Biochimica et Biophysica Acta, 1016 (1990) 339–343
Elsevier

BBABIO 43193

Inhibition by trimethylamine of methylamine oxidation by *Paracoccus denitrificans* and bacterium W3A1

Victor L. Davidson and M. Arun Kumar

Department of Biochemistry, The University of Mississippi Medical Center, Jackson, MS (U.S.A.)

(Received 6 November 1989)

Key words: Methylamine dehydrogenase; Methylotroph; Quinoprotein; Trimethylamine; (*P. denitrificans*)

Trimethylamine, a common substrate for methylotrophic growth, specifically inhibited methylamine-dependent respiration by *Paracoccus denitrificans* and bacterium W3A1. These effects were caused by the specific inhibition by trimethylamine of the periplasmic quinoprotein methylamine dehydrogenase. Steady-state kinetic analysis of the effect of trimethylamine on methylamine oxidation by methylamine dehydrogenase indicated that the inhibition was a mixed type. Apparent K_i values for trimethylamine of 1.1 mM and 4.7 mM, respectively, were obtained for the *P. denitrificans* and bacterium W3A1 enzymes. Methylamine-dependent oxygen consumption by each bacterium was inhibited either by preincubation of cells with trimethylamine prior to the addition of substrate or by addition of trimethylamine to actively respiring cells. Formate-dependent respiration was not inhibited by trimethylamine. A scheme is proposed which describes a regulatory role for trimethylamine in the metabolism and dissimilation of methylamine by methylotrophic bacteria.

Introduction

Methylamine dehydrogenase is a periplasmic pyrroloquinoline quinone (PQQ)-containing enzyme which catalyzes the oxidation of methylamine to formaldehyde and ammonia. It has been isolated from a variety of methylotrophic and autotrophic bacteria [1–8]. Many of the physical, redox and kinetic properties of the methylamine dehydrogenase from the facultative autotroph, *Paracoccus denitrificans*, have been characterized in this laboratory [3,9–12]. This enzyme reacted with a variety of *n*-alkylamines but not with amino acids or secondary, tertiary or aromatic amines [10]. Similar results [13] have been obtained with the methylamine dehydrogenase from the restricted methylotroph, bacterium W3A1. Those amines which did not react with methylamine dehydrogenase were subsequently assayed for their ability to inhibit methylamine oxidation. In this paper, we report that trimethylamine, which is not a substrate for this enzyme, inhibited the oxidation of methylamine by methylamine dehydrogenases from *P. denitrificans* and bacterium W3A1. The inhibition by trimethylamine of methylamine-dependent respiration by these two bacteria is described as well. In many methylotrophs, methylamine dehydrogenase is synthesized during growth on trimethylamine [14,15] and is required for the complete oxidation of this substrate. As such, the physiological significance of the inhibition of the bacterium W3A1 enzyme is discussed. The evolutionary implications of the analogous inhibition by trimethylamine of the enzyme from *P. denitrificans*, which does not grow on trimethylamine, are also discussed.

Materials and Methods

Bacterial strains and culture conditions

P. denitrificans (ATCC 13543) was grown aerobically at 30 °C in the medium of Kornberg and Morris [16] supplemented with 0.05% $NaHCO_3$, 0.01% yeast extract and 0.3% methylamine-HCl. Bacterium W3A1 (NCIB 11348) was grown aerobically at 30 °C in the medium of Owens and Keddie [17], supplemented with 0.3% methylamine-HCl.

Enzyme purification and assay

Methylamine dehydrogenase was purified from *P. denitrificans* as described previously [3] and from bacterium W3A1 according to Kasprzak and Steenkamp [18]. Methylamine dehydrogenase activity was

Abbreviations: PQQ, pyrroloquinoline quinone; PES, phenazine ethosulfate; DCIP, 2,6-dichloroindophenol.

Correspondence: V.L. Davidson, Department of Biochemistry, The University of Mississippi Medical Center, 2500 N. State St., Jackson, MS 39216-4505, U.S.A.

