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ISOLATION AND PROPERTIES OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM A STURGEON FROM THE CASPIAN SEA AND ITS INTERACTION WITH SPIN-LABELED NAD^+ DERIVATIVES *

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Key words: Spin label; NAD^+ derivative; Glyceraldehyde-3-phosphate dehydrogenase; Coenzyme conformation; Cooperativity

Summary

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) was isolated from a sturgeon, *Huso huso*, from the Caspian Sea. It is closely related to the enzyme from a Pacific sturgeon, *Acipenser transmontanus*, with respect to amino acid composition, steady-state kinetics and coenzyme binding. The latter, as studied by means of a spin-labeled derivative of NAD^+, is negatively cooperative exhibiting a Hill coefficient of 0.84 at 12°C.

Two derivatives of NAD^+ spin-labeled at N^6 or C_8 of the adenine ring were found to be active coenzymes with maximum velocities reaching 35 or 45% of the value for NAD^+ itself.

When more than two equivalents of either spin-labeled NAD^+ are bound to the enzyme spin-spin interactions are observed in the ESR spectra. Distances between the nitroxide radicals (8—9 Å) calculated from the observed splittings are in excellent agreement with data predicted from the crystal structure of the lobster enzyme when the coenzyme is bound in an anti-conformation of the adenine moiety about the glycosidic bond to all four subunits.

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* Dedicated to Professor Dr. Gerhard Pfleiderer on the occasion of his sixtieth birthday.

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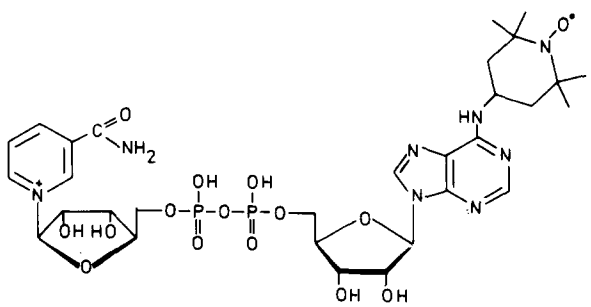
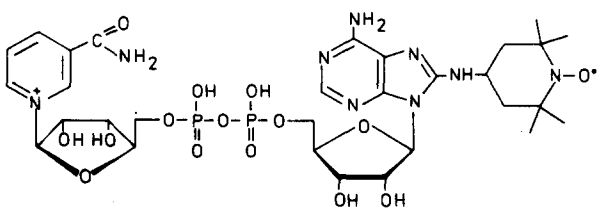
Abbreviations: N^6-SL and C_8-SL preceding NAD^+ refer to nicotinamide-purine-dinucleotide substituted at C_6 or C_8 with a 4-(2,2,6,6-tetramethyl-piperidinyl-1-oxyl)-amino group; ADPR, adenosine diphosphoribose; ϵ-NAD^+ and ϵ-ADPR refer to the N_1,N^6-etheno derivatives of these compounds; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid.

Introduction

Glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in glycolysis, has found wide interest because of cooperative phenomena between its four chemically identical subunits. Whereas, binding of the coenzyme NAD^+ to the yeast enzyme is positively cooperative, glyceraldehyde-3-phosphate dehydrogenases isolated from muscle of various species exhibit negative cooperativity. Two coenzyme binding sites each are in close association related by the *R* 2-fold molecular axis. The adenine binding pockets are opposite to one another, whereas the nicotinamide rings and thus, the catalytic sites are quite distant from one another, because NAD is bound in an open conformation [1]. The molecular mechanism by which information is transmitted from one subunit upon binding of the coenzyme to the adjacent active center is not yet fully understood in spite of extensive crystallographic and spectroscopic studies [2,3]. The close proximity between two adenine sites would suggest that the adenine ring of the coenzyme induces the conformational changes leading to cooperativity. However, modification of only two out of the four active center cystein residues with certain reagents leads to complete loss of activity [4]. This indicates that conformational changes at the catalytic site, can also be transmitted into the adjacent subunit. Spectroscopic evidence supporting either model has been provided [5,6]. By fluorescence life-time studies of ϵ-NAD^+ and ϵ-ADPR in complexes with the enzyme Gafni [7] has shown that the nicotinamide ring is required for charge transfer interactions of the adenine ring with aromatic residues in its binding pocket.



