Regulation of Citrate Transport and Pyruvate Dehydrogenase in Rat Kidney Cortex Mitochondria by Bicarbonate*

(Received for publication, November 8, 1976, and in revised form, March 21, 1977)

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1. Bicarbonate increased citrate and 2-oxoglutarate accumulation when rat kidney cortex mitochondria were incubated with pyruvate or L(-)-malpimoyl carnitine in the presence of t-malate.

2. Bicarbonate stimulated the exit of citrate from mitochondria. The $K_m$ for bicarbonate was 13.5 mM and the $V_{max}$ was 0.59 nmol/min/mg of protein at 10°C.

3. The bicarbonate-stimulated exit of citrate from the mitochondria was prevented by inhibitors of the tricarboxylate, dicarboxylate, and phosphate transport systems.

4. The activity of pyruvate dehydrogenase was significantly increased by preincubation of rat kidney mitochondria with bicarbonate. This bicarbonate-induced activation was not observed in the presence of inhibitors of citrate transport. Bicarbonate did not activate pyruvate dehydrogenase in rat heart mitochondria. Bicarbonate had no effect on pyruvate dehydrogenase activity in either broken mitochondria or whole tissue preparations.

5. The mechanism of activation is discussed in the light of the known regulatory properties of pyruvate dehydrogenase, pyruvate carboxylase, and citrate synthase.

After a sodium bicarbonate load, the concentrations of bicarbonate and citrate are elevated in plasma, urine, and the renal cortex, whereas in metabolic acidosis the converse occurs (1-5). In metabolic acidosis a much greater proportion of energy is derived from the oxidation of lactate which is extracted from mitochondria which indicate a regulatory link between bicarbonate and citrate synthase.

MATERIALS AND METHODS

Rat kidney cortex mitochondria were prepared by conventional centrifugation techniques using a medium containing 250 mM sucrose, 6 mM Tris chloride, 0.1 mM EGTA, 0.6% defatted bovine serum albumin, pH 7.4. The assay of various metabolites were carried out by the following methods: pyruvate, Bucher et al. (10); citrate, Moelling and Gruber (11); 2-oxoglutarate, Bergmeyer and Bernt (12).

*Citrate Loading – Kidney cortex mitochondria (100 mg of protein) were incubated in 10 ml of a medium containing 125 mM KCl, 20 mM Tris/chloride, pH 7.4, at 10°C for 10 min in the presence of 2 mM rotenone, 10 mM ATP, 1 mM NaHCO₃, 5 mM 2-oxoglutarate, and 20 μCi of NaH¹⁴CO₃. After this time the volume was made up to 100 ml with ice-cold buffer containing 550 mM sucrose and 5 mM Tris/chloride, pH 7.4, and the mitochondria were separated by centrifugation at 18,000 x g for 10 min. The adhering supernatant was decanted off as much as possible and the mitochondria were suspended in 2.5 ml of the sucrose/Tris buffer for use in isotopic exchange experiments. The incorporation of ¹⁴C label exclusively into citrate and isocitrate in position 6 was shown by the fact that incubation of a mitochondrial perchloric acid extract (neutralized with K₂CO₃) to remove remaining [¹⁴C]bicarbonate with aconitase hydratase and NADP-isocitrate dehydrogenase (13) yielded 98% of the counts as CO₂ by enzymic decarboxylation. The mechanism of labeling citrate by this method is assumed to be similar to that proposed for this procedure in rat liver mitochondria (14), i.e. reversal of intramitochondial isocitrate dehydrogenase (NADP).

The kinetics data of citrate exchange were determined with the inhibitor stop method (16), using 90 mM benzene 1,2,3-tricarboxylate as the exchange arresting inhibitor at times from 0 to 3 min at 20°C. The bicarbonate [¹⁴C]citrate exchange kinetic data were determined using mitochondria loaded with 11,5-¹⁴C]citrate (16).

Determination of Citrate and 2-Oxoglutarate Production Rates – Rat kidney mitochondria (3 mg of protein) were added to 5 ml of a medium containing 125 mM KCl, 20 mM Tris/Cl₄, 4 mM inorganic phosphate, pH 7.4 at 30°C. Oxygen consumption was measured polarographically with a Clark oxygen electrode for a period of 2 min. The reaction was stopped with 0.2 ml of 2 N H₂SO₄ and ¹⁴C activity measured in the supernatant.

