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Regulation of Bovine Kidney α-Ketoglutarate Dehydrogenase Complex by Calcium Ion and Adenine Nucleotides. Effects on $S_{0.5}$ for α-Ketoglutarate†

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ABSTRACT: Regulation of bovine kidney α-ketoglutarate dehydrogenase complex by energy-linked metabolites was investigated. Ca^{2+}, ADP, or inorganic phosphate markedly enhanced the activity of the complex, and ATP or, to a lesser extent, GTP decreased the activity of the complex. Initial velocity studies with α-ketoglutarate as the varied substrate demonstrated that these modulators induced large changes in $S_{0.5}$ for α-ketoglutarate (based on analysis in Hill plots) with no change in the maximum velocity (as determined by double-reciprocal plots). For all conditions studied, the Hill coefficients were significantly less than 1.0 with slopes that were linear over wide ranges of α-ketoglutarate concentrations, indicating negative cooperativity that probably resulted from multiple site–site interactions. Ca^{2+} (maintained at 10 μM by a Ca^{2+} buffer) decreased the $S_{0.5}$ for α-ketoglutarate 63-fold (from 25 to 0.40 mM); even in the presence of a positive effector, ADP or phosphate, Ca^{2+} decreased the $S_{0.5}$ for α-ketoglutarate 7.8- or 28-fold, respectively. Consistent with a mechanism of action independent of Ca^{2+}, ADP (1.60 mM) or phosphate (20 mM) reduced the $S_{0.5}$ for α-ketoglutarate in the presence of Ca^{2+} (i.e., 4.5- or 1.67-fold, respectively); however, these effectors elicited larger decreases in $S_{0.5}$ in the absence of Ca^{2+} (i.e., 37- or 3.7-fold, respectively). ATP (1.6 mM) increased the $S_{0.5}$ for α-ketoglutarate, and Ca^{2+} appreciably reduced the effect, lowering the $S_{0.5}$ 98-fold from 66 to 0.67 mM. Thus the activity of the kidney α-ketoglutarate dehydrogenase complex is poised to increase as the energy potential in mitochondria declines, and Ca^{2+} has a pronounced modulatory effect. Comparative studies on bovine heart α-ketoglutarate dehydrogenase complex and the effects of varying the ADP/ATP ratio in the presence or absence of Ca^{2+} or phosphate are also described.

In aerobic cells, the citric acid cycle serves a major catabolic energy-generating function, and the net flux through the cycle must respond to the energy needs of the cell. At the same time, the levels of intermediates of the cycle must be controlled independently of cycle flux for utilization in biosynthetic processes and also in the integrated shuttling of anions in and out of mitochondria. Thus an intricate system of regulation that reflects the phosphate potential and responds to other requirements of this amphibolic pathway would be anticipated. α-Ketoglutarate is a branch point metabolite that is generated as an intermediate in the citric acid cycle during the oxidation of carbohydrates and fatty acids and by glutamate dehydrogenase during the oxidative deamination of amino acids. In addition, α-ketoglutarate is produced by transamination of glutamate as part of the malate–aspartate shuttle which functions to transfer reducing equivalents from the cytoplasm into mitochondria. The flux through the α-ketoglutarate dehydrogenase complex would be expected to be tightly coupled to the energy state of mitochondria and to reflect the generation of α-ketoglutarate from glutamate. Thus, modulation of the α-ketoglutarate dehydrogenase complex in a manner independent of the preceding reactions in the citric acid cycle is anticipated.

Previous studies (Garland, 1964; Smith et al., 1974) have suggested that the α-ketoglutarate dehydrogenase complex from mammalian tissues is regulated by product inhibition and therefore by the NAD^+/NADH and CoA/succinyl-CoA ratios. Inhibition by GTP has also been suggested (Olson & Allgyer, 1973). Recently, McCormack & Denton (1979) have presented evidence that porcine heart α-ketoglutarate dehydrogenase complex is regulated by the level of Ca^{2+} and adenine nucleotides. They observed that ADP and micromolar levels of Ca^{2+} lowered the K_m for α-ketoglutarate and that ATP increased the K_m for α-ketoglutarate. Because Ca^{2+} serves unique roles in muscle tissue, we initiated studies to test whether similar effects were observable with the bovine kidney complex. We found similar effects with some qualitative differences. In addition, we have extended the studies of

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McCormack & Denton (1979) and report an effect of phosphate anion and significant negative cooperativity[1] that varies, depending on the nature of effectors. We have focused on the large variation in effects of several ligands in the presence vs. the virtual absence of Ca^{2+}. Elsewhere, we report that micromolar levels of Ca^{2+} also tend to decrease NADH inhibition of the α-ketoglutarate dehydrogenase complex (Lawlis & Roche, 1980, 1981).

Experimental Procedures

Materials. The reagents Mops,[2] DTT, NAD^+, thiamin pyrophosphate, cysteine hydrochloride, α-ketoglutarate, succinyl-CoA, EGTA, and GTP were purchased from Sigma. The nucleotides ADP, ATP, and coenzyme A were from P-L Biochemicals. All other common reagents used were of the highest quality commercially available.

Preparation of Enzymes. α-Ketoglutarate dehydrogenase complex from bovine kidney was prepared as described by Roche & Cate (1977); the enzyme from bovine heart was purified by the same procedure except that the mitochondria were ruptured by using a Parr bomb. Prior to decompression of the bomb, the mitochondria were incubated for 20 min (4 °C) at 1500 psi.

