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Preparations of Holodehydrogenases by Covalent Fixation of NAD^+-Analogs to Alcohol and Lactate Dehydrogenase

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Diazoniumaryl residues which are connected to the adenosine part of the coenzyme NAD^+ react with amino acid residues of dehydrogenases. These coenzyme analogs bind to the active site of the enzymes. The binary complexes are stabilized by the formation of ternary dead-end complexes with pseudosubstrates. After removal of these pseudosubstrates, the coenzyme analogs remain attached in the vicinity of the active sites by azo bridges. Addition of enzyme substrates to the synthesized holoenzymes causes an immediate reduction of the covalent-bound analogs. Reoxidation can be achieved by pH changes or by addition of adequate substrates. This modification does not cause a strong loss of enzymatic activity of the enzymes. The optical properties of the holoenzymes are in accordance with that of binary NAD^+ (NADH)–enzyme complexes.

INTRODUCTION

Unlike other prosthetic groups of redox enzymes, NAD^+ forms dissociable complexes with dehydrogenases. Holoenzymes should be obtained by the covalent fixation of NAD^+ on the enzymes. Such systems should be able to catalyze reactions in the absence of free coenzyme. Lately, we investigated the fixation of nicotinamide-(5-bromoacetyl-4-methylimidazole) dinucleotide and bromoacetophenone derivatives of NAD^+ on dehydrogenases (*1, 2*). Unfortunately, the bromoketone group reacts preferently with essential nucleophilic residues of the proteins which causes a nearly complete loss of enzymatic activities. A successful preparation of holodehydrogenases was reported by Mosbach *et al.* where NAD^+ was attached to alcohol dehydrogenase (ADH) via a bifunctional spacer (*3*). Recently we found the diazonium group suitable for reactions with a variety of amino acid residues (*4*). The introduction of this reactive group into the nonfunctional adenosine part of the NAD^+ molecule leads to a new class of compounds which can be used successfully for the preparations of holodehydrogenases. In this

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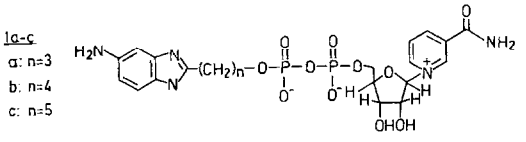
paper we describe the synthesis of such compounds and their application for the preparation of holoenzymes of ADH and lactate dehydrogenase (LDH).

RESULTS

A cyclohexylcarbodiimide condensation of 5-(amino-2-benzimidazolyl)alkyl phosphates with nicotinamide mononucleotide gave the according NAD^+ analogs **1a–c**. 4-Aminothiophenol reacted with 6-chloropurine riboside to give (4-aminophenyl)-6-thiopurine riboside in good yields. The N^6-substituted adenosine analogs **3** and **4** were obtained by reaction of 6-chloropurine riboside with 4-nitrobenzylamine and 4-aminophenylethylamine, respectively. The nucleosides **2**, **3**, and **4** were phosphorylated to their 5′-phosphate esters by the Yoshikawa method (*5*). N^6-(4-Nitrobenzyl)-AMP was reduced in nearly quantitative yield to N^6-(4-aminobenzyl)-AMP **6** with hydrazine/Raney nickel (*6*). The monophosphates were condensed with nicotinamide mononucleotide to yield the corresponding NAD^+ analogs **8**, **9**, and **10**.



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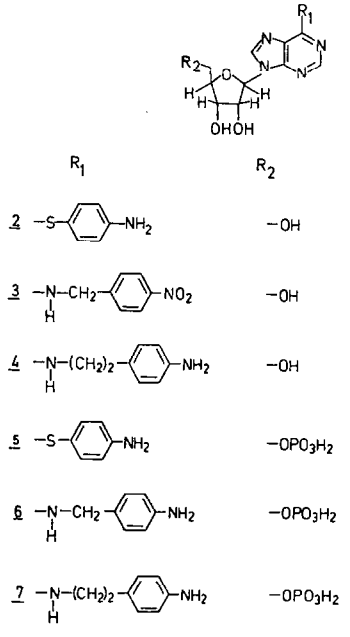

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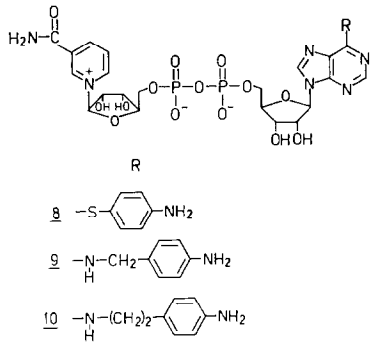

