Biochimica et Biophysica Acta, 567 (1979) 49—59

BBA 68672

THE NATURE OF THE SUBSTRATE INHIBITION IN LACTATE DEHYDROGENASES AS STUDIED BY A SPIN-LABELED DERIVATIVE OF NAD$^+$ *

WOLFGANG E. TROMMER [a,**], HELGA HUTH [a] and HERBERT R. WENZEL [b]

[a] *Institut für Organische Chemie, Biochemie und Isotopenforschung der Universität, Pfaffenwaldring 55, D-7000 Stuttgart 80, and* [b] *Abteilung Chemie, Lehrstuhl Biochemie der Ruhr-Universität, D-4630 Bochum (F.R.G.)*

(Received July 28th, 1978)

Key words: Lactate dehydrogenase; Spin label; Pyruvate binding; NAD$^+$ derivative; ESR

Summary

The formation of the ternary complex of lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) from pig heart and skeletal muscle with the adduct of pyruvate to NAD$^+$, spin-labeled at N^6 was studied by ultraviolet spectroscopy and ESR techniques. According to ultraviolet measurements we found identical binding characteristics for the natural coenzyme and its spin-labeled analog.

The rate by which the ESR signal of free spin-labeled NAD$^+$ decreased upon addition of pyruvate to the binary complexes was substantially different in the two isozymes. With the heart type an initial drop followed by a further linear decrease, zero order in the enzyme and coenzyme concentration was observed. In case of the skeletal muscle isozyme no immediate reaction and a first order process occurred. The initial reaction can be attributed to a non-covalent enzyme/spin-labeled NAD$^+$/pyruvate complex with a dissociation constant for pyruvate of 11 ± 1 mM, thus explaining the well-known substrate inhibition in the heart isozyme above 2 mM pyruvate. The further reaction is then determined by the buffer dependent enolization of pyruvate. In the muscle isozyme

---

* Taken in part from the doctoral thesis of H.R. Wenzel, Bochum 1976. Present address: Universität Bielefeld, Fakultät für Chemie, Postfach 8640, D-4800 Bielefeld 1, F.R.G.

** To whom correspondence should be addressed.

Pyr_E and Pyr_K denote the *enol* and *keto* forms of pyruvate in ternary complexes with A and B forms of lactate dehydrogenase, E and N^6-(2,2,6,6-tetramethylpiperidine-4-yl-1-oxyl)-NAD$^+$, N^6-SL-NAD$^+$ or NAD$^+$ itself. E · NAD$^+$ and E · N^6-SL-NAD$^+$ are the binary enzyme/coenzyme complexes and NAD-Pyr or N^6-SL-NAD-Pyr represent the adduct of pyruvate to NAD$^+$ or N^6-SL-NAD$^+$ respectively, as shown in Scheme I.

formation of the covalent adduct is not assisted by prior binding of pyruvate in a non-covalent ternary complex and therefore the rate depends on the binary complex concentration.

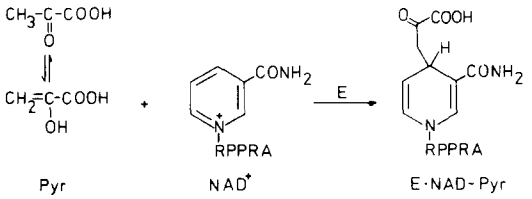
Introduction

The NAD-dependent lactate dehydrogenases catalyze the reversible interconversion of pyruvate and lactate. Two genetically distinct major isozymes have been isolated from most vertebrates, the A form (M_4) primarily from skeletal and the B form (H_4) from cardiac muscle [1]. An important difference between these isozymes is a pronounced substrate inhibition in the pyruvate → lactate reaction at pyruvate concentrations above 1—2 mM which is observed with the heart type only. Since the latter primarily functions as a lactate oxidase, this inhibition has been assumed to exert the metabolic control [2]. However, in vivo studies on perfused rat hearts seem to exclude this possibility [3].