0005-2728/90/$03.50 © 1990 Elsevier Science Publishers B.V. (Biomedical Division)

assayed spectrophotometrically as described previously [10] with the reoxidant phenazine ethosulfate (PES) and 2,6-dichloroindophenol (DCIP). The rates of reduction of DCIP were monitored at 600 nm and quantitated using an extinction coefficient for 21 500 $M^{-1} \cdot cm^{-1}$. Kinetic data were subjected to non-linear regression analysis with the Enzfitter computer program (Elsevier-BIOSOFT, Cambridge).

Assay of substrate-dependent rates of respiration

Rates of respiration by cell suspensions were measured polarographically at 30 °C with a Clark-type oxygen electrode and a Yellow Springs Instruments Model 5300 Biological Oxygen Monitor. Cells were harvested in mid-log phase growth, and washed and incubated in 10 mM potassium phosphate (pH 7.5) which contained 150 mM KCl. Freshly resuspended cells exhibited high rates of respiration. With these high background rates, it was not possible to measure rates of substrate-stimulated respiration accurately. It was necessary, therefore, to incubate cells for at least 30 min to reduce these basal rates of respiration from endogenous substrates sufficiently to observe substrate-dependent oxygen consumption. To determine the dry weight of cells, aliquots of the cell suspensions which were used in each study were dried in a Savant Speed Vac concentrator and directly weighed on an analytical balance.

Results

Inhibition by trimethylamine of methylamine dehydrogenase activity

The effect of trimethylamine on the catalytic activity of methylamine dehydrogenase was determined from steady-state kinetic experiments which were performed at different fixed concentrations of trimethylamine. The pattern of the family of reciprocal plots which was obtained with the *P. denitrificans* enzyme (Fig. 1A) is characteristic of a mixed-type of inhibition [19] in which trimethylamine is binding to both the free enzyme and the enzyme-substrate complex. Similar results were obtained with the enzyme from bacterium W3A1 (Fig. 1B). Although a clear point of intersection of these reciprocal plots was not obtained in the latter case, this pattern of inhibition is also best described as mixed. Several repetitions of these experiments yielded essentially the same pattern of reciprocal plots. Secondary plots of slope versus trimethylamine concentration yielded apparent K_i values of 1.1 mM and 4.7 mM, respectively, for the enzymes from *P. denitrificans* and bacterium W3A1 (Fig. 2). It should be noted that for mixed inhibition, these are not true K_i values, as the affinities for trimethylamine of the free enzyme and the enzyme-substrate complex may be different. These analyses do, however, provide a means of quantitatively expressing this inhibition and a frame of reference from which to compare the inhibitory effects of trimethylamine on the purified enzymes to the effects of trimethylamine on cellular respiration.

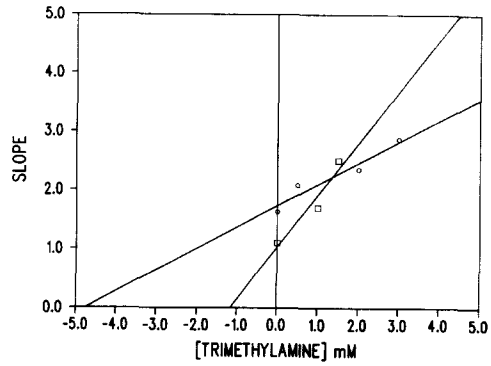

Fig. 2. Secondary plots of data taken from trimethylamine inhibition studies of methylamine dehydrogenase. The two lines represent data obtained with the enzymes which were isolated from (□) *P. denitrificans* and (○) bacterium W3A1. The units of slope (K_m/V) are $\mu M/\mu$mol per min per mg enzyme.

