

Photoaffinity Labeling of Lactate Dehydrogenase by the Bis-azido Analog of NAD⁺: P¹-N⁶-(4-azidophenylethyl)adenosine-P²-[4-(3-azidopyridinio)butyl]diphosphate

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Lactate dehydrogenase from pig heart is inactivated by the NAD⁺-analog P¹-N⁶-(4-azidophenylethyl)adenosine-P²-[4-(3-azidopyridinio)butyl]diphosphate (**6**) upon irradiation with UV light of wavelengths in the range from 300 to 380 nm. The decrease in enzyme activity can be prevented by the addition of NAD⁺ and oxalate. The modified enzyme shows a reduced binding capacity for its coenzyme as compared to native lactate dehydrogenase. The amount of incorporated coenzyme is deduced from the ribose content of inactivated enzyme. Tryptic digestion of the modified protein and separation of the peptides by HPLC yields 5 ribose-containing fractions. One of them, fraction 66, is split by treatment with nucleotide pyrophosphatase into two subfractions, 63 and 58. Only subfraction 63 contains ribose. Whereas peptide 58 shows a UV absorption spectrum similar to that of 4-(3-aminopyridinio)-butyl phosphate (**3**). Amino acid analyses of the peptides indicate that the inactivator forms covalent bonds with different parts of the protein: Peptide 63 is characterized by a great portion of hydrophobic amino acids whereas peptide 58 shows a high degree of hydrophilicity.

Introduction

Affinity labeling of dehydrogenases has been achieved with various reactive groups bound to nucleotide pyrophosphates. Bromoketones react preferentially with nucleophilic residues of the protein [1–4]. If such residues are not available in the vicinity of the nucleotide binding site no incorporation is observed. Hydrolysis of the bromoketo group occurs as a side reaction [5]. The use of highly reactive groups like diazonium residues, which react with several amino acid residues by forming azo bridges, does not lead to sufficiently stable fixation. During the protein analysis the nucleotide part is cleaved off [6]. In the literature several azido derivatives of the coenzymes NAD⁺ and NADP⁺ have been described as reagents for the modification of dehydrogenases [7–9]. These compounds are stable and decompose only upon irradiation with light of certain wavelengths to form highly reactive radicals capable of modifying different amino acid residues. Difficult syntheses and low yields impede the radioactive labeling of inactivators, which would facilitate the identification

of the site of attack. Non-radioactive affinity labels can only be located on the basis of molecule fragments of the reagent that are analytically distinguishable from protein constituents.

In spite of the frequent use of azido analogs of coenzyme NAD in inactivation experiments of dehydrogenases, protein sequencing was not carried out; only in few cases the amino acid modified by the inactivator was determined [10]. For the inactivation experiments we used lactate dehydrogenase from pig heart because the structure of this enzyme is known [11].

Results

For the preparation of a suitable azido compound we use N⁶-(4-aminophenylethyl)adenosine phosphate (**1**) and 4-(3-aminopyridinio)butyl phosphate (**3**) as starting materials [6]. The two phosphoric acid esters react with dicyclohexylcarbodiimide in aqueous pyridine to form the mixed anhydride (**5**) which is isolated by chromatography on DEAE Sephadex A-25. The bis-amino compound **5** is treated with nitrous acid and the resulting diazonium groups are then reacted with sodium azide to form the bis-azido derivative P¹-N⁶-(4-azidophenylethyl)adenosine-P²-[4-(3-azidopyri-

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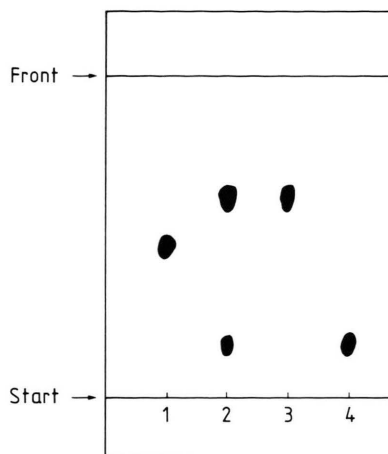
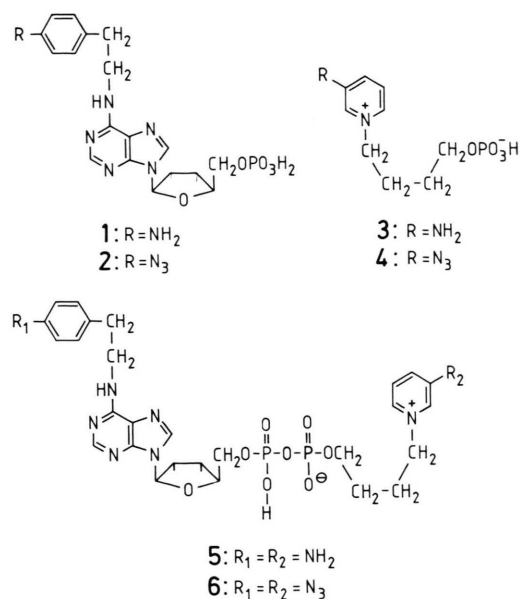


