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THE BINDING OF SPIN-LABELED DERIVATIVES OF NAD$^+$ AND ITS STRUCTURAL COMPONENTS TO PIG SKELETAL MUSCLE LACTATE DEHYDROGENASE

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Summary

The binding of spin-labeled derivatives of NAD$^+$ and its structural components to pig skeletal muscle lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) is described. In contrast to results previously obtained with the heart muscle isozyme (Wenzel, H.R., Pfleiderer, G., Trommer, W.E., Paschenda, K. and Redhardt, A. (1976) Biochim. Biophys. Acta 452, 292—301), no significant increase is observed in the binding constant of N^6-SL-ADP as compared N^6-SL-AMP. This different behavior can be explained by the substitution of glutamine-31 for alanine in the muscle isozyme, which has been proposed to account for the tighter binding of NADH to the heart type. In both isozymes the binding of the spin-labeled coenzyme itself is weaker than found for its structural components.

Introduction

In a preceding paper we described the binding of spin-labeled derivatives of NAD$^+$ and its structural components to the cardiac muscle isozyme (B form) of lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) [1]. It was found that the binding constant of SL-ADP showed more than a sixfold increase in comparison with SL-AMP. The primary sequences of various lactate

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Abbreviations: ADP-Rib: adenosine 5′-diphosphoribose; N^6-SL preceding AMP, ADP, ADP-rib and NAD$^+$ refers to the N^6-(2,2,6,6-tetramethyl-piperidin-4-yl-1-oxyl) derivatives of these compounds.

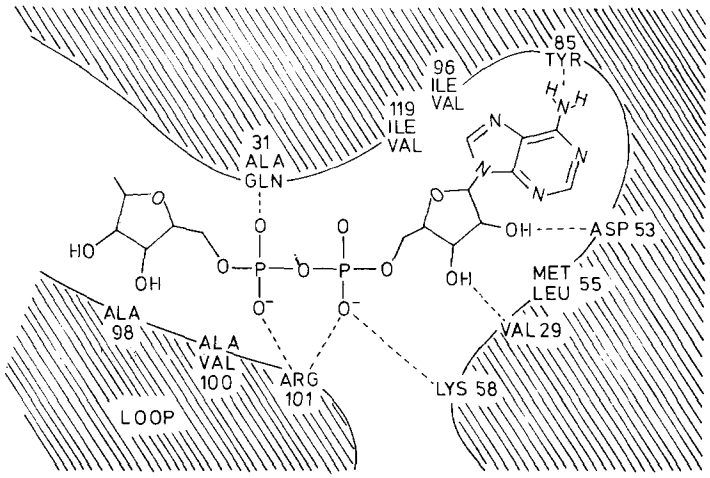

Fig. 1. Schematic representation of ADP-Rib in the nucleotide binding sites of lactate dehydrogenase isozymes A and B according to [2] indicating changes of the primary structure in this domain. Upper abbreviations for amino acids refer to the A form and lower to the B form of the enzyme. Figure after H.-H. Kiltz and reproduced with his kind permission.

dehydrogenases and the partial tertiary structure (X-ray diffraction measurements) have recently been published [2], and these can provide an alternative explanation of the above observation. An initial comparison of the coenzyme binding domains in cardiac and skeletal muscle reveals few changes of apparently sufficient significance to account for the well known physical and chemical differences. However, the much tighter binding of NADH in the B form can only be explained by the substitution of glutamine-31, (as opposed to alanine in the A form), as the amide group of glutamine-31 could provide additional binding energy by the formation of a hydrogen bond to the nicotinamide phosphate of the coenzyme (Fig. 1). This additional hydrogen bond could also explain the tighter binding of N^6-SL-ADP as compared to N^6-SL-AMP observed for the B form of the enzyme. Although the authors [2] state 'the evidence for this important change is not clear in the electron density maps' if this hypothesis (originally suggested to us by H.-H. Kiltz) is correct, it would be expected that with skeletal muscle lactate dehydrogenase, both spin-labeled derivatives should exhibit almost identical binding constants. In this paper we show that this is the case.

Methods

Pig skeletal muscle lactate dehydrogenase was purchased from Boehringer (Mannheim) and was further purified by affinity chromatography on an oxamate column eluted with NAD^+ [3] to remove any contaminating isozymes. Tightly bound nucleotides were subsequently removed by chromatography on Sephadex G-25 containing 5 mg finely powdered charcoal/ml gel [1] and a final separation from any denatured soluble protein was achieved by chromatography on Sephadex G-200 [4]. The enzyme was stored as $(NH_4)_2SO_4$ sus-

pension and freshly dialyzed against 67 mM phosphate buffer (pH 7.2) shortly before use (specific activity [5] 680 U/mg). Protein concentration was determined spectrophotometrically at 280 nm using a factor of 1.4 for a solution containing 1 mg/ml (69 μM) based on 144 000 molecular weight [6].

