New Reactive Coenzyme Analogues for Affinity Labeling of NAD^+ and $NADP^+$ Dependent Dehydrogenases

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Affinity Labeling, Dehydrogenases, NAD^+ Analogues, $NADP^+$ Analogues

Reactive coenzyme analogues ω-(3-diazoniumpyridinium)alkyl adenosine diphosphate were prepared by reaction of ω-(3-aminopyridinium)alkyl adenosine diphosphate with nitrous acid. In these compounds the nicotinamide ribose is substituted by hydrocarbon chains of varied lengths (*n*-ethyl to *n*-pentyl). The diazonium compounds are very unstable and decompose rapidly at room temperature. They show a better stability at 0 °C. Lactate and alcohol dehydrogenase do not react with any of the analogues. Glyceraldehyde-3-phosphate dehydrogenase reacts rapidly with the diazoniumpentyl compound. Decreasing the length of the alkyl chain significantly decreases the inactivation velocity. 3α,20β-Hydroxysteroid dehydrogenase reacts at 0 °C with the ethyl homologue and slowly with the propyl compound. The butyl- and pentyl analogues do not inactivate at 0 °C. Tests with ^{14}C-labeled 2-(3-diazoniumpyridinium)ethyl adenosine diphosphate show that complete loss of enzyme activity results after incorporation of 2 moles of inactivator into 1 mole of tetrameric enzyme. 4-(3-Acetylpyridinium)butyl 2′-phospho-adenosine diphosphate, a structural analogue of $NADP^+$, was prepared by condensation of adenosine-2,3-cyclophospho-5′-phosphomorpholidate with (3-acetylpyridinium)butyl phosphate, followed by hydrolysis of the cyclic phosphoric acid ester with 2′:3′-cyclonucleotide-3′-phosphodiesterase. Because of the redox potential (−315 mV) and the distance between the pyridinium and phosphate groups, this analogue is a hydrogen acceptor and its reduced form a hydrogen donor in tests with alcohol dehydrogenase from *Thermoanaerobium brockii*. The reduced form of the coenzyme analogue also is a hydrogen donor with glutathione reductase. With other $NADP^+$-dependent dehydrogenases the compound has been shown to be a competitive inhibitor against the natural coenzyme. The acetyl group reacts with bromine to form the bromoacetyl group. This reactive bromoacetyl analogue is a specific active-site directed irreversible inhibitor of isocitrate dehydrogenase.

Introduction

The formation of the active $NAD(P)^+$-enzyme complex is facilitated by binding of the non functional ADP moiety, which helps to position the functional nicotinamide ribose into the catalytic center of the enzyme. The coenzyme interacts with many protein side residues (Rossmann *et al.*, 1975). For labeling of amino acid side chains that participate in binding, various reactive coenzyme analogues were synthesized. These are able to form covalent bonds of different stabilities with side chains of the enzyme, causing a loss of enzymatic activity. Examples for reactive residues are azido-, haloketo- or diazonium groups fixed to the pyridinium ring of the coenzyme analogue (Biellmann *et al.*, 1974; Anderson and Kaplan, 1987; Woenckhaus and Filbrich, 1990). Azo bridges, formed by reaction of diazonium groups with sulfhydryl, hydroxy or amino groups, are unstable and decompose during the purification procedure of the modified protein (King *et al.*, 1991). Azo bridges with phenol or imidazole residues (Burkhard *et al.*, 1981) are more stable. Also nitrenes, formed by exposure of azido compounds to UV-light, rarely form bonds that are stable enough for protein analysis (Vogel *et al.*, 1992). Bromoacetyl compounds react favorably with sulfhydryl, imidazole or amino groups of proteins (Woenckhaus and

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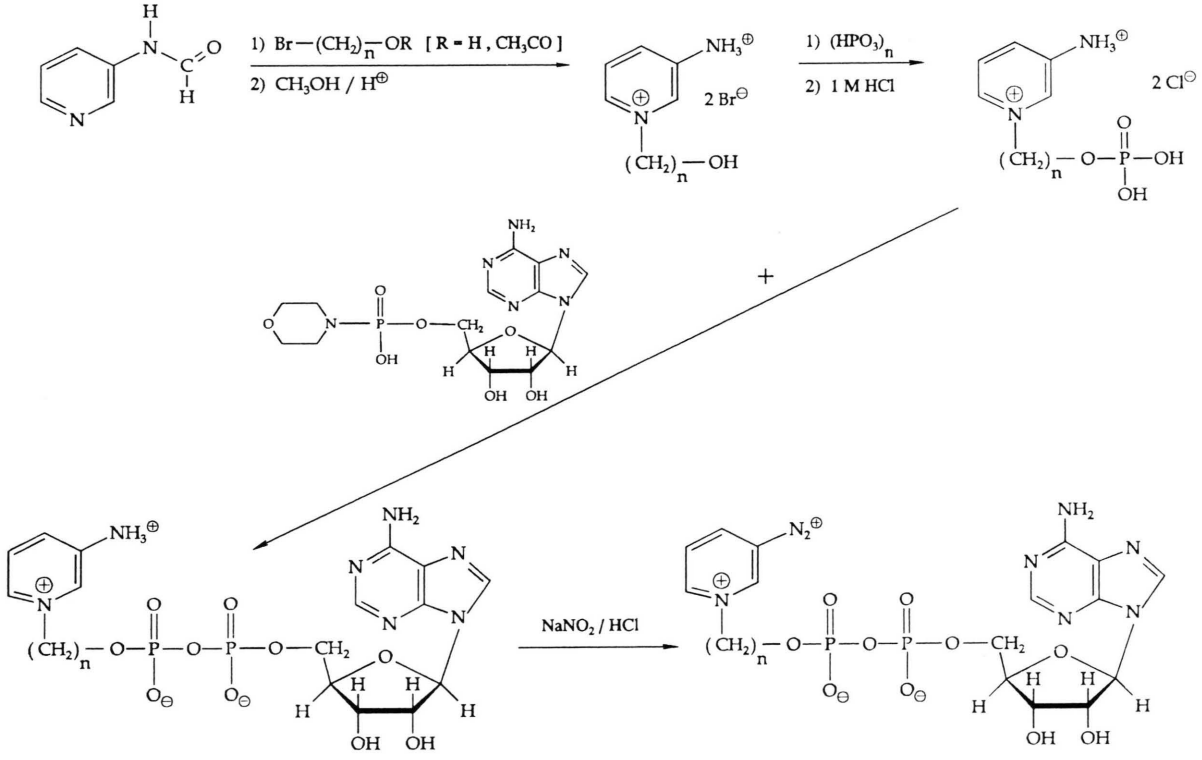
Jeck, 1977). In these cases the protein-coenzyme bonds were stabilized afterwards by reduction of the keto group to a carbinol (Jörnvall *et al.*, 1975).