N^6-SL-NAD^+



C-8-SL-NAD^+

Fig. 1.

Such questions could also be answered by means of spin-labeled NAD^+ derivatives which we introduced [8,9] (Fig. 1). Conformational changes in the environment of the nitroxide radicals would be reflected either in their conventional or saturation transfer ESR spectra, depending on the degree of immobilization [10,11].

Since coenzyme binding studies with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, which has been used in most investigations, are hampered by the extremely tight binding of NAD^+ to the first two sites and even more so by the high instability of the apoenzyme, Seydoux and coworkers [12] have applied the enzyme from a sturgeon, *A. transmontanus*, to investigate such interactions. Besides increased stability, NAD^+ binding is not quite as tight, whereas the cooperative effects are retained. Because of difficulties in obtaining alive or freshly frozen *A. transmontanus* we have isolated glyceraldehyde-3-phosphate dehydrogenase from a sturgeon, *H. huso* from the Caspian Sea. In this paper we describe its isolation and properties and its interactions with N^6 and C_8-SL-NAD^+.

Materials and Methods

Materials

N^6 and C_8-SL-NAD^+ were prepared as described previously [8,9]. However, C_8-SL-AMP, an intermediate in the synthesis of C_8-SL-NAD^+ was synthesized from 8-bromo-AMP in 68% yield by treatment with a 13-fold excess of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl in water, pH 11.0, for 13 h at 100°C. After adjustment to pH 7.0 the reaction product was purified by anion exchange chromatography on DEAE-Sephadex A-25 with a linear gradient of water against 0.9 M pyridine and 0.6 M acetic acid. Coenzymes and substrates were purchased from Boehringer, Mannheim. Glyceraldehyde-3-phosphate was prepared prior to use from the corresponding diethylacetal.

Isolation and purification of sturgeon muscle glyceraldehyde-3-phosphate dehydrogenase

Frozen sturgeon (*H. huso*) muscle (1 kg, white meat only) was homogenized in 2 vol. standard buffer 5 mM EDTA/1.5 mM mercaptoethanol (pH 7.0) for 3-times 10 s. Further steps were carried out at 4° C. The homogenate was stirred for 1 h, centrifuged at 23 000 × *g* for 40 min and filtered through glass wool.

Step 1: $(NH_4)_2SO_4$ precipitation. The precipitate forming at 70—100% satn. $(NH_4)_2SO_4$ was prepared and desalted, to yield 3.8 g with a specific activity of 120 U/mg.

Step 2: Cation exchange chromatography. The desalted enzyme (9.6 mg/ml) was loaded onto a CM-52 cellulose (Whatman) column (4 × 23 cm) equilibrated with standard buffer. The column was eluted with 2 l buffer, followed by a linear gradient of 1 l each of buffer and buffer/140 mM KCl. Active enzyme was eluted in buffer corresponding to approx. 60 mM KCl. The protein was desalted by chromatography on Sephadex G-50 and rechromatographed on the same CM-52 cellulose column, using a gradient of 0—60 mM KCl in buffer. The enzyme was eluted at 40 mM KCl (800 mg, 300 U/mg). The solution was concentrated by pressure dialysis (Berghof membranes, BM 500) and finally

slowly precipitated by dialysis against a gradient of $(NH_4)_2SO_4$ (0—100%) [13]. It was stored in this suspension at 4°C and dialysed against the appropriate buffer prior to use.

Protein determination and enzyme assay

Concentration of the apoenzyme was determined spectrophotometrically at 280 nm using a factor of 0.95 for solutions containing 1 mg/ml. This factor is based on a protein determination by quantitative amino acid analysis. The assay was carried out at 25°C by following the formation of NADH at 366 nm in 1 ml of 200 mM triethanolamine buffer, pH 8.9/1 mM EDTA/1.5 mM mercaptoethanol. The reaction was started by addition of 0.1 μg enzyme to the assay mixture containing 2.2 mM glyceraldehyde-3-phosphate/20 mM phosphate/2 mM NAD^+. All enzyme concentrations are based on a molecular weight of 144 000 for the tetramer.

