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# SOLUTION CONFORMATION OF LACTATE DEHYDROGENASE AS STUDIED BY SATURATION TRANSFER ESR SPECTROSCOPY *

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## Summary

Several binary and ternary inhibitor and 'dead end' complexes of pig heart lactate dehydrogenase (L-lactate:$NAD^+$ oxidoreductase, EC 1.1.1.27) were studied by saturation transfer ESR spectroscopy by means of an active NAD analog, spin-labeled at $N^6$. The mobility of the spin-label depends on the nature of small molecules bound at the remote catalytic end of the coenzyme. The spin-label was found to serve as a reporter group monitoring the conformation of the peptide loop that is folded down over the active cleft in crystals of ternary complexes. The data suggest a fluctuation of the loop between open and closed forms in solution.

The structure of the inhibitor molecules has been correlated with their ability to stabilize a more closed conformation of the loop.

## Introduction

A spin-labeled derivative of $NAD^+$ shown in Fig. 1, which we introduced [1], turned out to be rather useful to study the interaction of lactate dehydrogenase with its coenzymes and substrates [2,3,4]. The spin-label is attached to the exocyclic amino function of the adenine ring which is known to protrude somewhat out of the hydrophobic adenine binding pocket of this enzyme into

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Abbreviation: $N^6$-SL preceding NAD, $NAD^+$ and NADH refers to the $N^6$-(2,2,6,6-tetramethylpiperidine-4-yl-1-oxyl) derivatives of these compounds.

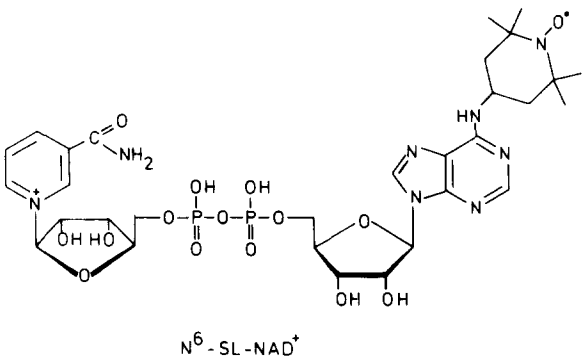


Fig. 1. Structural formula of $N^6$-SL-$NAD^+$.

the solution. Thus, the label causes little perturbation of the native protein structure as corroborated by the kinetic constants of the analog which hardly differ from those of $NAD^+$ itself.

The subject of the preceding paper has been the formation of ternary inhibitor complexes of lactate dehydrogenase as studied by ESR spectroscopy [5]. Considerable information about the mechanism of this enzyme has come from inhibitor studies, particularly with the substrate analogs oxalate and oxamate in presence of $NAD^+$ or NADH, respectively, and a dead end NAD-pyruvate complex. Another long known ternary complex is formed from lactate dehydrogenase, oxidized coenzyme and sulfite. For a comprehensive review see Holbrook et al. [6]. Binding data with $N^6$-SL-NAD have been in excellent agreement with results obtained by other techniques. An interesting finding of that investigation have been differences observed in the line shapes of the ESR signal of the bound spin-labeled coenzyme in different complexes, i.e., in the separation of the outer maxima. Although NAD is fixed to the enzyme in an open conformation, the spin-label witnesses changes at the remote end of $N^6$-SL-NAD when distinct small molecules are bound.

The interpretation, however, of such data is hampered by a principal limitation of conventional ESR spectroscopy. At rotation correlation times $\tau_R$ above $5 \cdot 10^{-8}$ s, typically encoutered when the label exhibits little motional freedom relatively to macromolecules of this size (tight binding), the spectrum approaches the rigid powder limit and becomes insensitive to a further decrease in the label's mobility [7]. Thus, mobility changes as possibly associated with conformational changes of the protein can not be observed. Moreover, even when changes in the separation of the outer maxima occur they may be due to polarity effects in the label's environment rather than to motion.

With the work of Dalton, Hyde and Thomas the spin-labeling technique has recently considerably been improved by extending the range of motions that can be studied by four orders of magnitude to times as long as $10^{-3}$ s [8,9]. This new technique, referred to as saturation-transfer spectroscopy, depends on the spectral diffusion of saturation of the sample in an intense microwave field. The spectral diffusion itself arises from rotational diffusion which must be comparable to spin-lattice relaxation $T_1$. Since $T_1$ for nitroxyl radical spin-

labels is in the order of $10^{-5}$ s, the rotation correlation time $\tau_R$ should be between $10^{-3}$ and $10^{-7}$ s, according to the following:

$$100\ T_1 > \tau_R > 0.01\ T_1$$

In this range considerable changes in line shape with $\tau_R$ may be observed which are well resolved from polarity effects. Spectra are usually recorded of first harmonic dispersion or second harmonic absorption signals, out of phase.

