Vol. 132, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1985 Pages 352-359

SUICIDE INHIBITION AS A LIKELY CAUSE OF VARIABLE SPECIFIC ACTIVITY IN TRIMETHYLAMINE DEHYDROGENASE FROM BACTERIUM W_3A_1

Daniel J. Steenkamp

Department of Chemical Pathology,
University of Cape Town Medical School,
Observatory 7925, South Africa

Received August 30, 1985

Summary: Trimethylamine hydrogenase isolated from bacterium W_3A_1 grown on dimethylamine was of variable, but low specific activity and had modified spectral properties. Chemical analyses for Fe, S and P indicated that the [4Fe-4S] clusters of the modified enzyme are intact and that the covalently bound flavin is probably present, but in modified form. A peptide with absorbance maximum at 358 nm and fluorescence excitation and emission maxima in dimethylformamide at 358 nm and 495 nm, respectively, was isolated by gel chromatography and HPLC of tryptic peptides of acetamidylated, modified trimethylamine dehydrogenase. These spectral properties are similar to those of 4a- or 5a-substituted flavins and suggest that the enzyme had been modified by in vivo reaction with a suicide inhibitor. This inhibitor, or a compound giving rise to it, seems to be present in a commercial source of dimethylamine.

Trimethylamine dehydrogenase is a homodimeric enzyme containing covalently bound flavin as the 6-S-cysteinyl-FMN derivative and a [4Fe-4S] cluster (1,2,3). The three-dimensional structure of the enzyme at 6.Ø A resolution has been reported (4).

In recent studies to be reported here, it was found that trimethylamine dehydrogenase isolated from bacterium W_3A_1 grown on dimethylamine had altered spectral properties and was of lower specific activity than the enzyme isolated from bacteria grown on trimethylamine. Two possible explanations could account for these observations. The altered spectral properties and decreased specific activity could either have been due to incomplete insertion of the covalently bound FMN into the enzyme or to inactivation of the enzyme by formation of an adduct of the 6-S-cysteinyl-FMN

0006-291X/85 $1.50

residue. The inactivation of flavoprotein dehydrogenases by suicide inhibitors often proceeds by modification of the flavocoenzyme with attendant alterations in their spectral properties (5,6,7,8,9,10).

In this communication evidence is presented that trimethylamine dehydrogenase isolated from bacterium W_3A_1 grown on dimethylamine had been partially inactivated by an inhibitor which occurs in low concentrations in dimethylamine obtained from a commercial source. The specificity of the inactivation and the conversion of the covalently bound flavin in the enzyme to an adduct suggests that the inhibition must have been mechanism based.

Methods:

Dimethylamine was obtained from BDH Chemicals, Poole, England as the anhydrous amine and from Sigma Chemical Company as the hydrochloride. Bacterium W_3A_1 was grown using either 0.2% trimethylamine or 0.2% dimethylamine as source of carbon and energy (11). Trimethylamine dehydrogenase was purified as described (1,12). Tryptic peptides of the acetamidylated enzyme were prepared as follows: Guanidinium hydrochloride was added to the enzyme to a final concentration of 6M. The denatured enzyme was dialyzed overnight at 4°C against 10 mM N-ethylmorpholinium acetate containing 3M urea and subsequently for 3h against 0.1 M $Na_2B_4O_7$ adjusted to pH 10.0 with sodium hydroxide. The preparation was incubated at 0°C for 3h with 0.2 M ethylacetimidate at a pH of 9.6 and then dialyzed overnight at 4°C against 10 mM N-ethylmorpholinium acetate, pH 7.8, containing 3 M urea. The acetamidylated enzyme was digested with trypsin at a 1:50 ratio by weight for 16h at 30°C and the reaction terminated by addition of phenylmethylsulfonyl fluoride to 0.1 mM.

Protein, non-heme iron and sulfur were determined by published methods (13,14,15). Phosphate was determined according to Bartlett (16) and also by a modification of the method of Itaya and Ui (17). Other methods are described in the figure legends.

