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The interaction between methanol dehydrogenase and the autoreducible cytochromes *c* of the facultative methylotroph *Pseudomonas* AM1

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Cytochromes c_H and c_L were autoreduced at high pH (pK greater than 10) and the autoreduced cytochromes reacted with CO. The autoreduction was first-order with respect to oxidized cytochrome *c* and was reversible by lowering the pH. Pure methanol dehydrogenase reduced cytochrome *c* (in the absence of methanol) by lowering the pK for autoreduction to less than 8.5. A mechanism is proposed for the autoreduction of cytochrome *c* and its involvement in the reaction with methanol dehydrogenase.

The methanol dehydrogenase of methylotrophs is unique in its unusual prosthetic group (Anthony & Zatman, 1967*a*,*b*; Westerling *et al.*, 1979; Salisbury *et al.*, 1979; Duine & Frank, 1980*b*), and it is also unusual in reacting with the electron-transport chain after the level of cytochrome *b* (Anthony, 1975; Bamforth & Quayle, 1978; Netrusov & Anthony, 1979). All that is known about its mechanism is that it may involve a free radical in some conditions (Duine *et al.*, 1979; Duine & Frank, 1980*a*). A direct reaction between pure methanol dehydrogenase and cytochrome *c* has not previously been possible to demonstrate because the cytochrome is reduced by the dehydrogenase even in the absence of added methanol (Anthony, 1975). During the purification of the two cytochromes *c* of *Pseudomonas* AM1 (O'Keeffe & Anthony, 1980) it was found that oxidized cytochrome *c* became rapidly reduced at high pH in the absence of added reductant. The present paper describes this process of autoreduction and concludes that it may be involved in the reaction of methanol dehydrogenase with the cytochromes *c* of methylotrophs.

Materials and methods

Chemicals, preparation of pure cytochromes c and methanol dehydrogenase and measurement of redox potentials

These were as described elsewhere (Anthony & Zatman, 1967*a*; O'Keeffe & Anthony, 1980).

Measurement of autoreduction of cytochromes c_H and c_L and reduction by pure methanol dehydrogenase

Autoreduction is defined for the purposes of the present paper as the reduction of cytochrome *c* occurring at alkaline pH in the absence of added reductant. Autoreduction was initiated by the addition of 20mM buffers to solutions of cytochrome *c* in 30% (v/v) glycerol containing 2–12nmol of cytochrome *c* in a total volume of 1.0ml. Cytochrome *c* (0.7–1.5μM) in 20mM buffers at various pH values was also reduced by the addition of methanol dehydrogenase (0.013–1.5μM) at 22°C under aerobic and anaerobic conditions. Spectra were recorded every minute, and the reduction of the cytochrome was calculated from the $\Delta(A_{550}-A_{575})$. The pH of the cytochrome was measured at the end of each experiment before the addition of $Na_2S_2O_4$ to obtain a value of 100% reduction. The zwitterionic buffers used in these experiments were as described elsewhere (O'Keeffe & Anthony, 1980).

Effects of ferricyanide, mercaptoethanol and methanol dehydrogenase on repetitive autoreduction of cytochrome c_L

Cytochrome c_L (40nmol) in 30% glycerol was autoreduced in buffer at pH 10.15 and then reoxidized with a minimum amount of $K_3Fe(CN)_6$. The ferricyanide was removed by gel filtration on Sephadex G-25 with distilled water, and the cytochrome was again autoreduced in 30% glycerol at pH 10.15. In parallel experiments mercaptoethanol (400nmol) or methanol dehydrogenase (200nmol) was present during the oxidation with ferricyanide; in these experiments the methanol dehydrogenase and ferricyanide were removed by gel filtration on Sephadex G-75 instead of G-25.

