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Effect of several anions on the activity of mitochondrial malate dehydrogenase from pig heart

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Abstract

Mitochondrial malate dehydrogenase (mMDH) shows a complex dependence upon ionic environment that includes kinetic and structural effects. We measured mMDH activity in several buffers (phosphate, MOPS, and MES) at pH 6.5 and 7.5, and in the presence of a number of anions, at highly diluted enzyme concentrations where mMDH showed significant loss of activity. Under these conditions, mMDH activity shows a non-linear dependence on enzyme concentration, in agreement with the existence of a dimer–monomer equilibrium, where only the dimeric form is active. According to this hypothesis, the dissociation constant of mMDH dimer has been determined to be 5.4 nM in the MES buffer at pH 6.5. Either the presence of a small anion like phosphate, or an increase of the pH from 6.5 to 7.5 shifts the equilibrium in favor of the dimeric form with the two effects appearing to be additive. To extend the study, we analysed the effect of a number of anions on the mMDH activity in 50 mM MOPS buffer at pH 7.5. All the anions had a dual effect: at low concentrations, they increased the activity of mMDH, while at high concentrations, they inhibited it. A more accurate analysis of the data revealed that the activation capacity of all the anions tested was similar, although they differed in their inhibitory influence. To show these differences more clearly, the experiment was repeated in 50 mM phosphate buffer at pH 7.5, under conditions where almost all activations were due to the buffer. The analysis of the results obtained under these conditions revealed the following sequence of inhibition potency: phosphate < acetate < sulfate < chloride < bromide < nitrate < perchlorate < thiocyanate, that was in good agreement with their chaotropicity. The behaviour of mMDH in the presence of different ions and/or ionic strength indicates that at low ionic concentrations, increased activity is likely due to a stabilization of the dimeric form of the enzyme. Further increases in the ionic concentration, especially if chaotropic ions are used, lead to a loss of enzyme activity that appears to be a consequence of structural changes in the enzyme rather than kinetic effects. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Mitochondrial malate dehydrogenase (mMDH); Monomer–dimer equilibrium; Anions; Enzyme inhibition and activation; Kinetic mechanism

1. Introduction

Many enzymes are multisubunit complexes made up of chemically identical monomers. Pig heart mitochondrial malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37, mMDH), which cat-

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alyzes the reversible oxidation of L-malate to oxaloacetate with NAD^+ as a coenzyme [1,2], consists of two identical subunits, each with 314 amino acids. While only the oligomeric form of mMDH is catalytically active, the role of the subunit interface in catalytic activity is controversial. It has been suggested that the dimer structure is critical for enzyme activity. Thus, the reciprocating compulsory ordered mechanism proposed by Harada and Wolfe [3] for mMDH predicts an inactive monomer. This hypothesis is supported by the drastic reduction in enzyme activity when the dimer dissociates to monomers at low pH and low enzyme concentrations [4–6]. In contrast, the report of active subunits in hybrid-modified mMDH [7] and the fact that monomers appear to be catalytically active when immobilized on Sepharose beads [8] suggest that they may be active in solution.

The dimer–monomer equilibrium of pig heart mMDH has been studied using gel filtration [4,5,9], fluorescence techniques [10–12], and stopped-flow kinetic studies [13,14]. Shore and Chakrabarti [10], by fluorescence polarization studies, reported a concentration-dependent dissociation consistent with a monomer–dimer equilibrium with a dissociation constant of 2×10^{-7} M. Bleile et al. [4], using gel filtration chromatography and sedimentation velocity ultracentrifugation, demonstrated that, at concentrations below 0.2 μM, the enzyme behaved as a monomer. In contrast, Frieden et al. [13], by sedimentation equilibrium and stopped-flow kinetic experiments, found no evidence for dissociation of mMDH even at 10^{-9} M. Jaenicke et al. [9], using gel filtration chromatography, failed to detect dissociation of the enzyme in the concentration range from 1.67×10^{-6} to 2.9×10^{-9} M at pH 7.6.

Wood et al. [5,6] and Hodges et al. [15] indicated that the dimeric form of mMDH dissociates to monomers at low protein concentration or at pH below neutrality. However, McKay and Jameson [11], using fluorescence, demonstrated that at pH between 5.5 and 8.0 and at ionic strengths near 0.1 M, the dissociation constant for the monomer–dimer equilibrium is subnanomolar. A recent report on pig heart mMDH, using both steady-state and time-resolved fluorescence methodologies, showed that the dimer–monomer dissociation constant is below 10^{-9} M and remains unchanged between pH 5.0 and 8.0 [12]. Some of the discrepancies in these results may be due to the tendency of the native protein to aggregate in certain experimental conditions [12], although these conflicting results suggest, in fact, that the structure of mMDH is strongly dependent on the solvent nature and concentration. Indeed, it has been indicated that the catalytic activity of mMDH is influenced by the ionic environment of the enzyme [16–21], but the structural reasons responsible for this sensitivity are not clearly established. A recent report by Jensen et al. [14] indicated that the effectiveness of salts of anions on the thermal stabilization of mMDH correlates with the Hofmeister series. They also detected significant amounts of monomeric mMDH at high bromide concentrations.

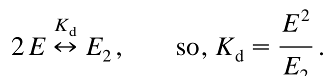
To clarify the effect of the pH and the composition of the ionic environment, here we analysed the kinetic behaviour of mMDH at pH 6.5 and 7.5 in the presence of a number of anions. The results enabled us to outline a model that could explain the influence of the anions on the monomer–dimer equilibrium and on the enzyme activity.

2. Theory

2.1. Dependence of mMDH activity on enzyme concentration

The rate equation used to fit the activities of mMDH measured in the buffers analyzed in this study was derived assuming the following conditions.

