NICOTINAMIDE: A NATURAL INHIBITOR OF tRNA METHYLASE

Richard M. Halpern, S. Q. Chaney, B. C. Halpern and Roberts A. Smith

Departments of Chemistry and Medicine and the Molecular Biology Institute, University of California at Los Angeles, California. Chemistry Department publication #2758.

Received December 7, 1970

SUMMARY: A dialyzable tRNA methylase inhibitor has been isolated from rat liver. By chromatographic and electrophoretic data as well as ultraviolet and mass spectral data, the inhibitor is identified as nicotinamide. Kinetic evidence indicates that nicotinamide is an inhibitor of tRNA methylase.

The occurrence of tRNA methylase inhibitors is of particular significance because the extent of tRNA methylation is very much greater in malignant cells than in normal cells (1). Experimentally it has been repeatedly shown that extracts of neoplastic cells methylate methyl poor E. coli tRNA at rates considerably greater than extracts of normal cells (2). Leboy (3) has ascribed this difference in the methylase activity to a variation in the concentration of polyamines present in the tissue extracts. Kerr (4) has shown that adult rat liver contains a methylase inhibitor. This inhibitory protein which is absent in fetal rat liver could also inhibit the tRNA methylase from Novikoff tumor.

Previously we reported (5) that rat liver contained a dialyzable tRNA methylase inhibitor which was not demonstrable in rapidly growing Walker-256 carcinosarcoma. The dialyzable inhibitor from liver was active against the tRNA methylase prepared from tumor. In this report we describe the isolation and purification of the inhibitor from rat liver and its identification as nicotinamide. A kinetic evaluation of the effect of nicotinamide on the methylase activity obtained both from liver and tumor is also described.

METHODS: Inhibitor isolation. Livers were rapidly removed from exanguinated ether anesthetized Sprague Dawley rats with or without subcutaneously implanted W-256 neoplasms, and immediately immersed in a cold buffer (0°) containing sucrose, 0.25 M; magnesium chloride, 0.01 M; tris, 0.01 M; pH 8.0 (SMT buffer). The liver was minced in two volumes of cold SMT buffer, then disrupted in a teflon-glass homogenizer. After centrifugation at 4°C for 10 min (17,300 xg) and after removing the surface lipids, the supernatant was centrifuged at 105,000 xg for 60 min at 2°C. The remaining surface lipid layer was again removed before reducing the pH to 5 by the careful addition of 1 N acetic acid. The acidified preparation was then centrifuged at 17,000 xg for 10 min at 4°C. The supernatant was dialyzed in the cold for 24 hours against 1 - 2 liters of distilled water and the dialysate was lyophilized. After redissolving the dialysate in 20 - 30 ml of distilled water, the pH of the solution was reduced to 4.5 by the addition of 1 N acetic acid. The dialyzed inhibitor was adsorbed onto oven-dried carbon which had been previously acid-boiled in 0.1 N HCl and thoroughly washed successively with 1 N KCl, 95% ethyl elcohol: 1 N NH_4OH (4:6), SMT buffer, and distilled water. To each ml of the pH 4.5 inhibitor solution, 30 mg of carbon were added and stirred for 30 min at 4°C. The preparation was then filtered in the cold through a 0.45 micron millipore filter, washed twice with two volumes of distilled water and then twice with two volumes of 0.1 N KCl. The carbon adsorbed material was eluted with 12 volumes of 95% ethyl alcohol: 1 N NH_4OH (4:6). The eluate was then concentrated at reduced pressure and dissolved in 0.5 - 1.0 ml of distilled H_2O. Final purification was achieved by chromatography on Whatman 3 mm paper using butanol: water (86:14) as the descending solvent system. Six ultraviolet absorbing and three fluorescing bands were found and eluted with distilled water. Each was lyophilized and redissolved in 0.2 ml of distilled water and tested in our standard tRNA methylase reaction (5). Only the ultraviolet absorbing band with an Rf value of 0.6 demonstrated a significant inhibitory effect on the tRNA methylation.

Enzyme Assay: Both the rat liver and Walker-256 tumor enzymes were isolated as previously described and the assays with or without added inhibitor were performed exactly as before (5). Duplicate 0.1 ml aliquots of each reaction were applied to 2.2 cm Whatman 3 mm discs and air dried. Following the cold TCA wash one set of discs was treated for 30 min with boiling 5% TCA while the other set remained in cold 5% TCA. The final washes of cold 5% TCA, ether-alcohol, and ether were the same for each set of discs as outlined by Mann and Novelli (6).