Fig. 1. Chromatography on TLC silica plates.
1) P¹-N⁶-(4-azidophenylethyl)adenosine-P²-[4-(3-azidopyridinio)butyl] diphosphate (**6**).
2) **6** after treatment with nucleotide pyrophosphatase.
3) N⁶-(4-azidophenylethyl)adenosine phosphate.
4) 4-(3-azidopyridinio)butyl phosphate.
Solvent: isobutyric acid – water – 30% ammonia, v:v:v, 70:29:1.

dinio)butyl]diphosphate (**6**) [12] (Formula scheme). The formation of the latter is monitored by thin-layer chromatography (Fig. 1). After cleavage of the pyrophosphate bond the fragments are compared with the mononucleotides N⁶-(4-azidophenylethyl)adenosine phosphate (**2**) and 4-(3-azidopyridinio)butyl phosphate (**4**). The structure is further confirmed by UV-spectroscopy: P¹-N⁶-(4-azidophenylethyl)adenosine P²-[4-(3-azidopyridinio)butyl]diphosphate (**6**) shows an absorbance maximum at 255 nm and a shoulder at 305 nm. The azido groups are identified on

the basis of the infrared absorbance at 2080 cm⁻¹ (Fig. 2a). The pyrophosphate compound **6** resembles the structure of NAD⁺. The adenine ring carries an azidophenylethyl group in N⁶-position whereas the nicotinamide riboside moiety of the natural coenzyme is replaced by a 3-azidopyridinio-butyl group. Earlier studies have shown that the replacement of the ribose by a *n*-butyl group does not alter the distance between the pyrophos-

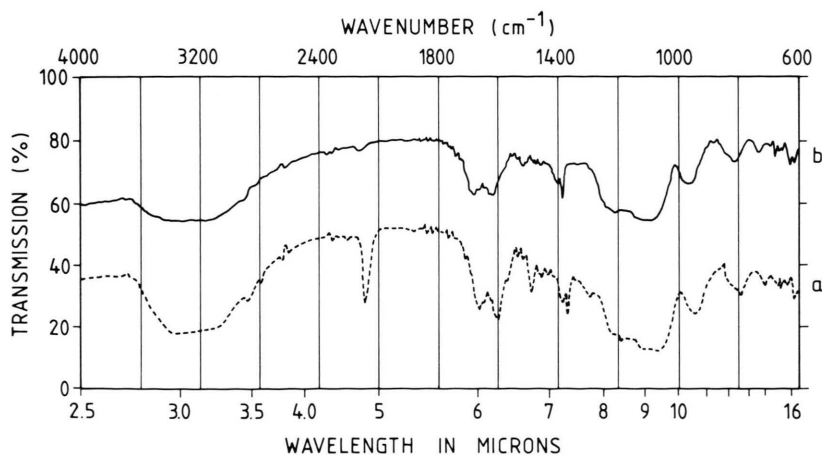


Fig. 2. IR-spectra in KBr of P¹-N⁶-(4-azidophenylethyl)-adenosine-P²-[4-(3-azidopyridinio)butyl] diphosphate (**6**). Dotted line: before; solid line: after irradiation.