Steady-state kinetic measurements. These were carried out at 25°C in 1 ml of 200 mM triethanolamine buffer, pH 8.9/1 mM EDTA/1.5 mM mercaptoethanol by following the formation of NADH or SL-NADH at 366 nm (ϵ_{366} = 3300). The reactions were started by addition of 80 ng enzyme to the following substrate concentrations: glyceraldehyde-3-phosphate from 0.1—4.4 mM at 10 mM phosphate and 1.84 mM NAD^+; phosphate from 1—200 mM at 2.2 mM glyceraldehyde-3-phosphate and 2.24 mM NAD^+; NAD^+ from 0.1—18 mM at 20 mM phosphate and 2.2 mM glyceraldehyde-3-phosphate.

Determination of the kinetic constants for the spin-labeled derivatives of NAD^+ was carried out in a total of 0.5 ml of the same buffer at 20 mM phosphate and 2.4 mM glyceraldehyde-3-phosphate. The reactions were started by addition of 40 ng enzyme. C_8-SL-NAD^+ was varied from 30—100 μM and N^6-SL-NAD^+ from 22—232 μM. Values for NAD^+ itself were redetermined with the same enzyme preparation and substrate solutions. It was varied from 20—700 μM.

Determination of NAD^+ bound to glyceraldehyde-3-phosphate dehydrogenase

After addition of 0.5 ml of 10 N NaOH to 0.5 ml enzyme solution (1.6 mg/ml) the mixture was kept at 100°C for 5 min. After it had cooled down 7 ml water were added. Fluorescence spectra were recorded at an emission wavelength of 455 upon excitation at 370 nm. The amount of NAD^+ was calculated by comparison with solutions of known NAD^+ concentration after correction for the blank value of buffer alone [14].

Electrophoreses. SDS -electrophoreses were carried out according to Lämmli [15] in 7.5 and 10% polyacrylamide gels. Low voltage electrophoreses were carried out on 25.5 × 145 mm cellulose acetate strips from Macherey and Nagel, Düren, at 250 V in either 50 mM phosphate buffer, pH 7.0/5 mM EDTA/1 mM mercaptoethanol or 30 mM barbiturate buffer, pH 8.6/5 mM EDTA/1 mM mercaptoethanol. Within 1 h the enzyme migrated 1.4 or 0.7 cm towards the cathode. For electrophoreses on agarose plates (6 × 6 cm) again a voltage of 250 V was applied. The 30 mM barbiturate buffer, pH 7.3/contained 1 mM EDTA/1 mM mercaptoethanol. For protein specific staining Coomassie brillant blue, R-250 (Serva) was applied in all cases.

Isoelectric focusing on Ampholine PAG-plates from LKB was carried out for 1 h over a pH gradient from 3.5—9.5. The stained protein band was cut out,

diluted with water and the pH value determined with a pH-meter.

Amino acid analyses. Analyses were carried out with a Biotronic LC 6000 amino acid analyser according to Moore et al. [16] after 24 and 72 h acid hydrolysis.

ESR experiments. These were performed with a Bruker B-ER 420 spectrometer operating in the X-band mode equipped with a Bruker B-ST 100/700 X variable temperature controller. 100 kHz modulation with amplitudes of 0.8 G and microwave powers from 6—20 mW were routinely applied. All experiments were carried out in a total volume of about 60—70 μl in micro flat cells equipped with a special Teflon adapter in order to minimize changes in the signal amplitude (below 1%) because of slightly different orientations of the cell when repositioned in the cavity [17]. Syringes with platinum needles and Teflon-tipped plungers were used for addition of the spin-labeled coenzymes to avoid reduction of the free radical by stainless steel. For the determination of the binding constant of C_8-SL-NAD^+ to glyceraldehyde-3-phosphate dehydrogenase the amplitudes of the high field peaks in solutions containing the enzyme were compared with those of solutions containing an equal amount of buffer (5 mM EDTA, pH 7.0, degassed under vacuum and saturated with argon). Since at the low protein concentrations required for determination of the binding constant the enzyme in the absence of thiols, which would reduce the spin-label, is rather unstable, all experiments were carried out in an atmosphere of argon and fresh enzyme solutions were used for every point of the titration curve. C_8-SL-NAD^+ was varied from 0.8—8 μM at 0.73 μM enzyme. Concentrations for ESR spectra of bound spin-label coenzymes are given in the legends to the figures.