The purified enzyme was stored in aliquots at −70 °C. After storage for 4 months at −70 °C, the heart enzyme maintained a high specific activity whereas storage of the kidney enzyme under identical conditions for 1 year resulted in a 50% decrease in its specific activity. After storage, the maximal velocities for the kidney and heart enzymes were 5.0 and 18.2 μmol min^{-1} mg^{-1}, respectively. Some studies were conducted with a fresh kidney preparation which had a specific activity of 12.5 μmol min^{-1} mg^{-1}. These values did not change throughout this study.

For all experiments described, the enzyme was rapidly thawed and diluted to 1 mg mL^{-1} in a buffer (at 4 °C) consisting of 40 mM Mops–K^+ (pH 7.5) and 1 mM DTT. To ensure that the activity of the enzyme remained stable, the specific activity was monitored at regular intervals during the experiment.

Enzyme Assays. The activity of the α-ketoglutarate dehydrogenase complex was measured by monitoring production of NADH at 340 nm (30 °C). The assay medium was 50 mM Mops–K^+, 2.0 mM $MgCl_2$, 2.0 mM NAD^+, 0.3 mM thiamin pyrophosphate, 0.12 mM coenzyme A, and 2.6 mM cysteine hydrochloride in a final volume of 1 mL. Unless otherwise indicated, the final pH was 7.6, and 15 μg of the kidney complex or 5 μg of the heart complex was added to assay mixtures. The concentration of α-ketoglutarate was varied as indicated. In general, assays in the presence of added Ca^{2+} were conducted with a Ca^{2+} buffer consisting of 5.0 mM EGTA–Ca^{2+} (pH 7.6). At pH 7.6, it was calculated that this would give a concentration of free Ca^{2+} of 10 μM by using the association constants and procedures of Portzehl et al. (1964). This was a saturating level of free Ca^{2+} for the effects described in that slight increases or decreases in the Ca^{2+} did not alter enzyme activity. In some assays in which nucleotide phosphates were varied, 1.0 mM Ca^{2+} was added to the assay mixture. For assays conducted in the virtual absence of Ca^{2+}, 1.0 mM EGTA–K^+ (pH 7.6) was added. Ca^{2+} from contamination of reagents was present in assay mixtures at a level less than 10 μM as determined by measurements with an atomic absorption spectrometer by using a procedure of extrapolation to basal level of a curve produced by addition of different levels of a Ca^{2+} standard. At pH 7.6, in the presence of 1 mM EGTA, the concentration of free Ca^{2+} was calculated to be $<10^{-9}$ M. All components of the assay mixtures, except enzyme, were equilibrated at 30 °C, and the reactions were initiated by the addition of enzyme.

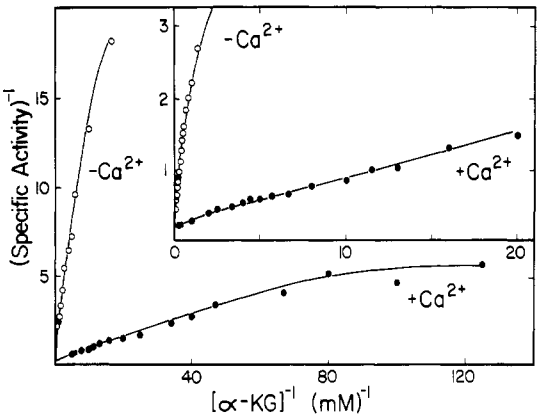

FIGURE 1: Lineweaver–Burk plot showing effect of Ca^{2+} on the activity of α-ketoglutarate dehydrogenase complex with varying α-ketoglutarate concentration. Studies were conducted in the presence (●) or absence (○) of Ca^{2+} at the α-ketoglutarate concentrations indicated. The insert is an expansion showing additional data at high α-ketoglutarate concentrations. Other conditions are described under Experimental Procedures.

Results

Effect of Ca^{2+} on the Activity of the α-Ketoglutarate Dehydrogenase Complex. Addition of 100 μM EGTA to assay mixtures lacking added Ca^{2+} reduced the activity of the bovine kidney α-ketoglutarate dehydrogenase complex to 40% of the control velocity with 2.0 mM α-ketoglutarate and saturating levels of the other substrates. Higher levels of EGTA (to 8.0 mM) caused no further inhibition, but addition of 20 mM α-ketoglutarate restored enzyme activity to 80–90% of control rates. This suggested that Ca^{2+} lowers the K_m for α-ketoglutarate for the bovine kidney complex, as was observed with the porcine heart complex (McCormack & Denton, 1979). Addition of EGTA, or addition of Ca^{2+} in excess of EGTA, caused an immediate decrease or increase, respectively, in the reaction rates, indicating that the effect of Ca^{2+} was rapid and reversible.

To further elucidate the effect of Ca^{2+} on catalysis, we investigated the kinetic response of the bovine kidney α-ketoglutarate dehydrogenase complex to changes in α-ketoglutarate concentration. Here and in subsequent experiments the addition of 1 mM EGTA in the absence of any added Ca^{2+} leads to free Ca^{2+} concentrations calculated to be <1.0 nM and will be referred to as the absence of Ca^{2+}. For each set of conditions, 20–30 concentrations of α-ketoglutarate were tested. Whenever possible, the concentration of α-ketoglutarate, which provided half of the maximal velocity ($S_{0.5}$), was bracketed. Concentrations of α-ketoglutarate higher than 10 mM were avoided because of the potential for chelation of Ca^{2+}; the EGTA–Ca^{2+} buffer used supplied adequate free Ca^{2+} in the presence of 10 mM α-ketoglutarate.

