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THERMAL STABILITY OF THE MOLECULAR FORMS OF GUINEA-PIG SKELETAL MUSCLE CYTOPLASMIC MALATE DEHYDROGENASE AND KINETIC MECHANISM OF THE THERMOSTABLE FORM

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Abstract—1. Guinea-pig skeletal muscle cytoplasmic malate dehydrogenase appears under two molecular forms; the heating of the dialyzed soluble fraction of the tissue shows that the A form is stable at 55°C, while the B form is inactivated. Under these conditions, the lactate dehydrogenase M_4 isoenzyme becomes considerably unstable; nevertheless, its activity is notably preserved with NADH.

2. The reaction kinetic mechanism of the isolated A form has been determined (pH 7.4), enabling the nature of the abortive complexes and the values of K_{eq} and of $\Delta G^{\circ\prime}$ of the reaction to be determined.

INTRODUCTION

Cytoplasmic malate dehydrogenase (s-MDH) (EC 1.1.1.37) from diverse sources is, generally, more thermostable than mitochondrial malate dehydrogenase (m-MDH) (Kitto & Kaplan, 1966; Kitto, 1967; Kitto & Lewis, 1967; Teague & Henney, 1973; Baró *et al.*, 1974; Francis & Coughlan, 1976; Hodnett *et al.*, 1976). Nevertheless, the opposite is true in the case of the mitochondrial enzyme of Opuntia (Mukerji & Ting, 1969), of Drosophila (McReynolds & Kitto, 1970) and of maize (Yang & Scandalios, 1974). Little attention has been paid to the thermostability of the molecular forms of s-MDH. It has been said that the pig heart enzyme forms have a similar stability (Kulick & Barnes, 1968), against what happens with Fasciola hepatica (Probert & Lwint, 1977) and chicken liver (Baró *et al.*, 1979) molecular forms.

At the same time as the stability of the forms of s-MDH present in the soluble fraction of guinea-pig skeletal muscle was determined, the relative stability of the five lactate dehydrogenase (LDH) (EC 1.1.1.27) isoenzymes present in it has also been determined. It has been widely shown that, in the case of LDH from various sources, the M_4 isoenzyme is the most thermolabile and that the stability of the remaining isoenzymes increases with the number of H subunits in the oligomeric molecule (Markert & Møller, 1959; Plagemann *et al.*, 1961; Strandjord *et al.*, 1962; Fondy *et al.*, 1964). In turn, the protective effect induced on the enzyme by the reaction substrates and by structural analogues is maximum with NADH (Pfleiderer *et al.*, 1957; Vesell & Yielding, 1968; Südi, 1970).

Two forms of s-MDH and the five LDH isoenzymes, with a predominance of M_4, are present in guinea-pig skeletal muscle (Puig *et al.*, 1980; Olsson, 1975). The reduction of the oxaloacetate (OAA) with NADH by the soluble fraction of this tissue causes deviations from the Michaelis–Menten kinetics if the keto acid concentrations are high. They are due to the capacity that the LDH isoenzymes have of catalysing this reaction, although with an affinity inferior to that shown by MDH (Puig *et al.*, 1980).

In this paper, it is shown that the different behaviour exhibited by the constituent molecular forms of both enzymes to temperature has an influence on the above mentioned kinetic anomalies. It has, moreover, been the basis for the development of a purification method for the thermostable molecular form of s-MDH, the kinetic characterization of which is also described.

MATERIALS AND METHODS

Reagents and chromatographic substances

Oxaloacetic acid, DL-malic acid, sodium DL-lactate, ATP and Tris (Merck); sodium pyruvate, NADH and NAD^+ (Boehringer); *p*-nitro bluetetrazolium chloride (Serva); phenazine methosulfate (Sigma). All other chemicals used were reagent grade. Ultrogel AcA 34 (LKB); diethyl aminoethyl Sephadex A-50 (DEAE-Sephadex) and Sephadex G-25 M (Pharmacia Fine Chemicals); acetate cellulose strips (cellogel) (Chemetron).

Enzyme activity and protein determination

The initial reaction velocities of the dehydrogenases under study were determined by measurement of the absorbance changes at 340 nm and 30 ± 0.1 C in a Beckman model 25 recording spectrophotometer, in 3 ml, 1 cm light path cells.

The standard reaction mixture, used in the determination of the MDH activity during the purification process and in the study of its thermal stability, contained 50 mM (pH 7.4) sodium phosphate buffer, 0.1 mM NADH and 0.09 mM oxaloacetate or 100 mM (pH 9.0) Tris–HCl buffer, 0.7 mM NAD^+ and 1.35 mM L-malate, according to the direction of the reaction to be determined. The enzyme concentration was the amount required to obtain a linear absorbance change during the first 2 min of the reaction. The LDH activity was determined in 50 mM (pH 7.4) sodium phosphate buffer, with 0.28 mM pyruvate and 0.07 mM NADH. The solutions used in the experiments were freshly prepared, dissolved in the corresponding buffer and adjusted to the appropriate pH. The oxaloacetate solutions used in the experiments were held at 0 C prior to carrying them out. The absence of pyruvate in the oxaloacetate solutions used was checked with a previously described process (Puig *et al.*, 1980).