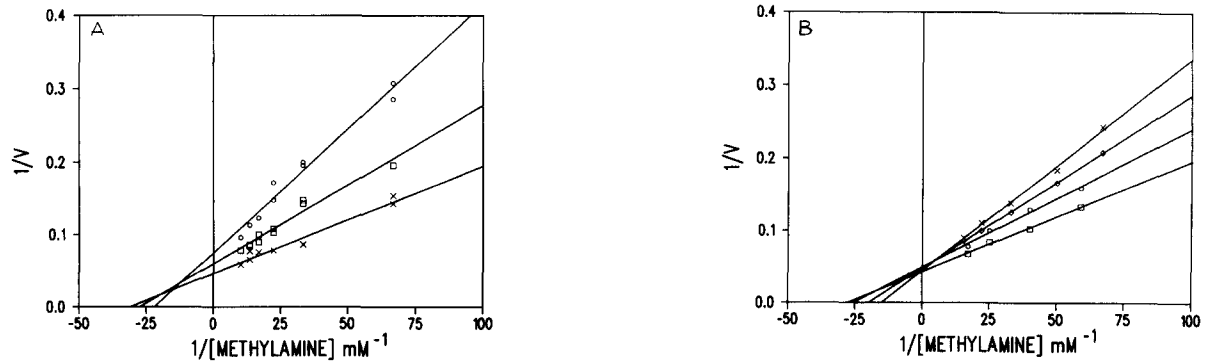

Fig. 1. Double-reciprocal plots of the reaction of methylamine and PES with methylamine dehydrogenase in the presence of trimethylamine. (A) *P. denitrificans* methylamine dehydrogenase. Initial rates of methylamine oxidation were measured at different concentrations of methylamine in the presence of 5 mM PES and (○) 2 mM, (□) 1 mM, and (×) no trimethylamine. (B) Bacterium W3A1 methylamine dehydrogenase. Initial rates of methylamine oxidation were measured at different concentrations of methylamine in the presence of 5 mM PES and (×) 3 mM, (◇) 2 mM, (○) 0.5 mM and (□) no trimethylamine. The units of V are μmol DCIP reduced per min per mg of enzyme.

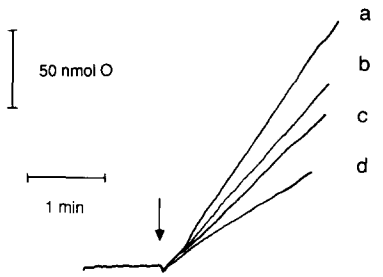

Fig. 3. The effect of preincubation with trimethylamine on methylamine-stimulated respiration by *P. denitrificans*. The arrow indicates the addition of 30 μM methylamine. Prior to addition of methylamine cells were incubated for 5 min with (a) ○, (b) 5 mM, (c) 10 mM and (d) 20 mM trimethylamine.

Inhibition by trimethylamine of methylamine-dependent respiration

As methylamine dehydrogenase is a periplasmic enzyme, it was possible to examine its activity in cells by monitoring rates of methylamine-dependent oxygen consumption and compare these data to the in vitro effects of trimethylamine on purified methylamine dehydrogenase. Methylamine and trimethylamine are small molecules which should be free to diffuse into the periplasm via outer membrane pores. As such, it is reasonable to assume that their concentrations in the periplasm are essentially the same as those in the medium in which the cells are suspended.

Inhibition by trimethylamine of methylamine-stimulated respiration was observed when trimethylamine was preincubated with cells prior to addition of methylamine (Fig. 3). The concentrations of trimethylamine required to inhibit oxygen consumption were dependent upon the concentration of methylamine used to drive respiration (Fig. 4). It was not possible to accurately determine the inhibition by trimethylamine at lower concentrations of methylamine because of low rates of

