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Synthesis of spin-labeled photoaffinity derivatives of NAD^+ and their interaction with lactate dehydrogenase

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The synthesis of NAD^+ derivatives spin-labeled at either N^6 or C8 of the adenine ring is described, in which the carboxamide function of the nicotinamide moiety is replaced by a diazirine ring. Irradiation of these compounds at 350 nm generates a carbene which will react with any functional group in its vicinity including hydrocarbons. Both NAD^+ derivatives form tight ternary complexes with lactate dehydrogenase and were covalently incorporated into this enzyme. They may be employed for ESR studies when non-covalent interactions are too weak for motionally restricted species to be observed.

Lactate dehydrogenase; Photoaffinity labeling; NAD; Diazirinopyridine; Spin labeling

1. INTRODUCTION

Derivatives of NAD spin-labeled at either N^6 or C8 of the adenine ring (N^6-SL-NAD, C8-SL-NAD) have been employed in studies of structure-function relationships in various dehydrogenases such as lactate dehydrogenase [1,2], glyceraldehyde-3-phosphate dehydrogenase [3], glutamate dehydrogenase [4], and D-3-hydroxybutyrate dehydrogenase [5]. Besides simple binding studies in which the signal amplitude of the freely tumbling spin-labeled ligand is utilized, important information can be obtained from the lineshape of the motionally restricted, enzyme-bound species. Changes in this lineshape have been correlated with conformational changes of the enzyme [6] or distances between two SL-NAD molecules bound to tetrameric glyceraldehyde-3-phosphate dehydrogenase could be determined [7]. The two immobilized radicals give rise to dipolar splitting of the spectra.

Such investigations are normally restricted to conditions of tight binding, because the much narrower, and hence, stronger signal of the free component would dominate the spectrum. Standring and Knowles [8] have described the synthesis of a 3-diazirino analogue of NAD^+, in which the diazirine ring replaces the carboxamide function in an almost isosteric substitution, as a photoaffinity reagent for dehydrogenases. Irradiation of the diazirine group yields a carbene under loss of nitrogen which may react with any functional group in its vicinity including hydrocarbons. Here, we describe the synthesis of the corresponding photoaffinity SL-NAD^+ derivatives N^6-SL-DAD^+ and C8-SL-DAD^+ (fig.1) and their covalent incorporation into lactate dehydrogenase.

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Abbreviations: DAD^+, 3-(3-*H*-diazirin-3-yl)pyridine adenine dinucleotide; N^6-SL preceding NAD^+ or DAD^+ refers to their N^6-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl) derivatives; C8-SL preceding NAD^+ or DAD^+ refers to their 8-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl)amino derivatives; HPLC, high-performance liquid chromatography

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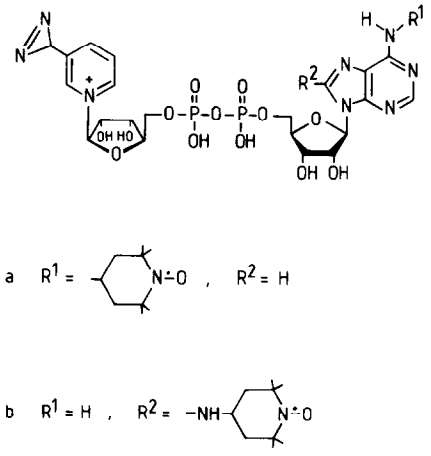

Fig.1. Structural formulas of N^6-SL-DAD$^+$ (a) and C8-SL-DAD$^+$ (b).

2. EXPERIMENTAL

Pig heart lactate dehydrogenase was from Boehringer Mannheim (FRG) and pig brain NAD$^+$ glycohydrolase from Sigma (München).

