

FEMSLE 05049

The mechanism of inhibition by EDTA and EGTA of methanol oxidation by methylotrophic bacteria

H.T.C. Chan and C. Anthony

SERC Centre for Molecular Recognition, Department of Biochemistry, University of Southampton, Southampton, UK

Received 5 June 1992

Accepted 24 June 1992

Key words: Methanol oxidation; Methylotrophs; Ethyleneglycol (aminoethylether) tetra-acetic acid; Methanol dehydrogenase; Calcium

1. SUMMARY

Ethyleneglycol (aminoethylether) tetra-acetic acid (EGTA) was shown to be a potent competitive inhibitor of electron transfer between methanol dehydrogenase (MDH) and its electron acceptor cytochrome c_L . Addition of Ca^{2+} ions relieved the inhibition by removal of the inhibitory EGTA. Removal of EGTA by gel filtration completely relieved the inhibition. EGTA did not remove the tightly bound Ca^{2+} present in the MDH. Indo-1, a fluorescent analogue of EGTA, bound tightly to MDH in a 1:1 ratio but not to cytochrome c_L ; binding was prevented by EGTA. It was concluded that EGTA inhibits methanol oxidation by binding to lysyl or arginyl residues on MDH thus preventing docking with cytochrome c_L .

2. INTRODUCTION

Methanol is oxidised in methylotrophic bacteria by a periplasmic quinoprotein methanol dehydrogenase (MDH) which catalyses electron transfer from methanol to its specific electron acceptor, cytochrome c_L [1,2]. In the first description of methanol oxidation by whole cells of the pink facultative methylotroph *Methylobacterium extorquens* [3] it was shown that 0.1 mM EDTA was a specific inhibitor. Because addition of metal ions did not relieve the inhibition, it was suggested that EDTA might act by binding to MDH; however, EDTA (10 mM) had no effect on purified MDH when assayed in a dye-linked assay system [4]. Using crude extracts of *Hyphomicrobium*, it was shown that EDTA inhibits the electron transport chain somewhere between methanol and cytochrome c [5]. This was confirmed when the interaction of MDH with cytochrome c_L was first demonstrated, this reaction being shown to be sensitive to low concentrations of EDTA [6]. This very potent inhibition (K_i , 20 μ M) in the cell-free system was also demonstrated using the complete 'methanol oxidase'

Correspondence to: C. Anthony, SERC Centre for Molecular Recognition, Department of Biochemistry, University of Southampton, Southampton SO9 3TU, UK.

electron transport chain reconstituted in vitro using proteins from the obligate methylotroph *Methylophilus methylotrophus* [7]. Subsequently, in an extensive study using whole cells of this same organism it was suggested that EDTA acts by chelating Mg^{2+} , which was thought to be required for the functional association of methanol dehydrogenase with one or more components of the membrane-bound electron transport chain [8,9]. A further mechanism has also been proposed in which the inhibition occurs by EDTA binding to the cytochrome *c* [10].

Thus three mechanisms of action have been suggested: (i) binding to MDH; (ii) binding to cytochrome *c_L*; and (iii) removal of ions required for binding electron transport proteins together. The recent demonstration that MDH requires a Ca^{2+} ion for activity [11,12] clearly suggests a fourth possibility: that EDTA and related inhibitors act by removal of this essential ion from the enzyme; the present paper explores this possibility.

3. METHODS

Growth of bacteria, and purification and assay of MDH are as described [13]. Purification of cytochrome *c_L*, was as described [14]. Unless otherwise stated proteins and reagents were prepared in calcium-free buffer as described previously [12]. Calcium was determined using a Perkin-Elmer 280 atomic absorption spectrometer. Indo-1 was obtained from Sigma and fura-2 was obtained from Cambridge Biolabs, UK. Fluorescence spectra were recorded on a Perkin-Elmer LS-3B spectrophotofluorimeter at a scan speed of 120 nm/min with fixed excitation and emission slits of 10 nm nominal bandpass. The emission maximum of indo-1 shifted from 470 nm to 405 nm on binding Ca^{2+} . A calibration curve was derived by mixing $CaCl_2$ with indo-1 (0.5 μ M) in 10 mM Ca^{2+} -free Mops buffer at pH 7.0. Binding of indo-1 to MDH was determined by mixing various amounts of wild-type or mutant MDHs to indo-1 and the binding quantified by the 405/470 ratio.