The purpose of this work was to synthesize a new group of reactive NAD^+ analogues that contain a diazonium group at the pyridinium moiety and whose pyridinium ribose are substituted by alkyl chains of different lengths. Furthermore, we prepared an $NADP^+$ analogue with a reactive bromoacetyl group on the pyridinium ring and an *n*-butyl chain instead of the pyridinium ribose. These compounds were tested for their reactivity with various $NAD(P)^+$ dependent dehydrogenases.

Results

For preparation of reactive coenzyme analogues of NAD^+ we changed two parts of the natural coenzyme. The reactive group at the pyridinium ring was formed from aminopyridinium by diazotization with nitrous acid. The diazonium group is very unstable; at room temperature it decomposes in few minutes. It is more stable at 0 °C; at this temperature it takes about one hour until the solution is completely colorless and the control reaction with α-naphthol is no longer observable. The second change is the substitution of the ribose, bound to the pyridinium ring, with alkyl chains. This change causes a weaker binding of the coenzyme to the enzyme, but enables the reactive part to be placed in the neighborhood of various side chains in the active site, which participate in coenzyme binding (Niekamp *et al.*, 1980). By using hydrocarbon chains of different lengths, the distance between reactive group and pyrophosphate residue becomes variable (Scheme I). All new compounds were shown to be affinity labeling agents of NAD^+-dependent alcohol dehydrogenases. Because of the instability of the diazonium compounds they were tested at an incubation temperature of 0 °C.

ω-(3-diazoniumpyridinium)alkyl adenosine diphosphates show different behavior with various dehydrogenases. At 0 °C none of the diazonium compounds affects the activity of lactate- and alco-



Scheme I. Synthesis of ω-(3-diazoniumpyridinium)alkyl adenosine diphosphates; $n = 2-5$.

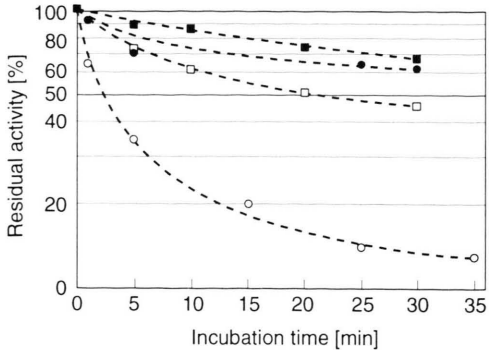


Fig. 1. Inactivation of 3α,20β-hydroxysteroid dehydrogenase with ω-(3-diazoniumpyridinium)alkyl adenosine diphosphates. ○ = 1 mM 2-(3-diazoniumpyridinium)ethyl adenosine diphosphate; □ = 1 mM 3-(3-diazoniumpyridinium)propyl adenosine diphosphate; ● = 1 mM 2-(3-diazoniumpyridinium)ethyl adenosine diphosphate and 1 mM NADH; ■ = 1 mM 3-(3-diazoniumpyridinium)propyl adenosine diphosphate and 1 mM NADH.

hol dehydrogenase. The activity of glyceraldehyde-3-phosphate dehydrogenase decreases in the presence of each of the four compounds. Here the inactivation with the pentyl- and butyl analogue rapidly leads to a complete loss of enzymatic activity, whereas the reaction with the propyl- and ethyl homologues, in slower reaction, causes a loss of activity of 50%.

We found different behavior of the diazonium alkyl derivatives in tests with 3α,20β-hydroxysteroid dehydrogenase. The butyl and pentyl compounds do not influence the enzyme activity at 0 °C, whereas 3-(3-diazoniumpyridinium)propyl adenosine diphosphate slowly inactivates. The ethyl compound rapidly decreased enzymatic activity. The presence of the natural cofactor decreases the velocity of inactivation in both cases (Fig. 1).

In order to describe the course of the inactivation reaction, we determined the inactivation constants K_I and k_2. Fig. 2 shows that the K_I values of both inactivators are about the same but the k_2 value for the ethyl analogue is four times higher than for the propyl compound.

