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PROTEIN LIGAND INTERACTIONS:ISOQUINOLINE ALKALOIDS AS INHIBITORS FOR LACTATE AND MALATE DEHYDROGENASE

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Kinetic analysis has shown that isoquinoline, papaverine and berberine act as reversible competitive inhibitors to muscle lactate dehydrogenase and mitochondrial malate dehydrogenase with respect to the coenzyme NADH. The inhibitor constants K_i vary from 7.5 μM and 12.6 μM berberine interaction with malate dehydrogenase and lactate dehydrogenase respectively to 91.4 μM and 196.4 μM with papaverine action on these two enzymes. Isoquinoline was a poor inhibitor with K_i values of 200 μM (MDH) to 425 μM (LDH). No inhibition was observed for both enzymes in terms of their respective second substrate (oxaloacetic acid – malate dehydrogenase; pyruvate – lactate dehydrogenase). A fluorimetric analysis of the binding of the three alkaloids show that the dissociation constants (K_d) for malate dehydrogenase are 2.8 μM (berberine), 46 μM (papaverine) and 86 μM (isoquinoline); the corresponding values for lactate dehydrogenase are 3.1 μM, 52 μM and 114 μM. In all cases the number of binding sites averaged at 2 (MDH) and 4 (LDH). The binding of the alkaloids takes place at sites close to the coenzyme binding site. No conformational non equivalence of subunits is evident.

KEY WORDS: Isoquinoline, papaverine, berberine, inhibition, malate, lactate, dehydrogenase, fluorimetry.

INTRODUCTION

The majority of the pyridine-nucleotide linked dehydrogenases have generally been found to be multisubunit complexes. A major goal in the elucidation of the mechanism of the action of these enzymes has been the determination of the role of subunit interactions in their catalytic function. Contradictory studies have appeared which demonstrates both anti-cooperative binding of ligands or $NADH/NAD^+$ to the dimeric enzyme[1] and an absence of cooperativity in which the $NADH/NAD^+$ binding sites on each subunit are independent and indistinguishable.[2]

It is of interest to investigate the character of subunit interactions in order to reveal if conformational changes which accompany catalysis in one active centre can be transmitted to the neighbouring subunit(s). In light of these observations the role of subunit interactions in enzyme function is of interest for both muscle lactate dehydrogenase and, in particular, mitochondrial malate dehydrogenase. The major difficulty which has limited progress in the study of subunit interactions is the very nature of these interactions themselves. In most cases the native oligomeric structure is not easily dissociated, thus making it almost impossible to investigate the native monomeric form of the enzyme. More drastic conditions utilising protein denatura-

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tion are unsatisfactory in that extensive subunit unfolding and loss of enzymatic activity also occur. Recent investigations reported that dehydrogenase enzymes can be dissociated into their constituent sub-units under rather mild conditions.[3,4] However, the presence of the enzymatic cofactors of these enzymes apparently facilitates the reassociation of the subunits thus making it impossible to investigate the kinetic parameters of the free monomeric form of the enzymes.

In order to answer the questions of the effect of $NADH/NAD^+$ on the subunit dissociation as well as the contradictory reports mentioned above it is of interest to investigate the character of subunit-ligand interactions. The purpose of the present work is to use a ligand with one or more features present in the enzyme system and attempt to mimic the key parameters of enzyme/coenzyme function. Such a molecule must, (a) fit the active cleft of the enzyme, (b) have a rigid and fixed conformation, (c) possess a quaternary nitrogen to stimulate the oxidised coenzyme, and (d) possess additional functional groups to act as electron donors for specific amino acids in the enzyme.

As a continuing study on the interaction of biologically active compounds on biochemical processes currently being undertaken in these laboratories[5] we envisaged certain natural products may fulfil our requirements for suitable organic molecules. Indeed the isoquinoline alkaloids, berberine and papaverine which exhibit a broad spectrum of biological activity, used as their hydrochloride salts, possess all the necessary features.