Analysis of changes in initial velocities as a function of the α-ketoglutarate concentration, in the presence or absence of Ca^{2+}, yielded nonlinear double-reciprocal plots (Figure 1). At

[1] The term negative cooperativity is used here to imply kinetic patterns which give Hill coefficients less than 1 and for which the ratio of $S_{0.9}$ to $S_{0.1}$ was significantly greater than 81. Since only kinetic observations are described, a mechanism requiring protein–protein interactions has not been established.

[2] Abbreviations used: Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; $S_{0.5}$, substrate concentration giving half-maximal velocity; *h*, Hill coefficient; M_r, molecular weight; $NaDodSO_4$, sodium dodecyl sulfate.

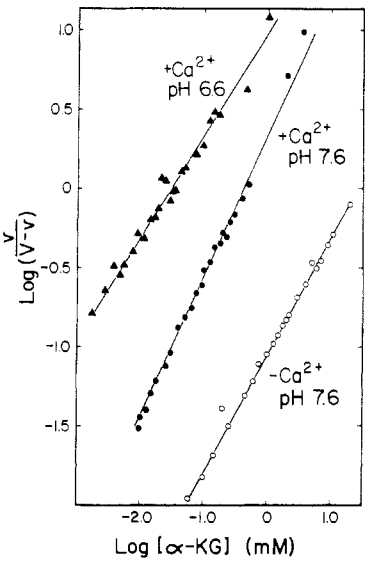

FIGURE 2: Hill plot showing the effects of Ca^{2+} and pH on cooperativity in the activity of the α-ketoglutarate dehydrogenase complex in response to varying α-ketoglutarate concentration. Enzymatic activity was determined at pH 6.6 (▲) or pH 7.6 in the presence (●) or absence (○) of Ca^{2+}. Maximal activities for assays at pH 6.6 and 7.6 were 3.8 and 5.0 μmol min^{-1} mg^{-1}, respectively. Other conditions are described under Experimental Procedures.

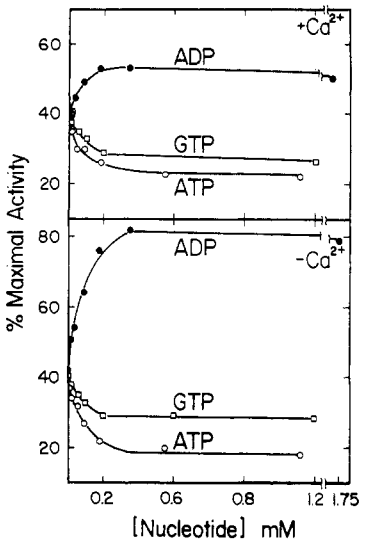

FIGURE 3: Effect of varying nucleotide phosphates on α-ketoglutarate dehydrogenase complex activity at suboptimal α-ketoglutarate concentration in the presence and absence of Ca^{2+}. The concentrations of ADP (●), GTP (□), and ATP (○) were as indicated. The α-ketoglutarate concentrations were 0.10 and 2.0 mM for the upper ($+Ca^{2+}$) and the lower portions ($-Ca^{2+}$), respectively. For the conditions of plus and minus Ca^{2+}, 1 mM $CaCl_2$ or 1 mM EGTA–K^+ was added to the reaction mixture. The final pH of the reaction mixture was 7.2. One hundred percent activity was considered to be that value obtained when enzyme was assayed with 2.0 mM α-ketoglutarate and in the presence of Ca^{2+}. This rate was 3.27 μmol min^{-1} mg^{-1}.

pH 7.6, Ca^{2+} had a large effect on $S_{0.5}$ for α-ketoglutarate, but little or no effect on the maximal velocity was observed (inset Figure 1). From Hill plots of these data (Figure 2), the $S_{0.5}$ in the presence or absence of Ca^{2+} was determined to be 0.40 or 25 mM, respectively. For all Hill plots, the values for V_m were determined from the double-reciprocal plots. Thus, 10 μM Ca^{2+} facilitated more than a 60-fold decrease in $S_{0.5}$ for α-ketoglutarate. We have previously shown with EGTA–Ca^{2+} buffers that a half-maximal increase in activity due to lowering in $S_{0.5}$ for α-ketoglutarate occurs at a concentration of free Ca^{2+} slightly less than 1.0 μM (Lawlis & Roche, 1980).

When the enzyme was assayed at pH 6.6 in the presence of Ca^{2+}, a similarly curved Lineweaver–Burk plot was observed (data not shown). From Figure 2, the $S_{0.5}$ was 0.032 mM α-ketoglutarate, which was 12.5-fold lower than the $S_{0.5}$ obtained at pH 7.6 in the presence of Ca^{2+}. The maximal velocities at pH 6.6 and 7.6 were 3.8 and 5.0 μmol min^{-1} mg^{-1} protein, respectively. Such a change in V_m with pH was not observed by McCormack & Denton (1979).