The enzyme unit (μkatal) is the amount of enzyme producing the conversion of 1 μmol of substrate per second under the experimental conditions. It was calculated using the molecular extinction coefficient of NADH 6220×10^3 cm^2 mol^{-1} (Horecker & Kornberg, 1948) at 340 nm.

The amount of protein in the solutions was determined by the method of Warburg & Christian (1941) by reading the absorbance at 260 and 280 nm in 1 cm light path cells. The soluble fraction protein content was measured after dialysing or filtering a sample through gels (Sephadex G-25 M) to remove the endogenous substances interfering in the determination.

Electrophoretic and kinetic methods

Electrophoresis was carried out on cellulose acetate strips (Cellogel), equilibrated in a 50 mM, pH 7.4 phosphate buffer medium, for 75 min at 4°C and 200 V. 0.01 units of each dehydrogenase were applied, measured in the sense of reduction of the NADH.

The molecular forms of the MDH and of the LDH were detected by specific staining according to the methods of Thorne *et al.* (1963) and Fine & Costello (1963), respectively. So as to compare the mobilities of the bands appearing in the different stainings accurately, at the end of the electrophoresis operation, the strips were cut longitudinally in two before being dipped in the corresponding staining mixture.

The determination of the kinetic parameters in the oxaloacetate reduction and L-malate oxidation processes was effected at pH 7.4 (50 mM phosphate buffer). The values of the initial velocities obtained according to the different substrate concentrations and cofactors were expressed by the double reciprocal plot ($1/v$ vs $1/[S]$). The kinetic parameters were calculated by the method of Florini & Vestling (1957). Substrate inhibition was studied by application of the Dalziel's (1957) method. The product inhibition method was applied for determination of the s-MDH (A) reaction mechanism and the results were analysed in accordance with Cleland's (1963) theoretical predictions.

Purification of the thermostable form of guinea-pig skeletal muscle s-MDH

Step 1. *Preparation of the soluble fraction.* The skeletal muscle was removed from freshly sacrificed male guinea-pigs (*Cavia porcellus*). The soluble fraction of the tissue was obtained by differential centrifugation as described (Puig *et al.*, 1980).

Step 2. *Thermal treatment.* The soluble fraction was dialyzed for 20 hr at 4 C with 10 times the amount of 5 mM (pH 7.4) phosphate buffer, with constant stirring. The dialyzed material was heated at 55°C for 25 min and cooled to 0 C by immersion into an ice bath. The suspension was centrifuged at 5000 *g* for 30 min and the supernatant was collected. The thermolabile form of guinea-pig skeletal muscle s-MDH (B form) was eliminated in this step and the amount of LDH was considerably reduced.

Step 3. *Ammonium sulfate fractionation.* The supernatant from the previous step was brought to 40% saturation with solid $(NH_4)_2SO_4$. The precipitate which formed was removed by centrifugation (5000 *g*, 30 min) and the supernatant was brought to 80% saturation. The suspension was allowed to rest at 4°C for 12 hr and was centrifuged under the usual conditions. The residue containing the s-MDH and LDH activities was collected.

Step 4. *Ultrogel AcA* 34 *chromatography.* The precipitate obtained from the saline fractionation was dissolved in the minimum amount of 0.1 M (pH 8.5) Tris–HCl buffer and was centrifuged at 15.000 *g* for 20 min at 4°C. The supernatant was chromatographed at 4 C in an Ultrogel AcA 34 column (2.6 × 100 cm) equilibrated with the same buffer, which as also used as eluent. 5 ml fractions were collected. Thus the s-MDH (A) (fractions 66–73) with a very low proportion of contaminating LDH was obtained (Fig. 6).

Step 5. *DEAE-Sephadex chromatography* (*pH* 8.5). The residual LDH contained in the Ultrogel eluates having MDH activity was removed by passing the eluates through a column of DEAE-Sephadex (1.6 × 40 cm) equilibrated with the buffer used in the previous step. Both enzymes were eluted with a linear concentration gradient with this buffer up to 0.2 M. 5 ml fractions were collected and the s-MDH (A), LDH free, was isolated under these conditions, as described by Puig *et al.* (1980) in the preparation of the s-MDH (A) from the same source by an alternative purification method.

The thus prepared s-MDH (A) was stored at −20°C, at which temperature it retained its activity unaltered for several months.