(1) The dimeric form (E_2) of the mMDH coexists with the monomeric (E) form. Assuming an equilibrium between them:



$$2E \overset{K_d}{\leftrightarrow} E_2, \qquad \text{so, } K_d = \frac{E^2}{E_2}.$$

(2) The total enzyme concentration (expressed as subunits): $E_T = 2E_2 + E$ is constant, then the algebraic expressions for the monomer and dimer con-

centrations are given by Eqs. (1) and (2), respectively:

$$E = \frac{-K_d + \sqrt{K_d^2 + 8K_d E_T}}{4}, \quad (1)$$

$$E_2 = \frac{4E_T + K_d - \sqrt{K_d^2 + 8K_d E_T}}{8}. \quad (2)$$

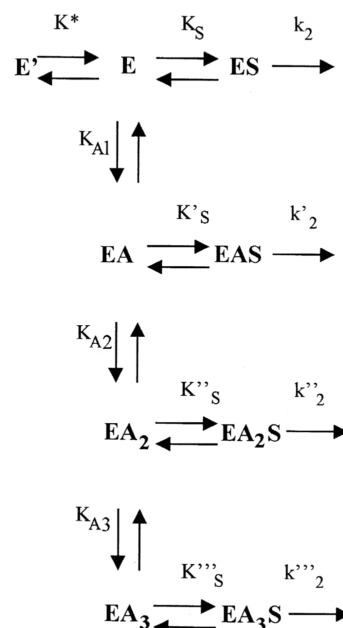
Finally, assuming that the activity of the monomer is negligible, the rate equation is given by:

$$v = k_{cat} \frac{4E_T + K_d - \sqrt{K_d^2 + 8K_d E_T}}{8}, \quad (3)$$

that predicts a non-linear relationship between activity and E_T, if a significant amount of the monomeric and dimeric species coexists.

2.2. Kinetic mechanism proposed to explain the dependence of mMDH activity on the anion concentration

The kinetic mechanism proposed to explain the effect of anions on mMDH activity appears in the Scheme 1. We considered a substrate (S), an anion (A), an active form (E) and an inactive form (E′) of the enzyme. Although the available evidence strongly suggests that E′ should be monomeric, to simplify the equations obtained, we represent the E → E′ process as an inactivation rather than a dissociation (E → 2E′). In addition, different complexes of the active form: ES (enzyme–substrate complex), EA (enzyme–anion complex), EAS (enzyme–anion–substrate complex), EA_2 (enzyme–di-anion complex), EA_2S (enzyme–di-anion–substrate complex), EA_3 (enzyme–tri-anion complex), EA_3S (enzyme–tri-anion–substrate complex) are considered. It is



Scheme 1. Kinetic mechanism proposed to explain the effect of different anions on the activity of the mMDH (50 mM MOPS buffer, pH 7.5). (A) Studied anion. S: substrate. E: active enzyme form. E′: inactive enzyme form. K^*, K_{A1}, K_{A2}, K_{A3}, K_S, K'_S, K''_S, K'''_S: dissociation equilibrium constants. k_2, k'_2, k''_2, k'''_2: catalytic constants.

assumed that an equilibrium among the intermediates is established. The dissociation equilibrium constants for the different complexes described above are: K^*, K_S, K'_S, K''_S, K'''_S, K_{A1}, K_{A2}, and K_{A3}, respectively. On the other hand, the catalytic constants are defined as: k_2, k'_2, k''_2 and k'''_2. From this scheme, we obtained a rate equation as a function of the equilibrium dissociation and the catalytic constants as well as substrate and anion concentrations:

$$\frac{v}{v_0} = \frac{1 + \frac{k'_2 K_S A}{k_2 K'_S K_{A1}} + \frac{k''_2 K_S A^2}{k_2 K''_S K_{A1} K_{A2}} + \frac{k'''_2 K_S A^3}{k_2 K'''_S K_{A1} K_{A2} K_{A3}}}{1 + \frac{(1 + S/K'_S) A}{(1 + K^* + S/K_S) K_{A1}} + \frac{(1 + S/K''_S) A^2}{(1 + K^* + S/K_S) K_{A1} K_{A2}} + \frac{(1 + S/K'''_S) A^3}{(1 + K^* + S/K_S) K_{A1} K_{A2} K_{A3}}}. \quad (4)$$

This can be written as:

$$v = \frac{P_3\left(1 + P_1 A + P_4 A^2 + P_7 A^3\right)}{1 + P_6 A + P_2 A^2 + P_5 A^3}, \quad (5)$$

where:

$$P_1 = \frac{k'_2 K_S}{k_2 K'_S K_{A1}}, \quad (6)$$

$$P_2 = \frac{(1 + S/K''_S)}{(1 + K^* + S/K_S) K_{A1} K_{A2}}, \quad (7)$$

$$P_3 = v_0, \quad (8)$$

$$P_4 = \frac{k''_2 K_S}{k_2 K''_S K_{A1} K_{A2}}, \quad (9)$$

$$P_5 = \frac{(1 + S/K'''_S)}{(1 + K^* + S/K_S) K_{A1} K_{A2} K_{A3}}, \quad (10)$$

$$P_6 = \frac{(1 + S/K'_S)}{(1 + K^* + S/K_S) K_{A1}}, \quad (11)$$

$$P_7 = \frac{k'''_2 K_S}{k_2 K'''_S K_{A1} K_{A2} K_{A3}}. \quad (12)$$

3. Experimental procedures

3.1. Reagents

Oxaloacetic acid (OAA), sodium acetate, sodium bromide, sodium chloride, sodium nitrate, sodium perchlorate, sodium sulfate, sodium thiocyanate, monosodium phosphate, disodium phosphate, 4-morpholinepropanesulfonic acid (MOPS) and 4-morpholineethanesulfonic acid (MES) were purchased from Sigma (St. Louis, MO, USA). NADH and pig heart mMDH (5mg/ml, solution in 50% glycerol) were supplied by Boehringer Mannheim (Mannheim, Germany).

