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TERNARY COMPLEX FORMATION OF PIG HEART LACTATE DEHYDROGENASE WITH SPIN-LABELLED COENZYMES AND INHIBITORS AS STUDIED BY ELECTRON SPIN RESONANCE

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Summary

The formation of ternary inhibitor and 'dead end' complexes of pig heart lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) was studied by means of two NAD derivatives, spin-labelled at N^6 and C-8 of the adenine ring. Dissociation constants calculated for the inhibitors oxamate and oxalate from their corresponding ternary complexes are in excellent agreement with data from literature derived from sedimentation experiments. However, the recently postulated enzyme-NADH-sulfite complex was not observed.

The mobility of the spin-label, i.e. the protein conformation near the adenine binding pocket in various ternary complexes depends on the type of inhibition or substrate employed.

Introduction

For the interconversion of pyruvate and lactate, catalyzed by the NAD-dependent lactate dehydrogenase, a compulsory order of binding, that is, coenzyme first and substrate second, has been established as a result of numerous experiments. Besides the two active ternary complexes, two abortive ternary complexes involving oxidized coenzyme and oxidized substrate or reduced coenzyme and reduced substrate were found to be formed from the

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Abbreviations: N^6SL- or 8SL- preceding NAD denote a nitroxyl spin-label attached to the N^6- or 8-position of the adenine moiety as shown in Fig. 2, SL signifies N^6SL or 8SL. The subscripts t, b, f with concentration data mean total, bound, and free respectively.

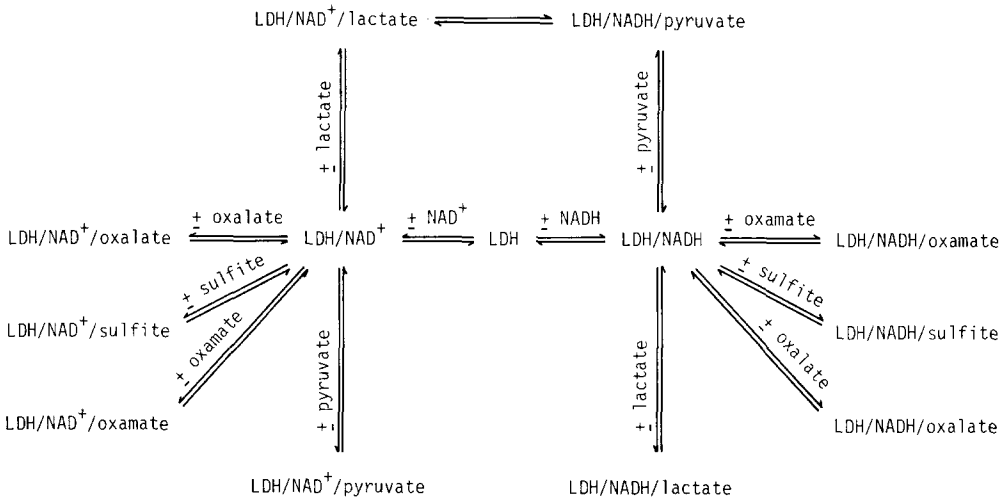

Fig. 1. Binary and ternary complex formation of lactate dehydrogenase.

corresponding binary complexes. Important information about the mechanism of the enzyme has come from inhibitor studies with carboxylic acids, particularly oxamate and oxalate, which participate in ternary complexes with lactate dehydrogenase and NAD^+ or NADH [1]. Another long-known ternary complex is formed from lactate dehydrogenase, oxidized coenzyme and sulfite [2]. An enzyme-NADH-sulfite complex was postulated recently [3]. These results are summarized in Fig. 1 (for review, see ref. 4).

The use of the spin-labelled coenzymes shown in Fig. 2, which we introduced [5,6], now offers a new method for studying the complex formation of lactate dehydrogenase. Binding constants and the stoichiometry of the complexes can be determined by titration experiments. Moreover, the lineshape of the 'bound' ESR spectrum contains indirect information on the protein conformation.