SCHEME 3

In the case of N^6-substituted AMP analogs it was necessary to protect the hydroxyl and amino groups of the nucleotides prior to condensation by trifluoroacetylation to obtain satisfactory yields. The protecting groups were removed easily during the chromatographic purification of the coenzyme analogs. Under these conditions no cleavage of the pyrophosphate bond was observed. The analogs **9** and **10** showed the same electrophoretic mobility as NAD^+. All analogs reacted with various NAD^+ dependent dehydrogenases as coenzymes. Table 1 shows the kinetic parameters of the analogs. The data for the analogs **1a–c** show that major structural changes at the adenosine part cause a large increase of the Michaelis constants K_M and a decrease of the turnover numbers. Compounds with a purine riboside (**8–10**) part showed only slightly changed coenzymatic properties compared with NAD^+, indicating that they are bound similarly to the active sites of the enzymes. Treatment of the aromatic amino derivatives with nitrous acid at 4°C yields the reactive diazonium compounds. Even at temperatures below 4°C these compounds were only fairly stable and decomposed within 24 h at pH 7.5.

ADH was modified with the diazonium analogs in the presence of pyrazole. Pyrazole acts as pseudosubstrate and stabilizes the binary enzyme–coenzyme complex by forming a ternary dead-end complex. In the case of LDH, oxalate was used as ternary complex ligand. After completion of the modification reaction

TABLE 1

COENZYMATIC PROPERTIES OF THE ANALOGS

	ADH from horse liver		LDH from pig heart	
Coenzyme	$K_M \times 10^4$ M	Turn-over number	$K_M \times 10^4$ M	Turn-over number
NAD^+	0.3	410	0.75	17,000
1a–c	2	36	1	10
8	0.5	410	1	11,000
9	0.5	410	1.5	11,000
10	0.4	340	2.1	10,000

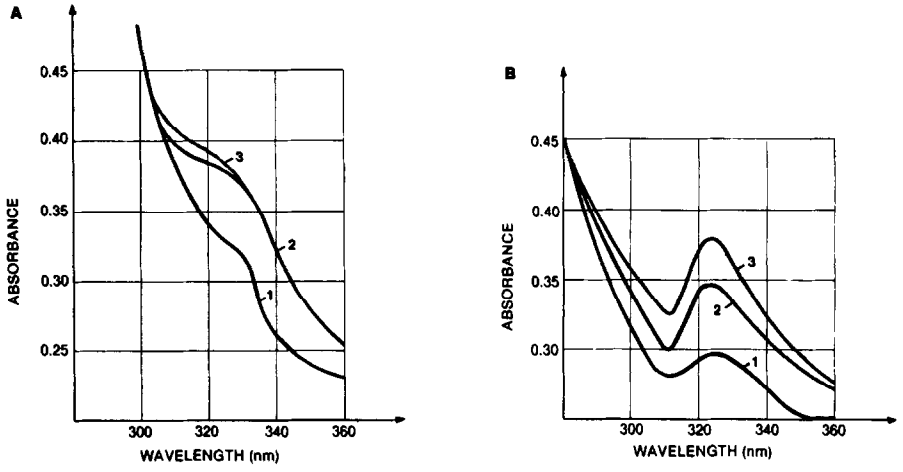

FIG. 1. (A) 1. Absorption spectrum of modified LDH; incorporation, 3.2 mol/mol tetrameric enzyme; protein concentration, 0.8 mg/ml in 0.1 *M* glycine/NaOH buffer, pH 9.5. 2. After addition of lithium lactate up to 0.05 *M*. 3. After addition of potassium cyanide up to 0.02 *M*. (B) 1. Absorption spectrum of modified ADH; incorporation 2 mol/mol dimeric enzyme; protein concentration, 0.8 mg/ml in 0.1 *M* glycine /NaOH buffer, pH 9.5. 2. After addition of ethanol up to 0.05 *M*. 3. After addition of potassium cyanide up to 0.02 *M*.

excess analogs were removed by exhaustive dialysis. The modified enzymes were orange colored after incubation, and their enzymatic activities were reduced by 30–100% of the starting activity.

