Kinetic studies of the regulation of mitochondrial malate dehydrogenase by citrate

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Mitochondrial malate dehydrogenase shows a complex regulation pattern in the presence of citrate. Previously published results indicate that this enzyme is activated by citrate in the $\text{NAD}^+ \rightarrow \text{NADH}$ direction and inhibited in the opposite direction. Moreover, high concentrations of L-malate or oxaloacetate produce deviations from the Michaelis–Menten behaviour. Results reported in this paper clearly show that citrate both activates and inhibits mitochondrial malate dehydrogenase in the same direction ($\text{NAD}^+ \rightarrow \text{NADH}$), and in the same reaction medium, depending on substrate concentration. This surprising effect has made it necessary to propose a new kinetic mechanism that extends those previously suggested and allows us to explain both the citrate effect (activating or inhibitory) and the effect of high concentrations of L-malate and oxaloacetate.

INTRODUCTION

Cellular regulator metabolites, which play an important part in metabolism, provide vital enzymes with information as to the state of the metabolism in specific regions of the metabolic pathway. It must be emphasized that such regulator metabolites fulfil a dual function, in that they are both intermediaries in certain metabolic pathways and also specific regulators of key enzymes, which may also be involved in other metabolic pathways.

Citrate is an important allosteric effector of several enzymes involved in fundamental pathways of cellular metabolism, such as acetyl-CoA carboxylase (Gregolin *et al.*, 1968a,b), pyruvate kinase (Miyana *et al.*, 1984; Chrispeels & Gaede, 1985; Storey, 1985; Evans & Ratledge, 1985) and phosphofructokinase (Passonneau & Lowry, 1963; Foe & Kemp, 1982; Narabayashi *et al.*, 1985). With reference to the tricarboxylic acid cycle, citrate activates isocitrate dehydrogenase (Gabriel & Plaut, 1984) and inhibits fumarate (Cennamo *et al.*, 1967). Further, its effect on malate dehydrogenase depends on the direction of the reaction, since it has been described as an activator of this enzyme in the $\text{NAD}^+ \rightarrow \text{NADH}$ direction and as an inhibitor in the reverse direction (Mullinax *et al.*, 1982; McEvily *et al.*, 1985).

Mitochondrial malate dehydrogenase (EC 1.1.1.37) is a dimeric enzyme that shows deviations from Michaelis–Menten kinetics at high concentrations of its substrates (i.e. L-malate activation and oxaloacetate inhibition) (Telegdi *et al.*, 1973; Bernstein *et al.*, 1978). Although it has not usually been considered to be a regulatory enzyme, recent data suggest that its activity can be regulated by citrate or by multienzyme interaction (Mullinax *et al.*, 1982; Fahien *et al.*, 1988). Owing to the importance of oxaloacetate concentration in the control of citrate synthase activity, and, in consequence, in the modulation of the overall tricarboxylic acid cycle activity, the regulation of mitochondrial malate dehydrogenase by citrate may be of physiological significance (McEvily *et al.*, 1985).

Previous kinetic data have demonstrated that both catalytic activity and regulation of mitochondrial malate dehydrogenase by effectors are properties of the individual subunits and are therefore independent of interactions between catalytically active subunits (Jurgensen *et al.*, 1981; McEvily *et al.*, 1985). Mullinax

et al. (1982) have proposed that L-malate activation, oxaloacetate inhibition and the citrate kinetic effect may be interpreted in relation to an allosteric conformationally specific binding model for mitochondrial malate dehydrogenase.

Preliminary studies have shown that NAD^+ concentration is a key factor in the activation of mitochondrial malate dehydrogenase by L-malate (Dordal *et al.*, 1990). At high NAD^+ concentrations activation by L-malate is observed, whereas Michaelis–Menten kinetic behaviour is observed when NAD^+ concentration is low.

In the present investigation the kinetic behaviour of mitochondrial malate dehydrogenase in the presence of citrate and the influence of substrate concentrations on the effect of this metabolite were established.

Steady-state data obtained from a wide range of concentrations of substrates reveal that the activating effect of citrate in the $\text{NAD}^+ \rightarrow \text{NADH}$ direction becomes inhibitory at low substrate concentrations. Our results suggest an alternative mechanism that explains the influence of substrate concentrations on the regulation pattern of citrate, which cannot be explained by the mechanisms previously proposed for mitochondrial malate dehydrogenase (Harada & Wolfe, 1968; Mullinax *et al.*, 1982; Müller, 1985). Determination of kinetic parameters that correspond to the mechanism proposed corroborates the validity of theoretical predictions made and permits simulation of the effect of citrate on mitochondrial malate dehydrogenase activity at all the experimental conditions assayed.

EXPERIMENTAL

Reagents

Sodium L-malate, sodium oxaloacetate and sodium citrate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and NAD^+ and NADH were supplied by Boehringer Mannheim (Mannheim, Germany). All other chemicals used were of standard reagent grade.

Homogeneous chicken liver mitochondrial malate dehydrogenase (specific activity 45 nkat/mg) was obtained by the method of Gelpí *et al.* (1988). Pig heart mitochondrial malate dehydrogenase was purchased from Boehringer Mannheim.

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Activity measurements

In order to state enzyme concentration during purification, and as routine checking of enzyme stock solutions, malate dehydrogenase activity was measured in a medium containing 0.14 mM-NADH and 0.3 mM-oxaloacetate in 100 mM-sodium phosphate buffer, pH 7.4, by monitoring the absorbance at 340 nm in a Hewlett-Packard 8450A spectrophotometer in a 1 cm-light-path cell thermostatically controlled at 30 ± 0.1 °C. Enzyme activities were expressed in katal (kat).

Theoretical rate equations

The rate equations derived from the mechanism proposed were deduced by application of the method of Cha (1968). Algebraic manipulations were performed by using the program REDUCE implemented on a IBM 3090 computer (Rayna, 1987).

