Independent Modulation of the Activity of α-Ketoglutarate Dehydrogenase Complex by Ca^{2+} and Mg^{2+} †

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ABSTRACT: The activity of α-ketoglutarate dehydrogenase complex (KGDHC), an important enzyme regulating several metabolic pathways, could be regulated by changes in the environment within the mitochondrial matrix. It has been postulated that the activity of this and other dehydrogenases *in vivo* could be modulated by changes in the intramitochondrial concentrations of Ca^{2+} or Mg^{2+}. Using a purified α-ketoglutarate dehydrogenase from pig hearts, the effect of Ca^{2+} and/or Mg^{2+} on the enzyme activity was investigated. Either Ca^{2+} or Mg^{2+} increased enzyme activity, and the effects were additive if the concentrations of free divalent cations were below 0.1 and 1 mM for Ca^{2+} and Mg^{2+}, respectively. In the presence of 1 mM α-ketoglutarate and other cofactors, the K_M for Mg^{2+} was 25 μM and less than 1 μM for Ca^{2+}. The K_M for α-ketoglutarate was a function of the divalent cation(s) present: 4 ± 1.1 mM in the absence of Ca^{2+}, with or without Mg^{2+}; 2.2 mM in the presence of 1.8 μM Ca^{2+} alone; and 0.3 mM in the presence of both Ca^{2+} and Mg^{2+}. Mg^{2+} increased KGDHC activity only in the presence of thiamine pyrophosphate (TPP) indicating that KGDHC requires both TPP and Mg^{2+} for enzyme's maximal activity. The affinity of KGDHC for NAD^+ is significantly changed by either Mg^{2+} or Ca^{2+}. The conclusions are that changes in both Ca^{2+} and Mg^{2+}, in concentrations possibly occurring within mitochondria, could control KGDHC activity and that thiamine pyrophosphate is required for maximal enzyme activity.

Mitochondrial α-ketoglutarate dehydrogenase complex plays an important role in the control of α-ketoglutarate concentration, which is a common metabolite of several enzymes of different metabolic pathways. Hence, changes in concentrations of α-ketoglutarate will affect oxidative metabolism of carbohydrates and fatty acids at the level of (i) isocitrate dehydrogenase, during oxidative metabolism of carbohydrates and fatty acids, (ii) glutamate−oxalacetic transaminase, during transfer of reducing equivalents by the malate−aspartate shuttle from the cytosol to the mitochondrial matrix, and (iii) glutamate dehydrogenase in the course of oxidative deamination of amino acids (Smith et al., 1974).

Experiments with isolated mitochondria and with the isolated α-ketoglutarate dehydrogenase complex have shown that the activity of KGDHC is controlled by various factors, including the redox state of the NAD couple, and by the concentration of succinyl-CoA (Smith et al., 1974; LaNoue & Williamson, 1971; LaNoue et al., 1972). There is also a large body of evidence using heart, kidney, and liver mitochondria demonstrating that KGDHC is activated by Ca^{2+} ions (Hansford, 1991; McCormack & Denton, 1989). Using purified KGDHC, it has been shown that Ca^{2+} increases the affinity of the enzyme for α-ketoglutarate (McCormack & Denton, 1979). The hypothesis that KGDHC activity and average rate of mitochondrial respiration can be regulated by changes in mitochondrial Ca^{2+} *in vivo* is controversial. While past and recent studies support this hypothesis (Hansford, 1991; McCormack & Denton, 1989), other studies have shown that mitochondrial dehydrogenases are activated *in vivo* without a detectable increase of mitochondrial Ca^{2+} (Moravec & Bond, 1991, 1992). Recent data have shown that Mg^{2+} content in mitochondria can change *in vivo* and *in vitro* in response to metabolic or hormonal stimulation, suggesting a possible role of Mg^{2+} in the regulation of the mitochondrial dehydrogenases or modulation of the calcium effects on the dehydrogenases (Romani et al., 1993; Brierley et al., 1987). Moreno-Sanchez et al. (1995) recently proposed that the spermine/Mg^{2+} ratio may control mitochondrial respiration without a concomitant increase in mitochondrial calcium.

To date, there is no consensus in the literature as to the effects of Mg^{2+} ions on the activity of isolated KGDHC. Mg^{2+} has been shown to increase (Hirashima et al., 1967; Hayakawa et al., 1966; Patel, 1974) or to have no effect on KGDHC activity (McCormack & Denton, 1979; Shylaja et al., 1990; Lai & Cooper, 1986). According to Hirashima et al. (1967), 5 μM Mg^{2+} increases the activity of KGDHC 2-fold, and 1 μM of Ca^{2+} increases the activity 3-fold when compared with the enzyme's activity without any added divalent cations. On the other hand, McCormack and Denton (1979) found no influence of EDTA and 1 mM Mg^{2+} on KGDHC activity isolated from pig heart, whereas Ca^{2+} was effective at less than 1 μM concentration. One of the reasons for such a discrepancy might be a very high affinity of KGDHC for Ca^{2+} and Mg^{2+}. Hayakawa et al. (1966) showed that, after dialysis of the isolated KGDHC against

