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EFFECT OF BICARBONATE AND OXALOACETATE ON MALATE OXIDATION BY SPINACH LEAF MITOCHONDRIA

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Summary

Mitochondria isolated from spinach leaves oxidized malate by both a NAD^+-linked malic enzyme and malate dehydrogenase. In the presence of sodium arsenite the accumulation of oxaloacetate and pyruvate during malate oxidation was strongly dependent on the malate concentration, the pH in the reaction medium and the metabolic state condition.

Bicarbonate, especially at alkaline pH, inhibited the decarboxylation of malate by the NAD^+-linked malic enzyme in vitro and in vivo. Analysis of the reaction products showed that with 15 mM bicarbonate, spinach leaf mitochondria excreted almost exclusively oxaloacetate.

The inhibition by oxaloacetate of malate oxidation by spinach leaf mitochondria was strongly dependent on malate concentration, the pH in the reaction medium and on the metabolic state condition.

The data were interpreted as indicating that: (a) the concentration of oxaloacetate on both sides of the inner mitochondrial membrane governed the efflux and influx of oxaloacetate; (b) the NAD^+/NADH ratio played an important role in regulating malate oxidation in plant mitochondria; (c) both enzymes (malate dehydrogenase and NAD^+-linked malic enzyme) were competing at the level of the pyridine nucleotide pool, and (d) the NAD^+-linked malic enzyme provided NADH for the reversal of the reaction catalyzed by the malate dehydrogenase.

Abbreviations: FCCP, *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

It is now well established since the pioneering work of Wiskich and Bonner [1] that plant mitochondria isolated from etiolated tissues [2] or leaves [3] oxidize malate more or less rapidly in the absence of either glutamate or a source of acetyl-CoA. This oxidation is attributed to malate dehydrogenase (L-malate : NAD^+oxidoreductase EC 1.1.1.37) and/or NAD^+-linked malic enzyme (L-malate : NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.39) discovered in plant mitochondria by Macrae and Moorhouse [4]. The oxidation of malate which is coupled to three sites of ATP formation is stimulated under certain conditions by NAD^+ and is inhibited by rotenone, but this inhibition is partially relieved by the addition of 1 mM NAD^+. Two interpretations of the phenomena have been offered. Coleman and Palmer [5], Brunton and Palmer [6] and Palmer and Arron [7] have suggested that NAD^+-linked malic enzyme, located presumably in the intermembrane space reduces externally added NAD^+ which is reoxidized by a specific NADH dehydrogenase located near the external face of the inner membrane [8,9]. This NADH dehydrogenase is linked to the respiratory chain (via the ubiquinone pool) and is specific for the 4 β-hydrogen atom of NADH [8]. In contrast, Day and Wiskich [10—12] indicated that malate had to penetrate through the inner membrane in order to be oxidized. Consequently they proposed that a transmembrane transhydrogenase was responsible for the reduction of external NAD^+.

In order to clarify the mechanisms of malate oxidation in intact plant mitochondria this report details the effects of bicarbonate * and oxaloacetate on malate oxidation in spinach leaf mitochondria.

Materials and Methods

Preparation of mitochondria. Spinach (*Spinacia oleracea* L.) leaves (about 8 weeks old) were freshly harvested from a local spinach field and used immediately. Leaves (0.8 kg after deribbing) were cut into 3 l of chilled medium containing 0.3 M mannitol, 2 mM β-mercaptoethanol, 1 mM EDTA, 30 mM morpholinopropanesulfonic acid buffer (pH 7.5), 0.2% defatted bovine serum albumin and 0.6% insoluble polyvinylpyrrolidone. The leaves were disrupted at low speed for 2 s in a 1 gallon Waring blendor. The homogenate was squeezed through six layers of cheesecloth and one layer of 50 μm nylon net. Mitochondria were isolated as fast as possible by differential centrifugation according to the method of Bonner [13]. We found that very short grinding times in a Waring blendor were the most successful. Longer grinding times had a deleterious effect on the mitochondrial oxidative and phosphorylative capacities [3]. Likewise the use of stored leaves is not advised. Mitochondria were only used if they were tightly coupled, with respiratory control ratios and ADP:O values close to those normally observed with intact purified mitochondria [14].