Results

For the preparation of glyceraldehyde-3-phosphate dehydrogenase from sturgeon from the Caspian Sea (*H. huso*), the procedure as described for a species from the North Pacific coast (*A. transmontanus*) was closely followed [12]. However, anion exchange chromatography could be omitted without affecting the final homogeneity of the preparation. Whereas the *A. transmontanus* enzyme is eluted in three active bands with KCl from CM-cellulose, only one active band was observed in our case. It was well separated from hemoglobin, a major contaminant of glyceraldehyde-3-phosphate dehydrogenase. The enzyme after this cation exchange chromatography was practically coenzyme free as determined fluorimetrically (about 0.01 mol NAD^+/mol subunit and a A_{280}/A_{260} ratio of 2.1 [14,12]. In contrast to mammalian muscle enzymes which retain most of the coenzyme unless treated with charcoal [12], the major band III from *A. transmontanus* is eluted as apoenzyme as well.

Our preparation exhibiting a specific activity of 320 U/mg after rechromatography from CM-cellulose was homogeneous as shown by preparative anion exchange chromatography on DEAE-Sephadex A-25, affinity chromatography on Blue Sepharose with NAD^+ or NaCl as eluent [19] and by gel chromatography on Sephadex S-300 in presence of 0.1 and 0.5 M NaCl to suppress any hydrophobic interactions. Electrophoreses in SDS-polyacrylamide gels, on

cellulose acetate strips (pH 7.0 and 8.6) and agarose plates (pH 7.3) yielded single bands upon protein specific staining. The amino acid composition (Table I) indicates high homology with the *A. transmontanus* enzyme. However, a somewhat higher content of basic amino acids may well explain the fairly high isoelectric point of 9.1 as determined by isoelectric focusing. Differences in the values for most hydrophobic amino acids are probably due to the fact that 72 h acid hydrolyses were carried out as compared to 48 h, as performed by Allison and Kaplan [18].

Even as apoenzyme the preparation is extremely stable. In concentrated solutions (10 mg protein/ml) at 8°C less than 10% loss of activity was observed over a period of 5 months.

Steady-state kinetics

The assay in the direction from NAD^+ to NADH when carried out according to Ferdinand [20] yielded specific activities below 220 U/mg. Whereas, NAD^+ and glyceraldehyde-3-phosphate exhibit normal Michaelis-Menten kinetics (NAD^+: K_m = 97 μM; glyceraldehyde-3-phosphate: K_m = 230 μM), a pronounced substrate inhibition by phosphate (Fig. 2) above 20 mM was observed. Therefore, the phosphate concentration as applied by Ferdinand had to be reduced to this value. Under these conditions specific activities reached 320 U/mg.

The spin-labeled NAD^+ analogs, C_8-SL-NAD^+ and N^6-SL-NAD^+ were shown to be active coenzymes, exhibiting fairly high maximum velocities (Table 2) of 45% or 35%, respectively, of the values for the natural system.

Binding studies with spin-labeled NAD^+ derivatives

Figs. 3 and 4 show ESR spectra of C_8 and N^6-SL-NAD^+ in binary complexes with the enzyme at a coenzyme/enzyme ratio of about 1 per tetramer. Some residual free signal results from a contamination with the enzymatically inactive diastereomer with an α-glycosidic linkage of the nicotinamide ring to its ribose. The bound spectra are both typical for a highly immobilized nitroxide radical [21]. The separation of 62.5 G, as observed for the C_8 analog

TABLE I

COMPARISON OF AMINO ACID COMPOSITION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES FROM TWO STURGEONS, *HUSO HUSO* AND *ACIPENSER TRANSMONTANUS* [18]

Values from Ref. 18 were recalculated for a molecular weight of 144 000, instead of 120 000 as originally assumed.