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[1] Abbreviations: BSA, bovine serum albumin; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid ; KGDHC, α-ketoglutarate dehydrogenase complex; MOPS, 3-(*N*-morpholino) propanesulfonic acid; NAD^+, β-nicotinamide adenine dinucleotide, oxidized form; NADH, β-nicotinamide adenine dinucleotide, reduced form; PDHC, pyruvate dehydrogenase complex; PDHCP, phosphorylated form of pyruvate dehydrogenase complex; TPP, thiamine pyrophosphate.

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10 mM EDTA at 0 °C for 48 h, the enzyme still contained 1.7 μmol each of Mg^{2+} and of Ca^{2+} per mg of protein. These authors (Hayakawa et al., 1966) found that the stimulatory effects of Ca^{2+} and Mg^{2+} were not additive, suggesting that either Mg^{2+} or Ca^{2+} can activate KGDHC through a similar mechanism.

The purpose of this study was to define the regulatory effect of Mg^{2+} on isolated KGDHC. We show that Mg^{2+} does affect the activity of KGDHC and also, in the presence of low Ca^{2+} concentration, strongly modifies the enzyme's affinities for α-ketoglutarate and NAD^+. Furthermore, we show that the effects of low concentrations of Ca^{2+} and Mg^{2+} are additive, and the binding of either cation to the enzyme has distinct effects, consistent with independent binding sites. Contrary to previous belief, we show that the effect of Mg^{2+} on KGDHC activity depends upon the presence of thiamine pyrophosphate. In the absence of TPP, Mg^{2+} inactivates whereas Ca^{2+} stimulates the enzyme's activity.

MATERIALS AND METHODS

Source and Characteristics of KGDHC. The effects of Mg^{2+} and Ca^{2+} ions on the activity of KGDHC were studied using commercially available enzyme (Sigma, St. Louis, lot 44H80801). The enzyme was isolated from the porcine hearts by the method of Stanley and Perham (1980) which was designed for the isolation and separation of KGDHC and PDHC from the beef heart. According to Bunik and Follman (1993), an alternative preparation method consisting in differential protein sedimentation with poly(ethylene glycol) is less effective in separation of KGDHC and PDHC isolated from the pig hearts. The Sigma preparation of KGDHC from porcine heart is a solution of 1 mg of the enzyme in 50% glycerol containing 10 mg of BSA/mL, 30% sucrose, 2.5 mM EDTA, 2.5 mM EGTA, 2.5 mM 2-mercaptoethanol, 0.5% Triton X-100, 0.005% sodium acetate, and 25 mM potassium phosphate. Total concentration of protein was 11 mg/mL. The designated activity of the enzyme was 0.25 units per mg of enzyme and contained less than 10% of the pyruvate dehydrogenase activity. According to the Sigma Company specification, 1 unit of the enzyme reduced 1 μmol of NAD^+ to NADH per min at pH 7.4 at 30 °C in the presence of saturating concentration of CoA and 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$.

Determination of Activity and Purity of the Isolated α-Ketoglutarate Dehydrogenase Complex. We have characterized purity and divalent metals content of this enzyme. Sodium dodecyl sulfate/polyacrylamide gel (12% w/v) electrophoresis of 100 μg of the Sigma KGDHC performed in the Tris/glycine system (Laemmli, 1970) revealed the presence, besides the major spot of albumin, of nine bands. When compared with the data of the SDS/polyacrylamide gel electrophoresis of the purified KGDHC and PDHC described by Stanley and Perham (1980) and Barrera et al. (1972), the bands were identified as follows according to their molecular weight distribution: 2-oxoglutarate decarboxylase (113 000), PDH phosphatase (100 000), dihydrolipoyl transacetylase (74 000), dihydrolipoyl dehydrogenase (53 000), lipoamide dehydrogenase (51 000), minor band of PDH phosphokinase (50 000), lipoate succinyltransferase (48 000), α-chain pyruvate decarboxylase (PDH) (42 000), and β-chain pyruvate decarboxylase (PDH) (37 000). Thus, the Sigma KGDHC preparation is a mixture of KGDHC and PDHC without significant contamination from other enzymes. Control experiments have shown that under conditions of maximum KGDHC activity (in the presence of 0.1 mM Ca^{2+} and 0.47 mM Mg^{2+}), the activity of pyruvate dehydrogenase in the presence of 1 mM pyruvate was 5.7% of the maximum KGDHC activity with 1 mM α-ketoglutarate, and there was no detectable glutamate dehydrogenase activity. With 0.5 mM Mg^{2+} present, the kinetic parameters of KGDHC were very similar to those observed by other authors (McCormack & Denton, 1979). The preparation contained 0.2 nmol of Mg^{2+}/mg of protein and 0.7 nmol of Ca^{2+}/mg of protein, as determined by absorption spectrophotometry. Hence this preparation is very similar, in terms of activity, subunit composition, and contaminants, to that described by McCormack and Denton (1979) and Hayakawa et al. (1966) and to that routinely used in the literature.