3.2. Kinetic measurements

mMDH activity was measured in 1 ml of a medium containing 0.14 mM of NADH and 0.2 mM of oxaloacetate in the different buffers assayed (50 mM MES pH 6.5, 50 mM phosphate pH 6.5, 50 mM MOPS pH 7.5 and 50 mM phosphate pH 7.5). Final enzyme concentration in the cuvette depended on the experiment. A stock solution of the enzyme, 500-fold more concentrated than the desired enzyme concentration in the cuvette, was prepared in the appropriate buffer. The reaction was started by addition of 2 μl of the corresponding enzymatic stock solution, cooled on ice. In these conditions, the stock solution remained stable during the experiment, and any loss of activity is produced at the time of the final dilution.

When the dependence of the initial reaction rates on the enzyme concentration was determined, several concentrated stock solutions of mMDH from 5 to 100 μg/ml were prepared in the activity assay buffer used. When the effect of different anions on the activity of the mMDH was studied, an enzyme stock solution of 50 μg/ml in the assay buffer was prepared and the final enzyme concentration in the cuvette was 0.1 μg/ml.

Initial reaction rates in the $NADH \rightarrow NAD^+$ direction were measured by monitoring the absorbance at 340 nm in a Unicam UV3 spectrophotometer in a 1-cm light path cell, thermostatically controlled at 30 ± 0.1°C. Enzyme activity is expressed in micromoles per minute liter.

A typical kinetic experiment consisted of 10–15 steady-state rates measured in quintuplicate, corresponding to the various anion concentrations in the buffers assayed. The values of the initial rates were analysed to detect and reject possible outliers by using the program, KAICOUT [22]. Kinetic parameters and predicted curves were obtained by fitting the appropriate rate equations (see Section 2) by non-linear regression, by using both KAICOUT on a Sun SparcClassic computer and a simplified version of the same program running under MS-DOS. Points were weighted according to the reciprocal of the squared SEM of experimental replicates. Parameters are expressed as value $\pm$ SE (calculated from the regression).

4. Results and discussion

4.1. Influence of the pH and buffer composition on the monomer–dimer equilibrium of mMDH

The monomer–dimer equilibrium can be analysed through the dependence of activity on enzyme concentration, if there are significant activity differences between the two forms, and they coexist in the conditions of the experiment. mMDH activity was measured by steady-state kinetics in several buffers: 50 mM MES or phosphate at pH 6.5; 50 mM MOPS

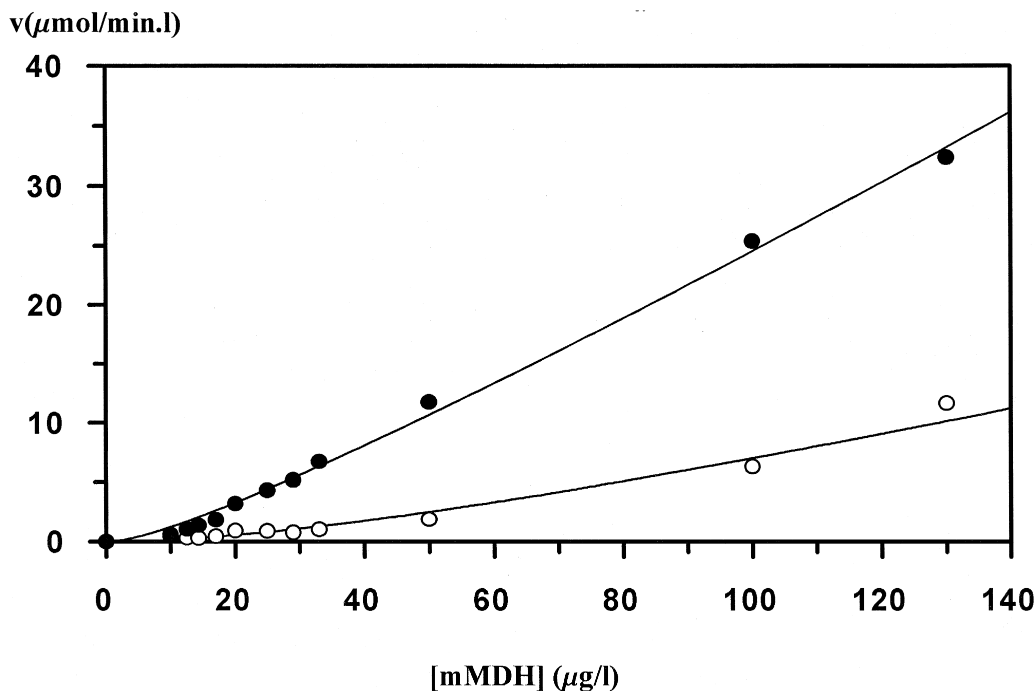


Fig. 1. Dependence of mMDH activity on enzyme concentration at pH 6.5. Initial rates were determined with [OAA]: 0.2 mM and [NADH]: 0.14 mM in 50 mM MES (○) or 50 mM phosphate (●) buffer at pH 6.5. Curve drawings were performed by a non-linear fitting to Eq. (3) as described in Section 3.

