system.

† Intramolecular transfer appears to be very fast and the rate measured reflects the rate of reduction of FAD by NADH (hence second-order rate constant).

‡ Obtained from the biphasic oxidation of cytochrome *c* (see the Results section).

$2.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. The data indicate there was no pre-equilibration of NADH with protein C. This contrasts with the observations by Lund *et al.* (1985), where pre-equilibration was observed.

During the investigations of protein C reduction by NADH the appearance of a new absorption band was noted, centred about 750 nm. The rate of disappearance of this new absorption was 67 s^{-1} and was independent of the concentration of NADH ($5\text{--}200 \mu\text{M}$). It seemed probable that this absorbance was associated with a charge-transfer interaction between the reduced FAD of protein C and NAD^+ . The decay of the absorption band at 750 nm could be attributed to the dissociation of NAD^+ from the complex.

Indeed, if the 750 nm absorbance is due to a charge-transfer intermediate, it should be possible to observe this species in static titrations. When an anaerobic solution of reduced protein C was added to NAD^+ a new absorbance band did appear. The similarity of the two spectra suggest they were the same species. Fig. 3 shows data obtained by titrating reduced protein C with NAD^+ . The K_{diss} , determined for the dissociation of NAD^+ from reduced protein C was 80×10^{-3} M.

Observation of the reduction of protein C at 550 nm allowed the reduction of the iron-sulphur centre of protein C to be investigated. The reduction proceeded with a second-order rate constant of $3.6 \times 10^{-6} \text{ M}^{-1} \cdot \text{s}^{-1}$.

Reoxidation of protein C by O_2 was very slow, the apparent first-order rate constant being 0.016 s^{-1} . All the experiments described here were performed both aerobically and anaerobically. The rate constants quoted are for 18°C ; the experiments were also performed at 25°C , but there was no difference in the mechanism of the reactions at the two temperatures.

Fig. 3. Benesi-Hildebrand plot of the titration of protein C_{red.} with NAD⁺

The calculated dissociation constant of the reduced-protein C-NAD⁺ complex was 80×10^{-3} M. The protein C concentration was $6.2 \mu\text{M}$, and the temperature was 18°C .

except that the rate constants measured were higher than those measured at 18°C .

Role of protein A

The role of protein A in the soluble methane mono-oxygenase complex is to effect the oxidation of methane to methanol, in which it acts as the hydroxylase component (Green & Dalton, 1985). The transfer of electrons from the iron-sulphur centre of protein C to the iron centre of protein A is slower than the reduction of protein C, the second-order rate constant for the reoxidation of protein C by protein A being $6.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 4). Pre-equilibration of protein A with protein C does not affect this rate, suggesting that association of the methane mono-oxygenase proteins is unlikely to be limiting the intermolecular electron-transfer rates at the protein concentrations used here. Reoxidation of protein C only proceeds to two-thirds of the absorbance change seen on reduction when equimolar proteins C and A are present. The amount of protein A present in the system does have a small effect on the rate of reduction of protein C, the second-order rate constant being $2.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ in the absence of protein A and $3.8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ in the presence of $5 \mu\text{M}$ -protein A ($5 \mu\text{M}$ -protein C was used in this experiment). The values are the averages for three experiments (each of which consisted of five determinations), which lay within $\pm 4\%$ of the values quoted. This provides some additional evidence for the co-operativity between proteins A and C observed during steady-state kinetic studies (Green & Dalton, 1986); however, this small difference in rate is probably insufficient to account fully for this co-operativity, since methane may play a significant role in the steady-state observations.

Role of protein B

The role of protein B in the soluble methane mono-oxygenase complex is to couple NADH oxidation to substrate oxidation (Lund *et al.*, 1985; Green & Dalton, 1985). The effect of protein B on the transfer of electrons from protein C to protein A was investigated. It is well established that protein B can shut down steady-state

Fig. 4. Dependence of the rate constant for transfer of electrons for protein C to protein A on the concentration of protein A

Protein C ($2 \mu\text{M}$) and NADH ($2 \mu\text{M}$) were allowed to react with protein A ($0-6 \mu\text{M}$) in the stopped-flow apparatus. The reoxidation of protein C was monitored at 465 nm (the reoxidation representing the transfer of electrons to protein A). A linear dependence of the reciprocal of the apparent first-order rate constant on the reciprocal of the concentration of protein A was observed with an abscissa intercept of 0, indicating that rapid equilibration of protein C with protein A followed by slow electron transfer is not the mechanism of this reaction. The temperature was 18°C .