Novoa et al. [4] initially suggested that inhibition was caused by a 'dead-end' ternary complex composed of the enzyme. NAD^+ and pyruvate, $E \cdot NAD^+ \cdot Pyr$, whereas subsequent studies led to the conclusion that a covalent adduct of pyruvate to position 4 of the nicotinamide ring produced inhibition [5,6]. The enzyme was found to catalyze formation of this tightly binding inhibitor according to Scheme I. The rate, however, is rather low and depends strongly on the type and the ionic strength of the buffer system.

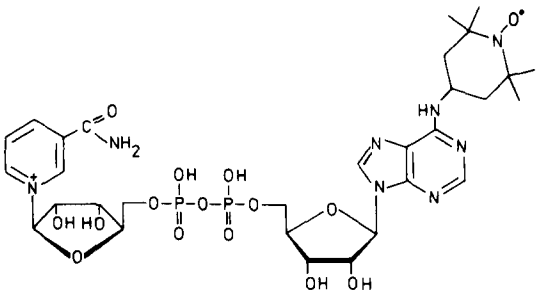
Since substrate inhibition is nearly buffer independent and because of the low rate of the adduct formation its role in the substrate inhibition has soon been questionated [7,8,9]. Very recently Burgner and Ray provided good evidence for either an $E \cdot NAD^+ \cdot Pyr_K$ or $E \cdot NAD^+ \cdot Pyr_E$ complex to produce the pyruvate induced inhibition patterns observed in initial velocity studies of the normal enzymic reaction [10]. Their data have been obtained from computer fits to progress curves of the E · NAD-Pyr formation and decomposition which is accompanied by changes in the absorption and fluorescence spectra. Besides its well-known 325 nm absorption binding of the adduct causes quenching of the tryptophan fluorescence in the protein. However, these fluorescence changes are non-linear with saturation of the protein and had to be corrected as orginally described by Holbrook [11].

In this paper we present independent evidence for the $E \cdot NAD^+ \cdot Pyr_K$ complex obtained from binding studies of a spin-labeled derivative of NAD^+ [12]



SCHEME I

(N^6-SL-NAD^+) in the presence of pyruvate to the B and A forms of lactate



dehydrogenases from pig. The spin-labeling technique offers the unique advantage to allow for direct measurement of the concentration of free and bound substrate and to follow the time course of changes in these concentrations [13].

Methods and Materials

N^6-SL-NAD^+ was prepared as previously described [14]. Its concentration was determined by enzymic reduction with alcohol dehydrogenase to the corresponding NADH derivative ($\epsilon_{340} = 6300$). NAD^+ was purchased from Boehringer, Mannheim, and was used without further purification. Lactate dehydrogenases (EC 1.1.1.27) from pig heart and skeletal muscle were obtained from Boehringer, Mannheim, as well but were further purified by affinity chromatography on an oxamate column [15] and subsequently by gel chromatography on Sephadex G-50 containing finely powdered charcoal to remove any thightly bound nucleotides [16]. After final chromatography on Sephadex S-200 [7,12] the protein was precipitated by ammonium sulfate. It was freshly dialyzed against the appropriate buffer and centrifuged at 40 000 $\times g$ shortly before use.

Enzyme assays

Standard procedures were used for determination of the enzymatic activity [17].

The protein concentration was determined spectrophotometrically at 280 nm using a factor of 1.34 for a solution containing 1 mg/ml (6.94 μM) of the heart muscle isozyme (420 U/mg) and a factor of 1.4 for the skeletal muscle isozyme (630 U/mg) [1].