Kinetic measurements

Kinetic studies were carried out in a medium containing 50 mM-Tris/HCl buffer, pH 8.1, at 30 ± 0.1 °C. The pH of substrate solutions was adjusted to 8.1 when necessary. The amount of mitochondrial malate dehydrogenase used was 1 nkat, determined as indicated. Initial reaction rates in the $\text{NADH} \rightarrow \text{NAD}^+$ direction were measured by monitoring the absorbance at 340 nm. The opposite direction was analysed by coupling 0.064 mM-phenazine methosulphate and 0.384 mM-Nitro Blue Tetrazolium and by monitoring the increase of absorbance at 548 nm due to formazan. This method obviates the unfavourable equilibrium and product inhibition by NADH, and gives linear responses even at very low substrate concentrations. The molar absorption coefficient of formazan at 548 nm is $10.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. When substrate concentration allows measurement of reaction rates by monitoring 340 nm absorbance, determinations by both 340 nm and 548 nm absorbance were routinely performed, in order to provide assurance that the coupled system does not affect enzyme activity.

A typical kinetic experiment consists in the measurement of 20–25 points, corresponding to the combination of different substrate and effector concentrations. A minimum of five replicates for each single experimental point were performed.

The values of initial rate were plotted in the Lineweaver-Burk plot, and analysed to detect and reject possible outliers, by using the program KAICOUT (López Cabrera *et al.*, 1988) implemented on a VAX 6310 computer. Kinetic parameters and curve drawings were obtained by fitting the appropriate rate equations to experimental data by non-linear regression, by using both KAICOUT on a VAX 6310 computer and a simplified version of the same program running under MSDOS. Points were weighted according to the reciprocal of squared s.e.m. of experimental replicates. Parameters are expressed as value $\pm$ s.e. (calculated from regression). Fitting goodness were established according to a standard *F* test (Pettersson & Pettersson, 1970).

RESULTS AND DISCUSSION

It is generally accepted that citrate is an activator of the oxidation of L-malate by NAD^+ , catalysed by mitochondrial malate dehydrogenase in a medium containing 50 mM-Tris/HCl buffer, pH 8.1. However, the mode of action of allosteric effectors (inhibitory or activating) depends in some instances on the experimental conditions (pH, substrate concentrations, the concentration of the effector itself etc.).

In the present paper we describe steady-state kinetic studies on the effect of citrate on mitochondrial malate dehydrogenase in a wide range of substrate concentrations. Experiments were carried out in a medium containing 50 mM-Tris/HCl buffer, pH 8.1. This medium allows us to analyse both reaction directions in the same conditions, which is necessary to achieve a global knowledge of the mechanism under study. Moreover, in this way, it is possible to compare our results with those previously published by others.

Fig. 1. Influence of citrate on malate dehydrogenase activity

Assays were performed as described in the Experimental section. Malate dehydrogenase activity measured in absence of citrate was designated as 100%. Values are expressed as means $\pm$ s.e.m. for five replicates ($P < 0.05$). NADH $\rightarrow$ NAD⁺ direction. [NADH]: (a) 0.02 mM; (b) 0.14 mM. [Oxaloacetate]: ●, 0.05 mM; ○, 125 mM. NAD⁺ $\rightarrow$ NADH direction. [NAD⁺]: (c) 0.2 mM; (d) 5 mM. [L-Malate]: ●, 2.5 mM; ○, 10 mM.

Fig. 2. Influence of citrate on pig heart malate dehydrogenase activity

Assays were performed as described in the Experimental section. Malate dehydrogenase activity measured in absence of citrate was designated as 100%. Values are expressed as means $\pm$ S.E.M. for five replicates ($P < 0.05$). $[NAD^+]$: 1 mM. $[L\text{-Malate}]$: ○, 1 mM; ●, 10 mM.

Fig. 1 shows the effect of increasing concentrations of citrate on both directions of the reaction catalysed by mitochondrial malate dehydrogenase. This metabolite inhibits the reduction of oxaloacetate independently of the NADH concentration assayed (Figs. 1a and 1b). In the opposite direction, at high NAD^+ concentrations (5 mM) mitochondrial malate dehydrogenase activity is enhanced at any L-malate concentration (Fig. 1d), in agreement with the results described for pig heart enzyme (Mullinax *et al.*, 1982). However, when NAD^+ concentration is low (0.2 mM) citrate acts as an inhibitor (Fig. 1c). Thus, in the same direction of the reaction ($NAD^+ \rightarrow NADH$) and in the same medium (50 mM-Tris/HCl buffer, pH 8.1), citrate is an activator at high coenzyme concentration and an inhibitor when the NAD^+ concentration is lower. This result has not been reported previously. However, Fahien *et al.* (1988) have described an inhibitory effect of citrate on the $NAD^+ \rightarrow NADH$ direction

at pH 6.5. These authors only report activation by citrate at pH 8.0. This complex effect of citrate is not a specific characteristic of chicken liver enzyme. A similar experiment performed with pig heart mitochondrial malate dehydrogenase reveals an identical dual effect (activation and inhibition) of citrate (Fig. 2). Neither activating nor inhibition effects can be detected in the presence of up to 200 mM-NaCl (results not shown); therefore the above indicated behaviour is due to a specific effect of citrate rather than an ionic-strength effect.

This surprising effect of citrate on the $NAD^+ \rightarrow NADH$ direction of the enzymic reaction implies a regulatory pattern that is much more efficient than a simple activation or inhibition. Subsequently a complete steady-state kinetic study was carried out of the effect of substrate concentration on the regulation of mitochondrial malate dehydrogenase by citrate. A range of substrate concentrations wide enough to include activation by excess L-malate and inhibition by excess oxaloacetate has been chosen.

Fig. 3 shows Lineweaver-Burk plots obtained when L-malate is the substrate whose concentration is varied and different fixed concentrations of NAD^+ are used. If NAD^+ is 5 mM, enzymic activity increases in the presence of citrate, and malate dehydrogenase activation at the higher concentrations of L-malate (> 10 mM) is eliminated (Fig. 3c). However, at low NAD^+ concentrations (0.2 mM) activation by excess L-malate is not detected and citrate appears as a competitive inhibitor (Fig. 3a). Finally when an intermediate NAD^+ concentration is used (1 mM) citrate acts as an inhibitor at low L-malate concentrations and as an activator at high concentrations of this substrate (Fig. 3b).