or phosphate at pH 7.5 (Figs. 1 and 2). A non-linear dependency of mMDH activity on enzyme concentration can be observed that confirms the co-existence of two forms of the enzyme. The shape of these curves is consistent with a monomer–dimer equilibrium where the dimeric form is much more active than the monomeric form. This assumption is compatible with the reported preparation of *Escherichia coli* MDH stable monomers whose measured activity was about 15,000-fold lower than the corresponding dimeric form [23]. To analyse quantitatively these results, we used Eq. (3), which was derived assuming an equilibrium between monomer–dimer, total enzyme concentration constant and activity of the monomer negligible (see Section 2). The values of K_d (app.) and k_{cat} (app.) obtained for the different conditions assayed appear in Table 1. The values of K_d (app.) obtained are consistent with recent results reported by Sánchez et al. [12] using steady-state and dynamic polarization methodologies. The K_d (app.) obtained at pH 6.5 in MES 50mM decreased in one order of magnitude in phosphate 50 mM at the same pH, indicating that the presence of phosphate shifted the equilibrium towards the dimeric form of the enzyme. A similar effect was observed when the pH was increased by one unit (MOPS 50 mM at pH 7.5). As MES and MOPS are closely related compounds, there is no reason to suggest a specific effect due to the change of buffer and the difference must be attributed to pH. This is in agreement with the previously reported dissociation at acid pH of mMDH [13] that has been correlated with the protonation of a His residue located at the subunit interface [13]. The effect of phosphate, a much smaller anion, should be considered at this point as a specific interaction with the protein that stabilizes the dimeric form in a way that cannot be fulfilled by MOPS or MES. The combination of the two conditions further reduces K_d (app.), leading to a situation where only the dimeric enzyme is present. This is indicative that the two effects — pH increase and presence of phosphate — are accu-

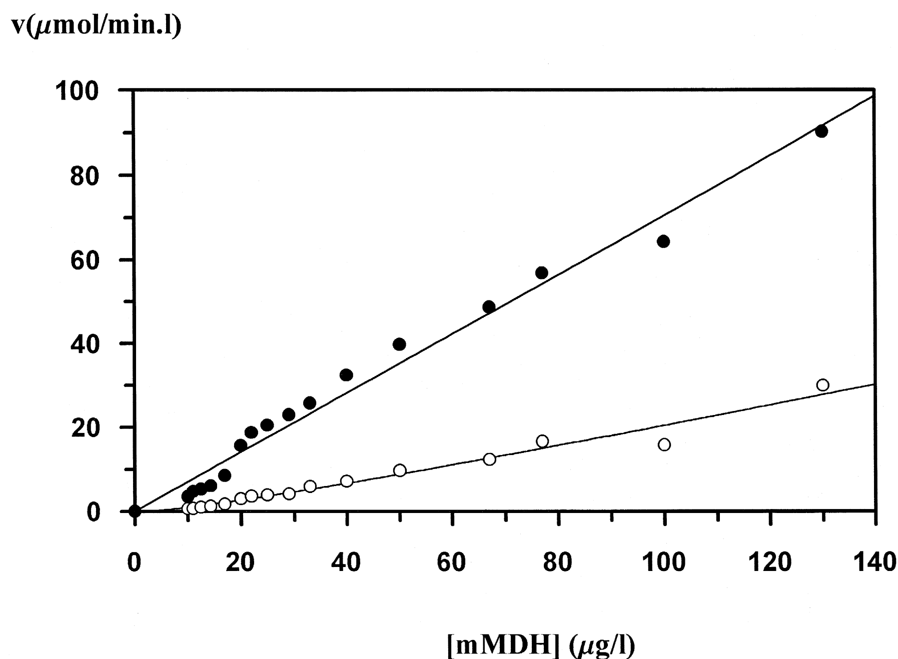


Fig. 2. Dependence of mMDH activity on enzyme concentration at pH 7.5. Initial rates were determined with [OAA]: 0.2 mM and [NADH]: 0.14 mM in 50 mM MOPS (○) or 50 mM phosphate (●) buffer at pH 7.5. Curve drawings were performed by a non-linear fitting to Eq. (3) as described in Section 3.

mulative in shifting the equilibrium to the dimeric form of the enzyme and come from different mechanisms.

On the other hand, the results of the fittings evaluated also the kinetic effect produced by the change of medium on the active form. The ratio between the k_{cat} (app.) observed in phosphate buffers and MES or MOPS buffers was low and approximately 2. Therefore, the kinetic effect of the phosphate in the catalysis was small and independent of the pH.

4.2. Influence of different anions on the mMDH activity (50 mM MOPS, pH 7.5 buffer)

In Section 3, we showed that the nature of the buffer affects the activity of the mMDH. Thus, when phosphate was used instead of MOPS or MES, the

Table 1
Parameters obtained from the study of the dependence of the mMDH activity on enzyme concentration[a]

	pH 6.5		pH 7.5	
	Phosphate	MES	Phosphate	MOPS
k_{cat} (app.) [μmol/min μg]	0.70 ± 0.04	0.35 ± 0.06	1.41 ± 0.05	0.60 ± 0.09
K_d (app.) [nM]	0.7 ± 0.3	5.4 ± 2.7	non-detectable	0.8 ± 0.6

[a]The equilibrium between the monomeric and dimeric forms of the mMDH was studied by steady-state kinetic in buffers of different pH values and ionic strengths. The apparent catalytic constant (k_{cat} (app.)) as well as the apparent dissociation constant (K_d (app.)) were determined from a non-linear fitting (see Eq. (3)) to the experimental data.