Formation of the E · NAD-Pyr and E · N^6-SL-NAD-Pyr complexes as studied by ultraviolet spectroscopy

A Beckman model 25 double beam spectrophotometer was used to measure the 325 nm absorption of the adduct complexes in 100 mM phosphate buffer, pH 8.0. NAD^+ or N^6-SL-NAD^+ were varied from 7—690 μM at 6 mM pyruvate and an enzyme concentration (B type) of 27.7 μm [7] based on the subunit molecular weight of 36 000. The reference cuvette contained the enzyme and pyruvate. The absorption of NAD^+ and of its analog at 325 nm was determined

separately against buffer and the absorption of the complex corrected accordingly.

ESR experiments

ESR spectra were recorded with a Bruker B-ER 420 spectrometer operating in the X-band mode. 100 kHz modulation with amplitudes of 0.8 G and a microwave power of 6 mW was routinely applied. All experiments were carried out at 24°C in a total volume of about 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 [12]. The solutions were added by means of Hamilton syringes equipped with a repeating dispenser (model PB 600-1) and platinum needles as well as teflon tipped plungers to avoid reduction of the free radical by stainless steel.

For determination of binding constants of N^6-SL-NAD^+ in binary complexes the amplitudes of the high field peak in solutions containing the enzymes were compared with those of solutions containing an equal amount of buffer (67 mM phosphate buffer, pH 7.2; 67 mM pyrophosphate buffer, pH 8.5; 100 mM glycine buffer, pH 9.5). The linearity between signal amplitude and N^6-SL-NAD^+ concentration was established by a blind titration. Concentrations of N^6-SL-NAD^+, pyruvate and the enzymes are given in Table I and the legends of Figs. 3 and 5.

For studying the time course of the ternary complex formation rather concentrated solutions of 0.5 M pyruvate were added to the binary mixtures in order to keep volume changes below 1—2%. Spectra were usually recorded within 30 s after addition of pyruvate. Because of the linear progress curves extrapolation back to zero time was easily achieved when the heart isozyme was investigated. In case of the A form linear semilogarithmic plots of the time course were computed in order to achieve extrapolation.

Results

Investigations with the B form of lactate dehydrogenase

Fig. 1 shows a binding curve for the E · NAD-Pyr and E · N^6-SL-NAD-Pyr complexes as expressed by the end values of their 325 nm absorption at various NAD^+ and N^6-SL-NAD^+ concentrations. The absorption values were derived from difference spectra with respect to enzyme, pyruvate and NAD^+ or N^6-SL-NAD^+. Data for NAD^+ and its spin-labeled analog are evenly distributed and can be fitted by a common non-linear least squares regression to a theoretical binding curve [18] computed for an end value of 0.186 and 4.1 independent binding sites per tetramer of the enzyme exhibiting a dissociation constant of 3.7 ± 0.4 μM each.

Formation of the E · NAD-Pyr complex as studied by ESR techniques

When pyruvate is added to a mixture of the B form of lactate dehydrogenase and N^6-SL-NAD^+ the signal intensity of free N^6-SL-NAD^+ immediately drops followed by a further decrease, linear with time, which eventually levels off

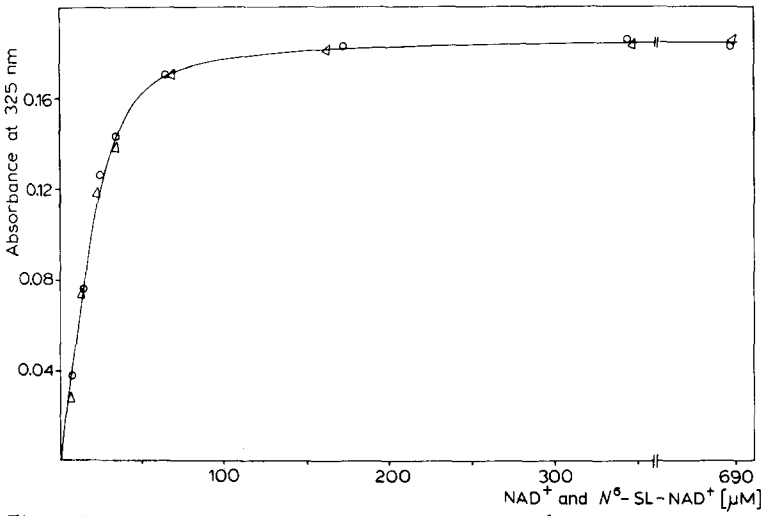


Fig. 1. Binding curve for the E · NAD-Pyr (○) and E · N^6-SL-NAD-Pyr (△) complexes. The solid line is a computer fit to a theoretical binding curve, 4.1 binding sites per tetramer of lactate dehydrogenase, B form, K_D = 3.7 μM.