The results shown in Fig. 4, where NAD^+ is the substrate whose concentration is varied, the initial-velocity patterns obtained at different fixed concentrations of L-malate, also corroborate the dual effect (inhibitory, activating or both) of citrate.

In contrast with what occurs in the $NAD^+ \rightarrow NADH$ direction, the steady-state kinetic data obtained with oxaloacetate and NADH as substrate demonstrate that the mode of action of citrate does not depend on the fixed NADH or oxaloacetate concentrations present in the reaction medium. Double-reciprocal plots indicate that citrate and NADH appear to be competitive and that citrate is a mixed inhibitor versus oxaloacetate (Fig. 5). Moreover, Fig. 5(b) shows that inhibition by excess oxaloacetate is diminished in the presence of citrate.

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Fig. 3. Effect of citrate on mitochondrial malate dehydrogenase activity: $[NAD^+]$ fixed concentrations

Mitochondrial malate dehydrogenase activity was measured as described in the Experimental section in the $NAD^+ \rightarrow NADH$ direction, with several fixed $[NAD^+]$ concentrations and varied concentrations of L-malate. Curve drawings were performed by fitting polynomial-equations of the same degree of model equations. $[NAD^+]$: (a) 0.2 mM; (b) 1 mM; (c) 5 mM. $[Citrate]$: ●, 0 mM; ○, 2.5 mM; ■, 10 mM; □, 25 mM.

Fig. 4. Effect of citrate on mitochondrial malate dehydrogenase activity: fixed L-malate concentrations

Mitochondrial malate dehydrogenase activity was measured as described in the Experimental section in the $\text{NAD}^+ \rightarrow \text{NADH}$ direction, with fixed L-malate concentrations and varied concentrations of NAD^+ . Curve drawings were performed by fitting polynomial equations of the same degree of model equations. [L-Malate]: (a) 2.5 mM; (b) 10 mM. [Citrate]: ●, 0 mM; ○, 2.5 mM; ■, 10 mM; □, 25 mM.

Fig. 5. Effect of citrate on mitochondrial malate dehydrogenase activity: $\text{NADH} \rightarrow \text{NAD}^+$ direction

Mitochondrial malate dehydrogenase activity was measured as described in the Experimental section. Curve drawings were performed by fitting polynomial equations of the same degree of model equations. (a) Fixed [oxaloacetate]: 0.3 mM. (b) Fixed [NADH]: 0.14 mM. [Citrate]: ●, 0 mM; ○, 2.5 mM; ■, 10 mM; □, 25 mM.

shown in the results reported here, and on activation by L-malate, shown in a previous work (Dordal *et al.*, 1990), have not been described elsewhere, and thus it is necessary to redesign the kinetic mechanism of mitochondrial malate dehydrogenase in order to justify these new findings.

In summary, a kinetic mechanism for mitochondrial malate dehydrogenase should explain the following experimental results. (1) The enzyme follows a compulsory ordered bi-bi mechanism, with coenzymes being added first, when citrate is not present in the reaction medium (Banaszak & Bradshaw, 1975). (2) The enzyme is activated by high concentrations of its substrate L-malate (Telegdi *et al.*, 1973; Mullinax *et al.*, 1982). This phenomenon disappears when NAD^+ concentration is low enough (Fig. 3). (3) The enzyme is inhibited by excess oxaloacetate (Fig. 5). This inhibition is not dependent on the NADH concentration present in the reaction medium. (4) In the physiological direction of the reaction catalysed by mitochondrial malate dehydrogenase

($\text{NAD}^+ \rightarrow \text{NADH}$) the effect of citrate (activating, inhibitory or both) depends on L-malate and NAD^+ concentrations (Figs. 3 and 4). (5) In the $\text{NADH} \rightarrow \text{NAD}^+$ direction citrate always produces a competitive inhibition with respect to NADH and a mixed inhibition versus oxaloacetate (Fig. 5). (6) The deviations from Michaelis-Menten kinetics observed at high concentrations of L-malate or oxaloacetate are not detected in the presence of citrate (Figs. 3 and 5).

The kinetic behaviour exhibited by the enzyme at high concentrations of oxaloacetate or L-malate cannot be explained by a Michaelis-Menten kinetic mechanism. Thus it seems evident that the mechanism of mitochondrial malate dehydrogenase should take into account the existence of conformational changes in its subunits induced by the binding of the appropriate effectors. In this sense, Mullinax *et al.* (1982) have pointed out the existence of an allosteric centre that is common for citrate, oxaloacetate and L-malate, and they have suggested an equilibrium between

two conformational states of the enzyme molecule. In brief, the mechanism proposed by Mullinax *et al.* (1982) suggests that a different conformation of the enzyme catalyses each direction of the reaction. Therefore binding of effectors to the conformation that catalyses NAD^+ reduction may lead to activation by citrate and L-malate and inhibition by oxaloacetate. However, the mechanism proposed by these authors is not compatible with the influence of substrate concentration on citrate action, and cannot explain any inhibitory effect of citrate in the $\text{NAD}^+ \rightarrow \text{NADH}$ direction. An analysis of this mechanism reveals a paradox. At high NAD^+ concentrations, when the enzyme shows a high reaction rate, it is reasonable to suppose that the enzyme is in the conformation that preferentially binds NAD^+ . In consequence, if citrate activation were due to a conformational change towards the NAD^+ -binding form, the effect of this metabolite would be either null or very small in these conditions. In contrast at low NAD^+ concentrations the effect of citrate would be more important owing to the displacement of the equilibrium between conformations towards the NADH -binding form. Derivation of theoretical rate equations for the mechanism proposed by Mullinax *et al.* (1982) confirms that this model cannot explain the effect of substrate concentration either on citrate action or on L-malate activation (results not shown).