RESULTS AND DISCUSSION

Thermal stability of the molecular forms of s-MDH and of the LDH isoenzymes in the guinea-pig skeletal muscle soluble fraction

In the guinea-pig skeletal muscle soluble fraction $A_{280}/A_{260} = 0.5$. When the fraction was dialyzed for 20 hr with 5 mM (pH 7.4) phosphate buffer or filtered through Sephadex G-25 M and eluted with the same buffer, the ratio rose to 1.4. By both processes there were removed endogenous metabolites, probably nucleotides, which are abundant in muscular tissue and give absorbance readings at 260 nm. Their presence may influence the action of the temperature on the MDH and the LDH activity and the stability of their molecular forms. An attempt has been made to check this aspect.

When operating with undialyzed guinea-pig skeletal muscle soluble fraction, the thermostability of s-MDH (A) at 55°C and 60°C is greater than that of the B form (Figs 1c & 2c). The effect of the heat is little noticeable on the LDH isoenzymes (Figs 1d & 2d).

When the dialyzed soluble fraction is heated at 55°C for 25 min, the B form of s-MDH is seen to be destroyed, whereas the s-MDH (A) is scarcely altered (Fig. 1g). Under these conditions, the amount of M_4 isoenzyme is greatly reduced, unlike what happened on heating the undialyzed soluble fraction under the same conditions (Figs 1d & 1h). The variations are even more noticeable with heating at 60°C (Fig. 2).

The study of the electrophoretic patterns of the samples heated at 55°C (Figs 1d & 1h) confirms the notable reduction of the LDH activity in the dialyzed soluble fraction in comparison with the undialyzed fraction (Figs 3i & 3k). The activity differences observed with the s-MDH are less significant (Figs 3b & 3d). The facts suggest that the elimination of the nucleotides by dialysis affects basically the stability of the LDH, in particular, the stability of the M_4 isoenzyme.

With variable oxaloacetate and constant NADH (0.1 mM), the dialyzed guinea-pig skeletal muscle soluble fraction heated at 55°C for 25 min shows a normal kinetic behaviour and excess oxaloacetate inhibition (Fig. 4c) unlike the behaviour of the undialyzed fraction under identical conditions (Fig. 4b).

The kinetic differences observed are attributable to the different relative amount of LDH in both cases.

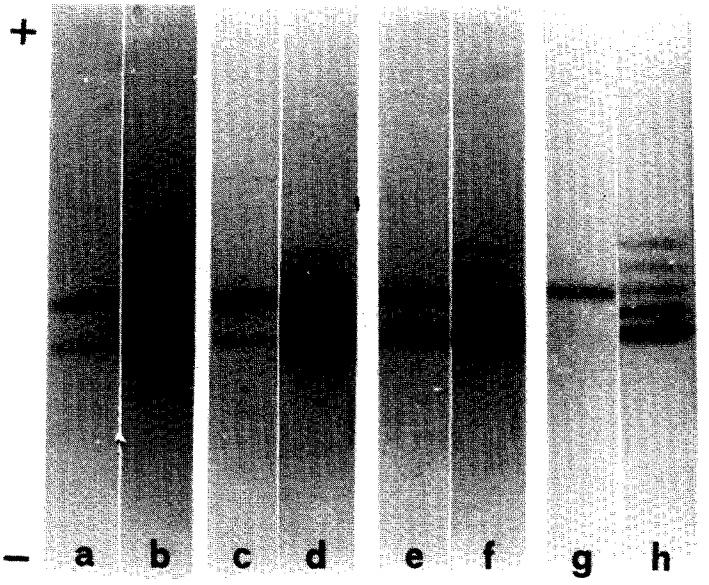


Fig. 1. Electrophoresis on cellulose acetate strips at pH 7.4 of the guinea-pig skeletal muscle soluble fraction. Electrophoresis was carried out for 75 min at 4°C in 50 mM phosphate buffer, pH 7.4. (a, c, e, g) Specific staining for MDH; (b, d, f, h) Specific staining for LDH. (a, b) Undialyzed soluble fraction; (c, d) Undialyzed soluble fraction heated at 55°C for 25 min; (e, f) Dialyzed soluble fraction; (g, h) Dialyzed soluble fraction heated at 55°C for 25 min.