Results:

The specific activity of trimethylamine dehydrogenase from cells grown on trimethylamine, 'authentic' trimethylamine dehydrogenase, is usually in the range 1.1 - 1.4 umol min^{-1} mg^{-1} while the specific activities of four enzyme preparations from cells grown on dimethylamine from BDH Chemicals were 0.27, 0.31, 0.67 and 0.87 umol min^{-1} mg^{-1}. Comparison of the spectral properties of a preparation with specific activity of only 0.27 umol min^{-1} mg^{-1}, 'low activity'

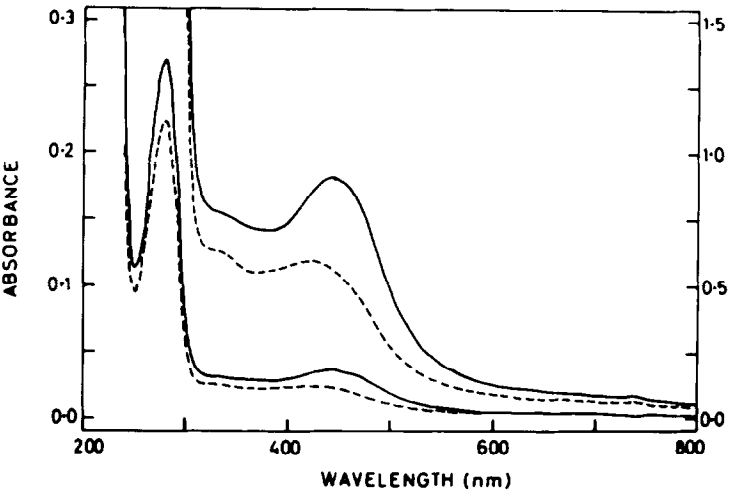

Fig. 1. Absorption spectra of trimethylamine dehydrogenase isolated from bacterium W_3A_1 grown on trimethylamine (——) as opposed to dimethylamine (---) shown at two different scale expansions. The protein concentration was Ø.58 mg ml^{-1} in both cases.

trimethylamine dehydrogenase, with those of 'authentic' trimethylamine dehydrogenase indicated a decrease in the intensity of the absorbance maximum at 443 nm. This coincided with a three fold larger decrease in absorbance at 278 nm for the 'low activity' enzyme relative to the 'authentic' enzyme at 278 nm (Fig. 1). Assuming a molar extinction coefficient of 54.2 mM^{-1} cm^{-1} for 'authentic' trimethylamine dehydrogenase at 443 nm (1) a molar extinction coefficient of 33.4 mM^{-1} cm^{-1} at 443 nm was estimated for the 'low activity' enzyme. 'Low activity' and 'authentic' trimethylamine dehydrogenase co-migrated on SDS disc gel electrophoresis (18) and peptide mapping by a two dimensional thin layer technique (19) or by HPLC (Fig. 2) failed to indicate significant differences between the two preparations.

The spectral differences between the two trimethylamine dehydrogenase preparations therefore seemed to be due either to a partial absence of 6-S-cysteinyl-FMN in 'low activity' trimethylamine dehydrogenase or to modification of the flavin moiety. Analysis of the 'low activity' enzyme for iron, sulfur and