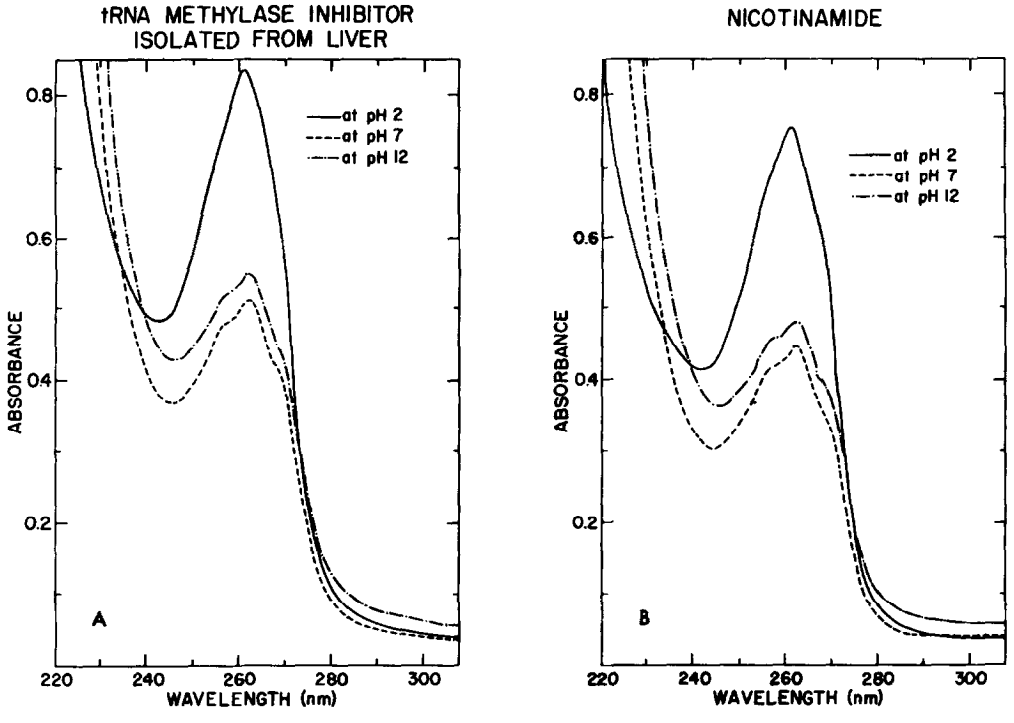

Figure 1: The ultraviolet absorption spectra of the A, tRNA methylase inhibitor isolated from rat liver and of B, nicotinamide. The inhibitor was isolated as described in the text.

Identification of Nicotinamide: The UV quenching inhibitory substance isolated from liver and purified as outlined above was subjected to paper electrophoresis at pH 3.5 (0.1 M Citrate), pH 7.0 (0.05 M triethylammonium bicarbonate), and pH 11.0 (0.005 M sodium phosphate). At the lowest pH (3.5) the substance moved toward the negative pole with a mobility expected

of a singly charged substance. It was immobile at the higher pH values.

The UV absorption spectra at acid, neutral and alkaline pH values were identical with nicotinamide and differed from nicotinic acid, α picolinic acid, and N-methyl nicotinamide (fig. 1). Examination of the inhibitory material with a scanning mass spectrometer revealed a maximum mass of 122 identical with that of nicotinamide. Paper chromatography of the inhibitor in two other solvent systems [butanol: acetic acid: water (3:2:5); and butanol: NH_4OH: water (7:1:2)] further established that the unknown was nicotinamide.

Nicotinamide obtained commercially and purified by the method described above was in every way indistinguishable from the inhibitor isolated from rat liver.

RESULTS AND DISCUSSION: Nicotinamide is inhibitory to the methylation of tRNA using either rat liver or Walker-256 tumor as the enzyme source. Kinetic studies using carefully dialyzed and lyophilized enzyme preparations were performed without and with nicotinamide in concentrations up to 5 mM (7). The results of these studies are summarized in Figure 2.

With both the liver and the tumor enzymes nicotinamide is a competitive inhibitor of S-adenosylmethionine (SAM) and a non-competitive inhibitor of tRNA. The Km for SAM with the liver enzyme is 2.8×10^{-6} M, whereas with tumor enzyme it is 1.60×10^{-6} M. The Km for tRNA (based on M. W. of 27,000) in each reaction, is also of the same order: with liver enzyme, 2.2×10^{-6} M; with tumor enzyme, 1.0×10^{-6} M. From the nicotinamide inhibited reactions in which the SAM concentration is varied, the Ki of nicotinamide with the liver enzyme is 3.1×10^{-3} M and with the tumor enzyme the Ki is 2.1×10^{-3} M. In the reactions where nicotinamide is a non-competitive inhibitor of tRNA, the Ki is 7.0×10^{-3} M with the liver enzyme and 5.3×10^{-3} M with the tumor enzyme.