The activity of KGDHC was measured fluorimetrically by following the production of NADH using 340 and 480 nm as excitation and emission wavelengths, respectively. The incubation conditions were essentially the same as described by McCormack and Denton (1979). The assay medium contained 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.1, 1 mM dithiothreitol, 1 mM thiamine pyrophosphate, 0.25 mM CoA, 1 mM NAD^+, and various concentrations of α-ketoglutarate. In some experiments, 25 mM α-ketoglutarate was used and NAD^+ concentration was varied. The reaction was initiated by adding 20 μL of the diluted enzyme solution (1:2 by volume) to 2 mL of reaction mixture (at 28 °C). In the presence of all reaction components and divalent cations, the rates of NAD^+ reduction were linear for 3−5 min. The linearity was lost earlier in the absence of metals, and no linearity was observed in the absence of TPP. Experiments on the effects of Ca^{2+} and Mg^{2+} on KGDHC activity were performed in the presence of 0.1 mM EGTA. The data are expressed as μmol of NADH formed during 1 min by 20 μL of diluted (1:2 vol/vol) enzyme. Since both KGDHC and PDHC have high specificity toward their corresponding substrates, their activities should not interfere with each other. Control experiments have shown that there was no formation of NADH when either α-ketoglutarate or CoAs was absent.

Measurement of Free Ca^{2+}. Determination of free Ca^{2+} in media with different Ca^{2+}/EGTA (0.1 mM) ratios and in the presence of various concentrations of Mg^{2+} was performed with the Ca^{2+}-sensitive electrode built at Bioanalytical Bioinstrumentation, Cleveland, OH, using ETH 1001 (calcium ionophore I) as a neutral carrier. The electrode and calibration solutions with different pCa were made according to Tsien and Rink (1980). The electrode displayed linear relationship between electric potential (mV) and pCa in the range between pCa 3 and pCa 7 with practically no hysteresis.

Chemicals. $MgCl_2$ and $CaCl_2$ of 99.995% purity were purchased from Aldrich Chemical Co. The exact concentrations of the stock solutions of these cations were determined by using atomic absorption spectrophotometry. Other chemicals were analytical grade.

RESULTS

Since Ca^{2+} was shown to be effective in activating KGDHC at concentrations less than 1 μM (McCormack & Denton, 1979), all experiments in this study were performed in the presence of 0.1 mM EGTA. The actual concentrations

Table 1: Comparison of Free Ca^{2+} Concentrations at Various Ca^{2+}/0.1 mM EGTA Ratios as Determined by the Ca^{2+}-Sensitive Electrode and as Computed using the "Chelator" Computer Program (Van Heeswijk et al., 1984)[a]

total $[Ca^{2+}]$ (μM)	free $[Ca^{2+}]$ (μM)			
	measured with Ca^{2+} electrode		computed	
	Ca^{2+} alone	Ca^{2+} + 1 mM Mg^{2+}	Ca^{2+} alone	Ca^{2+} + 1 mM Mg^{2+}
16.5	0.13 ± 0.01	0.12 ± 0.01	0.09	0.092
26.5	0.18 ± 0.02	0.15 ± 0.01	0.16	0.167
31.5	0.21 ± 0.03	0.17 ± 0.01	0.207	0.213
56.5	0.71 ± 0.2	0.63 ± 0.1	0.58	0.59
81.5	6.3 ± 1.2	7.0 ± 1.6	1.8	1.84
106.5	18.3 ± 1.6	18.4 ± 29	10.7	10.77
156.5	48.0 ± 6.4	45.1 ± 2.5	57.4	57.42
206.5	77.0 ± 9.3	74.9 ± 9.2	107	107.08
506.5	236 ± 43	333.3 ± 71	406.8	406.8

[a] Incubation conditions: 100 mM KCl, MOPS 50 mM, pH 7.1, 0.1 mM EGTA, and various concentrations of total $[Ca^{2+}]$ and $[Mg^{2+}]$ shown in the table.

of free Ca^{2+} in reaction mixtures containing variable Ca/EGTA ratios, both in the absence and in the presence of Mg^{2+}, were either determined using a Ca^{2+}-sensitive electrode or computed based on the program "Chelator" (Van Heeswijk et al., 1984). Measurements of free Ca^{2+} with a Ca^{2+}-sensitive electrode yielded higher values of free calcium at Ca^{2+}/EGTA ratios between 0.1 and 1.0 and lower values at Ca^{2+}/EGTA ratios higher than 1.0 than values obtained by computation (see Table 1). Using either method, 1 mM Mg^{2+} did not significantly displace Ca^{2+} from EGTA, and therefore concentrations of free Ca^{2+} at various Ca^{2+}/EGTA ratios were only minimally influenced by addition of Mg^{2+} and *vice versa*. Throughout the manuscript, values for free Ca^{2+} refer to values observed with the Ca^{2+}-sensitive electrode.

