8-SPIN-LABEL NICOTINAMIDE ADENINE DINUCLEOTIDE, SYNTHESIS AND PROPERTIES OF A NEW SPIN-LABELLED COENZYME

H. R. WENZEL

Ruhr-Universität Bochum, Institut für Biophysik, Postfach 102148, 4630 Bochum 1

and

W. E. TROMMER

Universität Stuttgart, Institut für Organische Chemie, Biochemie und Isotopenforschung, Pfaffenwaldring 55, 7000 Stuttgart 80, FRG

Received 24 March 1977

1. Introduction

Probing active sites of enzymes by electron spin resonance using spin-labelled coenzyme analogues has been exploited by several groups [1–6]. Useful information concerning binary complexes of the enzymes with the analogues and ternary complexes with additional substrates or substrate-like compounds can often be obtained by such studies.

NAD^+ with a spin-label linked to the amino group of the adenine moiety (N^6SL-NAD^+) was synthesized by us [4] and has been used in binding studies with lactate dehydrogenase [5] and glutamate dehydrogenase [6].

In this paper we describe the synthesis of 8SL-NAD^+ in which the spin-label is bound via an amino function to C-8 of the coenzyme (fig.1). The dinucleotide has been prepared by condensation of nicotinamide mononucleotide and 8SL-AMP in presence of dicyclohexylcarbodiimide [7].

This new attachment of the spin-label to the coenzyme is particularly interesting because bulky substituents at C-8 of adenine nucleotides are known to favour a *syn*-conformation of the base relative to the ribose ring, in contrast to N^6-substituents which obviously do not affect the predominant *anti*-conformation of the unsubstituted compounds [8]. Thus, 8SL-NAD^+ is expected to be a useful complement of N^6SL-NAD^+ in many cases.

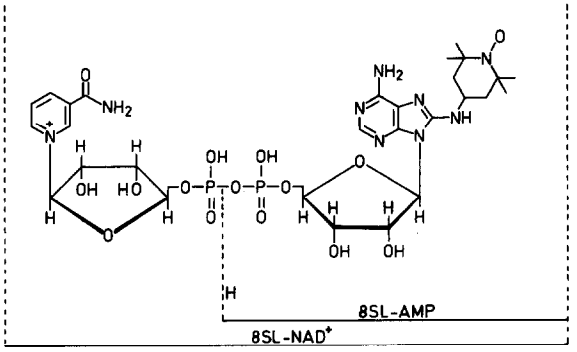

Fig.1. Structure of the 8-spin-labelled nucleotides.

2. Experimental

2.1. *Materials*

Lactate dehydrogenase (EC 1.1.1.27) from pig heart, alcohol dehydrogenase (EC 1.1.1.1) from yeast, nicotinamide mononucleotide (free acid) and NAD^+ were purchased from Boehringer, Mannheim.

Presented in part at the Jahrestagung der Deutschen Gesellschaft für Biophysik in Regensburg, 1976

2.2. *General procedures*

Thin-layer chromatography was carried out on precoated silica gel 60 F_{254} plates from Merck, Darmstadt, in the following systems: (A) Isopropanol/1% aqueous ammonium sulfate (2:1, v/v); (B) Isobutyric acid/conc. ammonia/water (66:1:33, v/v/v).

Coenzyme reductions, catalyzed by the two dehydrogenases, were followed by the increase in absorbance at 366 nm ($\epsilon = 3330\ M^{-1} . cm^{-1}$).

Reaction conditions: Buffer, 0.2 M glycine/NaOH, pH 9.5; Substrates, 0.1 M lactate or 0.3 M ethanol; Coenzymes, concentrations varied from 7×10^{-5} M to 8×10^{-4} M. The reactions were initiated by addition of 0.4 μg lactate dehydrogenase or 0.2 μg alcohol dehydrogenase, respectively. Total volume, 400 μl. Temperature, 25°C.

K_m- and V_{max}-values were determined according to the method of Lineweaver and Burk [9].

The concentration of lactate dehydrogenase (mol. wt tetramer = 144 000) was determined spectrophotometrically: $E^{1\ mg/ml}_{1\ cm}$ (280 nm) = 1.4 in 67 mM phosphate buffer, pH 7.2; ESR spectra were recorded on a X-band spectrometer at 25°C.

The ESR titration experiments were carried out as described previously [5].