We now have applied this method to study the mobility of the spin-label in $N^6$-SL-NAD when bound to pig heart lactate dehydrogenase in various complexes.

## Materials and Methods

$N^6$-SL-$NAD^+$ was prepared as previously described [1]. Its concentration was determined by enzymic reduction with alcohol dehydrogenase to the corresponding NADH derivative ($\epsilon_{340} = 6300$). The same enzymic reaction was performed in the ESR flat cell to prepare $N^6$-SL-NADH [10]. $NAD^+$ was purchased from Boehringer, Mannheim, and was used without further purification. Lactate dehydrogenase (EC 1.1.1.27) was obtained from Boehringer, Mannheim, as well but was further purified by affinity chromatography on an oxamate column [11] and subsequently by gel chromatography on Sephadex G-50 containing 5 mg finely powdered charcoal/ml gel to remove any tightly bound nucleotides [12]. After final chromatography on Sephadex S-200 [13,14] the protein was precipitated by ammonium sulfate. It was freshly dialysed against 67 mM phosphate buffer, pH 7.2, and centrifuged at 40 000 × *g* shortly before use.

*Enzyme assays*

Standard procedures were used for determination of enzymatic activity [15]. The protein concentration was determined spectrophotometrically at 280 nm using a factor of 1.34 for a solution containing 1 mg/ml of enzyme (420 U/mg). The same factor was found to apply to solutions in glycerol/buffer mixtures.

*Disc electrophoresis* were carried out according to the original procedure of Tamura and Ui [16] (7.5% gel). The stock solutions as defined in his paper had the following glycerol concentrations by volume: A, 60%; B, 60%; C, 10%; D, 27%; E, 85%; F, 60% (no sucrose added). Ammonium persulfate was dissolved in 85% aqueous glycerol.

*Viscosity measurements* were carried out with a rotation viscometer, Low Shear 30 from Contraves AG, Zürich, equipped with a Rheoscan 30 from the same company. Enzyme solutions in glycerol/buffer mixtures were obtained by dialysis of the solution in phosphate buffer against the appropriate mixtures.

*ESR measurements*

ESR spectra were recorded with a Bruker B-ER 420 spectrometer operating in the X-band mode. For first harmonic in phase absorption spectra 100 kHz modulation with amplitudes of 0.8 G and 5—20 mW microwave power was

routinely applied. Second harmonic out of phase absorption signals (50 kHz field modulation and 100 kHz signal detection) were obtained at 5.0 G modulation amplitude and about 90 mW microwave power. Sample temperature was controlled by means of a Bruker B-ST 100/700 variable temperature unit. The cavity itself was kept at 20°C by passing a stream of dry nitrogen through the radiation slots. Micro flat cells equipped with a special teflon adapter, as previously described [2], were used in order to ensure identical orientation of cell relatively to the magnetic field for all experiments. Concentrations are given in the legends to the figures.

## Results

In Fig. 2 is shown a conventional first harmonic ESR spectrum typical for $N^6$-SL-NAD bound to lactate dehydrogenase in a ternary inhibitor complex. The residual signal (high and low field components) of free label accounting for about 1% of the total coenzyme concentration is indicated with arrows. It results primarily from a contamination of $N^6$-SL-NAD$^+$ with the enzymatically inactive $\alpha$-diastereomer regarding the nicotinamide ribose. Computer simulations of the line shape according to the method of Freed yield a rational correlation time of about $1.5 \cdot 10^{-8}$ s [17] based on 71 G separation of the outer maxima as observed for a frozen solution. As outlined in the introduction optimal saturation transfer spectra are to be expected for correlation times near $10^{-5}$ s, $10^{-7}$ s being an upper limit. Moreover, spectra also depend on the frequency of the $H_0$ field modulation, being most sensitive to rotational diffusion when $1/\tau_R$ equals this frequency. As described in Methods, the highest frequency attainable with our spectrometer of 50 kilocycles corresponds to $\tau_R = 2 \cdot 10^{-5}$ s. Saturation transfer spectra of the various complexes were therefore recorded in highly viscous aqueous glycerol at 4°C in order to reduce the tumbling rate of the enzyme. Ethylene glycol is known to cause dissociation of lactate dehydrogenase [18]. However, even in highly diluted