According to McCormack and Denton (1979), the activity of KGDHC isolated from pig hearts is independent of the presence of thiamine pyrophosphate and the addition of 1 mM EDTA or 1 mM Mg^{2+}. Figure 1A and Table 2 show that, with 1 mM α-ketoglutarate as a substrate, the activity of KGDHC was 2−3 times lower in the presence of 0.1 mM EGTA (Table 1), as compared to the activity in the absence of the chelator. These data indicate that the amount of Mg^{2+} (0.2 nmol/mg of protein) and Ca^{2+} (0.7 nmol/mg of protein), bound with the enzyme plus BSA solution, was sufficient enough to permit relatively high activity of the isolated KGDHC and that 0.1 mM EGTA effectively removes these cations from the proteins and thus inhibits the activity of KGDHC. Figure 1A,B shows that the titration of the enzyme's activity in the presence of 1 mM α-ketoglutarate and 0.1 mM EGTA with Mg^{2+} and Ca^{2+} resulted in a dramatic increase of the KGDHC activity. Note the difference in the ordinate scale between Figure 1A and B. Figure 1A shows that activation of the enzyme by Mg^{2+} was more pronounced in the presence of 1 mM α-ketoglutarate than with 0.5 mM of α-ketoglutarate. This is because KGDHC has a low affinity for α-ketoglutarate in the absence of Ca^{2+}. Calculated from the results of five experiments, the average value of K_M for α-ketoglutarate was 4 ± 1.1 mM, ranging from 2.0 to 5.0 mM in the presence of 0.1 mM EGTA. These data are in a good agreement with the estimates published in the literature (Hirashima et al., 1967).

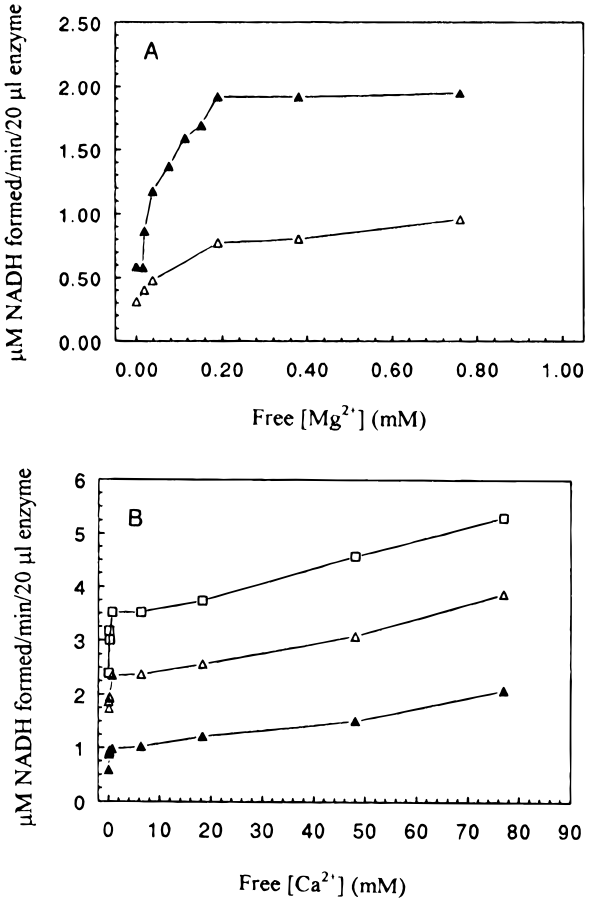

FIGURE 1: Effects of magnesium and calcium on activity of KGDHC. Incubation conditions as described in Materials and Methods. A. Effects of magnesium on the KGDHC activity: △, 0.5 mM α-ketoglutarate; ▲, 1 mM α-ketoglutarate. B. Effects of calcium and magnesium on the KGDHC activity; ▲, calcium alone; △, 76 μM Mg^{2+} present; □, 760 μM Mg^{2+} present.