4. RESULTS AND DISCUSSION

4.1. Effect of EDTA and its analogues in the dye-linked assay system

It was confirmed MDH was not inhibited in the dye-linked assay system by any of the chelating agents used in this work (at 15 mM): ethylenediamine tetra-acetic acid (EDTA), ethylene-glycol (aminoethylether) tetra-acetic acid (EGTA), diaminocyclohexane tetra-acetic acid (CDTA), ethylene diamine di-acetic acid (EDDA) and indo-1. Indo-1 is a fluorescent indicator that binds Ca^{2+} in a 1:1 ratio, giving a marked change in fluorescence spectrum; it contains a stilbene fluorophore (coupled to an indole ring) with the tetracarboxylate pattern of liganding groups characteristic of EGTA [15]. Fura-2 is a similar reagent [15].

4.2. Effect of EDTA and its analogues in the cytochrome-linked assay system

Fig. 1 shows the effect of EDTA on reduction of cytochrome *c_L* by MDH. When assayed using Ca^{2+} -free reagents and proteins the I_{50} value was 3 μ M (the concentration required for 50% inhibition). By contrast, when using buffer containing no added calcium but from which Ca^{2+} had not been removed, the ' I_{50} ' value was about 200 μ M. The shape of the curve in Fig. 1 indicates that if EDTA acts by binding to MDH or cytochrome *c_L*

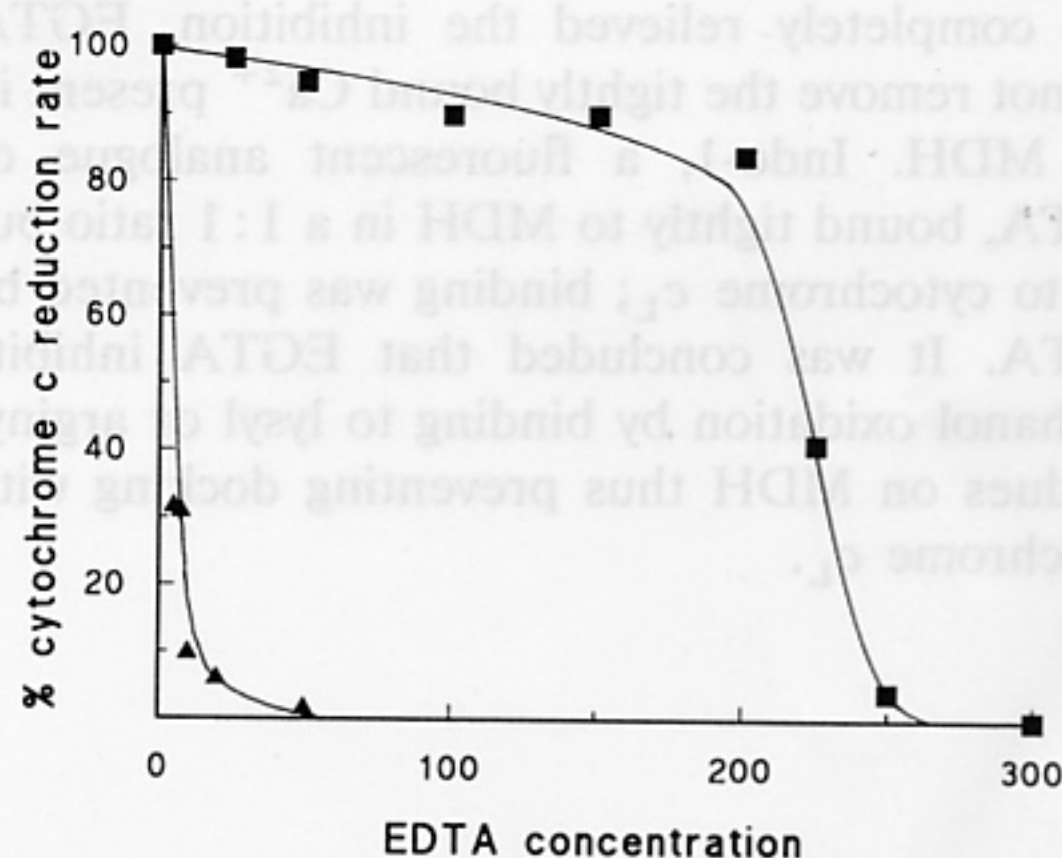


Fig. 1. The effect of EDTA on the reduction of cytochrome *c_L* (2 μ M) by MDH (2 μ M). (■) 12 mM MOPS buffer, pH 7.0; (▲) 12 mM Ca^{2+} -free MOPS buffer.