While the two binary complexes and the abortive enzyme-NAD^+-pyruvate complex were the subjects of previous reports [7,8], this paper deals primarily with the remaining ternary complexes of Fig. 1.

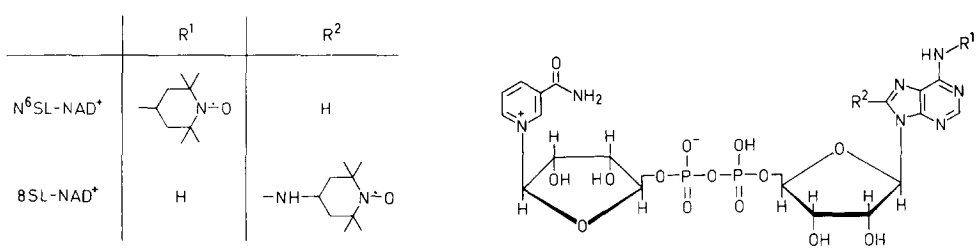

Fig. 2. Structural formula of the spin-labelled coenzymes.

Materials and Methods

Enzymes

Lactate dehydrogenase (EC 1.1.1.27) from pig heart was purchased from Boehringer (Mannheim, F.R.G.).

The enzyme crystals were removed from the suspension by centrifugation and dissolved in 67 mM phosphate buffer, pH 7.2. After gel chromatography on Sephadex G-25 containing finely powdered charcoal to remove any tightly bound nucleotides, the enzyme was precipitated again by addition of solid ammonium sulfate. It was dialyzed against the appropriate buffer (67 mM phosphate, pH 7.2, or 100 mM Tris-HCl, pH 9.5) and centrifuged before use.

Protein concentrations are based on the lactate dehydrogenase monomer (molecular weight 36 000) and were determined spectrophotometrically at 280 nm using an absorption coefficient of 1.4 $cm^2 \cdot mg^{-1}$.

Alcohol dehydrogenase (EC 1.1.1.1) from yeast for SL-NAD^+ reductions was also from Boehringer Mannheim.

Coenzymes

N^6SL-NAD^+ and 8SL-NAD^+ were synthesized as previously described [5,6]. Concentrations were measured after reduction to the corresponding NADH derivatives with ethanol and alcohol dehydrogenase ($\epsilon_{366} = 3330\ M^{-1} \cdot cm^{-1}$). The same enzymic reduction was performed in the ESR flat cell to prepare N^6SL-NADH and 8SL-NADH shortly before measurement.

ESR experiments

ESR spectra were recorded with an X-band spectrometer (9.36 GHz) at 25°C. The titration equipment and technique employed were described earlier [7]. The fraction of unbound spin-label in the various enzyme complexes was determined from the amplitude of the high field line by means of a diagram, amplitude versus concentration resulting from a blank titration without enzyme. No broadening of the 'free' triplet was observed with the protein concentrations used. Concentrations of the compounds are given in the legends of the corresponding figures.

Binding data

Dissociation constants describing the complex formation of lactate dehydrogenase with spin-labelled NAD^+ and oxamate were evaluated according to the method of Fisher and McGregor [9] with slight modifications. Assuming the following equilibria, which make allowance for a compulsory binding, LDH + SL-$NAD^+ \rightleftharpoons$ LDH/SL-NAD^+, LDH/SL-NAD^+ + oxamate $\rightleftharpoons$ LDH/SL-NAD^+/oxamate, the dissociation constants K_b of the binary complex and K_t for the dissociation of oxamate from the ternary complex can be defined:

$$K_b = \frac{[\text{LDH}]_f \cdot [\text{SL-NAD}^+]_f}{[\text{LDH/SL-NAD}^+]} \qquad K_t = \frac{[\text{LDH/SL-NAD}^+] \cdot [\text{oxamate}]_f}{[\text{LDH/SL-NAD}^+]/\text{oxamate}]}$$

