Fluorometric Assay of Nicotinamide Methyltransferase with a New Substrate, 4-Methylnicotinamide

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A fluorometric method for the assay of nicotinamide methyltransferase has been established using rat liver 9000 × *g* supernatant fluid as a model enzyme preparation. 1,4-Dimethylnicotinamide formed enzymatically from a new substrate, 4-methylnicotinamide, is quantified by means of its fluorescence reaction with 4-methoxybenzaldehyde in aqueous alkali. The lower limit of determination of 1,4-dimethylnicotinamide is 100 pmol in the enzymatic reaction mixture. The apparent K_m values for 4-methylnicotinamide and for nicotinamide, which is a known substrate for this enzyme, were 0.19 and 0.13 mM, respectively, whereas the relative activity of 4-methylnicotinamide as a methyl acceptor was about 1.5 times the value of nicotinamide.

Keywords nicotinamide methyltransferase; 4-methylnicotinamide; rat liver; fluorometry; 1,4-dimethylnicotinamide; 4-methoxybenzaldehyde

Nicotinamide methyltransferase (EC 2.1.1.1) catalyzes transmethylation from *S*-adenosylmethionine (AdoMet) to nicotinamide[1] (Fig. 1) and to some other azaheterocycles.[2] Its activity was demonstrated in the liver and kidney of mammalian omnivores and carnivores such as rat and pig[1-6] and in human tumor cells in culture.[3] The roles of this enzyme have been studied by several authors.[2,4-6]

For the assay of nicotinamide methyltransferase, conventional fluorometric methods[1,2,4,6] have been most widely used, in which 1-methylnicotinamide formed enzymatically from nicotinamide is converted to fluorescent 2,7-naphthyridine derivatives[7] by treatment with methyl ketones such as acetone[8] and acetophenone[9] in aqueous alkali followed by addition of acids (Fig. 1). The method for determining 1-methylnicotinamide is very sensitive when acetophenone is used as the ketone.[9] However, 1-methylnicotinamide is a common biological component, and some pyridine nucleotides such as nicotinamide-adenine dinucleotide (NAD) gave a positive response under these conditions.[9,10]

In this study, in order to improve the selectivity in the assay of nicotinamide methyltransferase, a new system consisting of 4-methylnicotinamide as the substrate and a fluorometric method for determining 1,4-dimethylnicotinamide with aromatic aldehydes was devised (Fig. 1). In the modified procedure, 1-alkylnicotinamide derivatives such as NAD would be expected to give a negative result. The utility of the proposed method was demonstrated using a rat liver 9000 × *g* supernatant fluid as a model enzyme preparation.

Experimental

Chemicals and Apparatus 4-Methylnicotinamide and 1,4-dimethylnicotinamide chloride were synthesized by the published method.[11] AdoMet *p*-toluenesulfonate and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. Water used was purified on a Milli RO-Milli Q system (Millipore Ltd.). Thin-layer chromatography (TLC) was carried out with pre-coated Silica gel 60 F_{254} TLC plates (E. Merck) and with a solvent system of acetone–methanol–formic acid (7:2:1). Spots were detected by visual inspection under 360-nm light. All other chemicals used were of analytical-reagent grade.

A Shimadzu RF-510 spectrofluorometer equipped with a xenon lamp was used with a 0.4 × 10 mm quartz cell at room temperature; spectral bandwidths of 5 and 10 nm were used in excitation and emission, respectively. All fluorescence excitation and emission spectra were uncorrected.

Tissue Preparation Male Sprague–Dawley rats (200—280 g) were killed by stunning and decapitation. The livers were removed, washed with cold 0.9% (w/v) sodium chloride and homogenized 1:4 (w/v) in cold 5 mM potassium phosphate buffer (pH 7.5) with a Teflon-glass homogenizer. The homogenates were centrifuged at 9000 × *g* for 20 min at 4 °C. The supernatant was kept in small aliquots at −20 °C until assayed. Protein concentration was determined by the method of Lowry *et al.*[12] using BSA as the standard, and adjusted to 500 μg/ml with the same buffer for routine use.

