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STUDIES OF FAD AND NADH IN THE PRESENCE OF N^1-DODECYLNICOTINAMIDE CHLORIDE MICELLES*

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SUMMARY

1. The effects of N^1-alkylnicotinamide chloride micelle formation on selected properties of FAD, FMN and NADH were studied.

2. Critical micelle concentrations were determined spectrophotometrically for N^1-decyl-, N^1-undecyl- and N^1-dodecylnicotinamide chlorides. Ionic strength effects were observed in the micellization of the dodecyl derivative.

3. The interaction of FAD with N^1-dodecylnicotinamide chloride micelles is accompanied by an enhancement of the FAD fluorescence and an increase in the polarization of FAD fluorescence. FMN fluorescence is unaffected by the presence of these micelles while the fluorescence of NADH is extensively quenched.

4. The rate of FAD-catalyzed oxidation of NADH is increased 3.6-fold through the interactions of these nucleotides with N^1-dodecylnicotinamide chloride micelles.

INTRODUCTION

The catalytic processes of enzymes are for the most part preceded by the selective binding and specific orientation of substrates. The enhancement of reaction rates through the binding of small molecular weight reactants to micelles has recently received attention as a possible model system for enzyme-catalyzed reactions[1–7]. Micellization is a versatile process and can be applied to the study of reaction rates in a variety of ways. Some of these possibilities have already been studied and include (a) the bringing together of two catalytically-active functional groups to mimic an enzyme active site orientation, (b) the bringing together of a catalytic group and a reactant, (c) the bringing together of two reactants, and (d) the insertion of catalytic groups or reactants into environments of differing dielectric constants.

The present study was initiated to investigate the effect of a micellization process on the oxidation of NADH as catalyzed by FAD. Nonenzymatic flavin catalysis of NADH oxidation was previously described by SINGER AND KEARNEY[8]. The use of N^1-dodecylnicotinamide chloride micelles in the present study was prompted by the observation of anomalous behavior of high concentrations of N^1-alkylnicotinamide chlorides in studies of NADH oxidation in submitochondrial particles. Due to a structural analogy to the pyridinium moiety of NAD, N^1-alkylnicotinamide chlorides

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have been used to investigate various properties of NAD binding sites of dehydrogenases. With certain dehydrogenases selective binding and coenzyme-competitive inhibition by these compounds was observed[9,10]. These inhibitory effects were obtained at relatively low concentrations of the nicotinamide derivatives. In cases where selective binding of N^1-alkylnicotinamide chlorides does not occur, as observed with rat-liver mitochondrial malate dehydrogenase[11], enzyme inhibition is observed only at high concentrations of the longer-chain nicotinamide derivatives. These effects correlate well with micelle formation of these compounds and result in protein denaturation and complications from the quenching of the fluorescence of NADH. These and other related studies of mitochondrial processes suggested the possibility that both flavin and reduced pyridine nucleotides were interacting with micelles formed from N^1-alkylnicotinamide chlorides having alkyl chains of ten or more carbons. The possibility that such interactions could form the basis of a micellar catalysis of reactions involving flavin and pyridine nucleotides has been investigated.

MATERIALS AND METHODS

FMN, FAD (Grade III) and NADH were obtained from the Sigma Chemical Co. N^1-Decylnicotinamide chloride, N^1-undecylnicotinamide chloride and N^1-dodecylnicotinamide chloride were prepared as described previously[9].

Measurements of fluorescence intensity were carried out at 25° in a temperature-controlled cell compartment of an Aminco–Bowman spectrophotofluorometer with a xenon lamp, Pacific photometric recording photometer, Model 15 fitted with an EMI 9502 photocell and a Mosely autograf Model 135A X–Y recorder.

Polarization of fluorescence measurements was performed using a photometer designed and described previously by CHURCHICH[12]. Illumination was provided by a xenon lamp (200 W) with wavelengths selected by a quartz prism monochromator (Schoeffel, QPM, 30S). The incident light was polarized by a Glan–Thompson prism (aperture 12 mm × 12 mm, Crystal Optics, Chicago) and focused onto the thermostated cell. An identical arrangement mounted at right angles to the excitation beam was used to select polarized light along vertical and horizontal axes.