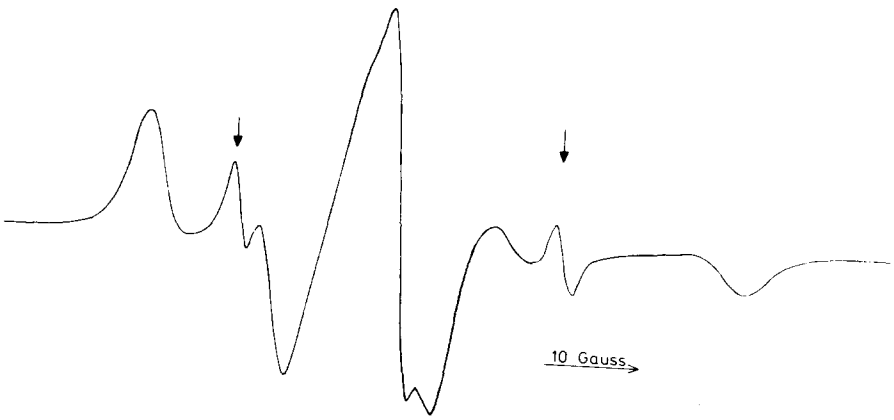


Fig. 2. Conventional ESR spectrum of 17 $\mu$M $N^6$-SL-NAD$^+$ in a ternary complex with 18 $\mu$M lactate dehydrogenase and 4.5 mM oxalate in 67 mM phosphate buffer at 25°C. The arrows indicate the high and low field contributions from free $N^6$-SL-NAD$^+$ (about 1%).

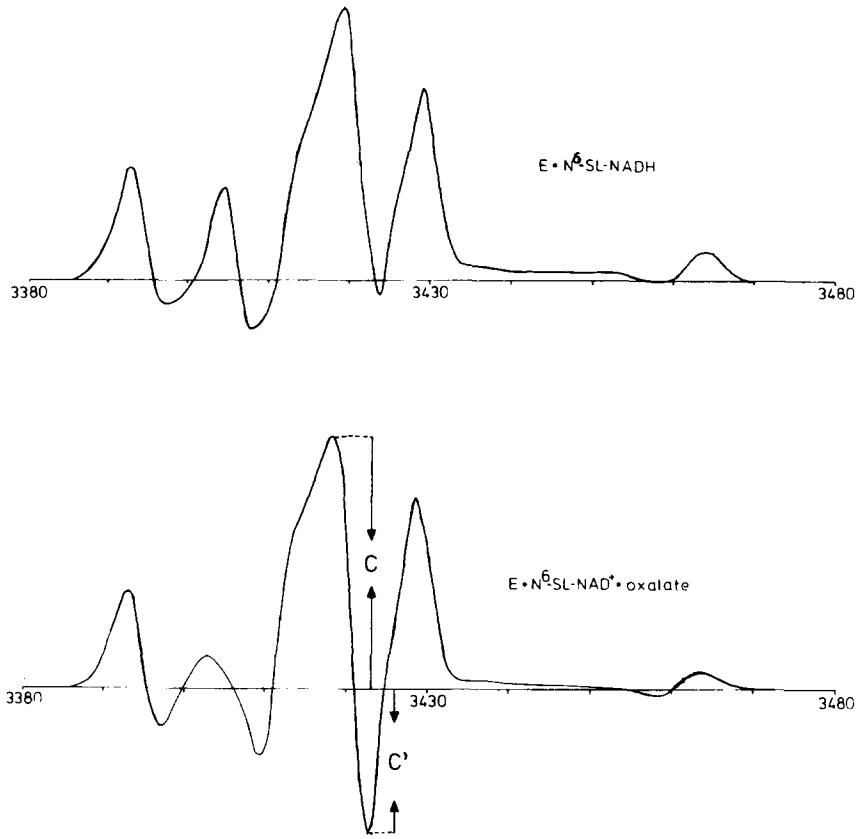


Fig. 3. Second harmonic out of phase saturation transfer ESR spectra of 110 $\mu$M $N^6$-SL-NADH in a binary complex with 270 $\mu$M lactate dehydrogenase and 130 $\mu$M $N^6$-SL-NAD$^+$, in a ternary complex with 230 $\mu$M enzyme and 2.8 mM oxalate. Spectra were recorded at 4° C of solutions in 41.4% glycerol/67 mM phosphate buffer, pH 7.2 for the oxidized coenzyme and 41.4% glycerol/0.2 M glycine buffer, pH 9.5 for the NADH derivative. They are corrected for contributions from free $N^6$-SL-NAD as outlined in the text.

solutions (50 $\mu$g/ml) the native tetrameric structure is not affected by 60% aqueous glycerol buffer. This could be demonstrated by comparison of initial progress cruves of standard assays started with enzyme from phosphate buffer solutions or from concentrated and diluted glycerol solutions. The identical curves observed are not due to immediate reassociation of the enzyme in the assay mixture as shown by disc electrophoresis of lactate dehydrogenase in 50% aqueous glycerol which gave no indication of monomers or dimers.