Charcoal treatment of holoenzymes prepared by reaction with diazo **10** did not remove the coenzyme analog. The incorporation ratio remained unchanged. Under these conditions added NAD^+ or **10** is removed completely. Modified LDH was very slowly denatured by 5 *M* urea. After incubation for 15 min the enzymatic activity was decreased to only 70%. In parallel control experiments native LDH was inactivated completely. In the case of ADH we found no difference between native and modified enzyme. Both enzymes were inactivated by 5 *M* urea within 15 min. Addition of the substrates ethanol and lactate, respectively, led to the reduction of the covalent-bound analogs (Fig. 1). The specific activities of the reduced forms of holoenzymes were further reduced to more than 50%.

The reduction could be easily monitored by the increase of absorbance at 340 nm in the case of LDH and 325 nm with ADH. The fluorescence excitation spectra showed an energy transfer band at 290 nm upon reduction (Fig. 2). The emission maxima were shifted to 420 nm compared with 455 nm for the free coenzyme analogs and the fluorescence intensities were strongly enhanced compared to the oxidized form (Fig. 3). The amount of modification could be determined by the increase of absorption at 325 or 340 nm as well as from the increase of fluorescence after reduction with substrates.

The number of fixed analogue molecules per enzyme subunit was calculated after protein determination (Table 2).

The products of the enzymatic reactions, propionaldehyde (in some experiments propanol was used instead of ethanol because of the lower volatility of

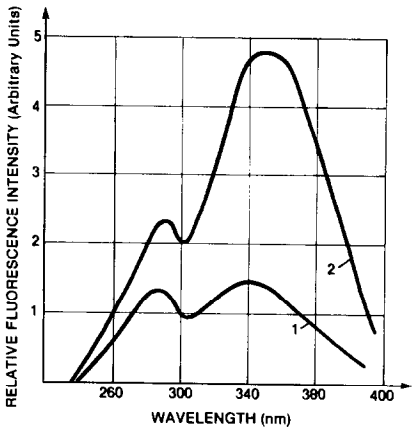

FIG. 2. 1. Fluorescence excitation spectrum of holo-LDH; 0.8 mg protein/ml in 0.1 *M* glycine/NaOH buffer, pH 9.5; 3.3 μmol diazo-**10** incorporated in 1 μmol tetrameric enzyme. 2. After adding 10 μmol lithium lactate. (Spectrum of dihydro-**10** incorporated in LDH).

propionaldehyde compared to acetaldehyde) and pyruvate, could be assayed by enzymatic reactions after denaturation and removal of the modified proteins.

The reduction rate of holoenzymes with bound diazo **8–10** was too rapid to be followed without rapid mixing methods. The reaction was completed within the time necessary for manual mixing of the holoenzymes and the substrates. However, when holoenzymes with the covalently fixed analogs **1a**, **1b**, or **1c** were used, the reaction rates of the covalent-bound coenzymes were found to be about 20-fold higher than those measured with the free amino analogs present in enzyme-saturating concentrations. The formation of NADH catalyzed by the native enzymes ADH or LDH is slower than the formation of the reduced form of covalent-bound analogs **8–10** under comparable conditions as shown in Figs. 4

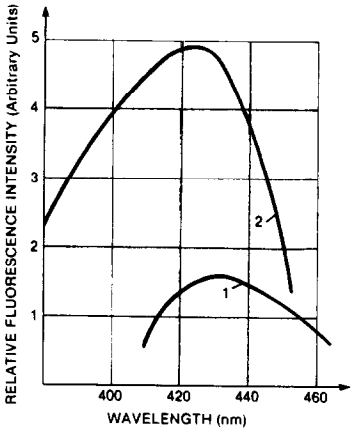

FIG. 3. 1. Fluorescence emission spectrum of holo-LDH, same as before. 2. After addition of 10 μmol lithium lactate; excitation wavelength, 340 nm.

TABLE 2

INCORPORATION OF DIAZO COENZYME ANALOGS IN DEHYDROGENASES

Coenzyme	ADH from horse liver[a]		LDH from pig heart[b]	
	Incorporation (μmol/μmol enzyme)	Percentage enzymatic activity[c]	Incorporation (μmol/μmol enzyme)	Percentage enzymatic activity[c]
Diazo-**8**	1.6	60	3.2	80
	0.8	80	2.0	90
Diazo-**9**	1.8	50	3.2	90
Diazo-**10**	1.6	60	3.2	90
	2.0	50	2.8	100
	1.2	70	1.8	100
Dihydroform of diazo-**10**	2.0	30	1.6	35

[a] Dimeric enzyme.
[b] Tetrameric enzyme.
[c] Determined with 0.01 *M* NAD^+.

