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EFFECT OF CALCIUM IONS ON PYRUVATE CARBOXYLASE FROM PIGEON LIVER

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Summary

Pigeon liver pyruvate carboxylase (pyruvate: CO_2 ligase (ADP forming), EC 6.4.1.1) shows allosteric properties similar to those of chicken or rat liver enzyme. Kinetic methods have been used to determine the effect of Ca^{2+} on this enzyme. The Ca^{2+} activation effect is absolutely dependent on the Mg^{2+} concentration; in the absence of Mg^{2+}, pyruvate carboxylase has no catalytic activity. Furthermore, Ca^{2+} cannot replace Mg^{2+} and also shows a paradoxical effect on the liver enzyme activity. It is an activator at low pyruvate or Mg^{2+} concentrations; at increased pyruvate concentrations, however, it becomes an inhibitor. At low levels of ATP a pronounced activation of pigeon liver pyruvate carboxylase by Ca^{2+} has been demonstrated. The results of this communication demonstrate pigeon liver pyruvate carboxylase to be different from pyruvate carboxylase from other sources.

Introduction

Several attempts have been made to elucidate the regulatory effect of nucleotides and some other compounds with activating or inhibitory effects on the pyruvate carboxylase (pyruvate: CO_2 ligase (ADP forming), EC 6.4.1.1) activity in preparations from various sources [1—8]. This enzyme catalyses the $MgATP^{2-}$-dependent carboxylation of pyruvate [5,8—13]. The Mg^{2+} requirement is known, but little light has been shed on the significance of Ca^{2+} in the following enzyme-catalyzed reaction:

$$\text{Pyruvate} + MgATP^{2-} + HCO_3^- \xrightarrow{\text{acetyl CoA, } Mg^{2+}} \text{Oxaloacetate} + MgADP + P_i$$

It is shown in this communication that Ca^{2+} induces both activation and inhibi-

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tion of pyruvate carboxylase from pigeon liver, depending on the experimental conditions. The fact that mitochondria are rich in Ca^{2+} suggests that this ion may have a regulatory function. Indeed, any enzyme regulated by an ion should exhibit altered activity with altered concentration of the ion in question. In this regard, pyruvate carboxylase is of special interest. Firstly, Mg^{2+} affects the pyruvate carboxylase activity in two ways, as a substrate complex in the form of $MgATP^{2+}$ and as an allosteric activator [9,13]. With the several reports of Ca^{2+}-induced inhibition of Mg^{2+}-activated enzymes in mind [3,6,7,14] and the mitochondrial capacity for Ca^{2+} [15] accumulation, we embarked on the study of the influence of Ca^{2+} on pyruvate carboxylase from pigeon liver. It became evident that like some others [16,17], this enzyme from pigeon liver is activated by Ca^{2+}.

Materials and Methods

Coenzymes and enzymes were purchased from Boehringer, Mannheim, G.F.R. All reagents were of the highest purity grade commercially obtainable. The activity of the purified enzyme [18] was measured by an optical assay, in which the pyruvate carboxylase-dependent formation of oxaloacetate is linked with the oxidation of $NADH_2$ by malate dehydrogenase [12,13,18]. The standard assay contained in 1.5 ml reaction mixture: 100 μmol Tris/HCl, pH 7.7; 20 μmol $KHCO_3^-$; 0.3 μmol $NADH_2$; 3 μmol sodium pyruvate; 10.5 units malate dehydrogenase; 0.5 mg serum albumin; 0.15 μmol acetyl-CoA and 2 μmol ATP. The concentrations of Ca^{2+}, Mg^{2+} and/or pyruvate varied as indicated in the figures. All assays were carried out along with controls lacking ATP and acetyl-CoA.

Results

The effect of Ca^{2+} on the activity of pyruvate carboxylase from pigeon liver

(a) In relation to pyruvate concentration. Ca^{2+} concentration shows a paradoxical effect on the Mg^{2+}-dependent activity of pigeon liver pyruvate carboxylase in that Ca^{2+} activates at low pyruvate concentrations and inhibits at higher ones (Fig. 1), compared to the controls without Ca^{2+}.

Activation was observed up to 1 mM pyruvate, the maximum activation in this region being dependent on both Ca^{2+} and pyruvate concentrations. Further addition of pyruvate beyond the stimulating range causes relative inhibition, i.e. a leveling of the Ca^{2+}-dependent activation of pyruvate carboxylase activity. However, the stimulatory effect of Ca^{2+} is observed only in the presence of Mg^{2+}. The enzyme is, in fact, catalytically inactive, regardless of Ca^{2+} concentration, when Mg^{2+} is absent [13], suggesting that Ca^{2+} probably does not exert its influence at the Mg^{2+}-active sites, and that Ca^{2+} is not a replacement for Mg^{2+}.