3α,20β-hydroxysteroid dehydrogenase forms a fluorescing complex with NADH. Partly inactivated enzyme shows decreased fluorescence corresponding to the loss of activity. By using [8-^{14}C]-

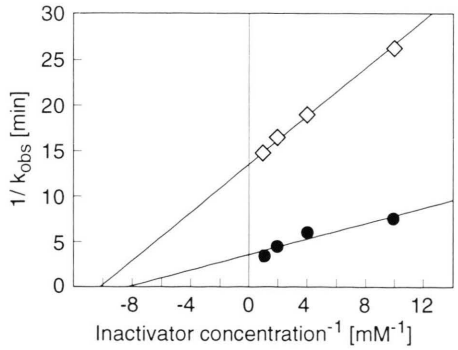
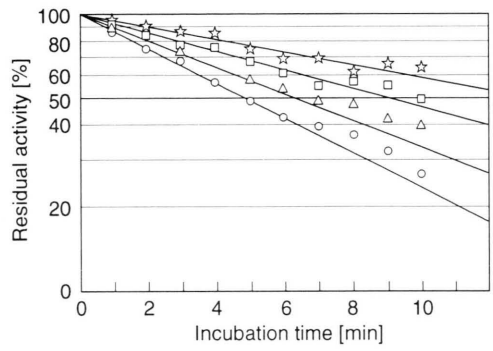


Fig. 2. Concentration dependence of the inactivation of 3α,20β-hydroxysteroid dehydrogenase with ω-(3-diazoniumpyridinium)alkyl adenosine diphosphates. ● = 2-(3-diazoniumpyridinium)ethyl adenosine diphosphate; ◇ = 3-(3-diazoniumpyridinium)propyl adenosine diphosphate.



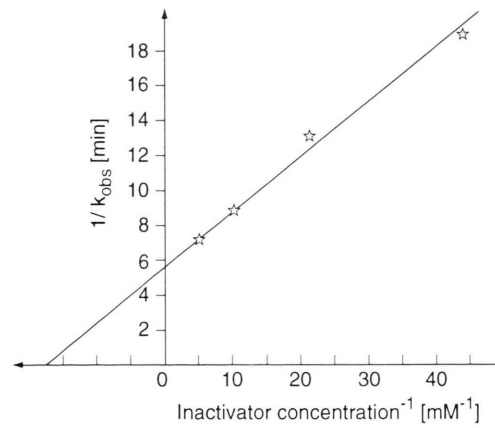
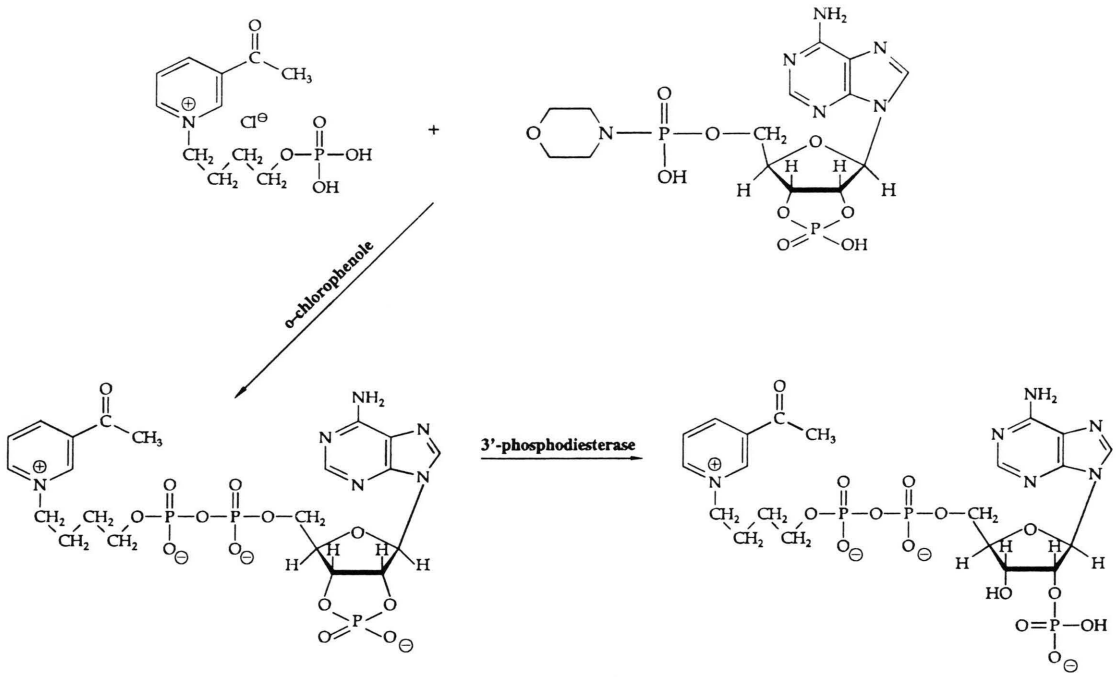


Fig. 3. Determination of the dissociation constant K_I of the enzyme-inactivator complex of isocitrate dehydrogenase and 4-(3-bromoacetylpyridinium)butyl 2'-phosphoadenosine diphosphate. A: Time course of the inactivation reaction in dependence of inactivator concentration ☆ = 0.225×10^{-4} M, □ = 0.45×10^{-4} M, △ = 0.90×10^{-4} M, ○ = 1.80×10^{-4} M. B: Dependence of the rate constant for pseudo-first order reaction upon inactivator concentration.



Scheme II. Synthesis of 4-(3-acetylpyridinium)butyl 2'-phospho-adenosine diphosphate.

adenine labeled 2-(3-diazoniumpyridinium)ethyl adenosine diphosphate we were able to determine the rate of incorporation into the enzyme. A total loss of enzymatic activity resulted after incorporation of 2 mol of inactivator into 1 mol of tetrameric enzyme.

For modification of NADP$^+$ dependent dehydrogenases, two of us (Jeck & Tischlich) synthesized 4-(3-acetylpyridinium)butyl 2′-phospho-adenosine diphosphate, which could be converted to the reactive bromoacetylpyridinium compound by bromination. For the synthesis of this compound adenosine-2′:3′-cyclophospho-5′-phosphomorpholidate was formed by reaction of a mixture of adenosine-2′,5′-diphosphate and adenosine-3′,5′-diphosphate with dicyclohexylcarbodiimide and morpholine (Moffatt and Khorana, 1961; Cocco and Blakley, 1979). Adenosine-2′:3′-cyclophospho-5′-phosphomorpholidate reacts in orthochlorophenol with (3-acetylpyridinium)butyl phosphate to form the nucleotide anhydride. By treating the cyclic ester with a specific 3′-phosphodiesterase, we obtained the coenzyme analogue in good yield (Scheme II).