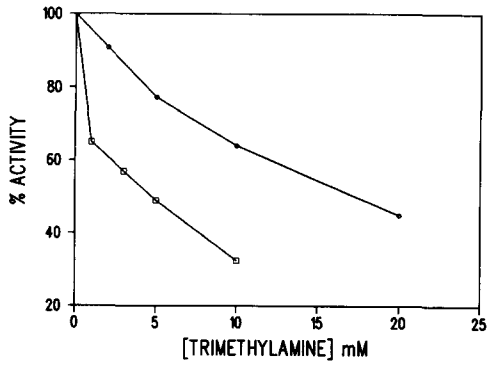

Fig. 4. Inhibition of enzyme activity and methylamine-dependent respiration by trimethylamine. Traces represent the rates of oxygen consumption by suspensions of *P. denitrificans* which were preincubated with trimethylamine for 5 min prior to the addition of methylamine. The concentrations of methylamine were (○) 60 μM and (□) 30 μM. 100% activity corresponds to 2.5 (○) and 2.8 nmol O/mg per min (□).

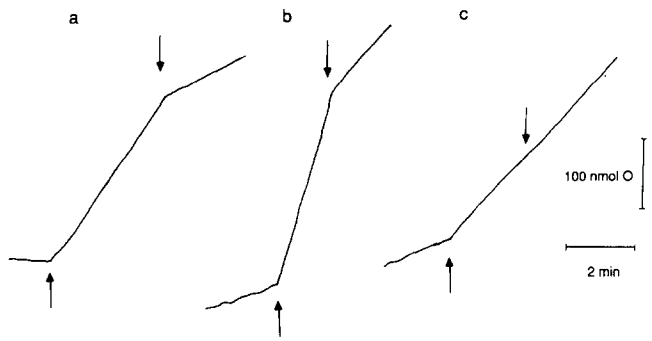

Fig. 5. The effect of added trimethylamine on substrate-stimulated respiration. (a) The first arrow indicates the addition of 300 μM methylamine to a suspension of bacterium W3A1. The second arrow indicates the addition of 40 mM trimethylamine. (b) The first arrow indicates the addition of 60 μM methylamine to a suspension of *P. denitrificans*. The second arrow indicates the addition of 40 mM trimethylamine. (c) The first arrow indicates the addition of 3 mM sodium formate to a suspension of *P. denitrificans*. The second arrow indicates the addition of 40 mM trimethylamine.

respiration which did not allow for reliable measurements.

With bacterium W3A1 and *P. denitrificans*, inhibition of oxygen consumption was also observed when trimethylamine was added to cells after the initiation of methylamine-stimulated respiration (Fig. 5a, b). When added after the addition of substrate, somewhat higher concentrations of trimethylamine were required to effect the same levels of inhibition observed for preincubated cells. Trimethylamine itself did not stimulate any significant levels of respiration in either bacterium. This was expected, as *P. denitrificans* does not utilize this compound and as bacterium W3A1 does not synthesize significant levels of trimethylamine dehydrogenase when grown under these conditions [15]. All of the experiments described in this section were performed with each bacterium. Most of the data presented are from studies with *P. denitrificans*. This is because bacterium W3A1 routinely exhibited much lower rates of respiration, which made precise quantitation of the effects of trimethylamine difficult. It should be noted that in each instance qualitatively identical results were obtained for *P. denitrificans* and bacterium W3A1.

To confirm that the inhibition of respiration by trimethylamine was specific for enzyme(s) involved in methylamine oxidation, the effect of trimethylamine on formate-dependent respiration by these bacteria was also examined. Formate oxidation by formate dehydrogenase is coupled to the reduction of NAD^+ [20]. If any of the observed decrease in the rate of oxygen consumption was caused by non-specific effects of trimethylamine on the membrane-bound respiratory chain or the cells in general, then formate-dependent oxygen consumption would be affected. No significant inhibition by trimethylamine of formate-dependent respiration was observed (Fig. 5c). The inhibition of methylamine-de-

pendent respiration, therefore, can be attributed to a specific interaction of trimethylamine with protein(s) involved in methylamine oxidation.

Discussion

Trimethylamine is a substrate for many methylotrophic bacteria. When methylotrophs are cultured with trimethylamine · HCl as a carbon source, concentrations of 0.1–0.5% are routinely used. This corresponds to trimethylamine levels of 10.5 to 52.3 mM. These concentrations are well within the range in which significant inhibition of enzyme activity and methylamine-dependent respiration by each bacterium was observed in this study.

