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A Fluorescent Analog of Nicotinamide Adenine Dinucleotide

(fluorescent coenzyme/quenching/enzyme activity/NAD$^+$/1,N^6-ethenoadenosine derivative)

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ABSTRACT **Nicotinamide 1,N^6-ethenoadenine dinucleotide, a fluorescent analog of the coenzyme nicotinamide adenine dinucleotide, has been synthesized by the reaction of chloroacetaldehyde with the coenzyme. The technical fluorescence emission maximum of the analog is 410 nm, upon excitation at 300 nm. Its fluorescence yield is about 8% of that of the 1,N^6-ethenoadenine 5'-phosphate, and its fluorescence lifetime is shorter. Upon hydrolysis of the modified coenzyme analog with *Neurospora crassa* NADase or phosphodiesterase I at room temperature, the intensity of fluorescence was increased 10-fold, corresponding to separation of the nicotinamide and ethenoadenine rings. The spectroscopic results with nicotinamide 1,N^6-ethenoadenine dinucleotide are consistent with the concept of an intramolecular interaction between the modified adenine and pyridine moieties of the dinucleotide that is disrupted by enzymatic hydrolysis. The fluorescent analog showed reasonable activity as a substitute for NAD$^+$ in four different dehydrogenase-catalyzed reactions.**

The coenzyme nicotinamide adenine dinucleotide (NAD$^+$) plays an indispensable role in living organisms. Its function in enzymatic oxidation–reduction reactions necessitates some type of enzyme–coenzyme interaction, and considerable information has been gathered concerning the mechanism of action of NAD$^+$ (1). A major question has centered on the conformations of NAD$^+$ in solution and in the enzyme-bound state. The conclusion from x-ray analysis at 5-Å resolution is that the coenzyme when bound to dogfish lactate dehydrogenase exists in the "open" form (2–4). Equilibria between "open" and "folded," or internally complexed, forms of reduced NAD$^+$ have been examined by nuclear magnetic resonance (5, 6), and synthetic models of NAD$^+$ and NADH have been provided that permit maximum intramolecular interaction between the adenine and nicotinamide moieties (7, 8).

The preparation of a fluorescent and enzymatically active derivative of NAD$^+$ would permit, at the oxidized level, the same kind of fluorescence studies that have been so useful with the reduced form, NADH (9). Accordingly, we have applied the chloroacetaldehyde reaction which, with adenine-containing moieties, introduces an etheno bridge between the 1- and N^6-positions of the adenine ring (10–15) and renders the molecule fluorescent. The coenzyme NAD$^+$ can be converted, thereby, to nicotinamide 1,N^6-ethenoadenine dinucleotide, abbreviated "ϵNAD$^+$"†, and shown in I as the doubly zwitterionic form. In the neutral pH range 6–8, the ethenoadenosine moiety will be unprotonated (15). Adenosine-modified analogs of NAD$^+$ previously examined include, *inter alia*, deamino (or desamino) NAD$^+$, which is the corresponding hypoxanthine compound (16–19), and the 1- and N^6-hydroxyethyl compounds (20).

MATERIALS AND METHODS

Technical Fluorescence Emission Spectra were measured on a Hitachi–Perkin Elmer MPF-2A spectrophotometer. *Ultraviolet spectra* were obtained on a Cary model 15 spectrophotometer.

Dehydrogenase Studies were performed at 23° in a total volume of 3.0 ml. The reaction was followed by monitoring the increase in absorbance at 340 nm as NADH or ϵNADH was produced.

Lactate Dehydrogenase (*EC 1.1.1.27*). Assay mixtures contained 0.05 M Tris·HCl buffer (pH 7.5), 83 μmol of hydrogen peroxide, 12 μmol of lactate, and 1 μmol of NAD$^+$ or ϵNAD$^+$. The reaction was initiated by addition of 0.5 μg of lactate dehydrogenase.

Glyceraldehyde-3-Phosphate Dehydrogenase (*EC 1.2.1.9*). Assay mixtures contained 0.05 M Tris·HCl buffer (pH 7.5), 0.1 ml of 5.3% sodium arsenate, 1 μmol of fructose 1,6-diphosphate, 0.1 mg of fructose diphosphate aldolase (EC 4.1.2.13), and 1 μmol of NAD$^+$ or ϵNAD$^+$. The reaction was initiated by addition of 2 μg of glyceraldehyde-3-phosphate dehydrogenase.