Because

$$[\text{LDH}]_f = [\text{LDH}]_t - [\text{LDH/SL-NAD}^+] - [\text{LDH/SL-NAD}^+/\text{oxamate}]$$

and

$$[\text{SL-NAD}^+]_b = [\text{LDH/SL-NAD}^+] + [\text{LDH/SL-NAD}^+/\text{oxamate}]$$

one obtains:

$$\frac{[\text{LDH}]_t}{[\text{SL-NAD}^+]_b} = 1 + \frac{K_b}{[\text{SL-NAD}^+]_f} \cdot \frac{1}{1 + \frac{[\text{oxamate}]_f}{K_t}}$$

If oxamate is present in great excess, $[\text{oxamate}]_f$ can be replaced by $[\text{oxamate}]_t$. Lactate dehydrogenase is titrated with SL-NAD$^+$ in the presence of different amounts of oxamate. The results are represented as plots of

$$\frac{[\text{LDH}]_t}{[\text{SL-NAD}^+]_b} \text{ vs. } \frac{1}{[\text{SL-NAD}^+]_f}.$$

According to the reaction mechanism straight lines with identical intercepts on the ordinate should be obtained. For the abscissa intercepts the following equation holds:

$$\frac{1}{[\text{SL-NAD}^+]_f} = \frac{-K_t - [\text{oxamate}]_f}{K_b \cdot K_t}$$

K_b can be derived from the intercept of a titration curve without oxamate

$$\left(\frac{1}{[\text{SL-NAD}^+]_f} = -\frac{1}{K_b}\right),$$

K_t from those of titration curves with oxamate.

The binary and ternary complexes of lactate dehydrogenase with SL-NADH were characterized by an amplitude ratio: high field line of the 'free' triplet (F) to high field line of the 'bound' spectrum (B) (see Fig. 6). The higher this ratio, the weaker the corresponding complex.

The distance A between the outer extrema of the 'bound' spectra was taken as a measure of the relative mobility of the nitroxyl moiety in the various complexes (see Fig. 6). For this purpose additional second derivative spectra were recorded to facilitate the determination of the splitting.

Results

Ternary enzyme-SL-NAD$^+$-inhibitor complexes

The two spin-labelled coenzymes in solution show a nearly symmetric ESR triplet, typical for freely tumbling nitroxyl radicals. When lactate dehydrogenase is added, the amplitudes of the three lines are reduced in the same measure as SL-NAD$^+$ is bound, and the components of a 'strongly immobilized' spectrum appear. The amplitude of the triplet high field line was used to calculate the concentration of unbound SL-NAD$^+$ in titration experiments, first with fixed amounts of enzyme and inhibitors and varying amounts of spin-labelled coenzymes. Fig. 3 shows the results for N^{6}SL-NAD$^+$; 8SL-NAD$^+$ exhibits principally the same behaviour.

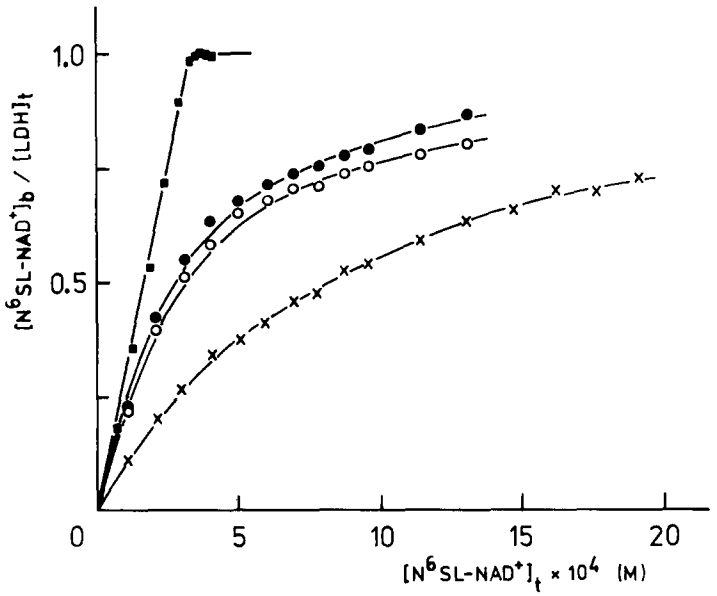

Fig. 3. Binding of N^6SL-NAD^+ to lactate dehydrogenase in binary and ternary complexes. Lactate dehydrogenase concentration: $4.0 \cdot 10^{-4}$ M, pH = 7.2. ×, without inhibitor; ○, with $1.6 \cdot 10^{-3}$ M oxamate; ●, with $2.5 \cdot 10^{-3}$ M oxamate; ■, with $3.0 \cdot 10^{-3}$ M oxalate.

