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Synthesis of 8-N_3-[2'-^{32}P]NADP(H), a new photoaffinity label for NADP(H)-specific enzymes

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The synthesis of the photoaffinity label 8-N_3-[2'-^{32}P]NADP$^+$ is described. The synthesis consists of the following steps: first 8-N_3-AMP is synthesized from AMP or ATP and then the 8-N_3-AMP is coupled with NMN, using DCCD as coupling reagent. Finally, the formed 8-N_3-NAD$^+$ is phosphorylated with [γ-^{32}P]ATP, using NAD-kinase. The formed product is purified and analysed and is shown to react well with NADP-specific enzymes and not to react with NAD-specific enzymes. Additionally, it is proposed that in the NAD-kinase reaction 2 mol ATP are used for the formation of 1 mol NADP: one molecule of ATP is hydrolyzed to ADP and phosphate while the other delivers the phosphate for the phosphorylation of NAD$^+$.

NADP-specific enzyme; Photoaffinity probe; NADP analog; [^{32}P]NADP$^+$, 8-azido; NAD-kinase

1. INTRODUCTION

Photoaffinity labelling of catalytic or regulatory sites of enzymes with azido analogues has become an important tool in the determination of catalytic subunits of multi-subunit enzymes and in the identification of the catalytic or regulatory regions of various enzymes. Especially the adenine nucleotide analogues play an important role in the study of the catalytic mechanism of the mitochondrial and chloroplast F_1-ATPase [1-5].

Also in the study of the mitochondrial transhydrogenase photoaffinity labelling has been successfully applied [6], but for a clear differentiation between the NAD- and NADP-binding sites the availability of a specific label is of relevance.

For the identification of the NADPH binding component of the respiratory burst oxidase of human neutrophils the 2',3'-dialdehyde analogue was used [7], but this analogue has several disadvantages, e.g. a relatively low specific activity, due to the introduction of radioactivity (^{3}H) by reduction with borohydride, and reactivity with lysine only. To obtain a specific analogue with high specific activity we decided to synthesize the 8-azido analogue of NADP(H), labelled with ^{32}P. An additional advantage of this analogue is the fact that the label can be introduced in the last step of the synthesis.

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Abbreviations: 8-N_3-AMP, 8-azido-adenosine-5'-monophosphate; 8-N_3-ATP, 8-azido-adenosine-5'-triphosphate; 8-N_3-NAD(H), nicotinamide-8-azido-adenine dinucleotide; 8-N_3-NADP(H), nicotinamide-8-azido-adenine dinucleotide phosphate; TEA·HCO_3, triethylamine-bicarbonate

2. METHODS AND MATERIALS

8-N_3-AMP was synthesized from AMP or was isolated as a hydrolytic by-product of the synthesis of 8-N_3-ATP from ATP, as described by Schäfer et al. [8]. 8-N_3-AMP was coupled to nicotinamide-mononucleotide (NMN) using dicyclohexylcarbodiimide (DCCD), as described by Christ et al. [9] for the synthesis of NAD-analogues.

NMN and NAD-kinase were obtained from Sigma (St. Louis, USA), pyridine and DMSO (dimethylsulfoxide) from Pierce (Rockford, USA), Sephadex DEAE A-25 from Pharmacia (Uppsala, Sweden), and [^{32}P]ATP from New England Nuclear (Dupont, Delaware, USA). All other chemicals were of analytical grade.

3. RESULTS AND DISCUSSION

3.1. *Synthesis of 8-N_3-NAD$^+$*

8-N_3-AMP, a starting material for the synthesis of 8-N_3-NAD$^+$, was synthesized from AMP or ATP as described by Schäfer et al. [8], without modifications. For the coupling of 8-N_3-AMP to NMN the method of Christ et al. [9] for the synthesis of NAD-analogues was used, not the method described by Michelson [10] and used by Koberstein [11] for the synthesis of 8-N_3-NAD$^+$. The procedure was as follows. To a 2 ml solution (in water) of 200 μmol of the lithium salt of 8-N_3-AMP 24 mg (70 μmol) NMN was added together with 12 ml pyridine. This solution was stirred at 4°C in the dark and 800 mg DCCD was added. After 20 h of stirring again 800 mg DCCD was added and after 40 h another 400 mg. Stirring was continued for another 24 h.