Spectrophotometric measurements were carried out in temperature-controlled cell compartments of a Zeiss PMQ II spectrophotometer or a Gilford Model 2000 recording spectrophotometer, with 1-cm light path cuvettes used in all studies. Measurements of pH were made at 25° with a Radiometer pH meter, PHM 4b, with a G-200-B glass electrode. Viscosity measurements were made at 25° in a thermostated Cannon–Fenske viscometer tube.

RESULTS

In previous studies of micelles formed with N^1-decyl- and N^1-dodecylpyridinium iodides, the critical micelle concentration was determined by measuring the concentration at which an abrupt change in the ultraviolet absorption of these compounds was observed[13]. This method was applied to the study of micelle formation with N^1-decyl-, N^1-undecyl- and N^1-dodecylnicotinamide chlorides. The effect of increasing concentrations on the ultraviolet absorption of these compounds is shown in Fig. 1. The critical micelle concentration values calculated from these plots were 0.039, 0.016, and

0.0058 M for the N^1-decyl, N^1-undecyl, and N^1-dodecyl derivatives, respectively. The concentration required for micelle formation with these compounds decreases with increasing ionic strength. The effect of ionic strength on the value of the critical micelle concentration for N^1-dodecylnicotinamide chloride is shown in Fig. 2. When N^1-dodecylnicotinamide chloride micelles are allowed to form in the presence of flavin or reduced pyridine nucleotides, the fluorescence of these nucleotides is affected in various ways. The fluorescence of NADH, for example, is quenched by the formation

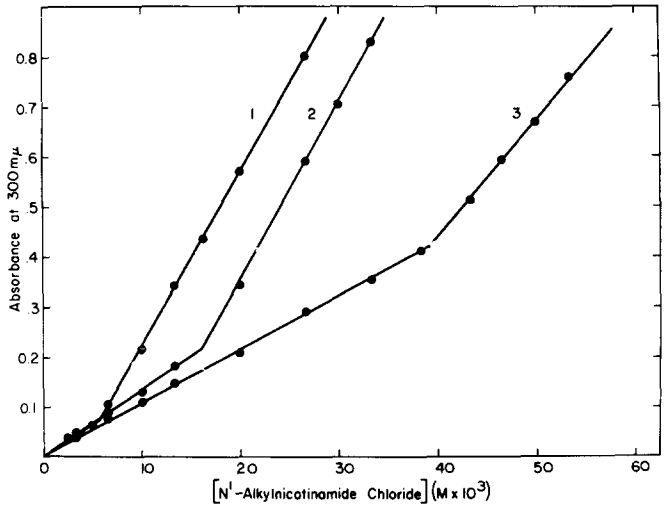

Fig. 1. The effect of micelle formation on the absorbance of N^1-alkylnicotinamide chloride solutions. Absorbance measurements were made in potassium phosphate buffer (pH 7.0), ionic strength 0.1 M. Curve 1, N^1-dodecylnicotinamide chloride; Curve 2, N^1-undecylnicotinamide chloride; Curve 3, N^1-decylnicotinamide chloride.

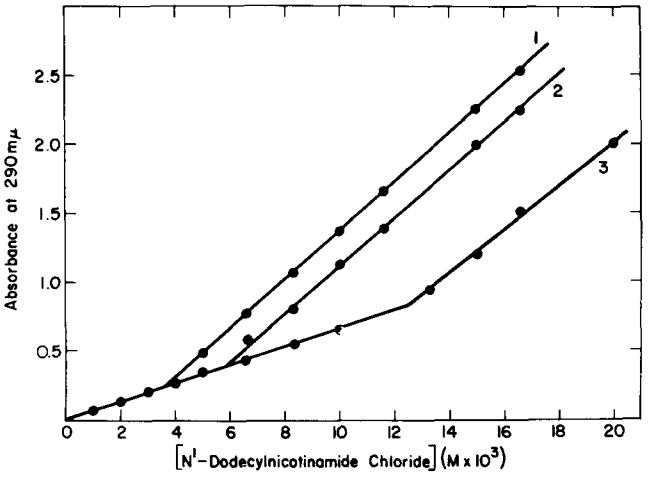

Fig. 2. The effect of micelle formation on the absorbance of N^1-dodecylnicotinamide chloride solutions. Absorbance measurements were made on 3-ml solutions of N^1-dodecylnicotinamide chloride in potassium phosphate buffer (pH 7.0), ionic strength 0.4 M (Curve 1); potassium phosphate buffer (pH 7.0), ionic strength 0.1 M (Curve 2); and in water (Curve 3).

of N^1-dodecylnicotinamide chloride micelles. The quenching of NADH fluorescence at two different ionic strengths is shown in Fig. 3 and correlates well with micelle formation. Although micelle formation with N^1-dodecylnicotinamide chloride is accompanied by a large increase in absorbance at wavelengths below 300 mμ, no absorbance at the excitation wavelength, 340 mμ, was observed.