Synthesis of 3-(3-*H*-diazirin-3-yl)pyridine N^6-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl)adenine dinucleotide (N^6-SL-DAD$^+$): A suspension of 46 mg NAD$^+$ glycohydrolase from pig brain (0.5 mU/mg) in 1 ml of 0.1 M phosphate buffer (pH 7.3) was added in the dark to a mixture of 60 mg (63.8 μmol) N^6-SL-NAD$^+$ [9] and 70 mg (0.59 mmol) 3-(3-*H*-diazirin-3-yl)pyridine [10] dissolved in 1.7 ml of the same buffer. The reaction mixture was stirred in the dark for 14 h at room temperature, followed by addition of another 14 mg enzyme in 0.8 ml buffer. After 10 h the suspension was centrifuged and the supernatant extracted with 9 ml chloroform. The aqueous phase was diluted with water to 300 ml and applied to a Fractogel TSK DEAE-650 (S) column (2 × 40 cm, Merck, Darmstadt) previously equilibrated with 5 mM ammonium bicarbonate (pH 8.4). Elution with 200 ml of the same buffer was followed by a linear gradient of 2 l of 5–200 mM ammonium bicarbonate. Fractions containing spin-labeled dinucleotides as revealed by ESR and UV spectroscopy were pooled and lyophilized. Removal of unchanged N^6-SL-NAD$^+$ was achieved by enzymatic reduction to its corresponding NADH derivative. The crude product was incubated with 1.5 mg yeast alcohol dehydrogenase in 15 ml of 0.1 M glycine buffer (pH 9.5), containing 0.5 M ethanol for 2 h and rechromatographed on Fractogel TSK DEAE-650 (S), yielding 36 mg (60%) N^6-SL-DAD$^+$. Traces of decomposition products were removed prior to any use by reversed-phase HPLC on LiChroprep RP-18 from Merck-Darmstadt (6300 theoretical plates) at 700 kPa with 0.15 M aqueous LiCl containing 5% isopropanol and methanol each. Spectral data: UV, λ_{max} = 268 nm, weak shoulders at 330 and 370 nm; NMR, H-3 of the diazirine ring, two singlets at 3.36 and 2.64 ppm in water, with relative intensities depending on the pH. NMR spectra were recorded after reduction of the spin label by acidification.

Synthesis of 3-(3-*H*-diazirin-3-yl)pyridine 8-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl)aminoadenine dinucleotide (C8-SL-DAD$^+$): The C8 derivative was obtained from 28 mg (34 μmol) C8-SL-NAD$^+$ [11] as described for N^6-SL-DAD$^+$ in 47% yield (13 mg).

Photolysis experiments were carried out in a Ryonet photoreactor (Middletown, CT), equipped with 16 concentric 350 nm lamps. Samples were kept in Duran vessels. ESR spectra were recorded at 20°C in 67 mM phosphate buffer (pH 7.2) with a Bruker ER-420 spectrometer operating in the X-band mode at 18 mW and a peak-to-peak modulation amplitude of 0.8 G.

Protein was determined with Coomassie blue [12] or photometrically at 280 nm employing a factor of 1.34 for 1 mg/ml lactate dehydrogenase. Ribose assay as needed for determination of covalently bonded SL-DAD$^+$ was carried out according to Brückner [13] by the orcinol method with slight modifications as described by Wenzel [14]. Tightly enzyme-bound nucleotides as NADH-X [15] were determined spectrophotometrically at 260 nm after denaturation and precipitation of lactate dehydrogenase by addition of 70% perchloric acid up to a final concentration of 10%.

For covalent labeling of lactate dehydrogenase with N^6-SL-DAD$^+$, 1.25 mg (8.7 nmol) enzyme in 100 μl phosphate buffer (pH 7.2) were irradiated for 5 min in the presence of 266 μM N^6-SL-DAD$^+$ and 2.4 mM oxalate. Separation of the protein from free ligands was achieved either by gel chromatography on Sephadex G-25 (3 × 30 cm) and two subsequent dilutions with 1 l each of buf-

fer and concentration to 100 μl in a micro-dialysis concentrator (Prodicon, model 310 from Bio-Molecular Dynamics, Beaverton, OR) or by repeated gel centrifugation chromatography on Sephadex G-25 at 600 × g for 2 min [5,16].