The enzyme responsible for the initial oxidation of trimethylamine is an inducible cytoplasmic trimethylamine dehydrogenase [14,21]. Also induced during growth on trimethylamine is a periplasmic methylamine dehydrogenase [14,15]. In bacterium W3A1, the latter is not present during growth on methanol and is present during growth on trimethylamine at approx. 10–20% the level which is observed during growth on methylamine [4,15]. A question which is raised by these data is why an enzyme which is synthesized in response to trimethylamine should be inhibited by that same substrate. The answer to this question may lie in the localization of these enzymes and in the manner by which they donate electrons which are derived from these oxidations to the membrane-bound respiratory chain (Fig. 6). In bacterium W3A1, and most obligate and restricted methylotrophs, the complete oxidation of 1 mol of trimethylamine to 3 mol of formaldehyde involves the sequential action of trimethylamine dehydrogenase, dimethylamine monooxygenase and methylamine dehydrogenase [14]. Dimethylamine monooxygenase is linked to the respiratory chain by NAD [22]. Trimethylamine dehydrogenase donates electrons to an electron transfer flavoprotein [21,23] which, by analogy to its mitochondrial counterpart [24], is thought to donate electrons to the respiratory chain at the level of ubiquinone. Methylamine dehydrogenase donates electrons, via other soluble redox proteins, to cytochrome oxidase [25] and the formaldehyde which is formed must be transported into the cell to be assimilated into biomass. Thus, methylamine oxidation is a much less efficient process for generating energy than the oxidations of trimethylamine or dimethylamine. Inhibition of methylamine dehydrogenase by trimethylamine may provide the cell with a mechanism for bypassing this relatively inefficient process when trimethylamine is abundant. This would be particularly important during growth under oxygen-limited conditions. As methylamine dehydrogenase is synthesized during growth on trimethylamine, methylamine-dependent respiration can proceed whenever trimethylamine

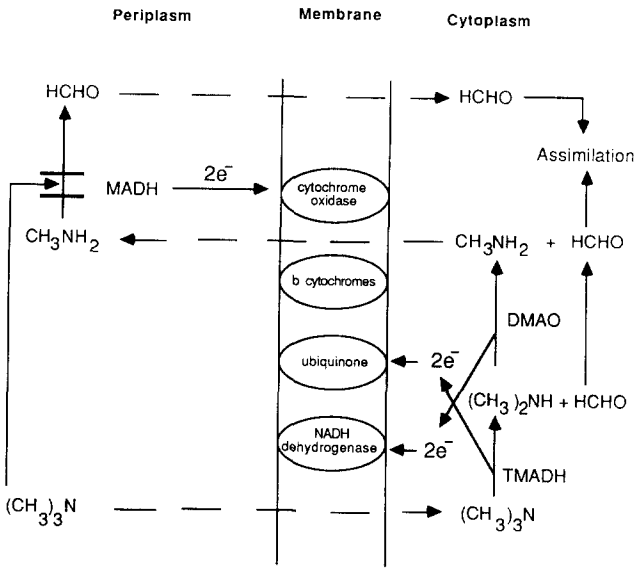

Fig. 6. Proposed scheme for the dissimilation of methylamines by bacterium W3A1. Dotted lines represent the transport of species across the cytoplasmic membrane. The major components of the membrane-bound respiratory chain are displayed in circles. The solid double line indicates the site of enzyme inhibition. TMADH, trimethylamine dehydrogenase; DMAO, dimethylamine monooxygenase; MADH, methylamine dehydrogenase.

levels drop relative to the methylamine present. Thus, trimethylamine may play a role in regulating the metabolism of methylamine by both controlling the levels of synthesis of methylamine dehydrogenase and the activity of the enzyme.

