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THE SYNTHESIS OF SPIN-LABEL DERIVATIVES OF NAD$^+$ AND ITS STRUCTURAL COMPONENTS AND THEIR BINDING TO LACTATE DEHYDROGENASE

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Summary

Spin-labelled derivatives of NAD$^+$ and its structural components (i.e. adenosine, adenine, AMP, ADP and ADPR) have been synthesized. Their binding to pig heart lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) has been studied and dissociation constants have been determined. The spin-labelled derivatives of ADP and ADPR exhibit a tighter binding than the corresponding NAD$^+$ derivative. This may be attributed to the repulsion of the positively charged nicotinamide ring by an histidine side chain in the active center of the enzyme.

Introduction

Most of the intricate organic molecules playing a role as coenzymes in biological systems have a structure far more complex than necessary for the chemical reaction itself. The functional part very often comprises only a minor constituent of the overall structure. Many investigators have therefore studied the importance of the so-called nonfunctional parts for biological activity. Mainly, this has been attempted by synthesizing structural analogs of the natural compound and the study of the effects of these alterations on their activity. In the case of NAD$^+$ numerous analogs with alkyl bridges substituting

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Abbreviations: ADPR: adenosine 5′-diphosphoribose; NMN: nicotinamide mononucleotide; N^6-SL preceeding NAD$^+$, ADPR, ADP, AMP, adenosine and adenine refers to the N^6-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl) derivatives of these compounds.

the ribose moieties and various aromatic systems substituting the adenine ring have been used [1,2].

A different approach would be binding studies of the structural components of the ligand under investigation. However, these components often lack the specific spectral changes which may be encountered upon binding of the entire natural substrate and which may be used as a means to determine the degree of binding. The spin-label technique provides a valuable tool for the study of such interactions where spectral changes when occuring at all are too weak to be observed. The ESR spectrum of a spin-labelled ligand will change drastically upon binding to a macromolecule. The decrease of the amplitude of the first derivative of the absorption spectrum is a direct measure of the fraction bound [3,4]. This is highly advantageous with respect to most other spectral techniques. Fluorescence titrations for instance yield the amount of protein bound as a fraction of the end value. An error in this end value which very often is experimentally not fully attainable greatly influences the results [5]. Moreover, in these techniques one has to assume that all possible binding sites contribute equally to the intensity of the emission or absorption respectively. This is particularly questionable in oligomers exhibiting different binding in the various subunits.

We have synthesized spin-labelled derivatives of NAD^+ [6] and its components and studied their binding to pig heart lactate dehydrogenase (EC 1.1.1.27) by ESR titration experiments. This enzyme is a tetramer of four identical subunits catalyzing the interconversion of L-lactate and pyruvate in presence of NAD^+ or NADH respectively. Spin-labelled NAD^+ has been shown previously to be enzymatically highly active in this system [6]. A different spin-labeled derivative of NAD^+ has previously been used with other dehydrogenases. [7]. However, it lacks the functional part of the coenzyme. Several investigators have studied the binding of the NAD^+ components to lactate dehydrogenase kinetically. The NAD^+-fragments are competitive inhibitors of the enzymic reaction with respect to NAD^+ [8—10].

Generally an increase in the degree of inhibition was observed as the structure of the inhibitors approached the structure of NAD^+ due to more complete binding at the active site of lactate dehydrogenase. Nevertheless all the inhibitor constants were higher than the Michaelis constant of NAD^+. It is well known, however, that the actual equilibrium dissociation constants may differ greatly from kinetic constants in systems with more than one substrate. The dissociation constants obtained in our investigation lead to different interpretations regarding the binding of these fragments.

Methods

Materials

Lactate dehydrogenase (EC 1.1.1.27) from pig heart, NAD^+ glycohydrolase (EC 3.2.2.5) from pig brain (acetone powder) and NAD^+ were purchased from Boehringer, Mannheim. A X-band ESR spectrometer constructed in our laboratories [11] was used. Spectra were recorded at a modulation amplititude of 0.7 gauss with a time constant of 0.1 or 0.3 s and a scan of 10 gauss/min at 25° C.