Assuming the existence of a common regulatory site that binds citrate, oxaloacetate and L-malate, and keeping a dual-conformation model for mitochondrial malate dehydrogenase, the catalytic mechanism of mitochondrial malate dehydrogenase can be revised in such a way that it can account for all the data presented in this paper. This mechanism is shown in Scheme 1.

The most remarkable difference of the present mechanism from the previously proposed by Mullinax *et al.* (1982) is that the normal conformation of the enzyme (conformation E) is able to catalyse both the oxaloacetate reduction and the L-malate oxidation. The activation and inhibition phenomena are not due

Scheme 1. Kinetic mechanism for mitochondrial malate dehydrogenase

Despite the dimeric nature of the enzyme, kinetics of each subunit are independent. Conformation E catalyses both the oxidation of L-malate and the reduction of oxaloacetate. Conformation F is characterized by a lower substrate affinity and a higher catalytic efficiency than conformation E. Because of the expected extremely low affinity of conformation F for NADH this conformation cannot catalyse the reduction of oxaloacetate. Allosteric effectors: C indicates citrate, and X represents either L-malate (Mal) or oxaloacetate (OAA). In the derivation of the theoretical rate equations, binding of any effector to the allosteric site has been considered as a rapid-equilibrium step.

to the change of conformation itself, as suggested by Mullinax *et al.* (1982), but to the special kinetic characteristics of the conformation that appears when a metabolite binds to the regulatory site (F).

Conformation F exhibits a lower substrate affinity than conformation E. With respect to NADH , the decrease of affinity is such that the binding of this coenzyme to conformation F becomes impossible (Scheme 1), as indicated by the inhibition patterns observed in the $\text{NADH} \rightarrow \text{NAD}^+$ direction (Fig. 5). This hypothesis is also supported by binding experiments (Mullinax *et al.*, 1982) and by X-ray-diffraction determinations (Roderick & Banaszak, 1986).

The competitive inhibition produced by citrate when the reaction is in the opposite direction, when the concentration of the fixed substrate is low (Figs. 3 and 4), is also in good agreement with a lower substrate affinity (L-malate and NAD^+) of conformation F with respect to that of conformation E. Nevertheless, the change in the citrate action (inhibition becomes activation) when the concentration of fixed substrate rises indicates, on one hand, that the decrease in the NAD^+ affinity is lower than for NADH and, on the other hand, that conformation F exhibits a higher catalytic efficiency than conformation E. However, activation only occurs when L-malate and NAD^+ concentrations are high enough to surmount their lower affinity for conformation F.

The mechanism shown in Scheme 1 also explains the deviations from Michaelis-Menten kinetics observed at high concentrations of oxaloacetate and L-malate when citrate is not present. Indeed, the conformational change ($E \rightarrow F$) promoted by the binding of L-malate or oxaloacetate to the allosteric centre produces, as in the case of citrate, an inhibitory effect in the $\text{NADH} \rightarrow \text{NAD}^+$ direction (inhibition by excess of oxaloacetate) and also an activating effect in the opposite direction (activation by excess of L-malate) when the NAD^+ concentration is high. Obviously, the competition between citrate, L-malate and oxaloacetate for the common centre agrees with the elimination of these deviations when the citrate is present in the reaction medium (Figs. 3 and 5).

In order to confirm that the proposed mechanism and its theoretical predictions can really explain the behaviour of mitochondrial malate dehydrogenase in the presence of citrate, the corresponding rate equations and kinetic parameters need to be determined.

Theoretical rate equations for the mechanism shown in Scheme 1 have been deduced. In order to make the equations more simple it has been necessary to define a number of kinetic parameters that combine the real rate constants present in the reaction scheme. Both the parameters defined and the final rate equations are indicated in the Appendix.

In the absence of citrate, initial rates were determined with substrate concentration margins in which no activation by L-malate (< 10 mM) and no inhibition by oxaloacetate (< 0.3 mM) were detected. Parameter values of the enzyme in absence of citrate were obtained by fitting the data to eqn. (1). Experiments performed in the presence of citrate (up to 25 mM) in the $\text{NAD}^+ \rightarrow \text{NADH}$ direction, either with fixed NAD^+ concentration (5 mM) and varied L-malate concentration (0.1–100 mM) or with constant L-malate concentration (10 mM) and varied NAD^+ concentration (0.05–2 mM), were analysed by fitting eqn. (4) and eqn. (5) respectively to the data. Measurements made in the opposite direction either with constant NADH concentration (0.15 mM) and varied oxaloacetate concentration (0.01–5 mM) or with fixed oxaloacetate concentration (0.125 mM) and varied NADH concentration (0.032–0.2 mM) were analysed by using eqn. (6) and eqn. (7) respectively.

Table 1 summarizes the different parameters of the mechanism that can be deduced from the experiments performed. Detailed

Table 1. Summary of the kinetic parameters of the mechanism of mitochondrial malate dehydrogenase

Parameter values were deduced by fitting eqns. (4)–(7) to experimental results. Results of regression runs are shown in the Appendix. Abbreviations: Mal, L-malate; OAA, oxaloacetate.