	H. huso	*A. transmontanus*		*H. huso*	*A. transmontanus*
Asx	38	39	Ile	18	20
Thr	21	21	Leu	23	19
Ser	19	18	Tyr	11	11
Glx	20	20	Phe	15	14
Pro	12	12	His	7	6
Gly	31	32	Lys	31	29
Ala	36	34	Arg	12	11
Val	39	36	Cys	—	—
Met	—	9	Trp	—	4

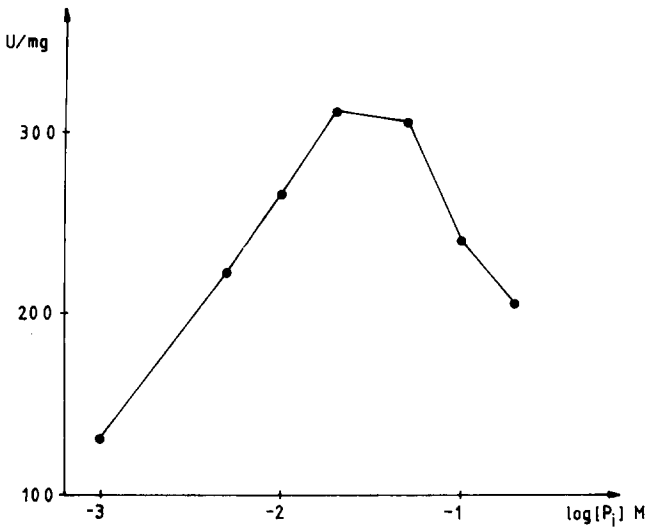


Fig. 2. Specific activity of sturgeon glyceraldehyde-3-phosphate dehydrogenase depending on the phosphate concentration.

corresponds to a rotational correlation time τ_r of about $1 \cdot 10^{-8}$ s, whereas 68.7 G in case of the N^6 derivative corresponds to $8 \cdot 10^{-8}$ s, when calculated according to Eqn. 1 for Brownian diffusion [22]. 2 A_z is the separation of the outer maxima of spectra in the rigid powder limit (71 G as determined at —15°C in buffer with lactate dehydrogenase substituting for glyceraldehyde-3-phosphate dehydrogenase under conditions where very little SL-NAD^+ is bound) and 2 A'_z is the corresponding value in solution.

$$\tau_r = 5.4 \cdot 10^{-10} (1 - A'_z/A_z)^{-1.36} \quad (1)$$

Based on the Stokes radius, of glyceraldehyde-3-phosphate dehydrogenase from lobster, of 41 Å a correlation time of $9 \cdot 10^{-8}$ s may be calculated for the enzyme itself. Thus, the spin-label in C_8-SL-NAD^+ when bound to the enzyme exhibits some mobility relatively to the protein.

The bound spectra of the spin-labeled cofactors (Figs. 3 and 4) change dramatically when more than two equivalents of SL-NAD^+ are added to the enzyme. Two additional peaks exhibiting a separation of 88.6 G (C_8-SL-NAD^+) or 75.2 G (N^6-SL-NAD^+) are formed, reaching their maximum amplitude at a coenzyme/enzyme ratio somewhat above 4 per tetramer (Figs. 5 and 6).

TABLE II

KINETIC CONSTANTS FOR NAD^+ AND ITS SPIN-LABELED ANALOGS N^6 AND C_8-SL-NAD^+ WITH GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM *HUSO HUSO*

	V (U/mg)	K_m (μM)
NAD^+	320	97
C_8-SL-NAD^+	144	61
N^6-SL-NAD^+	111	200

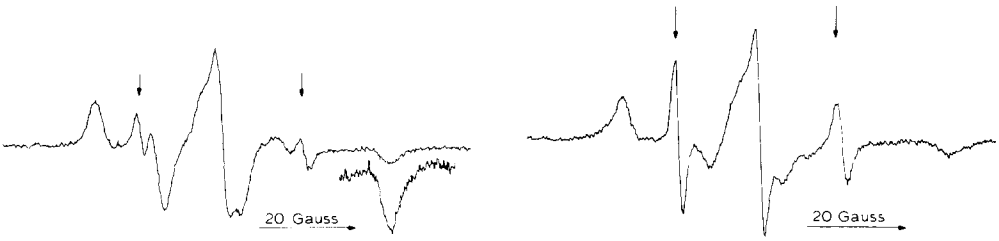


Fig. 3. ESR spectrum of 60 μM C_8-SL-NAD^+ in a binary complex with 60 μM sturgeon glyceraldehyde-3-phosphate dehydrogenase in 5 mM EDTA buffer, pH 7.0, at 10° C. The arrows indicate the high and low field contributions from free C_8-SL-NAD^+.