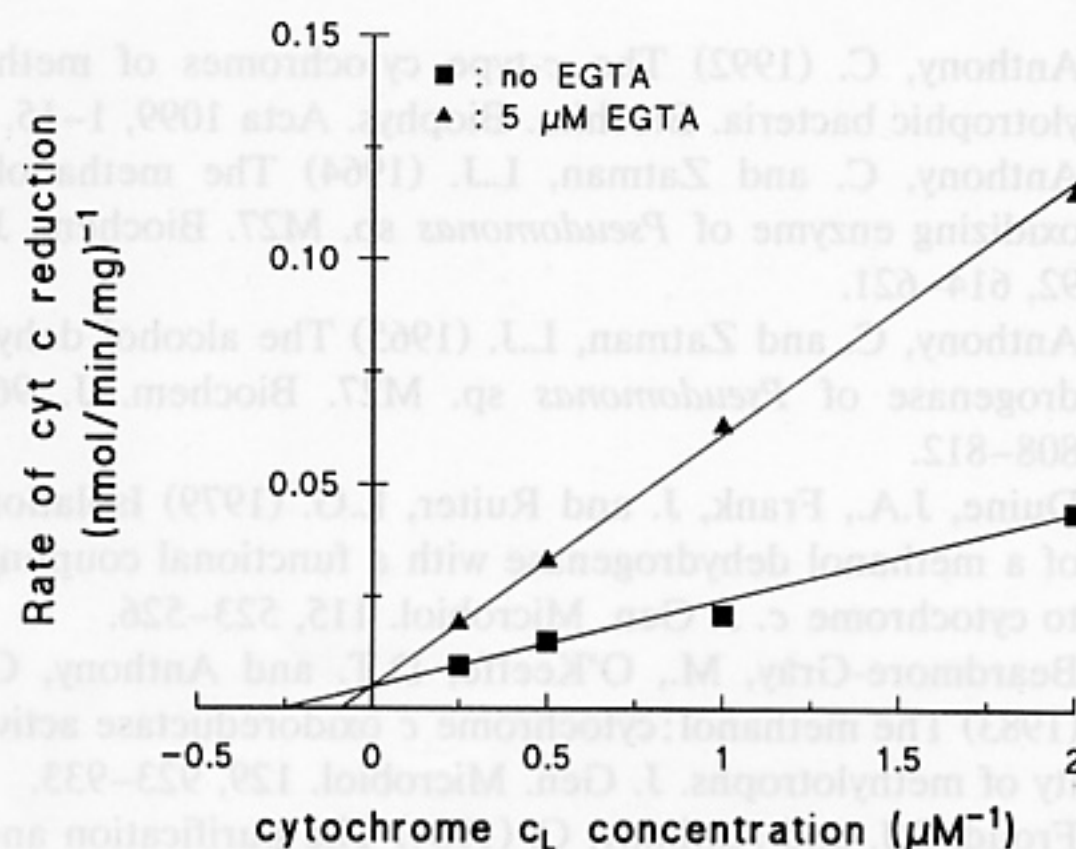


Fig. 2. The effect of EGTA on the affinity of MDH for cytochrome c_L . MDH (2 μM) was measured in the cytochrome-linked assay system in Ca^{2+} -free MOPS buffer as described in MATERIALS AND METHODS. (■), no EGTA, K_m , 3.3 μM . (▲), 5 μM EGTA, K_m , 10 μM .

then its affinity for the protein is less than its affinity for free calcium ions.

Similar curves were obtained for the following analogues (I_{50} values given in parentheses): EGTA (2.5 μM), CDTA (4.0 μM), indo-1 (40 μM) and EDDA (5 mM). This demonstrates that the four carboxylates are necessary for binding to protein or binding to Ca^{2+} . The inhibition by the higher concentration of the diacetic acid derivative (EDDA) is probably due to its high ionic strength ($I = 0.04$); this ionic strength, using a range of other salts has been shown previously to give 50% inhibition of the reaction between MDH and cytochrome c_L [16]. EGTA gave almost identical results to EDTA and is more closely related in structure to indo-1 used elsewhere in this work; EGTA was therefore used for the subsequent experiments described below. Fig. 2 shows that EGTA was a competitive inhibitor with respect to the electron acceptor cytochrome c_L , which is consistent with the suggestion that EGTA inhibits by binding to MDH in such a way as to prevent docking with cytochrome c_L . Similar competitive inhibition was shown by EDTA.