The redox potential of the compound is −315 mV, which is in the range of the natural cofactor (−320 mV). Therefore the compound is expected to have coenzymatic activity. The NADP$^+$ dependent alcohol dehydrogenase from *Thermoanaerobium brockii* was able to use 4-(3-acetylpyridinium)butyl 2′-phospho-adenosine diphosphate instead of NADP$^+$. The reduced form is a hydrogen donor. The K_m of the oxidized analogue was 60 μM and the turnover number 0.7 sec^{-1}; under the same conditions we determined a K_m of 8.7 μM and a turnover number of 40 sec^{-1} for NADP$^+$. The reduced form of the coenzyme analogue was shown to have a K_m of 50 μM and a turnover number of 0.07 sec^{-1}. Corresponding data of NADPH are K_m = 9.2 μM and a turnover number of 7 sec^{-1}. With glutathione reductase the reduced form worked as hydrogen donor with a K_m of 20 μM and a turnover number of 1 sec^{-1}. Corresponding values for NADPH are 9 μM and 215 sec^{-1}.

No coenzyme function could be found in tests with the following NADP$^+$ dependent enzymes: isocitrate, glucose-6-phosphate, gluconate-6-phos-

phate, glutamate dehydrogenase and malate enzyme. With these dehydrogenases both the oxidized and the reduced forms were shown to be competitive inhibitors with inhibition constants ranging from 25 to 500 μM.

With alcohol dehydrogenase from yeast, an NAD^+ dependent enzyme, the coenzyme analogue is neither a hydrogen acceptor nor a competitive inhibitor. These results show the structural specificity of the coenzyme analogue.

In acidic solution 4-(3-acetylpyridinium)butyl 2′-phospho-adenosine diphosphate reacts with bromine by formation of the bromoacetyl analogue. This compound was tested as a specific inactivator of isocitrate dehydrogenase. In presence of 30 μM of coenzyme analogue the enzyme lost 50% of its initial activity in 10 min.

The K_I of 6.2 μM (Fig. 3, p. 478) indicates stronger affinity of the inactivator molecule to the enzyme as compared to the non brominated analogue. The k_2 value of the inactivation reaction was 0.02 min^{-1}. $NADP^+$ or NADPH decreased the velocity of the inactivation reaction by factor 3 to 4.

Discussion

Coenzyme analogues, whose nicotinamide ribose part is substituted by diazotized 3-aminopyridinium-alkyl residues, showed different behavior in tests with dehydrogenases. Independent on the length of the alkyl chain of the analogue, neither alcohol dehydrogenase from horse liver nor lactate dehydrogenase from pig heart could be inactivated, although alcohol dehydrogenase, with two cysteine sulfhydryl groups as ligands of the essential zinc, has highly reactive amino acid residues in the active site (Brändén *et al.*, 1975). Those react with structurally similar bromoacetyl analogues in a fast reaction, forming thiomethyl ketones. Here Cys46 favorably reacts with 4-(3-bromoacetylpyridinium)butyl adenosine diphosphate, whereas the homologue 3-(3-bromoacetylpyridinium)propyl adenosine diphosphate reacts predominantly with Cys174 (Woenckhaus *et al.*, 1979).

Since diazonium compounds, compared to bromoacetyl compounds, are more reactive, the lack of inactivation reactions in the case of 3-diazoniumpyridinium compounds has to be explained with the structures of the binary enzyme complexes. An essential difference between both types of inactivators is the positive charge of the diazoniumgroup compared to the neutral bromoacetyl. It is characteristic for alcohol dehydrogenase and lactate dehydrogenase that NADH with its neutral dihydropyridine ring is fixed more tightly than the oxidized form with its positively charged pyridiniumsystem (Theorell and McKinley-McKee, 1961; Holbrook *et al.*, 1975). Therefore it is possible that the additional positive charge of the diazonium compounds prevents a correct complexation of the pyridinium ring to its binding site. Indeed with lactate dehydrogenase also sterical reasons cannot be excluded: as modifiable residue here is the essential His191, which is participated in substrate activation. This one could be modified by 3-(4-bromoacetylpyridinium)propyl adenosine diphosphate (Woenckhaus *et al.*, 1972), bromoacetylpyridine (Woenckhaus *et al.*, 1969), and bromopyruvate (Berghäuser *et al.*, 1973), but not by ω-(3-bromoacetylpyridinium)alkyl adenosine diphosphates.

The long chained diazonium compounds are the more effective inactivators of glyceraldehyde-3-phosphate dehydrogenase. The kinetics of the inactivation reaction are comparable to those of ω-(3-bromoacetylpyridinium)alkyl adenosine diphosphates. Therefore the diazonium group probably reacts with Cys149 of the enzyme. This residue is more easily reached by the butyl and pentyl analogues than by the ethyl and propyl compounds.

3α,20β-Hydroxysteroid dehydrogenase, a representative of the group of short chain alcohol dehydrogenases (Marekov *et al.*, 1990), only becomes inactivated at 0 °C by the short chain compounds 3-(3-diazopyridinium)propyl adenosine diphosphate and fastest by the ethyl analogue.