(b) In relation to Mg^{2+} concentration. Figs 2 and 3 show a distinct activation of pyruvate carboxylase by the ion, but the direction of the allosteric effect does not depend on the Ca^{2+} concentration (Fig. 2). The activation course followed by the enzyme with respect to Ca^{2+} is both Mg^{2+}- and pyruvate-concentration dependent (Fig. 3a,b,c). As Fig. 3b demonstrates, under our

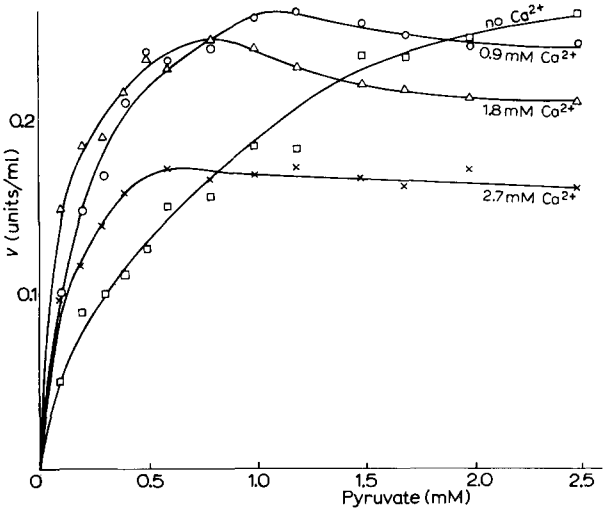

Fig. 1. Effect of Ca^{2+} on pyruvate carboxylase from pigeon liver at different pyruvate concentrations. Assays were carried out spectrophotometrically (see Materials and Methods). □———□, without $CaCl_2$; ○———○, 0.9 mM $CaCl_2$; △———△, 1.8 mM $CaCl_2$; X———X, 2.7 mM $CaCl_2$ (free ionic concentrations). The free Mg^{2+} concentration was kept constant at 6.2 mM.

assay conditions (see Material and Methods) optimal activation occurred at 3.6 mM Mg^{2+} (free ionic) concentration. With pyruvate concentration between 0.5 and 1.0 mM, enzyme activity is enhanced 280% when the assay mixture contained about 0.5 mM Ca^{2+}, over the value obtained in the absence of Ca^{2+} (Fig. 3b). In contrast, Mg^{2+} at the level of 8.6 mM exhibited the opposite effect — an inhibition.

(c) In relation to ATP. In connection with the evidence for the ATP requirement of pyruvate carboxylase by Utter et al. [19], other authors were able to demonstrate [3,6,8,9,20—24], that the substrate for this enzyme is $MgATP^{2-}$, under their particular experimental conditions. But this is incompatible with the finding that pyruvate carboxylases from pigeon liver and kidney do not show any activity with $MgATP^{2-}$ alone [13]. Furthermore, it is difficult to explain the Ca^{2+}-dependent activation in the presence of ATP by the assumption of a $CaATP^{2-}$ complex, analogously formed. The stability constants for $MgATP^{2-}$ and $CaATP^{2-}$ are not identical and since ATP is obviously complexed with Mg^{2+} a priori, the $MgATP^{2-}$ concentration can only be insignificantly changed by small Ca^{2+} additions. To support the suggestion that Ca^{2+} activates pyruvate carboxylase directly and via the substrate, activity determinations were performed at low ATP concentration. As Fig. 4 shows, 0.8 mM Ca^{2+} induces at 0.25 mM ATP and increasing Mg^{2+} concentrations a 6.25-fold activation of pyruvate carboxylase from pigeon liver, when all other reaction parameters were kept constant. This again demonstrated (Fig. 3) that the activating Ca^{2+} influence even at low ATP concentrations is affected by Mg^{2+}. However, the allosteric effect of Mg^{2+} is not altered by Ca^{2+}. Our results show, in addition, that Ca^{2+} together with $MgATP^{2-}$ alone does not produce sigmoidal kinetics of pyruvate carboxylase, Mg^{2+} also being required to observe these kinetics (Fig. 3c).