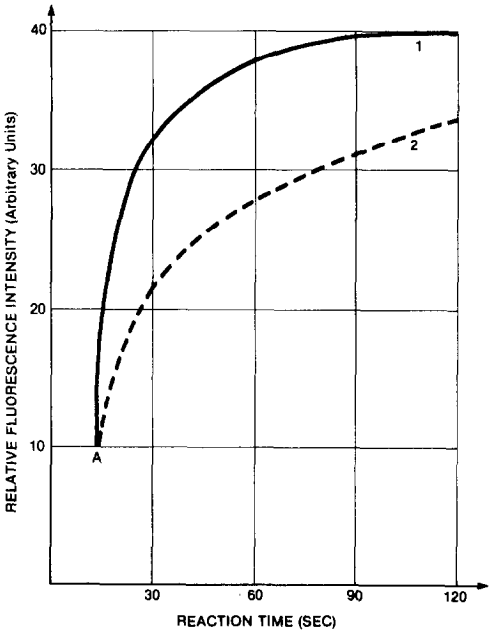

FIG. 4. 1. Time-dependent formation of the dihydro form of holo-ADH; 1 mg protein in 1 ml 0.1 *M* glycine/NaOH buffer pH 9.5; enzymatic activity 0.6 U/mg; incorporation, 1.2 μmol diazo-**10** in 1 μmol dimeric enzyme. The reaction was started by addition of 10 μl ethanol at point A. 2. Time-dependent formation of NADH by unmodified ADH; 0.5 mg protein in 1 ml 0.1 *M* glycine/NaOH, pH 9.5, containing 0.1 m*M* ethanol; enzyme activity, 1.5 U/mg. The reaction was started by adding 0.01 μmol NAD^+ at point A.

and 5. When the proteins were modified at conditions which preferentially lead to modifications of residues at the surface of the enzymes such as high concentrations of diazonium compounds, presence of protecting NAD^+, or absence of pseudosubstrates, the resulting holoenzymes showed biphasic reaction kinetics. The rapid reaction caused by the addition of substrates and measured by the increase in absorbance or fluorescence intensity was followed by a slow reaction. The total amount of bound coenzymes could be determined by addition of potassium cyanide and measurement of the absorbance of the cyanide complex at 325 nm.

In the presence of equimolar amounts of oxidized and reduced substrates the reaction of the fixed coenzyme was pH-dependent. The intensity of the fluorescence signals were in accordance with mixtures prepared from NAD^+, native enzymes, and both substrates.

The formation of complexes between modified enzymes and added NADH is disturbed. Concentrations of 0.1 m*M* NADH were required to obtain a significant increase of fluorescence with holo-LDH prepared with diazo **8–10**. In contrast, the same fluorescence intensity was reached with NADH concentrations of 0.01 m*M* when native enzyme was used. With modified ADH no additional fluorescence increase was observed even at very high NADH concentrations.

To determine the modified amino acid, model compounds from *N*-α-acetylamino acids and diazoniumnucleotides were prepared. These products were only fairly stable. After coupling with *N*-α-acetylcysteine a new absorption band at 330 nm appeared. In this range modified ADH absorbed as well. Modified LDH had an

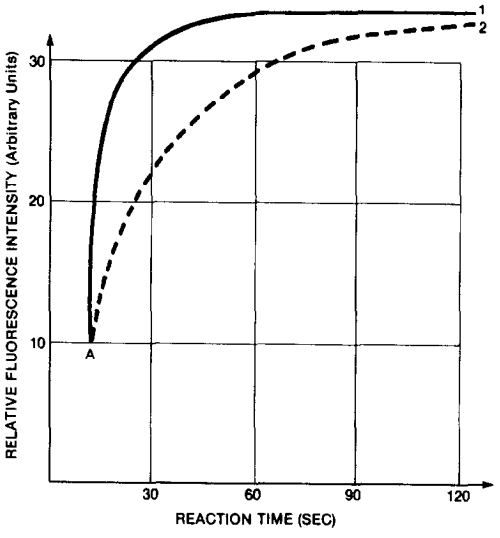

FIG. 5. 1. Time-dependent formation of dihydro form of holo-LDH; 0.5 mg protein in 1 ml 0.1 *M* glycine/NaOH buffer, pH 9.5; enzymatic activity, 350 U/mg; incorporation, 1.8 μmol diazo-**10** in 1 μmol tetrameric enzyme. The reaction was started by adding 100 μmol lithium lactate at point A. 2. Time-dependent formation of NADH by unmodified LDH, 0.5 mg; 350 U/mg in 0.1 *M* glycine/NaOH buffer, pH 9.5, containing 0.1 *M* lithium lactate. The reaction was started by adding 0.01 μmol NAD^+ at point A.

absorption maximum in the range 300–350 nm which corresponded with the maxima of histidine and tyrosine model compounds. When a tenfold excess of diazonium compounds was used or the modification was performed in presence of NAD^+, an increase at 330 nm was also observed.

