Oliveria, E., Martins, A., & Camargo, C. (1976) *Biochemistry 15*, 1967.
Pataki, G. (1963) *J. Chromatogr. 12*, 541.
Prasad, C., & Peterkofsky, A. (1976) *J. Biol. Chem. 251*, 3229.
Reichlin, S., Saperstein, R., Jackson, I., Boyd, A., & Patel, Y. (1976) *Annu. Rev. Physiol. 38*, 389.
Righetti, P., & Drysdale, J. W. (1971) *Biochim. Biophys. Acta 236*, 17.
Riley, R., & Coleman, M. (1968) *J. Lab. Clin. Med. 72*, 1974.
Schaeffer, J., Brownstein, M., & Axelrod, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3579.
Tate, S. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1780 (Abstract).
Taylor, W. L., & Dixon, J. E. (1976) *Biochim. Biophys. Acta 444*, 428.
Weber, J., Pringle, J., & Osborn, M. (1972) *Methods Enzymol. 26*, 3.
Yoshimoto, T., Fischl, M., Orlowski, R., & Walter, R. (1978) *J. Biol. Chem. 253*, 3708.

Structure of Lactate Dehydrogenase Inhibitor Generated from Coenzyme†

Jean-François Biellmann,* Claude Lapinte, Erich Haid, and Guenter Weimann

ABSTRACT: Two inhibitors of lactate dehydrogenase generated during NADH storage have been isolated by chromatography. One is a dimer of the dinucleotide where the AMP moiety is unmodified. The other is also generated from NAD^+ in the presence of a high concentration of phosphate ions at alkaline pH. This inhibitor was proved to be the addition compound of one phosphate group to position C-4 of the nicotinamide ring of NAD^+ by NMR spectroscopy, enzymatic cleavage, and dissociation to NAD^+ at neutral pH. This compound is a competitive inhibitor with respect to NAD^+ in the presence of the lactate dehydrogenase with a K_i of 2×10^{-7} M. The interaction of this inhibitor with lactate dehydrogenase is discussed relative to the structure of this enzyme.

Inhibitors of dehydrogenases appearing during storage of $NAD(P)^+$ and NAD(P)H have been known for a long time (Dalziel, 1961, 1962, 1963; Fawcett et al., 1961; Fine et al., 1962; Silverstein, 1965; Holman et al., 1966; Strandjord & Clayson, 1966; McComb & Gay, 1968; Härtel et al., 1968; Klotzsch et al., 1969; Babson & Arndt, 1970; Berry et al., 1973; Gerhardt et al., 1974; Gallati, 1976a,b,c; Loshon et al., 1977; Margolis et al., 1977).[1] The inhibitors are responsible for erratic kinetic results and falsify analytical determinations. Earlier, ADPR had been characterized as the inhibitor for alcohol dehydrogenase present in the NAD^+ preparation (Dalziel, 1961, 1962; Yonetani, 1963). For the dihydrofolate reductase from *Lactobacillus casei*, a dependence on the source and the treatment of NADPH has been noticed for the enzymic activity and was attributed to inhibitors present in NADPH (Williams et al., 1977).

Very strong inhibitors for lactate dehydrogenases from various origins appear during the storage of NADH in the presence of air or moisture as well as by repeatedly freezing and thawing NADH solutions (Fawcett et al., 1961; Silverstein, 1965; Strandjord & Clayson, 1966; McComb & Gay, 1968; Härtel et al., 1968; Klotzsch et al., 1969; Berry et al., 1973; Gerhardt et al., 1974; Gallati, 1976a,c). It is not known whether these different treatments yielded the same compounds. Since ADPR, adenosine diphosphate, and AMP are poor inhibitors for the lactate dehydrogenases (Geyer, 1968; McPherson, 1970), the inhibitors have presumably different structures. Isolation procedures of these compounds have been published but no definite structures have been established for them (Fine et al., 1962; Strandjord & Clayson, 1966; McComb & Gay, 1968).

Since lactate dehydrogenase is of major analytical importance in clinical diagnoses (Schmidt & Schmidt, 1976), it is therefore essential to avoid the formation of these inhibitors.[2] For this reason a structural determination of these inhibitors was undertaken. We shall report on the structure of one inhibitor, which was also formed from NAD^+ and phosphate at alkaline pH (Gallati, 1976a,b,c), and on the partial structure of another one. The third inhibitor has not been studied here.

Materials

NAD^+, NADH, NADPH, AMP, NMN, ADPR, sodium pyruvate, and alkaline phosphatase from calf intestine (grade I), phosphodiesterase from snake venom (Crotalus), lactate dehydrogenase from rabbit muscle, and yeast alcohol dehydrogenase were obtained from Boehringer Mannheim Corp.; Sephadex (R) G-15 and QAE-Sephadex (R) A-25, from Pharmacia, Uppsala, Sweden.