Fig. 4. ESR spectrum of 43 μM N^6-SL-NAD^+ in a binary complex with 43 μM sturgeon glyceraldehyde-3-phosphate dehydrogenase in 5 mM EDTA/3 mM HEPES buffer, pH 7.0, at 10° C. The arrows indicate high and low field contributions from free N^6-SL-NAD^+.

Changes in line shap are observed in the center parts of the spectra, too. However, they are partially buried under the increasing free signal. In presence of two equivalents of NAD^+ itself the addition of two equivalents of either spin-labeled cofactor gives rise to the normal bound spectra, only. However, at high excess (10-fold) of C_8 or N^6-SL-NAD^+ the additional peaks begin to show up again.

In order to study coenzyme binding a titration of the enzyme with the more active C_8SL-NAD^+ was carried out at 12°C. The amplitude of the high field signal of the free cofactor in equilibrium was used as a measure for binding (Fig. 7). In the concentration range studied the contribution from bound C_8-SL-NAD^+ to the amplitude of this peak was negligible. Since a Scatchard plot (Fig. 7) [23] is non-linear, data are also presented as a Hill plot [24] (Fig. 8). Calculation of the saturation fraction according to Hill was based on four binding sites per tetramer as derived from the direct plot by extrapolation

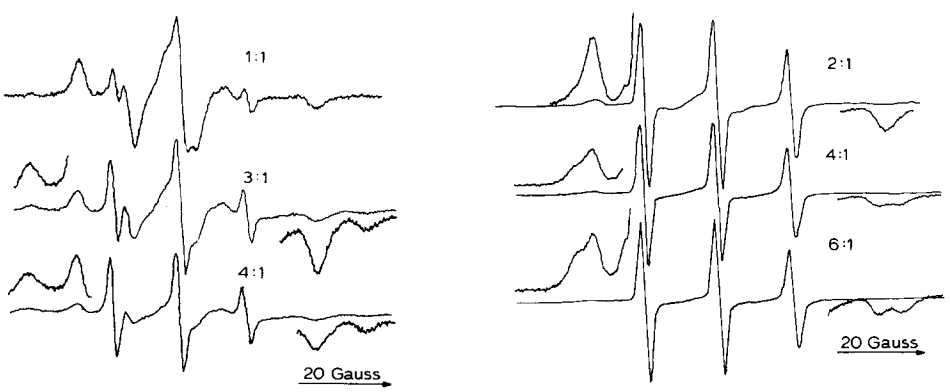


Fig. 5. ESR spectra of C_8-SL-NAD^+ in binary complexes with 54—60 μM sturgeon glyceraldehyde-3-phosphate dehydrogenase at the molar ratios per tetramer given in the fig in 5 mM EDTA buffer, pH 7.0, at 10° C.

Fig. 6. ESR spectra of N^6-SL-NAD^+ in binary complexes with 48—60 μM sturgeon glyceraldehyde-3-phosphate dehydrogenase at the molar ratios per tetramer given in the figure in 5 mM EDTA buffer, pH 7.0, at 10° C.