3. RESULTS AND DISCUSSION

N^6-SL-DAD$^+$ and C8-SL-DAD$^+$ were synthesized from 3-pyridyl-3*H*-diazirine and N^6-SL-NAD$^+$ or C8-SL-NAD$^+$, respectively, by enzymic transglycosidation with NAD$^+$ glycohydrolase [17]. Unreacted SL-NAD$^+$ was separated after reduction to the corresponding SL-NADH derivatives with ethanol in the presence of alcohol dehydrogenase [2,11]. Due to the altered redox potential of the pyridinium ring SL-DAD$^+$ is not reduced under these conditions as had been shown previously for DAD$^+$ itself [8]. Lactate dehydrogenase forms tight ternary complexes with NAD$^+$ or some of its analogues in the presence of the substrate-analogous inhibitor oxalate [18]. Correspondingly tight complexes are formed with N^6- and C8-SL-DAD$^+$ in the dark as shown by ESR spectroscopy (fig.2). An apparent dissociation constant well below 100 nM can be estimated from the residual signal of the freely tumbling species. Moreover, this free signal stems in part from contaminating decomposition products of the SL-DAD$^+$ preparations in the order of 1–2%, which do not bind to the enzyme. The spectrum of the bound fraction is typical for highly immobilized species with an outer peak separation of 63.7 G for N^6-SL-DAD$^+$ and 66 G for C8-SL-DAD$^+$. Similar values were found previously for N^6- and C8-SL-NAD$^+$, 63 G and 65 G, respectively [19,11]. The latter analogues are active coenzymes of lactate dehydrogenase (70% v_{max} in the case of N^6-SL-NAD$^+$), whereas the photoaffinity derivatives are inactive because of the redox potential.

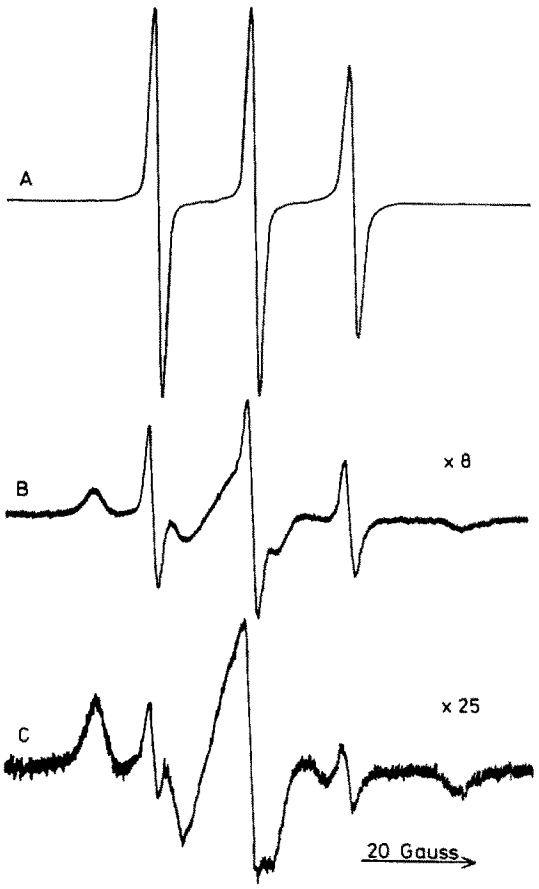

Fig.2. ESR spectra of N^6-SL-DAD$^+$. (A) 150 μM, (B) 25 μM in the presence of 92 μM lactate dehydrogenase and 27 mM oxalate, (C) covalently bonded to 92 μM lactate dehydrogenase.

Irradiation of the SL-DAD$^+$ derivatives leads to rapid decomposition of the diazirine ring under carbene formation and subsequent reactions as monitored spectrophotometrically by the decrease in the 310 nm absorption (fig.3) as described for 3-(3-*H*-diazirin-3-yl)pyridine [10]. A shoulder is formed at 300 nm immediately upon irradiation and is lost again upon further photolysis within 10 min. Under these conditions, i.e. irradiation for 5–10 min, lactate dehydrogenase lost less than 5% of its specific activity. When the ternary lactate dehydrogenase/N^6-SL-DAD$^+$/oxalate complex was irradiated, an immediate decrease in the signal amplitude of bound N^6-SL-DAD$^+$ was observed, followed by a decrease of the free signal as well (fig.4). This decomposition of the spin label is confined to the presence of oxalate and is not mediated by the diazirino group. Controls with N^6-SL-DAD$^+$ in the absence of oxalate showed no reduction of the spin label, whereas the spin label in normal N^6-SL-NAD$^+$ was destroyed at a similar rate in the presence of oxalate upon irradiation even in the absence of enzyme (fig.5).