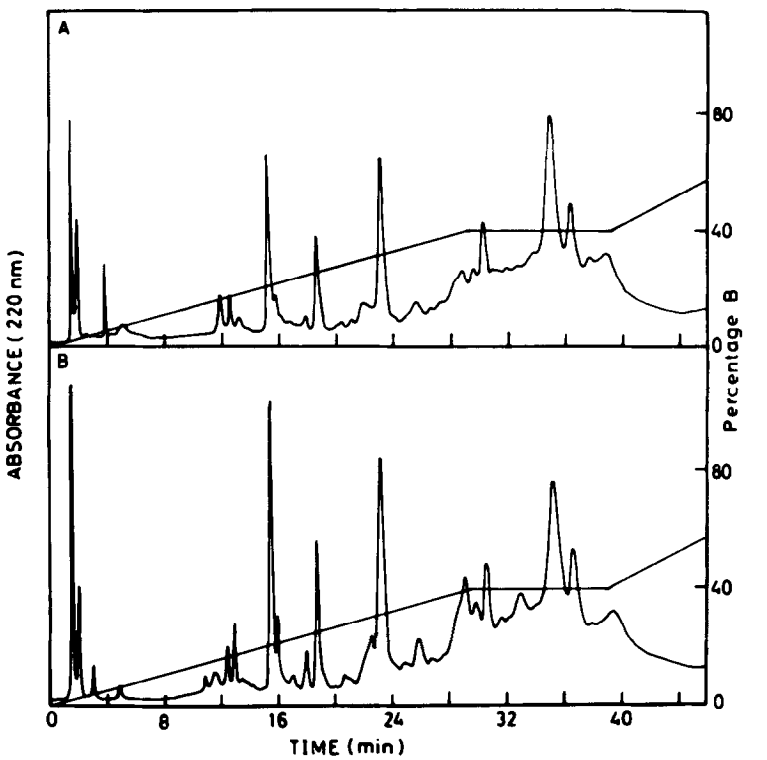

Fig. 2. HPLC of tryptic peptides of acetamidylated trimethylamine dehydrogenase from bacterium W_3A_1 grown on dimethylamine (A) as opposed to trimethylamine (B) using reverse phase chromatography on u Bondapak C18. The solvents were (A) Ø.1% ammonium acetate and (B) Ø.1% ammonium acetate, 8Ø% isopropanol and the flow-rate 2 ml min^{-1}.

phosphate gave values of 7.9, 6.6 and 1.85 moles per mole of the enzyme, respectively, based on a molar extinction coefficient of 33.4 mM^{-1} cm^{-1} at 443 nm. These analyses suggested not only that the [4Fe-4S] clusters are intact, but also that 6-S-cyteinyl-FMN was indeed present in the enzyme, but had been modified. In support of this thesis a bright yellow fluorescent spot with the same electrophoretic mobility, but a slightly higher Rf value in the chromatographic dimension, than the 6-S-cyteinyl-FMN peptide, could be detected on two-dimensional thin layer chromatograms of the tryptic peptides obtained from 'low activity' trimethylamine dehydrogenase. 6-S-Cysteinyl-FMN is non-fluorescent and only gives rise to a fluorescent photoproduct upon irradiation at pH values less than 7.Ø (2Ø).

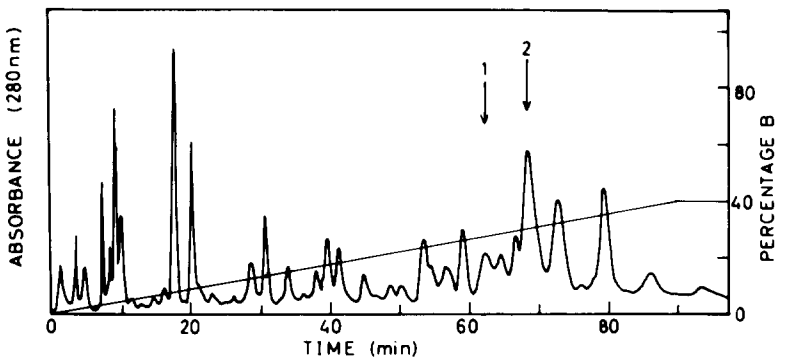

Fig. 3. HPLC of tryptic peptides of acetamidylated trimethylamine dehydrogenase on u Bondapak C18 after initial size separation on Sephadex G-25. Fractions which contained the 6-S-cysteinyl-FMN peptide were lyophilized, redissolved in Ø.1% ammonium acetate and applied to the HPLC column. The numbered arrows 1 and 2 indicate the positions of elution of 6-S-cysteinyl-FMN peptide and the modified flavin peptide, respectively. The solvents used were as in the legend to Fig. 2 and the flow rate was 3 ml min^{-1}.