Scatchard-plots for the binary complexes yield one binding site per enzyme subunit, dissociation constants of $5.6 \pm 0.4 \cdot 10^{-4}$ M for N^6SL-NAD^+ and $7.6 \pm 0.6 \cdot 10^{-4}$ M for 8SL-NAD^+ and no indication of cooperativity [6,7]. The presence of oxamate considerably strengthens the coenzyme binding as can be seen from Fig. 3. The effect of oxalate is still more pronounced: virtually all SL-NAD^+ added is bound until the enzyme is saturated.

These results clearly support the formulation of a weak enzyme-NAD^+-oxamate complex and a very strong enzyme-NAD^+-oxalate complex as deduced from ultracentrifuge experiments by Novoa and Schwert [1]. Quantitative data for the enzyme-SL-NAD^+-oxamate complexes can be gained from double reciprocal plots.

From Fig. 4 a dissociation constant K_t for oxamate from the ternary complex with N^6SL-NAD^+ of $4.5 \pm 0.5 \cdot 10^{-4}$ M can be calculated. The corresponding constant for the complex with 8SL-NAD^+ is $5.7 \pm 0.5 \cdot 10^{-4}$ M. These values are of the same order of magnitude as the dissociation constant of $1.7 \cdot 10^{-4}$ M derived from the sedimentation experiments, which, however, were performed with the beef heart enzyme [1].

A second series ESR titration experiments was carried out at constant enzyme and spin-labelled coenzyme concentrations, but varying the inhibitor concentrations. N^6SL- and 8SL-NAD^+ exhibit almost the same binding behaviour; the results for the N^6SL-analogue are shown in Fig. 5. Again, the comparably weak oxamate and the strong oxalate binding is obvious. From the oxalate plot the stoichiometry of the ternary complex involved can easily be deduced: a 1 : 1 : 1 adduct of subunit/SL-NAD^+/oxalate is formed at the binding sites of the tetrameric lactate dehydrogenase. From the non-linear part

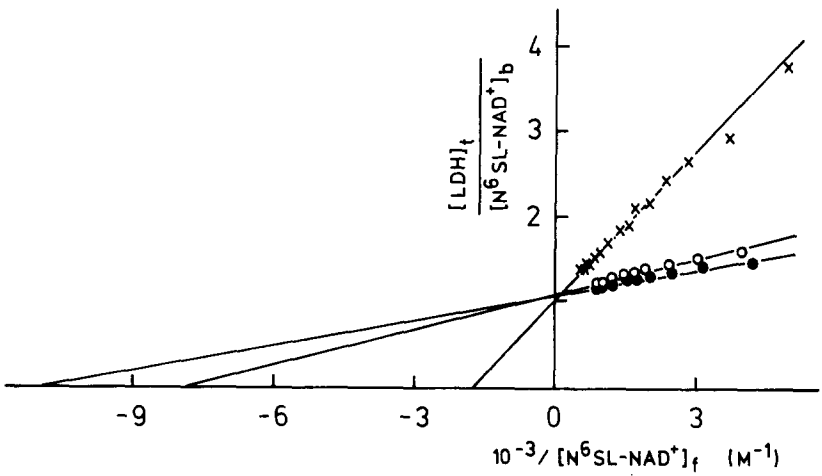

Fig. 4. Double reciprocal plot of the ESR titration data for lactate dehydrogenase and N^6SL-NAD^+ with and without oxamate, pH = 7.2. Lactate dehydrogenase, $4.0 \cdot 10^{-4}$ M; N^6SL-NAD^+ varied from $3.1 \cdot 10^{-4}$ M to $2.1 \cdot 10^{-3}$ M. X, without oxamate, ○, with $1.6 \cdot 10^{-3}$ M oxamate; ●, with $2.5 \cdot 10^{-3}$ M oxamate.

of the oxalate curve a dissociation constant for the inhibitor from the complex of about 10^{-6} M can be estimated, which compares favourably with the value of $2.1 \cdot 10^{-6}$ M determined for the natural coenzyme with optical measurements [10].