Table 2: Effects of Magnesium and Calcium on Activity of KGDHC in the Presence or Absence of Thiamine Pyrophosphate[a]

additions	1 mM TPP	TPP not added
No EGTA, No Mg^{2+} or Ca^{2+}	1.17 ± 0.05 (205%)	0.9 ± 0.30 (158%)
0.1 mM EGTA	0.57 ± 0.03 (100%)	0.4 ± 0.09 (70%)
0.1 mM EGTA, 0.47 mM Mg^{2+}	1.93 ± 0.17 (338%)	0.22 ± 0.05 (39%)
0.1 mM EGTA, 0.1 mM Ca^{2+}	1.86 ± 0.50 (326%)	1.4± 0.27 (245%)
0.1 mM EGTA, 0.1 mM Ca^{2+}, 0.47 mM Mg^{2+}	3.85 ± 0.6 (675%)	1.40 ± 0.56 (245%)

[a] Incubation conditions: MOPS 50 mM, pH 7.1, 1 mM dithiothreitol, 0.25 mM CoA-SH, 1 mM α-ketoglutarate, 1 mM NAD^+. Total volume 2 mL, 20 μL of diluted enzyme (1:2 vol/vol). Concentrations of Mg^{2+} and Ca^{2+} are presented as free cations. Activity is expressesd as μmol of NADH formed per 1 min by 20 μL of the diluted enzyme.

Figure 1B shows that the stimulatory effects of Mg^{2+} and Ca^{2+} are additive. The stimulating effect of Ca^{2+} can be seen only at relatively low concentrations of α-ketoglutarate. Since Ca^{2+} decreases the K_M of the enzyme for α-ketoglutarate, at high (25 mM) concentrations of α-ketoglutarate the effect of Ca^{2+} is absent. Even in the presence of 25 mM α-ketoglutarate, however, Mg^{2+} stimulates the activity of the dehydrogenase (not shown). Figure 1B shows that a sharp increase in the rate of KGDHC activity occurs at free Ca^{2+} concentrations below 2 μM, consistent with a K_M for

Ca^{2+} below 1 μM, as determined by McCormack and Denton (1979). In the presence of Ca^{2+}, Mg^{2+} increases KGDHC activity in a concentration-dependent manner (Figure 1B).

Table 2 shows the effect of thiamine pyrophosphate on the activity of KGDHC in the presence and absence of Ca^{2+} and Mg^{2+}. With 1 mM TPP and 1 mM α-ketoglutarate, the rate of NADH production is highly sensitive to the addition of EGTA, Mg^{2+}, and Ca^{2+}. When compared with the enzyme's activity in the presence of 0.1 mM EGTA (taken as 100%), the concomitant addition of Mg^{2+} and Ca^{2+} induces a nearly 7-fold increase in the rate of NADH production. The effects of Mg^{2+} and Ca^{2+} on the enzyme's activity are additive only at relatively low concentration of free cations. When free Ca^{2+} is greater than 0.2 mM and free Mg^{2+} is above 1 mM, the effects of the metals are no longer additive (not shown), suggesting that at high concentrations, each ion may occupy the binding site for the other cation.

In the absence of EGTA, Mg^{2+}, and Ca^{2+}, exclusion of thiamine pyrophosphate from the incubation medium had no effect on the initial enzyme's activity. When 0.1 mM EGTA was added, there was a 30% inhibition of NADH production and the reaction became nonlinear. In the absence of TPP, Mg^{2+} could no longer stimulate the activity of KGDHC (see Table 2). Moreover, Mg^{2+} inhibited the enzyme's activity, and production of NADH was highly nonlinear. The reaction could not be stimulated by subsequent addition of TPP. However, Ca^{2+} could still stimulate KGDHC in the absence of TPP. Taken together, the data presented in Figure 1A,B and Table 2 indicate that (i) KGDHC is stimulated by Mg^{2+} and the stimulatory effect of Mg^{2+} is TPP-dependent and that (ii) the effects of Mg^{2+} and Ca^{2+} on the activity of KGDHC have different mechanisms.

Figure 2A represents a double-reciprocal plot of the velocity of NADH production at two different concentrations of α-ketoglutarate versus [Mg^{2+}]. Figure 2B shows the plot of the reciprocal velocity of NADH production versus 1/[α-ketoglutarate] in the absence and in the presence of added Mg^{2+}. Dixon and Webb (1964) have presented a theoretical description of various types of activation of enzyme activities by metals, indicating that the K_M for metal activating an enzyme has a number of different interpretations, depending upon the mechanism of action and the experimental conditions. If the enzyme independently binds substrate and a metal ion, then the sequence of the reaction is as follows (Dixon & Webb, 1964):

$$E + M \leftrightarrow EM \quad (1)$$

$$E + S \leftrightarrow ES \quad (2)$$

$$EM + S \leftrightarrow EMS \quad (3)$$

$$ES + M \leftrightarrow EMS \quad (4)$$

$$EMS \Rightarrow EM + \text{products} \quad (5)$$

According to Dixon and Webb (1964) the rate of the reaction is

$$v = ke/(1 + K_S/s)(1 + K_A/a) \quad (6)$$

where K_A and K_S are the dissociation constants of the enzyme complexes for the activating metal and the substrate, respectively, and s and a are the concentrations of substrate and free activating ion. When metal ions and substrate bind to the enzyme independently, K_A will be equal for reactions 1 and 4 and K_S will be equal for reactions 2 and 3.