Mitochondria from potato (*Solanum tuberosum* L.) tubers and etiolated

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* The equilibrium mixture of CO_2, (H_2CO_3) and HCO_3^- will be referred to as bicarbonate.

mung bean (*Vigna radiata* L. Wilczek, formerly called *Phaseolus aureus* Roxb.) hypocotyls cut from bean seedlings grown for 5 days in the dark at 26° C and 60% relative humidity were prepared and purified by methods that have been previously described [14]. All operations were carried out at 0—4° C.

O_2 uptake measurements. O_2 uptake was measured at 25° C in a 1 ml stirred cell using a Clark-type O_2 electrode (Hansatech D.W. oxygen electrode unit). The reaction medium (medium A) contained: 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer, 0.1% defatted bovine serum albumin and known amount of mitochondrial protein. Unless otherwise stated all incubations were carried out at pH 7.2.

Assay of metabolic products. Products of malate metabolism by intact mitochondria were routinely assayed in a system containing medium A and known amounts of mitochondrial protein. The reaction was initiated by the addition of malate. Incubation temperature was 25° C. At various times 1-ml aliquots were taken and added to 0.3 ml of cold 20% $HClO_4$ containing 0.1 mM EDTA. After addition of 20 µl of an alcoholic solution of methyl orange (0.06%, w/v) the samples were quickly neutralized with KOH and centrifuged for 5 min at 5000 × *g* to remove $KClO_4$. The supernatant was used for pyruvate and oxaloacetate determination as described by Wedding et al. [15]. Simultaneously the O_2 consumption of 1-ml aliquot was measured. In some experiments, the reaction was stopped directly in the electrode cell. In good agreement with Wedding et al. [15] we have observed that, after treatment with $HClO_4$, a low malate dehydrogenase activity remained in the extract, making oxaloacetate determination with this enzyme difficult. Oxaloacetate was therefore quantitatively decarboxylated with 10 mM $NiCl_2$ [16] for 5 min at 45° C in a second aliquot of the centrifuged extract and pyruvate thus formed determined.

Assay of NAD^+. Extraction and enzymic estimation of endogenous mitochondrial NAD^+ was carried out according to Bergmeyer [17]. In order to maintain the total pyridine nucleotide pool in its oxidized state, prior to extraction of NAD^+, mitochondria were incubated in the following medium: medium A containing 150 µM ATP, 4 µM FCCP, 200 µg catalase, 100 µM H_2O_2.

Bicarbonate uptake. The uptake of labeled bicarbonate by intact purified mitochondria was initiated by adding 10 µl of the mitochondrial suspension (about 1 mg protein) to 200 µl medium A containing 2 mM NADH and ^{14}C-labeled compounds ($NaH^{14}CO_3$ or [^{14}C]sucrose) or ^3H_2O in a 400 µl capacity polypropylene microtube. The uptake was stopped by rapid centrifugation (Beckman, microfuge B) of the mitochondria through a layer of silicone oil (80 µl, Versilub F 50, General Electric) into 50 µl 2.5 M NaOH. In order to prevent the diffusion of CO_2 through the silicone oil layer 10 µl of 2.5 M NaOH was added to the supernatant at the end of the centrifugation. For details on the silicone layer filtering centrifugation technique and on the evaluation of the uptake into the sucrose impermeable space, which is the space surrounded by the inner mitochondrial membrane, see Ref. 18.

pH measurements. The external pH was determined potentiometrically on the supernatant obtained after rapid centrifugation of the mitochondrial suspension. The intramitochondrial pH (matrix space) was calculated on the

basis of the distribution of [^{14}C]acetate (1 μM, 10^6 dpm/200 μl) or 5,5-dimethyl-[2-^{14}C]oxazolidinedione (20 μM, 10^7 dpm/μl) or [^{14}C]methylamine (1 μM, 10^6 dpm/200 μl) between the matrix space and the medium. The intramitochondrial volume was estimated with [^{14}C]sucrose [19,20].