until a final constant end value is attained (Fig. 2). The rate of the linear decrease depends on the pyruvate concentration (Fig. 3) and the pH value of the solution but is zero order with respect to N^6-SL-NAD$^+$ and the enzyme in the concentration range studied. N^6-SL-NAD$^+$ was varied from 50 μM to 300

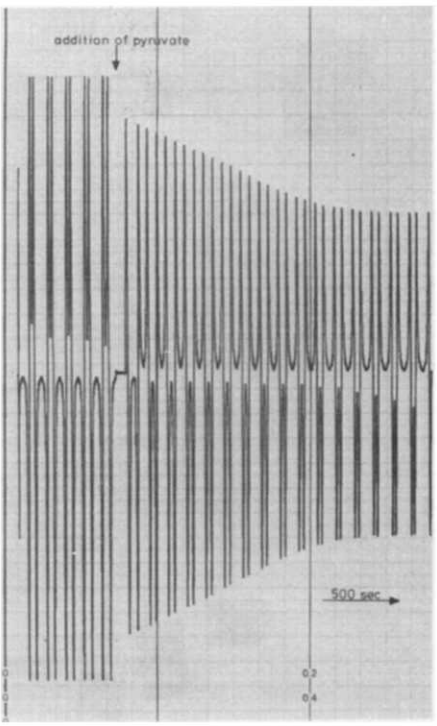


Fig. 2. Time dependent changes in the amplitude of the high-filed peak of the ESR spectrum of free N^6-SL-NAD$^+$ after addition of 22 mM pyruvate to a mixture of 130 μM lactate dehydrogenase, B form and 160 μM N^6-SL-NAD$^+$. Repetitive scans over 10 Gauss, upfield and downfield.

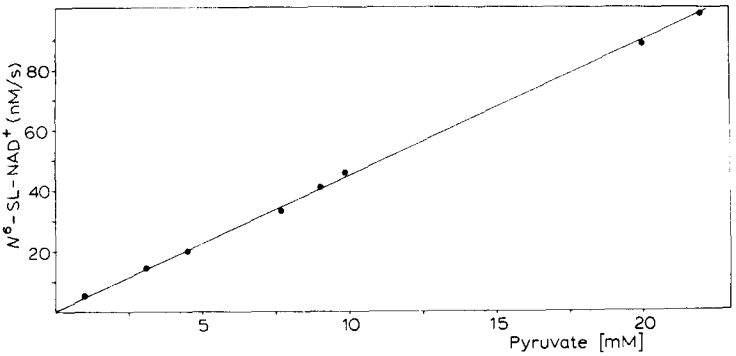


Fig. 3. Rate of N^6-SL-NAD$^+$ binding to lactate dehydrogenase, B form, depending on the pyruvate concentration in 67 mM phosphate buffer, pH 7.2.

μM and the enzyme from 60 μM to 150 μM (based on subunit molecular weight). Under these conditions the concentration of the binary E · N^6-SL-NAD$^+$ complex before addition of pyruvate varies from 6—57 μM as determined by ESR spectroscopy from the fraction of free N^6-SL-NAD$^+$ in the presence of enzyme, corresponding to a dissociation constant of 410 ± 30 μM.

The linear part of the time course, depending on the pyruvate concentration, varies slightly from 80 to 85% of the total reaction corresponding to a residual concentration of free enzyme below 30 μM. At high pH (9.5) only 65—70% of the reaction are of zero order, i.e deviation occurs below 80 μM free enzyme.