The kinetics data of the rate of pyruvate decarboxylation rat kidney mitochondria were incubated under the same conditions described above, except that 0.1 μCi/ml of [¹⁴C]pyruvate was included in the buffer. Incubations were carried out in stopped vessels fitted with small CO₂-collecting units containing 0.2 ml of hyamine. After 2 min the reaction was stopped by the addition of 0.5 ml of 2 N H₂SO₄ and ¹⁴C was collected for another 30 min. The hyamine was transferred to 10 ml of toluene/ethanol (9:1, v/v) scintillation fluid and the ¹⁴C activity measured in a Beckman scintillation spectrometer.

Pyruvate Dehydrogenase Activity – Rat kidney mitochondria were incubated for 2-min periods as described under “Determination of Citrate and 2-Oxoglutarate Production Rates.” At the end of this period a 3-ml sample was withdrawn by syringe and immediately syringed into a vessel immersed in liquid nitrogen. The frozen incubation was gradually thawed in an ice bath at 0°C and pyruvate activ
Kinetic parameters for rat kidney mitochondrial citrate-transporting dehydrogenase activity determined by the method of Taylor et al. (17) in duplicate in 0.5-ml aliquots of the incubation.

RESULTS

Citrate Transport in Rat Kidney Cortex Mitochondria—The rate of [14C]citrate exit from kidney mitochondria was measured using the inhibitor stop method with either malate, citrate, phosphoenolpyruvate, or bicarbonate as the countertransporting anion. Initial rates of exchange were measured and the kinetic parameters determined using double reciprocal plots of velocity and substrate concentration (Table I). The \( K_{m} \) values obtained for rat kidney mitochondria are similar to those obtained with rat liver mitochondria (18) and warrant only the comment that the \( K_{m} \) for l-malate appears to be significantly higher than for citrate or phosphoenolpyruvate. However, rat kidney mitochondria had much slower rates of transport at 10° than at 20°.

In order to measure the exchange of bicarbonate with [14C]citrate, we found it was not feasible to use the H^14CO_{3}^{-}loading technique to generate intramitochondrial citrate. Kidney cortex mitochondria were instead loaded with [1,5-\(^{14}\)C]citrate as described under "Materials and Methods" and rate of exchange with externally applied bicarbonate measured by the inhibitor stop method. A \( K_{m} \) of 13.5 mM and a \( V_{\text{max}} \) of 0.59 nmol/min/mg of protein were found at 10° (Fig. 1).

The presence of both benzene 1,2,3-tricarboxylate and 2-pentylmalonate prevented the observed stimulation of citrate exit (Table IV), the presence of the latter causing an apparent influx of citrate into the mitochondria as indicated by the negative value obtained for percentage exchange in the given time period. N-Ethylmaleimide also inhibited the bicarbonate/citrate exchange while having no inhibitory effect on the citrate/[14C]citrate exchange. None of the agents had a significant effect on the base-line state observed in the absence of added bicarbonate.

Effect of Bicarbonate on Rate of Citrate and 2-Oxoglutarate Accumulation—When rat kidney mitochondria were incubated with 0.5 mM pyruvate and 0.5 mM l-malate in the presence of ADP and phosphate to stimulate respiratory chain phosphorylation, citrate accumulated at a rate of 7.0 ± 0.6 nmol/min/mg of protein. This rate increased about 2-fold in the presence of bicarbonate (Table II). The production of 2-oxoglutarate also increased to a similar extent with bicarbonate. A similar but smaller stimulation of citrate and 2-oxoglutarate production was seen when l-palmitoyl carnitine plus l-malate were the substrates. In parallel experiments, the rates of pyruvate decarboxylation were measured and found to be rather high. These were thought to be overestimates of the rate of pyruvate decarboxylation because \(^{14}\)CO_{2} production rates were not abolished by 2 mM sodium arsenite which should completely inhibit pyruvate dehydrogenase (Table III).