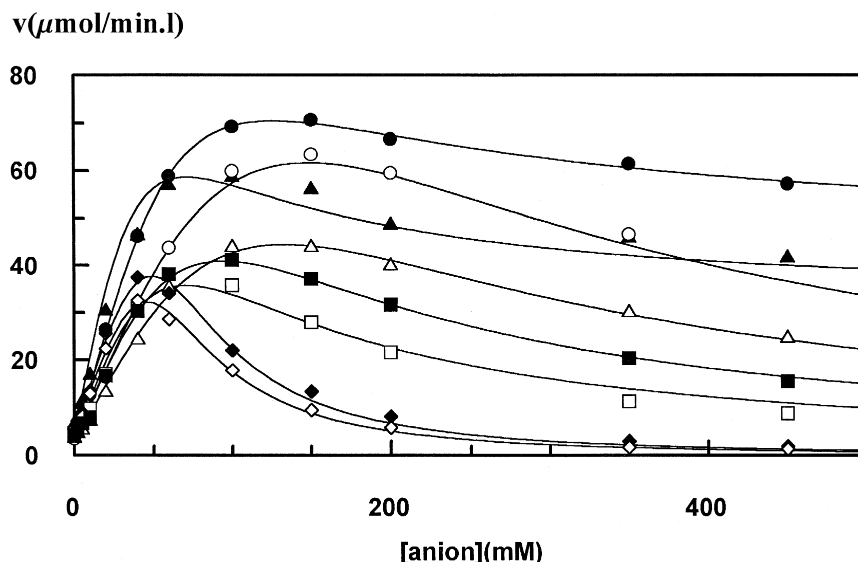


Fig. 3. Effect of several anions on mMDH activity in 50 mM MOPS buffer, pH 7.5. mMDH activity was measured in the presence of: (●) phosphate; (○) acetate; (▲) sulfate; (△) chloride; (■) bromide; (□) nitrate; (◆) perchorate; (◇) thiocyanate. The experimental values, determined in 50 mM MOPS buffer at pH 7.5; [OAA]: 0.2 mM and [NADH]: 0.14 mM, were fitted to Eqs. (13), (14) and (15).

K_d (app.) decreased in one order of magnitude or more, indicating that the presence of phosphate shifted the equilibrium towards the dimeric form of the enzyme. To analyse whether this is a specific effect of phosphate, we measured the effect of other anions on the activity of mMDH in 50 mM MOPS buffer at pH 7.5. The influence of phosphate, sulfate, acetate, chloride, bromide, nitrate, perchlorate and thiocyanate on mMDH activity is shown in the Fig. 3. Measures were performed at an enzyme concentration where a significant amount of monomer is present, and the activity is strongly reduced after dilution. For all the anions tested, at increasing anion concentrations, the mMDH activity increased until a maximum was reached. The magnitude of the apparent activation of the mMDH caused by the different anions followed the sequence: phosphate > acetate > sulfate > chloride > bromide > nitrate > perchlorate > thiocyanate. The maximum of mMDH activity was located depending on the anion, at concentrations between 40 and 150 mM. Interestingly, the activation factor observed at this concentration is much higher than the small kinetic activation detected in the previous experiment (Table 1), thus indicating that the effect is due to the recovery of the dimeric form of the enzyme, mediated by the presence of anions. In all the cases, a decrease in mMDH activity was observed at higher anion concentrations. The magnitude of this effect varied on the nature of the anion. The decrease was slow for the anions that had a high activation effect like phosphate or acetate and much faster for the anions that had a lower activation effect like perchlorate or thiocyanate. Sulfate, chloride, bromide and nitrate showed intermediate behaviour. Moreover, for perchlorate and thiocyanate, the inhibitory effect prevailed at concentrations between 50 and 400 mM.

In order to understand the activation and inhibition of mMDH by the anions at different concentrations from a quantitative point of view, the experimental data of the curves of the Fig. 3 were fitted using distinct rational polynomial functions of degree between 1:3 and 3:3. For the anions analysed, different rational polynomial functions gave the best fits. Thus, the experimental curves obtained in presence of phosphate and sulfate fitted to the 2:2 rational polynomial function:

$$v_1 = \frac{P_3\left(1 + P_1 A + P_4 A^2\right)}{1 + P_2 A^2}. \quad (13)$$

Acetate, chloride, bromide and nitrate fitted to the 1:2 rational polynomial function:

$$v_2 = \frac{P_3(1 + P_1 A)}{1 + P_2 A^2}. \quad (14)$$

Finally, perchlorate and thiocyanate fitted to the 1:3 rational polynomial function:

$$v_3 = \frac{P_3(1 + P_1 A)}{1 + P_5 A^3}. \quad (15)$$

The kinetic parameters P_1, P_2, P_3, P_4 and P_5 obtained from rate Eqs. (13), (14) and (15) are shown in Table 2 and the curves fitting experimental data are shown in Fig. 3. The fact, that the experimental curves corresponding to the anions tested fitted to different rational polynomial functions, is indicative that several interaction mechanisms may occur. To interpret the observed effects of the anions on the activity of mMDH, the kinetic mechanism of the Scheme 1 was proposed and the rate equation (Eq. (5)) was derived (see Section 2). This rate equation has a high degree (3:3) that, in general, cannot be detected experimentally [24]. In order to understand the meaning of the parameter values obtained from each experimental curve (Table 2), some simplifications in the Eq. (5) needed to be made. Due to the experimental facts reported earlier that mMDH is dimerized by the presence of anions and considering that at low concentrations the anion has little effect on the kinetics of the dimeric enzymatic form, the following approximations inside the model were made:

$$K_S = K'_S; \quad k_2 = k'_2.$$

According to this approximation, P_1 corresponds with the reciprocate of K_{A1} (Eq. (6)). In other words, P_1 represents the anion's ability to shift the equilibrium of the monomeric/dimeric (E′ vs. E + EA) forms of the enzyme towards the active one (Table 2), and consequently, accounts for the activation effects. Surprisingly, although the most chaotropic anions (perchlorate and thiocyanate) showed slightly smaller P_1 values compared with the rest of the values, this variation was not too big in comparison with the activation factor produced (Fig. 3). In fact, inspection of the K_{A1} values shows very little differences among all the anions assayed. Consequently, the reason for the differential behaviour must be in a distinct inhibitory capacity. The inhibitory effect observed at high anion concentration was represented by the parameters P_2 (Eqs. (13) and (14)) and P_5 (Eq. (15)). Values of P_2 (or P_5) are roughly in agreement with the differences observed in the effect of anions (Fig. 3), especially in those showing a intermediate effect, although the existence of both activation and inhibition behaviour makes these data difficult to analyse. Interestingly, the ratio P_4/P_2, which extrapolates the ratio v/v_0 (v_0 is the rate in absence of anion) when the anion concentration tends to infinity, is only significantly

Table 2
Parameters obtained from the study of the effect of anions on the mMDH activity in 50 mM MOPS buffer at pH 7.5[a]