Assay Conditions for Measurement of Nicotinamide Methyltransferase Activity The reaction mixture consisted of 25 μl of 2 mM dithiothreitol, 25 μl of 0.8 M Tris–HCl buffer (pH 8.6), 50 μl of 8 mM 4-methylnicotinamide, 100 μl of 0.4 mM AdoMet in 0.1 mM sulfuric acid and 200 μl of enzyme preparation. The mixture was incubated at 37 °C for 60 min. The reaction was terminated by heating in a boiling water bath for 2 min. In the case of blanks, the reaction was stopped immediately without incubation. After cooling, the mixture was centrifuged at 2000 × *g* for 10 min. A 100-μl aliquot of the supernatant was submitted to the fluorometric determination.

Fluorometric Determination of 1,4-Dimethylnicotinamide Aqueous sample solution (100 μl) was taken in a 1.5-ml glass-stoppered test tube, and 1 ml of 0.02 M 4-methoxybenzaldehyde in 35% (v/v) aqueous 2-methoxyethanol and 100 μl of 0.5 M aqueous sodium hydroxide were added. The tube was heated in a boiling water bath for 15 min. After cooling, the fluorescence intensity was measured with excitation at 418 nm and emission at 490 nm.

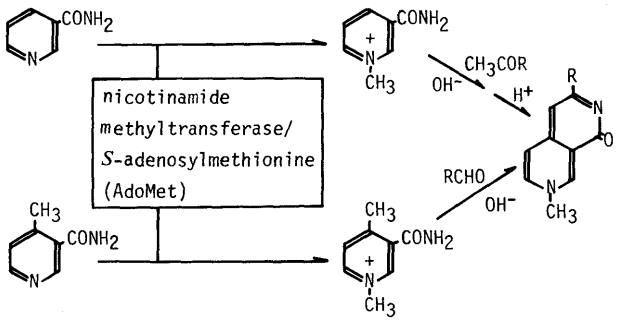

Fig. 1. Principle of the Nicotinamide Methyltransferase Assay System

Results and Discussion

Reaction Product The fluorescence reaction of 1,4-dimethylnicotinamide described here was designed by analogy with a fluorometric method for determining aromatic aldehydes with 1,4-dimethylnicotinamide chloride in aqueous sodium hydroxide.[11] However, there are marked differences between the fluorescent products obtained by the two methods with regard to fluorescence excitation and emission spectra, and chromatographic behavior.

The reaction product of 4-methoxybenzaldehyde treated by the original method[11] had the excitation maximum at

438 nm and emission maximum at 509 nm, and showed a green fluorescent spot on the TLC plate (*Rf* 0.03). The chemical structure of the fluorophore, however, has not been clarified.

On the other hand, the reaction product obtained by the present method had the excitation maximum at 418 nm and emission maximum at 490 nm and the fluorescence maxima changed reversibly to 395 nm (excitation) and 459 nm (emission) after addition of formic acid to the reaction mixture. TLC of the reaction product showed a blue fluorescent spot with the *Rf* value of 0.27. These characteristics are the same as those of the reaction product derived from 4-methoxyacetophenone and 1-methylnicotinamide by the method of Clark *et al.*,[9] which suggests that the same naphthyridine derivative is produced in the two methods (see Fig. 1).

Optimization of the Fluorescence Reaction for Determination of 1,4-Dimethylnicotinamide Various reaction conditions for the determination of 1,4-dimethylnicotinamide were studied by using 2 nmol/100 μl of the standard solution.

The effects of various aromatic aldehydes on the fluorescence intensity were examined (Table I). All reagents gave similar spectra as regards the maximal wavelengths for fluorescence excitation and emission. 4-Methoxybenzaldehyde was selected because it gave the highest fluorescence intensity. As almost constant fluorescence intensity was obtained at a concentration above 0.02 M 4-methoxybenzaldehyde, a 0.02 M solution was used. As shown in Fig. 2, aqueous 2-methoxyethanol was a good solvent for 4-methoxybenzaldehyde as compared with other solvent systems such as 2-ethoxyethanol– or 1,2-ethanediol–water mixtures, and 35% (v/v) 2-methoxyethanol was selected because the fluorescence intensity was maximum at the solvent concentration of 30—40%.