Alcohol Dehydrogenases (*Yeast and Horse Liver*) (*EC 1.1.1.2*). Assay mixtures contained 5 M ethanol, 0.05 M Tris·HCl buffer (pH 7.5), and 1 μmol of NAD$^+$ or ϵNAD$^+$. The reactions were initiated by addition of 2 μg of the appropriate enzyme.

Neurospora crassa NADase (*EC 3.2.2.6*). Experiments were done at 23° in 3-ml reaction mixtures containing 0.25 μmol of ϵNAD$^+$ in 0.1 M KH_2PO_4. The reactions were initiated by the addition of 0.32 unit of NADase. The reaction rate was determined by measurement of the increase of fluorescence intensity at 410 nm.

Crotalus adamanteus Venom Phosphodiesterase (*EC 3.1.4.1*). The assay was performed at 23° in 3-ml reaction mixtures containing 25 mM phosphate buffer (pH 7.0) and 0.50 μmol of ϵNAD$^+$ by following the increase in fluorescence intensity at 410 nm when phosphodiesterase I was added to a final con-

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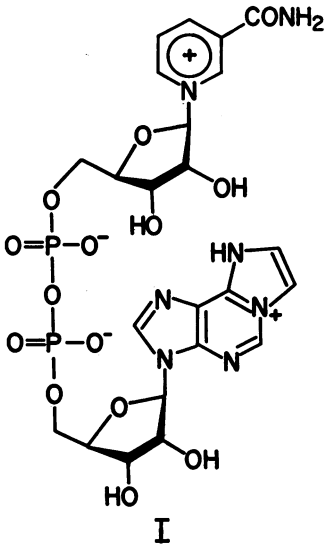
† In the abbreviation ϵNAD$^+$ for nicotinamide 1,N^6-ethenoadenine dinucleotide, ϵ stands for etheno and is also suggestive of the molar absorbance term and of fluorescence emission.

centration of 21 μg/ml. Under these conditions the reaction was complete in 30 min.

εNAD⁺ Preparation and Purification. A solution of 1 mmol of NAD^+ in 20 ml of 1.5 M aqueous chloroacetaldehyde at pH 4.0–4.5 was allowed to stand at room temperature until the NAD^+ had disappeared, as observed by thin-layer chromatography (15), and no further change was witnessed in the ultraviolet absorption spectrum with time (7–10 days). The solution was decolorized with charcoal, concentrated to 10 ml under reduced pressure, adjusted to pH 7.2, and applied to a Dowex 2X-8 (formate form) column (30 cm × 35 mm), which was then washed with water. Elution was performed with 0.1 N formic acid and continued until the fractions contained no fluorescence. Evaporation of fluorescent fractions, followed by precipitation with acetone, filtration, and drying under reduced pressure afforded ϵNAD^+ in about 20% yield as a practically colorless, hygroscopic powder, mp 158–160° (dec), with elemental analysis indicative of I·trihydrate, $C_{23}H_{27}N_7O_{14}P_2 \cdot 3H_2O$ (15).

RESULTS AND DISCUSSION

In contrast to the quantitative nature of the reaction with simpler coenzymes, when NAD^+ reacted with chloroacetaldehyde in aqueous solution at pH 4.5 some hydrolysis was noted, in addition to the formation of nicotinamide 1,N^6-ethenoadenine dinucleotide, ϵNAD^+. Pure ϵNAD^+ was isolated by the use of a Dowex 2X-8 (formate form) column, which was washed first with distilled water, followed by elution with 0.1 N formic acid to obtain the product. The analytical and spectroscopic data are consistent with structure I, and the compound was stable under neutral and mild-acidic conditions.