We present here an investigation aimed at both kinetic and binding studies of the interaction of muscle lactate dehydrogenase and mitochondrial malate dehydrogenase with berberine, papaverine and isoquinoline (Figure 1). It should be realised

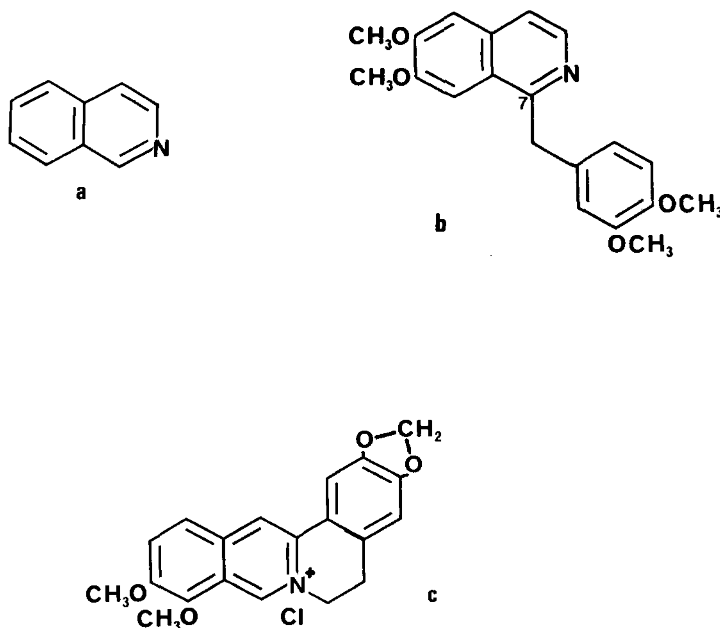

FIGURE 1 (a) Isoquinoline (b) Papaverine (c) Berberine.

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that the analysis reflects changes in optical properties due to the nature of the subunit ligand binding. Also binding and kinetic studies critically depend on the specificity of the ligands applied and the observed changes in fluorescence. Fluorescent probes have been valuable tools in the studies involving subunit cooperativity,[6] hydrophobic binding sites[7] and distance between two types of binding sites.[8]

MATERIALS AND METHODS

Materials

Muscle lactate dehydrogenase, mitochondrial malate dehydrogenase, NADH, sodium pyruvate and oxaloacetic acid were purchased from Boehringer Mannheim. Papaverine, berberine and isoquinoline were obtained from Aldrich Chemicals as their hydrochloride salts and they were used without purification. All other inorganic and buffer materials were of reagent grade.

Methods

Fluorimetric Analysis Fluorimetric measurements were made with a Hitachi fluorescence spectrophotometer using an excitation light source from a Xenon 150 lamp, the fluorescence measured through the cell at an angle of 90° to the incident beam. All fluorimetric titrations were carried out at 20° in 0.1 M phosphate buffer (pH 7.2).

For studies involving binding of the isoquinoline alkaloids to the enzyme increasing concentrations of ligand (5–100 μM) were added to a sample of the enzyme (1.2 μM). The mixture was excited at 280 nm and the emission measured at 305 nm. The fluorescence data was used to determine the dissociation constant (K_d) of the ligands with the complexes formed with the enzyme. The results were analysed according to equations (1)–(4).

$$K_d/1 - \theta = (L)_t/\theta - p(A)_t \ldots \tag{1}$$

$$\theta = \Delta F/\Delta F_{max} \ldots \tag{2}$$

$$\Delta F/\Delta F_{max} = v/p \ldots \tag{3}$$

$$v/(L)_f = p - v/Kd \ldots \tag{4}$$

where $(A)_t$ is the total concentration of acceptor in the system; p is the total number of binding sites; θ is the fractional occupancy of total acceptor sites by ligand; ΔF is the change in fluorescence in the presence of a known amount of ligand; ΔF_{max} is the chanbe in fluorescence at saturation with ligand; $(L)_t$ is the total concentration of ligand; Kd is the dissociation constant; v are the number of moles of bound ligand and $(L)_f$ is the concentration of free ligand.