The apparent dissociation constant of SL-NAD$^+$ as calculated from the end values of ESR signals of free N^6-SL-NAD$^+$ varies from 2 μM to 4 μM depending on the pyruvate concentration (7—20 mM). At 6 mM pyruvate, as utilized for the ultraviolet binding studies it is in excellent agreement with the value from those experiments. 3.2 ± 0.4 μM as compared to 3.7 ± 0.4 μM. Table I summarizes these results.

In contrast, however, to ultraviolet studies of pyruvate binding to the binary E · N^6-SL-NAD$^+$ complex a considerable immediate drop in the concentration of free N^6-SL-NAD$^+$ is observed after pyruvate addition. After appropriate corrections for dilution and extrapolation of the linear decrease to zero time as detailed in the methods section this initial drop can be used to calculate an apparent dissociation constant for N^6-SL-NAD$^+$ in a ternary E · N^6-SL-NAD$^+$ · Pyr complex (see discussion section; Table I). This $K_{D(app)}$ is related to the dissociation constant of the binary E · N^6-SL-NAD$^+$ complex, K_D and the concentration of free pyruvate by Eqn. 1 [19], where $K_{D(Pyr)}$ is the dissociation constant of pyruvate from the ternary complex. Since the concentration of pyruvate is practically identical to its total concentration (the initial complex never exceeded 0.15% of the total pyruvate) Eqn. 1 may be transformed to Eqn. 2.

$$K_{D(app)} = \frac{K_D}{1 + \frac{[\text{pyruvate}]_{free}}{K_{D(Pyr)}}} \quad (1)$$

$$K_{D(Pyr)} = \frac{K_{D(app)} \times [\text{pyruvate}]_{total}}{K_D - K_{D(app)}} \quad (2)$$

TABLE I

BINDING OF N^6-SL-NAD^+ AND PYRUVATE IN VARIOUS BINARY AND TERNARY COMPLEXES WITH A AND B FORMS OF LACTATE DEHYDROGENASE AT 24°C IN 67 mM PHOSPHATE BUFFER (pH 7.2), 67 mM PYROPHOSPHATE BUFFER (pH 8.5) AND 100 mM GLYCINE BUFFER (pH 9.5). THE STANDARD ERROR OF THE DISSOCATION CONSTANTS IS IN THE ORDER OF 10%.

pH	Concn. of lactate dehydrogenase B form (μM)	Concn. of N^6-SL-NAD^+ (μM)	Concn. of E · N^6-SL-NAD^+ (μM) *	K_D E · N^6-SL-NAD^+ (mM)	Concn. of pyruvate (mM)	$K_{D(app)}$ of N^6-SL-NAD^+ in E · N^6-SL-NAD^+ · Pyr_K (mM)	$K_{D(pyr)}$ in E · N^6-SL-NAD^+ · Pyr_K (mM)	K_D of N^6-SL-NAD^+ in E · N^6-SL-NAD-Pyr (μM)	Rate of E · N^6-NAD-Pyr formation (nM/s)
7.2	105 (130)	99 (160)	18 (32)	0.40	4.5	0.28	11	3.4	20
7.2	60—152 **	50—280 **	6—57	0.42	9.0	0.25	11	3.2	41
7.2	103(130)	97(160)	17(32)	0.40	20	0.15	12	2.0	88
8.5	130	160	—	—	4.5	—	—	—	40
8.5	130	160	21	0.70	9.0	0.37	10	—	78
9.5	130	160	18	0.90	4.5	0.90	—	—	59
	A form of lactate dehydrogenase								
7.2	90	97	15	0.41	9.9	0.41	—	—	—
7.2	90	97	14	0.45	20	0.35 ***	70 ***	—	—

* Before addition of pyruvate.