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The ultraviolet absorption spectrum of ϵNAD^+, as well as that of its fluorescent "half" molecule 5′-εAMP, are shown in Fig. 1. Just as in the case of the simpler modified coenzymes (11–15), excitation of the long-wavelength transition in the neighborhood of 295–300 nm leads to fluorescence emission with a maximum at about 410 nm in aqueous solution buffered at pH 7.0. The lowest-energy absorption band of ϵNAD^+, appearing as a shoulder at 294 nm (Fig. 1), is at sufficiently long wavelength that excitation can be done without interference from most ultraviolet-absorbing moieties in proteins and nucleic acids. The intensity of the ϵNAD^+ emission is considerably less than that of 5′-εAMP, undoubtedly due to the intramolecular quenching by the nicotinamide mononucleotide portion of the coenzyme. While the quantum yield of 5′-εAMP in aqueous buffered solution at pH 7.0 is 0.56, that of ϵNAD^+ is about 8% of this value. The quenching is consistent with the existence of a high proportion of folded or stacked conformation of the coenzyme in aqueous solution. This suggestion is borne out by a doubling of emission of ϵNAD^+ in 1,2-propanediol as compared to aqueous solution. In 1,2-propanediol, the population of molecules of NADH (9) or related models (8) is dynamically distributed among a large number of conformations, with a reduction in the probability of these molecules residing in the folded form. The fluorescence lifetime of ϵNAD^+ in aqueous solution buffered at pH 7.0 again reflects the base–base interaction in the preferred folded conformation in that solvent, since the predominant lifetime was shorter than the 23 nsec observed for 5′-εAMP, as well as for the other coenzymes containing only the modified adenosine chromophore (11, 12, 14, 15). The fluorescence properties of ϵNAD^+ are under thorough study and will be reported in a subsequent paper.

The enzyme *Neurospora crassa* NADase is fairly specific for catalysis of the cleavage of NAD^+ at the nicotinamide-ribose linkage, yielding free nicotinamide and adenosine diphosphate ribose (21–23). The incubation of ϵNAD^+ with this enzyme at room temperature resulted in similar cleavage, with the formation of 5′-εADP-ribose and nicotinamide. The enzyme reaction was followed by observation of the intensity of the fluorescence emission. Whereas the wavelength of the fluorescence maximum was unchanged, an increase in intensity of about 10-fold was observed, due to cleavage between the two interacting parts of the molecule (Fig. 2).

Cleavage of the ϵNAD^+ coenzyme analog could also be followed by means of the cyanide reaction. The ability of NAD^+ and NAD^+ analogs to form various addition products has been reported (24, 25), and the reaction of NAD^+ with cyanide can be followed by the appearance of a new ultraviolet maximum at 325 nm. When ϵNAD^+ was dissolved in 0.1 N KCN, a new maximum appeared at 316 nm, the blue shift probably resulting from an overlap with the original band at 294 nm for ϵNAD^+ (Fig. 1). As in the case of NAD^+, cyanide reacted with the quaternary nitrogen form of ϵNAD^+ and no reaction was detected after hydrolysis with *Neurospora*

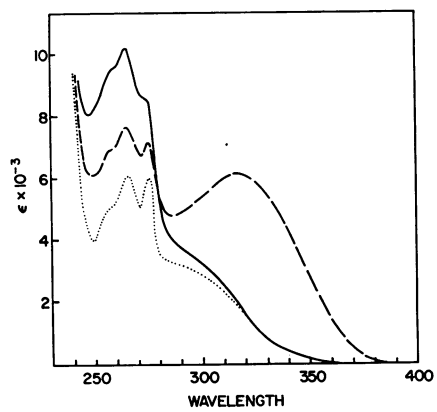

FIG. 1. Ultraviolet absorption spectra of ϵNAD^+ in aqueous buffered solution at pH 7.0 (——) and in 0.1 N KCN (— —), and of 5′-εAMP in aqueous buffered solution at pH 7.0 (· · ·).

crassa NADase. The ultraviolet absorption spectrum of 1,N^6-ethenoadenosine 5'-monophosphate (5'-εAMP) (11) was unchanged in going from pH 7 aqueous phosphate buffer to 0.1 N KCN, indicating that the cyanide reaction was taking place as expected with the quaternary nicotinamide moiety.

Phosphodiesterase I (*Crotalus adamanteus* venom) also cleaved εNAD$^+$. Incubation with this enzyme resulted in the formation of 5'-εAMP and NMN, and the increase in fluorescence intensity could again be used as an index of the progress of the enzymatic hydrolysis. After hydrolysis, the ultraviolet absorption spectrum showed a slight hyperchromic effect in the 265-nm maximum when the spectrum was compared with an appropriate blank of buffer and enzyme. The effect on the ultraviolet absorbance is like that observed for NAD$^+$ and NAD$^+$ analogs before and after enzymatic hydrolysis (26–28) and for NAD$^+$ models compared with their component half-molecules (7, 8). All of the accumulated evidence supports the existence of intramolecular interaction between the pyridinium and the modified adenine rings of εNAD$^+$ in folded conformation that is disrupted by hydrolysis catalyzed by enzymes that are also effective with this modified coenzyme.