Sulfite is known to form a rather strong complex with lactate dehydrogenase and NAD^+ [2]. The ratio of 'bound' to 'free' components in ESR spectra of enzyme-SL-NAD^+-sulfite complexes corroborates this finding and leads one to assume a similarly small dissociation constant as for the enzyme-SL-NAD^+-oxalate complexes. Quantitative experiments are hampered, however, by the slow reduction of the nitroxyl moieties by sulfite.

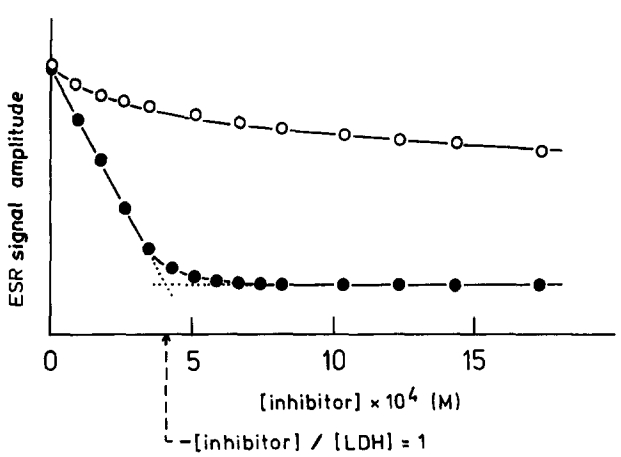

Fig. 5. Binding of oxamate (○) and oxalate (●) to the binary lactate dehydrogenase-N^6SL-NAD^+ complex (pH 7.2). Lactate dehydrogenase, $3.9 \cdot 10^{-4}$ M; N^6SL-NAD^+, $5.2 \cdot 10^{-4}$ M (ordinate: amplitude of the high field line of the 'free' triplet in arbitrary units).

Ternary enzyme-SL-NADH-inhibitor complexes

Fluorescence measurements have contributed primarily to our detailed knowledge about complexes of lactate dehydrogenase with the reduced coenzyme [11]. NADH is bound about 360-times more tightly to the enzyme than NAD^+. Oxamate leads to the strongest ternary complex; oxalate, lactate and sulfite were reported to bind as well.

Because of the limited stability of SL-NADH, due to slow redox reactions involving the dihydropyridine- and the nitroxyl part of the coenzyme analogues, an exact quantitative characterization of the enzyme-SL-NADH complexes cannot be achieved. Rough estimations, however, are possible with SL-NADH generated enzymatically shortly before the ESR experiments. The spectra in Fig. 6 show the result of experiments with 8SL-NADH. The N^6SL-analogue again leads to the same conclusions. The ratio of the amplitudes F and B, which decreases with decreasing dissociation constants, is 6.20 for the binary enzyme-8SL-NADH complex, representing a drastic diminuation of the value for the enzyme-8SL-NAD^+ complex, which exceeds 100. With oxalate added, the ratio of F/B drops to 3.40, and with oxamate to 0.37. These values represent upper limits, because the amplitude F may result to some extent from unbound paramagnetic impurities in the SL-NAD preparations. Neither with lactate nor with sulfite was a significant change of the F/B ratio observed.

With lactate this finding is in accordance with fluorescence measurements, which yield a dissociation constant of about 1 M for lactate from the ternary enzyme-NADH-lactate complex at pH 8.5 [4]. At pH 9.5, where our ESR measurements were performed, this constant is probably still higher.