2.3. *Syntheses*

2.3.1. 8-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl)Amino adenosine 5′-phosphate (8SL-AMP)

426 mg (1 mmol) 8-bromoadenosine 5′-phosphate (free acid) [10] were dissolved in 50 ml water, and the pH was adjusted to 6.9 with 1 M NaOH. After addition of 700 mg (4.1 mmol) 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl, the solution was kept in a sealed tube at 110°C for 40 h. The crude product was purified on DEAE–Sephadex A-25 (column, 2 × 25 cm, acetate form). Elution with water was followed by a linear gradient of 600 ml each of water and a pyridinium acetate buffer (1 M acetic acid, 1.5 M pyridine). 8SL-AMP eluted after 420 ml of the gradient and was lyophilized. Rechromatography under similar conditions and lyophilization yielded 90 mg (17%) of the pure product.

λ_{max} = 278 nm, $\epsilon = 18\ 400\ M^{-1} . cm^{-1}$ in 67 mM phosphate buffer, pH 7.2.

Anal. calcd. for $C_{19}H_{31}N_7O_8P$ (516.47):
C, 44.19; H, 6.05; N, 18.98; P, 6.00
Found: C, 44.10; H, 6.10; N, 18.82; P, 5.94

Thin-layer chromatography: System A, R_F = 0.53; System B, R_F = 0.50.

2.3.2. Nicotinamide 8-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl)amino adenine dinucleotide (8SL-NAD$^+$)

10 ml pyridine and then 0.5 g *N,N′*-dicyclohexylcarbodiimide were added to a solution of 37 mg (72 μmol) 8-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl) amino adenosine 5′-phosphate and 33 mg (99 μmol) nicotinamide mononucleotide in 2.5 ml water. The solution was stirred for 24 h at room temperature and then separated from precipitated dicyclohexylurea which was extracted with 0.5 ml hot water. Another 0.5 g carbodiimide was added to the combined filtrate and extract which was stirred for further 24 h. This process was repeated four times, the last two additions of carbodiimide being 0.2 g only.

Finally the solution was poured into 70 ml water. After 3 h the solution was filtered and carefully extracted with chloroform. The organic phase was washed with water and the combined aqueous phases were concentrated to about 10 ml under reduced pressure and separated on DEAE–Sephadex A-25 (column, 1.5 × 95 cm, chloride form). Elution was carried out with water and subsequently with a linear gradient of 900 ml water and 900 ml 0.2 M lithium chloride. 8SL-NAD$^+$ eluted after 680 ml of the gradient. The fraction was lyophilized and triturated with ethanol/ether (1:3) to remove the LiCl. The remaining crude dinucleotide was rechromatographed under similar conditions and isolated as described above. A final purification was achieved by precipitation from methanol with a 5-fold volume of ether at 0°C.

Yield: 6 mg (10%).

λ_{max} = 274 nm, $\epsilon = 22\ 300\ M^{-1} . cm^{-1}$ in 67 mM phosphate buffer, pH 7.2.

Thin-layer chromatography: System A, R_F = 0.37; System B, R_F = 0.43.

Efforts to isolate enzymatically reduced spin-labelled NADH were unsuccessful, due to slow redox reactions involving the dihydropyridine- and the nitroxyl-part of the coenzyme analogue. Qualitative ESR measurements of SL-NADH could, however, be carried out when the compound was prepared in situ in the ESR flat cell from SL-NAD$^+$ by reduction with ethanol and catalytic amounts of alcohol dehydrogenase at alkaline pH.

3. Results and discussion

3.1. *Enzyme kinetic data*

A crucial question inherent to spin-labelling is to what extent an attached spin-label perturbs the biological system under investigation [11]. In the case of spin-labelled coenzymes one obvious way to answer this question is to determine their kinetic parameters. The results with the NAD^+-analogues and two dehydrogenases are summarized in table 1.

In the lactate dehydrogenase system N^6SL-NAD^+ proves to be a nearly perfect coenzyme analogue; K_m- and V_{max}-values are very similar to those of NAD^+. The correspondence in the case of alcohol dehydrogenase from yeast, which is known to be a rather specific enzyme, is good as well.

A different behaviour is observed with 8SL-NAD^+. Its Michaelis constants are of the same magnitude as those of NAD^+; however, the maximum velocities are considerably smaller. A likely explanation for these differences is the preferred *syn*-conformation of the 8SL-adenosine moiety in solution.