ESR titration experiments

The ammonium sulfate suspension of lactate dehydrogenase was dialyzed extensively against 67 mM phosphate buffer, pH 7.2. After centrifugation at 44 000 × *g* the supernatant was passed over Sephadex G-25 fine containing 5 mg of finely powdered charcoal (Nuchar C-190) per ml of gel.

Titration experiments were carried out by addition of 1 µl portions of 10^{-2} M solutions of N^6-SL-AMP and N^6-SL-NAD^+ or $3 \cdot 10^{-3}$ M N^6-SL-ADP and N^6-SL-ADPR in phosphate buffer, pH 7.2, to 100 µl of $8 \cdot 10^{-5}$ M enzyme solutions in the same buffer by means of Hamilton syringes equipped with a repeating dispenser (model PB 600-1). Platinum needles and Teflon-tipped plungers had to be used due to reduction of the free radical by stainless steel (Robillard, G. and Zantema, A., private communication).

The concentration of the free spin-labelled compounds was determined from the amplitude of the high field peak by means of a diagram, amplitude versus concentration resulting from a blank titration without enzyme.

For the determination of the binding constants of N^6-SL-adenine and N^6-SL-adenosine 5 µl of a solution of $2.4 \cdot 10^{-2}$ M NAD^+ and 0.12 M oxalate were added to the mixture of 10^{-4} M lactate dehydrogenase and $4 \cdot 10^{-4}$ M of the spin-label analog in 100 µl of phosphate buffer pH = 7.2.

For the additions during titration the ESR cells were taken out of the cavity. To avoid changes in the signal amplitude due to slightly different orientations of the cell when repositioned in the cavity a special sample holder was used. During the course of the experiment the flat cell (AEG, Berlin, type E 777-097-672) was fixed in a Teflon adapter which fitted tightly into the sample holder as shown in Fig. 1.

Protein determination

The protein concentration was determined spectrophotometrically at 280 nm using a factor of 1.4 for a solution containing 1 mg/ml (6.94 µM) [12] lactate dehydrogenase in phosphate buffer, pH 7.2.

Thin-layer chromatography

Thin-layer chromatography was carried out on precoated silica gel 60 F-254 plates from Merck, Darmstadt. The following systems were used: System A: *n*-propanol/conc. ammonia/water (20 : 12 : 3); System B: isopropanol/1% aqueous ammonium sulfate (2 : 1); System C: isobutyric acid/conc. ammonia/water (66 : 1 : 33).

Syntheses

N^6-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl) adenine (N^6-SL-adenine). 77.3 mg (0.5 mmol) of 6-chloropurine and 256.5 mg (1.5 mmol) of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl were refluxed in 50 ml of ethanol for 40 h. The solvent was then evaporated under reduced pressure and the oily reddish residue purified by preparative thin-layer chromatography on silica gel with methanol as eluent (R_F = 0.6). Recrystallization from ethyl acetate and subsequently from toluene yielded 65 mg (45%) of N^6-SL-adenine, f.p. = 218° C. Thin-layer chromatography: System A: R_F = 0.76; System B: R_F = 0.66; System C: R_F = 0.87.