DISCUSSION

For the preparation of functional dehydrogenase holoenzymes, reactive NAD^+ analogs must be covalently attached in the vicinity of the active site. Amino acids of the catalytic nicotinamide-binding site may not be modified by the reactive analogs.

Substitution at the N^6-position of the adenine part does not interfere strongly with the binding of such analogs as is shown by the extensive use of this class of compounds as ligands for affinity chromatography or their coenzymatic activities.

As most dehydrogenases follow an ordered reaction mechanism with coenzyme binding first, the binary complexes can be stabilized by the formation of ternary dead-end complexes with pseudosubstrates. In this case the highly reactive diazonium group of the prepared analogs can react with amino acid residues near the active site. Due to the flexible spacer group the covalent-bound analog can leave and reenter the active site.

We propose a structure of these holoenzymes as is shown in Fig. 6. The coenzymes can not be removed by treatment with charcoal which indicates that they are covalently fixed. The optical properties of the holoenzymes, especially those modified by the dihydroform, point out that the analogs are, at least partially, bound in the active site. The fluorescence spectral data are in accordance with the formation of binary coenzyme enzyme complexes.

This view is furthermore supported by the enhanced stability of LDH holoenzymes against denaturation by urea and the faster reaction rates of holoenzymes

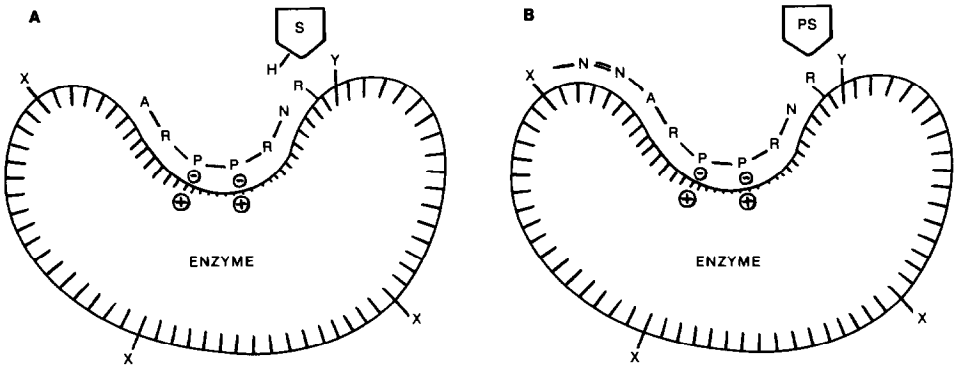

FIG. 6. (A) Diagrammatic representation of the natural ternary complex. (B) Diagrammatic representation of the enzyme modification. X, reactive amino acid residues; A–R–P–P–R–N, NAD; S, substrate; R and Y, amino acid residues which are involved in the enzymatic reaction; PS, pseudosubstrate; and, N = N–A–R–P–P–R–N, incorporated coenzyme analog.

with substrates when compared to the reaction of comparable amounts of free NAD^+ and native enzymes.

The high NAD^+ concentrations necessary to obtain optimal reaction rates as well as the high NADH concentrations needed to achieve fluorescence signals indicate a competition of free and covalent-bound coenzymes for the active center.

No clear linear relationship between the amount of modification and the decrease of enzymatic activity is detected. As expected, the activity of dihydroholoenzymes is decreased compared to the oxidized forms. When the modification is performed under conditions where the binding of the analogs to the active site is hindered as in the presence of natural coenzyme, or when high concentrations of diazonium analogs are used, one expects an indiscriminating modification of amino acid residues which can react chemically with diazonium compounds. Analogs which are bound outside the region of the active site can only be reduced very slowly by reacting with the active site of another enzyme molecule. There is no detectable decomposition of the holoenzymes after standing for several days in buffered solutions. However, the stability of the azobridge was not sufficient to allow the determination of the amino acid by means of protein analytical methods.

The appearance of absorption bands at 330 nm and between 300 and 350 nm suggests labeling of cysteine, histidine, and tyrosine residues, respectively.