α_1	0.13 ± 0.017	α_2	0.04 ± 0.009
α_3/K_{c3}	$0.049 \pm 0.005 \text{ mM}^{-1}$	α_{-1}	0.244 ± 0.021
α_{-2}/K_{c3}	$1.6 \pm 0.8 \text{ mM}^{-1}$	$\gamma(\text{NAD}^+)/K_{c3}$	0.0155 ± 0.005
δ_1	0.18 ± 0.05	δ_2/K_{c3}	$1.53 \pm 0.8 \text{ mM}^{-1}$
$\gamma(\text{NADH})/K_{c3}$	$0.45 \pm 0.2 \text{ mM}^{-1}$		
K_{c1}	$2.1 \pm 0.13 \text{ mM}$	K_{c2}	$1.81 \pm 0.19 \text{ mM}$
$\beta_2(\text{Mal})$	10.2 ± 1.1	$\beta_3(\text{Mal})$	0.21 ± 0.08
$\beta_3(\text{OAA})$	19 ± 9	$\beta_3(\text{OAA})$	2.7 ± 1
$k'_{\text{cat.}}/k_{\text{cat.}}(\text{NAD}^+ \rightarrow \text{NADH})$	3.26 ± 1.4		
$k'_{\text{cat.}}/k_{\text{cat.}}(\text{NADH} \rightarrow \text{NAD}^+)$	0.3 ± 0.1		
K_{NAD^+}	$1.21 \pm 0.4 \text{ mM}$	K'_{NAD^+}	$2.27 \pm 0.3 \text{ mM}$
$K_m(\text{NAD}^+)$	$60 \pm 1 \text{ }\mu\text{M}$	$K'_m(\text{NAD}^+)$	$1.45 \pm 0.5 \text{ mM}$
$K_m(\text{Mal})$	$0.145 \pm 0.002 \text{ mM}$	$K'_m(\text{Mal})$	$4.75 \pm 1 \text{ mM}$
$K_m(\text{NADH})$	$44 \pm 1.6 \text{ }\mu\text{M}$	$K'_m(\text{NADH})$	$38.1 \pm 20 \text{ }\mu\text{M}$
$K_m(\text{OAA})$	$17 \pm 8 \text{ }\mu\text{M}$	$K'_m(\text{OAA})$	$12 \pm 8 \text{ }\mu\text{M}$

Fig. 6. Simulation of kinetic behaviour of mitochondrial malate dehydrogenase in different experimental conditions

Lineweaver-Burk plots were performed by using eqn. (1) and the parameter values shown in Table 1. Experimental conditions correspond to the experimental results shown in Fig. 3. L-Malate concentration varied. $[\text{NAD}^+]$: (a) 0.2 mM; (b) 1 mM; (c) 5 mM. [Citrate]: ●, 0 mM; ○, 2.5 mM; ■, 10 mM; □, 25 mM.

results and statistics of the experiments performed are shown in the Appendix. The values indicated show a 25-fold increase of NAD^+ and L-malate K_m values for conformation F with respect to conformation E. Moreover, the catalytic constant calculated for the conformation produced by the binding of citrate is 3.62-fold higher than that for conformation E. These results agree both with the decrease in the affinity of the enzyme for NAD^+ and L-malate and also the increase in catalytic efficiency that the mechanism predicts as effects of citrate binding. The increase of L-malate K_m also agrees with the results reported by Telegdi *et al.* (1973) in their study of L-malate activation. No significant differences were found in the K_m values of conformations E and F for NADH and oxaloacetate, as can be expected from the mechanism proposed, where conformation F does not participate in the binding of these substrates to the enzyme in the course of the reaction (Scheme 1). The difference in catalytic constants of the two conformations is in good agreement with the results reported by Harada & Wolfe (1968) and is consistent with the variation of this parameter detected when oxomalonate is replaced by oxaloacetate as substrate.

The stimulation of the behaviour of mitochondrial malate

dehydrogenase in all experimental conditions, performed with all the parameters obtained from independent experiments at saturating concentrations of the different substrates, is a final proof of the validity of the kinetic mechanism proposed. For instance, Fig. 6 shows the simulation of $\text{NAD}^+ \rightarrow \text{NADH}$ direction measurements in the experimental conditions of Fig. 3 (fixed NAD^+ concentrations 0.2, 1, and 5 mM; varied L-malate concentration). The simulation of the $\text{NADH} \rightarrow \text{NAD}^+$ direction is also in good agreement with the behaviour shown in Fig. 5.

Finally, it is important to note that the citrate dissociation constants (about 2 mM; see Table 1) are within the range of physiological concentrations reported for this metabolite (Siess *et al.*, 1977; Tischler *et al.*, 1977; Watkins *et al.*, 1977). The citrate effect described in the present paper leads to an important increase in the influence of substrate concentrations on malate dehydrogenase activity (i.e. inhibition/activation at low/high substrate concentrations) and subsequently could make the enzyme more adaptable to metabolic requirements.

The importance of oxaloacetate concentration in the control of citrate synthase activity and the existence of citrate synthase-malate dehydrogenase complexes (Srere, 1985; Fahien &

Kmiotek, 1983) reinforce the suggestion that the kinetic regulation of mitochondrial malate dehydrogenase by citrate reported here may be of physiological significance.

This work was supported by a grant of the Comissió Interdepartamental de Recerca i Innovació Tecnològica (C.I.R.I.T.).

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APPENDIX

Theoretical rate equations

Theoretical rate equations were deduced according to the method of Cha (1968). Real rate constants are grouped to define the following kinetic parameters:

$$\alpha_1 = k_{+11}/k_{+1} \quad \alpha_3 = k_{+13}/k_{+3} \quad \alpha_{-2} = k_{-12}/k_{-2}$$

$$\alpha_2 = k_{+12}/k_{+2} \quad \alpha_{-1} = k_{-11}/k_{-1}$$

NAD⁺ → direction:

$$\gamma = \frac{k_{+4}k_{+5} + k_{-3}k_{+5} + \alpha_3k_{+3}k_{+3} + \alpha_3k_{+3}k_{+5}}{k_{+4}k_{+5} + k_{-3}k_{+5} + k_{+3}k_{+4} + k_{+3}k_{+5}}$$

$$k_{\text{cat.}} = \frac{k_{+3}k_{+4}k_{+5}}{k_{+4}k_{+5} + k_{-3}k_{+5} + k_{+3}k_{+4} + k_{+3}k_{+5}}$$

$$k'_{\text{cat.}} = \frac{\alpha_3k_{+3}k_{+4}k_{+5}}{k_{+4}k_{+5} + k_{-3}k_{+5} + \alpha_3k_{+3}k_{+4} + \alpha_3k_{+3}k_{+5}} = k_{\text{cat.}}\alpha_3/\gamma$$