Consistent with the hyperbolic nature of double-reciprocal plots, the slopes from Hill plots (Figure 2) are significantly less than unity, indicating negative cooperativity. For the conditions of $+Ca^{2+}$ (pH 6.6), $-Ca^{2+}$ (pH 7.6), and $+Ca^{2+}$ (pH 7.6), the Hill coefficients [h; see Atkinson et al. 1965] were 0.65, 0.72, and 0.89, respectively. For these three conditions, the plots appeared linear throughout the concentration range tested. Hill coefficients less than 1 have not been reported previously in the studies on α-ketoglutarate dehydrogenase complex from mammalian sources, perhaps reflecting tissue specific differences or because not nearly as many α-ketoglutarate concentrations were tested.

When 10 mM NAD^+ rather than 2.0 mM NAD^+ was included in assays, the $S_{0.5}$ for α-ketoglutarate was decreased more than 3-fold in the absence of Ca^{2+} (from 25 to 7.4 mM), but it was changed only slightly in the presence of Ca^{2+} (from 0.40 to 0.32 mM). Consistent with NAD^+ lowering the $S_{0.5}$ for α-ketoglutarate, 10 mM NAD^+ also stimulates the activity of the resolved α-ketoglutarate dehydrogenase component (Lawlis & Roche, 1981).

Effects of ADP, ATP, and GTP on Bovine Kidney α-Ketoglutarate Dehydrogenase Complex. The regulatory effects of nucleotides important in mitochondrial metabolism were explored. α-Ketoglutarate concentrations of 0.10 or 2.0 mM were selected for studies in the presence or absence of Ca^{2+}, respectively, since these concentrations gave about 40% of maximal activity. As shown in Figure 3, ATP inhibited the enzyme with a maximum decrease of 1.92-fold or 2.33-fold in the presence (upper panel) or absence (lower panel) of Ca^{2+}, respectively. GTP, a product generated by succinyl thiokinase reaction, also inhibited, but to a lesser extent than ATP. In contrast, ADP increased enzyme activity with a maximum effect of 1.25- or 1.9-fold in the presence or absence of Ca^{2+}, respectively (Figure 3). For all three nucleotides, a concentration of about 0.3 mM was saturating in giving the various effects. For ATP or ADP, the magnitude of the effects was greater in the absence than in the presence of Ca^{2+}.

Effect of Inorganic Phosphate on the Activity of the Bovine Kidney α-Ketoglutarate Dehydrogenase Complex. In a concentration range of 10–20 mM, inorganic phosphate activated the α-ketoglutarate dehydrogenase complex, but to a lesser extent than ADP (data not shown). As noted above, the $S_{0.5}$ for α-ketoglutarate was decreased with decreasing pH, and, thus, the potassium phosphate stock solution was prepared so that it maintained the pH at 7.6 upon dilution into the reaction mixture. That the stimulation of activity upon addition of potassium phosphate was not due to the presence of additional K^+ (which was already present in assays at a concentration higher than 60 mM) was established by the observation that addition of 40 mM KCl had no effect on $S_{0.5}$ for α-ketoglutarate.

As shown in Figure 4, the maximum velocity for studies in the presence of phosphate was 5.0 μmol min^{-1} mg^{-1}, which is identical with the value observed in the absence of phosphate (Figure 3). This indicates that phosphate stimulated the activity of the complex by lowering the $S_{0.5}$ for α-ketoglutarate.

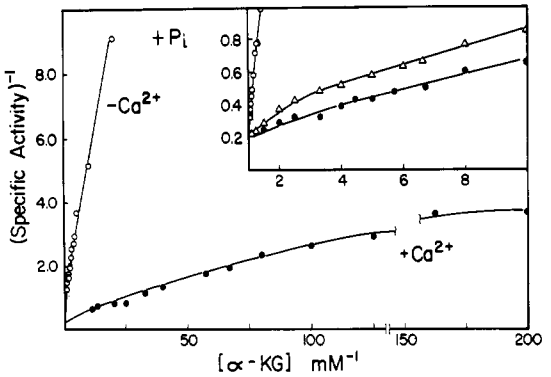

FIGURE 4: Lineweaver–Burk plot showing effect of inorganic phosphate, in the presence and absence of Ca^{2+}, on the α-ketoglutarate dehydrogenase activity with varying α-ketoglutarate concentration. Studies were conducted in the presence of (●) or absence (○) of Ca^{2+} with 20 mM potassium phosphate at the indicated concentrations of α-ketoglutarate. The insert is an expansion showing additional data at high α-ketoglutarate concentrations and includes data for kinetics in the presence of Ca^{2+} with no phosphate (▲), for which a complete profile is shown in Figure 1. Other conditions were as described under Experimental Procedures.

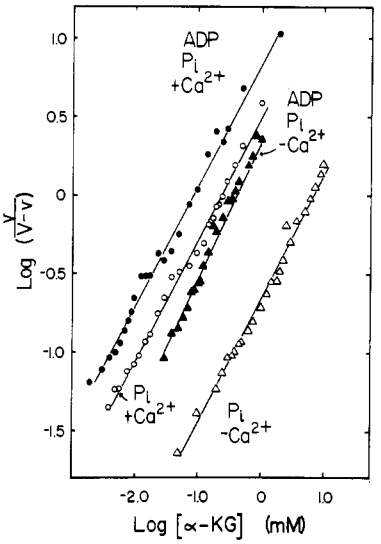

FIGURE 5: Hill plot showing the effect of inorganic phosphate and inorganic phosphate plus ADP on cooperativity in the activity of the α-ketoglutarate dehydrogenase complex in response to varying α-ketoglutarate concentration. The potassium phosphate concentration was 20 mM, and, where applicable, the ADP concentration was 1.6 mM. The maximal velocity for all four conditions was 5.0 μmol min^{-1} mg^{-1}. For other conditions see Figure 4 and Experimental Procedures.