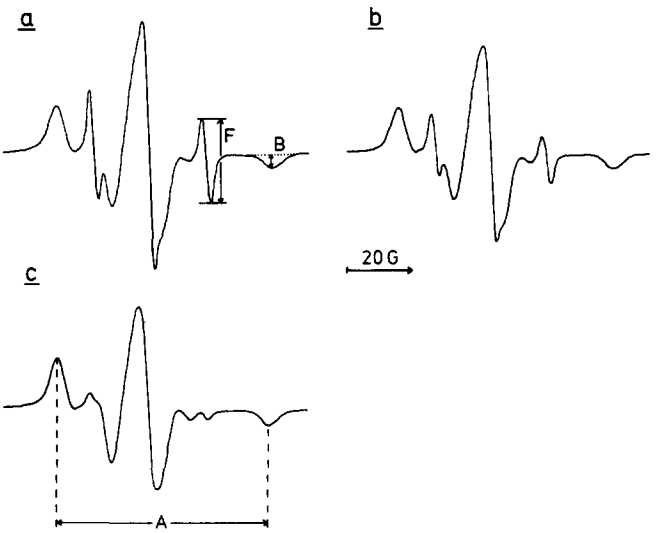

Fig. 6. ESR spectra of binary and ternary lactate dehydrogenase complexes with 8SL-NADH; concentrations: (a) Lactate dehydrogenase, $2.8 \cdot 10^{-4}$ M; ethanol, $3.4 \cdot 10^{-1}$ M; 8SL-NAD, $2.1 \cdot 10^{-4}$ M; alcohol dehydrogenase, $8 \cdot 10^{-7}$ M, (b) lactate dehydrogenase, $2.6 \cdot 10^{-4}$ M; ethanol, $3.1 \cdot 10^{-1}$ M; 8SL-NAD, $1.9 \cdot 10^{-4}$ M; alcohol dehydrogenase, $7 \cdot 10^{-7}$ M; oxalate, $4.1 \cdot 10^{-3}$ M, (c) lactate dehydrogenase, $2.6 \cdot 10^{-4}$ M; ethanol, $3.1 \cdot 10^{-1}$ M; 8SL-NAD, $1.9 \cdot 10^{-4}$ M; alcohol dehydrogenase, $7 \cdot 10^{-7}$ M; oxamate, $4.1 \cdot 10^{-3}$ M.

TABLE I

DISTANCE A BETWEEN THE OUTER EXTREMA OF THE 'IMMOBILIZED' ESR SPECTRA FOR BINARY AND TERNARY LACTATE DEHYDROGENASE COMPLEXES

LDH complex with	A (gauss) ± 0.3		pH
	N^6SL-	8SL-	
-AMP	63.2	—	7.2
-ADP	63.3	—	7.2
-ADPR	63.4	—	7.2
-NAD^+	63.5	65.1	7.2
-NAD^+	63.3	65.0	9.5
-NADH	63.8	65.5	9.5
-NAD^+/oxalate	62.5	64.9	7.2
-NAD^+/sulfite	63.7	65.3	7.2
-NAD^+/pyruvate	62.2	64.5	7.2
-NADH/oxamate	63.3	65.0	9.5

The existence of a ternary enzyme-NADH-sulfite complex, which was deduced from experiments with chemically modified lactate dehydrogenase [3], could not be corroborated by our spin-label method.

Enzyme conformation during ligand binding

The lineshape of the 'immobilized' components of the ESR spectra observed with the different enzyme-SL-NAD complexes reflect the mobility of the nitroxyl moiety [12] and the polarity of its direct environment [13]. A convenient lineshape parameter is the distance between the two outer peaks, denoted with A in Fig. 6, which are not overlapped by the 'free' nitroxyl triplet. Differences in A between the various complexes may indicate differences in the protein conformation around the adenine binding site, which at least partly accomodates the nitroxyl spin-label [6].