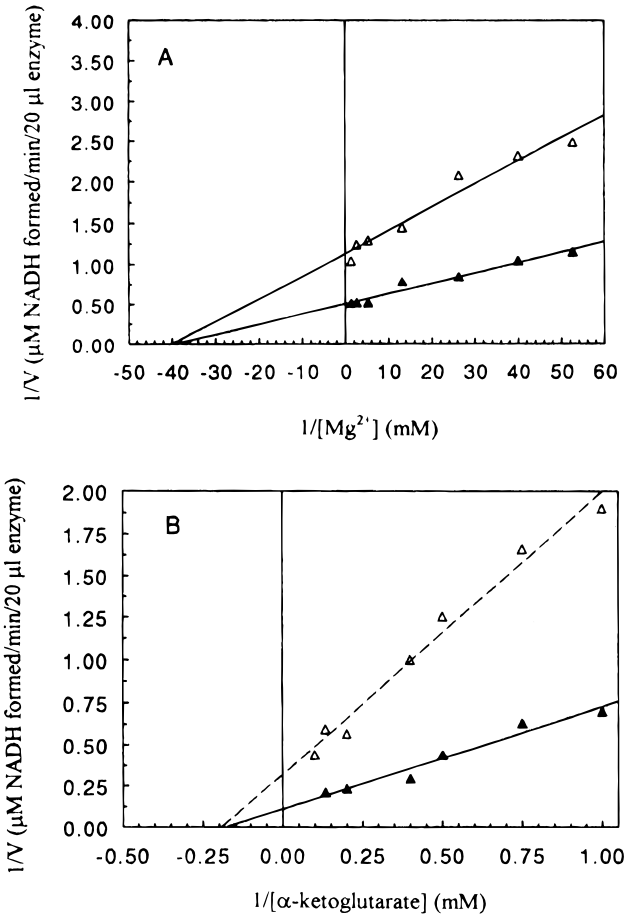

FIGURE 2: Double-reciprocal plots showing KGDHC activation by magnesium (0.1 mM EGTA was present in the incubation medium). A. △, 0.5 mM α-ketoglutarate; ▲, 1 mM α-ketoglutarate. B. △, Control; ▲, 0.38 mM Mg^{2+}.

Therefore, in the case of independent binding of an activating metal ion and a substrate, the double-reciprocal plot of activity versus a will yield the same value for K_A at all concentrations of a given substrate. In this case K_A is a true dissociation constant for the complex EM. Since eq 6 is symmetrical with respect to s and a, the double-reciprocal plot, with respect to s, will yield a K_M value that does not depend on the concentration of the activating ion. As can be seen from Figure 2A,B, the above considerations apply to the effect of Mg^{2+} on the activity of KGDHC at various Mg^{2+} and α-ketoglutarate concentrations. In our experiments, the K_M for α-ketoglutarate varied between 2 and 5 mM (average 4 ± 1.1 mM) and did not depend on the presence of Mg^{2+}. K_A for Mg^{2+} was 25 μM and was independent of α-ketoglutarate concentration (see Figure 2A). Thus, K_A for Mg^{2+} is a true dissociation constant for the KGDHC−Mg complex. As can be seen from Figure 2B, Mg^{2+} increases the V_{max} of the reaction without affecting the enzyme's affinity toward α-ketoglutarate.

On the other hand, the K_A for Ca^{2+} depended on the concentration of α-ketoglutarate (McCormack & Denton, 1979). From Figure 3A, it is evident that K_M for α-ketoglutarate depends on the concentration of free Ca^{2+}. Moreover, Figures 1B and 3B show that the effect of Ca^{2+} on the affinity of KGDHC for α-ketoglutarate depended on the presence of Mg^{2+}. Figure 3B demonstrates that in the