EXPERIMENTAL

Materials and Methods

ADH from horse liver, LDH from pig heart, NAD^+, and NADH were from Boehringer (Mannheim, FRG). Nicotinamide mononucleotide (*7*) and ω-(5-amino-2-benzimidazolyl) alkyl phosphates (*4*) were prepared as described elsewhere. Thin-layer chromatography was carried out on Merck silica gel F254 plates with ethylacetate : methanol, 10 : 1 (v : v) (solvent 1) for nucleosides; and isobutyric acid : water : concentrated ammonia, 70 : 29 : 1 (v : v) (solvent 2) for nucleotides. High-voltage paper electrophoresis was performed in 0.1 *M* Tris/HCl buffer, pH 8.2, and 30 V/cm. Spots were located under UV light and developed with a spray (*8*). UV and visible spectra were recorded with a Perkin–Elmer 555 or a Cary 14 spectrophotometer. Fluorescence measurements were performed with a Hitachi MPF 4. Enzymatic activities of ADH and LDH were assayed as described (*9*). Protein concentrations were determined by the microbiuret method (*10*), according to Lowry *et al.* (*11*) or to Bradford (*12*).

Chemical Synthesis

P^2 - (2 - (3 - Hydroxypropyl) - benzimidazole - 5 - ylamine)nicotinamide - D-ribofuranosyl 5'-diphosphate ***1a****; P^2-(2-(4-Hydroxybutyl)-benzimidazole-5-ylamine)-nicotinamide-D-ribofuranosyl 5'-disphophate* ***1b****; and P-(2-(5-Hydroxypentyl)-benzimidazole-5-ylamine)-nicotinamide-D-ribofuranosyl 5'-diphosphate* ***1c***. Equimolar amounts of nicotinamide mononucleotide and ω-(5-amino-2-benzi-

midazolyl)alkyl monophosphates were condensed to give corresponding nucleotide anhydrides as described by Todd (*13*). The nucleotide solutions were chromatographed on Dowex 1-X8 columns (formiate form, 200–400 mesh, 2 × 25 cm) and eluted with formic acid (convex gradient formed by 0.01 *M* formic acid and 500 ml water in the mixing chamber). The product fractions were pooled and lyophilized. Typical yields were 6–8%. UV (0.1 *M* glycine/NaOH buffer, pH 9.5) λ_{max} 290 (6200); (0.1 M K_2CO_3, 0.01 M KCN aquous) λ_{max} 295 (8300), λ_{max} 325 (6500).

(4-Aminophenyl)-6-thiopurine riboside **2.** 6-Chloropurineriboside (2.86 g, 10 mmol), 1.75 g (4 mmol) 4-aminothiophenole, and 3.3 ml (14 mmol) tributylamine were suspended in 100 ml ethanol and refluxed for 5 hr. The resulting clear solution was evaporated *in vacuo* to a 10 ml volume. The product precipitated after cooling at −15°C overnight and was recrystallized from ethanol. Yield 3.15 g, mp 151°C; UV (ethanol) λ_{max} 260 (20,000) 281 (22,400). *Anal.* Calcd for $C_{16}H_{17}N_5O_4S$ (375.4): C, 51.19; H, 4.56; N, 18.65 Found: C, 51.11; H, 4.66; N, 18.18.

(4-Aminophenyl)-6-thiopurine riboside 5'-phosphate **5.** One gram (2.66 mmol) of the riboside **2** was dissolved in 10 ml triethyl phosphate and the mixture was cooled to −20°C. One milliliter (8.95 mmol) of freshly distilled phosphoroxytrichloride was added dropwise to the stirred solution over a period of 30 min. Stirring was continued for 6 hr at −15°C. The solution was poured into 50 ml of cold 1 *M* triethylammonium hydrogencarbonate solution and the triethyl phosphate was extracted with four 50-ml portions of ether. The aquous phase was evaporated *in vacuo,* the residue was dissolved in 10 ml water, and the pH was adjusted to 7.4 with ammonia. The sample was applied to a DEAE A 25 column (4 × 30 cm, HCO_3^- form). The column was washed with water and then with a linear gradient of triethylammonium hydrogencarbonate (0–0.4 *M*, 4 liters). The product fractions were pooled and evaporated *in vacuo* at 30°C. The residue was repeatedly dissolved in 50% aquous methanol and evaporated. Finally, the nucleotide was dissolved in a minimal amount of water and the pH was adjusted to 3.5 with 0.5 *N* HCl. The product crystallized. Yield 450 mg, 37%; UV (H_2O) λ_{max} 255 (17,400) 288 (21,400). *Anal.* Calcd for $C_{17}H_{18}N_6O_6S$ P (439.4): C, 42.01; H, 4.40; N, 15.31; 0, 24.48; S, 7.01; P, 6.77. Found: C, 42.05; H, 4.07; N, 15.32; O, 24.56; S, 6.98; P, 6.65.