For lactate dehydrogenase it could be shown with molecular models of 8SL-NAD^+ and the enzyme 2.5 Å resolution that binding of the analogue can occur exclusively in the *anti*-conformation (Eventoff, W. and Rossmann, M. G., personal communication). Thus, the isomerization may be rate limiting.

3.2. *ESR Spectra*

8SL-NAD^+ in buffer shows the expected 'nitroxyl-triplet' with the slightly broadened high-field line typical for spin-labelled molecules of that size (fig.2).

The addition of lactate dehydrogenase causes the amplitudes of all three lines to decrease. With increased instrumental gain the components of an 'immobilized spectrum', resulting from the fraction of 8SL-NAD^+ bound to the enzyme, can be seen (fig.3a).

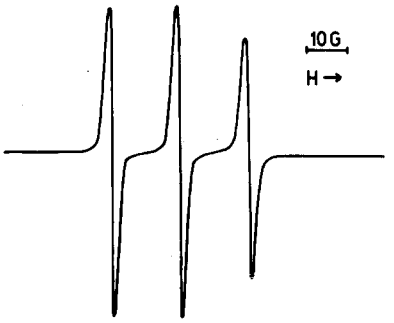

Fig.2. ESR spectrum of 4.4×10^{-4} M 8SL-NAD^+ in 67 mM phosphate buffer, pH 7.2. Relative instrument gain: 1.

When the spin-labelled coenzyme is reduced by ethanol and catalytic amounts of alcohol dehydrogenase the contribution from the 'immobilized spectrum' drastically increases (fig.3b), indicative of an almost complete binding of 8SL-NADH to lactate dehydrogenase under these conditions, i.e., 1.35-fold excess of possible binding sites over the coenzyme analogue. The distance between the outer peaks in fig.3 is about 65 G, still 2 G higher than in the corresponding complexes of N^6SL-NAD [5]. Splitting of this size shows a highly restricted environment of the nitroxyl group on the enzyme. This again is in accordance with the X-ray structure. There is sufficient space for the spin-label when 8SL-NAD is bound in the *anti*-conformation. However, movement of the nitroxyl group relative to the adenine base is reduced to a minor wobbling. In the case of lactate dehydrogenase/N^6SL-NAD complexes the motional freedom of the spin-label seems to be somewhat less constrained.

3.3. *Binding parameters*

The amplitude of the triplet high-field line is pro-

Table 1
Kinetic parameters of NAD^+ and its spin-labelled analogues

Coenzyme	Lactate dehydrogenase		Alcohol dehydrogenase	
	K_m (M)	V_{max} (%)	K_m (M)	V_{max} (%)
NAD^+	0.7×10^{-4}	100	2.4×10^{-4}	100
N^6SL-NAD^+	0.8×10^{-4}	71	5.6×10^{-4}	65
8SL-NAD^+	1.2×10^{-4}	7	6.7×10^{-4}	13

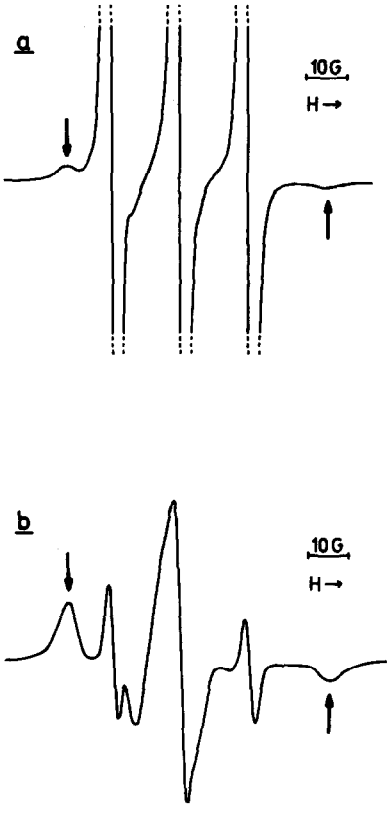

Fig.3. ESR Spectra of the binary lactate dehydrogenase complexes with 8SL-NAD$^+$ and 8SL-NADH in 0.1 M Tris/HCl buffer, pH 9.5. Relative instrument gain: 53. Curve a: 2.2×10^{-4} M 8SL-NAD; 7.5×10^{-5} M lactate dehydrogenase; 3.6×10^{-1} M ethanol. Curve b: 2.1×10^{-4} M 8SL-NAD; 7.1×10^{-5} M lactate dehydrogenase; 3.4×10^{-1} M ethanol; 8×10^{-7} M alcohol dehydrogenase. The vertical arrows indicate the positions of the outer peaks of the 'immobilized spectra'.

portional to the concentration of unbound radical because the 'immobilized spectrum' does not contribute significantly to this line. Hence it is possible to titrate lactate dehydrogenase with the spin-labelled coenzymes [5]. The results for 8SL-NAD$^+$ are shown as a Scatchard plot [12] in fig.4.