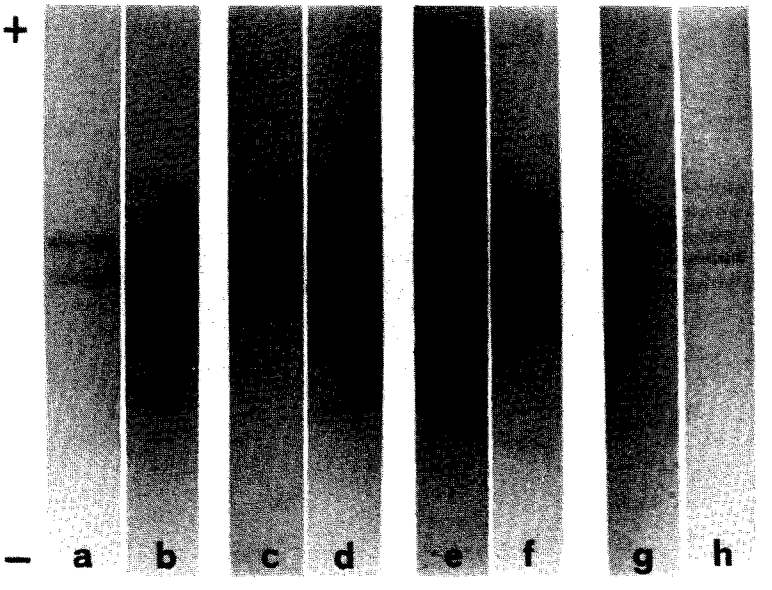


Fig. 2. Electrophoresis on cellulose acetate stripts at pH 7.4 of the guinea-pig skeletal muscle soluble fraction. The conditions used were as described in Fig. 1 but the thermal treatment was effected at 60°C.

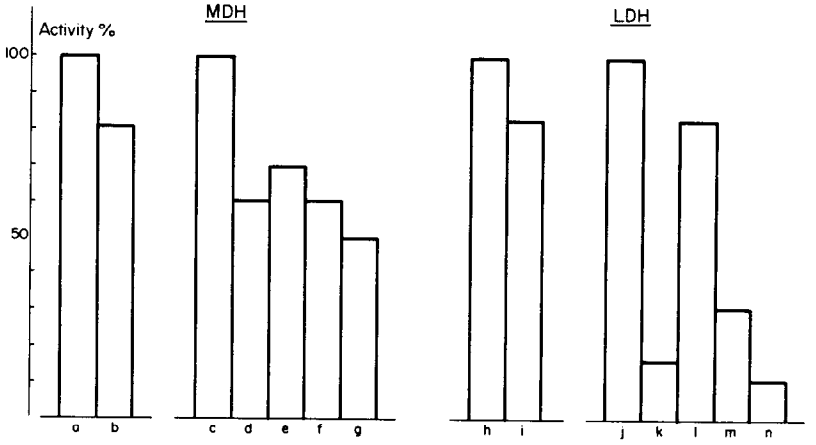


Fig. 3. MDH and LDH activities after different treatments of the guinea-pig skeletal muscle soluble fraction. Undialyzed soluble fraction: (a, h) Control; (b, i) Thermal treatment at 55°C for 25 min. Dialyzed soluble fraction: (c, j) Control; (d, k) Thermal treatment at 55°C for 25 min. Thermal treatment of the dialyzed soluble fraction at 55°C for 25 min containing: (e, l) 0.1 mM NADH; (f, m) 0.1 mM NAD^+; (g, n) 0.1 mM ATP. (a–g) MDH activity with L-malate and NAD^+; (h–n) LDH activity with pyruvate and NADH.

This enzyme is responsible for the kinetic anomalies at high oxaloacetate concentrations (Puig *et al.*, 1980).

Influence of the nucleotides

The influence of NADH, NAD^+ and ATP on the stability of the molecular forms of s-MDH and of LDH has been determined, working with the dialyzed guinea-pig skeletal muscle soluble fraction. Mixtures of the soluble fraction containing, in each case, NADH, NAD^+ or ATP (0.1 mM) were prepared and heated at 55°C, 25 min.

The s-MDH activity is slightly protected by NADH (Figs 3d & 3e), NAD^+ has no effect (Fig. 3f) and ATP is weakly inhibiting (Fig. 3g). On the other hand, LDH is noticeably stabilised by NADH (Figs 3k & 3l), the effect of NAD^+ is very much weaker (Fig. 3m) and ATP slightly inhibits the enzyme (Fig. 3n). The noticeable stabilising effect of NADH on LDH is in agreement with Vessell *et al.* (1968) and by Südi (1970) with the heart and skeletal muscle enzyme of the pig and the rabbit, respectively.

The kinetic behaviour of the above samples is different. In fact, those containing NADH continue to show anomalies (Fig. 5b), since the LDH activity, which also reduced oxaloacetate with NADH (Puig *et al.*, 1980), is preserved under these conditions (Fig. 3l). The apparent activation disappears in the NAD^+ containing sample (Fig. 5c) and even an increase of excess oxaloacetate inhibition is observed in the ATP containing sample (Fig. 5d); this is attributed to the fact that the LDH activity has been notably reduced in both cases (Figs 3m & 3n).

Purification of the thermostable molecular form of s-MDH

The heating of the dialyzed soluble fraction at 55°C for 25 min was a step in the isolation and purification of the thermostable s-MDH molecular form (step 2).

The treatment supernatant was fractionated with ammonium sulfate (step 3) and the thus obtained residue was eluted through an Ultrogel column leading to the preparation of the s-MDH (A) practically free from LDH (Fig. 6).