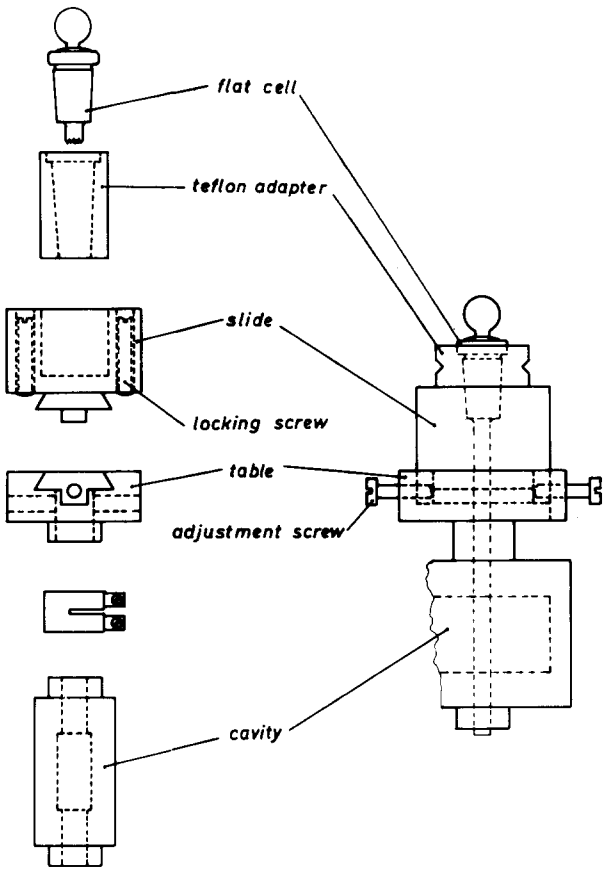

Fig. 1. Schematic representation of the cell holder used for the ESR titration experiments.

Mass spectrum (70 eV): M^+ for $C_{14}H_{21}N_6O$: calculated *m/e* = 289.17767, found *m/e* = 289.1777.

N^6-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl) adenosine (N^6-SL-adenosine). A suspension of 100 mg (0.35 mmol) of 6-chloro-9-β-D-ribofuranosylpurine and 134 mg (0.79 mmol) of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl in 5 ml of ethanol was refluxed under stirring for 5 h. The clear solution was concentrated under reduced pressure and the solid residue was triturated repeatedly with a total of 100 ml of anhydrous ether. N^6-SL-adenosine formed reddish crystals on concentration: 45 mg (31%). Thin-layer chromatography: System A: R_F = 0.66; System B: R_F = 0.68; System C: R_F = 0.69. Mass spectrum after trimethylsilylation (70 eV) : M^+ for the Tris-trimethylsilylated derivative *m/e* = 637.

N^6-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl) adenosine-5'-diphosphate (N^6-SL-ADP). (a) 6-Chloro-9-β-D-ribofuranosylpurine-5'-diphosphate. 251 mg (0.5 mmol) of the barium salt of 6-chloro-9-β-D-ribofuranosylpurine-5'-phosphate [6] were converted to the pyridinium salt by the passage over SE-Sephadex C-25, pyridinium form. The lyophilized product was dissolved in 4 ml of methanol. 220 μl of tri-*n*-octylamine were added and shaking was continued until a clear

solution had formed. After evaporation of the solvent the residue was repeatedly taken up in 3 ml of dimethyl formamide and evaporated. The tri-*n*-octyl ammonium salt was dissolved in 3.5 ml of dioxane and kept at room temperature for 3 h after addition of 0.15 ml of diphenyl phosphorochloridate and 0.225 ml of tri-*n*-butylamine. After evaporation of the solvent the residue was triturated with ether, dissolved in dioxane and evaporated again. 283 mg (0.1 mmol) of the primary tri-*n*-butylammonium salt of phosphoric acid prepared by the method of Moffatt and Khorana [13] were added, the mixture dissolved in 2 ml of pyridine and kept at room temperature for 18 h. After evaporation and trituration with ether the remaining brown syrup was dissolved in 20 ml of water and extracted twice with ether. The organic phase was re-extracted with water and the combined aqueous phases were concentrated and separated over DEAE-Sephadex A-25 (2 × 30 cm, chloride form). After elution with water a linear gradient of 700 ml each of water and 0.5 M lithium chloride was applied. The diphosphate eluted at 0.25 M lithium chloride. It was used directly for the conversion to N^6-SL-ADP after lyophilization and trituration with ethanol/ether 1 : 3. 72 mg (31%). Thin-layer chromatography: System A: R_F = 0.28; System B: R_F = 0.32; System C: R_F = 0.24.

(b) 46.5 mg (0.1 mmol) of the product from (a) and 136.8 mg (0.8 mmol) of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl were dissolved in 30 ml of water and kept at 50°C for 20 h under stirring. N^6-SL-ADP was purified by chromatography on DEAE-Sephadex A-25, chloride form by elution with water and subsequently with a linear gradient of 0—0.3 M lithium chloride. The fraction at 0.2 M containing the diphosphate was lyophilized, triturated with ethanol/ether 1 : 3 and precipitated from methanol in the cold on addition of ether. 20 mg (33%). Thin-layer chromatography: System A: R_F = 0.36; System B: R_F = 0.39; System C: R_F = 0.45. For $C_{19}H_{28}Li_3N_6O_{11}P_2 \cdot 3H_2O$ (653.28), calculated: C = 34.9, H = 5.3, N = 12.8, P = 9.5; found: C = 34.7, H = 5.3, N = 12.2, P = 9.6.