4.3. Reversibility of EGTA inhibition

Addition of Ca^{2+} ions to the assay mixture containing EGTA led to immediate recovery of activity suggesting that EGTA inhibits by removal of Ca^{2+} ions. That this is not the case was

demonstrated by treatment of MDH with a very high concentration of EGTA (10 mM) followed by its removal by gel filtration on a Pharmacia PD10 column. The enzyme completely regained its activity and the concentration of calcium present (1.1 $\text{Ca}^{2+}/\alpha_2\beta_2$ tetramer of MDH) was unaffected. It was concluded that the observed reversal of inhibition by addition of Ca^{2+} to the assay system was due to removal of inhibitory EGTA.

4.4. Reaction with indo-1

Indo-1 inhibited reduction of cytochrome c_L by MDH with an I_{50} value of 40 μM . This reagent is usually used as an assay for free Ca^{2+} , the assay depending on the change in fluorescence emission maximum that occurs on its bind-

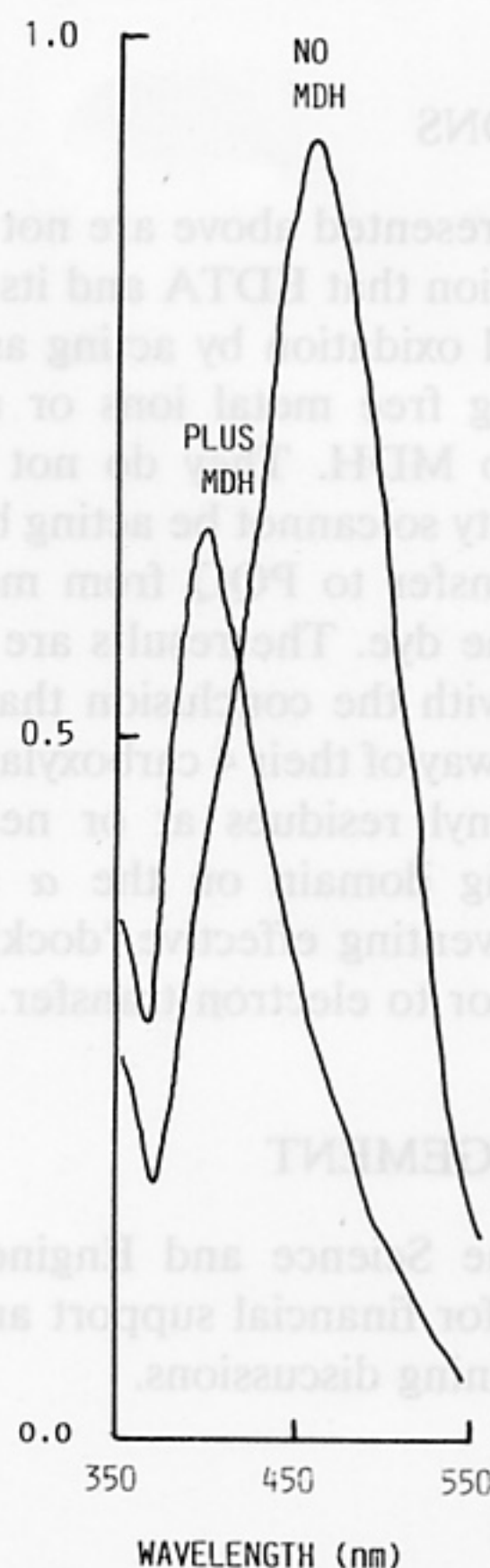


Fig. 3. Fluorescence (emission) spectra of indo-1 and its complex with MDH. Spectra were recorded in 10 mM Ca^{2+} -free MOPS buffer pH 7.0 (excitation wavelength of 355 nm) using indo-1 (0.5 μM) $\pm$ MDH (0.25 μM).

ing to Ca^{2+} [15]. The results in Fig. 3 show that indo-1 is able to bind to MDH. There was no binding of indo-1 to cytochrome c_L . If it is assumed that the magnitude of the fluorescence change on binding to MDH is similar to that when binding to Ca^{2+} then the ratio of indo-1 to MDH in the complex was 0.8–1.05 (1 mol/mol). The binding was inhibited by EGTA (4 μM), showing that the binding site is the same as that causing inhibition by these reagents. That indo-1 was not chelating tightly-bound calcium in MDH was demonstrated by showing that exactly the same change in fluorescence was observed when indo-1 was reacted with MDHs from the mutant strains MoxK and MoxL which contain no calcium [12]. A similar 1:1 ratio of binding was recorded for a second fluorescent analogue, fura-2 (results not shown).