Chromatography of the combined A form containing fractions through a column of DEAE-Sephadex (pH 8.5) gave a peak containing this form, free from LDH and highly purified, with a specific activity, measured in the direction of oxaloacetate reduction, of 6.3 μkat/mg, in agreement with the results obtained by Puig *et al.* (1980) for s-MDH (A) from the same source by an alternative purification method.

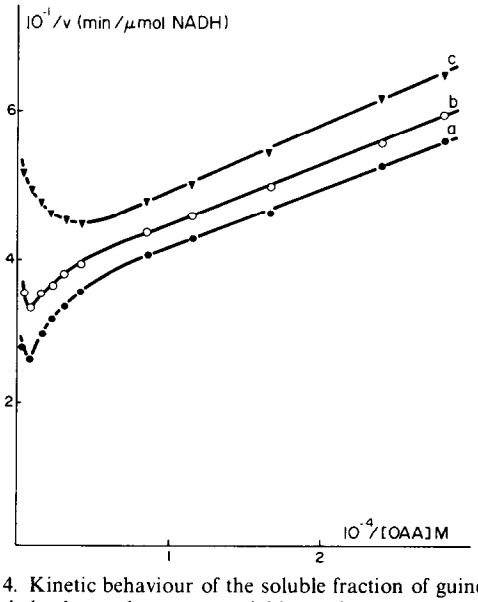


Fig. 4. Kinetic behaviour of the soluble fraction of guinea-pig skeletal muscle versus variable oxaloacetate concentrations. Reactions were carried out in 50 mM, pH 7.4, phosphate buffer. [NADH] = 0.1 mM. (a) Undialyzed or dialyzed soluble fraction; (b) Undialyzed soluble fraction heated at 55°C for 25 min; (c) Dialyzed soluble fraction heated at 55°C for 25 min.

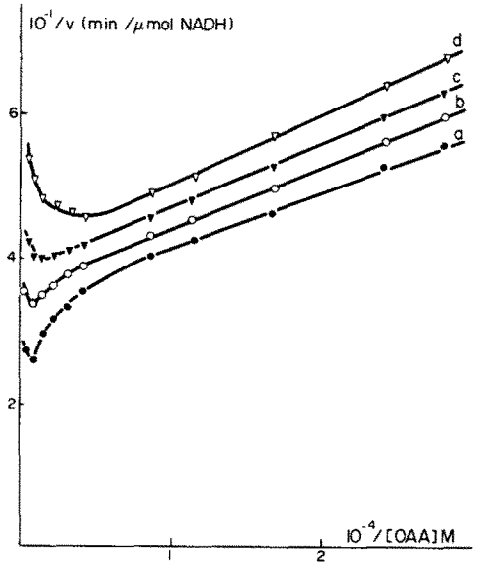


Fig. 5. Kinetic behaviour of the dialyzed soluble fraction of guinea-pig skeletal muscle versus variable oxaloacetate concentrations. (a) Dialyzed soluble fraction. Thermal treatment of the dialyzed soluble fraction at 55°C for 25 min containing: (b) 0.1 mM NADH; (c) 0.1 mM NAD^+; (d) 0.1 mM ATP. The conditions used were as described in Fig. 4.

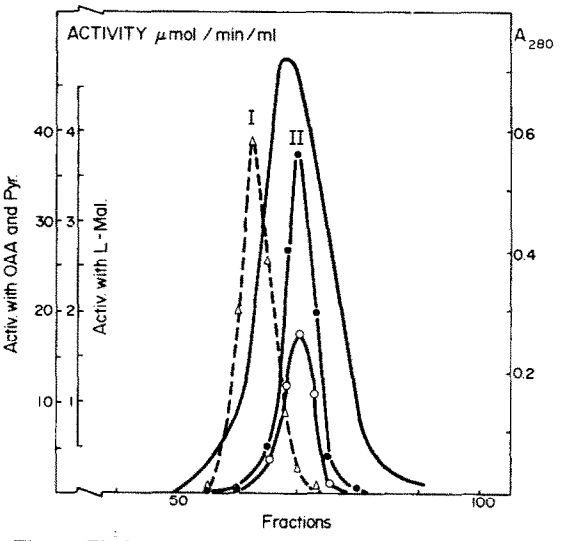


Fig. 6. Elution pattern of cytoplasmic malate dehydrogenase and lactate dehydrogenase obtained in step 3 of the purification procedure (described in Materials and Methods) from Ultrogel AcA 34. The column (2.6 × 100 cm) was equilibrated and eluted with Tris–HCl buffer 0.1 M, pH 8.5 at 4°C. Flow: 35 ml/hr. Activities: (●) with OAA and NADH; (△) with pyruvate and NADH; (○) with L-malate and NAD^+; (—) Absorbance at 280 nm. The fractions collected were 5 ml.