For formation of the ternary enzyme/NAD$^+$/oxalate complex, oxalate concentrations above

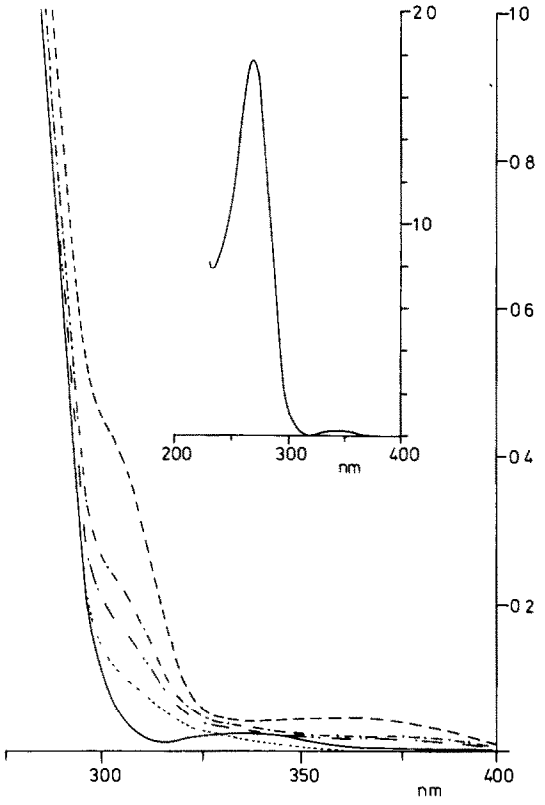

Fig.3. UV spectrum of 83 μM N^6-SL-DAD$^+$ (——) in phosphate buffer (pH 7.2) and after irradiation at 350 nm for 2 min (– – –), 3 min (– · –), 4 min (– · · · –) and 7 min (· · ·).

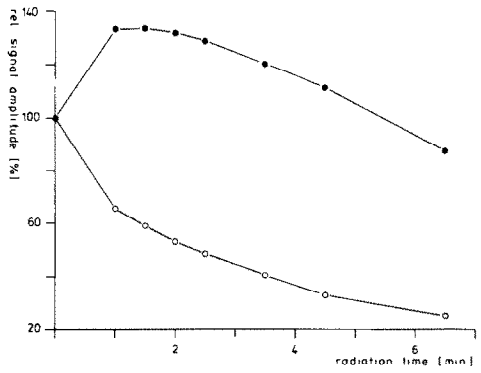

Fig.4. Decrease in the low-field ESR signal amplitude with time of 260 μM N^6-SL-DAD$^+$ in a complex with 44 μM lactate dehydrogenase upon irradiation at 350 nm. Signal amplitude of bound (○) and free (●) N^6-SL-DAD$^+$.

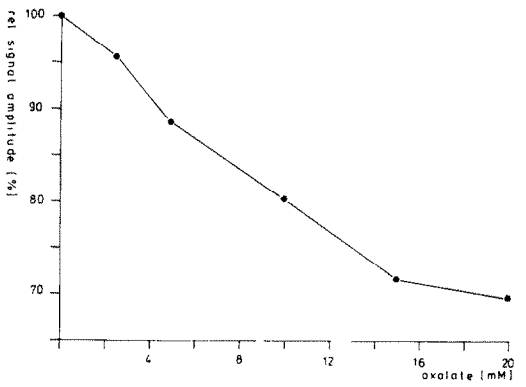

Fig.5. Decrease in the low-field signal amplitude of 0.7 mM N^6-SL-NAD$^+$ upon irradiation at 350 nm for 5 min in the presence of various oxalate concentrations.