N^6-(4-Nitrobenzyl)adenosine **3.** A suspension of 2.86 g (10 mmol) 6-chlorpurine riboside, 2.64 g (14 mmol) 4-nitrobenzylamine hydrochloride, and 4.75 ml (20 mmol) tributylamine in 80 ml ethanol was refluxed for 5 hr. The solvent was evaporated *in vacuo* at 30°C, and the residue was dissolved in 50 ml hot water, treated with charcoal, and filtered. N^6-(4-Nitrobenzyl)adenosine precipitated from the filtrate upon cooling. Yield 2.39 g, mp 125°C, UV (ethanol) λ_{max} 268 (20,900) *Anal.* Calcd for $C_{17}H_{18}N_6O_6 \times \frac{1}{2} H_2O$ (411.4): C, 49.62; H, 4.66; N, 20.24. Found: C, 49.38; H, 4.66; N, 19.95.

N^6-(4-Aminobenzyl)adenosine 5'-phosphate **6.** N^6-(4-Nitrobenzyl)adenosine was phosphorylated as described for N^6-(aminophenyl)-6-thiopurine riboside. Yields were 50–55%. A 520-mg (1 mmol) amount of the obtained N^6-(4-nitrobenzyl)adenosine 5'-phosphate was dissolved in 6 ml 20% aquous ethanol and 0.35 ml hydrazine hydrate. A 0.5-ml aliquot of a freshly prepared Raney-nickel suspen-

sion was added, and after the nitrogen evolution had finished the mixture was refluxed for 90 min. Raney-nickel was filtered off from the hot suspension and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in water and the pH was adjusted to 3.5 with 1 *N* HCl. A 360-mg amount of N^6-(4-aminobenzyl)adenosine 5′-phosphate (80%) precipitated. UV (H_2O) λ_{max} 264 (20,900) *Anal.* Calcd for $C_{17}H_{21}N_6P \times H_2O$ (470.4): C, 43.40; H, 4.93; N, 17.81; P, 6.59. Found: C, 43.79; H, 4.99, N; 17.71; P, 6.69.

N^6-(4-Aminophenylethyl)adenosine 5′-phosphate **7**. N^6-(4-Aminophenylethyl)adenosine **4** was obtained in nearly quantitative yield by reacting 6-chloropurine riboside with β-(4-aminophenyl)ethylamine as described for (4-aminophenyl)-6-thiopurine riboside. UV (0.01 *N* HCl) λ_{max} 267 (19,500). *Anal.* Calcd for $C_{18}H_{22}N_6O_4$ (386.5): C, 55.95; H, 5.74; N, 21.75. Found: C, 55.72; H, 5.50; N, 21.54. The phosphorylation was performed as described above but the reaction time had to be extended to 5 days. A 350-mg amount of N^6-(4-aminophenylethyl)adenosine 5′-phosphate **7** was obtained from 1 g **4**. UV (H_2O) λ_{max} 267 (19,500). *Anal.* Calcd for $C_{18}H_{23}N_6O_7P$ (466.4): C, 46.35; H, 4.97; N, 18.02; P, 6.64. Found: C, 46.10; H, 4.89; N, 18.16; P, 6.64.

Synthesis of the NAD^+ analogues ***8, 9, and 10***. One millimole of the nucleotide **5** was dissolved in aquous pyridine, 400 mg (1.3 mmol) nicotinamide mononucleotide was added, and the reaction was performed in the presence of dicyclohexylcarbodiimide as described (*13*). Prior to the condensation the nucleotides **6** or **7** and equimolar amounts of nicotinamide mononucleotide were treated with trifluoroacetic acid anhydride at 0°C. Excess of solvent was removed by evaporating the mixtures *in vacuo*. The residues were used for condensation with dicyclohexylcarbodiimide (*13*) without further purification. The reaction mixtures were worked up as described and the coenzyme analogs were purified by chromatography on DEAE A 25 (column 4 × 30 cm, HCO_3^- form). The columns were washed with a linear gradient of triethylammonium hydrogencarbonate (0–0.2 *M*, 4 liters). Product fractions were collected, pooled, and lyophilized. Salts were removed by repeated evaporation of the aquous methanolic solutions below 30°C. Yields were 30% with respect to the mononucleotides. The NAD^+ analogs were enzymatically reduced to the corresponding dihydro forms (*14*).