Nevertheless, bicarbonate stimulated the rate of \(^{14}\)CO_{2} production from \(^{11}\)Clpyruvate by 39% and this stimulation was prevented by benzene 1,2,3-tricarboxylate. Benzene 1,2,3-tricarboxylate did not significantly inhibit the rate of \(^{14}\)CO_{2} production in the absence of bicarbonate.

Effect of Bicarbonate on Pyruvate Dehydrogenase Activity—The possibility that increased production of citrate was caused by the bicarbonate on pyruvate dehydrogenase activity, 2-oxoglutarate, and citrate production and oxygen consumption in rat kidney cortex mitochondria

Experiments were carried out as described under "Materials and Methods" at 30° and at pH 7.5 to minimize the effect of bicarbonate on pH at high concentration.

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\begin{array}{cccc}
\text{Substrate} & \text{Pyruvate dehydrogenase} & \text{Citrate production} & \text{2-Oxoglutarate production} & \text{Consumed oxygen} \\
& \text{nmol/min/mg} & \text{nmol/min/mg} & \text{nmol/min/mg} & \text{nmol/min/mg} \\
\hline
\text{P, pyruvate malate ADP (13)} & 23.1 ± 2.9 & 7.0 ± 0.6 & 7.1 ± 0.5 & 168 ± 19 \\
+10 mM \text{ HCO}_{3}^{-} (7) & 41.7 ± 3.3 & 11.4 ± 1.1 & 11.7 ± 1.4 & 195 ± 12 \\
+20 mM \text{ HCO}_{3}^{-} (7) & 48.2 ± 3.9 & 15.1 ± 0.8 & 12.9 ± 1.1 & 171 ± 15 \\
+50 mM \text{ HCO}_{3}^{-} (7) & 57.7 ± 3.8 & 17.5 ± 2.6 & 15.6 ± 0.5 & 145 ± 17 \\
\text{P, palmitoyl carnitine malate ADP (5)} & 10.7 ± 1.4 & 4.88 ± 0.3 & 5.3 ± 0.5 & 138 ± 14 \\
+10 mM \text{ HCO}_{3}^{-} (5) & 5.7 ± 1.1 & 7.1 ± 1.9 & 144 ± 14 \\
+20 mM \text{ HCO}_{3}^{-} (5) & 7.73 ± 1.5 & 9.0 ± 1.1 & 140 ± 12 \\
+50 mM \text{ HCO}_{3}^{-} (6) & 10.7 ± 2.8 & 13.6 ± 1.7 & 99 ± 12 \\
\end{array}
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\( ^{a} p < 0.01 \) as compared to no bicarbonate.
not only by a stimulation of citrate exit from the mitochondria by bicarbonate but also by an increased pyruvate dehydrogenase activity was investigated by monitoring pyruvate dehydrogenase activity under various conditions of incubation. The activity of pyruvate dehydrogenase increased with increasing bicarbonate concentration (Table II). This effect could not be demonstrated with rat heart mitochondria incubated with pyruvate as substrate.

When isolated freeze-thawed rat kidney mitochondria were preincubated with bicarbonate, no effect of bicarbonate was observed on pyruvate dehydrogenase activity, although ATP was found to inactivate and Ca^2+ or Mg^2+ found to activate the enzyme as was found previously (Table V) (19). Similarly, no effect of bicarbonate was found on rat kidney cortex pyruvate dehydrogenase activity in fresh whole tissue homogenized in 10 volumes of 10 mM phosphate, 1 mM dithiothreitol, pH 7.0.

When the exit of citrate from mitochondria metabolizing pyruvate was inhibited by benzene 1,2,3-tricarboxylate (Table VI), the effect of bicarbonate in stimulating pyruvate dehydrogenase was prevented both in State 3 and State 4 oxidation states as defined by Chance and Williams (20). As expected, the presence of pyruvate itself caused a stimulation of pyruvate dehydrogenase activity. Benzene 1,2,3-tricarboxylate, added in the absence of bicarbonate ions, produced a slight but not significant inhibition of pyruvate dehydrogenase activity.