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The formed dicyclohexylurea was removed by centrifugation as precipitate and the clear solution, containing the formed addition product, was diluted with 125 ml water. After 1 h of stirring the solution was treated with chloroform to extract the residual dicyclohexylurea. The volume of the residual water layer was reduced with a rotary evaporator to 3 ml and brought on a DEAE-A-25 Sephadex column. The column was eluted with a TEA · HCO_3 gradient (pH 7.8) from 25 to 400 mM salt and the eluate at about 50 mM contained the 8-N_3-NAD^+ in a 15% yield. NMN eluted before the 8-N_3-NAD^+ and 8-N_3-AMP after. The 8-N_3-NAD^+-containing fractions were pooled and the solution was lyophilised with a rotary evaporator and 3 times coevaporated with 10 ml ethanol. The final 8-N_3-NAD^+-TEA salt was stored in dry dimethylsulfoxide. The spectral properties were identical with the ones reported by Koberstein [11]. Further evidence for the nature of the obtained compound was given by the appearance of a 340 nm band upon reaction with potassium cyanide, accompanied with a shift of the maximum at 274 nm to 283 nm. The millimolar extinction coefficient at 274 nm was 15 cm^{-1} (see also [11]).

Thin-layer chromatography on cellulose (Macherey Nagel Polygram Cel 300) with solvent A, consisting of *n*-butanol/acetic acid/water (5:2:3) gave one spot at $R_f = 0.32$. For the synthesis of [2-^{3}H]-8-N_3-NAD^+ the same procedure was followed, using [2-^{3}H]-8-N_3-AMP as starting material.

3.2. *Synthesis of 8-N_3-[2'-^{32}P]NADP$^+$*

To obtain the 8-N_3-analogue of $NADP^+$ the NAD^+-analogue was phosphorylated with an enzymatic procedure, involving NAD-kinase. To a 500 μl solution of 3.0 mM 8-N_3-NAD^+ in 100 mM Hepes-NaOH (pH 7.8) 250 μl of a 10 mM ATP solution was added, as well as 10 μl of a 1 M $MgCl_2$ solution and 100 μl of a [^{32}P]ATP solution (1 mCi). Under continuous stirring 5 mg NAD-kinase (70 units, [12]) was added. The reaction mixture was kept at 37°C in the dark for 6 h with continuous stirring. After 6 h the mixture was brought on a DEAE-A-25 Sephadex column (height 15 cm, diameter 1 cm) and 8-N_3-[2'-^{32}P]$NADP^+$ was eluted with a 25–400 mM TEA · HCO_3 block gradient at 225 mM (fig.1A). Another fraction containing ^{32}P was eluted at about 175 mM (inorganic phosphate) and at 400 mM the residual ATP was still bound to the column. The fractions containing 8-N_3-[2'-^{32}P]$NADP^+$ were pooled, evaporated and 3 times coevaporated with ethanol. The final product was stored as a solution in 20 mM Tris-HCl (pH 7.5) at −20°C.

Calculated from the 8-N_3-NAD^+ the yield of the procedure was 80% and of the total ^{32}P added about 45% was incorporated into the 8-N_3-$NADP^+$. Another 45% of the added radioactivity was present as inorganic phosphate and about 10% as unreacted ATP. When the synthesis of 8-N_3-$NADP^+$ was performed without

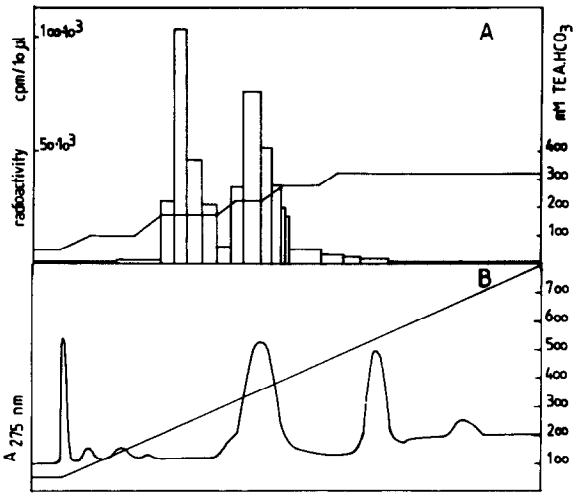

Fig.1. Elution profile of the DEAE-A-25 Sephadex column for the purification of 8-N_3-$NADP^+$. In (A) the column was stepwise eluted with a gradient of TEA · HCO_3 and the radioactivity in each fraction was measured and plotted. At 175 mM salt inorganic phosphate was eluted and at 225 mM the 8-N_3-$NADP^+$. Some ATP was still on the column at 400 mM. In (B) the absorbance profile is shown of a non-radioactive preparation, eluted with a linear gradient of TEA · HCO_3 from 50 to 800 mM. At 350 mM salt 8-N_3-$NADP^+$ is eluted, at 500 mM the ADP and at 650 mM the residual ATP.

radioactivity it was found by analysis of the column fractions that at about 400 mM salt ADP was eluted (fig.1B). The amount of ADP appeared to be twice the amount of the formed 8-N_3-$NADP^+$ (and that of inorganic phosphate). Our data, then, indicate that for each phosphate transferred from ATP to 8-N_3-NAD^+ by the kinase, one ATP is hydrolysed to ADP and inorganic phosphate. Also the data in [12] fit with the hydrolysis of one extra ATP for each phosphate that is transferred from ATP to NAD^+. It should be interesting to investigate whether the kinase contains two binding sites for ATP, the hydrolysis of one ATP being required for the correct orientation of the bound (azido-)NAD^+ relative to the second bound ATP.