Kinetic characteristics of s-MDH (A)

The optimum pH of the enzyme was determined with 0.1 mM oxaloacetate and 0.1 mM NADH in 50 mM sodium phosphate buffer in the pH range of 6–9 and was found to be 7–7.2. 100 mM (pH 8.5–10.5) glycine–NaOH buffer was used with 2.7 mM L-malate and 0.6 mM NAD^+ and in this case the optimum pH was found to be 9.8–10.2.

There was established the particular reaction mechanism of s-MDH (A) in both directions of the reaction it catalyses, by application of the reaction product inhibition method. Likewise, the initial reaction velo-

Table 1. Reaction product inhibition of guinea-pig skeletal muscle s-MDH (A)

	Variable substrate			
	NADH (0.02–0.1 mM)		Oxaloacetate (0.06–0.4 mM)	
Inhibitor	OAA non-saturating 0.08 mM	OAA saturating 0.4 mM	NADH non-saturating 0.03 mM	NADH saturating 0.1 mM
NAD^+ (1.5–14 mM)	C K_{is} = 0.65 mM	C K_{is} = 0.8 mM	NC[a]	NC K_{is} = 103 mM K_{ii} = 8.95 mM
L-Malate (0.5–3 mM)	NC K_{is} = 5.34 mM K_{ii} = 1.35 mM	UC K_{ii} = 2.25 mM	NC K_{is} = 0.65 mM K_{ii} = 5.6 mM	NC K_{is} = 0.8 mM K_{ii} = 2.7 mM
	Variable substrate			
	NAD^+ (0.24–1.5 mM)		L-Malate (0.5–2.7 mM)	
Inhibitor	L-Mal non-saturating 0.7 mM	L-Mal saturating 2.7 mM	NAD^+ non-saturating 0.35 mM	NAD^+ saturating 1.5 mM
NADH (0.8–2.7 μM)	C K_{is} = 0.46 μM	C K_{is} = 0.45 μM	NC K_{is} = 1.1 μM K_{ii} = 3.78 μM	NC K_{is} = 6.44 μM K_{ii} = 11.55 μM
Oxaloacetate (1.8–7.4 μM)	NC K_{is} = 3.5 μM K_{ii} = 6.6 μM	UC K_{ii} = 5.6 μM	NC K_{is} = 1.56 μM K_{ii} = 21.75 μM	NC K_{is} = 8 μM K_{ii} = 8 μM

[a] NAD^+ inhibition versus oxaloacetate (NADH non-saturating) gives rise to deviations from linearity at high NAD^+ and oxaloacetate concentrations. C, competitive; NC, non-competitive; UC, uncompetitive.

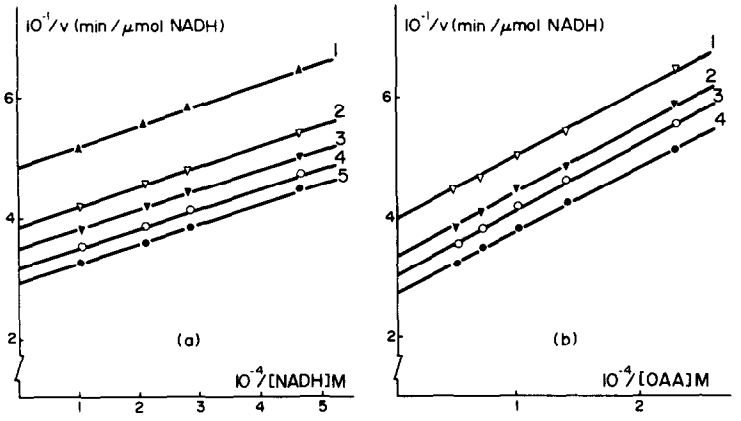


Fig. 7. Initial reaction rates of oxaloacetate–s-MDH (A)–NADH system. Reactions were carried out in 50 mM, pH 7.4 phosphate buffer. (a) [Oxaloacetate]: 1, 0.043 mM; 2, 0.07 mM; 3, 0.1 mM; 4, 0.143 mM; and 5, 0.2 mM. (b) [NADH]: 1, 0.021 mM; 2, 0.036 mM; 3, 0.05 mM; and 4, 0.1 mM.

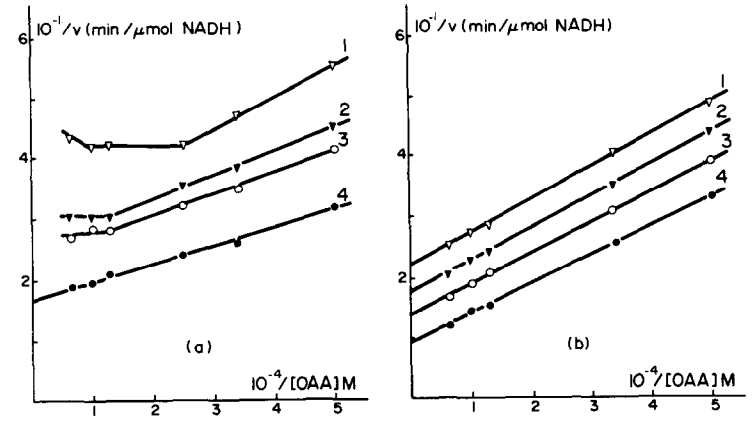


Fig. 8. Inhibition of the oxaloacetate–s-MDH (A)—NADH system by NAD^+. (a) [NADH] non sat. = 0.03 mM; (b) [NADH] sat. = 0.1 mM. (a) [NAD^+]: 1, 7 mM; 2, 3.5 mM; 3, 2.1 mM; and 4, 0 mM. (b) [NAD^+]: 1, 14 mM; 2, 10 mM; 3, 7 mM; and 4, 0 mM. The conditions used were as described in Fig. 7.