When the concentration of L-malate was increased 10-fold, the rate of citrate accumulation was found to increase with a small concomitant increase in pyruvate dehydrogenase activity. Bicarbonate increased both the pyruvate dehydrogenase activity and the rate of citrate accumulation. The addition of the uncoupling agent CCCP (1 μM) to rat kidney mitochondria caused a small increase in pyruvate dehydrogenase activity without increasing citrate accumulation rates. Bicarbonate again doubled both citrate accumulation rate and pyruvate dehydrogenase activity. When L-palmitoyl carnitine was used as the substrate instead of pyruvate, the observed activities of pyruvate dehydrogenase were low in both State 3 and State 4. The presence of bicarbonate induced an increase of pyruvate dehydrogenase both in State 3 and State 4 while the rate of citrate accumulation increased in State 3 by 65%. This compares with a bicarbonate stimulated increase of 116% with pyruvate as the substrate.

**DISCUSSION**

The control of intramitochondrial citrate synthesis has been the subject of much investigation in liver (21, 22) but not in kidney. The relative activities of the mitochondrial aconitate hydratases are such that whereas in rat liver mitochondria most of the citrate that is produced has to exit from the mitochondria, in rat kidney mitochondria only a small proportion of that which is produced leaves the mitochondria, most of it being oxidized through the Krebs cycle (23).

The observation that the rate of citrate accumulation in suspensions of rat kidney mitochondria oxidizing pyruvate in the presence of L-malate is markedly increased in the presence of bicarbonate is open to a number of interpretations. Firstly, it could be due to feedback inhibition of one or both isocitrate dehydrogenases or 2-oxoglutarate dehydrogenase by CO_2 so that flow through this step is restricted and citrate accumulates. Secondly, it could be due to increased transport of citrate out of the mitochondrial compartment under the influence of bicarbonate, and thirdly, it could be due to increased citrate synthesis brought about by bicarbonate through some mechanism unknown, such that citrate, surplus to the flow through isocitrate dehydrogenase, accumulates. Each of these possibilities will be discussed in turn.

**Feedback Inhibition of Isocitrate Dehydrogenase—Al-**
though NADP-isocitrate dehydrogenase is a reversible enzyme under the right conditions and is inhibited by its end product CO2 (24), the likelihood of the bicarbonate-stimulated citrate accumulation occurring because of this is remote. The concomitant increase in the accumulation of 2-oxoglutarate (Table IV) would not have occurred if the flow through isocitrate dehydrogenase had been curtailed. It is feasible that bicarbonate could cause product inhibition of 2-oxoglutarate oxidation though this step is usually considered to be non-equilibrium and irreversible.

Stimulation of Citrate Transport—Although the probable stimulation of citrate transport by bicarbonate has been demonstrated by Simpson (8), the data reported herein represent the first kinetic analysis. We have demonstrated (a) that the observed rates of citrate transport in response to bicarbonate are of the same order of magnitude as with other stimulators such as L-malate (Table I); and (b) that the $K_m$ (13.5 mM) obtained for bicarbonate closely approximates its intracellular concentration. Thus, administration of bicarbonate to rat kidney mitochondria may increase accumulation of citrate in the suspending medium by stimulating export from the intramitochondrial compartment. The fact that 2-oxoglutarate also accumulates, means either that bicarbonate also stimulates 2-oxoglutarate transport or that the rate of pyruvate or palmitoyl carnitine oxidation has been increased, giving rise to increased 2-oxoglutarate synthesis.

The mechanism whereby bicarbonate stimulated the efflux of citrate from the intramitochondrial compartment can be evaluated from the observations reported in Table IV. The inhibition of bicarbonate-induced citrate efflux by benzene 1,2,3-tricarboxylate, 2-pentylmalonate, and N-ethylmaleimide suggests involvement of the tricarboxylate, dicarboxylate, and phosphate-hydroxyl transporters as these compounds are reported to be specific transport inhibitors at the concentrations used (14, 23, 25). Bicarbonate also causes an efflux of L-malate from mitochondria loaded with that substrate. We propose that bicarbonate first stimulates phosphate egress from the mitochondria via a bicarbonate/phosphate exchange on the phosphate carrier. Phosphate exchanges for malate on the dicarboxylate carrier and the malate then exchanges for citrate on the tricarboxylate carrier. Thus a net exchange of bicarbonate for citrate is accomplished. The stoichiometry of this exchange cannot at this time be documented because of the mobility of CO2 in rapid equilibrium with bicarbonate. However, there is another implication which can be derived from this data. It is that in such an exchange as the bicarbonate/citrate exchange, described here, three separate carriers appear to work in an integrated manner to carry out the net exchange with a velocity approaching that of direct exchanges like the malate/citrate or citrate/citrate exchanges.