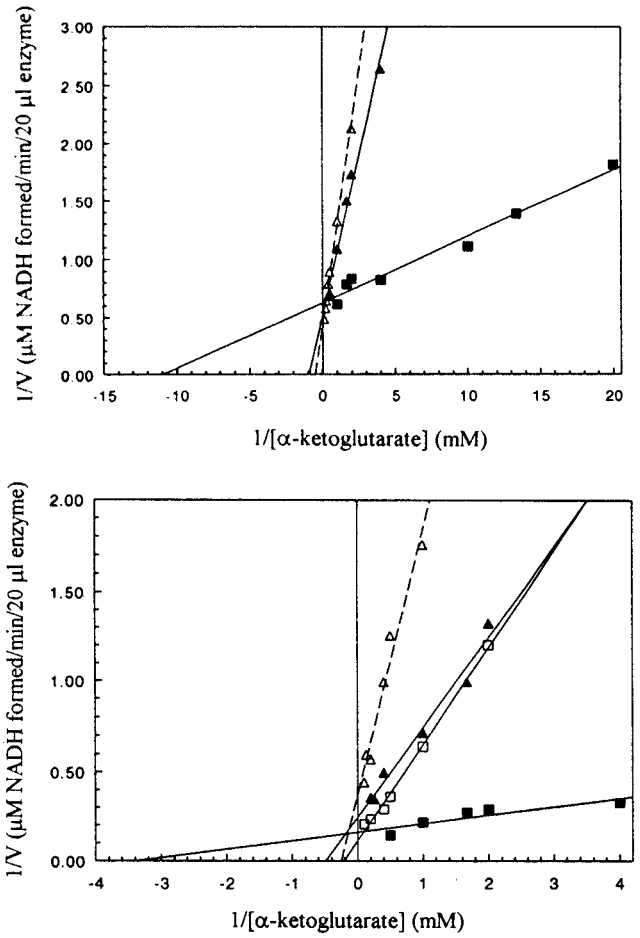

FIGURE 3: Double-reciprocal plots showing acivation of KGDHC by calcium and modulation of its effects by magnesium (0.1 mM EGTA was present). A. △, Control; ▲, 0.7 μM free Ca^{2+} present; ■, 7.7 μM free Ca^{2+} present. B. △, Control; □, −0.5 mM Mg^{2+}; ▲, 1.8 μM free Ca^{2+}; □, 1.8 μM free Ca^{2+} and 0.5 mM Mg^{2+} present.

absence of Mg^{2+}, 1.8 μM Ca^{2+} decreased the K_M for α-ketoglutarate from 5 to 2.2 mM. In the presence of 0.47 mM Mg^{2+}, however, the same concentration of free Ca^{2+} decreased the K_M for α-ketoglutarate to 0.3 mM, a 16-fold change. Therefore, K_A for the binding of Ca^{2+} to KGDHC is an apparent dissociation constant. Thus, Mg^{2+} increases the ability of Ca^{2+} to affect the enzyme's affinity for α-ketoglutarate. The data presented show that the affinity of KGDHC toward α-ketoglutarate depends on the formation of complexes with both Mg^{2+} and Ca^{2+}, with the possible order $EMg^{2+}Ca^{2+} \gg ECa^{2+} > E = EMg^{2+}$.

Figure 4 shows the effects of Mg^{2+} and Ca^{2+} on the affinity of the enzyme toward the second substrate for KGDHC, NAD^+. In the absence of added divalent cations, the affinity of KGDHC displays a two-branch kinetic pattern that disappears in the presence of either divalent metals. Ca^{2+} appears to increase the affinity of KGDHC for NAD^+. In the presence of Mg^{2+} , regardless of the presence of Ca^{2+}, the K_M for NAD^+ is relatively high, 66 μM, a value similar to the K_M for NAD^+ of 79 μM determined in the presence of 0.5 mM $CaCl_2$ obtained by Hamada et al. (1975).

DISCUSSION

At variance from the previous work of McCormack and Denton (1979), we found that KGDHC could be activated by Mg^{2+} and/or Ca^{2+} and that its activity could be influenced

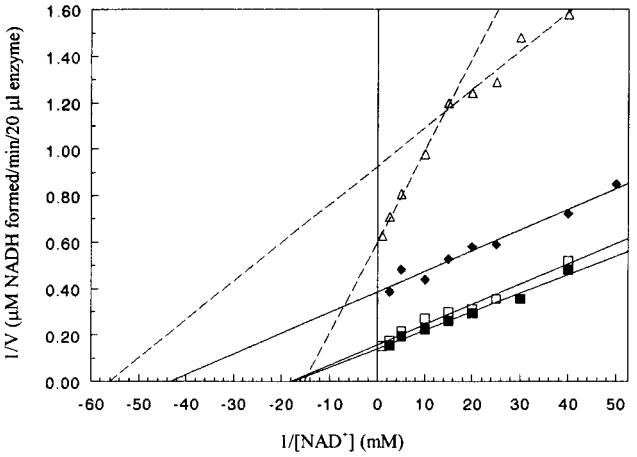

FIGURE 4: Double-reciprocal plot showing effects of calcium and magnesium on affinity of KGDHC for NAD^+. 0.1 mM EGTA, 25 mM α-ketoglutarate. △, Control; □, 0.47 mM Mg^{2+}; ◆, 48 μM Ca^{2+}; □, 48 μM Ca^2 and 0.47 mM Mg^{2+} present.

by divalent metal chelators. McCormack and Denton (1979) did not observe any effect on KGDHC activity following the addition of 5 mM EDTA or 1 mM Mg^{2+}, although they observed that at low (0.2 mM) α-ketoglutarate concentration, either EDTA or EGTA caused a marked decrease in the activity of the purified pig heart KGDHC. The effect was attributed solely to the complex formation between Ca^{2+} and chelators. This discrepancy with respect to the effects of chelators and Mg^{2+} could be accounted for by the endogenous Mg^{2+} present in the purified enzyme complex (Hayakawa et al., 1966). Another possibility for this discrepancy might be connected with the fact that we used the enzyme which has been isolated by solubilization of the proteins with the non-ionic detergent Triton X-100. This is the first study of the kind, whereas earlier works on the Mg^{2+} effects on KGDHC published in the literature used the enzyme released by freezing and thawing of the isolated mitochondria. Thus, either the detergent solubilizes some additional isoform of the enzyme with different sensitivity to Mg^{2+} or the detergent modifies the accessibility of the enzyme's Mg^{2+} binding sites to EGTA and Mg^{2+}.