Methods

Spectral Data. *^1H NMR spectra* were determined at 250 MHz with a Cameca spectrometer or at 90 MHz with a

† From the Institut de Chimie, Université Louis Pasteur de Strasbourg, Strasbourg, France (J.-F.B. and C.L.), and from Boehringer Mannheim GmbH, Biochemica Werk Tutzing, Tutzing, West Germany (E.H. and G.W.). *Received May 1, 1978.*

[1] Abbreviations used: AMP, adenosine monophosphate; NMN^+, nicotinamide mononucleotide; ADPR, adenosine diphosphate ribose; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Inh_1, inhibitor eluted first after NADH by chromatography on a QAE-Sephadex column; Inh_2, inhibitor eluted second after NADH by chromatography on a QAE-Sephadex column.

[2] Generally, a ratio of absorbance at 260–340 nm of less than 2.32 is a good indication of an inhibitor-free NADH preparation.

0006-2960/79/0418-1212$01.00/0

Brucker WH 90 spectrometer; *^{13}C NMR spectra*, at 62.86 MHz with a Cameca spectrometer or at 22.63 MHz with a Brucker WH 90 spectrometer; and *^{31}P NMR spectra*, at 36.43 MHz with a Brucker WH 90 spectrometer or at 62.86 MHz with a Cameca spectrometer. The nucleotide for the ^{1}H, ^{13}C, and ^{31}P NMR spectral determinations was transferred in D_2O solution in the following way: the nucleotide was absorbed on a QAE-Sephadex column (2 × 15 cm), which was first washed with D_2O, and then eluted with 1.2 M sodium chloride solution in D_2O. The nucleotides were eluted after the void volume.

The absorption spectral data were measured with a Cary 118 spectrophotometer.

Gel Filtration. The molecular weight was determined by gel filtration on a Sephadex G-15 fine column (1.5 × 35 cm) with 0.1 M Tris buffer, pH 8.5, as an eluant: flow rate 1 mL/min (Burnett & Underwood, 1968). The reference compounds were NADH, NADPH, and the 4,4′ dimer of NAD^+ produced by Zn–Cu reduction (Biellmann & Lapinte, 1978). Absorption at 260 nm was used for the detection.

Electrophoresis. Electrophoretic mobility was determined in an apparatus similar to the one described by Markham & Smith (1952) on 13 cm wide Whatman 3 MM strips with 450 V for 60 min. The buffer was 0.05 M triethylammonium bicarbonate, pH 7.5. The compounds were detected with a UV lamp at 254 nm.

Inhibition Test. In order to locate the inhibitors, a simple test was devised. The inhibition was determined by the initial rate of the reduction of pyruvic acid under the following conditions: 2.5 mL of 10 mM potassium phosphate buffer, pH 7.5; 0.7 mM of sodium pyruvate; 10 μL of 15 mM NADH solution; 10 μL of an inhibitor solution approximately 15 mM (determined by using the extinction coefficient of NADH at 260 nm); and 5 μL of a lactate dehydrogenase from rabbit muscle about 500 UI. The inhibition power is defined as the difference in rates in the absence and presence of inhibitors expressed as a percentage of the rate when the inhibitor is absent.

$$\frac{V_{(NADH)} - V_{(NADH+Inh)}}{V_{(NADH)}} \times 100$$

Isolation Method of Inh_1 and *Inh_2*. NADH (20 g), stored in the dark in a water-saturated atmosphere for 2–3 days, was used in order to increase the yield of inhibitors (inhibition power of about 20%). The yellow product was applied at 4 °C to a QAE-Sephadex column, and the chromatography was performed as published (Haid et al., 1975), except that a gradient of 0.1–0.3 M sodium chloride (10 L:10 L; pH 8) was applied. NMN, NAD^+, AMP, and ADPR were eluted before NADH; the inhibitors appeared after NADH. Our elution diagram was similar to the published one except that better resolution was obtained. At least three inhibitors were eluted. The first was called Inh_1 and the second Inh_2. A third inhibitor was present in minute and variable amounts and its structure was not further investigated. The solutions of Inh_1 and of Inh_2 could not be concentrated under reduced pressure without changing the ratio of absorption at 260–340 nm. Therefore the inhibitors were precipitated as barium salts. A 1.3 equiv of barium acetate (calculated on the basis of an equal molar extinction coefficient for the inhibitors and NADH) and twice its volume of ethanol were added. After 1 h at 4 °C, the precipitate was centrifuged, washed with ethanol, and dried under reduced pressure. Solutions of the inhibitors were prepared by suspending the barium salts in water, then adding stoichiometric amounts of sodium sulfate while stirring, holding the pH between 7.5 and 8, and finally removing the barium sulfate by centrifugation.

The UV absorption spectrum of both inhibitors showed maxima at 260 and at 340 nm. A freshly prepared solution of Inh_1 showed an OD_{260}/OD_{340} ratio of about 5. The lability of Inh_1 is indicated by an increase of this ratio with time as well as by the decrease of the inhibition in the lactate dehydrogenase assay. Freshly prepared solutions of inhibitors were used for the molecular weight determinations by gel filtration, for electrophoresis, and for the cleavage by alkaline phosphatase and phosphodiesterase.