Results and discussion

The spectrum and other properties of the pure enzyme were the same as those of methanol

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dehydrogenase from other methylotrophs (Anthony & Zatman, 1967*a*,*b*; Bamforth & Quayle, 1978). The specific activity was 1.2 μmol of methanol oxidized/min per mg of protein. The pH optimum was between pH 9.0 and 9.5, and there was an absolute requirement for added ammonium salts for activity. Isoelectric focusing showed that it was a completely pure basic protein with an isoelectric point of 8.8. The molecular weight was about 120000 as measured by gel filtration, and 60400 when measured by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, indicating that, like other methanol dehydrogenases, it is a dimer.

When the pH of pure cytochromes *c* in water was raised by addition of KOH, the cytochromes *c* became reduced (autoreduction), and addition of HCl to the autoreduced cytochromes reversed this process. The same results were obtained under strictly anaerobic conditions.

The autoreduction of cytochromes c_H and c_L occurred with first-order kinetics for at least 90% of the reaction in the presence of 30% glycerol, which presumably stabilized the autoreduced form of the cytochromes. In the absence of glycerol results were not always reproducible; no more than 25% reduction occurred and rates of autoreduction were lower. The rate constants for autoreduction were independent of the cytochrome *c* concentrations (2–12 μM). The autoreduced cytochromes reacted with CO at similar rates and to similar extents when the cytochromes were reduced by dithionite before reaction with CO (see O'Keeffe & Anthony, 1980).

The first-order kinetics of the autoreduction process and the increase in the rate constants at high pH (Fig. 1) suggest that the internal electron donor for the intramolecular reduction of the haem arises from the dissociated form of a weakly acidic group with a p*K* greater than 10.

Tanaka *et al.* (1978) proposed that the intramolecular autoreduction of cytochrome *f* (a thylakoid-bound *c*-type cytochrome) was due to a thiol group donating an electron to the haem. Cytochromes c_H and c_L differed from the autoreducible cytochrome *f* in that the autoreduction was not inhibited by thiol-blocking reagents (5,5′-dithiobis-2-nitrobenzoate, *p*-chloromercuribenzoate, monoiodoacetate and monoiodoacetamide) and no thiol groups were detected in the cytochromes *c* in the presence of urea or sodium dodecyl sulphate by titrations with 5,5′-dithiobis-2-nitrobenzoate.

Reoxidation of autoreduced cytochrome c_L by ferricyanide at high pH appeared to cause the cytochrome some damage that affected the autoreduction process; after this treatment the rate and extent of autoreduction of the cytochrome at pH 10.15 was diminished. Mercaptoethanol and pure methanol dehydrogenase protected the cytochrome c_L of *Pseudomonas* AM1 against the damaging