Of the many analogs of NAD$^+$ that have been synthesized and tested for enzymatic activity (16–20, 27–41), none has been described as appreciably fluorescent, so that εNAD$^+$ is the first coenzyme analog at the oxidized level that permits utilization of the power of fluorescence techniques to study interactions between enzymes and oxidized coenzymes in dehydrogenase systems. The value of fluorescence techniques in the study of enzyme binding has been amply demonstrated in the case of the fluorescent reduced form of the natural coenzyme NADH (42–45). For εNAD$^+$ to be useful for the acquisition of information on enzyme–coenzyme interactions, it must show some activity in the dehydrogenase reactions. We have tested the fluorescent analog with four enzymes: horse-liver alcohol dehydrogenase, yeast alcohol dehydrogenase, lactate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase. In all four reactions, εNAD$^+$ exhibited activity, at levels of 7, 7, 31, and 19%, respectively, of the activity of NAD$^+$ itself. Conditions were selected to give consistent results for both NAD$^+$ and εNAD$^+$ without seeking to attain those of maximal activity for either coenzyme. The activities shown by εNAD$^+$ are consistent with the earlier findings that neither the presence of an unsubstituted amino group at the 6-position (16–20) nor the absence of a 1-substituent (20) on the adenine of NAD$^+$ is essential for activity with dehydrogenases. The different levels of activity of εNAD$^+$ reflect, as did the earlier analogs, heterogeneity among similar enzymes.

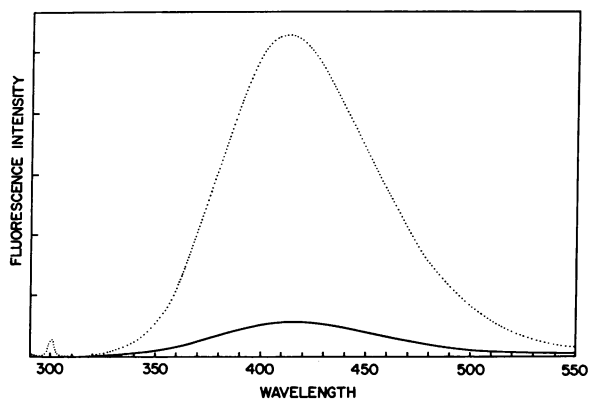

FIG. 2. Fluorescence emission spectra of εNAD$^+$ in 0.1 M KH_2PO_4 (——) and after enzymatic hydrolysis with NADase (· · ·).