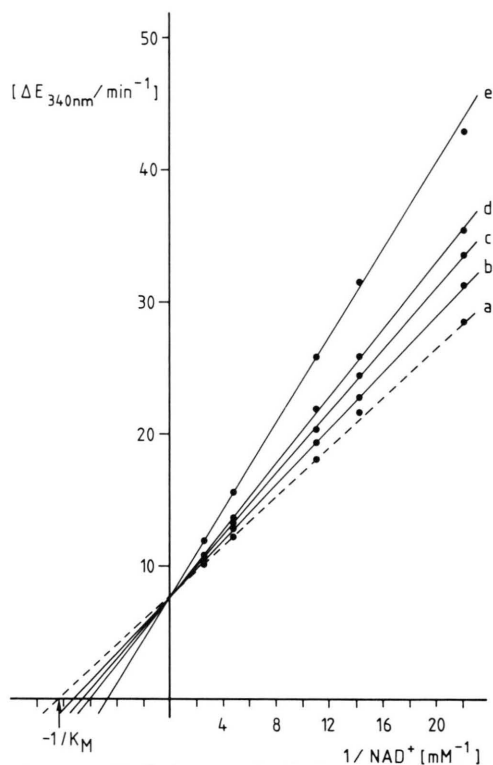


Fig. 3. Competitive inhibition of LDH by **6**. Double reciprocal plot $1/v$ versus $1/c$; for details see text. Concentration of inhibitor **6**: a = 0, b = 2.4, c = 4.53, d = 5.2 and e = 6.79 μM .

phate and the pyridinium ring [13]. $\text{P}^1\text{-N}^6$ -(4-aminophenylethyl)adenosine- P^2 -[4-(3-aminopyridinio)butyl]diphosphate (**5**) and the bis-azido derivative (**6**) are competitive inhibitors with respect to NAD^+ in tests with lactate dehydrogenase (Fig. 3). The inhibition constant (K_i) for **6** is 0.02 mM.

The Michaelis constant for NAD^+ is under the same conditions determined 0.1 mM.

Upon irradiation with UV light of 300–380 nm wavelength the azido groups are cleaved to form radicals which react to inactive compounds in aqueous medium. The optical properties change in parallel with the irradiation. The shoulder at 305 nm disappears, the absorption at short wavelength is reduced, and the maximum is shifted to 275 nm. In the IR spectrum of the irradiated compound no band could be detected at 2080 cm^{-1} (Fig. 2b).

When **6** was irradiated in the presence of equimolar amounts of lactate dehydrogenase the enzyme activity was reduced to about 20% of the initial value after 30 min. The rate of inactivation depends on the temperature, pH and concentration of the inactivator **6**. Using up to threefold molar excess of **6** over lactate dehydrogenase the decrease of activity is linear with time. Assuming that the reaction mechanism follows simple saturation kinetics we obtain a rate constant k_2 of 0.83 min^{-1} and a dissociation constant K_i^* of 5 mM [14]. In the presence of NAD^+ the reaction of **6** causes only a slight decrease in LDH activity after irradiation; the presence of both NAD^+ and oxalate prevents any loss of activity even after prolonged irradiation (Fig. 4). Enzyme activity can not be recovered neither by exhaustive dialysis nor by charcoal

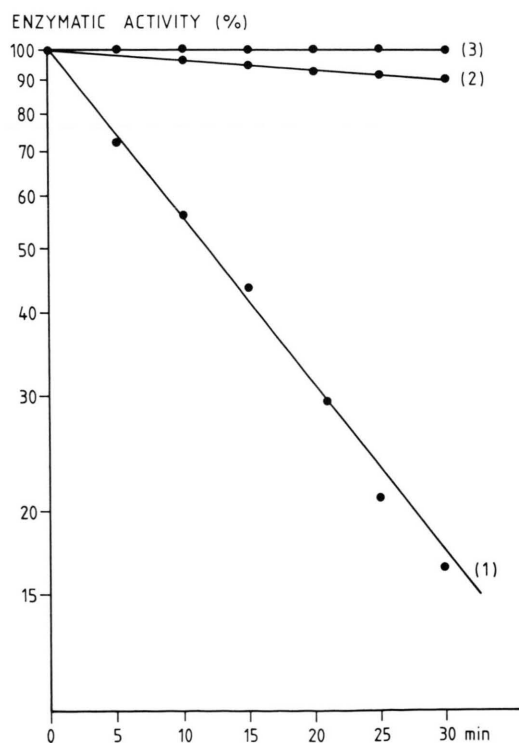


Fig. 4. Inactivation of LDH in presence of:
1) 0.1 mM **6**,
2) 0.1 mM **6** and 0.15 mM NAD^+ ,
3) 0.1 mM **6** and 0.15 mM NAD^+ + 4 mM oxalate,
in 0.1 M Tris/HCl buffer pH 8, 4 °C; wavelength: 300–380 nm; abscissa: irradiation time; ordinate: enzyme activity 100% = nonirradiated.

