ENZYMATIC METHYLATIONS, II[+]

IN VITRO INHIBITION OF tRNA AND PROTEIN METHYLATION BY NICOTINAMIDE AND ISONICOTINIC ACID HYDRAZIDE: ACTIVATION OF A S-ADENOSYLMETHIONINE-SPLITTING ENZYME IN RAT LIVER.

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Recently, nicotinamide (1) and nicotinamide analogs (2) have been shown to inhibit tRNA methylation in vitro, and a competitive or "mixed type" inhibition has been postulated (1,2). In our preceeding paper we could exclude these inhibition mechanisms (3).

This report describes the detection in rat liver methylase fractions of a weakly active, S-adenosylmethionine (SAM)-splitting enzyme, which is strongly activated by nicotinamide and isonicotinic acid hydrazide (INH). Nicotinamide may possibly function as a coenzyme of the SAM-splitting enzyme.

For this enzyme an assay system has been developed which allowed the partial separation from methylase activity. Methylase fractions free of SAM-splitting enzyme were not inhibited at all by nicotinamide or INH.

MATERIALS AND METHODS:

Hydroxylapatite chromatography and assay for rat liver methylases were performed as previously described (3,4). Precoated thin-layer chromatography (TLC) plates (cellulose F) were

[+]I: ref. (3)

obtained from Merck AG Darmstadt.
The following solvents were used for TLC: A (n-butanol/acetic acid/H_2O -60/15/25 - v/v/v); B (isopropanol/88 % formic acid/ H_2O - 70/10/20 - v/v/v) and C (isopropanol/conc. NH_3/H_2O - 70/10/20 - v/v/v).
SAM-splitting enzyme activity and its stimulation by nicotinamide or INH was measured in two ways:
1.) 120 µl incubation volume contained $MgCl_2$: 1 µmole; Tris·HCl pH 7.8: 10 µmoles; (methyl-^{14}C) SAM (spec. act. 13.9 ci/M): 1.44 mµmoles; aliquots of methylase fraction I (1.8 mg protein) containing SAM-splitting enzyme and nicotinamide (1 µmole) or INH (5 µmoles). 10 µl aliquots were placed on TLC plates after 0, 5, 15 and 30 min. incubation at 35°C, and the plates were developed in solvents, A, B or C. The solvent front was allowed to move 11.5 cm. For the evaluation of the thin-layer chromatograms a half automatic scraper (5) was used. Radioactivity from the plates was measured in 10 ml toluene/cellosolve/PPO (1000/500/6 - v/v/w).
2.) Assay for SAM-splitting enzyme: 150 µl incubation mixture contained a 50 µl aliquot from hydroxylapatite column fractions (4), all components of the methylase assay (3,4) and additional methylase III (4): 700 µg, and nicotinamide: 1 µmole (or INH: 5 µmoles). After one hour incubation at 35°C, 100 µl aliquots were placed onto paper filter disks which were processed as described by Mans and Novelli (6).

RESULTS AND DISCUSSION:

Recently (3) we have shown that the action of nicotinamide (or INH) on the enzymatic methylation of protein and tRNA in vitro can not be explained by a competitive or "mixed type" inhibition as suggested by other authors (1,2).
Our results (3) might be explained if one of the following possible reactions occurs:
a.) SAM is used for rapid methylation of nicotinamide or INH by an accompanying nicotinamide methyltransferase (E.C.2.1.1.1.)(7), this causing a decrease in tRNA and protein methylation;