$$K_{\text{NAD}^+} = \frac{k_{-1}}{k_{+1}}$$

$$K'_{\text{NAD}^+} = \frac{\alpha_{-1}k_{-1}}{\alpha_1k_{+1}} = K_{\text{NAD}^+}\alpha_{-1}/\alpha_1$$

$$K_{\text{m}}(\text{Mal}) = \frac{(k_{+4}k_{-2} + k_{+3}k_{+4} + k_{-2}k_{-3})k_{+5}}{k_{+2}(k_{+4}k_{+5} + k_{-3}k_{+5} + k_{+3}k_{+4} + k_{+3}k_{+5})}$$

$$K'_{\text{m}}(\text{Mal}) = \frac{(\alpha_{-2}k_{+4}k_{-2} + \alpha_3k_{+3}k_{+4} + \alpha_{-2}k_{-2}k_{-3})k_{+5}}{\alpha_2k_{+2}(k_{+4}k_{+5} + k_{-3}k_{+5} + \alpha_3k_{+3}k_{+4} + \alpha_3k_{+3}k_{+5})}$$

$$K_{\text{m}}(\text{NAD}^+) = \frac{k_{+3}k_{+4}k_{+5}}{k_{+1}(k_{+4}k_{+5} + k_{-3}k_{+5} + k_{+3}k_{+4} + k_{+3}k_{+5})}$$

$$K'_{\text{m}}(\text{NAD}^+) = \frac{\alpha_{-3}k_{+3}k_{+4}k_{+5}}{\alpha_{-1}k_{+1}(k_{+4}k_{+5} + k_{-3}k_{+5} + \alpha_3k_{+3}k_{+4} + \alpha_3k_{+3}k_{+5})} = K_{\text{m}}(\text{NAD}^+)\alpha_3/\alpha_{-1}\gamma$$

NADH → NAD⁺ direction:

$$\begin{aligned}\gamma &= \frac{\alpha_{-1}k_{-1}k_{-3} + \alpha_{-2}k_{-2}k_{-3} + \alpha_{-1}\alpha_{-2}k_{-1}k_{-2} + \alpha_3\alpha_{-1}k_{+3}k_{-1}}{k_{-1}k_{-3} + k_{-2}k_{-3} + k_{-1}k_{-2} + k_{+3}k_{-1}} \\ k_{\text{cat.}} &= \frac{k_{-1}k_{-2}k_{-3}}{k_{-1}k_{-3} + k_{-2}k_{-3} + k_{-1}k_{-2} + k_{+3}k_{-1}} \\ k'_{\text{cat.}} &= \frac{\alpha_{-1}\alpha_{-2}k_{-1}k_{-2}k_{-3}}{\alpha_{-1}k_{-1}k_{-3} + \alpha_{-2}k_{-2}k_{-3} + \alpha_{-1}\alpha_{-2}k_{-1}k_{-2} + \alpha_3\alpha_{-1}k_{+3}k_{-1}} = k_{\text{cat.}}\alpha_{-1}\alpha_{-2}/\gamma \\ K_{\text{NAD}} &= \frac{k_{+5}}{k_{-5}} \\ K_m(\text{OAA}) &= \frac{(k_{+4}k_{-2} + k_{-2}k_{-3} + k_{+3}k_{+4})k_{-1}}{k_{-4}(k_{-1}k_{-3} + k_{-2}k_{-3} + k_{-1}k_{-2} + k_{+3}k_{-1})} \\ k'_m(\text{OAA}) &= \frac{(\alpha_{-2}k_{+4}k_{-2} + \alpha_3k_{+3}k_{+4} + \alpha_{-2}k_{-2}k_{-3})\alpha_{-1}k_{-1}}{k_{-4}(\alpha_{-1}k_{-1}k_{-3} + \alpha_{-2}k_{-2}k_{-3} + \alpha_{-1}\alpha_{-2}k_{-1}k_{-2} + \alpha_3\alpha_{-1}k_{+3}k_{-1})} \\ K_m(\text{NADH}) &= \frac{k_{-1}k_{-2}k_{-3}}{k_{-5}(k_{-1}k_{-3} + k_{-2}k_{-3} + k_{-1}k_{-2} + k_{+3}k_{-1})} \\ K'_m(\text{NADH}) &= \frac{\alpha_{-1}\alpha_{-2}k_{-1}k_{-2}k_{-3}}{k_{-5}(\alpha_{-1}k_{-1}k_{-3} + \alpha_{-2}k_{-2}k_{-3} + \alpha_{-1}\alpha_{-2}k_{-1}k_{-2} + \alpha_3\alpha_{-1}k_{+3}k_{-1})} = K_m(\text{NADH})\alpha_{-1}\alpha_{-2}/\gamma \\ \delta_1 &= \frac{\alpha_{-1}k_{-1}k_{-3} + \alpha_{-2}k_{-2}k_{-3} + \alpha_{-1}k_{-1}k_{-2} + \alpha_3k_{+3}k_{-1}}{k_{-1}k_{-3} + k_{-2}k_{-3} + k_{-1}k_{-2} + k_{+3}k_{-1}} \\ \delta_2 &= \frac{k_{-1}k_{-3} + k_{-2}k_{-3} + \alpha_{-2}k_{-1}k_{-2} + \alpha_{-1}k_{+3}k_{-1}}{k_{-1}k_{-3} + k_{-2}k_{-3} + k_{-1}k_{-2} + k_{+3}k_{-1}}\end{aligned}$$

Parameters noted as K'_m , $k'_{\text{cat.}}$, K'_{NAD^+} and K'_{NADH} correspond to the conformation F. Mal denotes L-malate and OAA denotes oxaloacetate. Dissociation constants of citrate from F-citrate, F-NAD⁺-Citrate and F-NAD⁺-L-malate-citrate are indicated as K_{c1} , K_{c2} and K_{c3} respectively. Dissociation constants of the substrate bound to allosteric centre (either L-malate or

In order to determine the values of the different parameters by non-linear regression, these equations were adapted to the different experimental conditions. Parameters were grouped to form polynomial expressions for making computer calculations. The final equations used in parameter determinations are listed below.