Stimulation of Pyruvate Oxidation by Bicarbonate—When the production of $^{14}$CO2 from [1-14C]pyruvate was monitored under the same conditions used to monitor citrate accumulation, it was found that the rate of pyruvate decarboxylation was increased by the presence of bicarbonate. That bicarbonate was able at the same time to promote activation of pyruvate dehydrogenase is most interesting since in vivo bicarbonate loading causes an increase in kidney utilization of pyruvate and lactate (6, 7). The fact that the activating effect of bicarbonate was not seen either in broken cell preparations or in freeze-thawed mitochondria (Table III), indicated that the activation was not a direct effect of bicarbonate but rather a secondary effect. This was further demonstrated by the fact that benzene 1,2,3-tricarboxylate, an inhibitor of citrate transport (14, 15) was able to prevent the activation of pyruvate dehydrogenase by bicarbonate, and also the stimulation of pyruvate decarboxylation in respiring mitochondria. Similarly, the effect of bicarbonate on pyruvate dehydrogenase activity could not be observed in rat heart mitochondria. It has been previously shown that the citrate transporter has very low activity in rat heart mitochondria (26). These data strongly suggest that removal of citrate from the intramitochondrial compartment might allow activation of pyruvate dehydrogenase either by (a) increasing the concentration of the free valent metal cations, Ca2+ and Mg2+; (b) by removing possible citrate inhibition of pyruvate dehydrogenase as shown in muscle and adipose tissue (9, 27); or (c) the effect of bicarbonate could be to supply pyruvate carboxylase with sufficient substrate to become active thereby increasing the synthesis of oxaloacetate. The increased oxaloacetate by driving citrate synthesis would lower the acetyl-CoA/CoA ratio thereby reducing the activity of pyruvate dehydrogenase kinase (28) and increasing the percentage of the total pyruvate dehydrogenase in the active form. This explanation cannot account for all the data for three reasons. Firstly, increasing the L-malate concentration 10-fold, which should increase ox-
alacetate accordingly, did not activate pyruvate dehydrogenase although it did increase citrate accumulation. Secondly, in the presence of uncoupling agent which would be expected to increase oxalacetate concentration, there again was little increase in pyruvate dehydrogenase activity. Adding bicarbonate in either of these situations still gave a healthy increase in both citrate accumulation rate and pyruvate dehydrogenase activity. Thirdly, bicarbonate causes an activation of pyruvate dehydrogenase when L-palmitoyl carnitine is substrate. Unless there is some form of compartmentation of oxalacetate increase oxalacetate concentration, there again was little increase in pyruvate dehydrogenase activity. Adding bicarbonate the presence of uncoupling agent which would be expected to both citrate accumulation rate and pyruvate dehydrogenase activity.

mitochondria while bringing about an activation of pyruvate dehydrogenase, these results do not support the sole mechanism as one involving increased activity of pyruvate carboxylase.

It is possible that bicarbonate stimulated citrate egress from the mitochondria and relieved feedback inhibition by citrate at citrate synthase (29) thereby promoting increased citrate synthesis. The lower acetyl-CoA/CoA ratio resulting from increased flow through both citrate synthase and pyruvate dehydrogenase by intramitochondrial citrate transport, by lowering intramitochondrial citrate concentration, relieves constraint placed on the activity of both citrate synthase and pyruvate dehydrogenase by intramitochondrial citrate. This provides the basis for an important mechanism governing the regulation of substrate utilization by the kidney such that elevation of intravascular bicarbonate in metabolic alkalosis has been shown to increase the uptake of pyruvate and lactate (6, 7) as energy substrates. This switches renal metabolism away from ammonia production using glutamine as the precursor, a process which is not required for acid-base balance under these conditions. At the same time, the amount of citrate produced by the kidney becomes elevated, a situation which may be designed to minimize the formation of calciferous renal stones under conditions of alkaline urine excretion.

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