Assuming that inactivator and enzyme form a binary complex leads to the following mechanism analogous to that of Michaelis and Menten (Gold and Fahrney, 1964).

$$E + I \leftrightarrow E:I \rightarrow EI$$

The inactivation kinetics show that both compounds bind about the same to the enzyme. The three dimensional structure of the ternary enzyme-NADH-substrate complex shows a histidine located in the active site (Ghosh *et al.*, 1991). This residue is easily reached by the ethyl compound, whereas the diazotized butyl and pentyl analogue

extend beyond this residue. The incorporation of only two labeled coenzyme analogues into the tetrameric enzyme agrees with observations of Sweet *et al.* (1978)., who were able to modify two residues with a radioactive substrate analogue in the ternary enzyme-coenzyme complex.

4-(3-Acetylpyridinium)butyl 2′-phospho-adenosine diphosphate was prepared as a structural analogue of $NADP^+$. The acetylpyridinium group causes a more positive redox potential compared to the natural carbamoyl pyridinium group. Replacing the ribose by a *n*-butylgroup compensates, so that the redox potential is in the same range as that of the natural coenzyme (Jeck, 1977). The length of the butyl chain is about the same as the ribose in natural coenzyme, and therefore the compound is expected to have coenzymatic activity. The turnover numbers in the tests with alcohol dehydrogenase from *Thermoanaerobium brockii* are highly decreased, compared to those of NADP(H), which should result from decreased activation of the functional part because hydrogen bonds cannot be formed to the hydrophobic butyl moiety. We also observed highly decreased catalytic activity in tests with glutathione reductase and the reduced form of the coenzyme analogue. The competitive inhibition of $NADP^+$-dependent dehydrogenases also indicates that the analogue binds to the active site. 4-(3-Bromoacetylpyridinium)butyl 2′-phospho-adenosine diphosphate inactivated isocitrate dehydrogenase in an irreversible reaction. The kinetics of inactivation in the absence or presence of cofactors and substrates indicate a specific attack at the active site. The pH dependence of the reaction indicates the participation of an amino acid residue with a pK value larger than 7 and leads to the conclusion, that a cysteine or lysine residue reacts. A possible partner for the reaction also might be a methionine residue discovered by Colman, which can be carboxymethylated (Colman, 1968).

Materials and Methods

Enzymes and coenzymes

Alcohol dehydrogenase from *Thermoanaerobium brockii* (EC 1.1.1.2), glutathione reductase from yeast (EC 1.6.4.2), malate enzyme from chicken liver (1.1.1.40), NAD^+-kinase from chicken liver (EC 2.7.1.23), 2′:3′-cyclonucleotide-3′-phosphodiesterase from bovine brain (EC 3.1.3.37) and 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (EC 1.1.1.53) were purchased from Sigma, Munich. Alcohol dehydrogenase from horse liver and yeast (EC 1.1.1.1), lactate dehydrogenase from pig heart (EC 1.1.1.27), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (EC 1.2.1.12), isocitrate dehydrogenase from yeast (EC 1.1.1.42), phosphogluconate dehydrogenase from yeast (1.1.1.44), glutamate dehydrogenase from bovine liver (1.4.1.3) and glucose-6-phosphate dehydrogenase (1.1.1.49) and NAD^+, $NADP^+$, NADH, NADPH, AMP and adenosine were products from Boehringer Mannheim. [8-^{14}C]-AMP was delivered by Amersham, Braunschweig. Ion exchanger and separation gels were products from Serva, Heidelberg, or Pharmacia, Uppsala, Sweden.

Column chromatography were observed with LKB-Uvicord 4700 at 254 nm. UV spectra were made with a Perkin-Elmer photometer 555, fluorescence spectra with a Perkin-Elmer spectrofluorometer MPF 4. NMR spectra were made by the Institut für Organische Chemie der Universität Frankfurt with 500, 400, 270 and 162 MHz spectrometers.

Synthesis

4-(3-acetylpyridinium)butyl phosphate was prepared as described earlier (Woenckhaus and Jeck, 1977).

ω-(3-aminopyridinium)alkyl phosphates

31.5 g 3-amino pyridine was dissolved in 200 ml formic acid and refluxed for 3 h. The solution was evaporated under reduced pressure and the procedure repeated two times. The remainder was brought to pH 8 at 0 °C with aqueous ammonia. The solution was extracted 4 times each with 30 ml of chloroform, the organic extracts were dried with sodium sulfate and the solvent evaporated under reduced pressure. 31 g of the colorless crystalline product were recrystallized from methanol (mp. 93 °C).

10.81 g (100 mmol) 3-formylaminopyridine were dissolved in 100 mmol of each alkyl component: 7.09 ml bromoethanol, 9.04 ml 1-bromo-3-propanol, 14.47 ml 1-bromobutyl-4-acetate or 16.66 ml 1-bromopentyl-5-acetate. After 7 days at room

temperature the solution crystallized completely. The removal of the acetyl ester group from butyl- and pentyl compounds and the phosphorylation of all hydroxyalkyl pyridinium salts was executed as described earlier (Woenckhaus and Jeck, 1977). The yields of the monophosphates decreased with increasing length of the alkyl chain and were in the range of 30 and 10%. The ω-(3-aminopyridinium)alkyl phosphates absorb light at 259 nm, ε = 9800 (pH 5).

ω-(3-aminopyridinium)alkyl adenosine diphosphates were prepared the same way as the (3-acetylpyridinium)alkyl adenosine diphosphates and eluted from a Dowex 1×8 column (100–200 mesh, 2×50 cm, formate form) with a convex gradient of 500 ml water and 5 L 25 mM formic acid. The dinucleotide derivatives appeared after about 300 ml in a volume of 800 to 1600 ml. The solutions were evaporated at 30 °C under reduced pressure and the coenzyme analogues were precipitated with cold isopropanol. The yields were in the range of 40 to 50%.