Enzymatic Assays The enzymes activity were determined spectrophotometrically at 25°C in 0.1 M sodium phosphate buffer (pH 7.2) by measuring the decrease in absorbance at 340 nm associated with NADH oxidation, with a Bausch and Lomb 1001 spectrophotometer. The assay mixtures (3.0 ml) contained NADH (0.2 mM) and oxaloacetic acid (0.25 mM for mitochondrial malate dehydrogenase) or sodium pyruvate (0.76 mM for muscle lactate dehydrogenase). Aliquots were made up to 2.9 ml with distilled water and the reaction was initiated by adding 0.1 ml diluted enzyme.

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The concentrations of the enzymes were determined at 280 nm using an absorption coefficient ($A^{1\%}_{1\,cm}$) of 14.4 (LDH) and 2.53 (MDH).[9]

Inhibition Studies Inhibition of the enzymes by interaction with the isoquinoline alkaloids was studied in the presence of phosphate buffer (0.1 M, pH 7.2), 0–0.2 mM NADH, oxaloacetic acid (0.25 mM, for malate dehydrogenase) or sodium pyruvate (0.76 mM, for lactate dehydrogenase) and ligand (0–0.1 mM). Aliquots (2.9 ml) of such reaction solutions were initiated by the addition of the eyzme (0.1 ml).

Concentrations of isoquinoline, papaverine and berberine were determined spectrophotometrically using $E_{220} = 6.31 \times 10^4 M^{-1}.cms^{-1} (M_R = 129)$; $E_{240} = 7.94 \times 10^4 M^{-1}.cms^{-1} (M_R = 376)$ and $E_{263} = 5 \times 10^4 M^{-1}.cms^{-1}$) ($M_R = 353$) respectively.

RESULTS

Fluorimetric Studies

Typical binding curves and the analysis as plots of $1/1 - \theta$ versus (ligand)/θ are shown for the effect of the alkaloids on mitochondrial malate dehydrogenase in the

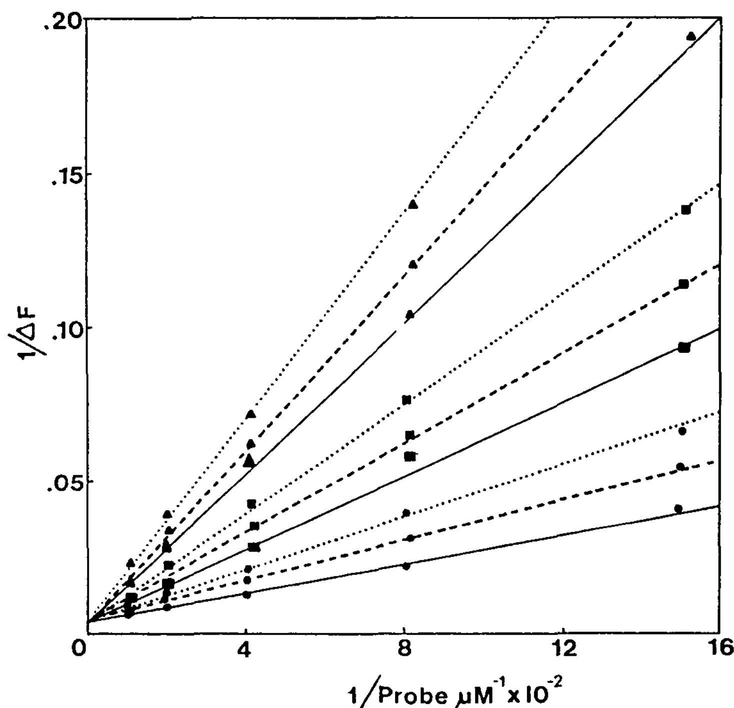

FIGURE 2 Double reciprocal plots of fluorescence change on binding of isoquoline (▲), papaverine (■) and berberine (●) to mitochondrial malate dehydrogenase (1.2 ng ml^{-1}) in the presence of 100 mM phosphate buffer (pH 7.2) and 0 μM (———), 45 μM (– – – –) and 90 μM (· · · · ·) NADH. The mixtures were excited at 280 nm and emission measured at 305 nm.