The spin-labeled derivatives of AMP, ADP, ADP-Rib and NAD^+ were prepared as previously described [1,7]. However, a few minor changes were found to improve the yields. A LiCl gradient was used as eluent for all N^6-SL-nucleotides except N^6-ADP-Rib. Removal of this salt could be easily achieved by pressure dialysis in Amicon cells with UM 05 membranes (Robillard, G.T. and Zantema, A., personal communication). For N^6-SL-AMP activation as applied to the synthesis of N^6-SL-ADP, the general procedure of Furusawa et al. [8] was followed. Concentrations of SL-nucleotides were determined photometrically using molar extinction coefficients of $\epsilon_{268} = 21\,500$ for N^6-SL-AMP, N^6-SL-ADP and N^6-SL-ADP-Rib and of $\epsilon_{340} = 6300$ for N^6-SL-NAD^+ after enzymic reduction to the corresponding NADH derivative with ethanol in glycine buffer pH 9.5 catalyzed by alcohol dehydrogenase [9].

Steady state kinetic measurements for NAD^+ and N^6-SL-NAD^+ were carried out at 25°C in 0.2 M glycine buffer pH 9.5 at 40 mM L-lactate. The coenzyme and its analog were varied from 30—300 μM. Kinetic constants were computed according to the method of Cornish-Bowden [10].

X-Band ESR spectra were recorded with a Bruker B-ER 420 spectrometer with 100 kHz modulation of 0.8 G and 6 mW microwave power. Binding experiments were carried out at 25°C in 67 mM phosphate buffer pH 7.2 in micro cells (70 μl) equipped with Teflon adapters for a modified sample holder [1]. Determination of free and bound spin-label concentrations was carried out, as previously described [1], by comparing the signal height of the high field peak in samples with and without enzyme. In the concentration range studied the contribution of bound label to this signal again was below 1%. However, in this investigation, separate solutions were prepared for every single point of the titration curves in order to eliminate protein denaturation due to prolonged stirring.

Results and Discussion

N^6-SL-NAD^+ is an active coenzyme of pig skeletal muscle lactate dehydrogenase. The kinetic constants differ only slightly from the values for the natural coenzyme, i.e. almost identical Michaelis constants (N^6-SL-NAD^+ : $K_m = 100$ μM; $NAD^+ = 130$ μM) and only 30% decrease of the maximum velocity. This finding gives an obvious answer to the crucial question inherent to any reporter group. The spin-label does not disturb the enzyme structure significantly so that data obtained with the analog should be valid for NAD^+ as well. This was to be expected from X-ray data which show that the amino function of the adenine moiety protrudes somewhat from a hydrophobic pocket [6] into the solution. There is ample space for the spin-label and indeed, there are very few interactions with the enzyme (Rossmann, M.G. and Eventoff, W., private communication).

N^6-SL-NAD^+ and its structural components N^6-SL-ADP-Rib, N^6-SL-ADP and N^6-SL-AMP form binary complexes with the enzyme as studied by ESR

spectroscopy. Spectra of the bound N^6-SL-nucleotides are typical for highly immobilized nitroxyl radicals with rotational correlation times of about $3 \cdot 10^{-8}$ S [11,12] and are virtually identical to those described previously for complexes with the B isozyme [1].

Plotting of the binding data of the N^6-SL-nucleotides according to the method of Scatchard [13] shows linear relationships with the exception of N^6-SL-ADP-Rib. Least squares fits yield 4 ± 0.3 identical and independent binding sites with dissociation constants as summarized in Table I. Fig. 2 shows a Hill plot [14] of the same data including N^6-SL-ADP-Rib. As would be expected from evaluation according to Scatchard, N^6-SL-NAD$^+$, N^6-SL-ADP and N^6-SL-AMP yield straight lines almost parallel to one another with Hill coefficients n_H equal or close to one. However, N^6-SL-ADP-Rib data may be linearized for $n_H = 1.7$. Table I summarizes these results including the dissociation constants computed according to Hill based on 1.0 binding sites per subunit.

The most interesting finding, of course, is the fact that indeed N^6-SL-AMP and N^6-SL-ADP exhibit similar dissociation constants in contrast to the rather dramatic decrease observed with the B isozyme. As pointed out above, this strongly supports the hypothesis that a hydrogen bond between glutamine-31 and the nicotinamide phosphate provides the additional binding energy for NADH in cardiac muscle lactate dehydrogenases.