treatment. The optical properties of the enzyme are altered, too: lactate dehydrogenase emits light with a maximum of 340 nm when irradiated at 290 nm. This fluorescence is reduced in the presence of NAD^+ or other nucleotide pyrophosphates [15]. This quenching of the protein fluorescence is also observed in the presence of equimolar amounts of **6**. After addition of **6** and irradiation, the fluorescence of the protein at 340 nm drops even further.

The incorporation of **6** into the protein cannot be monitored spectrophotometrically in a direct manner by recording UV spectra because the chromophoric groups of all reactants absorb in the same region between 240 nm and 300 nm. Therefore, the degree of enzyme modification is derived from coenzyme binding studies using column chromatography on a Sephadex G-10 column with NADH containing buffer [16]. One mol of the native lactate dehydrogenase binds four mol of NADH. After inactivation of the enzyme to a residual activity of 30–40% only 1.6 to 1.7 mol of NADH are still bound per mol of tetrameric lactate dehydrogenase. A similar correlation of residual activity and coenzyme binding capacity has been obtained from fluorescence titration experiments of the native and partially inactivated enzyme (Fig. 5). The phosphate and ribose content of a similar enzyme preparation with 40% residual activity indicates an incorporation of 2.2 mol of **6** per mol enzyme tetramer.

In order to identify the binding site of modified lactate dehydrogenase the protein is unfolded in urea solution and digested with trypsin. The peptides are separated by reversed phase HPLC. All fractions are tested for their ribose content [17]. Ribose is detected in five fractions, but two of these are nucleotide fragments that do not contain amino acids. Subsequent treatment of the nucleotide containing peptides with nucleotide pyrophosphatases causes a change in the chromatographic properties in only one case: Peptide 66 of the fractions as shown in Fig. 6 [18]. Chromatography of the latter fraction yields two UV-absorbing sub-fractions: 63 and 58. One of them, peptide 63 contains ribose and phosphoric acid, whereas the other, peptide 58, contains phosphoric acid only.

The ribose-containing pyrophosphatase-treated peptide 63 predominantly contains hydrophobic amino acids whereas the other one peptide 58, con-

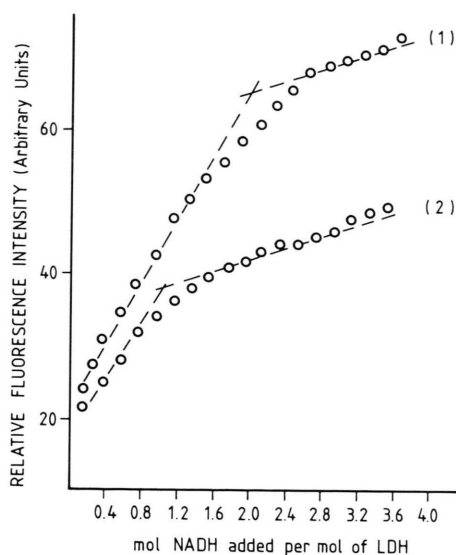


Fig. 5. Fluorescence titration of 2 ml samples containing 0.8 mg native (1) or modified (2) LDH (residual activity 45%) with 2 mM NADH solution in 0.1 M phosphate buffer pH 7 at 20 °C. Ordinate: Fluorescence increase at 420 nm after exciting with light of 340 nm.

tains mostly hydrophilic amino acids (Table I). It is indicated in the table that the original tryptic peptide 66 is still contaminated by traces of other peptides, which could not be separated even by repeated column chromatography. Therefore, the amino acid analysis of peptide 66 shows some amino acids in excess of the sum of the two pyrophosphatase peptides.

Table I. Amino acid content of the modified peptides.