Chromatographic analyses, using a solvent containing butanol, acetic acid and water (5:2:3), of reaction mixtures containing only SAM and nicotinamide revealed that methylase preparations from liver contained an enzyme

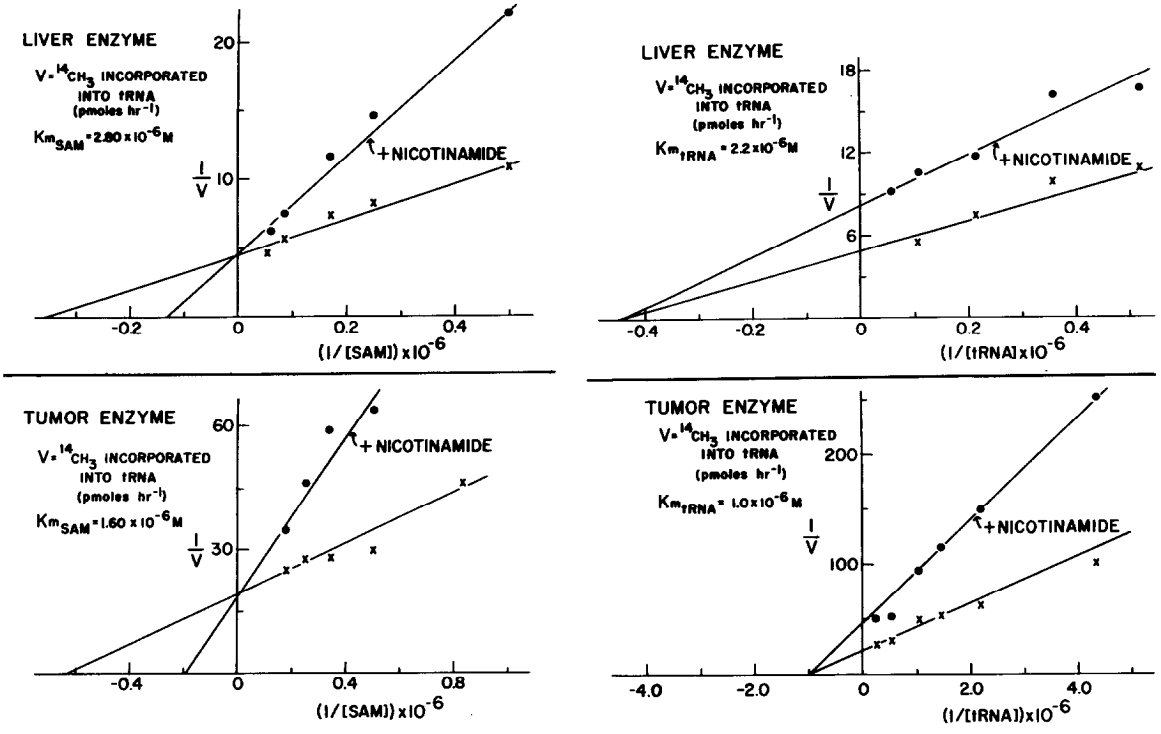

Figure 2: Reciprocal plots for the methylation of tRNA by enzymes prepared from adult rat liver and the Walker-256 carcinosarcoma without and with nicotinamide (5 mM). The tRNA methylase assay was carried out as previously described (5).

capable of transferring a methyl group from SAM to nicotinamide forming N-methylnicotinamide. In similar analyses with the tumor enzyme no N-methyl nicotinamide could be detected. A further analysis of the liver enzyme reaction revealed that of the 20 nmoles of SAM originally added, 1.09 nmoles of methyl group were transferred to nicotinamide forming N-methylnicotinamide. Additional experiments showed that N-methylnicotinamide was not an inhibitor in the tRNA methylase system.

Since only about 5% of the SAM is used in methylating nicotinamide with the liver enzyme and since no detectable methylation of nicotinamide takes place with the tumor enzyme there is no real competition for the methyl

donor between the two enzyme systems. The data suggest to us that the inhibitory effect of nicotinamide on tRNA methylase is consequently regulatory in nature.

ACKNOWLEDGMENT: This investigation was supported in part by grants from the Julius and Dorothy Fried Research Foundation and the United States Public Health Service (GM 13407).

REFERENCES

1. Berquist, P. L., and Mathews, R. E. P., Biochemical Journal 85, 305 (1962).

2. Tsutsui, E., Srinivasan, P. R., and Borek, E., Proc. Nat. Acad. Sci, U.S. 56, 1003 (1966).

3. Leboy, P. S., Biochemistry 9, 1577 (1970).

4. Kerr, S. J., Biochemistry 9, 690 (1970).

5. Chaney, S. Q., Halpern, B. C., Halpern, R. M., and Smith, R. A., Biochemical and Biophysical Research Communications 40, 1209 (1970).

6. Manns, R. J., and Novelli, G. D., Arch. Biochem. Biophys. 94, 48 (1961).

7. Greengard, P., Petrack, B., Craston, A., and Kalinsky, H. J., Biochemical and Biophysical Research Communications 13, 478 (1963).