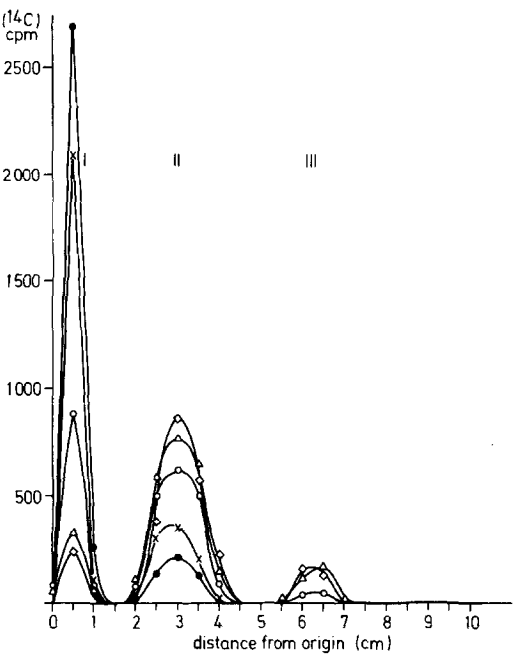

Fig. 1: Thin-layer chromatography in solvent A (11) of (methyl-^{14}C)SAM after incubation (details in "materials and methods") with methylase I (4), which contained SAM-splitting enzyme and INH (5 µmoles). 10 µl aliquots were removed from the incubation mixture after 0 ●——●, 5 x——x, 15 o——o and 30 Δ——Δ min., and immediately placed on TLC plates for chromatography. The same procedure was performed in the presence of nicotinamide (1 µmole), and the chromatogram after 5 min. incubation is shown here ◇——◇. Peak I: (methyl-^{14}C)SAM (RF = 0.09); peak II: unidentified (RF = 0.26); peak III: unknown (RF = 0.56); this material is a by-product produced slowly from the substance in peak II. RF-values of nicotinamide: 0.7; INH: 0.57.

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b.) nicotinamide or INH themselves catalyze the rapid breakdown of SAM, thus again decreasing tRNA and protein methylation;

c.) a SAM-splitting rat liver enzyme present in methylase fractions is activated by nicotinamide (or INH) or needs it as a coenzyme.

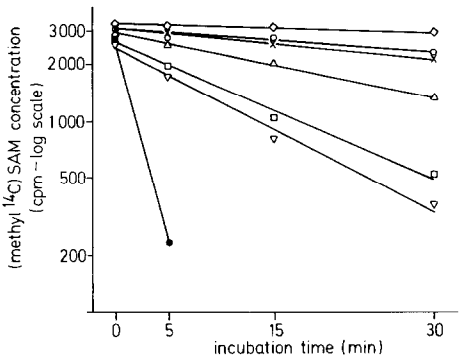

Fig. 2: Quantitative evaluation of thin-layer chromatograms as described in "material and methods" and in fig. 1. The (methyl-^{14}C) SAM concentration (peak I in fig. 1) was measured after 0, 5, 15, and 30 min. in the following incubation mixtures: (methyl-^{14}C) SAM with or without nicotinamide (1 µmole) or INH (5 µmoles): ◇——◇; (methyl-^{14}C) SAM with protein fraction (1.8 mg, methylase I containing SAM-splitting enzyme): o——o; (methyl-^{14}C) SAM with nicotinamide (1 µmole) and protein fraction as above (1.8 mg): ●——●; (methyl-^{14}C) SAM + INH (5 µmoles) with increasing amounts of the above protein fraction: 0.3 mg x——x; 0.6 mg Δ——Δ; 1.2 mg □——□; 1.8 mg ▽——▽.

The first possibility is easily ruled out, since nicotinamide and INH upon incubation with methylase I (4) from rat liver and (methyl-^{14}C) SAM gave completely identical radioactive products in thin layer chromatography (fig. 1), even if different solvents were used for TLC (not shown here). Since nicotinamide and INH have different RF values (legend to fig. 1), methyl-nicotinamide and methyl-INH should separate in any of the solvents used.

The second explanation is ruled out as shown in fig. 2: nicotinamide or INH alone does not catalyze the breakdown of (methyl-^{14}C) SAM.