Sequential ordered mechanism (Segel, 1975):

$$v = \frac{\phi_1[A][B]}{\phi_2\phi_3 + \phi_3[A] + \phi_4[B] + [A][B]} \quad (3)$$

oxaloacetate) are referred to those of citrate by means of the constants $\beta_i = K_i(\text{citrate})/K_i(\text{substrate})$.

A, NAD⁺ or NADH; B, L-malate or oxaloacetate; ϕ_1 , $k_{\text{cat.}}[E_T]$; ϕ_2 , K_A ; ϕ_3 , $K_m[B]$; ϕ_4 , $K_m[A]$.

NAD⁺ → NADH direction: high and fixed NAD⁺ concentration:

$$v = \frac{[B]\phi_1\{1 + \phi_2([C] + \phi_8[B])\}\{1 + \phi_3([C] + \phi_9[B])\}}{\{\phi_4 + \phi_5([C] + \phi_9[B])\}\{1 + \phi_6([C] + \phi_8[B])\} + [B]\{1 + \phi_7([C] + \phi_9[B])\}\{1 + \phi_2([C] + \phi_8[B])\}} \quad (4)$$

B, L-malate; C, citrate; ϕ_1 , $k_{\text{cat.}}[E_T]$; ϕ_2 , α_2/K_{c2} ; ϕ_3 , α_3/K_{c3} ; ϕ_4 , $K_m(\text{Mal})$; ϕ_5 , $K_m(\text{Mal})\alpha_2\gamma/K_{c3}$; ϕ_6 , $1/K_{c2}$; ϕ_7 , γ/K_{c3} ; ϕ_8 , $\beta_2(\text{Mal})$; ϕ_9 , $\beta_3(\text{Mal})$.

With the above indicated parameters the final rate equations for the kinetic mechanism proposed are:

(i) NAD⁺ → NADH direction (A, NAD⁺; B, L-malate; C, citrate):

$$v = \frac{[A][B][E_T]k_{\text{cat.}}\{1 + \alpha_1([C] + \beta_1[B])/K_{c1}\}\{1 + \alpha_2([C] + \beta_2[B])/K_{c2}\}\{1 + \alpha_3([C] + \beta_3[B])/K_{c3}\}}{K_A\{K_m(B) + K'_{m(B)}\alpha_2\gamma([C] + \beta_3[B])/K_{c3}\}\{1 + ([C] + \beta_1[B])/K_{c1}\}\{1 + \alpha_{-1}([C] + \beta_2[B])/K_{c2}\} + [A]\{K_m(B) + K'_{m(B)}\alpha_2\gamma([C] + \beta_3[B])/K_{c3}\}\{1 + ([C] + \beta_2[B])/K_{c2}\}\{1 + \alpha_{-1}([C] + \beta_1[B])/K_{c1}\} + [B]\{K_m(A)\{1 + ([C] + \beta_1[B])/K_{c1}\}\{1 + \alpha_2([C] + \beta_2[B])/K_{c2}\}\{1 + \alpha_3([C] + \beta_3[B])/K_{c3}\} + [A][B]\{1 + \gamma([C] + \beta_3[B])/K_{c3}\}\{1 + \alpha_1([C] + \beta_1[B])/K_{c1}\}\{1 + \alpha_2([C] + \beta_2[B])/K_{c2}\}\}} \quad (1)$$

(ii) NADH → NAD⁺ direction (Q, NADH; P, oxaloacetate; C, citrate):

$$v = \frac{[P][Q][E_T]k_{\text{cat.}}\{1 + \alpha_{-1}([C] + \beta_2[P])/K_{c2}\}\{1 + \alpha_{-2}([C] + \beta_2[P])/K_{c3}\}}{K_Q\{K_m(P) + K'_{m(P)}\gamma/\alpha_{-1}([C] + \beta_3[P])/K_{c3}\}\{1 + ([C] + \beta_1[P])/K_{c1}\}\{1 + \alpha_{-1}([C] + \beta_2[P])/K_{c2}\} + [Q]\{K_m(P) + K'_{m(P)}\gamma/\alpha_{-1}([C] + \beta_3[P])/K_{c3}\}\{1 + \alpha_{-1}([C] + \beta_2[P])/K_{c2}\} + [P]\{K_m(Q)\{1 + ([C] + \beta_1[P])/K_{c1}\}\{1 + \alpha_{-1}([C] + \beta_2[P])/K_{c2}\}\{1 + \alpha_{-2}([C] + \beta_2[P])/K_{c3}\} + [P][Q]\{1 + \delta_1([C] + \beta_2[P])/K_{c2} + \delta_2([C] + \beta_3[P])/K_{c3} + \gamma([C] + \beta_2)/K_{c2}([C] + \beta_3[P])/K_{c3}\}\}} \quad (2)$$

NAD⁺ → NADH direction: high, fixed and non-activating L-malate concentration:

$$v = \frac{[A]\phi_1(1 + \phi_2[C])(1 + \phi_3[C])}{\phi_4(1 + \phi_5[C])(1 + \phi_3[C]) + [A](1 + \phi_6[C])(1 + \phi_2[C])} \quad (5)$$

A, NAD⁺; B, citrate; ϕ_1 , $k_{\text{cat}}[E_T]$; ϕ_2 , α_1/K_{c1} ; ϕ_3 , α_3/K_{c3} ; ϕ_4 , $K_m(\text{NAD}^+)$; ϕ_5 , $1/K_{c1}$; ϕ_6 , γ/K_{c3} .