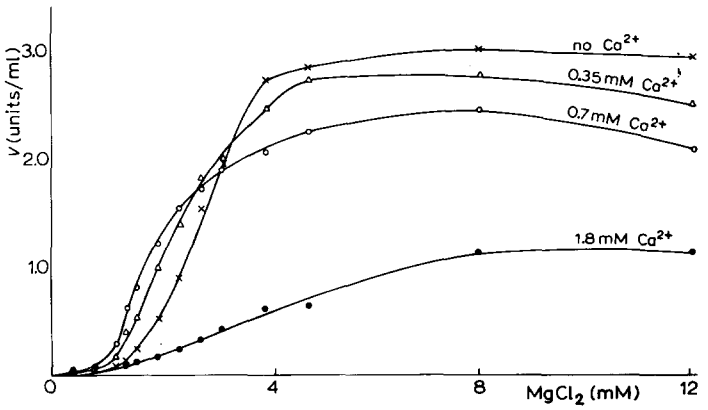

Fig. 2. Effect of Ca^{2+} on pyruvate carboxylase from pigeon liver at different $MgCl_2$ concentrations. For details of spectrophotometric assay see Materials and Methods. X———X, $CaCl_2$ omitted; △———△, 0.35 mM $CaCl_2$; ○———○, 0.7 mM $CaCl_2$; ●———●, 1.8 mM $CaCl_2$ (free ionic concentration).

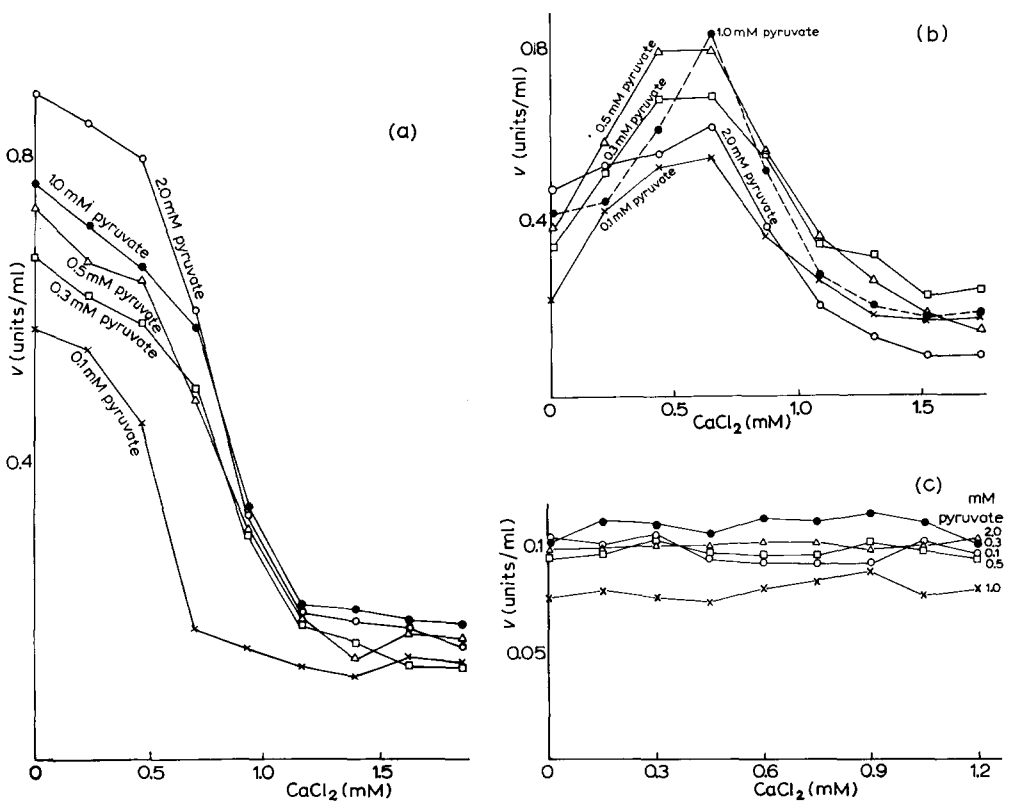

Fig. 3. Effect of Ca^{2+} on pigeon liver pyruvate carboxylase showing an apparent enzyme activation with various $MgCl_2$ concentrations. Assays were carried out spectrophotometrically (see Materials and Methods). The enzyme activity was measured at: 8.6 mM $MgCl_2$ (a); 3.6 mM $MgCl_2$ (b); 0.36 mM $MgCl_2$ (c) (free ionic concentrations).

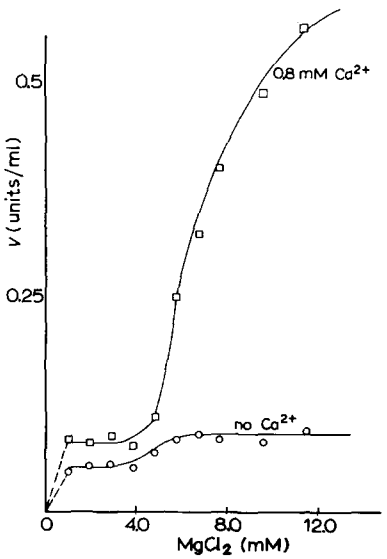

Fig. 4. Activation of pyruvate carboxylase from pigeon liver by $CaCl_2$. The enzymic activity plotted as a function of $MgCl_2$ concentrations at 0.25 mM ATP (suboptimal concentration). o———o, without $CaCl_2$; □———□, 0.8 mM $CaCl_2$ (free ionic concentrations).