cities were determined in absence of products and with excess substrate inhibition. The operating pH was 7.4 in all cases, in a 50 mM sodium phosphate buffer medium.

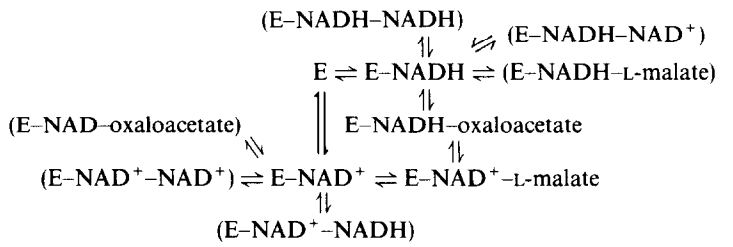
The results of the products inhibitions (Table 1) show that s-MDH (A) has a behaviour similar to the theoretically proposed one for an ordered bi–bi ternary complex mechanism (Cleland, 1963), with formation of E-coenzyme binary complexes.

There are, nevertheless, certain discrepancies relative to a simple ordered ternary mechanism. The apparent parallelism of the straight lines obtained in the double reciprocal plot with variable oxaloacetate and variable NADH (Fig. 7) should be observed. There is NAD^+ inhibition vs oxaloacetate for saturating NADH and NADH inhibition vs L-malate for saturating NAD^+, something which, theoretically, should not occur. Finally, in the noncompetitive inhibition of NAD^+ vs oxaloacetate for non-saturating NADH, there is a loss of the linearity of the straight lines inhibited at high NAD^+ and oxaloacetate concentrations (Fig. 8a).

With a view to explaining these anomalies, there has been proposed for s-MDH (A) a reaction mechanism (Scheme 1) in which, as well as the basic ordered sequential scheme, there has been taken into account the possible formation of several abortive complexes, appearing in brackets and which have kinetic significance under defined experimental conditions.

The uncompetitive excess oxaloacetate and L-malate inhibitions are justified, respectively, by the formation of the abortive E–NAD^+–oxaloacetate and E–NADH-L-malate complexes, while the non-competitive excess coenzyme inhibition takes place through the abortive E–NAD^+–NADH and E–NADH–NADH (excess NADH) complexes and E–NADH–NAD^+ and E–NAD^+–NAD^+ (excess NAD^+) complexes. All the abortive complexes cited are significant for substrate concentrations above the saturating concentrations.

L-malate is a non-competitive inhibitor of the enzyme vs NADH when the oxaloacetate concentration in the medium is non-saturating. Under these conditions, the L-malate binds to the E–NAD^+ complex, acting as product inhibitor. When the oxalo-



Scheme 1

acetate concentration is saturating, the inhibition is uncompetitive, due to the existence of an irreversible step between the binding sites of the variable substrate and of the inhibitor.

The binding of the L-malate to the E–NAD^+ complex justifies the non-competitive inhibition it has vs the oxaloacetate, irrespectively as to whether the NADH concentration in the medium is saturating or not.

Conclusions similar to those described are obtained on studying oxaloacetate inhibition. Oxaloacetate binds to the E–NADH form, which agrees with the non-competitive inhibition it has on the L-malate, irrespectively of the NAD^+ concentration. In turn, the keto acid is a non-competitive inhibitor of the enzyme vs NAD^+ when the L-malate is non-saturating, with the inhibition being uncompetitive when the latter concentration is saturating in view of the presence, under these conditions, of an irreversible step between the addition point of the inhibitor and that of the coenzyme.

The binding of the NADH and the NAD^+ to the free form of the enzyme justifies that the inhibition exercised by the coenzymes between them is competitive.

When determinations are made of the NAD^+ inhibition with variable oxaloacetate and non-saturating NADH, there are deviations from linearity in the inhibited straight lines (Fig. 8a), the more noticeable the higher the NAD^+ and oxaloacetate concentrations are. This phenomenon is due to the fact, under these conditions, there is an advance in the excess oxaloacetate inhibition, with formation of the abortive E–NAD^+-oxaloacetate complex, since the inhibitor increases the level in the steady-state of the E–NAD^+ form, facilitating the formation of the above mentioned abortive complex. In the presence of saturating NADH, there are not deviations from linearity in the inhibited straight lines (Fig. 8b).