NADH → NAD⁺ direction: high and fixed NADH concentration:

$$v = \frac{[P]\phi_1\{1 + \phi_2([C] + \phi_9[P])\}\{1 + \phi_3([C] + \phi_{10}[P])\}}{\{\phi_4 + \phi_5([C] + \phi_{10}[P])\}\{1 + \phi_2([C] + \phi_9[P])\} + [P]\{1 + \phi_6([C] + \phi_9[P]) + \phi_7([C] + \phi_{10}[P]) + \phi_8([C] + \phi_9[P])([C] + \phi_{10}[P])\}} \quad (6)$$

P, oxaloacetate; c, citrate; ϕ_1 , $k_{\text{cat}}[E_T]$; ϕ_2 , α_{-1}/K_{c2} ; ϕ_3 , α_{-2}/K_{c3} ; ϕ_4 , $K_m(\text{OAA})$; ϕ_5 , $K_m(\text{OAA})\alpha/\alpha_{-1}K_{c3}$; ϕ_6 , δ_1/K_{c2} ; ϕ_7 , δ_2/K_{c3} ; ϕ_8 , $\gamma/K_{c2}K_{c3}$; ϕ_9 , $\beta_2(\text{OAA})$; ϕ_{10} , $\beta_3(\text{OAA})$.

NADH → NAD⁺ direction: high, fixed and non-inhibitory oxaloacetate concentration:

$$v = \frac{[Q]\phi_1(1 + \phi_2[C] + \phi_3[C]^2)}{\phi_4(1 + \phi_5[C])(1 + \phi_2[C] + \phi_3[C]^2) + [Q](1 + \phi_6[C] + \phi_7[C]^2)} \quad (7)$$

Q, NADH; C, citrate; ϕ_1 , $k_{\text{cat}}[E_T]$; ϕ_2 , $\alpha_{-1}/K_{c2} + \alpha_{-2}/K_{c3}$; ϕ_3 , $\alpha_{-1}\alpha_{-2}/K_{c2}K_{c3}$; ϕ_4 , $K_m(\text{NADH})$; ϕ_5 , $1/K_{c1}$; ϕ_6 , $\delta_1/K_{c2} + \delta_2/K_{c3}$; ϕ_7 , $\gamma/K_{c2}K_{c3}$.

Determination of kinetic parameters

In order to estimate the values of the parameters that characterize the model proposed, several experiments were performed. Measures and equation fitting were performed as indicated in the Experimental section of the main paper. Each experiment shown consists of 20 experimental points, i.e. combinations of five substrate and four effector concentrations. Owing to the size of the equations used, it was impossible to perform a further optimization of the experimental design. In consequence, a geometric distribution within the available concentration margin was chosen.

According to the *F* test, all the results shown are above the 99.9 % confidence limit.

[L-Malate], 10 mM; [NAD⁺], 0.05–2 mM (eqn. 5). $\phi_1 = 0.223 \pm 0.0009 \mu\text{M/s}$; $\phi_2 = 0.060 \pm 0.0046 \text{ mM}^{-1}$; $\phi_3 = 0.051 \pm 0.005 \text{ mM}^{-1}$; $\phi_4 = 60.4 \pm 1 \mu\text{M}$; $\phi_5 = 0.45 \pm 0.026 \text{ mM}^{-1}$; $\phi_6 = 0.015 \pm 0.005 \text{ mM}^{-1}$, Standard deviation = $3.7 \times 10^{-3} \mu\text{M/s}$; $F = 807$ ($n = 20$).

[NAD⁺], 5 mM; [L-malate], 0.1–100 mM (eqn. 4). $\phi_1 = 0.25 \pm 0.022 \mu\text{M/s}$; $\phi_2 = 0.022 \pm 0.0021 \text{ mM}^{-1}$; $\phi_3 = 0.049 \pm$

0.0053 mM^{-1} ; $\phi_4 = 0.145 \pm 0.002 \text{ mM}$; $\phi_5 = 0.003 \pm 0.0009$; $\phi_6 = 0.55 \pm 0.06 \text{ mM}^{-1}$; $\phi_7 = 0.015 \pm 0.04 \text{ mM}^{-1}$; $\phi_8 = 10 \pm 1.1$; $\phi_9 = 0.21 \pm 0.08$. Standard deviation = $7 \times 10^{-3} \mu\text{M/s}$; $F = 1207$ ($n = 20$).

[NADH], 0.14 mM; [oxaloacetate], 0.043–2 mM (eqn. 6).

$\phi_1 = 0.3 \pm 0.046 \mu\text{M/s}$; $\phi_2 = 0.13 \pm 0.017 \text{ mM}^{-1}$; $\phi_3 = 1.6 \pm 0.8 \text{ mM}^{-1}$; $\phi_4 = 17.7 \pm 8 \mu\text{M}$; $\phi_5 = 0.068 \pm 0.003$; $\phi_6 = 0.1 \pm 0.08 \text{ mM}^{-1}$; $\phi_7 = 1.53 \pm 0.8 \text{ mM}^{-1}$; $\phi_8 = 0.45 \pm 0.2 \text{ mM}^{-2}$; $\phi_9 = 19 \pm 9.8$; $\phi_{10} = 2.7 \pm 1$. Standard deviation = $1.36 \times 10^{-2} \mu\text{M/s}$; $F = 202$ ($n = 20$).

[Oxaloacetate], 0.125 mM; [NADH], 0.032–0.2 mM (eqn. 7). $\phi_1 = 0.306 \pm 0.0004 \mu\text{M/s}$; $\phi_2 = 1.73 \pm 0.4 \text{ mM}^{-1}$; $\phi_3 = 0.22 \pm 0.10 \text{ mM}^{-2}$; $\phi_4 = 44 \pm 1.6 \mu\text{M}$; $\phi_5 = 0.376 \pm 0.019 \text{ mM}^{-1}$; $\phi_6 = 1.62 \pm 0.05 \text{ mM}^{-1}$; $\phi_7 = 0.22 \pm 0.02 \text{ mM}^{-2}$. Standard deviation = $2.1 \times 10^{-3} \mu\text{M/s}$; $F = 550$ ($n = 20$).

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Received 12 March 1991/9 September 1991; accepted 25 September 1991