Peptide fraction Nos.	66	63	58
Asp	9	1	5
Thr	3	0	1
Ser	6	1	3
Glu	7	2	5
Pro	3	1	2
Gly	6	1	2
Ala	3	1	1
Val	7	1	1
Ile	2	2	0
Leu	7	2	2
Phe	1	1	0
Lys	2	1	1
Arg	0.3	0	0

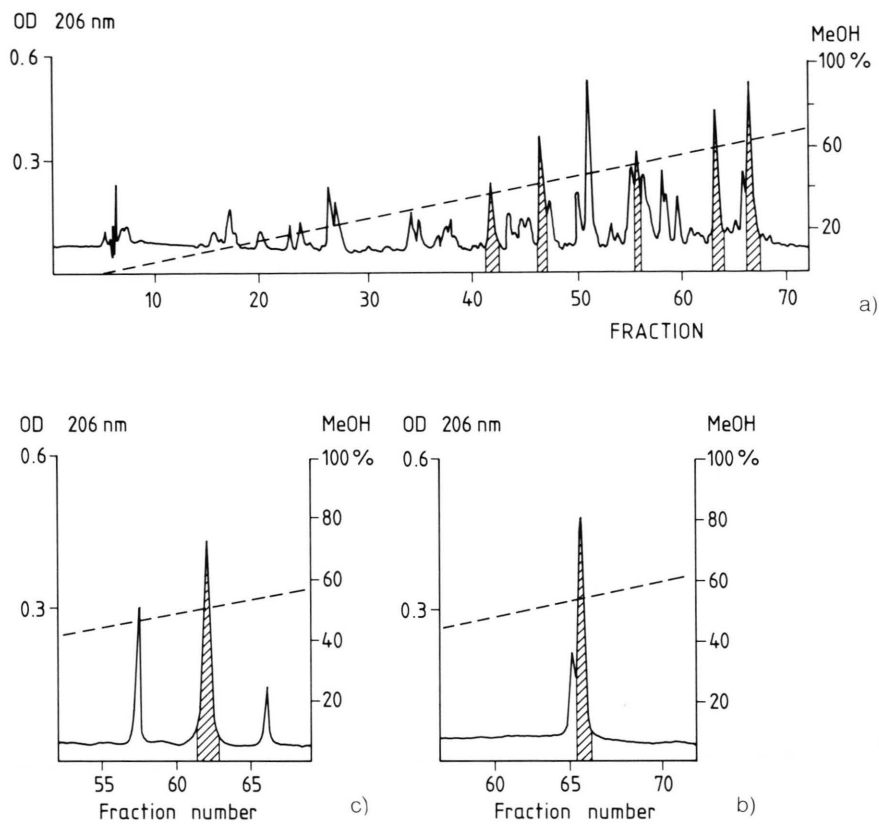


Fig. 6. a) HPLC elution profile of the tryptic digest of LDH (residual activity 40%) modified by **6** on a Waters RP 18 column, for details see text. Hatched: ribose containing fractions; ---- methanol concentration
 b) Rechromatography of fraction 66 before treatment with nucleotide pyrophosphatase and of
 c) fraction 66 after treatment with nucleotide pyrophosphatase.
 Ordinate: fraction number.

Discussion

P¹-N⁶-(4-azidophenylethyl)adenosine-P²-[4-(3-azidopyridinio)butyl]diphosphate (**6**) contains two azido groups that are bound to different parts of the molecule. The compound resembles the co-enzyme NAD⁺ structurally, but forms a tighter complex with lactate dehydrogenase and is not capable to transfer hydrogen.

In the NAD⁺-LDH complex the hydrophobic adenine ring is fixed to a hydrophobic binding pocket, whereas the more hydrophilic nicotinamide moiety is located in the catalytic center that is formed by hydrophilic amino acid residues [11]. **6** is also able to form a complex with this enzyme, but the small dissociation constant obtained from the competitive inhibition of **6** against NAD⁺ indi-

cates a different kind of binding. This can be explained by the presence of three different ring systems in **6**: benzene, adenine and nicotinamide. The benzene and the neighboring adenine ring could compete for the hydrophobic binding site, and thereby tighten the binding of the inhibitor. The irradiation of the bisazido compound **6** leads to the formation of radicals which immediately react with amino acid residues of the enzyme, thereby forming covalent bonds. The incorporation is accompanied by a loss of enzymic activity. The correlation of both phenomena indicate a stoichiometric binding of 1 molecule inactivator per active site of the enzyme. The optical properties of the modified enzyme are similar to those of the lactate dehydrogenase NAD⁺ complex. Evidence that the

coenzyme binding site of LDH is attacked by the irradiation derived radicals is indicated by the inactivation experiments in the presence of NAD^+ and of the pseudosubstrate oxalate. In this case the amino acid side chains are involved in binding the natural cofactor and are unable to react with the light induced radicals of added **6**, therefore, the enzymic activity remains unchanged. Further support comes from the fact that the NADH-binding capacity of partially inactivated LDH is linearly correlated with the residual activity.