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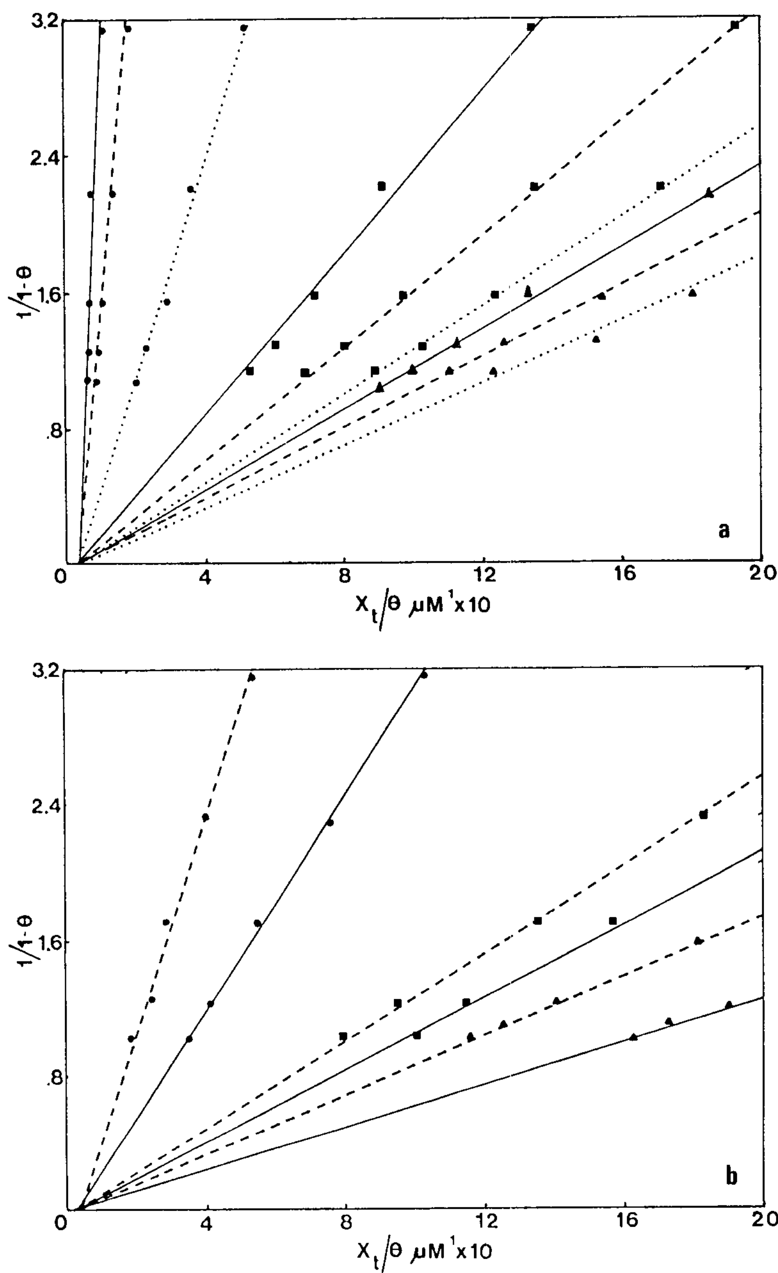

FIGURE 3 (a) Analysis data for the binding of isoquinoline (▲), papaverine (■) and berberine (●) to malate dehydrogenase in 100 mM phosphate buffer (pH 7.2) and 0 μM (———), 45 μM (- - - -) and 90 μM (· · · · ·) NADH. (b) Comparative analysis data for the binding of isoquinoline (▲), papaverine (■) and berberine (●) to malate dehydrogenase (- - - -) and lactate dehydrogenase (———) in the presence of NADH (90 μM).

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TABLE I

Binding constants obtained by competitive inhibition and fluorimetric titrations of isoquinoline, papaverine and berberine on muscle lactate and mitochondrial malate dehydrogenase. $K_{m_{app}}$ are the respective Michaelis constants; K_{i_c} and K_{i_g} are the inhibitor complexes calculated from $K_{m_{app}} = K_m(1 + K_{i_c}^{-1}.I)$ and from the extrapolation of the graph-slope versus alkaloid concentration respectively. K_d represents the dissociation constants for a single binding site. p is the number of binding sites.