Preparation of Inh_2. The pH of a solution of NAD^+ (2.5 g) and dipotassium hydrogen phosphate (3.5 g) in doubly distilled water (10 mL) was adjusted to 11 by the addition of potassium hydroxide. After 13 h of incubation in the dark, the solution was diluted with water to a volume of 350 mL and the pH adjusted to 10 by adding hydrochloric acid. Inh_2 was isolated by ion-exchange chromatography on a QAE-Sephadex column (3 × 50 cm) with a gradient of sodium chloride, 0.1 M–0.4 M (0.9 L:0.9 L), buffered at pH 10.0 with 5 mM sodium carbonate. The elution was followed by absorption measurements at 260 nm and by the inhibition test described above. Inh_2 eluted at 0.3 M sodium chloride. A yield of 350 mg of Inh_2 was obtained by this procedure.

The product prepared from NAD^+ and phosphate at a high pH, as described above, and Inh_2 were identical as shown by electrophoresis, thin-layer chromatography, and identification of the cleavage products by alkaline phosphatase and by phosphodiesterase.

Decomposition of Inh_2. When a 5×10^{-4} M solution of Inh_2 was kept in 0.05 M Tris buffer, pH 7, NAD^+ was produced and determined with yeast alcohol dehydrogenase. After 18 h of incubation, 30% NAD^+ was formed; after 90 h the amount of NAD^+ increased to 54%. At pH 1, 30% of NAD^+ was found after 1 h of incubation.

Action of Alkaline Phosphatase on Inh_2. Inh_2 barium salt (10 mg) was dissolved in 10 mM sodium sulfate solution (5 mL) while stirring. After removal of the barium sulfate by centrifugation, 1 mL of the supernatant was mixed with 1 mL of 1 M Tris buffer, pH 9, and 10 μL of a suspension of alkaline phosphatase from calf intestine. After 1 h at 37 °C, 3% inhibition with lactate dehydrogenase was observed instead of 72% obtained with an equivalent sample which was not treated with alkaline phosphatase. The electrophoretical properties of the treated Inh_2 were very close to those of NADH. However, no enzymatic reaction of this product could be observed with yeast alcohol dehydrogenase and rabbit muscle lactate dehydrogenase.

Action of Phosphodiesterase on Inh_2. One milliliter of 0.02 M Tris buffer, pH 8.9, and 10 μL of phosphodiesterase from snake venom were added to 1 mL of the solution of Inh_2 prepared as described above. After 1 h at 37 °C, 5% inhibition with lactate dehydrogenase was observed instead of 72% with a sample of the untreated inhibitor. After electrophoresis two spots were detected, one with a light blue UV fluorescence like NADH but migrating faster than either NADH or NADPH and one migrating exactly like AMP. The AMP concentration could be determined enzymatically (Bergmeyer, 1974): 0.95 mol of AMP was found per mol of Inh_2.

Results and Discussion

A test procedure was devised, for the presence of inhibitors of lactate dehydrogenase, on the assumption that the inhibitors had the same molar extinction coefficient at 260 nm as NADH. From NADH stored in a moist atmosphere at least three compounds showing inhibitory properties were isolated

Table I: Electrophoretic Mobilities of Inhibitors and Related Nucleotides[a]

nucleotide	NAD^+	NADH	NADPH	ADPR	AMP	NMN	4,4′ dimer[d]	Inh_1	Inh_2	Inh_2[b]	Inh_2[c]
mobility	1	2.0	3.1	2.1	2.5	1.5	2.5	2.7	3.1	1.9	2.5, 3.6

[a] Performed on Whatman 3 MM paper in 0.05 M trimethylammonium bicarbonate buffer, pH 7.5. Voltage 450 V, time 1 h. Detection by UV lamp. NAD^+ used as a standard. [b] Inh_2 after alkaline phosphatase treatment. [c] Inh_2 after phosphodiesterase treatment. [d] 4,4′ dimer produced by zinc–copper or electrochemical reduction of NAD^+ (Biellmann & Lapinte, 1978).

by chromatography on QAE-Sephadex. Two of them were investigated in more detail. The product that eluted closest to NADH was called Inh_1 and the second, Inh_2.

Freshly prepared Inh_1 showed an inhibition power of over 90%. On gel filtration it eluted before NADH, at the same position as the dimer of NAD^+ prepared by Zn–Cu or electrochemical reduction (Burnett & Underwood, 1968; Biellmann & Lapinte, 1978), and was therefore assumed to be a dimeric substance. The mobility of the dimers and of Inh_1 was the same on electrophoresis. This inhibitor was not modified by alkaline phosphatase as shown by electrophoresis and enzymic properties. Phosphodiesterase cleaved Inh_1 to AMP and to a nucleotide migrating faster than NAD^+ or NADH, different from NMN^+, NMNH, and ADPR and showing a light blue fluorescence like NADH. The UV spectral data suggested the presence of a 1,4-dihydronicotinamide chromophore. The occurrence of AMP after enzymic cleavage indicated that the nicotinamide–ribose moiety was involved in the dimeric linkage. The 4,4′ dimers of NAD^+ produced electrochemically or by Zn–Cu reduction do not show any inhibition in the lactate dehydrogenase reaction. Thus Inh_1 is not a 4,4′ dimer. Inh_1 proved to be quite unstable; for this reason its structural determination was discontinued.