N^6-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl) adenosine-5'-diphosphoribose (N^6-SL-ADPR). 2 mg (2.4 μmol) of N^6-SL-NAD$^+$ [6] were dissolved in 2 ml of 67 mM phosphate buffer pH = 7.2 and kept at 25°C for 4 h after addition of 25 mg of NAD$^+$ glycohydrolase from pig brain. The enzyme was separated by centrifugation at 44000 × *g* and the supernatant purified by paper chromatography on Whatman 3 MM with 67 mM phosphate buffer, pH 7.2/isopropanol (5 : 6) as eluent. N^6-SL-ADPR (R_F = 0.73) was eluted with water and lyophilized. Thin-layer chromatography: System A: R_F = 0.48; System B: R_F = 0.53; System C: R_F = 0.41.

Results

We have studied the binding of N^6-SL-NAD$^+$ and its components to pig heart lactate dehydrogenase. The various spin-label derivatives used in this investigation are shown in Fig. 2. N^6-SL-NAD$^+$ and N^6-SL-AMP had been synthesized by us previously [6]. All other analogs were synthesized as described in the Methods section. For N^6-SL-ADP the general procedure of Michelson was followed [14]. N^6-SL-ADPR was obtained by enzymatic hydrolysis of N^6-SL-NAD$^+$ by NAD$^+$ glycohydrolase [15]. They gave satisfactory combustion

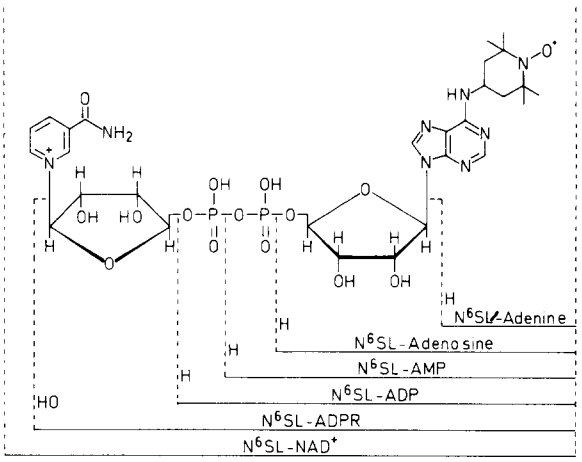

Fig. 2. Spin-labelled derivatives of NAD^+ and its structural components.

analyses or high-resolution mass spectra. All compounds showed single spots by thin-layer chromatography in various systems and when applicable eluted as sharp bands from ion-exchange resins. We also synthesized the spin-label derivative of the reduced coenzyme, N^6-SL-NADH. However, it proved to be too unstable for binding studies. The nitroxyl radical is reduced slowly by the dihydronicotinamide ring. Fig. 3 shows ESR spectra of N^6-SL-NAD^+ free in solution and when bound to lactate dehydrogenase. They are typical for the structural components as well. The spectra of the bound labels are characteristic for highly immobilized species corresponding to a rotational correlation time of about $3 \cdot 10^{-8}$ s [16]. The outer maxima are separated by 63 G. The ESR spectrum of N^6-SL-NAD^+ reveals a considerably reduced tumbling rate as compared to 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl. The ratios of the high-field line to the middle peak are 0.70 and 0.97 respectively. The NAD^+ fragments exhibit intermediate values.

Prior to the ESR titration experiments the enzyme was treated with charcoal to remove any tightly bound nucleotides [17].

Fig. 4 shows Scatchard plots [18] of data obtained for the spin-labelled derivatives of AMP, ADP, ADPR and NAD^+ when ESR titrations as described in the Methods section were carried out. Strict linearity is observed in all cases indicating four independent and identical binding sites in the tetrameric enzyme. Titrations with N^6-SL-AMP were repeated in the presence of nicotinamide mononucleotide. However, any differences in its binding were within experimental error.