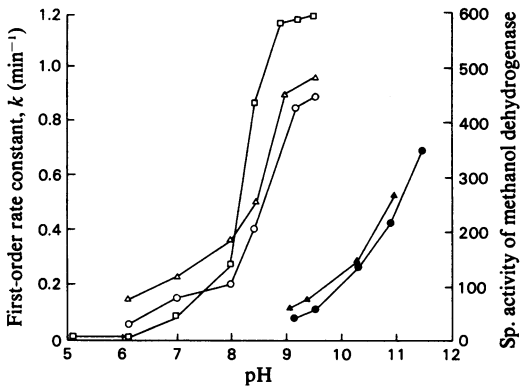
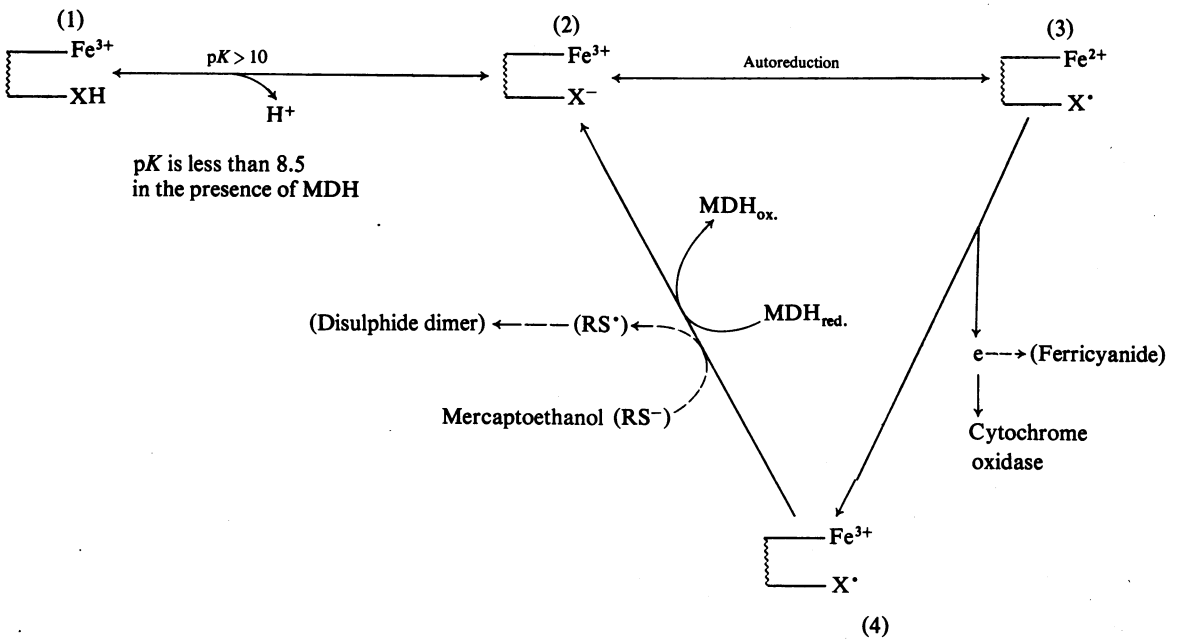


Fig. 1. *Effect of pH on the rate constants for autoreduction of cytochromes c_H and c_L in the presence and absence of methanol dehydrogenase*

The cytochromes (±methanol dehydrogenase) were incubated in the following buffers: acetic acid/sodium acetate (pH 4.0–5.8); sodium phosphate (pH 5.8–6.5); 4-morpholinepropanesulphonic acid (pH 6.5–7.9); 4-(2-hydroxyethyl-1-piperazine-ethanesulphonic acid (pH 6.8–8.2); Tris (pH 7.5–8.8); 2-(*N*-cyclohexylamino)ethanesulphonic acid (pH 9.0–10.0); 3-(*N*-cyclohexylamino)propanesulphonic acid (pH 10.0–11.2). Methanol dehydrogenase activity was assayed as described in the Materials and methods section. □, Methanol dehydrogenase specific activity (nmol of methanol oxidized/min per mg of protein); ○, cytochrome c_H (1.2 μM) autoreduction in the presence of 0.75 μM-methanol dehydrogenase; △, cytochrome C_L (0.7 μM) autoreduction in the presence of 1.13 μM-methanol dehydrogenase; ●, autoreduction of cytochrome c_H (4.0 μM); ▲, autoreduction of cytochrome c_L (6.9 μM).

effects of oxidation by ferricyanide occurring at high pH.

A mechanism involving electron transfer between a dissociable group (XH) and the haem iron of cytochrome *c* that is consistent with all the available evidence is presented in Scheme 1. The essential feature of this scheme is that a free radical is produced and stabilized by sharing an electron with the iron atom; this proposal has the advantage that electron transfer to the iron and stabilization of the resulting radical do not have to be explained separately. The electron-donating group (X^-) must clearly be within the usual atomic distance to the iron, and it is conceivable that it might indeed replace the usual (assumed) methionine as the sixth ligand to the iron. There is considerable evidence that electron transfer between a free radical and iron can occur. Thus the thiyl radical has been proposed as an intermediate in the reversible electron transfer between ferrous and ferric complexes of cysteine during the iron-catalysed oxidation of cysteine