Comparing the Hill plots in Figure 5 to those in Figure 2 shows that phosphate decreased the $S_{0.5}$ to a lesser extent in the presence of Ca^{2+} (from 0.40 to 0.24 mM) than in the absence of Ca^{2+} (from 25 to 6.8 mM). Clearly, 10 μM Ca^{2+} had a pronounced effect on $S_{0.5}$ for α-ketoglutarate in the presence of 20 mM inorganic phosphate, causing about a 28-fold decrease from 6.8 to 0.24 mM. In the presence of phosphate, the Hill coefficient was 0.78 in the presence of Ca^{2+} or 0.80 in the absence of Ca^{2+}; again, Hill plots were linear over 2 orders of magnitude of α-ketoglutarate concentrations.

Since, as noted below, ADP caused larger reductions in $S_{0.5}$ for α-ketoglutarate than phosphate, studies were conducted to evaluate whether ADP and inorganic phosphate reduce the $S_{0.5}$ for α-ketoglutarate through competing or complementary mechanisms. The effect of the combination of these modulators was compared to that for each alone, and these comparisons were made in the presence or absence of Ca^{2+}. Figure 5 shows Hill plots for data from initial velocity studies conducted with 20 mM potassium phosphate alone or together

Table I: Summary of Kinetic Constants of Bovine Kidney α-Ketoglutarate Dehydrogenase Complex and Effects of Calcium, Phosphate, ADP, and ATP; Attenuation of Effects of Adenylates and Phosphate by Ca^{2+} [a]

additions				ratio of $S_{0.5}$ values	
Ca^{2+}	phospho-effector(s)	$S_{0.5}$	h [c]	$-Ca^{2+}/+Ca^{2+}$	−phosphoeffector(s)/ +phosphoeffector(s)
+		0.40	0.89		
−		25	0.72	63	
+ [b]		0.032	0.65		
+	P_i	0.24	0.78		1.7
−	P_i	6.8	0.80	28	3.7
+	ADP	0.087	0.80		4.6
−	ADP	0.68	0.94	7.8	37
+	ADP + P_i	0.085	0.77		4.7
−	ADP + P_i	0.41	0.85	4.8	62
+	ATP	0.67	0.81		0.60
−	ATP	66	0.75	99	0.38

[a] Maximum velocity was 5.0 μmol min^{-1} mg^{-1} under all conditions except as indicated in *b*. [b] This experiment was performed at pH 6.6, and V_m of 3.8 was observed. [c] All Hill plots from which h values were determined had correlation coefficients greater than 0.98 and a mean value of 0.995.

with Ca^{2+} (10 μM), ADP (1.6 mM), or the combination of these levels of Ca^{2+} and ADP. None of these conditions changed V_m, which was 5.0 μmol min^{-1} mg^{-1}. ADP caused a significant reduction in $S_{0.5}$ in the presence of phosphate (Figure 5). However, in the presence of Ca^{2+} and ADP, phosphate caused little, if any, decrease in $S_{0.5}$ below that achieved with ADP alone (cf. below; data summarized in Table I). In the absence of Ca^{2+}, phosphate caused a relatively small decrease below that for ADP from 0.68 to 0.41 mM. For further evaluation of whether the latter effect was significant, the effects of saturating levels of phosphate (20 mM), ADP (1.6 mM), or the combination of these positive effectors were compared at a fixed α-ketoglutarate concentration of 0.5 mM in the absence of Ca^{2+}. Phosphate, ADP, or the combination caused a 2.7-, 10.0-, or 16.6-fold increase in activity, respectively. Thus, phosphate caused a 66% increase in activity, indicating that, in the absence of Ca^{2+}, phosphate decreases $S_{0.5}$ by a mechanism that is at least partially distinct from ADP.

Effects of Adenylates in Initial Velocity Studies. Initial velocity studies on the effects of 1.6 mM ADP or 1.6 mM ATP demonstrated that the maximum velocity was not changed (in double-reciprocal plots) and that the $S_{0.5}$ (in Hill plots) was appreciably decreased by ADP and increased by ATP (data not shown). In the presence of 10 μM Ca^{2+}, ADP decreased $S_{0.5}$ for α-ketoglutarate from 0.40 to 0.087 mM, and, in the absence of Ca^{2+}, ADP caused a 37-fold decrease in $S_{0.5}$ from 25 to 0.68 mM. ATP increased the $S_{0.5}$ for α-ketoglutarate 1.7- or 2.6-fold in the presence or absence of Ca^{2+}, respectively. Table I summarizes these and previous results. As in the studies above, Hill plots were linear over a wide range of α-ketoglutarate concentrations, and Hill coefficients were less than 1.