It can be deduced that 8SL-NAD$^+$ is bound to the four coenzyme-binding sites of the tetrameric enzyme with a dissociation constant of 7.6×10^{-4} M. This value is slightly higher than the value of 5.6×10^{-4} M found for N^{6}SL-NAD$^+$ [5], in accordance with the kinetic results. The dissociation constant for NAD$^+$ is about 3×10^{-4} M [13].

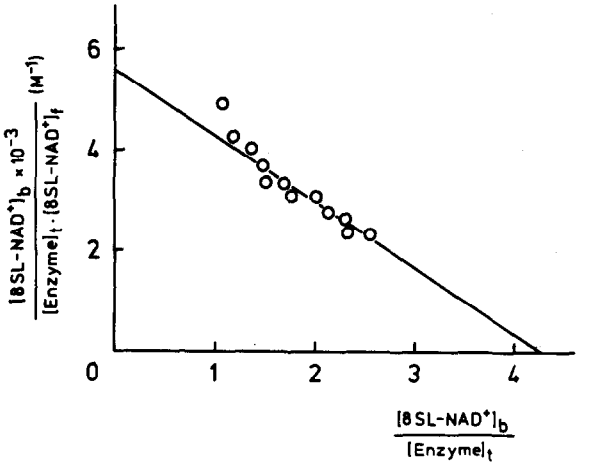

Fig.4. Scatchard plot of the ESR titration data for 8SL-NAD$^+$ binding to lactate dehydrogenase. 67 mM phosphate buffer, pH 7.2. Enzyme concentration: 6.0×10^{-5} M. 8SL-NAD$^+$ concentration varied from 2.8×10^{-4} M to 1.2×10^{-3} M.

8SL-NADH is bound tighter by about two orders of magnitude by lactate dehydrogenase than the oxidized analogue, as can be estimated from the ESR-signal amplitudes. Similar results have been obtained with N^{6}SL-NADH. A detailed binding study could not be carried out due to slow reduction of the spin-label by the dihydronicotinamide ring. The natural coenzyme NADH itself is known to bind about 360-times more tightly to the enzyme than NAD$^+$ [13], which is in accordance with our findings. Spin-labelled coenzymes have been extremely valuable for studying various ternary complexes of lactate dehydrogenase [14]. The results will be summarized elsewhere.

Acknowledgement

This work has been supported by the Deutsche Forschungsgemeinschaft.

References

[1] Weiner, H. (1969) Biochemistry 8, 526–533.
[2] Weidman, S. W., Drysdale, G. R. and Mildvan, A. S. (1973) Biochemistry 12, 1874–1883.
[3] Misharin, A. Yu., Polyanovsky, O. L. and Timofeev, V. P. (1974) FEBS Lett. 41, 131–134.

[4] Trommer, W. E., Wenzel, H. and Pfleiderer, G. (1974) Liebigs Ann. Chem. 1357–1359.
[5] Wenzel, H. R., Pfleiderer, G., Trommer, W. E., Paschenda, K. and Redhardt, A. (1976) Biochim. Biophys. Acta 452, 292–301.
[6] Zantema, A., Trommer, W. E., Wenzel, H. and Robillard, G. T. (1977) Eur. J. Biochem. 72, 175–184.
[7] Hughes, N. A., Kenner, G. W. and Todd, A. (1957) J. Chem. Soc. 3733–3738.
[8] Hoppe, J. and Wagner, K. G. (1974) Eur. J. Biochem. 48, 519–525.
[9] Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658–666.
[10] Ikehara, M., Tazawa, I. and Fukui, T. (1969) Chem. Pharm. Bull. 17, 1019–1024.
[11] McConnell, H. M. and McFarland, B. G. (1970) Quart. Rev. Biophys. 3, 91–136.
[12] Scatchard, G. (1948) Ann. NY Acad. Sci. 51, 660–672.
[13] Stinson, R. A. and Holbrook, J. J. (1973) Biochem. J. 131, 719–728.
[14] Wenzel, H. (1976) Doctoral Thesis, University of Bochum.