electron transfer in the absence of a hydroxylatable substrate (Lund *et al.*, 1985; Green & Dalton, 1985). However, it has been reported that protein B does not affect quantitatively the transfer of electrons between proteins C and A (Lund *et al.*, 1985). The data presented in Fig. 5 illustrate the effect of the concentration of protein B on the rate of intermolecular electron transfer between proteins C and A (both $5 \mu\text{M}$). Protein B slows the rate of electron transfer between proteins C and A. However, if the same experiment is performed in the presence of saturating concentrations of methane the rate of electron transfer when proteins A, B and C are in equimolar concentration rises from 0.35 s^{-1} in the absence of methane to 5.8 s^{-1} . Thus in the presence of methane intermolecular electron transfer, which had been shut down by protein B, is restored, and indeed the rate is higher than that observed in the presence of proteins C and A alone.

By trapping the methanol formed from methane by the complete soluble methane mono-oxygenase complex by using alcohol oxidase and then coupling the H_2O_2 produced by this enzyme to the reduction of a dye by using horseradish peroxidase we have been able to observe the rate of methanol formation by soluble methane mono-oxygenase in a stopped-flow apparatus. This method was necessary since no spectral changes

Fig. 5. Effect of the concentration of protein B on the apparent first-order rate constant for electron transfer from protein C to protein A

Protein C (5 μM), protein B (0–5 μM) and protein A (5 μM) were mixed with NADH (5 μM) at 18 °C. Protein B lowers the rate of electron transfer between protein C and protein A, confirming previous steady-state data (Green & Dalton, 1985).

could be observed in protein A. With saturating concentrations of both O₂ and methane the observed rate constant for the production of methanol was 0.26 s⁻¹ at 18 °C. This value is equivalent to a specific activity of soluble methane mono-oxygenase of approx. 250 munits/mg of protein A (at 45 °C); this value is close to the specific activity for the protein A used in these experiments (approx. 195 munits/mg).

Kinetic isotope effects

It was apparent that the rate-limiting step in the oxidation of methane lay not in the intermolecular electron-transfer reactions between the proteins of the enzyme complex but in the conversion of methane into methanol. Thus possible limiting steps were the breakage of the C–H bond, the activation of O₂ or the release of the product, methanol. Therefore steady-state assays were performed with methane and [2H₄]methane as substrates for soluble methane mono-oxygenase to assess the contribution of C–H-bond cleavage to the rate-determining step in the conversion of methane into methanol. A kinetic isotope effect of $K_H/K_D = 11.8$ was measured, indicating that C–H-bond cleavage is probably the rate-limiting step in methane oxidation.

DISCUSSION

Many aromatic and alkane oxygenases are multi-component enzymes that utilize external electron donors, usually NAD(P)H. One or more of the proteins of which these enzymes are composed form a short electron-transport chain from the electron donor to the oxygenase protein of the enzyme, usually via two redox centres. The two redox centres may be contained within the one protein, as is the case for protein C of soluble methane mono-oxygenase (Colby & Dalton, 1978, 1979). Others, such as the benzene dioxygenase of *Pseudomonas putida* (Axcell & Geary, 1975), have the two redox centres on two separate proteins. The function of these electron-

transport chains may be to take the high-potential electron pairs from NAD(P)H and convert them into constant-potential single electrons and pass these to the redox centre of the oxygenase component of the enzyme (Kamin *et al.*, 1980). Thus protein C acts as a two-electron–one-electron transformase (Hemmerich, 1977; Lund *et al.*, 1985), with the FAD centre accepting two electrons from NADH, but the Fe₂S₂ centre can only accept one electron from FAD (Lund *et al.*, 1985).

