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MALEIMIDE INACTIVATION OF LACTATE DEHYDROGENASE ISOZYMES

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SUMMARY

The homologous beef lactate dehydrogenase (EC 1.1.1.27) isozymes H_4 and M_4 were observed to be effectively inactivated by a homologous series of *N*-alkylmaleimides. With each isozyme, the second-order rate constants of inactivation increased with increasing chain length of the alkyl group of the maleimide derivative. The rate of inactivation of lactate dehydrogenase (H_4) by *N*-heptylmaleimide was decreased in the presence of NADH while no protective effect by NADH was noted in the inactivation of lactate dehydrogenase (M_4) by this maleimide.

Adenosine, AMP, ADP and adenosine diphosphoribose were shown to be coenzyme-competitive inhibitors of both lactate dehydrogenase (H_4) and lactate dehydrogenase (M_4). Binding patterns of these compounds were very similar with the two isozymes studied.

INTRODUCTION

The classification of dehydrogenases as "sulfhydryl enzymes" is an interesting generalization that leads to many questions concerning the actual role of sulfhydryl groups in the functioning of these enzymes. The literature concerning the inactivation of dehydrogenases by sulfhydryl reagents is voluminous; however, variability in the sensitivity of different dehydrogenases to the same reagent and in the effectiveness of different sulfhydryl reagents with the same dehydrogenase has been noted frequently.

In order to investigate properties of the environment of "essential" sulfhydryl groups of dehydrogenases, a homologous series of *N*-alkylmaleimides, varying in the alkyl chain from *N*-ethyl to *N*-decyl, was synthesized [1] to provide sulfhydryl reagents having a varying degree of non-polarity. The positive chain-length effects observed in the rate constants for inactivation of yeast alcohol dehydrogenase by these maleimides suggested the functionally important sulfhydryl groups to be located in non-polar regions [1]. Second-order rate constants for reactions of *N*-alkylmaleimides with cysteine and glutathione do not vary with chain length [1]. More recently, positive chain-length effects have also been observed in the *N*-alkylmaleimide inactivation of D-amino acid oxidase [2], L-α-glycerophosphate dehydrogenase [3] and

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papain [4]. In these studies, it was suggested that reversible non-polar interactions between the enzyme and the alkyl chains of the maleimides occurred prior to the irreversible inactivating reaction between the maleimide ring and the enzyme sulfhydryl groups. Papain inactivation was an excellent test system for the application of *N*-alkylmaleimide reactions since crystallographic studies had revealed the essential sulfhydryl group to be located in a non-polar cleft region of papain [5].

The present study of reactions of *N*-alkylmaleimides with lactate dehydrogenase (EC 1.1.1.27) isozymes was initiated to investigate the immediate environment of functional sulfhydryl groups of these enzymes.

MATERIALS AND METHODS

NAD, NADH, AMP, ADP, ADP–ribose, ribose 5-phosphate, glucose 6-phosphate and crystalline beef heart lactate dehydrogenase (H_4 isozyme) were obtained from Sigma Chemical Co. Crystalline beef muscle lactate dehydrogenase (M_4 isozyme) was obtained from Boehringer Mannheim Corp. *N*-Alkylmaleimides were synthesized as previously described [1].

The incubation of lactate dehydrogenase isozymes with *N*-alkylmaleimides was carried out at 25 °C in 3-ml reaction mixtures containing 67 mM potassium phosphate buffer (pH 7.6), 20% ethanol and either 10 μg of the H_4 isozyme or 1 μg of the M_4 isozyme. The concentrations of *N*-alkylmaleimides were varied in ranges dependent on the alkyl derivative used. For enzyme assays, 0.33 μg of H_4 isozyme or 0.033 μg of M_4 isozyme was transferred from the incubation mixture to a 3-ml assay mixture containing 50 mM Tris–HCl buffer (pH 8.1), 40 mM lithium lactate, and 0.17 mM NAD. Initial velocities of NAD reduction were measured spectrophotometrically at 340 nm.

RESULTS

The binding of adenosine, AMP, ADP and ADP–ribose to lactate dehydrogenase isozymes was evaluated through inhibition analysis. Each of these compounds was observed to be a coenzyme-competitive inhibitor of both the H_4 and M_4 isozyme. The inhibitor dissociation constants (K_i) calculated from data obtained in double reciprocal [6] and Dixon [7] plotting methods were averaged and listed in Table I. During these studies, 16 values of the K_m for NAD with each isozyme were obtained, averaged, and included in Table I for comparison with K_i values. Ribose 5-phosphate and glucose 6-phosphate showed no inhibition of either lactate dehydrogenase isozyme up to 5 mM, the maximum concentration studied.