Table I. Effect of Aldehydes on the Fluorescence Reaction of 1,4-Dimethylnicotinamide

Compound[a]	λ_{max} (nm)		RFI[b]
	Ex	Em	
4-Methoxybenzaldehyde	418	490	100
4-Methylbenzaldehyde	418	488	83
3-Methoxybenzaldehyde	418	489	65
Benzaldehyde	415	488	64
3-Methoxy-4-hydroxybenzaldehyde	421	503	63
4-Hydroxybenzaldehyde	421	502	54
4-Chlorobenzaldehyde	418	490	54
2-Methoxybenzaldehyde	410	492	45
Pyridine-3-carboxyaldehyde	412	492	38

a) Reagent concentration: 0.02 M. *b*) Relative fluorescence intensity at optimal wavelengths; 4-methoxybenzaldehyde is arbitrarily taken as 100.

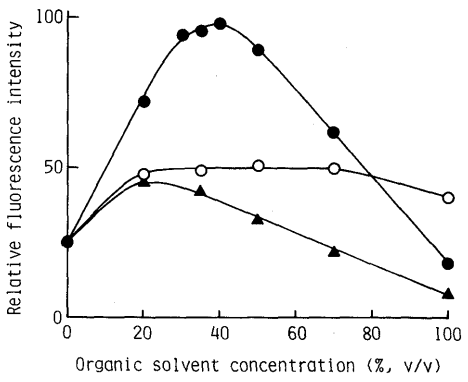

Fig. 2. Effect of Organic Solvent Concentration in 4-Methoxybenzaldehyde Solution on the Fluorescence Reaction of 1,4-Dimethylnicotinamide

1,4-Dimethylnicotinamide (2 nmol/100 μl) was treated by the recommended procedure except that 4-methoxybenzaldehyde was dissolved in various solvent systems. ●, 2-methoxyethanol–water mixture; ▲, 2-ethoxyethanol–water mixture; ○, 1,2-ethanediol–water mixture.

Sodium hydroxide was used as the alkali because it gave a high fluorescence intensity; it was superior to potassium hydroxide, sodium carbonate and piperidine. Almost constant fluorescence intensity was obtained at sodium hydroxide concentrations above 0.3 M; hence, a 0.5 M solution was used.

The relationship between the fluorescence intensity and the reaction time was studied in the temperature range of 80—95 °C. The reaction of 1,4-dimethylnicotinamide was complete at 95 °C (on a boiling water bath) after 15—30 min, but longer times were necessary below 80 °C. Thus, reaction in a boiling water bath for 15 min was used. The fluorescence was stable for at least 3 h at room temperature.

Determination of 1,4-Dimethylnicotinamide A linear calibration curve was obtained over the concentration range of 0.02—2 nmol of 1,4-dimethylnicotinamide in 100 μl of sample. The relative standard deviations ($n=10$) were 1.9% at 0.1 nmol and 2.7% at 2 nmol.

Pyruvic acid, α-ketoglutaric acid, 8 different amino acids (alanine, asparagine, histidine, methionine, phenylalanine, proline, tryptophan and tyrosine), glutathione, glucose, saccharose, nicotinic acid, nicotinamide, 4-methylnicotinamide, AdoMet and dithiothreitol showed no fluorescence at a concentration of 0.2 μmol/100 μl. 1-Methylnicotinamide and NAD gave a weak fluorescence, probably because of their alkaline degradation reaction to give unknown fluorescent products. However, the fluorescence intensities relative to 1,4-dimethylnicotinamide were only 0.16% for the former and 0.03% for the latter. A 100-fold molar ratio (to 1,4-dimethylnicotinamide) of the compounds described above did not interfere in the determination of 2 nmol/100 μl of 1,4-dimethylnicotinamide, except that the molar tolerance ratios of glucose, NAD, dithiothreitol and AdoMet were 10, 10, 25 and 50, respectively. In the present method, the selectivity for 1-alkylpyridinium derivatives of nicotinamide was greater than in the reported fluorometric methods for determining 1-methylnicotinamide.[9,10]