Several two-electron–one-electron transformases have been proposed to operate a three-electron–one-electron catalytic cycle (Siegel *et al.*, 1972; Lambeth & Kamin, 1977). It has been suggested that protein C may operate with either a three-electron–one-electron catalytic cycle or a two-electron–zero-electron cycle (Lund *et al.*, 1985), both cycles fitting the data available at that time. Making use of the capacity of protein C to reduce a number of electron acceptors, including cytochrome *c* at the level of FAD, the data in Table 1 indicate that protein C undergoes a three-electron–one-electron catalytic cycle, and we suggest that this is probably the case when protein C is operating as part of the soluble methane mono-oxygenase complex (Fig. 1). Protein C first reacts with NADH with an apparent first-order rate constant of 38 s⁻¹ to produce NAD⁺–protein C–FAD–Fe₂S₂ species, a form containing two electrons in the reduced flavin of protein C as a charge-transfer complex with NAD⁺ that decays with a rate constant of 76 s⁻¹. Analysis of the kinetics of the reduction of protein C by NADH indicates that there was no rapid pre-equilibration between NADH and protein C before reduction, as was previously suggested (Lund *et al.*, 1985), but rather NADH binds to protein C very rapidly, but leaves very slowly.

The transfer of one electron from the fully reduced FAD to the Fe₂S₂ centre of protein C yields the species protein C–FAD–Fe₂S₂. The rate of this intramolecular transfer is rapid and appears to be limited by the rate of reduction of FAD by NADH. The semiquinone form of protein C has been observed previously (Lund & Dalton, 1985). This species is now capable of reducing two equivalents of cytochrome *c*. When presented with a single cytochrome *c* moiety a single-phase rapid reduction (approx. 19 s⁻¹) of cytochrome *c* was observed, equivalent to the oxidation of the flavin centre of protein C; the Fe₂S₂ centre remains reduced. However, when a second cytochrome *c* molecule is present a second slow phase of cytochrome *c* reduction is observed ($k = 1.62$ s⁻¹), equivalent to the transfer of the electron from the Fe₂S₂ centre back to FAD and then to cytochrome *c*. The one-electron species is thus only slowly reactive with cytochrome *c*. However, inclusion of a second equivalent of NADH allows rapid reduction of second and third cytochrome *c* molecules, indicating that the reduction of the one-electron species of protein C by NADH proceeds rapidly.

Thus NADH–cytochrome *c* reduction is catalysed by protein C of soluble methane mono-oxygenase cycling between one-electron and three-electron reduced forms. Fully oxidized protein C is only present before the reaction of the first NADH molecule or after slow oxidation after the reaction of the last NADH molecule. The first NADH molecule thus primes the catalytic cycle as indicated in Fig. 1.

When protein C is functioning as part of the soluble methane mono-oxygenase, we propose that it operates the same three-electron–one-electron catalytic cycle, only

in this case electrons are transferred from the Fe_2S_2 centre of protein C to protein A of soluble methane mono-oxygenase (represented by $\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$ in Fig. 1).

The transfer of electrons from protein C to protein A proceeds at a lower rate than does the reduction of protein C. The reoxidation of protein C by protein A does not reach completion, since only two-thirds of the absorbance change at 465 nm is recovered. This provides additional evidence for protein C cycling through three-electron-one-electron forms, the remaining absorbance representing the one-electron reduced form of protein C.

Protein B shuts down electron transfer between protein C and protein A, thus confirming observations made during a steady-state kinetic study of this enzyme (Green & Dalton, 1986). Electron transfer is restored in the presence of methane and proceeds with an apparent first-order rate constant of 5.9 s^{-1} . The conversion of methane into free methanol occurs with an apparent rate constant of 0.26 s^{-1} and thus appears to be the rate-limiting step of the enzymic conversion. It is not known if the actual rate-limiting step is the activation of O_2 , the cleavage of the C-H bond or the release of the product, methanol. The K_{diss} for the enzyme-methanol complex was $4.2 \times 10^{-3} \text{ M}$; although this tells nothing of the rate of dissociation, it is perhaps unlikely that product release is rate-limiting, since this value compares with that for the reduced-protein C-NAD⁺ complex, which breaks down quite readily. The kinetic isotope effect measured here indicates that C-H-bond cleavage is probably the rate-limiting step in methane oxidation by soluble methane mono-oxygenase. A kinetic isotope effect for methane oxidation of $K_{\text{H}}/K_{\text{D}} = 5$ was observed for a solubilized

particulate methane mono-oxygenase (Gvozdev *et al.*, 1984), again indicating that C-H-bond cleavage is rate-limiting for this reaction.

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