The observation that ATP inhibition leveled off with increasing ATP (Figure 3) and that ATP only altered the $S_{0.5}$ for α-ketoglutarate indicates that ATP is a partial competitive inhibitor. Inhibition by ATP and activation by ADP, which results from a decrease in $S_{0.5}$, can be described by[3]

$$v/V_m = \left(\frac{S}{K_m} + \frac{SL}{\alpha K_m K_l}\right) \Big/ \left(1 + \frac{S}{K_m} + \frac{L}{K_l} + \frac{SL}{\alpha K_m K_l}\right)$$

where S = α-ketoglutarate, L = ADP or ATP, K_I represents either activation constant (K_a) for ADP or inhibition constant (K_i) for ATP, and $\alpha > 1$ for ATP and $\alpha < 1$ for ADP.

Studies conducted in the presence of Ca^{2+} to evaluate these parameters[3] confirmed that ATP is a partial competitive inhibitor with a K_i of 0.10 ± 0.02 mM and α = 1.6 ± 0.3 and established that ADP had a K_a of 0.33 ± 0.03 mM with α = 0.20 ± 0.03. Since the intramitochondrial levels of adenine nucleotides are considerably higher than the K_i and K_a for ATP and ADP, respectively, the α values should indicate the relative effectiveness of such saturating levels of ATP and ADP. These values imply that ADP will be significantly more effective as an activator, consistent with studies described below in which the ADP/ATP ratio was varied.

Summary of Kinetic Studies: Ca^{2+} Attenuation of Regulation by Adenylates and Phosphate. Table I summarizes the kinetic parameters studied for the bovine kidney α-ketoglutarate dehydrogenase complex. As emphasized, Ca^{2+}, ADP, or phosphate reduced the $S_{0.5}$ for α-ketoglutarate whereas ATP increased this parameter. Under all conditions, Ca^{2+} caused large changes in $S_{0.5}$ for α-ketoglutarate. In the presence of phosphate, ADP, or the combination of these effectors, 10 μM Ca^{2+} decreased $S_{0.5}$ 28-, 7.8-, or 4.8-fold, respectively. Interestingly, in the absence of effectors, or in the presence of the negative effector ATP, Ca^{2+} reduced the $S_{0.5}$ 63- or 99-fold, respectively. Under all conditions, there was little or no effect on V_m at a constant pH.

Discernible trends are evident for changes in the Hill coefficient in the presence and absence of the different effectors (Table I). Removal of Ca^{2+} with EGTA–K^+ in the presence of no effectors, or in the presence of the negative effector ATP, resulted in a decrease in the Hill coefficient of 0.17 or 0.14, respectively. However, removal of Ca^{2+} in the presence of positive effectors resulted in an increase in h ranging from 0.08 to 0.14, depending on the conditions. Therefore, the presence or absence of Ca^{2+} appeared to have an opposite effect on the Hill coefficient, depending on whether a positive or negative regulator was present.

Studies with freshly prepared kidney complex, for which the maximum velocity was 12.5 μmol min^{-1} mg^{-1}, gave similar $S_{0.5}$ values and slightly lower Hill coefficients. Thus, the negative cooperativity observed was not a consequence of changes in the enzyme during storage.

The last column in Table I describes the ratio of the $S_{0.5}$ for α-ketoglutarate in the absence of effector to that in the presence of effector, thus reflecting the impact of the effector on $S_{0.5}$. Clearly, all these effectors caused larger changes in the absence than in the presence of Ca^{2+}. ATP was 1.6-fold more effective in increasing the $S_{0.5}$ for α-ketoglutarate; phosphate, ADP, or ATP plus phosphate was 2.2-, 8.0-, or 13.2-fold more effective in decreasing the $S_{0.5}$ for α-ketoglutarate. Therefore, Ca^{2+} appreciably altered regulation of the kidney α-ketoglutarate dehydrogenase complex, by adenylates or inorganic phosphate but through an independent rather than a competitive mode of action.

Table II: Comparison of Bovine Heart and Kidney α-Ketoglutarate Dehydrogenase Complex Activities in the Presence of 10 μM Ca^{2+} [a]

altered condition	enzyme source	$S_{0.5}$ (mM)	h	V_m (μmol min^{-1} mg^{-1})
	heart	0.67	0.88	18.2
	kidney	0.40	0.89	5.0
pH 6.6	heart	0.081	0.84	12.5
pH 6.6	kidney	0.032	0.65	3.8
+P_i	heart	0.32	0.90	18.2
+P_i	kidney	0.24	0.78	5.0

[a] Except as indicated (lines 3 and 4), assays were conducted at pH 7.6. Where indicated, phosphate was present at 20.0 mM.

Comparison of Bovine Heart and Kidney α-Ketoglutarate Dehydrogenase Complexes. As mentioned, McCormack & Denton (1979) did not report negative cooperativity or regulation by inorganic phosphate. However, these experiments were performed with porcine heart complex instead of bovine kidney complex. Our observations may reflect the organ from which the complex was isolated or possibly a species difference. For the determination of whether the former were the case, comparative studies were performed with the purified bovine heart complex, and Table II summarizes the results of these studies.

For all the conditions studied, the heart complex had a $S_{0.5}$ for α-ketoglutarate that was somewhat higher (1.6- to 2.5-fold) than was observed with the kidney enzyme. For the kidney or heart complex, inorganic phosphate in the presence of Ca^{2+} reduced the $S_{0.5}$ 1.7- or 1.8-fold, respectively. Also, inorganic phosphate had no effect on the V_m of the heart enzyme. Contrary to McCormack & Denton (1979), we observed a substantial decrease in V_m with a change in pH from 7.6 to 6.6.