Since no ribose is present in the native LDH apoenzyme, the ribose content of the modified protein allows to determine the incorporation of the nucleotide analog **6**.

After tryptic digestion of the modified protein five ribose containing cleavage products are obtained. Analysis of two of these fractions indicates the presence of both nucleotide and peptide structures, but, both compounds are not significantly changed by the action of a nucleotide pyrophosphatase. This behaviour precludes the possibility that any of these compounds contains 2 peptide structures crosslinked by the nucleotide diphosphate.

Only one of the ribose containing fractions is cleaved into two peptides by the action of a nucleotide pyrophosphatase. In this case two different peptides are formed. Ribose is detected only in one of the two peptides that resulted from nucleotide pyrophosphatase catalyzed hydrolysis. The other one shows an absorbance pattern resembling that of (3-aminopyridinio)butyl phosphate (**3**).

The orcin method used to identify ribose-containing peptides lead to a big loss of the substance, so that the sequencing of the rest peptide was not possible.

The three-dimensional structure of the NAD^+ -LDH complex reveals that the coenzyme moieties are bound to different binding sites, which are perfectly adapted to the different physicochemical characters of corresponding coenzyme parts [11]. The hydrophobic adenine moiety is placed in a hydrophobic pocket formed by amino acids which bear aliphatic or aromatic rests. In contrast to this region, the positively charged nicotinamide riboside moiety is bound at a more hydrophilic region, formed by polar amino acid side chains. The high content of hydrophobic amino acids in the ribose-containing peptide indicates that the adenine bind-

ing site is labeled, whereas the hydrophilic residues in the ribose-free peptide point to a modification of the nicotinamide binding site.

In the original tryptic peptide both structures appear to be linked by the pyrophosphate anhydride bond.

Materials and Methods

Nucleotides

Preparation of $\text{P}^1\text{-N}^6$ -(4-aminophenylethyl)-adenosine- P^2 -[4-(3-aminopyridinio)butyl]diphosphate (**5**).

The synthesis of N^6 -(4-aminophenylethyl)-adenosine phosphate (**1**) has been described earlier [6]. For the synthesis of 4-(3-aminopyridinio)butyl phosphate (**3**), 12.7 g of 3-formylaminopyridine (104 mmol) were added to 20.3 g of 4-bromobutyl acetate (104 mmol), mixed and left at 80 °C for 5 days. An oil formed which after cooling and repeated treatment with methanol-ether (v:v = 10:1) could be crystallized. The yield was 32 g or 99% of the theoretical value, m.p. 104 °C.

30 g of crude 1-(3-formylaminopyridinio)butyl acetate were dissolved in 350 ml of methanol and 35 ml of concentrated HCl were added. After three days at room temperature the solvent was evaporated and the residue redissolved in methanol and HCl. This procedure was repeated until ester was no longer detectable [5]. A positive ninhydrin reaction showed that the formyl group had been removed.

25 g of 4-(3-aminopyridinio)butanol were phosphorylated with 200 g polyphosphoric acid as described earlier [19]. The reaction mixture containing polyphosphoric acid ester was hydrolyzed by treatment with 1000 ml 1 M HCl. After evaporation to 350 ml the crude reaction product was placed on a Dowex 50 × 8 column (H^+ -Form, 200–400 mesh, 4 × 7.5 cm). The column was washed with water. 4-(3-aminopyridinio)butyl phosphate (**3**) appeared after 8 l in a volume of 18 l. The solvent was removed at 30 °C in vacuo until the final volume was 10 ml. After cooling the solution to 4 °C acetone was added until a turbidity appeared. **3** crystallized in colorless needles. The yield was 8.26 g or 36% with respect to 3-formylamino-pyridine, m.p. 72 °C.