The two lactate dehydrogenase isozymes were incubated at 25 °C and pH 7.6 with *N*-ethylmaleimide and a series of *N*-alkylmaleimides varying in the sidechain alkyl group from butyl to decyl, inclusive. Enzyme activity was studied as a function of the time of incubation. Inactivation was observed to follow pseudo first-order kinetics. The reactions of lactate dehydrogenase (H_4) with *N*-pentylmaleimide are shown in Fig. 1. Under identical conditions and in the absence of *N*-alkylmaleimides, no loss in catalytic activity of either isozyme was observed during a 60-min incubation. First-order rate constants for lactate dehydrogenase (H_4) inactivation were calculated from data of Fig. 1 using the equation $k_1 = 0.693/t^1/_2$ and show a linear dependence

TABLE I

INHIBITION CONSTANTS FOR LACTATE DEHYDROGENASE ISOZYMES

The experimental conditions are described in the text.

Inhibitor	K_i (mM)	
	Lactate dehydrogenase (M_4)	Lactate dehydrogenase (H_4)
Adenosine	7.07	11.0
AMP	0.55	0.96
ADP	0.77	1.07
ADP–ribose	0.19	0.13
NAD	0.036*	0.0096*
Ribose 5-phosphate	n.i.	n.i.
Glucose 6-phosphate	n.i.	n.i.

* K_m values.

n.i., no inhibition at 5 mM inhibitor concentration.

on *N*-pentylmaleimide concentration permitting the calculation of a second-order rate constant (k_2) of 20.5 $M^{-1} \cdot min^{-1}$. Inactivation of both lactate dehydrogenase isozymes by *N*-alkylmaleimides was studied and second-order rate constants along with actual concentration ranges studied are listed in Table II. In both cases, the second-order rate constants increase with increasing chain length of the maleimide derivative and a linear relationship exists between the logarithm of these constants and the number of carbons in the alkyl sidechain of the maleimide derivatives (Fig. 2). For comparison purposes, the second-order rate constants for inactivation of both isozymes by *N*-phenylmaleimide were included in Table II.

The inactivation of the lactate dehydrogenase isozymes by *N*-heptylmaleimide

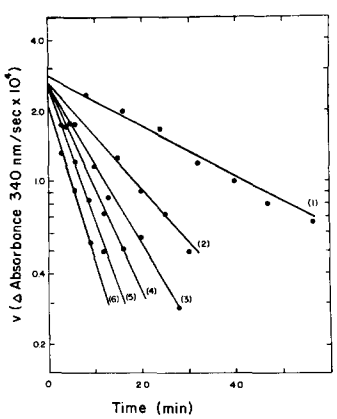

Fig. 1. Time-dependent inactivation of beef lactate dehydrogenase (H_4) by *N*-pentylmaleimide at 25 °C. Reaction conditions are discussed under Materials and Methods. Lines 1–6 represent different concentrations of *N*-pentylmaleimide as follows: (1) 1.2 mM, (2) 2.4 mM, (3) 3.61 mM, (4) 4.81 mM, (5) 6.01 mM and (6) 7.21 mM.

TABLE II

RATES OF *N*-ALKYLMALEIMIDE INACTIVATION OF LACTATE DEHYDROGENASE ISOZYMES

The reactions of *N*-alkylmaleimides with lactate dehydrogenase isozymes were carried out at 25 °C in 3-ml reaction mixtures containing 20% ethanol, 67 mM potassium phosphate buffer (pH 7.6) and maleimide concentration as indicated.

N-Alkylmaleimide	Isozyme			
	Lactate dehydrogenase (M_4)		Lactate dehydrogenase (H_4)	
	Concentration range of maleimide (mM)	k_2 ($M^{-1} \cdot min^{-1}$)	Concentration range of maleimide (mM)	k_2 ($M^{-1} \cdot min^{-1}$)
Ethyl	1.67 –10.0	7.7	1.67 –10.0	9.9
Butyl	0.690 – 8.29	12.8	1.40 – 8.38	18.2
Pentyl	0.491 – 7.36	29.4	1.20 – 7.21	20.5
Hexyl	0.259 – 2.59	35.0	0.556 – 3.34	27.5
Heptyl	0.167 – 1.00	84.5	0.167 – 1.00	47.5
Octyl	0.0333– 0.333	210	0.0648– 0.389	55.0
Nonyl	0.0133– 0.0800	359	0.0667	*
Decyl	0.0267– 0.0667	455	0.0667	*
Phenyl	0.360 – 2.16	18.0	0.77 – 4.62	40.0

* Inadequate solubility of inhibitor prevented k_2 determination.

was studied in the presence of NAD and NADH. At a concentration (0.18 mM) approximately five times the K_m value listed in Table I, NAD had no protective effect on the rate of inactivation of lactate dehydrogenase (M_4). NADH at 39 μM likewise had no effect on the rate of inactivation of lactate dehydrogenase (M_4) by *N*-heptylmaleimide. In the case of *N*-heptylmaleimide inactivation of lactate dehydrogenase (H_4) 43 μM NAD (five times K_m) showed no protective effect; however, the presence of 39 μM NADH decreased the rate of inactivation by 50%.