Scheme 1. *Speculative mechanism for the autoreduction of cytochrome c and its involvement in reaction with methanol dehydrogenase*

The electron donor in autoreduction must be a weakly acidic group (XH) dissociating at high pH to give a negatively charged species able to donate an electron to the haem. Species (1) is the undissociated cytochrome *c*; this is dissociated to form species (2) at high pH values (or in the presence of methanol dehydrogenase); species (3) is the radical complex of ferrous iron, isoelectronic with the ferric species (2); species (4) is the ferric form of this radical; it is not autoreducible, but is reducible to the autoreducible species (2) by reaction with mercaptoethanol (RS^-) or methanol dehydrogenase. The damage to the autoreduction mechanism by ferricyanide (at high pH in the absence of mercaptoethanol or methanol dehydrogenase) is presumably because species (4) is unstable. If the autoreduction process is involved in the physiological reaction with methanol dehydrogenase and with cytochrome oxidase, then the ferrous radical species (3) would be oxidized by cytochrome oxidase to the ferric radical (4), which would then be reduced by methanol dehydrogenase to the ferric ion species (2). Abbreviation: MDH, methanol dehydrogenase.

(Mathur *et al.*, 1966; Gray, 1964), and this idea has been extended by Atherton *et al.* (1966) to the mechanism of reduction of rubredoxin.

Both pure cytochromes c_H and c_L were completely reduced by pure methanol dehydrogenase in the absence of glycerol at pH 7.0. Addition of methanol and/or ammonium salts had no effect on the rate or extent of this reduction, and methanol dehydrogenase (4.2 μM) had no effect on the midpoint redox potential of the cytochromes measured at pH 7.0. The dehydrogenase was able to reduce a 54-fold molar excess (at least) of cytochrome c_L, but only a 5.4-fold molar excess of cytochrome c_H. The rate of reduction of the cytochrome *c* in the presence of methanol dehydrogenase was roughly proportional to the dehydrogenase concentration, and it involved a rate-limiting reaction showing first-order kinetics with respect to the oxidized cytochrome. First-order kinetics were demonstrated at all pH values tested, and the variation of the rate constants for cytochrome reduction with varied pH reflected the variation of methanol dehydrogenase activity with varied pH value (Fig. 1). These results suggest that the methanol dehydrogenase reduces the cytochromes by way of the autoreduction mechanism shown to operate at high pH values (see above, Scheme 1). Thus the cytochromes in the presence of methanol dehydrogenase may undergo a conformational change, resulting in a decrease in the p*K* of the group responsible for autoreduction from greater than 10 to less than 8.5 (Fig. 1). That the dehydrogenase caused the reduction of a 54-fold excess of cytochrome c_L must mean that after 'dehydrogenase-induced' autoreduction the cytochrome must remain in the autoreduced state.

It should be emphasized that the reduction of cytochrome *c* by methanol dehydrogenase measured above does not require transfer of electrons from methanol to the cytochrome *c*. The demonstration of methanol-dependent cytochrome *c* reduction with pure methanol dehydrogenase and pure cytochrome *c* is clearly very difficult, because of the auto-

reduction of cytochrome *c* facilitated at physiological pH by methanol dehydrogenase. However, it has been shown that when methanol dehydrogenase is prepared anaerobically from *Hyphomicrobium* X some of its properties are different from the aerobically prepared enzyme (Duine *et al.*, 1978, 1979) and that methanol-independent cytochrome *c* reduction could be observed in anaerobically prepared crude extracts.

In similar preliminary experiments (suggested by Dr. J. A. Duine and Dr. J. Frank) we have confirmed that, in crude anaerobically prepared extracts of *Pseudomonas* AM1, the rate of reduction of cytochrome *c* was greater in the presence of methanol than in its absence.

In our opinion it is probable that the reaction between methanol dehydrogenase and cytochrome *c* described in the present paper is physiologically important, but involvement of other unknown factors in this reaction has not yet been unequivocally ruled out.

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