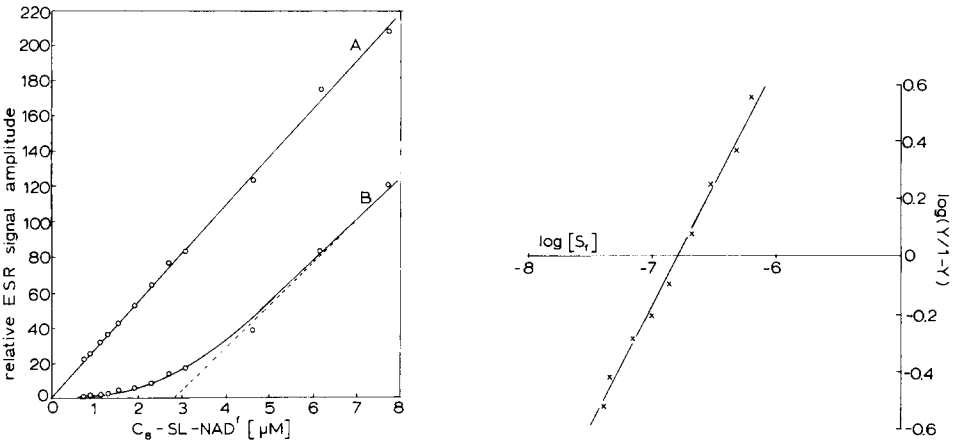


Fig. 7. ESR titration data for C_8-SL-NAD$^+$ (A) binding to 0.73 μM glyceraldehyde-3-phosphate dehydrogenase (B) at 12°C in 5 mM EDTA buffer pH 7.0. Curve B is calculated for n_H = 0.84 and K_d 0.16 μM as derived from a Hill plot (Fig. 7).

Fig. 8. Hill plot of ESR titration data for C_8-SL-NAD$^+$ binding to glyceraldehyde-3-phosphate dehydrogenase at 12°C in 5 mM EDTA buffer, pH 7.0.

(2.82 μM bound C_8-SL-NAD$^+$ at 0.73 μM enzyme) (Fig. 7). The Hill coefficient of 0.84 (apparent K_d 0.16 μM) is characteristic for moderate negative cooperativity as has been observed fluorimetrically for NAD$^+$ with the *A. transmontanus* enzyme at comparable temperature [25].

Discussion

The glyceraldehyde-3-phosphate dehydrogenase from two *Acipenserinae*, *A. transmontanus* and *H. huso* obviously are highly homologous. The isolation procedure as described for *A. transmontanus* could closely be followed yielding an *H. huso* enzyme of almost identical specific activity (320 U/mg as compared to 330 U/mg) and, again, practically free of coenzyme. In these aspects, particularly the high activity, both enzymes differ considerably from glyceraldehyde-3-phosphate dehydrogenases from mammalian species [1]. However, most important for our investigations is their high stability in solution as apoenzyme, the dehydrogenase from *H. huso* being even more stable.

The crucial question inherent in the application of any reporter group is the extent to which this group, e.g., the spin-label, causes perturbations of the protein thus altering its normal behavior. For conclusions to be drawn from data obtained with structural analogs of natural ligands one has to establish that these effects are negligibly small. As has been shown previously for lactate dehydrogenases [26,27] and glutamate dehydrogenase [28] our spin-labeled coenzyme derivatives, N^6 and C_8-SL-NAD$^+$ appear to be appropriate models for NAD$^+$ itself. Not only could they be shown to be active coenzymes but the kinetic constants do not differ drastically from values for the natural system. Additional evidence stems from the equilibrium binding data of C_8-SL-NAD$^+$. Coenzyme binding to *A. transmontanus* glyceraldehyde-3-phosphate dehydrogenase is highly temperature dependent, most pronounced in the degree of

negative cooperativity. Hill coefficients vary from 0.76 at 10°C to virtually 1.0 at 35°C [25]. In this investigation a temperature of 12°C was chosen which could approach the actual temperature in the natural environment of *H. huso*. The Hill coefficient of 0.84 for C_8-SL-NAD^+ binding at this temperature with an apparent K_d of 0.16 μM is in excellent agreement with data for *A. transmontanus* ($n_H = 0.82$, $K_d = 0.15$ μM at 16°C). Thus, the spin-label clearly does not interfere with coenzyme binding and the important subunit interaction. This result also demonstrates at the same time the similarity between the two glyceraldehyde-3-phosphate dehydrogenases from different *Acipenserinae*.

Structural models of N^6 and C_8-SL-NAD^+ when built into the model of holo-glyceraldehyde-3-phosphate dehydrogenase from lobster, as obtained by X-ray crystallography (Rossmann, M.G. and Grau, U., personal communication), would suggest that for sterical reasons the adenine ring has to be in the *anti*-conformation with respect to its ribose. This is the predominant conformation of N^6-SL-NAD^+ in solution, however, C_8-SL-NAD^+ has been shown to exhibit *syn* conformation [9]. The tetramethyl piperidine ring in C_8-SL-NAD^+ would then be fairly unrestricted, rotation about the C-N bond between the adenine ring and the label should be possible. In contrast, the spin-label in the N^6 derivative would be highly restricted due to close steric interaction, particularly with phenylalanine-34. This is in excellent agreement with the rotational correlation times derived from the ESR spectra of the bound analogs, which allow for considerable mobility of the spin-label relative to the enzyme, in the case of the C_8 analog only.