When N^6-SL-adenine and N^6-SL-adenosine were added to an enzyme solution the decrease of the ESR signal as compared to that of the same concentration in buffer alone was too small for meaningful titration experiments. An increase in the enzyme concentration does not eliminate this problem because the saturation fraction will get too low for an accurate evaluation of the data [19]. Moreover, at the high concentrations of these analogs necessary for measurable effects considerable non-specific binding may occur. We have therefore employed a different technique. The increase of the ESR signal

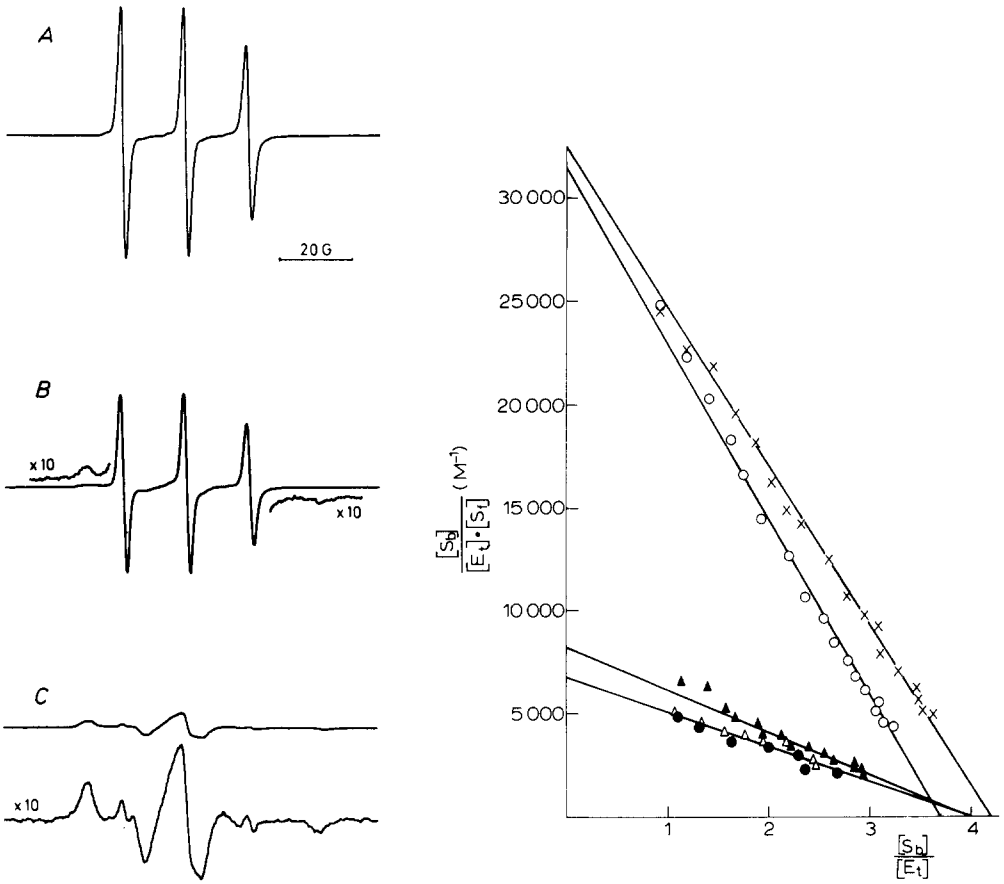

Fig. 3. ESR spectra of $2.3 \cdot 10^{-4}$ M N^6-S-NAD$^+$ in phosphate buffer, pH 7.2, A: in absence of lactate dehydrogenase; B: in presence of $7.2 \cdot 10^{-5}$ M lactate dehydrogenase; C; in presence of $7.2 \cdot 10^{-5}$ M lactate dehydrogenase and $2.1 \cdot 10^{-3}$ M oxalate.