After an initial size separation of the tryptic peptides from 'low activity' trimethylamine dehydrogenase two peptides with absorbance above 3ØØ nm could be separated by HPLC (Fig. 3). The first species to elute was identified as the 6-S-cysteinyl-FMN peptide. The second species had an absorbance maximum at 358 nm and was non-fluorescent in aqueous solution, but fluorescent when examined on thin layer chromatograms and in dimethylformamide. These absorbance and fluorescence properties are consistent with those of a 4a or 5 substituted flavin or a 4a, 5 di-substituted flavin (21) and suggest that the low specific activity of trimethylamine dehydrogenase from cells grown on dimethylamine was due to a modification of the 6-S-cysteinyl-FMN moiety.

The properties of the modified flavin peptide were reminiscent of the phenyl adduct of 6-S-cysteinyl-FMN which was obtained after suicide inhibition of 'authentic' trimethylamine dehydrogenase with phenylhydrazine (1Ø). The 4a-phenyl adduct of the 6-S-cysteinyl-FMN peptide was, therefore, prepared as described (1Ø), and was found to comigrate with the modified flavin peptide obtained from the 'low activity' enzmye on thin layer chromatography on microcrystalline

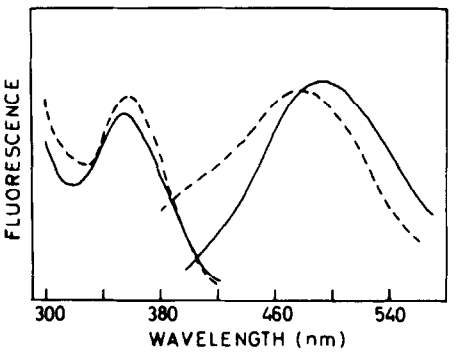

Fig. 4. Fluorescence excitation and emission spectra of the modified flavin isolated from 'low activity trimethylamine dehydrogenase' (——) as opposed to the 4 a-phenyl-6-S-cysteinyl-FMN peptide (----) obtained by inactivation of the enzyme with phenylhydrazine. The excitation spectrum was recorded while measuring emission at 495 nm and the emission spectrum using exciting radiation at 360 nm. Spectra were recorded on an Aminco-Bowman ratio recording spectrofluorimeter and corrected for background.

cellulose with butanol:pyridine:acetic acid:water::50:33:1:40 as the mobile phase. The fluorescence properties of the two modified flavin peptides were, however, different (Fig. 4). The fluorescence emission maxima were located at about 480 nm and 495 nm for the 4a-phenyl-6-S-cysteinyl-FMN peptide and the unknown adduct, respectively.

It was of interest to establish whether the inhibitor which gave rise to the modified 6-S-cysteinyl-FMN in the 'low activity' enzyme was a natural product produced by bacterium W_3A_1 itself or a contaminant present in the chemicals used in the growth medium. No decrease in activity of the authentic' enzyme was observed upon prolonged incubation with or dialysis of the enzyme against 10 mM dimethylamine obtained from BDH. Under similar conditions 0.5 mM phenylhydrazine gave complete inhibition. However, when bacterium W_3A_1 was grown with dimethylamine obtained from Sigma Chemical Company as carbon source unmodified 'authentic' trimethylamine dehydrogenase was isolated after harvesting the cells. Both the yield of cells (about 0.5g/l) and of purified enzyme (0.5-0.75 umoles from 35g wet cells) was similar when dimethylamine from

either source was used in the growth medium. These observations suggest that dimethylamine, which is a poor substrate, protects the enzyme against inactivation during in vitro incubation. During growth of the cells dimethylamine is probably taken up by active transport as is the case for methylamine (22) and trimethylamine (23) and is rapidly utilized. One possibility is that the intracellular concentration of dimethylamine was too low to protect the enzyme inside the cells against inactivation by the inhibitor.