Fig. 3 shows two second harmonic absorption saturation transfer spectra typical for the complexes investigated. They represent the two extreme line shapes observed, with all other spectra being of intermediate type. The differences can be best conceived when comparing the ratio of the center peak heights above and below the base line (C'/C). This ratio can be correlated with the rotational correlation time of the label, i.e. with the differences in its mobility relatively to the protein [9]. Similar relationships hold for high field and low signals, which lead in principal to the same results, however, being less accurate for these correlation times.

TABLE I

ROTATIONAL CORRELATION TIMES OF $N^6$-SL-NAD IN VARIOUS BINARY AND TERNARY COMPLEXES WITH LACTATE DEHYDROGENASE

Data derived from C'/C ratios of saturation transfer ESR spectra in 41.4% glycerol/67 mM phosphate buffer, pH 7.2, for the oxidized and 41.4% glycerol/0.2 M glycine buffer, pH 9.5, for the reduced coenzyme analog at 4°C.

| Complex | C'/C | $\tau_R$ ($s \times 10^{-6}$) |
|---|---|---|
| E/$N^6$-SL-NADH | —0.07 | 8 |
| E/$N^6$-SL-$NAD^+$/sulfite | —0.23 | 5 |
| E/$N^6$-SL-NADH/oxamate | —0.28 | 5 |
| E/$N^6$-SL-NAD · pyruvate | —0.40 | 3 |
| E/$N^6$-SL-$NAD^+$/oxalate | —0.58 | 1 |

Anyway, as will be seen in the discussion section the absolute values of the correlation times derived from C'C are not required for an interpretation of the data. Of interest is their relative order for different complexes, which is highly reproducible as has been demonstrated by reruns of several spectra on a different ESR spectrometer (Bruker ER220DSR). Table I summarizes the center peak ratios and corresponding correlation times for five complexes. The data in fact result from difference spectra to correct for contributions from free $N^6$-SL-NAD arising from the $\alpha$-diastereomer and the free fraction in equilibrium. The free spin-label concentration was determined from the height of the low field signal in conventional ESR spectra. Corresponding saturation transfer spectra with the appropriate $N^6$-SL-NAD concentration in the absence of enzyme were subsequently recorded. The viscosity of these solutions was adjusted to its value in presence of the enzyme by additional glycerol by means of a rotation viscometer. However, all these correction did not significantly alter the results because measurements were restricted to strong complexes with small apparent dissociation constants for the spin-label coenzyme [6]. For investigation of the binary complex, $N^6$-SL-NADH was used instead of $N^6$SL-$NAD^+$, again because of its much tighter binding.

The gradual increase in rotational mobility (Table I) of the spin-label when going from the binary $N^6$-SL-NADH complex to the ternary enzyme/$N^6$-SL-$NAD^+$/oxalate complex is corroborated by the differences in the separation of the outer maxima reported in the previous paper. In that investigation the binary complex with the oxidized coenzyme was included which fitted in well with $N^6$-SL-NADH.

## Discussion

X-ray analysis of lactate dehydrogenases from various sources has revealed a major conformation change in the protein structure upon comparison of apo enzyme crystals and those of ternary complexes [6]. In the latter a peptide loop of 17 amino acids is folded down over the active cleft. Arginine-101 is part of this loop and forms an ionic bond with the pyrophosphate moiety of the coenzyme, thus changing its position in the apo enzyme by 13 Å. Formation of this bond has been postulated to trigger the conformational change

[19] and one would therefore predict the loop to be closed in binary complexes. However, low resolution data of a binary complex reveal an open conformation [20]. Moreover, subsequent binding of substrates (for which a compulsory order mechanism has been established) is difficult to envisage when the loop is down. Very recently Rossmann and Musick have solved the structure of mouse testicular lactate dehydrogenase at 2.9 Å resolution, in the apo enzyme crystals of which the loop is down (Musick, W.D.L. and Rossmann, M.G., personal communiation; J. Biol. Chem. submitted for publication). This led these authors to postulate that in solution the loop must fluctuate between open and closed forms because coenzyme binding to a closed loop conformer would be impossible.