Enzyme	Inhibitor	Conc (μM)	$K_{m_{app}}$ (μM)	K_{i_c} (μM)	K_{i_g} (μM)	NADH (μM)	K_d (μM)	p
MDH	Berberine	33.3	215.4	8.8		0	2.8	
		66.7	367.7	9.3	7.5	45	4.6	2.4
		100	600	8.1		90	16.9	
	Papaverine	33.3	62.44	86		0	46	
		66.7	78.2	90.4	91.4	45	61	2.2
		100	95.7	88.7		90	78	
	Isoquinoline	33.3	52.7	194.7		0	86	
		66.7	60	200	198.6	45	100	2.3
		100	68.1	195.2		90	113	
LDH	Berberine	33.3	75.3	13.4		0	3.1	
		66.7	112.8	15.8	12.6	45	8.2	4.3
		100	191.7	12.7		90	33.0	
	Papaverine	33.3	25.2	202		0	52	
		66.7	29.2	190	196.4	45	81	4.5
		100	53.8	205		90	96	
	Iscquinoline	33.3	23.2	456		0	114	
		66.7	25.0	430	427.1	45	146	4.1
		100	26.5	441		90	160	

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presence and absence of NADH (Figure 2 and 3). A similar set of curves were obtained for muscle lactate dehydrogenase. From the plots and equations (1) and (2) the dissociation constants (K_d) for a single binding site and the total number of binding sites for the alkaloids can be estimated and represented (Table 1). Papaverine binds to malate dehydrogenase and lactate dehydrogenase with K_d values of 46 μM and 52 μM respectively while isoquinoline showed values of 86 μM and 114 μM. The more complex berberine molecule appeared to bind relatively tightly with K_d values of 2.8 μM and 3.1 μM respectively. In every case studied the number of binding sites (p) for muscle lactate dehydrogenase was 4 while that for malate dehydrogenase was 2.

The data are also represented through Scatchard plots (Figure 4a,b) and equations (3) and (4) and it is noted that both enzymes contain a single class of binding sites.

The effect of increasing NADH concentrations showed a corresponding decrease in the relative fluorescence of binding of the ligand to the enzyme. This indicated that NADH is strictly competitive towards the enzyme-alkaloid complex. The dissociation constants (K_d) may be calculated as described earlier and are also represented (Table 1).

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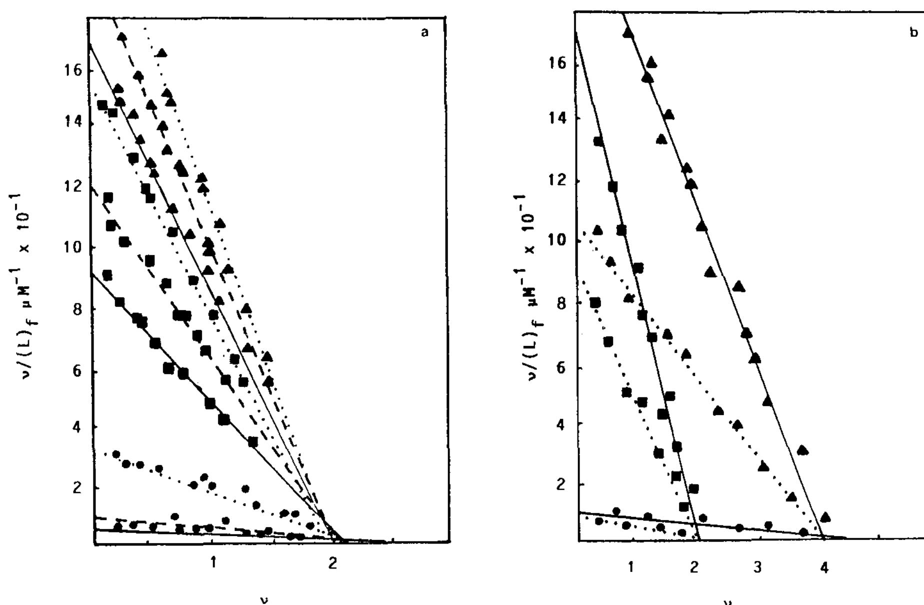

FIGURE 4 (a) Scatchard plot for the binding of isoquinoline (▲), papaverine (■) and berberine (●) to malate dehydrogenase as determined by fluorimetry. The concentrations of NADH are 0 μM (——), 45 μM (----) and 90 μM (·····) $(L)_f$ is the concentration of free alkaloid. ν are the number of moles of bound alkaloid. (b) Comparative Scatchard plots for the binding of isoquinoline (▲), papaverine (■) and berberine (●) to malate dehydrogenase (·····) and lactate dehydrogenase (——) in the absence of NADH.