** A total of five different concentrations.

*** Values uncertain, lower limits.

The values of $K_{D(Pyr)}$ obtained at various pyruvate, N^6-SL-NAD$^+$ and enzyme concentrations are summarized in Table I.

Investigations with the A form of lactate dehydrogenase

Binding of N^6-SL-NAD$^+$ to the muscle isozyme was studied by ESR spectroscopy as described in the methods section. The dissociation constant of 430 ± 30 μM is virtually identical to that of the heart type enzyme (410 ± 30 μM) and is in good agreement with data from the literature for NAD$^+$ itself as determined by fluorescence measurements studying competition with NADH (500 ± 200 μM) [20,21]. Moreover, it has been shown [23] that binding of N^6-SL-NAD$^+$ is strictly hyperbolic to four independent binding sites, as had been demonstrated for the heart isozyme [12]. However, addition of pyruvate to this E · N^6-SL-NAD$^+$ complex leads to a rather different time course of N^6-SL-NAD$^+$ binding as compared to the cardiac muscle enzyme. There is no initial drop in the concentration of free N^6-SL-NAD$^+$ (after correction for dilution) and a first order reaction rather than the linear decrease is observed (at least under conditions comparable to those used for the heart isozyme). Fig. 4 shows the time course of such a reaction. From the semilogarithmic plot of the data inserted in Fig. 4, it can be seen that the reaction follows first order kinetics over 30% of the time course.

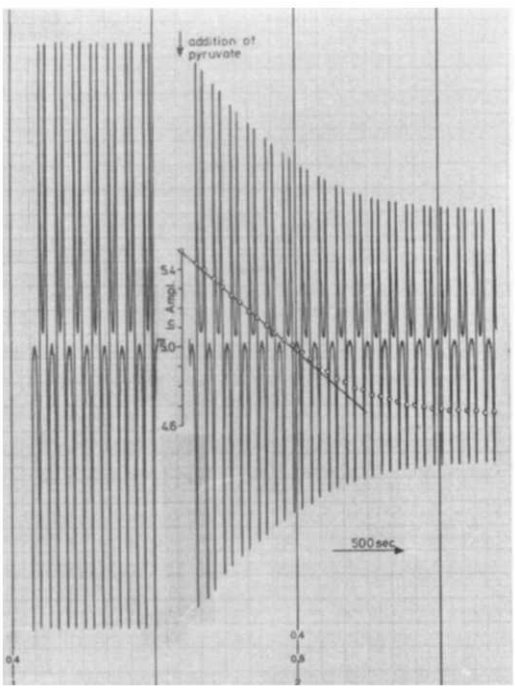


Fig. 4. Time dependent changes in the amplitude of the high-field peak of the ESR spectrum of free N^6-SL-NAD$^+$ after addition of 9.9 mM pyruvate to a mixture of 90 μM lactate dehydrogenase, A form and 97 μM N^6-SL-NAD$^+$. Repetitive scans over 10 Gauss, upfield and downfield. Insert: semilogarithmic plot of the signal amplitude versus time.

Discussion

A crucial question inherent in the application of any reported group is the extent to which this group causes perturbations in a particular 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 negligible. In the lactate dehydrogenase system N^6-SL-NAD^+ indeed, is an appropriate model for NAD^+ itself. As has previously been shown the kinetic constants are very similar [14]. Moreover, in the formation of the ternary E · NAD-Pyr complex N^6-SL-NAD^+ is indistinguishable from NAD^+ as demonstrated by their identical binding properties (Fig. 1). When the ESR signal intensity is used to calculate the concentration of free spin-labeled ligand one has to consider another possible error. Once a considerable fraction of the ligand is bound, its broadened absorption will contribute to the signal height of the free label even when calculations are based on the high-field peak for which this effect is minimal [13]. The ESR spectrum of N^6-SL-NAD^+ in the E · N^6-SL-NAD-Pyr complex is indeed almost identical to that observed in the E · N^6-SL-NAD^+ oxalate complex which has been described previously [12].