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1. Sund, H. (1968) in *Biological Oxidations*, ed. Singer, T. P. (Interscience Publishers, New York), pp. 603–705, and references therein.
2. Adams, M. J., McPherson, A., Jr., Rossman, M. G., Schevitz, R. W., Smiley, I. E. & Wonacott, A. J. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases*, ed. Sund, H. (Springer-Verlag, Heidelberg, Berlin), pp. 157–174.
3. Adams, M. J., McPherson, A., Jr., Rossmann, M. G., Shevitz, R. N. & Wonacott, A. J. (1970) *J. Mol. Biol.* **51**, 31–38.
4. McPherson, A., Jr. (1970) *J. Mol. Biol.* **51**, 39–46.
5. Oppenheimer, N. J., Arnold, L. J. & Kaplan, N. O. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 3200–3205.
6. Jacobus, J. (1971) *Biochemistry* **10**, 161–164.
7. Secrist, J. A., III & Leonard, N. J. (1972) *J. Amer. Chem. Soc.* **94**, 1702–1706.
8. Scott, T. G., Spencer, R. D., Leonard, N. J. & Weber, G. (1970) *J. Amer. Chem. Soc.* **92**, 687–695.
9. Weber, G. (1957) *Nature* **180**, 1409.
10. Kochetkov, N. K., Shibaev, V. N. & Kost, A. A. (1971) *Tetrahedron Lett.* 1993–1996.
11. Barrio, J. R., Secrist, J. A., III & Leonard, N. J. (1972) *Biochem. Biophys. Res. Commun.* **46**, 597–604.
12. Secrist, J. A., III, Barrio, J. R. & Leonard, N. J. (1972) *Science* **175**, 646–647.
13. Leonard, N. J., Barrio, J. R. & Secrist, J. A., III (1972) *Biochim. Biophys. Acta* **269**, 531–532.
14. Secrist, J. A., III, Barrio, J. R., Leonard, N. J., Villar-Palasi, C. & Gilman, A. G. (1972) *Science*, in press.
15. Secrist, J. A., III, Barrio, J. R., Leonard, N. J. & Weber, G. (1972) *Biochemistry*, in press.
16. Stockwell, A. (1959) *J. Biol. Chem.* **234**, 1293–1296.
17. Schlenk, F., Hellström, H. & von Euler, H. (1938) *Ber.* **71**, 1471–1479.
18. Kaplan, N. O., Colowick, S. P. & Ciotti, M. M. (1952) *J. Biol. Chem.* **194**, 579–591.
19. Pullman, M. E., Colowick, S. P. & Kaplan, N. O. (1952) *J. Biol. Chem.* **194**, 593–602.
20. Windmueller, H. G. & Kaplan, N. O. (1961) *J. Biol. Chem.* **236**, 2716–2726.
21. Kaplan, N. O., Colowick, S. P. & Nason, A. (1951) *J. Biol. Chem.* **191**, 473–483.
22. Nason, A., Kaplan, N. O. & Colowick, S. P. (1951) *J. Biol. Chem.* **188**, 397–406.
23. Kaplan, N. O. (1955) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, Inc., New York), Vol. II, pp. 660–666.
24. Colowick, S. P., Kaplan, N. O. & Ciotti, M. M. (1951) *J. Biol. Chem.* **191**, 447–459.
25. Anderson, B. M., Ciotti, C. J. & Kaplan, N. O. (1959) *J. Biol. Chem.* **234**, 1219–1225.
26. Siegel, J. M., Montgomery, G. A. & Bock, R. M. (1959) *Arch. Biochem. Biophys.* **82**, 288–299.
27. Woenckhaus, C. (1964) *Chem. Ber.* **97**, 2439–2446.
28. Pfleiderer, G., Woenckhaus, C., Scholz, K. & Feller, H. (1964) *Justus Liebigs Ann. Chem.* **675**, 205–212.
29. Walter, P. & Kaplan, N. O. (1963) *J. Biol. Chem.* **238**, 2823–2830.
30. Fawcett, C. P. & Kaplan, N. O. (1962) *J. Biol. Chem.* **237**, 1709–1715.

31. Kaplan, N. O., Ciotti, M. M. & Stolzenbach, F. E. (1956) *J. Biol. Chem.* **221**, 833–844.
32. Hoffman, D. J. & Whistler, R. L. (1970) *Biochemistry* **9**, 2367–2372.
33. Freda, C. E. & Stoppani, A. O. M. (1970) *Enzymologia* **38**, 225–242.
34. Woenckhaus, C. & Zumpe, P. (1968) *Z. Naturforsch. B.* **23**, 484–490.
35. Leonard, N. J. & Laursen, R. A. (1965) *Biochemistry* **4**, 365–376.
36. Pfleiderer, G., Woenckhaus, C. & Scholz, K. (1963) *Angew. Chem.* **75**, 92.
37. Honjo, M., Furukawa, Y., Moriyama, H. & Tanaka, K. (1962) *Chem. Pharm. Bull.* **10**, 73–75.
38. Nisselbaum, J. S. & Bodansky, O. (1961) *J. Biol. Chem.* **236**, 323–327.
39. Schwarcz, M. N. & Stoppani, A. O. M. (1960) *Rev. Soc. Argent. Biol.* **36**, 319–333.
40. Van Eys, J., Ciotti, M. M. & Kaplan, N. O. (1958) *J. Biol. Chem.* **231**, 571–582.
41. Kaplan, N. O. & Ciotti, M. M. (1954) *J. Amer. Chem. Soc.* **76**, 1713–1714.
42. Fisher, H. F. & McGregor, L. L. (1960) *Biochim. Biophys. Acta* **38**, 562–563.
43. Shifrin, S., Kaplan, N. O. & Ciotti, M. M. (1959) *J. Biol. Chem.* **234**, 1555–1562.
44. Winer, A. D., Novoa, W. B. & Schwert, G. W. (1957) *J. Amer. Chem. Soc.* **79**, 6571–6572.
45. Boyer, P. D. & Theorell, H. (1956) *Acta Chem. Scand.* **10**, 447–450.