Conclusion:

Mechanism-based inhibitors of enzymes have been extensively investigated since discovery of the phenomenon by Bloch et al (25). Such investigations frequently stem from the observed toxicity or antimicrobial properties of the suicide inhibitor and entail inactivation of an enzyme with a radiolabeled suicide inhibitor and subsequent determination of the stoichiometry of incorporation of the label into the enzyme. The situation described here, in which an enzyme isolated from biological material is found to be already partially inactivated by an inhibitor of unknown structure, has apparently not been reported, although numerous natural and synthetic suicide inhibitors occur in the environment.

Although prolonged incubation of trimethylamine dehydrogenase of normal specific activity with the same batch of dimethylamine as was used in the growth medium did not inhibit the enzyme, unmodified trimethylamine dehydrogenase was produced in cells grown on dimethylamine from an alternate source. Impurities occur to variable extents in the chemicals used to prepare microbial growth media or in animal feed and the question whether partial inactivation of enzymes in vivo by adduct formation with suicide inhibitors can be regarded as a widespread phenomenon is, therefore, a relevant one. There is no clear answer to this question, since adduct formation with a suicide inhibitor is unlikely to be detected

in an enzyme preparation unless the spectral properties of a prosthetic group in the enzyme had been modified or the inhibitor itself carries a detectable label.

Acknowledgement:

This work was supported by the South African Council for Medical Research. The technical assistance of Miss S-J. Cour-Palais is appreciated.

References:

1. Kasprzak, A.A., Papas, E.J. and Steenkamp, D.J. (1983) Biochem. J. 211, 535-541.
2. Steenkamp, D.J., McIntyre, W. and Kenney, W.C. (1978) J. Biol. Chem. 253, 2818-2824.
3. Hill, C.L., Steenkamp, D.J., Holm, R.H. and Singer, T.P. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 547-551.
4. Lim, L.W., Shamala, N., Mathews, F.S. and Steenkamp, D.J. (1984) J. Biol. Chem. 259, 14458-14462.
5. Pompon, D. and Lederer, F. (1985) Eur. J. Biochem. 148, 145-154.
6. Maycock, A.L., Abeles, R.H., Salach, J.I. and Singer, T.P. (1976) Biochemistry 15, 114-125.
7. Ghisla, S., Olson, S.T., Massey, V. and Lhoste, J-M. (1979) Biochemistry 18, 4733-4742.
8. Wenz, A., Thorpe, C. and Ghisla, S. (1981) J. Biol. Chem. 256, 9809-9812.
9. Schonbrunn, A., Abeles, R.H., Walsh, C.T., Ghisla, S., Ogata, H. and Massey, V. (1976) Biochemistry 15, 1798-1807.
10. Nagy, J. and Kenney, W.C. (1979) J. Biol. Chem. 254, 2684-2688.
11. Colby, J. and Zatman, L. (1973) Biochem. J. 132, 101-112.
12. Steenkamp, D.J. and Mallinson, J. (1976) Biochim. Biophys. Acta 429, 705-719.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
14. Massey, V. (1957) J. Biol. Chem. 229, 763.
15. Lowenberg, W., Buchanan, B.B. and Rabinowitz, J.C. (1963) J. Biol. Chem. 238, 3899-3913.
16. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
17. Itaya, K. and Ui, M. (1966) Clin. Chim. Acta 14, 361-366.
18. Laemmli, U.K. and Favre, M. (1973) J. Mol. Biol. 80, 575-599.
19. Gracy, R.W. (1977) Methods Enzymol. 47, 195-204.
20. Steenkamp, D.J., Kenney, W.C. and Singer, T.P. (1978) J. Biol. Chem. 253, 2812-2817.
21. Ghisla, S., Massey, V., Lhoste, J.M. and Mayhew, S.G. (1974) Biochemistry 13, 589-597.
22. Dijkhuizen, L., De Boer, L., Beers, R.H., Harder, W. and Konings, W.N. (1982) Arch. Microbiol. 133, 261-266.
23. Barton, S.M., Byron, D., Carver, M., Jones, G.D.D. and Jones, C.W. (1983) FEMS Microbiol. Lett. 17, 185-190.
24. Bloch, K. (1969) Acc. Chem. Res. 2, 193-202.