Synthesis of diazonium NAD^+ analogs. A 10-μmol of the NAD^+ analogs **8**, **9**, or **10** was dissolved in 2 ml 0.5 *N* HCl and cooled in ice. In dim light, the solutions were vigorously stirred and 50 μl of 1 *M* $NaNO_2$ was added. After 30 min the mixtures were neutralized with solid sodium hydrogencarbonate and excess nitrite was decomposed with amidosulfonate (*4*). The diazonium analogs were only slightly stable. An isolation as solid substances was impossible, UV light decomposed the analogs within a few minutes. In daylight, the lifetime was about 6 hr. In the case of diazonium **8**, the decay could be followed easily by absorption measurements at 360 nm.

N-α-Acetylamino acid azo products. A 1-ml aliquot of a 10 m*M* solution of *N*-α-acetylamino acid in 0.1 *M* phosphate buffer, pH 6.5, or 0.1 *M* borate buffer, pH 8 or 10, was cooled to 4°C and 1 ml 7 m*M* diazonium coenzyme solution was added. The solutions were kept in the dark and 10-μl aliquots were removed to follow the reaction spectrophotometrically and by TLC on silica gel and solvent 2. The

optical properties were determined by measurements of the difference spectra against unreacted amino acids.

Preparation of the holoenzymes. All reactions were carried out at 4°C and in dim light. To 1 ml of a solution containing 5 mg ADH in 0.1 *M* phosphate buffer, pH 7.4, which was 0.24 m*M* in pyrazole, the diazonium coenzymes were added to give final concentrations of 0.01 to 0.04 m*M*. The reaction was monitored spectrophotometrically. After 12 hr standing at 4°C, the excess of diazonium coenzymes, decomposition products, and pyrazole were removed by exhaustive dialysis. Modified LDH was prepared similarly with 0.2 m*M* oxalate as pseudosubstrate. In control experiments the diazonium analogs were replaced by the analogs **8**, **9**, or **10**. For studies of the protecting effect of NAD^+, the natural coenzyme was added in concentrations from 0.1 to 10 m*M*. In further experiments pseudosubstrates were omitted from the reaction mixtures.

Reduction of the holoenzymes. To prepare the dihydroform of covalent-bound analogs, the modified enzymes were dialyzed against 0.1 *M* glycine/NaOH buffer, pH 9.5. The solutions contained 1 mg protein/ml. A 0.1-ml aliquot of ethanol was added to 1 ml modified ADH solution. LDH holoenzyme was reduced by adding 0.1 ml 1 *M* lithium lactate/ml protein solution. After heat denaturation of the modified enzymes for 30 min at 100°C in a sealed tube and removal of the proteins, the products could be identified by enzymatic tests at pH 7.6 with ADH and LDH (*9*). Investigation of the pH dependence of the holoenzyme reduction was performed in 0.1 *M* glycine /NaOH buffer, pH 9 and 10, respectively. The buffers were 0.1 *M* in propanol and propanal for measurements with modified ADH or 0.1 *M* in pyruvate and lactate with LDH. The fluorescence emission at 420 nm was monitored after excitation at 340 nm. Controls contained unmodified enzymes and NAD^+.

The enzymatic activities of the modified enzymes in the presence of free NAD^+ were determined with an assay system described earlier. In this experiment the NAD^+ concentration was varied between 0.1 and 2 m*M*.

Activity determinations of the modified enzymes in absence of NAD^+ were performed as follows: 0.025 to 0.5 mg modified protein was dissolved in 2 ml 0.1 *M* glycine/NaOH buffer, pH 9.5. A 0.05-ml aliquot of ethanol or 0.1 ml of 0.5 *M* lithium lactate was added and the fluorescence increasements at 420 nm were registered. The excitation wavelength was 340 nm. Controls contained unmodified enzymes of identical concentrations and 1 n*M* of the analogs **8**, **9**, or **10**. The reverse reaction was studied with the dihydroform of the holoenzymes in 0.1 *M* phosphate buffer, pH 7, and propanal or sodium pyruvate as substrates.

The formation of binary NADH enzyme complexes were measured by fluorescence titration (*15*).

Charcoal treatment was performed as described by Velick (*16*). The time course of denaturation by urea was monitored according to Pfleiderer (*17*).

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