Bovine heart α-ketoglutarate dehydrogenase complex exhibited weak negative cooperativity with the Hill coefficients for the heart enzyme generally higher than for the kidney enzyme. Indeed, when assayed at pH 6.6, the kidney complex had a Hill coefficient of 0.65 whereas 0.85 was observed for the heart complex.

Effect of ADP/ATP Ratio on the Activity of the Bovine Kidney α-Ketoglutarate Dehydrogenase Complex. In mitochondria, the ATP and ADP concentrations do not change independently, and inorganic phosphate is an important parameter in the potential for oxidative phosphorylation. The effects of changes in the ADP/ATP ratio (at a constant adenine nucleotide pool of 1.6 mM) in the presence or absence of inorganic phosphate or Ca^{2+} are shown in Figure 6. For the various conditions, α-ketoglutarate concentrations were selected which gave, in the presence of ATP alone, about 20% of the maximum activity (measured in the presence of Ca^{2+} and 10 mM α-ketoglutarate).

For all four conditions studied, enzyme activities exceeded those of controls conducted in the absence of adenine nucleotides for the various α-ketoglutarate concentrations at ADP/ATP ratios less than 0.2 (data not shown) and reached or were approaching the maximum activity at a ratio of 1. With the assumption of competitive (rather than mutual) binding of ATP and ADP, the K_i, K_a, and α values predict a velocity approximately equal to that in the absence of ATP, and ADP will be achieved at an ADP/ATP ratio of 0.25 for studies in the presence of Ca^{2+}. This is close to the observed result.

In the presence or absence of phosphate, Ca^{2+} reduced the degree of response to increasing the ADP/ATP ratio, and, in

[3] Although this equation does not account for the observed negative cooperativity, only small errors are introduced in evaluating kinetic parameters by the following approach. The α-ketoglutarate concentration was varied over a relatively narrow range which bracketed the $S_{0.5}$ value in the presence and absence of allosteric effector (ATP or ADP). The resultant double-reciprocal plots were nearly linear. From double-reciprocal plots, α was determined from the ratio of the slope in the presence of a saturating level of allosteric effector to the slope in the absence of the effector. The change in slope of the double-reciprocal plot was a hyperbolic function of the effector concentration (L), and the inhibition or activation constant was determined from a replot of $1/\Delta$ slope vs. $1/L$.

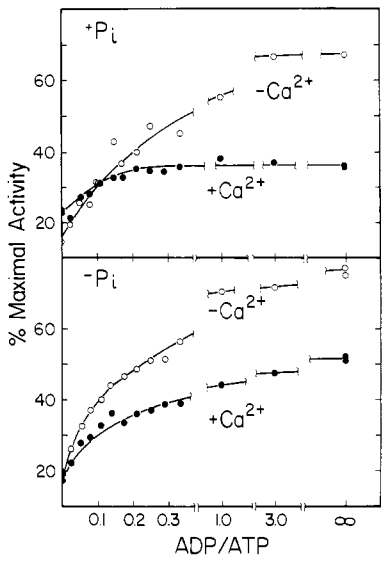

FIGURE 6: Effect of varying the adenylate ratio on α-ketoglutarate dehydrogenase activity, at suboptimal α-ketoglutarate concentration, in the presence and absence of both inorganic phosphate and Ca^{2+}. Assays were conducted in the presence (●) or absence (○) of Ca^{2+} at the indicated ATP/ADP ratio. The total concentration of ADP and ATP was 1.6 mM. In the presence of potassium phosphate (20 mM), the α-ketoglutarate concentrations were 0.050 and 1.0 mM for plus or minus Ca^{2+}, respectively. For assays in the absence of added phosphate, the α-ketoglutarate concentrations were 0.10 and 10.0 mM for plus or minus Ca^{2+}, respectively. Maximal activity was considered to be the rate from an assay in the presence of Ca^{2+} and 10 mM α-ketoglutarate, and this rate was 4.40 μmol min^{-1} mg^{-1}. Other conditions are described under Experimental Procedures.

the presence of the positive effectors, Ca^{2+} plus phosphate, the effect of increasing the ADP/ATP ratio on activity saturated at a ratio less than 0.3. The reduced degree of response is consistent with the reduced effects on the $S_{0.5}$ which would be lowered by Ca^{2+} and phosphate. Saturation of the response at lower ratios suggests that ADP may have enhanced effectiveness vs. ATP in the presence of Ca^{2+} and phosphate.

Discussion

Our studies on the bovine kidney α-ketoglutarate dehydrogenase complex establish that the $S_{0.5}$ for α-ketoglutarate is decreased markedly by micromolar Ca^{2+}, and this parameter is also decreased appreciably by ADP and phosphate. All of these effects appear to be independent. ADP lowers the $S_{0.5}$ for α-ketoglutarate in the presence or absence of Ca^{2+}, and the reciprocal also occurs. The effect of phosphate is much greater in the absence of Ca^{2+} or ADP but is not eliminated when either ADP or Ca^{2+} is present. However, phosphate has little, if any, effect on the $S_{0.5}$ for α-ketoglutarate when both Ca^{2+} and ADP are present.