Finally, fig. 1 and especially fig. 2 clearly demonstrate:
a.) (methyl-^{14}C)SAM is rather stable in the presence of nicotinamide (or INH) or methylase I (which contains SAM-splitting enzyme);
b.) both nicotinamide (or INH) and methylase fraction together cause rapid breakdown of SAM;
c.) under our conditions the SAM breakdown rate in the presence of nicotinamide (not shown in detail) and INH depends on the amount of protein ("methylase I") fraction added;
d.) nicotinamide is a much more efficient activator for SAM-splitting enzyme than INH. This explains the stronger effect of nicotinamide on tRNA and protein methylation as compared to INH (3).

These results indicate the existence of a nicotinamide-dependent SAM-splitting enzyme. In order to locate this enzyme in the fractions of hydroxylapatite chromatography of rat liver supernatant (4) we developed a more suitable assay than TLC. Column fractions (4) were incubated with all components of the methylase assay and additional nicotinamide and methylase III (4). This methylase III does not contain SAM-splitting enzyme and hence is not affected by nicotinamide. Thus all fractions to be assayed show incorporation of ^{14}C-methyl groups into TCA-insoluble material. In those fractions however, which contain nicotinamide-dependent SAM-splitting enzyme, the degree of methylation should be considerably reduced because of rapid (methyl-^{14}C)SAM breakdown. The result of this experiment is shown in fig. 3. The fractions with decreased methylation of TCA-insoluble material contain the nicotinamide-dependent SAM-splitting enzyme. Methylase fractions free of this enzyme are not inhibited by nicotinamide (fig. 3). Nicotinamide is not an

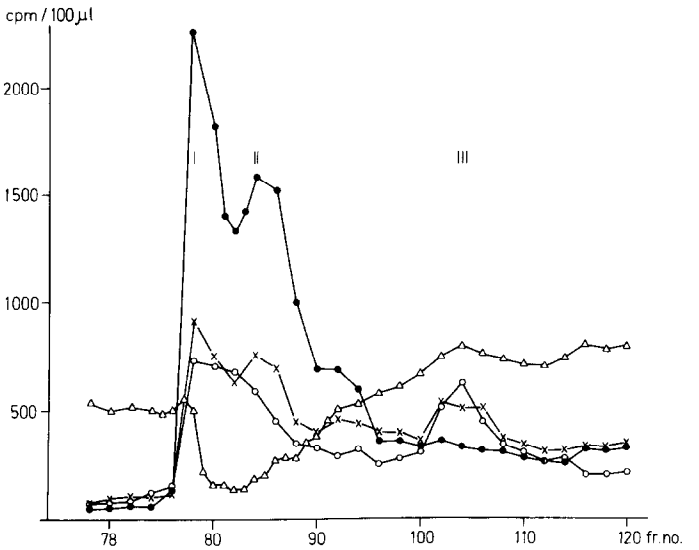

Fig. 3: Fractionation of rat liver methylase and nicotinamide-dependent SAM-splitting enzyme on hydroxylapatite. A linear gradient from 0 (fr. 60) to 0.5 m (fr. 175) potassium phosphate was used (fr. vol. 3.3 ml).
Assay for tRNA methylase activity with substrates $tRNA^{fMet}_{E.coli}$ ●——●, $tRNA^{Val}_{1\ E.coli}$ o——o and unfractionated E.coli tRNA x——x. Assay for nicotinamide-dependent SAM-splitting enzyme: Δ——Δ; fractions 80-84 contained the SAM-splitting enzyme and were pooled and processed like methylase fractions as described previously (3). Methylase fractions which were obtained free of nicotinamide-dependent SAM-splitting enzyme were not inhibited by nicotinamide or INH (not shown here).

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inhibitor of tRNA methylase as stated by other authors (1,2), and the "non-dialyzable inhibitor of tRNA methylase (8,9,10) may in fact be identical with the nicotinamide-dependent SAM-splitting enzyme described here. Experiments for further characterization of this enzyme are in progress.

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