Table I lists the *A* values for the binary enzyme-SL-NAD complexes and for the hardly dissociated ternary complexes, where the contribution of the binary complexes to the lineshape is negligible. Values for complexes with spin-labelled structural components of NAD, namely adenosine 5′-monophosphate, adenosine 5′-diphosphate and adenosine 5′-diphosphoribose [7] are included as well for comparison. It can be seen that all binary enzyme-N^6SL-ligand complexes exhibit nearly the same splitting of 63.5 ± 0.3 G. This value also holds for enzyme-N^6SL-NAD^+-sulfite and enzyme-N^6SL-NADH-oxamate. Significantly lower values, however, are found with enzyme-N^6SL-NAD^+-oxalate and with the abortive ternary complex involving pyruvate [8]. The corresponding values of the 8SL-series are about 2 G higher, indicative of a still more restricted environment. The differences of the splitting within this series seem to show the same tendency as those within the N^6SL-series. They are, however, smaller and, due to the error interval of 0.6 G, not equally significant.

Discussion

The various complexes of lactate dehydrogenase have long been the subject of extensive studies. Besides steady state kinetic measurements numerous

equilibrium experiments have been carried out using different techniques. These include ultracentrifugation [1], gel filtration [11], dialysis [14], calorimetry [14] and spectroscopic methods [11]. The latter were mainly confined to complexes involving NADH, because the optical properties of the oxidized coenzyme are unsuitable for binding studies. Since equilibrium experiments are not feasible with the active complexes, studies have often been performed with the substrate analogous inhibitors oxamate and oxalate. With the synthesis of pyridine nucleotides having a nitroxyl radical attached [5,6] the lactate dehydrogenase complexes have become accessible to the spin-label method. From the ESR spectra concentrations of bound and free SL-NAD can directly be evaluated; no extrapolations or questionable corrections are necessary as are often associated with spectrophotometric methods. Once the ESR spectrometer is set, the measurements are easy and rapid. Complex formation involving both the oxidized and, with some restrictions because of its limited stability, the reduced coenzyme can be followed.

When using analogues in place of natural compounds, it has to be shown that the modification made does not significantly perturb the natural conditions before generalizing the results. With lactate dehydrogenase and N^6SL-NAD, kinetic measurements and molecular models have shown that the nitroxyl group seems to create only minor perturbations upon complex formation [7]. Thus, data obtained with this analogue should be valid for NAD as well. 8SL-NAD apparently is a somewhat less appropriate coenzyme analogue, probably because of the syn conformation of the adenine ring relatively to its ribose moiety in solution [6]. Nevertheless it can be a useful complement of N^6SL-NAD. The ESR measurements of this study on the whole corroborate the results of previous reports: lactate dehydrogenase from pig heart forms strong complexes with NAD^+-oxalate, NAD^+-sulfite and NADH-oxamate and weak complexes with NAD^+-oxamate and NADH-oxalate. The dissociation constants evaluated, which consistently are somewhat lower with N^6SL- than with 8SL-NAD, are in reasonable agreement with literature values. An enzyme-NADH-sulfite complex, [3] could not be found by our spin-label method.

The potentially most promising aspect of our approach probably lies in ESR line shape measurements. The rather rough measurements described herein already reveal some interesting features of the protein conformation associated with coenzyme binding: the spin-label attached to the adenine moiety obviously witnesses the same environment in all binary complexes, even when only fragments of the coenzymes are involved. When distinct small molecules, namely oxalate and pyruvate, are bound at the remote nicotinamide end of $SL\text{-}NAD^+$, fixed in an open conformation to lactate dehydrogenase, the spin-label environment changes. The motional freedom of the spin-label in these two ternary complexes seems to be less constrained than in the binary ones. This statement does not hold for the ternary complexes enzyme-$SL\text{-}NAD^+$-sulfite and enzyme-SL-NADH-oxamate.

These differences in the protein conformation of various ternary complexes have been studied in more detail by saturation-transfer ESR spectroscopy (Trommer, W.E. and Glöggler, K.G., unpublished data). This method is especially sensitive to changes of rather slow molecular movements [15], as they are observed for enzyme-bound SL-NAD. Comparison with X-ray data

reveals that the spin-label monitors the movement of a peptide 'loop', which is known to fold down over the active cleft during catalysis [4].

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