The inhibitor Inh_2, present in rather small amounts, was shown not to originate directly from NADH but from NAD^+. Actually, incubation of NAD^+ alone at pH 14 resulted in a higher inhibition than incubation of an equimolar mixture of NAD^+–NADH. We soon realized that the inhibition increased when incubation of NAD^+ was performed in the presence of phosphate at a high pH. Therefore a simple preparation of the inhibitor Inh_2[3] could be achieved by the incubation of NAD^+ in the presence of phosphate ions.

Fortunately the inhibitor Inh_2 proved to be stable enough for a structural investigation. The barium salt could be isolated and was estimated to be 95% pure on the basis of the AMP determination after enzymatic degradation with phosphodiesterase. The UV absorption data determined for Inh_2 were λ max 260 nm (ϵ 15 000) and 343 (5000) (Figure 1). It was found to be related to that of NADH. The molecular weight of Inh_2 was close to that of NADH, thus excluding a dimeric structure for Inh_2. The identity of Inh_2 with α-NADH, which is known to inhibit lactate dehydrogenase (Pfleiderer et al., 1965), was rejected because Inh_2 has an electrophoretical behavior like NADPH (Table I), suggesting that Inh_2 has additional charges compared to NADH. The cleavage experiments with alkaline phosphatase and phosphodiesterase unambiguously showed that the AMP part was not modified (Table I). Therefore a structure derived by the addition of a phosphate anion to the C-4 position of the nicotinamide ring of NAD^+ was proposed. It has been stated that one NAD^+ analogue phosphorylated on the nicotinamide ribose has no inhibitory properties for lactate dehydrogenase (Kuwahara et al., 1971). Also X-ray diffraction data for the lactate dehydrogenase show that there is no space for an additional group at the C-2 or C-3 position of the nicotinamide ribose (Holbrook et al., 1975).

[3] The identity of Inh_2 and the inhibitor isolated and studied by Gallati (1976a,c) relies on a similar mode of preparation. However the UV spectrum of Gallati's inhibitor shows an OD_{260}/OD_{340} ratio of 26 (Gallati, 1976c) instead of 3 for Inh_2.

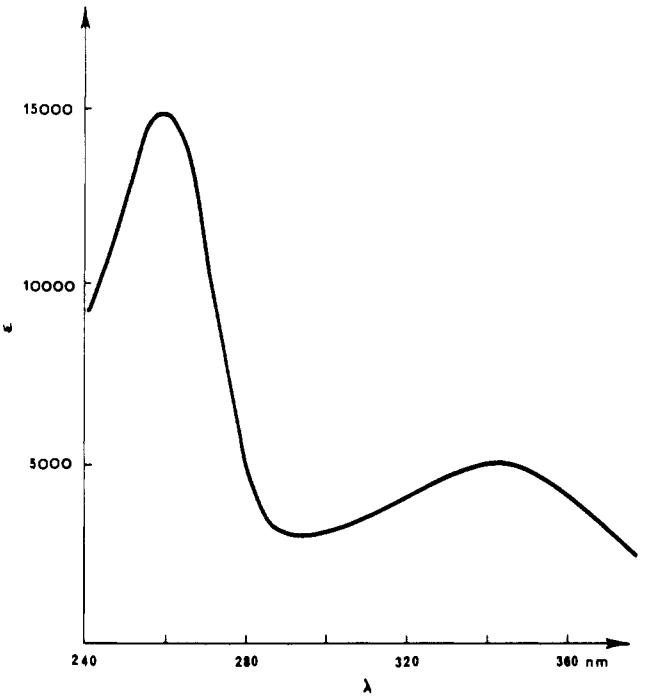


FIGURE 1: UV absorption spectrum of Inh_2 barium salt in 40 mM phosphate buffer, pH 6.9.

Table II: ^1H NMR Chemical Shifts[a]

	nicotinamide ring				adenine ring	
	$H-N_2$	$H-N_4$	$H-N_5$	$H-N_6$	$H-A_2$	$H-A_8$
NAD^+	9.32	8.84	8.23	9.20	8.10	8.30
NADH	6.94	2.73	4.52	5.99	8.20	8.47
$NAD^+ + CN^-$	7.22	4.49	4.73	6.08	8.15	8.42
Inh_2	7.42 7.36	–	4.60	6.39	8.18	8.58

[a] Measured at 250 or 90 MHz in D_2O (1000 scans). Chemical shifts expressed in parts per million from sodium trimethylsilylpropionate-d_4 as an internal standard. pH 10.0 (not corrected) at 25 °C. Concentration 6–10 mg of dinucleotide/mL (Fourier transform).