However, the contribution from the bound signal may obviously be disregarded because the dissociation constants calculated for the E · NAD-Pyr complex from the final values in ESR experiments, i.e. at high saturation fractions, are identical to the ultraviolet data within the limits of error (3.2 ± 0.4 μM and 3.7 ± 0.4 μM). Following the decrease in the N^6-SL-NAD^+ signal height after addition of pyruvate (at only low saturation fractions) therefore provides an excellent tool to measure coenzyme binding in any feasible enzyme complex. Following the increase in the 325 nm absorption or the decrease of the tryptophan fluorescence as being applied by Burgner and Ray [10], however, allows for determination of E · NAD-Pyr formation only.

Comparison of the N^6-SL-NAD^+ binding curves after pyruvate addition to the binary E · N^6-SL-NAD^+ complexes of the A and B forms of the enzyme reveals two important differences. Insteady of a simple first order reaction with the A type an immediate drop in the free N^6-SL-NAD^+ concentration after pyruvate addition and a subsequent zero order process is observed with the B type. A zero order reaction with a rate constant dependent exclusively on the pyruvate concentration has been postulated by Burgner and Ray for the dogfish skeletal muscle enzyme at high E · NAD^+ to pyruvate ratios [8], conditions where enolization of pyruvate is rate limiting. Obviously the enolization is rate limiting in case of the cardiac muscle enzyme even at very low E · N^6-SL-NAD^+ concentrations. As would be expected the enolization rate and therefore the rate of N^6-SL-NAD^+ binding increases considerably with pH and deviation from linearity is observed much earlier (65—70% conversion as compared to 80—85%), i.e. already at higher free enzyme concentrations. How may the immediate reaction in case of the B enzyme be explained? Part of it could indeed result from complex formation with the enol form of pyruvate present at equilibrium. However, its concentration never exceeded $8 \cdot 10^{-8}$ M as estimated from the equilibrium constant of the enolization of pyruvate ($4 \cdot 10^{-6}$) [8]. This would account for 0.2—0.6% of the total initial reaction only, which may therefore be attributed to formation of a E · N^6-SL-NAD^+ · Pyr_K complex. For

calculating the dissociation constant of pyruvate from this complex (11 ± 1 mM) we have tacitly assumed one binding site per subunit of the enzyme. This seems justified on the basis of our data derived from the ultraviolet experiments (Fig. 1). Burgner and Ray [10] have estimated this constant from kinetic experiments to be in the order of 5 mM. However, they state that this values is rather uncertain because of 'inner filter effects in the fluorescence assay at sufficiently high pyruvate concentrations'. Our value does well explain the substrate inhibition observed in standard enzyme assays when pyruvate concentrations above 1—2 mM are used. In vivo, pyruvate concentration is unlikely to be sufficiently high to allow for the E · NAD^+ · Pyr_K complex to play a regulatory role in the cardiac muscle lactate dehydrogenase. This is suggested by perfusion experiments with rat hearts, which indicate that the lactate/pyruvate ratio is at its equilibrium value, except when unnormally high pyruvate concentrations (10 mM) are used in the perfusate [22].

At high pH we did not observe the immediate reaction, although this does not exclude formation of an E · N^6-SL-NAD^+ · Pyr_K complex. The enolization and subsequent adduct formation under these conditions, however, is rapid making low pyruvate concentrations necessary for extrapolation to zero time. At these low concentrations, however, a complex with such a rather high dissociation constant can not be observed.

A similar argument holds for the skeletal muscle enzyme. Clearly there is no E · N^6-SL-NAD^+ · Pyr_K complex being formed that exhibited a dissociation constant comparable to that in the B form. A complex with a considerably higher dissociation constant can not be excluded. At sufficient high E · N^6-SL-NAD^+ or pyruvate concentrations adduct formation would be too fast in order to determine the 'dead end' ternary complex by our method. However, at 20 mM pyruvate we may have observed such a complex. Limiting values for its dissociation constant (>70 mM) are given in Table I.