UV λ_{max} = 249 nm; log ϵ = 3.88

UV λ_{max} = 320 nm; log ϵ = 3.51

Analysis:

Calcd 43.9 C, 6.09 H, 11.38 N, 12.58 P

Found 43.7 C, 6.08 H, 11.43 N, 12.46 P

For the preparation of the pyrophosphate **5**, 100 mg of N⁶-(4-aminophenylethyl)adenosine phosphate (**1**) and 100 mg of **3** were dissolved in 3.5 ml of trifluoroacetic anhydride. After 8 h at room temperature the solvent was removed at 30 °C *in vacuo*. The amorphous residue was dissolved in 75% aqueous pyridine and treated with dicyclohexylcarbodiimide as described before [6]. The pyrophosphate **5** was isolated by chromatography on DEAE Sephadex A-25 (HCO₃⁻-form, 3.5 × 25 cm). The column was washed with 2 l of water followed by a linear gradient of 4 l 0–0.2 M ammonium bicarbonate. **5** appeared in the eluate at 0.12 M NH₄HCO₃ in a volume of 0.4 l. After removing the solvent at 30 °C *in vacuo* the excess of ammonium bicarbonate was removed by repeated dissolving in ethanol and evaporating. 48 mg of a colorless amorphous product were obtained.

For the formation of the bisazido derivative **6**, 48 mg **5** were dissolved in 10 ml 1 N HCl at 0 °C and 23 ml of 1 M sodium nitrite solution were added. The solution was stirred for 10 min, 0.5 ml of 6 M sodium azide was added and stirred for another hour at 0 °C. The reaction mixture was allowed to warm up to room temperature while stirring. After 8 h the pH was adjusted to 6.5 with 1 M ammonia at 0 °C and the mixture was applied to a DEAE Sephadex A-25 column (HCO₃⁻-form, 3.5 × 25 cm). The following steps were performed at 4 °C and in dim light. After washing with 2 l of water a linear gradient of 4 l 0–0.3 M ammonium bicarbonate was used to develop the column. **6** appeared at 0.14 M ammonium bicarbonate in a volume of 1.9 l which was then reduced at 30 °C *in vacuo*. Further purification was achieved by chromatography on a Sephadex G-10 column (2 × 105 cm). 30 mg of a colorless amorphous product were obtained. **6** was stored at –20 °C in the dark.

UV λ_{\max} = 255 nm; log ϵ = 4.41

IR: band at 2080 cm⁻¹

In solution, the compound is not stable and decomposes even at 4 °C and a brown precipitate appears after 8 h. The concentration of **6** in freshly prepared solutions was determined from the absorbance at 255 nm.

Analogously N⁶-(4-azidophenylethyl)adenosine phosphate (**2**) was prepared from **1**; the yield was 61% with respect to the amino compound **1**.

m.p.: 192–194 °C (decomposition)

UV λ_{\max} = 265 nm; log ϵ = 4.42

Analysis: C₁₈H₂₁N₈O₇P × 1/2 ethanol (MW 515.4)

Calcd 44.27 C, 4.66 H, 21.7 N, 6.02 P

Found 44.09 C, 4.67 H, 21.8 N, 6.04 P

In accordance with the described synthesis of **2** 4-(3-aminopyridinio)butyl phosphate (**3**) was transformed into the azide (**4**). This compound could only be obtained as an oil. The yield was 40% with respect to **3**.

UV λ_{\max} = 250 nm; log ϵ = 4.11

λ_{\max} = 295 nm; log ϵ = 3.70

IR: band at 2080 cm⁻¹

Enzymes

Nucleotide pyrophosphatase from *Crotalus atrox* (EC 3.6.1.9) was obtained from Serva, Heidelberg. Lactate dehydrogenase from pig heart (EC 1.1.1.27) and trypsin (EC 3.4.21.4) were products of Boehringer, Mannheim. Natural co-enzymes were purchased from Pharma Waldhof, Düsseldorf.

For the *cleavage of the pyrophosphate bond*, 0.5 mg of **6** were dissolved in 1 ml 0.1 M Tris-HCl buffer, 50 mM sodium acetate, pH 7.5, and incubated with 0.2 mg of pyrophosphatase for 3 h at 20 °C. The reaction was monitored by thin-layer chromatography. (TLC silica plates 60F₂₅₄ were obtained from Merck, Darmstadt; The solvent was isobutyric acid – water – 30% ammonia (v:v:v) 70:29:1 [20]).