Fig. 4. Scatchard plots of the ESR titration data for the spin-labelled derivatives of NAD$^+$ and its structural components binding to lactate dehydrogenase. ▲, N^6-SL-NAD$^+$, varied from $3.1 \cdot 10^{-4}$ M to $2.1 \cdot 10^{-3}$ M, enzyme concentration: $9.6 \cdot 10^{-5}$ M. X, N^6-SL-ADPR, varied from $1.0 \cdot 10^{-4}$ M to $8.9 \cdot 10^{-4}$ M, enzyme concentration: $7.0 \cdot 10^{-5}$ M. ○, N^6-SL-ADP, varied from $1.1 \cdot 10^{-4}$ M to $9.4 \cdot 10^{-4}$ M, enzyme concentration: $7.7 \cdot 10^{-5}$ M. △, N^6-SL-AMP, varied from $3.5 \cdot 10^{-4}$ M to $1.8 \cdot 10^{-3}$ M, enzyme concentration: $6.5 \cdot 10^{-5}$ M; ●, with addition of $1.1 \cdot 10^{-3}$ M NMN.

TABLE I

Dissociation constants, ± standard error of the mean, of the spin-labelled derivatives of NAD$^+$ and its structural components binding to lactate dehydrogenase.

Compound	K_D
N^6-SL-adenine	$8.6 \pm 1.8 \cdot 10^{-3}$ M
N^6-SL-adenosine	$5.0 \pm 0.9 \cdot 10^{-3}$ M
N^6-SL-AMP	$7.4 \pm 0.4 \cdot 10^{-4}$ M
N^6-SL-ADP	$1.20 \pm 0.02 \cdot 10^{-4}$ M
N^6-SL-ADPR	$1.25 \pm 0.02 \cdot 10^{-4}$ M
N^6-SL-NAD$^+$	$5.6 \pm 0.2 \cdot 10^{-4}$ M

observed upon addition of NAD^+ and oxalate to a solution containing enzyme and N^6-SL-adenine or N^6-SL-adenosine was used to calculate the corresponding dissociation constants assuming four binding sites. Lactate dehydrogenase forms a very tight ternary complex with NAD^+ and the substrate analog oxalate [20]. This complex will displace any adenine or adenosine bound to the active centers.

Table I gives a summary of the various dissociation constants of N^6-SL-NAD^+ and its fragments.

Discussion

A rather spectacular result of the present investigation is the discovery that N^6-SL-ADP and N^6-SL-ADPR are bound more tightly to lactate dehydrogenase than the N^6-SL-coenzyme itself. Before we try to give a possible explanation for these findings let us consider the feasible errors of our method. When one uses structural analogs as a model for the investigation of the properties of natural ligands one has to prove that the altered structure does not cause perturbations in the protein which may lead to erroneous conclusions. In this particular case N^6-SL-NAD^+ appears to be a rather good model of NAD^+ itself. As could be shown previously [6] it behaves kinetically practically the same as NAD^+ in the lactate dehydrogenase system. Not only the Michaelis constants are identical but the maximum velocities are very similar (14 000 as compared to 17 000).

Using the crystallographic data for the dogfish enzyme [21] we built a model which clearly shows that there is ample space for the label at the adenine-binding site of the enzyme. The amino group of the adenine moiety is protruding somewhat out of a hydrophobic pocket.

A different kind of error can result from the ESR measurements itself. When a considerable fraction of the spin-labelled derivative is bound, its broadened absorption will contribute to the signal height of the free label. However, this effect is minimal for the high-field peak [22] which was used for our calculations. Moreover, the percentage of bound spin label never exceeded 63% of the total spin-label concentration during the titration experiments, leaving the contribution from the bound species to the signal height below 1%.

The dissociation constant of $5.6 \cdot 10^{-4}$M for N^6-SL-NAD^+ correlates well with values obtained by Schwert by the ultracentrifugal sedimentation technique [23] and data from fluorescence titrations by displacement of NADH [24]. As shown in Table I the corresponding values for N^6-SL-ADP and N^6-SL-ADPR are smaller by a factor of 5. N^6-SL-AMP, the next less complete structural component is bound almost as tightly as N^6-SL-NAD^+. These results are in marked contrast to data from kinetic measurements, where a continuous increase in the degree of inhibition was observed as the structure of the inhibitors more closely resembled that of the complete coenzyme. We find maximum binding for ADP and ADPR whereas the inhibitor constants of the NAD^+ components are always higher than the Michaelis constant of NAD^+. However, how could an increased affinity of the enzyme for ADP and ADPR with respect to NAD^+ be explained? The reduced coenzyme NADH does bind about 400 times more tightly to lactate dehydrogenase than NAD^+ [12]. This