From the model studies, however, an additional important effect may be predicted. Glyceraldehyde-3-phosphate dehydrogenase is a dimer of dimers with two pairs each of coenzyme binding domains in which the adenine moieties of two NAD^+ molecules are in close association related by the *R* 2-fold molecular axis [2].

The first 2 mol NAD^+ combine with non-adjacent sites of high affinity, before, at higher excess of the cofactor, the neighboring, interacting sites are saturated. When the spin-labeled cofactors are indeed bound in the *anti* conformation, the nitroxide radicals of two SL-NAD^+ molecules bound to adjacent subunits should be separated from one another by about 8 Å only, thus allowing for spin-spin interactions to be observed. Indeed, two additional peaks in the ESR spectra of bound N^6 and C_8-SL-NAD^+ show up when more than 2 mol of the coenzyme analogs are added to the enzyme, unless the tight sites have previously been saturated with unlabeled NAD^+. Since electron-nuclear interactions cannot account for the large separations observed between these new bands (88.6 G for C_8-SL-NAD^+), in a highly simplified model [29] they may be attributed to the stronger electron-electron interaction. Such a dipolar splitting will lead to considerable doublet separations. The largest splitting, *2D* defines the *z* axis of the dinitroxide. The intramolecular distance between the two N-O groups may then be calculated according to:

$$r = ((5.56 \cdot 10^4)/2D)^{1/3} \qquad (2)$$

This model of course applies to biradicals only. However, the orientation relative to one another of the nitroxide groups of two spin-labeled coenzymes bound to the enzyme does not change upon the molecular motion of the

protein and should be almost independent of the rotation of the spin-label about the C-N bond between the adenine ring and the label. This rotation, however, is the only possible mobility of the spin-label relative to the enzyme as revealed by the molecular model studies.

According to Eqn. 2 N-O/N-O distances of 8.6 Å for C_8-SL-NAD^+ and 9.0 Å for N^6-SL-NAD^+ can be calculated, which are in excellent agreement with the values predicted from the structure of lobster glyceraldehyde-3-phosphate dehydrogenase, when the coenzyme analogs are bound in the *anti* conformation. Several reports in the literature have led to the assumption that in contrast to glyceraldehyde-3-phosphate dehydrogenase from mammals the enzyme from *A. transmontanus* binds the coenzyme with the adenosine moiety in *syn* conformation. ϵ-NAD^+ which in solution exhibits *anti* conformation, is a rather poor coenzyme of the sturgeon enzyme but is highly active in the case of rabbit [6]. In contrast, 8-bromo-NAD^+, which in solution exhibits *syn* conformation is an excellent coenzyme analog for the coenzymes from sturgeon and lobster [30]. The X-ray structure of lobster holoenzyme at 2.9 Å resolution was interpreted as *anti* conformation of the adenine about the glycosidic bond in the so-called red and yellow subunits but did allow for *syn* conformation in the green and blue subunits [2]. Our current data would suggest that the coenzyme is bound in an open *anti* conformation to all four subunits. Thus, the interactions between the adenine ring and amino acid residues in its binding pocket likely to be required for cooperativity could be identical in adjacent active centers.

The observed large outer peak separations due to spin-spin interactions are very sensitive to small conformational changes, i.e., changes in the distances of several tenths of an Å, only, between the two nitroxide radicals as can be seen from Eqn. 2. Consequently, they provide an excellent means for studying conformational changes in the adenine moiety of the coenzyme due to alterations at the catalytic site. This would help to answer the question about the role of the nicotinamide portion of NAD^+ in the subunit interactions leading to negative cooperativity. Such an investigation applying spin-labeled derivatives of the structural components of NAD^+ and NADH [17] has been carried out for both the sturgeon enzyme and glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle in collaboration with Jane H. Park et al. at Vanderbilt University and will be the subject of a following paper.

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