The ^{31}P NMR spectrum of Inh_2 reinforced this hypothesis; the singlet at $\delta = -21.7$ ppm (phosphoric acid as an external standard) was assigned to the pyrophosphate ($\delta = -22.0$ ppm for NADH) and the multiplet at $\delta = 9.3$ ppm was attributed to a phosphoric monoester ($\delta = 7.7$ ppm for monophosphate of NADPH). The ratio of the integration of these two signals was 2/1. In the ^1H NMR spectrum of Inh_2 (Table II) the signals attributed to the protons at N_2, N_5, and N_6 were very close to those of NADH and to those of the NAD^+–cyanide adduct. There were two signals for $H-N_2$, and the signal of $H-N_6$ had a complex pattern like those of the NAD^+–cyanide and 3-acetylpyridine AD^+–cyanide adducts (Oppenheimer et al., 1971). This is probably due to diastereoisomerism at C-4. The signal corresponding to $H-N_4$ was hidden by the signals

Table III: ^{13}C NMR Chemical Shifts[a]

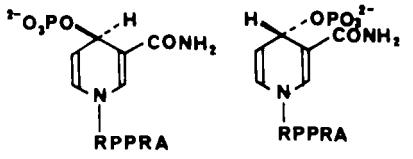
	N_2	N_3	N_4	N_5	N_6	C=O	N_1'	N_2', N_3'	N_4'	N_5'
NADH	140.9	102.9	24.7	108.0	127.0	175.1	98.0	73.7	84.9	68.7
Inh_2	147.0 143.9	101.1	78.7	103.6	136.3 132.7	172.8	97.6	71.9	84.6	65.9
	A_8	A_6	A_5	A_4	A_2	A_1'	A_2'	A_3'	A_4'	A_5'
NADH	142.3	157.9	121.1	153.1	155.4	90.1	73.2	77.3	86.3	68.0
Inh_2	141.6	156.5	119.6	150.0	154.1	88.5	71.6	75.7	85.06	66.7

[a] Expressed in parts per million from sodium trimethylsilyl propionate-d_4 as an internal standard. Measured at 15.08 or 62.86 MHz in D_2O. pH 10.0 (not corrected) at 25 °C. Concentration, 70–200 mg/mL (Fourier transform).

of the ribose protons. The ^1H decoupled ^{13}C NMR spectrum of Inh_2 was very similar to that of NADH (Table III), except for two features. The signal attributed to N_4 of the dihydronicotinamide had a chemical shift of 78.7 ppm for Inh_2 and 24.7 ppm for NADH. The difference of chemical shift, 54 ppm when going from C–H to C–OPO_3^{2-}, compares quite well with the published data: 50 ppm for such a change (Dorman & Roberts, 1970). On the other hand, the signals of the N_2 and N_6 of the dihydronicotinamide appeared as doublets due to the stereoisomerism at N_4 (Biellmann & Lapinte, 1978).

The ^1H–^{31}P coupling was hardly detectable in the ^1H NMR spectrum of Inh_2; this is due to the coupling with other protons. In ^{13}C NMR, signals attributed to the dihydronicotinamide carbons appeared as doublets; coupling constants were for N_4, 2 Hz, for N_3 and N_5, 5 Hz, and for N_2 and N_6, 2 Hz.

From these data, structure **1** was attributed to Inh_2 which

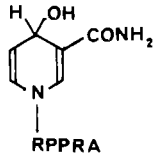


1

RPPRA = ribose-pyrophosphate-adenosine

is formed by the addition of one phosphate ion to position 4 of the nicotinamidinium ring of NAD^+. The nucleophilicity of phosphate trianion PO_4^{3-} must be quite high but has not been determined. The synthesis of phosphoric monoester by the reaction of a halide with PO_4^{3-} is indicative of a strong nucleophilicity of this trianion (Bailly, 1919a,b). In contrast to hydroxide ion which is a hard base and reacts with NAD^+ at position 2, PO_4^{3-} behaves like a soft base and reacts with NAD^+ at position 4 like cyanide and dithionite (Guilbert & Johnson, 1977; Klopman, 1968).

The action of alkaline phosphatase on Inh_2 retains the original dihydropyridine structure according to the UV absorption spectrum. This compound is not oxidized by acetaldehyde in the presence of yeast alcohol dehydrogenase. This compound could have the structure **2**, which turns out to be



2

stable, hydroxide ion being a very poor leaving group. The chemical behavior of **2** is worthwhile for further investigations. A similar adduct has been found to be stable (Wallenfels & Hanstein, 1965).

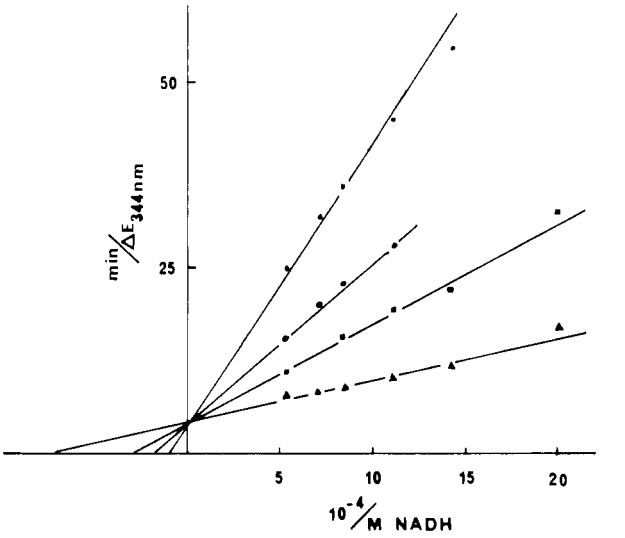
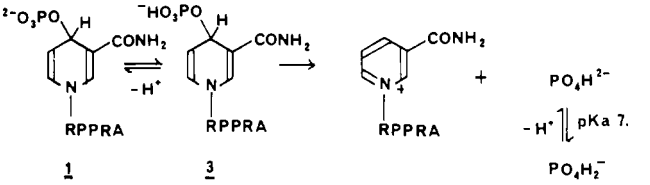