3.3. *Properties of 8-N_3-[2'-^{32}P]NADP$^+$*

The specific radioactivity of the synthesized 8-N_3-[2'-^{32}P]$NADP^+$ was 1000 dpm/pmol and with such a specific activity the analogue is very useful for detection of low amounts of NADP-binding proteins and for labelling studies aimed at the identification of NADP-binding sites. The enzymatic procedure for the coupling of phosphate is also attractive because of the low waste of radioactivity and high yield of product formation.

The absorbance spectrum shows a maximum at 270 nm (cf. 274 nm for the 8-N_3-NAD^+) and upon addition of potassium cyanide the expected band at 340 nm appears (fig.2). The millimolar extinction coefficient at 270 nm is 15 cm^{-1}. Thin-layer chromatography on cellulose with solvent A (see above) gave one single spot at an R_f value of 0.20, at which position also all radioactivity was recovered.

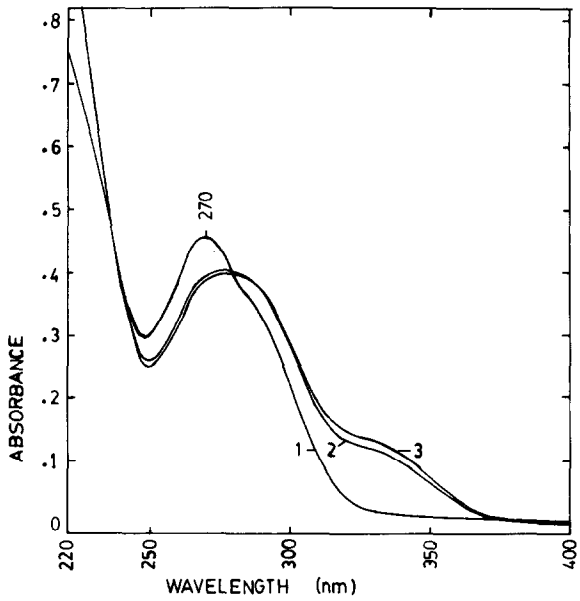

Fig.2. Absorbance spectrum of 30 μM 8-N_3-NADP$^+$ in 10 mM Tris-HCl (pH 7.0). Spectrum 1 represents the original spectrum, and spectra 2 and 3 the spectrum 10 and 20 min, respectively, after addition of 5 mM potassium cyanide.

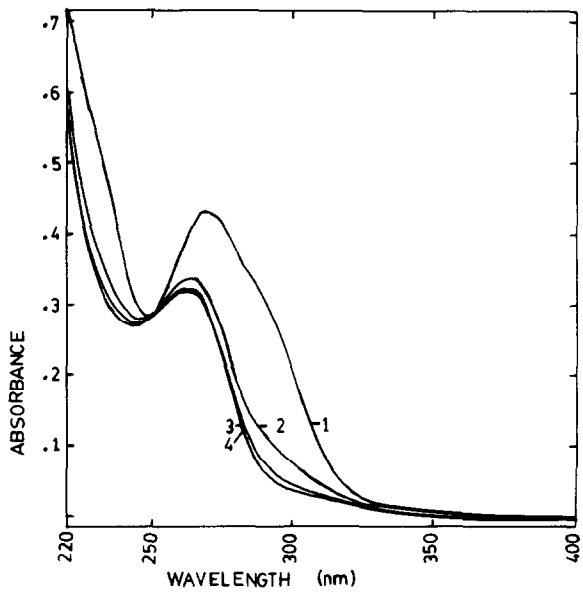

Fig.3. Effect of photo-irradiation on the spectrum of 8-N_3-NADP$^+$. A 30 μM solution of 8-N_3-NADP$^+$ in 10 mM Tris-HCl (pH 7.0) was irradiated for 10, 20 and 30 min (lines labelled 2, 3 and 4, respectively) with a CAMAG universal UV-lamp (type TL 900, 8 W lamp) at 360 nm at a distance of 1 cm from the cuvette.