The rapidly equilibrating E · NAD^+ · Pyr_K complex in the B enzyme offers a reasonable explanation for the differences in the reaction order observed with the two isozymes. Formation of the covalent adduct complex, E · N^6-SL-NAD-Pyr is assisted by prior formation of the 'dead-end' complex leaving the enolization of pyruvate rate limiting over a wide E · NAD^+ concentration range. This implies that enolization of pyruvate bound to the enzyme is possible which is in accordance with the kinetic model proposed by Burgner and Ray [10]. In the A form such an acceleration of E · NAD-Pyr formation does not occur in the concentration range studied and the E · NAD^+ concentration thus becomes rate limiting.

Acknowledgement

This work has been supported by the Deutsche Forschungsgemeinschaft.

References

1 Holbrook, J.J., Liljas, A., Steindl, S.J. and Rossmann, M.G. (1975) in The Enzymes (Boyer, P.D., ed.), 3rd edn., Vol. III, pp. 191—292, Academic Press, New York

2 Everse, J. and Kaplan, N.O. (1973) Adv. Enzymol. Relat. Areas Mol. Biol. 37, 66
3 Burgner, J.W., Ainslie, G.R., Cleland, W.W. and Ray, W.J. (1978) Biochemistry 17, 1646—1653
4 Novoa , W.B., Winer, A.D., Glaid, A.J. and Schwert, G.W. (1959) J. Biol. Chem. 234, 1143—1154
5 Di Sabato, G. (1968) Biochem. Biophys. Res. Commun. 33, 688—695
6 Everse, J., Barnet, R.E., Thorne, C.J.R. and Kaplan, N.O. (1971) Arch. Biochem. Biophys. 143, 444—460
7 Jeckel, D., (1975) Habilitationsschrift, Universität Heidelberg
8 Burgner, J.W. and Ray, W.J. (1974) Biochemistry 13, 4229—4237
9 Sugrabova, N.P., Kurganov, B.I. and Yakovlev, V.A. (1975) Biokhimiya 40, 281—289
10 Burgner, J.W. and Ray, W.J. (1978) Biochemistry 17, 1654—1661
11 Holbrook, J.J. (1972) Biochem. J. 128, 921—931
12 Wenzel, H.R., Pfleiderer, G., Trommer, W.E., Paschenda, K. and Redhardt, A. (1976) Biochim. Biophys. Acta 452, 292—301
13 Berliner, L.J. (1976) Spin Labeling, Theory and Applications, 1st edn. Academic Press, New York
14 Trommer, W.E., Wenzel, H.R. and Pfleiderer, G. (1974) Liebigs Ann. Chem. 1357—1359
15 O'Carra, P., Barry, S. and Corcoran, E. (1974) FEBS Lett. 43, 163—168
16 Wieland, T., Duesberg, P., Pfleiderer, G., Stock, A. and Sann, E. (1962) Arch. Biochem. Biophys. Suppl. 1, 260—263
17 Bergmeyer, H.U. (1970) Methoden der Enzymatischen Analyse, 2nd edn., Verlag Chemie, Weinheim/Bergstrasse
18 Engel, G. (1974) Anal. Biochem. 61, 184—191
19 Schmid, F., Hinz, H.-J. and Jaenicke, R. (1976) Hoppe Seyler's Z. Physiol. Chem. 357, 241—245
20 Stinson, R.A. and Holbrook, J.J. (1973) Biochem. J. 131, 719—728
21 Hinz, H.-J., Steininger, G., Schmid, F. and Jaenicke, R. (1978) FEBS Lett. 87, 83—86
22 Williamson, J.R. (1965) J. Biol. Chem. 240, 2308—2321
23 Deparade, M.P. and Trommer, W.E. (1979) Biochim. Biophys. Acta, in press