has been attributed to the repulsion of the positively charged nicotinamide moiety by the positively charged histidine-138 in the nicotinamide binding domain of the enzyme [12]. As revealed by X-ray crystallography there are the following major binding interactions between lactate dehydrogenase and its coenzyme. The adenine binds in a hydrophobic pocket. The adenine ribose is positioned by two hydrogen bonds to its hydroxyls. A charge interaction with the guanidinium group of an arginine which moves 13 Å with respect to its position in the apoenzyme fixes the pyrophosphate. This arginine is part of a loop that folds down over the active center pocket in the ternary complexes. The nicotinamide ribose again forms hydrogen bonds besides some hydrophobic interactions and the nicotinamide ring is positioned by a hydrogen bond of the amide function with a lysine residue thus determining the A-side specifity of the enzyme [12]. ADP, ADPR and their spin-label analogs can undergoe almost all of these interactions but lack the unfavorable positive charge at the pyridine ring. It is therefore quite conceivable that the dissociation constants should be somewhere between the values for NAD^+ and NADH, which we found to be the case: $1.3 \cdot 10^{-4}$M for N^6-SL-ADPR and $1.2 \cdot 10^{-4}$M for N^6-SL-ADP. The small difference in these values is within experimental error of our method.

N^6-SL-AMP indeed is bound considerably less tightly than N^6-SL-ADP and N^6-SL-ADPR. As indicated above, the pyrophosphate forms an ion pair with arginine-101. Its formation has been postulated to be a trigger for the conformational change of the loop upon coenzyme binding. However, one negative charge of the pyrophosphate is compensated by the interaction with arginine-101 while the other is possibly solvated. From our results the nicotinamide phosphate seems to exhibit the charge interaction which would explain a marked decrease in the dissociation constant when going from AMP to ADP.

The binding of N^6-SL-AMP is comparable to N^6-SL-NAD^+. This correlates well with results of Mosbach [25] and Kaplan [26] who have used a Sepharose-linked aminohexyl derivative of AMP for the affinity chromatography of lactate dehydrogenase. This AMP-Sepharose proved to be as effective for the separation of this enzyme as the corresponding NAD^+ derivative.

Addition of nicotinamide mononucleotide does not affect the binding of N^6-SL-AMP. This would seem reasonable if one considers the fact that N^6-SL-NAD^+ itself shows no marked differences in binding to the enzyme as compared to N^6-SL-AMP. In view of the adverse effects caused by the positive charge of the nicotinamide ring one might assume that it is hardly bound at all. The possible charge interaction with arginine-101 could be compensated by the additional negative charge of AMP as compared to the AMP moiety in the dinucleotide. As can be seen from Table I, the binding of N^6-SL-adenine and N^6-SL-adenosine is very weak, thus demonstrating the outstanding importance of the phosphate moieties and their contribution to the total binding energy of NAD^+ to lactate dehydrogenase. For the calculation of the dissociation constants of N^6-SL-adenine and N^6-SL-adenosine four binding sites per tetramer as found for the other analogs had to be assumed. Moreover, since we have determined their binding from displacement experiments with NAD^+/-oxalate, which are known to occupy four binding sites, any adenine or adenosine, possibly binding at additional sites, is not included.

Note Added in Proof (received October 4th, 1976)

Due to the revision of the sequence of pig heart lactate dehydrogenase (Kiltz, H.H., Keil, W., Meyer, H., Poth, E. and Trost, G., I.U.B. Meeting, Hamburg, 1976) the special functions of lysine-250 and histidine-138 are eliminated. A possible reason for the weaker binding of NAD^+ as compared to NADH may be the hydrophobicity of the nicotinamide binding pocket (Rossmann, M.G. and Eventoff, B., private communication) which could also account for our results.

Acknowledgements

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