When determining the influence of the presence of the reaction products on the value of the saturating concentration of the coenzymes (obtained experimentally in absence of products), by deducing this value theoretically by derivation of the rate equation drawn up according to the method of King & Altman (1956), it is observed that the saturating concentration of NADH remains unaltered in the presence of L-malate, but is significantly altered in the presence of NAD^+. Similarly, the saturating concentration of NAD^+ remains unaltered in the presence of oxaloacetate, but increases appreciably on adding NADH to the medium (Table 2). This result is in agreement with the competition between both coenzymes for the free form of the enzyme and justifies the subsistance of the noncompetitive inhibitions induced by the NAD^+ vs the oxaloacetate in the presence of a saturating concentration of NADH (determined in absence of products) and by the NADH vs the L-malate with saturating NAD^+ (concentration obtained in absence of products).

Finally, the fact that apparently parallel straight lines are obtained in the double reciprocal plot with oxaloacetate and NADH as substrates (Fig. 7) is due to the very low value of the E–NADH complex dissociation constant ($\bar{K}_{NADH}$) in comparison with its Michaelis constant (Table 3). This causes the intercept of the straight lines to be far removed from the ordinate axis (Garces & Cleland, 1969). This anomaly does not occur with L-malate (0.5–2.7 mM) and NAD^+ (0.24–1.5 mM) as substrates, since in this case the $\bar{K}_{NAD+}$ and $K_{m(NAD+)}$ values are similar (Table 3) and the straight lines obtained in the double reciprocal plot are not parallel.

Table 2. Influence of the reaction products on the saturating concentrations of the coenzymes

Substrate	Product	Saturating[a] concentration (mM)	Saturating[b] concentration (mM)
	—	0.1	0.1
NADH	L-Malate (2 mM)	0.12	
	NAD^+ (7 mM)	0.36	
	—	2	1.5
NAD^+	Oxalacetate (2 μM)	2.1	
	NADH (1 μM)	5.2	

[a] Theoretical value obtained by derivation of the appropriate rate equation.

[b] Experimental value obtained in absence of the products.

Table 3. Kinetic parameters of guinea-pig skeletal muscle s-MDH (A) at pH 7.4

Substrate	K_m (mM)	$\bar{K}$[a] (mM)
Oxaloacetate	0.041	—
NADH	0.018	0.00046
L-Malate	0.36	—
NAD^+	0.13	0.7

[a] Dissociation constant of the binary complexes.

The ordered bi–bi ternary complex mechanism, deduced for the guinea-pig skeletal muscle s-MDH (A), is similar to the one obtained for s-MDH from other sources (Silverstein & Sulebele, 1969; Frieden & Fernández-Sousa, 1975) and differs from that postulated by Cassman & Englard (1966) for beef heart s-MDH. By application of Haldane's ratio for ordered ternary complex mechanism, a K_{eq} value of $2.24 \times 10^{12}\,M^{-1}$ (at pH 7.4 and $30 \pm 0.1°C$) is obtained, to which there corresponds a $\Delta G^{\circ\prime}$ of -17.06 Kcal/mol, values very similar to those calculated with the enzyme from other sources (Yoshida, 1965).

CONCLUSION

Heating of the dialyzed guinea-pig skeletal muscle soluble fraction at 55°C for 25 min eliminates the kinetic anomalies observed with the undialyzed control sample in the reduction of oxaloacetate by NADH at high keto acid concentrations (>0.1 mM). This is due to the high thermal instability exhibited by the LDH M_4 isoenzyme under these conditions. The presence of NADH stabilises the LDH, the effect of NAD^+ is less and the effect of ATP is nil. The thermostable form of the s-MDH is adapted to an ordered bi–bi complex ternary mechanism, with the existence of certain abortive complexes.

SUMMARY

The thermal stability of the molecular forms of s-MDH from different sources have not received much study in comparison with the LDH isoenzymes, of which the H_4 is the most thermostable.

Two molecular forms of s-MDH and the five LDH isoenzymes, with predominance of the M_4, are found in guinea-pig skeletal muscle, the latter also catalysing the reduction of oxaloacetate by NADH. Their presence causes deviations from the Michaelis–Menten kinetics vs high keto acid concentrations.

The study of the thermal stability of both enzymes has evidenced the existence of a thermostable molecular form of the s-MDH (A form) and a notable instability of the M_4 isoenzyme of the LDH and has enabled the conditions under which the said kinetic anomalies disappear to be determined.

Highly purified s-MDH (A) is adjusted to an ordered bi–bi complex ternary mechanism, with the existence of the abortive E–NADH–NADH, E–NADH–NAD^+, E–NAD^+–NAD^+, E–NAD^+–NADH, E–NADH–L-malate and E–NAD^+–oxaloacetate complexes, which have kinetic significance under certain experimental conditions.

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