FIGURE 2: Inhibition of pyruvate reduction with NADH by Inh_2 in 40 mM phosphate buffer, pH 6.9; pyruvate, 0.26 mM; lactate dehydrogenase from rabbit muscle, 1.1×10^{-8} M (SU); NADH varied from 5 to 19 μM; Inh_2 was 0 (▲), 0.25 (■), 0.75 (○), and 1.5 μM (●). K_m of NADH = 1.2×10^{-5} M and K_i of Inh_2 = 2×10^{-7} M.

The dissociation of Inh_2 to NAD^+ at pH 7 might be due to the equilibrium with the protonated species **3** which dis-



1 **3**

sociates to NAD^+ and phosphate anions PO_4H^{2-} and $PO_4H_2^-$ which are not very nucleophilic. The pK_{a2} of phosphoric monoesters $ROPO_3^{2-} + H^+ \rightleftharpoons ROPO_3H^-$ is 6.1–6.2 (Dawson et al., 1969).

The treatment of Inh_2 at pH 1 also generates NAD^+ but at a lower yield. The modification of the 1,4-dihydropyridine structure under acidic conditions may compete with the C–O cleavage of the phosphate ester. The dissociation of Inh_2 to NAD^+ is reminiscent of the dissociation of the cyanide adduct of NAD^+ to NAD^+ and cyanide at neutral pH (Colowick et al., 1951).

This dissociation is comparable to the C–O cleavage of the *p*-nitrophenyl phosphate–amine adduct with loss of the phosphate group, yielding a *p*-nitroaniline derivative (Kirby & Jencks, 1965).

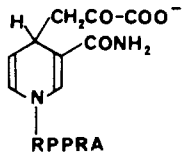
Under the more or less alkaline conditions where NADH is manufactured and stored, hydrolysis of NADH occurs, giving rise to phosphate ions. These ions subsequently react with NAD^+, which is present in minute amounts in even extremely purified NADH preparations (Eisner & Kuthan,

1972) and is further increased on prolonged storage, to form the adduct Inh_2.

The inhibition constant of Inh_2 was determined, the enzyme added last, in contrast to the conditions under which Gallati (1976c) performed his measurements, i.e., after incubation of the inhibitor with the enzyme for 10 min. Inh_2 was found to be a competitive inhibitor with respect to NADH with $K_i = 2 \times 10^{-7}$ M as shown in Figure 2. When Inh_2 was preincubated with the enzyme, the inhibition was increased as reported by Gallati (1976c).

This behavior as a coenzyme competitive inhibitor suggested that Inh_2 binds at the coenzyme binding site. According to the structure of lactate dehydrogenase from Dogfish (Holbrook et al., 1975), the pyruvate–lactate site is constituted essentially by Arg-171 and His-195. The guanidinium group of Arg-171 interacts with the carboxylate of lactate or pyruvate while the imidazole of His-195 is responsible for the catalysis by proton transfer. Strong inhibition appears on incubation of lactate dehydrogenase with NAD^+ and pyruvate at high pyruvate concentrations. This has been attributed to the formation of an abortive ternary complex: NAD^+, pyruvate, and the enzyme.

The product **4** arises from the addition of the enol form of



4

pyruvate to NAD^+ (Arnold & Kaplan, 1974).

The structure of the complex of this adduct with the lactate dehydrogenase has been determined (Adams et al., 1973). The carboxylate interacts with the guanidinium group of Arg-171. The binding constant of this adduct, **4**, with lactate dehydrogenase has been estimated to be less than 10^{-9} M (Arnold & Kaplan, 1974). This value compares well with the value obtained with Inh_2, suggesting that the binding of one of the stereoisomers of the phosphate–NAD^+ adduct is reinforced by the additional phosphate group interacting with Arg-171.

Added in Proof

Since this paper was submitted for publication, Godfredsen & Ottesen (1978) reported that 1,6-dihydro-NAD^+ is a potent inhibitor for lactate dehydrogenase and is presumably identical with a humidity-induced inhibitor.

Acknowledgments

We thank F. Hemmert, R. Graff (Strasbourg), and G. Beranger (Gif) for the NMR spectra and P. Weigert (Tutzing) for technical assistance.

References

Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A., Rossmann, M. G., Smiley, I. E., Allison, W. S., Everse, J., Kaplan, N. O., & Taylor, S. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1968–1972.

Arnold, L. J., Jr., & Kaplan, N. O. (1974) *J. Biol. Chem. 249*, 652–655.

Babson, A. L., & Arndt, E. G. (1970) *Clin. Chem. (Winston-Salem, N.C.) 16*, 254–255.

Bailly, O. (1919a) *C. R. Hebd. Seances Acad. Sci. 168*, 560–563.