Micelle formation in the presence of FAD results in a dramatic enhancement of fluorescence as shown in Fig. 4. The 4-fold increase in FAD fluorescence appears to be initiated at the critical micelle concentration. Under the same conditions, the fluorescence of FMN is unaffected by the presence of micelles (Fig. 4).

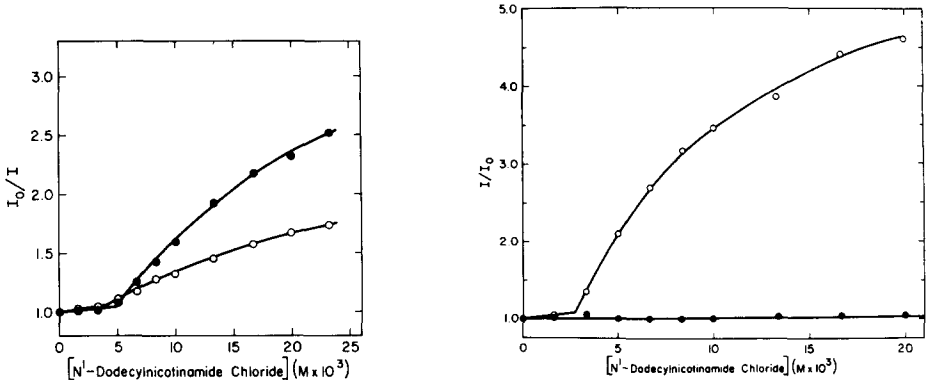

Fig. 3. Quenching of NADH fluorescence by N^1-dodecylnicotinamide chloride micelles. I_0 equals the fluorescence intensity of 18 μM NADH in potassium phosphate buffer (pH 7.0), ionic strength either 0.1 or 0.4 M. I equals the fluorescence intensity of the above solutions with N^1-dodecylnicotinamide chloride added at the concentrations shown. ●, observations made at an ionic strength of 0.1 M; ○, at ionic strength 0.4 M. Samples were excited at 340 mμ and fluorescence emission measured at 460 mμ.

Fig. 4. The effect of N^1-dodecylnicotinamide chloride micelles on the intensity of fluorescence of FAD and FMN solutions. I_0 equals the intensity of fluorescence of FAD or FMN in potassium phosphate buffer (pH 7.0), ionic strength 0.4 M. I equals the intensity of fluorescence of these flavin solutions with N^1-dodecylnicotinamide chloride added at the concentrations shown. ○, 8.33 μM FAD; ●, 6.67 μM FMN. Samples were excited at 475 mμ and fluorescence emission measured at 520 mμ.

The interaction of FAD with N^1-dodecylnicotinamide chloride micelles, in addition to fluorescence enhancement, is also accompanied by an increase in the polarization of fluorescence of the FAD (Fig. 5). Similar, but less dramatic changes were observed with FMN and no abrupt changes in the polarization of fluorescence were observed with NADH (Fig. 5). As a check on the polarization measurements, values of the polarization of fluorescence of FAD, FMN, and NADH in glycerol were determined to be 0.429, 0.416, and 0.399, respectively. These values agree well with those reported previously by WEBER[14].

The viscosities of N^1-dodecylnicotinamide chloride solutions were measured over a concentration range where micelle formation occurs. Solutions identical to those described in Fig. 2 at an ionic strength of 0.4 were used and less than 6 % change in viscosity was observed over this concentration range. Solutions of 3.3 mM and 6.7 mM N^1-dodecylnicotinamide chloride in 0.2 M potassium phosphate buffer (pH 7.0) were prepared containing either 8.33 μM FAD, 6.67 μM FMN, or 18 μM NADH.

In no case did the nucleotide have a significant effect on the viscosity of the solution.