Our data (Figure 2A) show that Mg^{2+} has a rather high affinity for KGDHC, $K_M = 25$ μM, and Figure 2A,B shows that the K_A for Mg^{2+} is a true dissociation constant for the complex EMg^{2+} (Dixon & Webb, 1964). Ca^{2+}, evidently, has a much lower K_M, less than 1 μM (McCormack & Denton, 1979). However, the V_{max} for the stimulation of KGDHC by Ca^{2+} strongly depends on the presence of Mg^{2+} (Figure 1B, Table 2). Hence, the K_A for the complex ECa^{2+} is an apparent dissociation constant. Thus, the data presented demonstrate that Mg^{2+} has a regulatory effect on the activity of KGDHC which is distinct from that of Ca^{2+}, and, in fact, Mg^{2+} is necessary for attenuation of the Ca^{2+}-dependent control of the activity of KGDHC.

It has been recognized that KGDHC and PDHC have analogous structural organization of the high molecular weight complex and catalyze similar reactions (Tanaka et al., 1972; Sakurai et al., 1970; Sanadi, 1963); thus it was suggested that the two enzymes might have common mechanisms of regulation. On the other hand, significant differences in catalysis and regulation between the two enzymes have been reported in the literature. The activity of PDHC is controlled by a phosphorylation−dephospho-

rylation cycle (Sanadi, 1963) and requires TPP (Walsh et al., 1976). PDHC also requires Mg^{2+} for TPP binding (Walsh et al., 1976; Wieland et al., 1969) and for the PDHCP phosphatase (Thomas et al., 1986). To date, evidence in the literature indicates that, unlike PDHC, KGDHC is not controlled by a phosphorylation−dephosphorylation mechanism (Hamada et al., 1975), and "does not require" TPP (McCormack & Denton, 1979) or Mg^{2+} for activity (McCormack & Denton, 1979; Lai & Cooper, 1986). The data presented in this paper demonstrate that KGDHC does require both TPP and Mg^{2+} for the enzyme's maximal activity. Hence, KGDHC has a greater similarity with PDHC than previously thought (McCormack & Denton, 1979).

Our data (see Figures 1B, 3, and 4, and Table 2) clearly indicate that both Ca^{2+} and Mg^{2+}, in the range of concentrations resembling those physiologically occurring within the mitochondria, are necessary for full KGDHC activity. Additional evidence for activation of mitochondrial dehydrogenases by both Mg^{2+} and Ca^{2+} is present in the literature, even if not specifically acknowledged. For instance, McCormack and Denton (1979) observed that, in the absence of Mg^{2+}, Ca^{2+} decreased the apparent K_M of the isolated citrate synthase for oxaloacetate from about 10 to 5 μM. However, in the presence of Mg^{2+} the K_M was further decreased and the effect of Ca^{2+} was no longer detectable. In spite of these observations, the authors (McCormack & Denton, 1979) did not ascribe any physiological significance to the role of Mg^{2+} in the dehydrogenase activation, yet these data and those reported in this manuscript are consistent with the hypothesis that Mg^{2+} is a physiological modulator of Ca^{2+} effects on mitochondrial dehydrogenases. This hypothesis is also consistent with the data that show that Mg^{2+} content in mitochondria rapidly changes in response to hormonal stimulation (Romani et al., 1993) and to changes in the mitochondrial metabolic state (Brierley et al., 1987).

The site of action of Mg^{2+} in the KGDHC complex is a matter of speculation. Biochemical and electron microscopic data indicate that the KGDHC is a mosaic structure comprising one molecule of lipoate succinyltransferase (E_2), six molecules of α-ketoglutarate dehydrogenase (E_1), and six molecules of lipoamide dehydrogenase (E_3). α-Ketoglutarate dehydrogenase and lipoamide dehydrogenase do not associate with each other directly but combine with lipoate succinyltransferase (Tanaka et al., 1972). TPP participates in the first reaction catalyzed by KGDHC when α-ketoglutarate reacts with the TPP−E_1 complex and undergoes oxidative decarboxylation (Hamada et al., 1975). Since Mg^{2+} exerts its stimulatory effect on KGDHC only in the presence of TPP, we may infer that Mg^{2+} is necessary for binding of TPP to E_1. Alternatively, Mg^{2+} might be of importance for "consolidation" of the multienzyme mosaic structure which is necessary for KGDHC's full activity.

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