Photodecomposition of the formed compound can be seen in fig.3 as the decrease of the absorbance and a shift of the maximum back to 260 nm, the absorbance maximum of NADP$^+$. A very similar effect is seen when 8-N_3-NAD$^+$ is illuminated. As an NADP$^+$-analogue, the newly synthesized compound has the advantage that the covalent linkage with a protein occurs with the adenine ring, the moiety of NADP$^+$ that is mostly directly involved in the binding. In contrast, in other types of analogues, like the arylazido analogues, there is a long distance between the formed covalent bound and the heterocyclic part of the molecule. Relative to the dialdehyde derivative the advantage is that not only lysine, but also other amino acids are able to form the covalent linkage.

The 8-azido analogues of ADP and ATP have been shown to be largely present (in solution) in the syn-configuration, while the ADP and ATP themselves are in the anti-configuration [13]. This difference in configuration is responsible for the lower affinity of these analogues for some specific sites (binding to these sites requires the anti-configuration [14]) and the fact that 8-N_3-ATP is a bad substrate for the F_1-ATPase [3] and 8-N_3-ADP a bad substrate for the ATP-synthase [15], just as for pyruvate kinase. Our data show that the 8-N_3-NAD$^+$ is a good substrate for the NAD-kinase. This is probably not due to a low specificity of this enzyme for the configuration of the adenine moiety of the substrate, but to the fact that the anti-configuration is maintained after binding of the azido-group at the 8-position of the adenine moiety. Abdallah et al. [16] have shown that NAD, even when the 8-position contains bromide, always assumes the anti-configuration.

Preliminary experiments have been carried out with the oxidase system of human neutrophils and we found that the same protein was labelled as has been reported by Smith et al. [7] for the dialdehyde derivative. For that experiment the compound had to be in the reduced form and it appeared that the 8-N_3-derivative of NADP$^+$ is a good substrate for glucose-6-phosphate dehydrogenase, although the K_m was slightly higher. On the other hand, the compound did not react with alcohol dehydrogenase or with lactate dehydrogenase, indicating that the analogue is indeed a real NADP-analogue.

Finally, it may be expected that with the same procedure also the 2-N_3-[2'-^{32}P]NADP$^+$ can be synthesized with the 2-N_3-AMP as starting material.

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REFERENCES

[1] Wagenvoord, R.J., van der Kraan, I. and Kemp, A. (1977) Biochim. Biophys. Acta 460, 17-24.
[2] Hollemans, M., Runswick, M.J., Fearnley, I.M. and Walker, J.E. (1983) J. Biol. Chem. 258, 9307-9317.
[3] Van Dongen, M.B.M., de Geus, J.P., Korver, T., Hartog, A.F. and Berden, J.A. (1986) Biochim. Biophys. Acta 850, 359-368.
[4] Garin, J., Boulay, F., Issartel, J.P., Lunardi, J. and Vignais, P.V. (1986) Biochemistry 25, 4431-4437.

[5] Cross, R.L., Cunningham, D., Miller, C.G., Xue, Z., Zhou, J.M. and Boyer, P.D. (1987) Proc. Natl. Acad. Sci. USA 84 5715-5719.
[6] Hu, P.S., Persson, B., Carlenor, E., Hartog, A.F., Höög, J.O., Jörnvall, H., Berden, J.A. and Rydström, J. (1988) EBEC Rep. 5, 65.
[7] Smith, R.M., Curnutte, J.T. and Babior, B.M. (1989) J. Biol. Chem. 264, 1958-1962.
[8] Schäfer, H.G., Scheurich, P. and Dose, K. (1978) Liebigs Ann. Chem. 1978-II, 1749-1753.
[9] Christ, W. and Coper, H. (1980) Methods Enzymol. 66, 71-80.
[10] Michelson, A.M. (1964) Biochim. Biophys. Acta 91, 1-13.
[11] Koberstein, R. (1976) Eur. J. Biochem. 67, 223-229.
[12] Blomquist, C.H. (1980) Methods Enzymol. 66, 101-104.
[13] Czarnecki, J.J. (1984) Biochim. Biophys. Acta 800, 41-51.
[14] Garin, J., Vignais, P.V., Gronenborn, A.M., Clore, G.M., Gao, Z. and Bauerlein, E. (1988) FEBS Lett. 242, 178-182.
[15] Sloothaak, J.B., Berden, J.A., Herweijer, M.A. and Kemp, A. (1985) Biochim. Biophys. Acta 809, 27-38.
[16] Abdallah, A., Biellmann, J.F., Nordström, B. and Brändén, C.I. (1975) Eur. J. Biochem. 50, 475-481.