5. CONCLUSIONS

The results presented above are not consistent with the suggestion that EDTA and its analogues inhibit methanol oxidation by acting as chelating agents, removing free metal ions or metal ions tightly bound to MDH. They do not affect the dye-linked activity so cannot be acting by preventing electron transfer to PQQ from methanol or from PQQ to the dye. The results are all consistent, however, with the conclusion that these inhibitors bind by way of their 4 carboxylate residues to lysyl or arginyl residues at or near the cytochrome-binding domain on the α subunit of MDH, thus preventing effective 'docking' of the two proteins prior to electron transfer.

ACKNOWLEDGEMENT

We thank the Science and Engineering Research Council for financial support and Dr. Jon Cox for entertaining discussions.

REFERENCES

- [1] Anthony, C. (1986) The bacterial oxidation of methane and methanol. *Adv. Microb. Physiol.* 27, 113–210.

- [2] Anthony, C. (1992) The *c*-type cytochromes of methylophilic bacteria. *Biochim. Biophys. Acta* 1099, 1–15.
- [3] Anthony, C. and Zatman, L.J. (1964) The methanol-oxidizing enzyme of *Pseudomonas* sp. M27. *Biochem. J.* 92, 614–621.
- [4] Anthony, C. and Zatman, L.J. (1965) The alcohol dehydrogenase of *Pseudomonas* sp. M27. *Biochem. J.* 96, 808–812.
- [5] Duine, J.A., Frank, J. and Ruiter, L.G. (1979) Isolation of a methanol dehydrogenase with a functional coupling to cytochrome *c*. *J. Gen. Microbiol.* 115, 523–526.
- [6] Beardmore-Gray, M., O'Keeffe, D.T. and Anthony, C. (1983) The methanol:cytochrome *c* oxidoreductase activity of methylophilic bacteria. *J. Gen. Microbiol.* 129, 923–933.
- [7] Froud, S.J. and Anthony, C. (1984) The purification and characterization of the *o*-type oxidase from *Methylophilus methylotrophus*, and its reconstitution into a 'methanol oxidase' electron transport chain. *J. Gen. Microbiol.* 130, 2201–2212.
- [8] Carver, M.A., Humphrey, K.M., Patchett, R.A. and Jones, C.W. (1984) The effect of EDTA and related chelating agents on the oxidation of methanol by the methylophilic bacterium *Methylophilus methylotrophus*. *Eur. J. Biochem.* 138, 611–615.
- [9] Carver, M.A. and Jones, C.W. (1984) The role of *c*-type cytochromes in the terminal respiratory chain of the methylophilic bacterium *Methylophilus methylotrophus*. *Arch. Microbiol.* 139, 76–82.
- [10] Frank, J., Dijkstra, M., Balny, C., Verwiel, P.E.J. and Duine, J.A. (1989) Methanol dehydrogenase: mechanism of action. In: PQQ and quinoproteins (Jongejans, J.A. and Duine, J.A., Eds.), pp. 13–22. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- [11] Adachi, O., Matsushita, K., Shinagawa, E. and Ameyama, M. (1990) Purification and properties of methanol dehydrogenase and aldehyde dehydrogenase from *Methylobacillus glycogenes*. *Agric. Biol. Chem.* 54, 3123–3129.
- [12] Richardson, I.W. and Anthony, C. (1992) Characterization of mutant forms of the quinoprotein methanol dehydrogenase lacking an essential calcium ion. *Biochem. J.* (in press).
- [13] Day, D.J. and Anthony, C. (1990) Methanol dehydrogenase from *Methylobacterium extorquens* AM1. *Methods Enzymol.* 188, 210–216.
- [14] Day, D.J. and Anthony, C. (1990) Soluble cytochromes *c* of methanol-utilising bacteria. *Methods Enzymol.* 188, 298–303.
- [15] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3450.
- [16] Cox, J.M., Day, D.J. and Anthony, C. (1992) The interaction of methanol dehydrogenase and its electron acceptor, cytochrome c_L in the facultative methylophilic *Methylobacterium extorquens* AM1 and in the obligate methylophilic *Methylophilus methylotrophus*. *Biochim. Biophys. Acta* 1119, 97–106.