Bailly, O. (1919b) *Bull. Soc. Chim. Fr. 25*, 240–260.

Bergmeyer, H. U. (1974) *Methoden der Enzymatischen Analyse*, 3rd Ed., pp 2178–2181, Verlag Chemie, Weinheim/Bergstr., Germany.

Berry, A. J., Lott, J. A., & Grannis, G. F. (1973) *Clin. Chem. (Winston-Salem, N.C.) 19*, 1255–1258.

Biellmann, J. F., & Lapinte, C. (1978) *Tetrahedron Lett.*, 683–686.

Burnett, R. W., & Underwood, A. L. (1968) *Biochemistry 7*, 3328–3333.

Colowick, S. P., Kaplan, N. O., & Ciotti, M. M. (1951) *J. Biol. Chem. 191*, 447–459.

Dalziel, K. (1961) *Nature (London) 191*, 1098–1099.

Dalziel, K. (1962) *Biochem. J. 84*, 240–244.

Dalziel, K. (1963) *J. Biol. Chem. 238*, 1538–1543.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1969) *Data for Biochemical Research*, pp 103–121, Oxford University Press, London.

Dorman, D. E., & Roberts, J. D. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 19–26.

Eisner, U., & Kuthan, J. (1972) *Chem. Rev. 72*, 1–42.

Fawcett, C. P., Ciotti, M. M., & Kaplan, N. O. (1961) *Biochim. Biophys. Acta 54*, 210–212.

Fine, I., Fawcett, C. P., Everse, J., & Kaplan, N. O. (1962) *Abstracts of Papers, National Meeting of the American Chemical Society*, 55C.

Gallati, H. (1976a) *Z. Klin. Chem. Klin. Biochem. 14*, 3–8.

Gallati, H. (1976b) *Z. Klin. Chem. Klin. Biochem. 14*, 9–13.

Gallati, H. (1976c) *Z. Klin. Chem. Klin. Biochem. 14*, 15–22.

Gerhardt, W., Kofoed, B., Westlund, L., & Pavlu, B. (1974) *Scand. J. Clin. Lab. Invest. 33*, 3–51.

Geyer, H. (1968) *Hoppe-Seyler's Z. Physiol. Chem. 349*, 293–296.

Godfredsen, S. E., & Ottesen, N. (1978) *Carlsberg Res. Commun. 43*, 171–175.

Guilbert, C. C., & Johnson, S. L. (1977) *Biochemistry 16*, 335–344.

Haid, E., Lehmann, P., & Ziegenhorn, J. (1975) *Clin. Chem. (Winston-Salem, N.C.) 21*, 884–887.

Härtel, A., Helger, R., & Lang, H. (1968) *Z. Klin. Chem. Klin. Biochem. 6*, 259–262.

Holbrook, J. J., Liljas, A., Steindel, S. J., & Rossmann, M. G. (1975) *Enzymes, 3rd Ed. 11*, 191–292.

Holman, M. J., Willis, J., & Siegel, J. (1966) *Clin. Chem. (Winston-Salem, N.C.) 12*, 543.

Kirby, A. J., & Jencks, W. P. (1965) *J. Am. Chem. Soc. 87*, 3217–3224.

Klopman, G. (1968) *J. Am. Chem. Soc. 90*, 223–234.

Klotzsch, S. G., Klotzsch, H. R., & Haus, M. (1969) *Clin. Chem. (Winston-Salem, N.C.) 15*, 1056–1061.

Kuwahara, M., Tachiki, T., Tochikura, T., & Ogata, K. (1971) *Agric. Biol. Chem. 35*, 177–183.

Loshon, C. A., McComb, R. B., Bond, L. W., Bowers, G. N., Jr., Coleman, W. H. O., & Gwynn, R. H. (1977) *Clin. Chem. (Winston-Salem, N.C.) 23*, 1576–1580.

Margolis, S. A., Howell, B. F., & Schaffer, R. (1977) *Clin. Chem. (Winston-Salem, N.C.) 23*, 1581–1584.

Markham, R., & Smith, L. D. (1952) *Biochem. J. 52*, 552.

McComb, R. B., & Gay, R. J. (1968) *Clin. Chem. (Winston-Salem, N.C.) 14*, 754–763.

McPherson, A., Jr. (1970) *J. Mol. Biol. 51*, 39–46.

Oppenheimer, N. J., Arnold, L. J., & Kaplan, N. O. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 3200–3205.

Pfleiderer, G., Woenckhaus, C., & Nelböck-Hochstetter, M. (1965) *Justus Liebigs Ann. Chem. 690*, 170–176.

Schmidt, E., & Schmidt, F. W. (1976) *Kleine Enzym-Fibel*, Schriftenreihe Diagnostica Boehringer, Mannheim.
Silverstein, E. (1965) *Anal. Biochem. 12*, 199–212.
Strandjord, P. E., & Clayson, K. J. (1966) *J. Lab. Clin. Med. 67*, 144–153.
Wallenfels, K., & Hanstein, W. (1965) *Angew. Chem. 77*, 861–862.
Williams, T. J., Lee, T. K., & Dunlap, R. B. (1977) *Arch. Biochem. Biophys. 181*, 569–579.
Yonetani, T. (1963) *Acta Chem. Scand. 17*, S69–S101.