Linearity and Recovery of 1,4-Dimethylnicotinamide Added to the Enzymatic Reaction Mixture The calibration curve for 1,4-dimethylnicotinamide was linear in the range of 0.1—8 nmol in the enzymatic reaction mixture. The lower limit of determination was defined as the concentration giving a fluorescence intensity twice the blank. The recoveries of 0.4 and 2 nmol of 1,4-dimethylnicotinamide added to the incubation mixture of the blank were 100.0 ± 3.8% and 102.2 ± 7.9% (mean ± standard deviation, $n=5$ in each case), respectively. The sensitivity of the present method, defined as the minimum detectable amount of the product in the enzymatic reaction mixture, is almost equal to that obtained by the method of Clark *et al.*[4] and higher than those of the methods reported by Cantoni[1] and by Shibata.[6]

Assay of Rat Liver Nicotinamide Methyltransferase The enzyme reaction conditions reported by Clark *et al.*[4] using nicotinamide as the substrate were modified for our purpose. In this study, a mixture of $9000 \times g$ supernatant fluids prepared from 3 rats was used as the enzyme source.

When the assay mixtures were incubated at 37 °C, the amounts of 1,4-dimethylnicotinamide increased linearly with the incubation time up to 75 min and with the protein concentration up to 200 μg. However, 1,4-dimethylnicotinamide was not produced in the reaction mixture unless AdoMet was added. Further evidence for the formation of 1,4-dimethylnicotinamide is provided by the following facts. The fluorescence spectra of the reaction mixture in the recommended procedure had the excitation maximum at 418 nm and emission maximum at 490 nm, and were identical to those observed in the reaction mixture resulting from a 1,4-dimethylnicotinamide standard solution. TLC of these reaction mixtures showed a single fluorescent spot having the same *Rf* value (*Rf* 0.27) in each case.

The effects of other enzyme reaction conditions such as substrate and AdoMet concentrations, buffer systems and their pH were examined, and those of the original method[4] were found to be optimal also for the assay with 4-methylnicotinamide. Thus, the assay conditions used were essentially the same as those of the original method except that the blank was stopped by heating in a boiling water bath, and consequently the actions of the enzyme on the two substrates could be compared directly.

The apparent Michaelis–Menten constant (K_m) was found to be 0.19 mM (mean of 2 experiments) with the maximum velocity (V_{max}) value of 0.56 nmol of 1,4-dimethylnicotinamide formed/mg protein/min, whereas those for nicotinamide determined by the method of Clark *et al.*[4] were 0.13 mM and 0.32 nmol of 1-methylnicotinamide formed/mg protein/min, respectively. The K_m value for nicotinamide determined in this study is similar to the value (0.08 mM) reported by Hoshino *et al.*[5]

The enzyme activities in livers of 3 individual rats measured with 4-methylnicotinamide and with nicotinamide as the substrates were 0.47 ± 0.12 nmol of 1,4-dimethylnicotinamide/mg protein/min and 0.31 ± 0.10 nmol of 1-methylnicotinamide/mg protein/min (mean $\pm$ standard deviation), respectively.

On the basis of the above findings, 4-methylnicotinamide is considered to be a good substrate for nicotinamide methyltransferase. The present method is useful for the assay of the methyltransferase because of its sensitivity, selectivity and simplicity.

Rat liver nicotinamide methyltransferase has not been purified more than 20-fold,[1,4] while porcine liver nicotinamide methyltransferase has recently been purified to homogeneity by Alston and Abeles,[2] though there is at least one quantitatively minor isozyme, which has not been purified. It has been clarified that the homogenous enzyme acted on several azaheterocycles such as thionicotinamide, 3-acetylpyridine, quinoline and isoquinoline, as well as nicotinamide. Thus, the assay of nicotinamide methyltransferase is of interest because quaternization of amines sometimes increases their chemical reactivity and toxicity.[2] The application of the present method to various tissues of several animals is in progress.

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