The scheme described above provides a physiologically relevant role in methylotrophs for the inhibition by trimethylamine of methylamine dehydrogenase. It is not, however, applicable to *P. denitrificans*, which does not utilize trimethylamine as a substrate for growth. In *P. denitrificans*, methylamine dehydrogenase is synthesized only when methylamine is present as a sole source of carbon [3]. *P. denitrificans* is an extremely versatile organism which is capable of growth on a variety of carbon sources [26]. The likelihood that it would encounter methylamine as a sole source of carbon outside of the laboratory is remote, and it is possible, therefore, that the methylamine oxidation system of this bacterium is an evolutionary artifact. This notion seems further supported by the observations that *P. denitrificans* methylamine dehydrogenase is inhibited by trimethylamine, a feature which may have evolved in obligate methylotrophs to serve a useful purpose but which has no role in *P. denitrificans* and other facultative autotrophs.

Acknowledgments

The authors thank Limei Hsu Jones and Mechelle Edmond for technical assistance. This work was sup-

ported by grant GM-41574 from the National Institutes of Health.

References

1 Eady, R.R. and Large, P.J. (1968) Biochem. J. 106, 245–255.
2 Haywood, G.W., Janschke, N.S., Large, P.J. and Wallis, J.M. (1982) FEMS Microbiol. Lett. 15, 79–82.
3 Husain, M. and Davidson, V.L. (1987) J. Bacteriol. 169, 1712–1717.
4 Kenny, W.C. and McIntire, W. (1983) Biochemistry 22, 3858–3868.
5 Lawton, S.A. and Anthony, C. (1985) Biochem. J. 228, 719–726.
6 Matsumoto, T. (1978) Biochim. Biophys. Acta 522, 291–302.
7 Shirai, S., Matsumoto, T. and Tobari, J. (1978) J. Biochem. 83, 1599–1607.
8 Vellieux, F.M.D., Frank, J., Swarte, M.B.A., Groendijk, H., Duine, J.A., Drenth, J. and Hol, W.G.J. (1986) Eur. J. Biochem. 154, 383–386.
9 Chen, L., Lim, L.L., Mathews, F.S., Davidson, V.L. and Husain, M. (1988) J. Mol. Biol. 203, 1137–1138.
10 Davidson, V.L. (1989) Biochem. J. 261, 107–111.
11 Davidson, V.L. and Neher, J.W. (1987) FEMS Microbiol. Lett. 44, 121–124.
12 Husain, M., Davidson, V.L., Gray, K.A. and Knaff, D.B. (1987) Biochemistry 26, 4139–4143.
13 McIntire, W.S. (1987) J. Biol. Chem. 262, 11012–11019.
14 Colby, J. and Zatman, L.J. (1975) Biochem. J. 148, 513–520.
15 Davidson, V.L. (1985) J. Bacteriol. 164, 941–943.
16 Kornberg, H.L. and Morris, J.G. (1968) Biochem. J. 95, 577–586.
17 Owens, J.D. and Keddie, R.M. (1969) J. Appl. Bacteriol. 32, 338–347.
18 Kasprzak, A.A. and Steenkamp, D.J. (1983) J. Bacteriol. 156, 348–353.
19 Segal, I.H. (1975) Enzyme Kinetics, pp. 170–202, John Wiley & Sons, New York.
20 Van Verseveld, H.W. and Stouthamer, A.H. (1978) Arch. Microbiol. 118, 21–26.
21 Davidson, V.L., Husain, M. and Neher, J.W. (1986) J. Bacteriol. 166, 812–817.
22 Eady, R.R., Jarman, T.R. and Large, P.J. (1971) Biochem. J. 125, 449–459.
23 Steenkamp, D.J. and Gallup, M. (1978) J. Biol. Chem. 253, 4086–4089.
24 Ruzicka, F.J. and Beinert, H. (1977) J. Biol. Chem. 252, 8440–8445.
25 Davidson, V.L. and Kumar, M.A. (1989) FEBS Lett. 245, 271–273.
26 John, P. and Whatley, F.R. (1977) Biochim. Biophys. Acta 463, 129–153.