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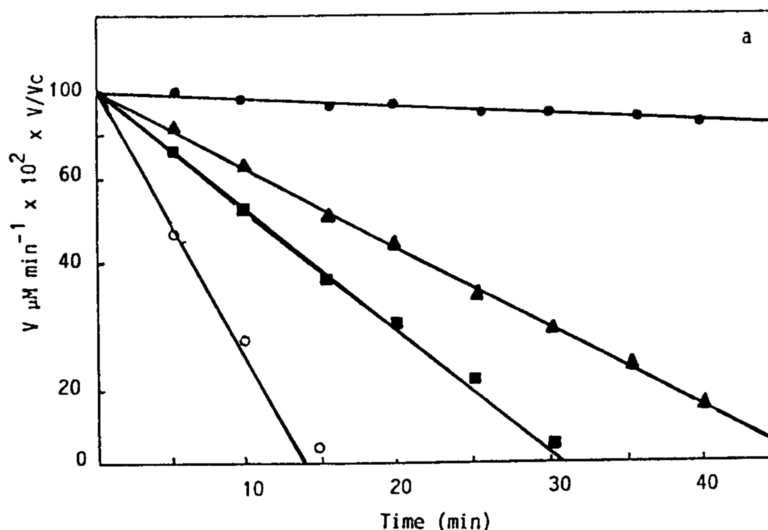

FIGURE 5 Inactivation of malate dehydrogenase by isoquinoline (▲), papaverine (■) and berberine (○) at pH 7.2. The concentration of each inhibitor was 50 µM. V and V_c were enzyme activities of experimental and control.

Kinetic Studies

Incubation of malate dehydrogenase with each of the isoquinoline alkaloids resulted in a progressive loss of enzyme activity (Figure 5). As shown the inactivation rate of the enzyme was dependent on the particular alkaloid and on the incubation time. When the enzyme was mixed with isoquinoline, 18% of the control activity remained after 40 min while in the presence of berberine it took only 12 min to reach the same activity.

The inactivation followed pseudo first-order kinetics as indicated by typcial semilogarithmic plots of enzyme activity vs time. Double log plots of k_1 as a function of berberine concentration (data not shown) were also linear, yeilding a slope of 1 and hence a reaction order (n) of 1 with respect to berberine (Equation (6)). The second-order rate constant (k_2) can be obtained from the slope of the linear plots of pseudo first-order rate constant (k_1) vs berberine concentraion.

The evidence of a reversible inhibitory mechanism was realised for both malate and lactate dehydrogenase after characteristic Lineweaver and Burk plots[10] (Figure 6) indicating that all the isoquinoline alkaloids were competitive inhibitors of the enzyme with respect to the coenzyme NADH. A replot of the slopes of the double reciprocal plot versus ligand concentration is linear (Figure 6, inset). The relevant kinetic parameters for the alkaloids are represented (Table 1). $K_{m_{app}}$ are the respective Michaelis constants; K_{i_c} and K_{i_g} are the inhibitor constants of the enzyme inhibitor complex calculated from equation (5) and from extrapolation of the graph-slope versus alkaloid concentration (Figure 6, inset) respectively.