Quaternary Structure, Subunit Activity, and in Vitro Association of Porcine Mitochondrial Malic Dehydrogenase†

Rainer Jaenicke,* Rainer Rudolph, and Ingrid Heider

ABSTRACT: The native, dimeric quaternary structure of porcine mitochondrial malic dehydrogenase (m-MDH) remains unchanged upon dilution in the concentration range from 114 to 0.2 μg/mL, as shown by gel chromatography in 0.2 M phosphate buffer, pH 7.6, at 20 °C. In the given concentration range the enzyme can be reassociated and reactivated after dissociation and denaturation at acid pH, as well as in 6 M urea or 6 M guanidine hydrochloride. Removal of the denaturants and separation of inactive "wrong aggregates" lead back to a final yield of about 60% of renatured enzyme. Reactivated m-MDH is found to be indistinguishable from the native enzyme as far as quaternary structure, enzymatic activity, Michaelis constants, and spectral properties are concerned. The concentration dependence of the rate of reactivation and the sigmoidicity of the kinetic profiles may be described by a consecutive first-order transconformation and second-order association reaction. One set of rate constants, $k_1 = 6.5 \times 10^{-4}$ s^{-1} and $k_2 = 3 \times 10^4$ M^{-1} s^{-1} (20 °C), is sufficient to describe the kinetics of reconstitution, independent of the mode of denaturation used to disrupt the native structure of the enzyme. This implies (1) that there exists a common, folded monomeric intermediate which is formed prior to the rate-limiting reactions described by k_1 and k_2 and (2) that this monomer does not show full enzymatic activity. The presence of NAD^+ does not influence the kinetics of reactivation, when one uses the apoenzyme as a reference.

The acquisition of the native three-dimensional structure of proteins as the "kinetically accessible minimum of the potential energy" is an intrinsic property of the polypeptide chain which is determined by kinetic and thermodynamic factors (Wetlaufer & Ristow, 1973). In the case of oligomeric enzymes, composed of two or more subunits, it is of interest to know whether the isolated chains are able to maintain the enzymatically active tertiary structure, i.e., whether or not intersubunit interactions are required to attain the native overall configuration of the molecule. For a number of oligomeric enzymes, isolated monomers have been reported to be accessible at low enzyme concentrations; however, most oligomers are dissociable only by strong denaturants such as guanidine hydrochloride or urea or by extremes of pH. Under these conditions the isolated subunits do not show catalytic activity because dissociation is accompanied by deactivation and a more or less pronounced loss of secondary and tertiary structure. In general all three processes, dissociation, denaturation, and deactivation, are found to be reversible. Therefore, under certain conditions structured monomers are accessible as intermediates during reconstitution. To characterize their properties regarding the correlation of folding, association, and catalytic function, the kinetics of reactivation and renaturation can be applied (Jaenicke, 1978a).

Previous investigations on the reconstitution of some tetrameric dehydrogenases have proved this approach to be useful (Jaenicke, 1974, 1978b; Rudolph, 1977). However, in the case of the tetrameric enzymes no clear-cut information regarding the functional properties of association intermediates was obtained because the dimer or tetramer could equally well be considered the active species fitting the time course of reconstitution in a quantitative way (Jaenicke & Rudolph, 1977; Hermann et al., 1979).

In order to provide an unambiguous correlation between the state of association and the processes of folding and reactivation, a *dimeric* enzyme has been investigated in the present study. Since there is a close relationship regarding the tertiary structure and the spatial arrangement of the subunits in the tetrameric lactic dehydrogenases (which were studied in detail in previous experiments) and in dimeric malic dehydrogenase, the latter enzyme was chosen to elucidate the mechanism of reconstitution. Besides the expected deeper understanding of the general mechanism of folding and association which may be deduced from the close evolutionary relationship of the two enzymes, there are two aspects of interest in a comparative study of LDH[1] and MDH.

While in the case of LDH earlier findings indicating concentration dependent dissociation were clearly disproved, recent reports for MDH strongly suggest monomers to predominate under the conditions of the enzymatic test. However, this observation is still controversial. Reconstitution experiments are expected to shed light on this problem.

† From the Institut für Biophysik und Physikalische Biochemie, FBB, Universität Regensburg, D-8400 Regensburg, West Germany. *Received November 8, 1978.* Dedicated to Professor Hermann Hartmann on the occasion of his 65th birthday. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

[1] Abbreviations used: m-MDH, pig heart mitochondrial malic dehydrogenase; LDH (LDH-H_4 and LDH-M_4), lactic dehydrogenase (H_4 and M_4 refer to the isoenzymes from heart and skeletal muscle, respectively); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAD, nicotinamide adenine dinucleotide; *c*, enzyme concentration; ϑ_i, denatured state; D and D′, monomeric intermediate states; N′, dimeric intermediate state; N and N*, dimeric native and renatured states; DTE, dithioerythritol; oxac, oxaloacetate.

0006-2960/79/0418-1217$01.00/0