Molecular model building has now revealed that the spin-label in $N^6$-SL-NAD may serve as a reporter group monitoring the loop conformation. W.D.L Musick (personal communication) has fitted the spin-label derivative into the coenzyme binding site of the model of the pig heart lactate dehydrogenase NADH/oxamate complex [21]. Severe steric hindrance with residues valine-54, valine-119 and phenylalanine-122 would result with the spin-label when the $NAD^+$ moiety is axially bonded to the piperidinium ring. Thermodynamically more favorable is the equatorial conformation about this bond. Although rotation about the adenine-$N^6$ and $N^6$-piperidine bonds again produces collision with the protein, the spin-label may protrude into solution. In fact, a rather unrestricted spin-label is in excellent agreement with its correlation time of about $1.5 \cdot 10^{-8}$ s derived from conventional ESR spectra at 25°C (Fig. 2). The corresponding rotational correlation time for the enzyme itself, based on its Stokes radius of 42 Å is about $7 \cdot 10^{-8}$ s. The separation of the outer maxima in saturation transfer spectra should depend only on the polarity of the label's environment. Assuming the label protrudes into solution this polarity and the separation must be very similar for all complexes. Indeed, the separation is constant (71 G) in all spectra.

The rotational correlation time of the various complexes (Table I) can be discussed in terms of steric intereference of the spin-label with the protein in the vicinity of the adenine pocket including loop residues glycine-99 and valine-100. In the crystal structure of ternary complexes the loop is folded down over the active cleft whereas the loop was found in the open conformation in a binary complex [20]. A high rotational correlation time can therefore be assigned to an open conformation and vice versa. In fact additional interactions of the spin-label with loop residues glycine-99 and valine-100 might occur when the loop is 'open' or 'up'. However, we did not observe two sets of data corresponding to open and closed forms but rather a gradual change between two extreme values. This may be best understood on the basis of a fluctuating loop as proposed by Musick and Rossmann. When loop oscillations are fast on an ESR time scale the label would sense an average conformation, i.e., a predominantly open form in the binary complex and a predominantly closed form in the $N^6$-SL-$NAD^+$/oxalate complex. If the interconversion rate between open and closed conformations, however, is slow, the spectra will reflect the sum of different forms and mobilities and again will be determined by the ratio between these conformations.

It is important to note that the polar interaction between arginine-101 and

the pyrophosphate moiety of the coenzyme is feasible even when the loop moves up with the adenine part of the coenzyme sliding slightly further into its binding pocket and tilting within it. Thus the important hydrophobic interaction would also be maintained upon this motion (Musick, W.D.L. and Grau, U., personal communication).

How can the differences in the predominant loop conformation be related to structural information? The carboxyl groups of the substrates are known to bind to the positively charged guanidium group of arginine-171. Whereas arginine-171 is a core amino acid, arginine-109 is part of the loop and is close to the substrate binding domain only when the loop is folded down [6]. Oxalate possibly forms an ion bridge between these two positive charges [19] which could be interpreted as 'glueing' the loop to the enzyme core. In the $NAD^+$/pyruvate complex the label exhibits a somewhat lower mobility. It has long been established that pyruvate adds to position 4 of the nicotinamide ring of $NAD^+$ under formation of a neutral dihydropyridine structure [22,23]. The carboxyl group of this adduct is known to form an ion bridge with arginine-171. By this the coenzyme is fixed to the enzyme core and consequently the loop should still be in a primarily closed conformation. Sulfite, like pyruvate adds to the nicotinamide ring, however, it is not likely to extend far enough from the coenzyme into the catalytic site to cause significant ionic interaction with arginine-171, thus the loop could open without immediate breakdown of a strong interaction as indicated by a higher correlation time, comparable to the binary complex. From the model an ion bridge between sulfite and arginine-109 appears to be possible. However, arginine-109 is part of the loop which may still be flexible with sulfite and the coenzyme bound to it. In the NADH/oxamate complex the carboxyl group of the inhibitor binds to arginine-171 [21]. On the other hand, since there is no covalent bond between the coenzyme and oxamate a possible loop oscillation will hardly be affected. Consequently, within the limits of error, there are virtually no differences in the correlation times observed for the oxamate and sulfite complexes.

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