$$K_{m_{app}} = K_m (1 + Ki_c^{-1}.I)\ldots \quad (5)$$

$$\log k_1 = \text{nlog[Berberine]} + \log k_2\ldots \quad (6)$$

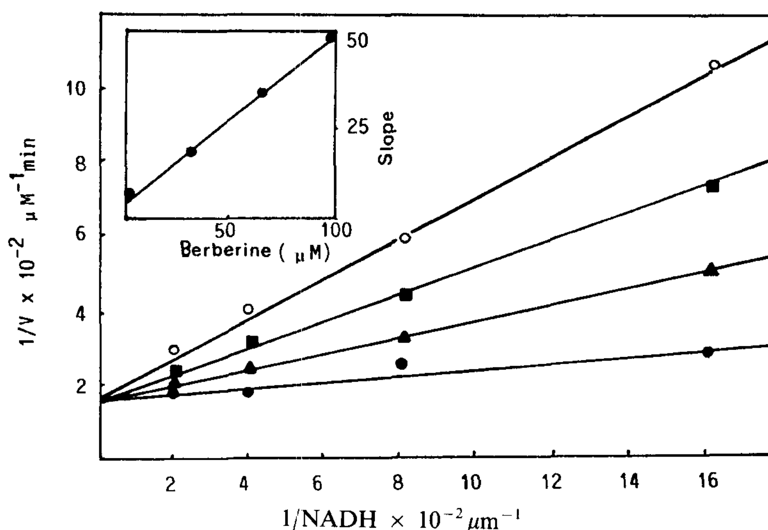

FIGURE 6 Competitive inhibition of malate dehydrogenase by berberine at pH 7.2. Concentration of berberine = (●), 0 μM; (▲), 33 μM; (■), 67 μM; and (○), 100 μM. The secondary plot represents the slopes of the Lineweaver-Burk lines versus berberine concentration.

I is the concentration of the particular alkaloid. With variable NADH concentrations values for K_m and V_{max} for malate and lactate dehydrogenase are 45 μM, 58 μM min^{-1} and 21.6 μM, 90 μM.min^{-1} respectively.

The pentacyclic berberine alkaloid showed a marked inhibition with both enzymes with K_{i_c} of 9.0 μM (MDH) and 14.0 μM (LDH). The other isoquinoline alkaloids were less potent as inhibitors with K_{i_c} values ranging between 90 μM for papaverine (MDH) to 200 μM for isoquinoline (MDH). The corresponding K_{i_c} values for lactate dehydrogenase ranged between 190 μM (papaverine) to 450 μM (isoquinoline).

No inhibition was observed for both enzymes with respect to the other substrate (oxaloacetic acid – MDH; sodium pyruvate – LDH).

DISCUSSION

This article has shown that the alkaloids are capable of binding to both malate and lactate dehydrogenase. A kinetic analysis reveals that the alkaloids also inhibit the enzymes, reversibly and competitively with respect to the coenzyme NADH, hence are capable of binding at the coenzyme binding site of the enzyme. Since malate and lactate dehydrogenase consist of two and four subunits respectively it is practically proved that each enzyme subunit bears identical binding sites for the probes. Our data support that each subunit of the dimeric malate dehydrogenase or tetrameric lactate dehydrogenase can bind one molecule of the alkaloids. Thus no conformational non equivalence of subunits is revealed by the probes. This is in direct contrast to yeast alcohol dehydrogenase[11] and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase.[12] The potency of a particular ligand in the interaction with the dehydrogenase enzymes is determined by the dissociation constant for the enzyme-ligand complex. The fit into the binding sites of the enzyme, reflected by the values of K_d (or

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K_i) is largely determined by the size, structure and configuration of the inhibiting molecule. The capability of the inhibitor to bind non-covalently at, or near, the active centre could also influence these values.

Although no evidence of active site cooperativity was obtained in the present study, the results could imply a transmission of conformational changes induced by the binding of ligands in the active centre of one subunit to an adjacent subunit. These changes are reflected in the alterations of the structure of a certain domain on the protein surface where a probe binding site is located. The aqueous isoquinoline alkaloid solutions are weakly fluorescent, the fluorescence intensity dramatically increasing after their binding to the enzyme (Figure 2). It may be established that these alkaloids bind strongly to a hydrophobic domain of the enzyme molecule. A contribution to the increase in fluorescence may be from an increase in the rigidity of the probe molecule on binding. Further more the addition of even small amounts of alkaloid results in quenching suggests that a conformational change opens up new tyrosine residues capable of fluorescing.