2-(3-Aminopyridinio)ethyl adenosine diphosphate

λ_{max} 259 nm; ε = 25000 (pH 5). – ^{1}H-NMR (D_6-DMSO/D_2O; 270 Mhz) δ (ppm) = 8.40 and 8.20 (2s, 2H, Adenin-CH); 8.11 (s, 1H, $CNH_2-C\underline{H}-N^+$); 7.65 (dd, 1H, $CNH_2-CH-C\underline{H}-CH-N^+$), J = 8.2 Hz); 7.57 (m, 1H, $CNH_2-CH-CH-C\underline{H}-N^+$); 7.40 (m, 1H, $CNH_2-C\underline{H}-CH-CH-N^+$); 5.94 (d, 1H, $C_{1'}H$, J = 7.9 Hz); 4.65 (m, 1H, $C_{2'}H$); 4.55 (m, 1H, $C_{3'}H$); 4.25 (m, 1H, $C_{4'}H$); 4.07 (m, 2H, $C_{5'}H$); 3.75 (m, 2H, $N^+-CH_2-C\underline{H_2}-O$, J = 7.1 Hz); 3.47 (m, 2H, $N^+-C\underline{H_2}-CH_2-O$).

3-(3-Aminopyridinio)propyl adenosine diphosphate

λ_{max} 259 nm; ε = 23000 (pH 5). – ^{1}H-NMR (D_6-DMSO/D_2O; 270 Mhz) δ (ppm) = 8.51 and 8.24 (2s, 2H, Adenin-CH); 8.19 (s, 1H, $CNH_2-C\underline{H}-N^+$); 8.08 (dd, 1H, $CNH_2-CH-C\underline{H}-CH-N^+$), J = 8.25 Hz); 7.61 (d, 1H, $CNH_2-CH-CH-C\underline{H}-N^+$, J = 8.3 Hz); 7.54 (d, 1H, $CNH_2-C\underline{H}-CH-CH-N^+$); 5.93 (d, 1H, $C_{1'}H$, J = 8.2 Hz); 4.56 (m, 1H, $C_{2'}H$); 4.51 (m, 1H, $C_{3'}H$, J = 7.8 Hz); 4.47 (m, 1H, $C_{4'}H$); 4.09 (m, 2H, $C_{5'}H$); 3.81 (m, 2H, $N^+-CH_2-CH_2-C\underline{H_2}-O$); 3.47 (m, 2H, $N^+-C\underline{H_2}-CH_2-CH_2-O$); 2.17 (m, 2H, $N^+-CH_2-C\underline{H_2}-CH_2-O$, J = 8.9 Hz).

4-(3-Aminopyridinio)butyl adenosine diphosphate

λ_{max} 259 nm; ε = 26000 (pH 5). – ^{1}H-NMR (D_6-DMSO/D_2O; 270 Mhz) δ (ppm) = 8.50 and 8.26 (2s, 2H, Adenin-CH); 7.95 (s, 1H, $CNH_2-C\underline{H}-N^+$); 7.87 (dd, 1H, $CNH_2-CH-C\underline{H}-CH-N^+$), J = 6.20 Hz); 7.51 (d, 1H, $CNH_2-CH-CH-C\underline{H}-N^+$, J = 6.2 Hz); 7.50 (d, 1H, $CNH_2-C\underline{H}-CH-CH-N^+$, J = 6.0 Hz); 6.10 (d, 1H, $C_{1'}H$, J = 6.1 Hz); 4.84 (m, 1H, $C_{2'}H$); 4.54 (m, 1H, $C_{3'}H$, J = 6.1 Hz); 4.42 (m, 1H, $C_{4'}H$); 4.34 (m, 2H, $C_{5'}H$); 4.27 (m, 2H, $N^+-CH_2-CH_2-CH_2-C\underline{H_2}-O$); 3.97 (m, 2H, $N^+-C\underline{H_2}-CH_2-CH_2-CH_2-O$); 1.97 (m, 2H, $N^+-CH_2-C\underline{H_2}-CH_2-CH_2-O$, J = 6.1 Hz); 1.64 (dt, 2H, $N^+-CH_2-CH_2-C\underline{H_2}-CH_2-O$, J = 6.2 Hz).

5-(3-Aminopyridinio)pentyl adenosine diphosphate

λ_{max} 259 nm; ε = 24000 (pH 5). – ^{1}H-NMR (D_6-DMSO/D_2O; 270 Mhz) δ (ppm) = 8.45 and 8.21 (2s, 2H, Adenin-CH); 8.18 (s, 1H, $CNH_2-C\underline{H}-N^+$); 8.12 (m, 1H, $CNH_2-CH-C\underline{H}-CH-N^+$), J = 6.80 Hz); 7.63 (d, 1H, $CNH_2-CH-CH-C\underline{H}-N^+$, J = 7.8 Hz); 7.53 (m, 1H, $CNH_2-C\underline{H}-CH-CH-N^+$); 5.92 (d, 1H, $C_{1'}H$, J = 8.1 Hz); 4.57 (m, 1H, $C_{2'}H$, J = 7.1 Hz); 4.40 (dd, 1H, $C_{3'}H$, J = 6.1 Hz); 4.24 (m, 1H, $C_{4'}H$); 4.05 (m, 2H, $C_{5'}H$); 3.78 (m, 2H, $N^+-CH_2-CH_2-CH_2-CH_2-C\underline{H_2}-O$, J = 5.8 Hz); 3.45 (m, 2H, $N^+-C\underline{H_2}-CH_2-CH_2-CH_2-CH_2-O$, J = 6.8 Hz); 1.85 (m, 2H, $N^+-CH_2-C\underline{H_2}-CH_2-CH_2-CH_2-O$); 1.57 (m, 2H, $N^+-CH_2-CH_2-CH_2-C\underline{H_2}-CH_2-O$, J = 9.0 Hz); 1.31 (m, 2H, $N^+-CH_2-CH_2-C\underline{H_2}-CH_2-CH_2-O$, J = 9.5 Hz).