The oxidation of NADH was studied at 25° in 3-ml reaction mixtures containing 0.2 M potassium phosphate (pH 7.0), 45 μM NADH, and 4.3 μM FAD. The oxidation of NADH was followed spectrophotometrically by measuring the decrease in the absorbance of NADH at 340 mμ. As previously reported[8], the flavin-catalyzed oxidation of NADH followed first-order kinetics and under the conditions described above, a first-order rate constant of $9.5 \cdot 10^{-3}$ min^{-1} was obtained. The addition of low concentrations of N^1-dodecylnicotinamide chloride (less than 2 mM) to these reaction mixtures did not alter the observable first-order rate constant of the NADH oxidation. How-

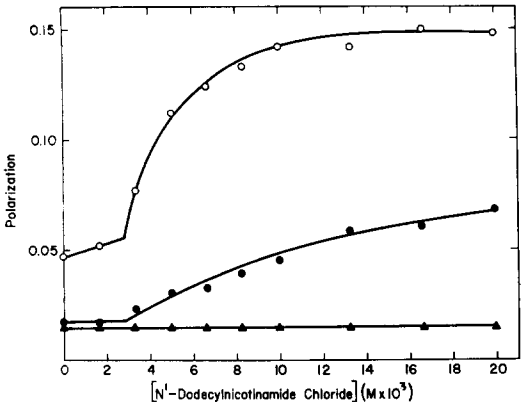

Fig. 5. The effect of N^1-dodecylnicotinamide chloride on the polarization of fluorescence of FAD, FMN and NADH solutions. Polarization measurements were made at 25° in potassium phosphate buffer (pH 7.0), ionic strength 0.4 M. ○, 8.33 μM FAD; ●, 6.67 μM FMN; ▲, 18 μM NADH. Flavin solutions were excited at 470 mμ with a band width of 2 mμ and emitted light was filtered through a Corning glass filter, CS 3-69. NADH solutions were excited at 340 mμ with a band width of 10 mμ and emitted light was filtered through a Corning glass filter, CS 3-74.

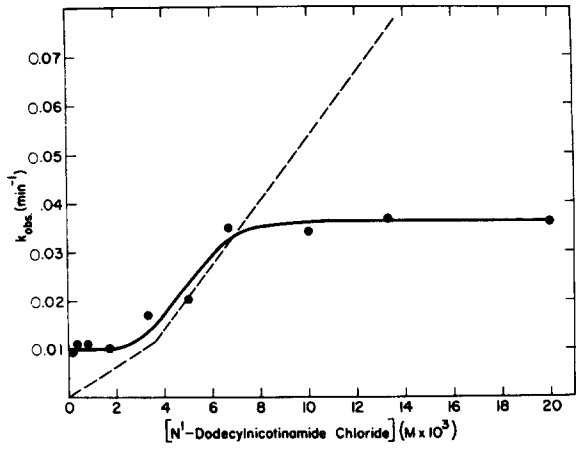

Fig. 6. The effect of N^1-dodecylnicotinamide chloride on the FAD-catalyzed oxidation of NADH. First-order rate constants were calculated from the equation, $K_{obs} = 0.693/t_{½}$. The dashed line represents the region of micelle formation of N^1-dodecylnicotinamide chloride solutions as described in Fig. 2, Curve 1.

ever, in the concentration region of micelle formation, a 3.6-fold increase in the rate of NADH oxidation was observed. The effect of N^1-dodecylnicotinamide chloride concentration on the rate of NADH oxidation is shown in Fig. 6. Micellar concentrations of N^1-dodecylnicotinamide chloride did not cause an oxidation of NADH in the absence of FAD.

DISCUSSION

The application of N^1-alkylnicotinamide chlorides as structural analogs of the pyridinium moiety of NAD to study coenzyme binding processes of dehydrogenases has been carried out for the most part at low concentrations of these compounds[9,10]. In the concentration ranges employed, these compounds exist in monomeric form and selective binding to dehydrogenases has been observed. The effective binding of N^1-alkylnicotinamide chlorides to yeast alcohol dehydrogenase[9] and rabbit muscle L-α-glycerophosphate dehydrogenase[10] is facilitated by nonpolar interactions and has been attributed to the presence of hydrophobic regions at the NAD binding sites of these enzymes. When such specific interactions are absent as in the case of rat-liver mitochondrial malate dehydrogenase[11], inhibition of the enzyme does not occur at low concentrations of the nicotinamide derivatives and when larger concentrations of the longer-chain derivatives were employed, extensive denaturation of this enzyme was observed. It is now known that these denaturing effects correlate well with micelle formation of these compounds as described in the present study (Fig. 1). The determination of the critical micelle concentrations for the N^1-decyl-, N^1-undecyl- and N^1-dodecylnicotinamide chlorides provides the information necessary for recognizing the concentration range in which anomalous behavior of these compounds is to be expected. As previously mentioned, protein denaturation is one effect observed in these micellar regions.