	Equation	P_1 [mM^{-1}]	P_2 [mM^{-2}]	P_3 [μmol/min l]	P_4 [mM^{-2}]	P_5 [mM^{-3}]	K_{A1} [mM]	P_4/P_2
Phosphate	13	0.33 ± 0.05	1.7e − 4 ± 1e − 5	3.2 ± 0.5	2.4e − 3 ± 5e − 4	–	3.0 ± 0.1	13.96
Acetate	14	0.4 ± 0.1	4.2e − 5 ± 2e − 6	2.2 ± 0.6	0	–	2.5 ± 0.2	0
Sulfate	13	0.27 ± 0.05	4e − 4 ± 5e − 5	5.5 ± 0.8	2.3e − 3 ± 6e − 4	–	3.7 ± 0.2	5.86
Chloride	14	0.28 ± 0.09	5.4e − 5 ± 3e − 6	2.2 ± 0.6	0	–	3.5 ± 0.3	0
Bromide	14	0.31 ± 0.09	1.05e − 4 ± 7e − 6	2.6 ± 0.7	0	–	3.3 ± 0.3	0
Nitrate	14	0.4 ± 0.2	1.9e − 4 ± 2e − 5	2.5 ± 1.3	0	–	2.5 ± 0.5	0
Perchlorate	15	0.22 ± 0.05	0	4.6 ± 0.8	0	3.8e − 6 ± 4e − 7	4.5 ± 0.2	–
Thiocyanate	15	0.20 ± 0.03	0	4.6 ± 0.5	0	4.4e − 6 ± 3e − 7	5.0 ± 0.2	–

[a] The parameters: P_1, P_2, P_3, P_4 and P_5 were deduced from the fitting of the experimental data (Fig. 3) to 2:2 (Eq. (13)), 1:2 (Eq. (14)) and 1:3 (Eq. (15)) rational polynomial functions. The anions' ability to shift the inactive (E′)/active (E) forms of the mMDH is given by K_{A1}. P_2 and P_5 quantify the inhibitory effect at high concentrations of the anions. Enzyme activity when no anion is present (v_0) is given by P_3. The ratio P_4/P_2 corresponds to the quotient between the enzyme activities, v/v_0, when the anion concentration tends to infinity.

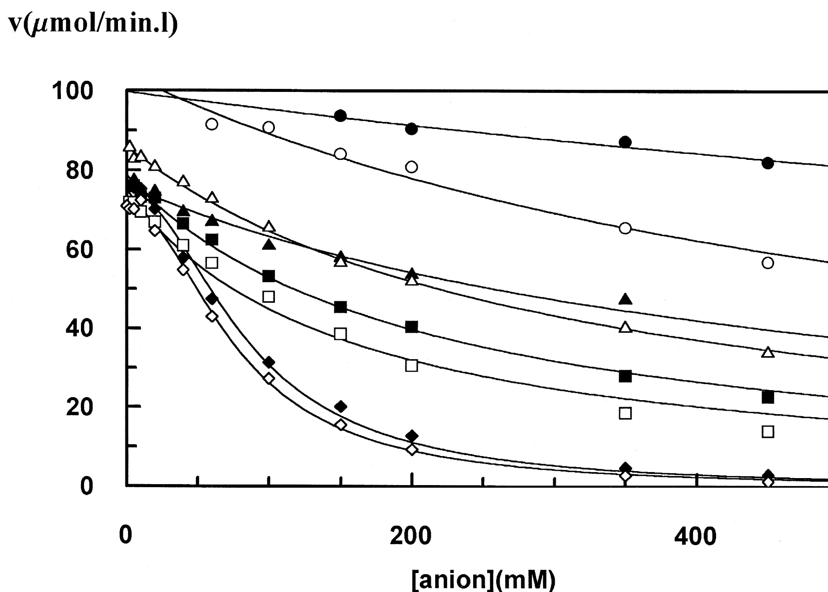


Fig .4. Effect of several anions on mMDH activity in 50 mM phosphate buffer, pH 7.5. mMDH activity was measured in the presence of: (●) phosphate; (○) acetate; (▲) sulfate; (△) chloride; (■) bromide; (□) nitrate; (◆) perchorate; (◇) thiocyanate. The experimental values, determined in 50 mM phosphate buffer at pH 7.5; [OAA]: 0.2 mM and [NADH]: 0.14 mM, were fitted to Eqs. (16) and (17).

different from zero in the case of the less chaotropic anions: phosphate and sulfate (Table 2). However, even in these two cases, the values are far below the maximum of activity measured. This fact indicates that a high-enough ionic concentration can inhibit completely the activity of mMDH, irrespective of the nature of the anion. The differences observed in the activity profile from one anion to another come basically from their differences in their inhibitory capacity.

4.3. Influence of different anions on the mMDH activity (50 mM phosphate buffer at pH 7.5)

To isolate the inhibition side of the effect from the activation process, we performed a similar experiment using 50 mM phosphate buffer at pH 7.5, instead of MOPS. As reported in Section 1, in phosphate, the monomer–dimer equilibrium of mMDH is completely shifted to the active dimeric form. Besides, according to the results obtained in Section 3, phosphate is the anion that requires the highest concentration to show inhibitory effects. Thus, in these buffer conditions, we do not need to consider the equilibrium monomer–dimer displayed in the upper part of the Scheme 1 that must be, in fact, already shifted by the presence of phosphate. Then, the observed effects will correspond only to the inhibitory section of the mechanism. In turn, it will reduce the size of the equations required. Fig. 4 shows the effects of phosphate (above 50 mM), acetate, sulfate, chloride, bromide, nitrate, perchlorate and thiocyanate. Inspection of the Fig. 4 shows that the order of inhibition capacity is coincident with that observed in the inhibitory part of the Fig. 3. The results confirm that the activation process was indeed completed by the presence of a constant concentration of phosphate. The analysis of these data required again two different equations — one for the less chaotropic anions and other for the more chaotropic anions — suggesting once more that at least two mechanisms occur. Thus, the experimental curves obtained in presence of phosphate, acetate, sulfate, chloride, bromide, and nitrate fitted to the 0:1 rational polynomial function:

$$v = \frac{P_1'}{1 + P_2' B}, \tag{16}$$

Table 3
Parameters obtained from the analysis of the anions' inhibitory capacity on mMDH activity (50 mM phosphate buffer, pH 7.5)[a]