[8-^{14}C]-Adenosine labeled 2-(3-aminopyridinium)-ethyl adenosine diphosphate

180 mg (0.5 mmol) [8-^{14}C]-AMP (250 mCi, 9.2 MBq) were converted to the morpholidate (Moffatt and Khorana, 1961) and made to the dinucleotide by the common procedure. – Yield: 130 mg; 40%, specific radioactivity 18.4 MBq/mmol.

Diazotization of the ω-(3-aminopyridinium)alkyl adenosine diphosphates

20 mg of the coenzyme analogue was dissolved in 1 ml 1M HCl. At 0 °C 3.5 mg sodium nitrite was

added, and the solution became yellow. Excess nitrite was decomposed by addition of ammonium amidosulfonate (Burkhard *et al.*, 1981), and the pH was brought to 4 by addition of solid sodium bicarbonate. All compounds were used immediately after preparation, because they decompose at 0 °C in one hour.

4-(3-acetylpyridinium)butyl 2′:3′-cyclophospho-adenosine diphosphate

Adenosine-2′(3′),5′-diphosphate was prepared after a procedure of Cocco and Blakley (1979) and, by a procedure of Moffat and Khorana (1961), converted to the adenosine-2′,3′-cyclophospho-5′-phospho morpholidate. 3.5 g (4 mmol) of the morpholidate and 1.28 g 4-(3-acetylpyridinium)butyl phosphate were dissolved in 10.5 ml freshly distilled ortho-chlorophenol and allowed to react 10 days at room temperature. 80 ml of water was added and the solution was washed three times each with 150 ml of diethyl ether. The first ether layer was reextracted with 30 ml of water. The combined aqueous phases were evaporated to a volume of 7 ml and applied to a DEAE Sephadex A25 column (HCO_3^- form, 3.6×45 cm). The column was washed with water, until no UV absorption was detectable in the eluate any more. Thereafter a linear gradient (3 L H_2O and 3 L 0.3 M NH_4HCO_3) was used. The coenzyme analogue eluted at 0.2 M NH_4HCO_3 in a volume of 700 ml. The fraction was brought to pH 6 with carbon dioxide and evaporated to a volume of 100 ml under reduced pressure at 30 °C. Salt and remaining solvent were removed by lyophilization. – Yield: 680 mg (24%).

^{1}H-NMR (D_6-DMSO/D_2O; 400 Mhz) δ (ppm) = 9.37 (s, 1H, CO–C–C<u>H</u>–N^+); 9.03 (s, 1H, CO–C–CH–CH–C<u>H</u>–N^+); 8.91 (d, 1H, CO–C–C<u>H</u>–CH–CH–N^+, J = 6.1 Hz); 8.43 and 8.27 (2s, 2H, Adenin-CH); 8.15 (dd, 1H, CO–C–CH–C<u>H</u>–CH–N^+, J = 6.1 Hz); 6.30 (d, 1H, $C_{1'}$H, J = 4.2 Hz); 5.41 (m, 1H, $C_{2'}$H, J = 6.8 Hz); 5.22 (m, 1H, $C_{3'}$H, J = 4.0 Hz); 4.70 (m, 2H, N^+–C<u>H_2</u>–CH_2–CH_2–CH_2–O, J = 7.6 Hz); 4.63 (m, 1H, $C_{4'}$H, J = 3.8 Hz); 4.27 (m, 2H, $C_{5'}$H, J = 5.0 Hz); 3.98 (m, 2H, N^+–CH_2–CH_2–CH_2–C<u>H_2</u>–O); 2.74 (s, 3H, CH_3); 2.1 (m, 2H, N^+–CH_2–C<u>H_2</u>–CH_2–CH_2–O, J = 7.0 Hz); 1.70 (m, 2H, N^+–CH_2–CH_2–C<u>H_2</u>–CH_2–O, J = 7.0 Hz). – ^{31}P-NMR, [^{1}H-broad band decoupled], (D_2O; 162 Mhz) δ (ppm) = 10.81 and 10.15 (2P, 2d, O–P–O–P–O, $J_{P_1P_2}$ = 22 Hz); 20.4 (1P, dd, $C_{2'}$–O–PO_2–O–$C_{3'}$, $J_{PO-C_{2'}}$ = 8.2 Hz, $J_{PO-C_{3'}}$ = 10.4 Hz).

4-(3-acetylpyridinium)butyl 2′-phospho-adenosine diphosphate

340 mg of 4-(3-acetylpyridinium)butyl 2′:3′-cyclophospho adenosine diphosphate was dissolved in 5.7 ml water and after addition of 15 units 2′:3′-cyclic-nucleotide-3′ phosphodiesterase incubated at 37 °C. The hydrolysis was monitored with HPLC at a RP C18 column and was almost completed after 75 min. Thereafter the complete solution was applied to a DEAE Sephadex A25 column (HCO_3^- form, 1×30 cm). The column was developed with water, followed by a linear gradient (2×400 ml, 0–0.4 M NH_4HCO_3). The product eluted at 0.25 M NH_4HCO_3 in a volume of 180 ml and was obtained in solid form by lyophilization. – Yield: 300 mg (86%); λ_{max} = 259 nm; ε = 17200 cm^{-1} M^{-1} (pH 6).