The binding of the alkaloids with the enzyme-inhibitor complex is also influenced by the solvating properties of the catalytic site at which the reaction occurs. The acceleration of this process can be attributed to a change of the solvating conditions in the active centre resulting from the binding of the alkaloids with the NADH binding site. The alkaloids themselves cannot increase the basicity of the micro-environment of the binding locus so it is assumed that they induce a conformational change in the enzyme molecule leading to the exposure of the some new basic groups inside the active centre.

Detection of the intrinsic fluorescent change in a protein upon binding with ligands is one of the simplest and most direct methods to study ligand induced conformational changes. In the present investigation the quenching of fluorescence of the enzyme upon binding with the alkaloids is used to study the enzyme-ligand interactions. The decrease in relative fluorescence when the binding to the enzyme takes place may be due to the presence of non-polar regions in or around the NADH binding sites of the enzyme. Our investigations provide substantial amount of evidence indicating that the binding sites for both malate and lactate dehydrogenase are independent and indistinguishable. The linearity of the Scatchard plots (Figures 4a and b) from the binding of the isoquinoline alkaloids to the enzyme further supports the absence of subunit interactions.

Since the inhibitors tested in this investigation gave cofactor-competitive kinetic plots it is assumed that they all interact exclusively at the same binding site of the enzyme. The necessary structures which the alkaloids must have in order to be able to exert an inhibitory effect may be defined as follows. A polar or ionic locus must be present which interacts with the positive quaternary nitrogen in a planar arrangement ($=N^+$). Several lines of evidence indicate hydrophobic interactions. There was an increase in inhibitory potency upon systematic increases in the hydrophobicity of the alkaloids studied. The simple isoquinoline molecule has limited hydrophobic groups when compared to papaverine and berberine and therefore has a greater dissociation constant (K_d). Alternatively although the hydrophobic groups on papaverine and berberine may occupy similar positions the dimethoxybenzyl group of the former possesses more freedom of rotation than the C and D rings of berberine.

The aromatic character of rings A and B is not entirely necessary for the inhibition since simple isoquinoline molecule has a very poor inhibitory effect. (450 μM for lactate dehydrogenase; 200 μM for malate dehydrogenase). The angle between rings

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A and D (roughly 20° from Dreiding models) seems to be crucial for inhibition since berberine, possessing such a structure, is a powerful inhibitor of the enzymes. The geometry of the berberine molecule appears to compensate well to that of the coenzyme molecule thereby fitting snugly into the cofactors binding site. The presence of a methylene dioxy group on ring D appears to enhance inhibitory power of the alkaloid. Papaverine is a weaker inhibitor than berberine as it has two methoxy groups in place of the methylene dioxy substituent of berberine. This may be ascribed to the opposite conjugation effects. There is cooperation of the two oxygen atoms in the case of the methylene dioxy group since the non-bonding electrons of the second oxygen can transfer via the methylene bridge thus making it considerably more easier for electrons of the methylenedioxy group to join the conjugated π-electron system of the aromatic rings.[13] Since the inhibitor and binding power of the alkaloid increases as the polarity of substituents on C_7 increases it can be assumed that the binding site of the enzyme for these alkaloids has hydrophobic character and that this hydrophobic group of the enzyme comes into the neighbourhood of C_7 during the interaction of the enzyme with the inhibitor.

Substituents on C_7 (Figure 1b) with a negative inductive effect remove electrons from the C and D rings and thus increase the positive charge in the nitrogen. This is reflected by the participation of mobile electrons of these substituents (free electron pairs of oxygen or electron contribution of alkyls by hyperconjugation) in conjugation with π electrons of ring D. This in turn leads to an extension of the conjugated system which may facilitate the interaction of the alkaloids with the electron donor parts of the enzyme.

In conclusion we can say that the NADH binding sites of malate and lactate dehydrogenase are independent. Fluorimetric titrations and steady state kinetics further supports the absence of NADH linked subunit interactions since the same K_d were obtained from either technique. The isoquinoline alkaloids are rigid, they bind at the coenzyme binding site and they possess quaternary nitrogen functional groups. Thus they have adequately satisfied all the necessary features and parameters for a ligand described above.

Acknowledgements

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