Anion	Equation	P'_1 [μmol/min l]	P'_2 [mM^{-1}]	P'_3 [mM^{-2}]	[mM] (C — Eq. (20))	[mM] (NC — Eq. (21))
Phosphate	16	99.7 ± 1.9	$4.5 \times 10^{-4} \pm 7 \times 10^{-5}$	–	116 ± 18	2222 ± 346
Acetate	16	104.1 ± 3.1	$1.7 \times 10^{-3} \pm 2 \times 10^{-4}$	–	30.7 ± 3.6	588 ± 69
Sulfate	16	76.2 ± 1.0	$2.0 \times 10^{-3} \pm 2 \times 10^{-4}$	–	26.1 ± 2.6	500 ± 50
Chloride	16	85.9 ± 0.5	$3.3 \times 10^{-3} \pm 9 \times 10^{-5}$	–	15.8 ± 0.5	303 ± 8
Bromide	16	78.8 ± 0.8	$4.9 \times 10^{-3} \pm 2 \times 10^{-4}$	–	10.6 ± 8.9	204 ± 17
Nitrate	16	75.1 ± 1.6	$6.7 \times 10^{-3} \pm 6 \times 10^{-4}$	–	7.8 ± 0.7	149 ± 13
Perchlorate	17	74.7 ± 1.1	–	$1.4 \times 10^{-4} \pm 10 \times 10^{-6}$	4.4*	84.5*
Thiocyanate	17	70.8 ± 0.6	–	$1.7 \times 10^{-4} \pm 8 \times 10^{-6}$	4.0*	76.7*

[a] The parameters: P'_1, P'_2 and P'_3 were obtained from the fitting of the experimental data to 0:1 (Eq. (16)) and 0:2 (Eq. (17)) rational polynomial functions. K_{A2} values were deduced either from Eq. (20) (competitive case) or Eq. (21) (non-competitive case).

* K_{A2} values for perchlorate and thiocyanate were estimated from $P_3^{0.5\prime}$ using Eqs. (20) and (21). This approach is not rigorous and hence, the values should be taken as qualitative.

and the most chaotropic ions — perchlorate and thiocyanate — fitted to the 0:2 rational polynomial function:

$$v = \frac{P'_1}{1 + P'_3 B^2}. \quad (17)$$

The parameters obtained are summarized in Table 3. To understand these parameters, it is necessary to further simplify the mechanism indicated in Scheme 1, reducing it to a single inhibition step (Scheme 2). From this scheme, we could interpret Eq. (16) using:

$$P'_1 = \frac{k_2 S}{K_S + S} \quad (18)$$

and

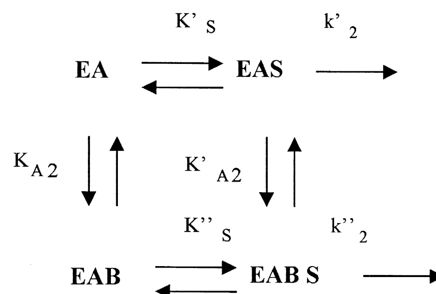
$$P'_2 = \frac{1}{K_{A2}} \frac{K_S + S(K_S/K'_S)}{K_S + S}. \quad (19)$$

Two basic mechanisms can be proposed to account for the inhibitory effect: a competition between the anion and the substrate (constant apparent V_{max}, increasing apparent K_M, i.e., competitive inhibition) and a decrease of enzyme concentration due to a structural alteration (decreasing apparent V_{max}, constant apparent K_M, i.e. non-competitive inhibition). Both interpretations are compatible with Eq. (19); however, the values estimated for the K_{A2} parameter must be different: a competitive inhibition is characterized by K'_S = infinity (Eq. (20)), whereas non-competitive inhibition occurs when $K_S = K'_S$ (Eq. (21)):

$$P'_2 = \frac{1}{K_{A2}} \frac{K_S}{K_S + S}, \quad (20)$$

$$P'_2 = \frac{1}{K_{A2}}. \quad (21)$$

Under these assumptions, the values of K_{A2} ranged from 150 to 2200 mM in the non-competitive case and from 7.8 to 116 mM in the competitive case (see Table 3), following the order thiocyanate < perchlorate < nitrate < bromide < chloride < sulfate < acetate < phosphate. The reason for this marked difference between the two sets of parameters must



Scheme 2. Kinetic mechanism proposed to explain the inhibitory effect on the mMDH activity of several anions in 50 mM phosphate buffer at pH 7.5. (A) Phosphate of the buffer. (B) Studied anion. S: substrate. E: active enzyme form. K_{A2}, K'_{A2}, K'_S, K''_S: dissociation equilibrium constants. k'_2, k''_2: catalytic constants.

be found in the fact that the experiments were performed at high substrate concentrations ($\sim 20 K_M$). All the values are above K_{A1} ($\sim$ 3 mM; see Table 2), that is consistent with the fact that the first bound anion is responsible of the activation effect, whereas higher concentrations are needed to reveal an inhibitory behaviour.