ATP and, to a lesser degree, GTP inhibit the kidney α-ketoglutarate dehydrogenase complex, and the effect of ATP was shown to be due to an increase in $S_{0.5}$ for α-ketoglutarate. These effects are greatly reduced in the presence of Ca^{2+}. All of the above effects result in an exceedingly wide range of potential $S_{0.5}$ values for α-ketoglutarate. Indeed, the $S_{0.5}$ for α-ketoglutarate under the condition +ATP ($-Ca^{2+}$) was 775-fold greater than under the condition +ADP, +phosphate ($+Ca^{2+}$).

Even in the presence of Ca^{2+}, the $S_{0.5}$ for α-ketoglutarate was decreased 12.5-fold for a decrease in pH from 7.6 to 6.6. This response of the kidney α-ketoglutarate dehydrogenase complex may contribute to the activation of this complex in response to acute acidosis. Activation of the kidney complex in rat kidney mitochondria and perfused kidney under acidotic conditions was proposed to result from a decrease in intramitochondrial pH and to lead to an accelerated utilization of α-ketoglutarate under conditions of increased gluconeogenesis and ammoniagenesis from glutamine (Lowry & Ross, 1980).

Thus, the data indicate that several allosteric effectors and pH alter the affinity of the α-ketoglutarate dehydrogenase component for α-ketoglutarate. Indeed, it is difficult to envision an alternative mechanism in which changes in the rate constants for a catalytic step such as decarboxylation would be involved since such a mechanism must account for a lack of change in V_m.

The observation of Hill coefficients less than 1 suggests negative cooperativity involving site–site interactions that influence the binding of α-ketoglutarate. Hill plots of our data are linear over a wide range of α-ketoglutarate concentrations and have correlation coefficients very near unity. The observed Hill plots could not be generated by a low and a high K_m form of the α-ketoglutarate dehydrogenase component or by site–site interactions involving only two subunits of a dimeric α-ketoglutarate dehydrogenase component.[4] Although the possibility of multiple (>3) K_m forms of the α-ketoglutarate dehydrogenase component (i.e., microheterogeneity) cannot be eliminated, it seems most likely that site–site interactions involving several protein subunits produce the observed negative cooperativity.

As reported by Linn (1974), an additional band ($M_r \sim$ 88 000) was present in our preparations of the kidney α-ketoglutarate dehydrogenase complex but not in preparations of the heart complex. That this component is probably derived by proteolytic cleavage of the α-ketoglutarate dehydrogenase component is indicated by the observation that treatment of the complex with low levels of papain (which we have used to resolve the complex) converts all of the higher molecular weight band to a species that comigrates with the lower molecular weight material (Lawlis & Roche, 1981). Possibly the additional protein subunit in the kidney complex contributes to the lower Hill coefficients observed for the bovine kidney than for the bovine heart complex.

The effect of negative cooperativity is to reduce the tendency for the activity of the α-ketoglutarate dehydrogenase complex to respond to changes in α-ketoglutarate concentration under conditions of a fixed Ca^{2+} concentration, phosphate potential, and NADH/NAD^+ ratio. However, large changes in the $S_{0.5}$ for α-ketoglutarate occur with changes in these parameters, which is consistent with the activity of the complex responding to the energy state of the cell rather than the availability of α-ketoglutarate dehydrogenase complex in the citric acid cycle. Clearly, the $S_{0.5}$ for α-ketoglutarate is most sensitive to a transition from the presence to the absence of significant free Ca^{2+}, as this results in a 63-fold change in $S_{0.5}$. Furthermore, in the presence of ATP, Ca^{2+} greatly reduces the tendency of ATP to increase $S_{0.5}$ and causes a 98-fold decrease in $S_{0.5}$ for α-ketoglutarate. The effect of Ca^{2+} on the activity of the complex is even more pronounced in that Ca^{2+} also reduces NADH inhibition (Lawlis & Roche, 1980, 1981). Based on our results, it would seem that a large increase in ATP would not appreciably decrease the activity of the complex unless this was associated with a concomitant decrease in intramitochondrial Ca^{2+} to a submicromolar level. Since both ADP

[4] The α-ketoglutarate dehydrogenase component resolved from porcine heart was reported to have a sedimentation coefficient of about 10 S (Tanada et al., 1972). It seems most likely that this represents a dimer, as had been established for this component resolved from the structurally similar *E. coli* complex (Pettit et al., 1973). The dihydrolipoyl transsuccinylase and dihydrolipoyl dehydrogenase components have M_r's of about 45 000 and 55 000, respectively, on $NaDodSO_4$ gels.

and phosphate independently lower the $S_{0.5}$, this strongly suggests that enzyme activity responds to the phosphate potential rather than just to the ADP/ATP ratio. Consistent with this hypothesis, phosphate was observed (Figure 6) to alter significantly the profile of enzyme activity at increasing ADP/ATP ratios.

Thus, our results indicate that changes in $S_{0.5}$ for α-ketoglutarate in the kidney α-ketoglutarate dehydrogenase complex are induced by energy-linked compounds with a clear trend toward activity being enhanced by a decreasing $S_{0.5}$ for α-ketoglutarate at a lower energy state, as reflected by an increasing ADP/ATP ratio and increasing inorganic phosphate. Furthermore, there is a pronounced modulatory role of Ca^{2+} which may serve either to amplify or reverse, under certain metabolic conditions, the response to changes in the energy state of the mitochondria. Since similar effects were observed with the bovine kidney and heart complexes as well as the procine heart complex (McCormack & Denton, 1979), it seems likely that these effects may be widely, if not uniformly, distributed in mammalian tissues.

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