For the *modification experiments*, 2 ml of lactate dehydrogenase suspension, 10 mg/ml, was centrifuged and the pellet dissolved in 1 ml of Tris-HCl buffer, pH 8.

Four mg charcoal were added to the enzyme solution and stirred for 30 min at 4 °C. The charcoal was removed by filtration and the solution exhaustively dialyzed against 0.1 M Tris/HCl buffer pH 8. The *protein content* was determined according to Bradford [21].

The *binding capacity for NADH* was determined according to a procedure of Pfleiderer [16]. The *fluorescence titrations* of modified and unmodified

lactate dehydrogenase were performed as described by Velick [15].

Lactate dehydrogenase activity was assayed in 0.2 M glycine-NaOH buffer pH 9.5 containing 2 mM NAD⁺ and 100 mM lithium lactate at 20 °C.

The *inhibitory* properties of **5** against NAD⁺ were determined in the same test system. The concentrations of NAD⁺ in these experiments were varied between 45 and 420 µM, those of **6** were 2.26, 4.5, 5.2 and 6.8 µM [22, 23].

For the *inactivation* experiments, 0.1 mg of **6** and 4.74 mg of lactate dehydrogenase were dissolved in 0.4 ml of 0.1 M Tris-HCl buffer, pH 8, and the solution was irradiated for 20 min using a xenon lamp Xe 1045 and an Oriel UG 5 filter. The temperature was kept at 4 °C using a temperature controlled cuvette holder. To investigate the temperature dependence the inactivation reaction was repeated at 10 and 20 °C. After the irradiation decomposition products of **6** were removed by charcoal treatment [24]. The decrease of enzymatic activity was determined in 1 µl samples. The effect of natural coenzyme was investigated by adding 5 mM NAD⁺, and 4 mM oxalate to the reaction mixture. The pH was adjusted to 7.0 with 0.1 M phosphate buffer, or to 9.5 with 0.1 M glycine-NaOH buffer.

For the protein analysis, 20 mg of modified enzyme protein were first unfolded by addition of urea to a final concentration of 9 M, dialyzed repeatedly against 1 l of 0.1 M ammonium bicarbonate and lyophilized. 19 mg of the protein were dissolved in 1.9 ml of 0.1 M ammonium bicarbonate and treated with 0.9 mg trypsin at 37 °C for 20 h. The resulting peptide mixture was lyophilized, dissolved in aqueous 0.1% trifluoroacetic acid and centrifuged. Portions corresponding to 0.5 mg of the peptide mixture were applied to a Waters RP

18 30 cm HPLC column. Using a flow rate of 2 ml/min the column was developed with 0.1% trifluoroacetic acid with increasing percentage of methanol (0–100%). The *ribose content* of the modified enzyme or the tryptic peptide was determined by the method of Volkin and Cohn [16]. Briefly, 1 ml solution, containing ca. 0.01 µmol of ribose was heated at 90 °C with 3 ml conc. HCl containing 2% (w:v) orcin and 1.35% (w:v) ferriammonium sulphate. After 30 min the absorption of the resulting color was read at 620 nm. NAD⁺ served as control, and AMP was used as standard, unmodified lactate dehydrogenase served as blank.

For the *amino acid analysis*, the peptides were dissolved in 6 N HCl, heated to 110 °C in a sealed tube for 16 h and dried. The residue was dissolved in 0.2 M citrate buffer, pH 2.0, and the amino acids were determined on an automatic analyzer Kontron Liquimat 3.

Each tryptic ribose-containing peptide was dissolved in 0.1 M Tris-HCl buffer pH 8 containing 50 mM magnesium acetate, pH 7.5 and treated with 0.2 mg of nucleotide pyrophosphatase at 37 °C for 3 h.

A Waters RP 18 30 cm HPLC column was used to analyse the samples. The elution was performed as described before. The HPLC system consisting of two pumps, controller, variable wavelength monitor, 2-channel recorder and Superrac was purchased from LKB.

UV spectra were recorded on a Perkin-Elmer photometer 555 and *fluorescence spectra* on a fluorometer MPF 4 of the same brand.

Phosphate was determined according to Martland and Robison [25]. *IR spectra* were recorded on a Perkin-Elmer infrared spectrophotometer 735.

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