There are, however, several additional points worth mentioning. The dissociation constants for N^6-SL-AMP with the A and B isozymes differ considerably (A form 0.2 mM, B form 0.7 mM). This again can be discussed on the basis of the different primary sequences. As stated by Eventoff et al. [2], the exchange

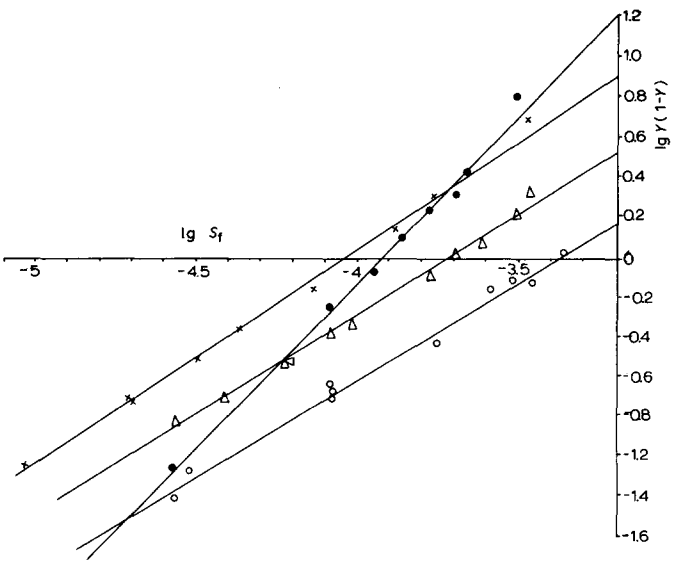

Fig. 2. Hill plots of ESR data for the spin-labeled derivatives of NAD$^+$ and its structural components binding to the A isozyme of lactate dehydrogenase. ○, N^6-SL-NAD$^+$; ●, N^6-SL-ADP-Rib; X, N^6-SL-ADP and △, N^6-SL-AMP. All spin-labeled derivatives were varied from about 40—600 μM at 41—48 μM of the enzyme based on 144 000 molecular weight.

TABLE I

Dissociation constants and Hill coefficients n_H of spin-labeled derivatives of NAD^+ and its structural components binding to pig skeletal muscle lactate dehydrogenase. Standard error of the mean about 10%.

Compound	K_D (mM) from Scatchard plot	Binding sites per tetramer	K_D (mM) from Hill plot	n_H
N^6-SL-AMP	0.20	4.0	0.20	1.0
N^6-SL-ADP	0.11	4.3	0.09	1.1
N^6-SL-ADP-Rib			0.12	1.7
N^6-SL-NAD^+	0.39	3.8	0.44	1.0

of three amino acids in the hydrophobic adenine binding pocket (indicated in Fig. 1) tends to reduce its size in the A form thus allowing for better contact with the adenosine moiety. The ionic interaction of the phosphate group with arginine-101 would appear to be the same in both isozymes.

The dissociation constant for N^6-SL-NAD^+ of 0.42 ± 0.05 mM correlates well with data from literature (0.5 ± 0.2 mM) for NAD^+ itself as determined by fluorescence spectroscopy from competition experiments with NADH [15,16]. On the other hand, this dissociation constant is higher than that found for any structural component of the spin-labeled coenzyme. This finding, although reasonably well understood from the repulsion of the positively charged nicotinamide ring in its hydrophobic environment [2,6], is contradictory to data from literature. Several laboratories have reported dissociation constants for AMP, ADP and ADP-Rib derived, however, from kinetic inhibition experiments. The values of these fragments are always higher than the dissociation constant for NAD^+ itself [17—20]. The actual figures, however, for any given inhibitor differ up to tenfold depending on the investigator. Moreover, whereas kinetic inhibition constants may be equal to the equilibrium dissociation constant on the premises of Michaelis-Menten, this becomes rather questionable in more complicated systems.

From the tertiary structure (Fig. 1) similar binding constants might be expected for N^6-SL-ADP and N^6-SL-ADP-Rib. An additional hydrogen bond between the nicotinamide ribose and the backbone at alanine-98 is not very well established. In addition, structural analogs of NAD^+ containing alkyl chains instead of the ribose moiety [21] exhibit rather high coenzymic activity. Indeed, the dissociation constant for N^6-SL-ADP-Rib and N^6-SL-ADP are virtually identical within the experimental error. For N^6-SL-ADP-Rib, however, a Hill coefficient of $n_H = 1.7$ is observed. At present we have no convincing explanation for this behavior (the corresponding experiments were repeated several times with different batches of the enzyme and N^6-SL-ADP-Rib. Our results may be discussed with respect to a controversial point in the literature, i.e. the non linearity in NADH binding to this isozyme [22,23]. In fact, N^6-SL-ADP-Rib can undergoe all interactions with the enzyme known for NADH [2] and therefore resembles NADH much more than N^6-SL-NAD^+, because repulsion of the positively charged pyridinium ring of the latter may dominate the binding characteristics.

Acknowledgements

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