^{1}H-NMR (D_6-DMSO/D_2O; 400 Mhz) δ (ppm) = 9.32 (s, 1H, CO–C–C<u>H</u>–N^+); 8.98 (s, 1H, CO–C–CH–CH–C<u>H</u>–N^+); 8.87 (d, 1H, CO–C–C<u>H</u>–CH–CH–N^+, J = 6.2 Hz); 8.45 and 8.18 (2s, 2H, Adenin-CH); 8.16 (m, 1H, CO–C–CH–C<u>H</u>–CH–N^+, J = 6.1 Hz); 6.17 (d, 1H, $C_{1'}$H, J = 5.8 Hz); 5.07 (m, 1H, $C_{2'}$H, J = 5.4 Hz); 4.67 (dt, 2H, N^+–C<u>H_2</u>–CH_2–CH_2–CH_2–O, J = 8.0 Hz); 4.62 (dd, 1H, $C_{3'}$H, J = 4.1 Hz); 4.40 (m, 1H, $C_{4'}$H); 4.25 (m, 2H, $C_{5'}$H); 3.98 (m, 2H, N^+–CH_2–CH_2–CH_2–C<u>H_2</u>–O, J = 6.6 Hz); 2.74 (s, 3H, CH_3); 2.1 (m, 2H, N^+–CH_2–C<u>H_2</u>–CH_2–CH_2–O); 1.70 (m, 2H, N^+–CH_2–CH_2–C<u>H_2</u>–CH_2–O, J = 6.8 Hz). – ^{31}P-NMR, [^{1}H-broad band decoupled], (D_2O; 162 Mhz) δ (ppm) = 10.81 and 10.18 (2P, 2d, O–P–O–P–O, $J_{P_1P_2}$ = 22 Hz); 0.46 (1P, d, $C_{2'}$–O–PO_3–, $J_{PO-C_{2'}}$ = 9.2 Hz).

The redox potential was determined by a procedure of Wallenfels and Diekmann (1959).

4-(3-Acetyl-1,4-dihydropyridine)butyl 2′-phospho-adenosine diphosphate was prepared as described for the corresponding coenzyme analogue without the 2′-phosphate group (Woenckhaus and Jeck, 1977).

λ_{max_1} = 259 nm, ε = 14600 cm^{-1} M^{-1}; λ_{max_2} = 380 nm, ε = 10600 cm^{-1} M^{-1} (pH 9.5).

4-(3-Bromoacetylpyridinium)butyl 2'-phospho adenosine diphosphate was prepared as described for the NAD$^+$ analogue (Woenckhaus and Jeck, 1977). The formation of the bromoacetyl group was observed according to a procedure of Bieber *et al.* (1984).

Enzyme Assays

The activities of individual enzymes were assayed with established spectrophotometrical procedures: lactate dehydrogenase (Bergmeyer *et al.*, 1962), alcohol dehydrogenase (Bonnichsen, 1962), glyceraldehyde-3-phosphate dehydrogenase (Racker, 1962), isocitrate dehydrogenase (Siebert, 1962), glucose-6-phosphate dehydrogenase (Hohorst, 1962a), 6-phosphogluconate dehydrogenase (Hohorst, 1962b), glutamate dehydrogenase (Klingenberg, 1962) and malate enzyme (Amy *et al.*, 1977).

The activity of 3α,20β-hydroxysteroid dehydrogenase was determined in 0.2 M sodium phosphate buffer, pH 7, in presence of 0.125 mM NADH and 0.1 mM cortisone at 25 °C (Hübener, 1962). The decrease of extinction was observed at 366 nm.

For the tests of alcohol dehydrogenase from *Thermoanaerobium brockii* 0.1 M Tris-HCl buffer was used, containing 15 mM isopropanol (Lamed and Zeikus, 1981). For the determination of the Michaelis constants, the concentration of the coenzyme was varied from 2.2 to 22 μM and the concentration of coenzyme analogue between 10 and 200 μM. After addition of 0.3 units of enzyme the increase of extinction at 40 °C was observed. The reaction was followed in case of NADP$^+$at 366 nm and for the 3-acetylpyridinium analogue at 380 nm. The oxidation of the dihydro coenzymes was observed at the same wavelengths in Tris-HCl buffer, pH 7.8, containing 1.7 mM acetone. For the determination of the kinetic constants, the dihydro coenzyme concentration was varied between 3 and 100 μM.

The test buffer of the glutathione reductase contained 0.1 mM Tris-HCl pH 7.8, 0.8 mM glutathione and 1.32 mM EDTA (Horn, 1962). The concentration of the dihydro coenzyme analogue was varied between 5 and 190 μM. The reaction was started at 25 °C by addition of 2.5 units of enzyme.

Inactivation studies with ω-(3-diazopyridinium)-alkyl adenosine diphosphates were made at 0 °C. Enzyme concentrations were: 2 mg/ml of alcohol dehydrogenase from horse liver or 3α,20β-hydroxysteroid dehydrogenase and 1 mg/ml of lactate or glyceraldehyde-3-phosphate dehydrogenase. The concentration of diazonium salt was between 30 and 1000 μM. For the observation of the enzyme activity, samples were taken at different times from the incubation solution and either directly or after dilution in 0.1 M cysteine solution tested as described above.

The determination of the radioactive incorporation of ^{14}C-labeled 2-(3-diazoniumpyridinium)-ethyl adenosine diphosphate was made as described earlier (Berghäuser, 1975) after TCA precipitation by using Whatman GF/C filter in the Packard Tricarb scintillation counter. Inactivated 3α,20β-hydroxysteroid dehydrogenase was titrated fluorometrically as described earlier (Velick, 1958). Determination of protein followed Bradford (1976).

Isocitrate dehydrogenase was inactivated in 0.1 M sodium phosphate buffer pH 7.6, containing 5 mM $MgCl_2$; the enzyme concentration was 2 mg/ml, the concentration of bromoacetyl analogue was varied from 22 to 180 μM. For the investigation of the pH dependence 0.3 M sodium acetate was used up to pH 5.5, 0.3 M sodium phosphate up to 8 and 0.15 M sodium pyrophosphate was used up to pH 8.5.

For the investigation of coenzyme protection NADP$^+$ or NADPH were added up to a concentration of 1 mM.

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