It has also been observed that properties of pyridine and flavin nucleotides can be altered by the presence of N^1-dodecylnicotinamide chloride micelles. A 4-fold increase in the intensity of fluorescence of FAD was observed in the N^1-dodecylnicotinamide chloride concentration range corresponding to micelle formation (Fig. 4), suggesting the binding of FAD to the micellar particles. The enhancement of FAD fluorescence associated with this interaction may arise from a disruption of the intramolecular ring–ring interactions of the FAD molecule. The increased fluorescence of FAD at low pH was previously attributed to changes in the intramolecular interactions of the purine and isoalloxazine moieties[15]. Alternately, an enhancement of fluorescence would also be expected on the binding of the fluorescing isoalloxazine moiety in a medium of lower dielectric constant. An increase in the fluorescence of flavins upon lowering of the dielectric constant of the solvent has been reported[16]. In this respect, if the insertion of the isoalloxazine ring of FAD into the nonpolar matrix of the micelle was of importance in the binding process, one would expect a similar fluorescence enhancement with FMN, which was not observed (Fig. 4). Thus, the binding of FAD to N^1-dodecylnicotinamide chloride micelles may require an interaction between the AMP moiety of the FAD and the externally oriented pyridinium rings of the micelle. Interactions between pyridinium and purine derivatives have been described previously[17–19].

The binding of FAD to N^1-dodecylnicotinamide chloride micelles is accompanied

by an increase in the polarization of the FAD fluorescence (Fig. 5). This increase in polarization is consistent with a restricted rotation of the FAD molecule upon binding to the larger micellar particle. An increase in polarization of fluorescence due to an abrupt change in viscosity of the medium as a result of micelle formation was considered but was ruled out on the basis that the viscosity was observed to remain essentially constant over the N^1-dodecylnicotinamide chloride concentration range used.

The changes in the polarization of fluorescence of FMN in the presence of N^1-dodecylnicotinamide chloride micelles are very small (Fig. 5), and require measurements for which reproducibility is poor. It is therefore difficult to determine whether or not FMN interacts with the micelles. The absence of abrupt changes in polarization of fluorescence in the micellar region would argue against the binding of FMN.

The interaction of NADH with N^1-dodecylnicotinamide chloride micelles results in quenching of fluorescence (Fig. 3). The complete absence of any change in the polarization of fluorescence of NADH (Fig. 5) during this interaction would suggest that the binding process results in extensive, if not complete, quenching of the NADH fluorescence.

The indication that both FAD and NADH interact with micelles of N^1-dodecylnicotinamide chloride suggested the possibility that any reaction between these two nucleotides might be facilitated by these binding processes. It has been shown (Fig. 6) that the rate of the FAD-catalyzed oxidation of NADH can be increased almost 4-fold in the presence of N^1-dodecylnicotinamide chloride micelles. These rate effects are very similar to those observed by BEHME *et al.*[2] in the cetyltrimethylammonium bromide micellar catalysis of the hydrolysis of *p*-nitrophenylhexanoate. However, in the FAD-catalyzed NADH oxidation, rate increases appear to be directly related to micelle formation. The lack of any change in reaction rates at concentrations of N^1-dodecylnicotinamide chloride lower than the critical micelle concentration would indicate that a FAD- or NADH-induced micellization does not occur. This was also suggested by the observation that the critical micelle concentration of N^1-dodecylnicotinamide chloride determined spectrophotometrically in the presence of FAD or NADH was the same as that observed in the absence of these nucleotides.

The fact that N^1-alkylnicotinamide chlorides continue to be of importance as structural analogs of NAD and NADP for the study of pyridine nucleotide interactions in a variety of biological processes requires that possibilities for selective interactions of nucleotides with micelles of these compounds be recognized. For example, the unusual inhibition of bovine liver glutamate dehydrogenase by high concentrations of NADH in the presence of 6.67 mM N^1-dodecylnicotinamide chloride[20] can now be attributed to a selective binding of NADH to micelles formed under these conditions. Also, care must be taken in the interpretation of results obtained in studies of the effects of N^1-alkylnicotinamide chlorides on flavin-catalyzed oxidations in submitochondrial particles since nonenzymatic oxidations may be catalyzed by high concentrations of these compounds.

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