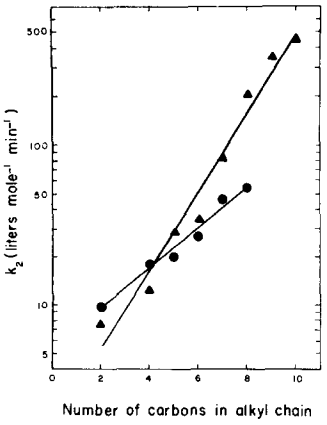

Fig. 2. The relationship of the logarithm of the second-order rate constants for lactate dehydrogenase inactivation by *N*-alkylmaleimides. (●—●) lactate dehydrogenase (H_4), (▲—▲) lactate dehydrogenase (M_4).

DISCUSSION

Adenosine, AMP, ADP and ADP–ribose were shown to be coenzyme-competitive inhibitors of both homologous beef lactate dehydrogenase isozymes. From the K_i values, one can conclude that the four adenine derivatives are essentially equally effective with both lactate dehydrogenase isozymes. With each inhibitor, only small differences were observed in the K_i values obtained with the two isozymes. Also, the binding patterns of the adenine derivatives with the two isozymes were essentially the same. Adenosine, although a coenzyme-competitive inhibitor of both enzymes, was bound very poorly in each case. The additional negatively charged phosphate and pyrophosphate groups of AMP and ADP, respectively, enhanced binding of these derivatives to each isozyme approx. 10-fold in each case. This enhancement of binding was observed with AMP with no further enhancement by the additional phosphate group of ADP. The only major difference in overall binding patterns was in the effectiveness of the binding of ADP–ribose relative to that of ADP. With the H_4 isozyme, ADP–ribose is bound 10 times better than ADP while with the M_4 isozyme only a 4-fold difference is observed.

The second-order rate constants for inactivation of beef lactate dehydrogenase H_4 and M_4 isozymes by *N*-alkylmaleimides increase with increasing chain length of the maleimide derivative. The sensitivity of beef lactate dehydrogenase isozymes to inactivation by sulfhydryl reagents has been well documented [8–15]. It has been assumed that maleimide inactivation involves the modification of sulfhydryl groups of these enzymes. Further study of the modified enzymes will be required to confirm this point since maleimides can react with other enzyme functional groups [16–19]. The chain-length effects observed in these inactivation processes (Fig. 2) are very similar to effects observed in the inactivation of several other enzymes by these reagents [1–4]. In these earlier studies, the chain-length effects were attributed to non-polar interactions between reagents and enzyme prior to the irreversible inactivation step. Non-polar interactions of the maleimide derivatives with lactate dehydrogenase isozymes are indicated by the chain-length effects observed (Fig. 2) and the M_4 isozyme appears to be more sensitive to these effects than the H_4 isozyme. Again, these differences between the two isozymes are minor ones since in each case one can suggest that the functional groups reacting with maleimides exist in a relatively non-polar environment. This environment may be somewhat more extensive in the case of the M_4 isozyme. A greater difference can be noted in the protection experiments. The rate of inactivation of the H_4 isozyme by *N*-heptylmaleimide is greatly decreased by the presence of NADH while under the same conditions NADH does not protect the M_4 isozyme from inactivation by *N*-heptylmaleimide. NADH protection of beef lactate dehydrogenase (H_4) from inactivation by *N*,*N*′-dimethylamino-3,5-nitrophenylmaleimide [13] and *p*-hydroxymercuribenzoate [11] was reported previously. The NADH effect could arise through a stabilization of the quaternary structure of the enzyme, as suggested by Cho and Swaisgood [15]. The contrasting absence of a NADH protective effect in the inactivation of the M_4 isozyme may reflect a difference in the position of the maleimide sensitive groups relative to the coenzyme binding sites of the different isozymes. Differences in the binding of bis(1-anilino-8-naphthalenesulfonate) to the five beef lactate dehydrogenase isozymes have been reported [20] and have been related to differences in the binding properties of the two types of pro-

tomers. In the inactivation of rabbit muscle L-α-glycerophosphate dehydrogenase by *N*-alkylmaleimides, effective protection by NADH but not by NAD was likewise observed [3].

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