Discussion

According to the hypothesis of Kimmich et al. [26], pyruvate carboxylase activity in intact rat liver mitochondria might be regulated by Ca^{2+}. Carafoli et al. [27] reported Ca^{2+} accumulation in mitochondria of different types of tissue during electron transport. The evidence presented by these investigators as well as the suggestion that Ca^{2+} could act as a competitive inhibitor analogously to other bivalent metal ions such as Zn^{2+}, Cu^{2+} Ni^{2+} etc. with regard to free Mg^{2+}, seemed to warrant further investigation of the regulation of pyruvate carboxylase.

Contrary to previous reports, our findings demonstrate an enhancement of pigeon liver pyruvate carboxylase activity in the presence of Ca^{2+} under conditions of low Mg^{2+} concentrations (Figs 2 and 3). This activation is manifest at low Ca^{2+} concentrations. The curve shown in Fig. 4 excludes the other possible explanation, that high Ca^{2+} values depress the $MgATP^{2-}$ concentration yielding an apparent inhibition due to substrate depletion. At a low level, 0.8 mM Ca^{2+} dramatically increases the pyruvate carboxylase activity, suggesting an activation by Ca^{2+} alone and not by the $CaATP^{2-}$ complex. Moreover, a possible competition between Mg^{2+} and Ca^{2+} for ATP^{4-} can reasonably be excluded, considering the stability constants [28] and results presented here. Fig. 4 also demonstrates the absolute necessity of free Mg^{2+} in this system, i.e. Ca^{2+} requires the presence of free Mg^{2+} to exert its effect and cannot simply replace it. This is also supported by Fig. 3C where at 0.36 mM Mg^{2+} (free ionic concentration) activation by Ca^{2+} is not even perceptible but is quite dramatic upon simply raising the Mg^{2+} concentration to 3.6 mM Mg^{2+} (Fig. 3B). There is no Ca^{2+} activation at very high (8.6 mM) Mg^{2+} concentrations (Fig. 3a). Possibly

the inhibitory effect of high Mg^{2+} concentrations supresses the activating effect of Ca^{2+}.

Our results, exhibiting that Ca^{2+} activates pigeon liver pyruvate carboxylase do not agree with the findings of Ca^{2+} sensitivity of this enzyme isolated from other species. Additional properties differentiating this enzyme from other sources have already been reported elsewhere [18,25]. Another observation of great interest is that provided that the Ca^{2+} level is adequate, low pyruvate concentrations show a higher rate of activation of the enzyme than is the case under saturating pyruvate conditions. This would suggest that Ca^{2+} is either capable of making the pyruvate carboxylase independent of the substrate concentration, or alternatively it enhances the enzyme affinity towards pyruvate, and after exceeding the optimum range for pyruvate and/or Ca^{2+}, becomes inhibitory. At concentrations of 1.0 mM or less pyruvate and above 1.5 mM Ca^{2+} the enzyme activity is not dependent on the pyruvate level, but is rather a function of the Ca^{2+} concentration (Fig. 3). An increase of Ca^{2+} from 0.5 mM to 1.5 mM (or even up to 12 mM, not shown here) induces an activity decrease of pyruvate carboxylase to a value which is identical to all pyruvate concentrations tested. The activation of the enzyme by Ca^{2+}, which is maintained over a rather wide range of pyruvate concentration, might be of greater importance, because this mechanism allows the maintenance of constant enzyme activity during pyruvate oscillation under physiological conditions. Additional indications for a regulation of gluconeogenesis at the level of pyruvate carboxylase, are given by the reports of the high mitochondrial affinity for Ca^{2+} [26]. Such an accumulation capacity could regulate the Ca^{2+} concentration in the mitochondrion and therefore possibly the pyruvate carboxylase activity. Further aspects concerning the role of Ca^{2+} in regulation processes were also discussed by other authors [29,30]. However, our results strongly support the conclusion that Ca^{2+} is not a competitive inhibitor for pyruvate carboxylase from pigeon liver, as was shown to be the case for Mg^{2+}-activated enzymes from other species [3,6,7,14], and that this pyruvate carboxylase is one of the most complex allosteric enzymes yet studied.

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