10 mM are routinely employed. In our experiments even 14 mM oxalate was utilized. However, the apparent dissociation constant of NAD$^+$ in this complex is hardly changed when the oxalate concentration is reduced. Irradiation of an enzyme/N^6-SL-DAD$^+$ complex in the presence of 2.4 mM oxalate did not result in a significant loss of spectral intensity. After removal of oxalate and excess N^6-SL-DAD$^+$ by repeated gel centrifugation or gel chromatography and dialysis 0.13 equiv. were found to be covalently incorporated per subunit as measured by means of ribose determination. Non-covalently bonded N^6-SL-NAD$^+$ is totally removed under these conditions.

Lactate dehydrogenase preparations contain tightly bound decomposition products of NADH which block a certain fraction of the active sites [20]. Hence, based on the available sites even a 17% incorporation of N^6-SL-DAD$^+$ was observed. Fig.2 shows an ESR spectrum of covalently bonded highly immobilized N^6-SL-DAD$^+$ with an outer peak separation of 63 G. A small fraction, less than 2%, exhibits a typical free spectrum which apparently arises from N^6-SL-DAD$^+$ bonded non-specifically to the surface of the enzyme. A rather similar spectrum was obtained with covalently bonded C8-SL-DAD$^+$, however with an outer peak separation of 65 G and containing about 4% of the mobile component (not shown).

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REFERENCES

[1] Trommer, W.E., Huth, H. and Wenzel, H.R. (1979) Biochim. Biophys. Acta 567, 49–59.
[2] Wenzel, H.R. and Trommer, W.E. (1979) Biochim. Biophys. Acta 568, 287–296.
[3] Glöggler, K.G., Balasubramanian, K., Beth, A.H., Park, J.H. and Trommer, W.E. (1982) Biochim. Biophys. Acta 706, 197–202.
[4] Zantema, A., Trommer, W.E., Wenzel, H. and Robillard, G.T. (1977) Eur. J. Biochem. 72, 175–184.
[5] Fritzsche, T.M., McIntyre, J.O., Fleischer, S. and Trommer, W.E. (1984) Biochim. Biophys. Acta 791, 173–185.
[6] Trommer, W.E. and Glöggler, K. (1979) Biochim. Biophys. Acta 571, 186–194.
[7] Beth, A.H., Robinson, B.H., Cobb, C.E., Dalton, L.R., Trommer, W.E., Birktoft, J.J. and Park, J.H. (1984) J. Biol. Chem. 259, 9717–9728.
[8] Standring, D.N. and Knowles, J.R. (1980) Biochemistry 19, 2811–2816.
[9] Trommer, W.E., Wenzel, H. and Pfleiderer, G. (1974) Liebigs Ann. Chem. 1357–1359.
[10] Smith, R.A.G. and Knowles, J.R. (1975) J. Chem. Soc. Perkin Trans. II, 686–694.
[11] Wenzel, H.R. and Trommer, W.E. (1977) FEBS Lett. 78, 184–188.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[13] Brückner, J. (1955) Biochem. J. 60, 200–205.
[14] Wenzel, H. (1976) Doctoral Thesis, Ruhr Universität Bochum, FRG.
[15] Oppenheimer, N.J. and Kaplan, N.O. (1974) Biochemistry 13, 4675–4685.
[16] Penefsky, H. (1979) Methods Enzymol. 56, 527–530.
[17] Zatman, L.J., Kaplan, N.O. and Colowick, S.P. (1953) J. Biol. Chem. 200, 197–212.
[18] Novoa, W.B. and Schwert, G.W. (1961) J. Biol. Chem. 236, 2150–2153.
[19] Wenzel, H.R., Pfleiderer, G., Trommer, W.E., Paschenda, K. and Redhardt, A. (1976) Biochim. Biophys. Acta 452, 292–301.
[20] Wieland, T., Duesberg, P., Pfleiderer, G., Stock, A. and Sann, E. (1962) Arch. Biochem. Biophys., suppl.1, 260–263.