The effectiveness in the inhibition of mMDH activity of the anions assayed is in agreement with the stability studies carried out by Jensen et al. [14] on pig heart mMDH by different salts in the range from 0.05 to 2 M. These authors reported that the protective ability to reduce the extent of thermal inactivation of mMDH at 40°C followed the order citrate > sulfate > tartrate > phosphate > fluoride > acetate > chloride > bromide. Thus, a higher inhibitory capacity would correlate with a lower stabilizing effect. In both cases, the effectiveness of the anions in either effect followed the Hofmeister [25] series that would suggest that the loss of activity would be a structural (non-competitive inhibition) rather than a kinetic effect. Indeed, if a kinetic inhibition involving the binding of anions to the active site was produced (competitive inhibition), the order of inhibition power would be related to the structural similarity to the natural substrate, a carboxylic acid, being in that case phosphate, sulfate or acetate as the best inhibitors. To further clarify this point, the effects of phosphate, chloride and thiocyanate on the apparent kinetic parameters of mMDH (Table 4) have been analyzed in the experimental conditions where the activation process is already completed. These results show that most of the inhibitory effects appear as a decrease of V_{max} parameter while K_M values remain almost constant. To determine which of the inhibitory mechanisms is in better agreement with the experimental data, the theoretical V_{max} and K_M parameters have been calculated (Table 4) using the inhibition constants shown in Table 3 for either case. The comparison of theoretical and experimental values suggests that the mechanism of inhibition is mainly non-competitive, although the variation of apparent K_M (pure non-competitive must have a constant value for apparent K_M) indicates a small contribution of a competitive inhibition.

The most probable interpretation, a non-competitive mechanism, is consistent with a decrease of the concentration of active enzyme instead of an active site competition against the substrate. The nature of a structure-related inhibition cannot be determined from the present data. Dissociation, changes in the protein flexibility or even the formation of wrong aggregates are possible reasons for the decrease of activity, and some of them have been already proposed for malate dehydrogenases. Thus, the presence of chaotropic anions in freezing–thawing experiments produced an important loss in the enzymatic activity. The analysis of the species generated in the different conditions assayed gave evidence for the existence of monomeric structures which have a high tendency to form wrong aggregates rather than correct intrachain or intradomain interactions [26–28]. In turn, Jensen et al. [14] were able to identify monomeric forms of mMDH in the presence of bromide, the most chaotropic anion assayed in their study. Although further studies are necessary to clarify this point, the results analyzed in this study suggest that changes in activity of mMDH in the presence of small anions are related to the monomer–

Table 4
Apparent kinetic parameters of mMDH in the presence of several anions in 50 mM MOPS buffer at pH 7.5[a]

Anion [100 mM]	Apparent V_{max} [μmol/min l]	Apparent K_M [μM]	Theoretical K_M for competitive inhibition (V_{max} constant)	Theoretical V_{max} for non-competitive inhibition (K_M constant)
Phosphate	132.1 ± 8	21 ± 6	21	132.1
Chloride	96.1 ± 7	25 ± 7	82.6	103.8
Thiocyanate	38.0 ± 2	46 ± 11	706	51.2

[a]Apparent kinetic parameters for oxaloacetate were determined in the presence of a concentration 100 mM of either anion. At this concentration, activation process is already completed. Theoretical values were determined using K_{A2} values indicated in Table 3, taking phosphate as the control value. For the competitive case, apparent V_{max} must be a constant value, whereas in the non-competitive case, apparent K_M must be the constant one.

dimer equilibrium in such a way that at low concentrations, anions would act as associating agents, helping to recover a fully active dimer of the protein. Higher concentrations of the chaotropic anions would revert this effect yielding inactive proteins, which possibly are in the monomeric state and can further evolve to the formation of inactive aggregates.

Little datum is available to understand this behaviour from the structural point of view. Crystal structures of several 2-oxoacid dehydrogenases are available that include anions bound to the protein. In most cases, a sulfate ion is placed in the active site [29,30] or in specific anion binding sites [31]. In the case of mMDH and *E. coli* MDH, a citrate ion is bound to the active site [32–34]. However, citrate is a close structural analog of L-malate and its presence mimics, in some degree, the binding of L-malate via its two carboxylate moieties; the third has been found to intrude upon the NAD^+ binding site [32]. These structures confirm that anion binding to the active site is related to the possibility of establishing strong interactions with the Arg residue (Arg 149 in mMDH) which is responsible for substrate binding [32–35]. No evidence for other anionic sites in mMDH that can be correlated with the dimeric interface is available. mMDH has indeed a number of positive charged residues located in the subunit interface [34]. Some of them are required to form ionic bridges between subunits (Glu 50, Arg 243), but most of them would repel each other in the dimeric state. Protonation of His 46, originally part of a hydrogen bond across the subunits and has been suggested as the main reason for dissociation at acid pH, stresses the importance of the charge state of the interface [2]. A certain amount of anions may play a role in the neutralization of the excess of positive charge, and hence stabilize the dimeric state in a sort of “salting-in” effect. Higher ionic concentrations would, in turn, weaken otherwise necessary ionic interactions and result in a loss of activity.

5. Conclusions

Based on the results presented in this paper, we would like to propose the following views concerning the action of anions on mMDH activity.

(1) The behaviour of mMDH is consistent with a monomer–dimer equilibrium with a K_d in the nanomolar range that is strongly influenced by the ionic environment. The equilibrium is shifted to the dimeric active form at pH values slightly above the neutrality or in the presence of small anions like phosphate. Thus, mMDH has significant amounts of monomeric form at pH 6.5 (50 mM MES buffer) and 7.5 (50 mM MOPS buffer).

(2) The increase in mMDH activity observed in 50 mM MOPS buffer at pH 7.5, in the presence of low concentrations of different anions, can be explained by an increase in the amount of the dimeric active form of the enzyme. In these conditions, the anions studied act as associating agents with a similar equilibrium constant (about 3 mM). At higher concentrations, all the anions assayed inhibited the enzyme activity. When the studies described above were made in 50 mM sodium phosphate buffer at pH 7.5, increasing amounts of anions only produced inhibition of the enzyme activity. In contrast with the activating capacity, the effectiveness in this inhibition follows the order: thiocyanate > perchlorate > nitrate > bromide > chloride > sulfate > acetate > phosphate; i.e. a higher inhibitory capacity correlates with a higher chaotropicity of the anion.

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