NAD^+-linked malic enzyme assay. The malic enzyme activity was measured by following the reduction of NAD^+ and the appearance of pyruvate in a medium containing 0.1 M Hepes buffer (pH 6.5—8), 2 mM $MnCl_2$, 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 1 mM NAD^+, 5 μM antimycin A and appropriate levels of malate in 3 ml total volume. Mitochondrial membranes were dispersed with 0.02% Triton X-100 and eliminated by centrifugation. Under these conditions the bulk of the matrix enzymes and especially malic enzyme were released in the supernatant.

In good agreement with Mannella [21] and Day et al. [22] we have also verified that the malic enzyme is specifically localized inside the matrix space. There is no malic enzyme activity in the intermembrane space.

Mitochondrial protein determination. Total protein was determined by the Folin-Ciocalteau phenol reagent [23]. Chlorophyll was extracted from the mitochondrial pellet (spinach leaves) in 80% acetone and measured according to Arnon [24]. If we assume a protein to chlorophyll ratio of 7 in broken thylakoids [25], the amount of mitochondrial protein has to be corrected for the contribution of the broken thylakoids.

Results

Products of malate metabolism

In order to inhibit the pyruvate dehydrogenase complex 3 mM sodium arsenite was routinely added to the incubation medium. We have verifed that sodium arsenite was practically without effect on malate dehydrogenase and NAD^+-linked malic enzyme activities. The effect of various malate concentrations on O_2 consumption and on accumulated products (pyruvate and oxaloacetate) in spinach leaf mitochondria is shown in Fig. 1. In the presence of 3 mM sodium arsenite, there is a good correlation between O_2 uptake and oxaloacetate + pyruvate formed (Fig. 1). Under state 4 conditions (Fig. 1A) at malate concentrations up to 15 mM and at pH 7.2 the fraction of total O_2 uptake which can be accounted for by accumulated pyruvate is high and the fraction which appears as oxaloacetate is low. However, as the concentration of malate (above 15 mM) increases the fraction of O_2 uptake appearing as oxaloacetate increases progressively (see also Ref. 26). Under state 3 conditions (Fig. 1B), more oxaloacetate is formed at all malate concentrations. When the same experiment is carried out at pH 6.5 either in state 3 or state 4 there is an increase in pyruvate production which is accompanied by a marked decrease in oxaloacetate formation. In contrast, at pH 7.5 the situation is dramatically reversed. Under these conditions, especially in state 3, at all malate concentrations, the rate of oxaloacetate production is higher than that of pyruvate.

We have systematically verified that oxaloacetate is excreted by the mitochondria during the course of malate oxidation. For example, the oxaloacetate level in the matrix of spinach leaf and mung bean hypocotyl mitochondria

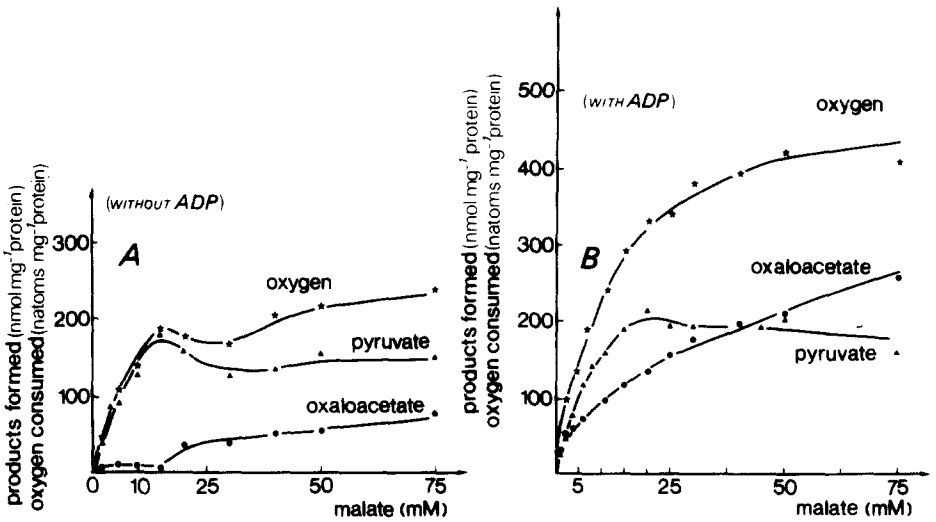

Fig. 1. Production of pyruvate (▲) and oxaloacetate (●) and O_2 consumption (*) during malate oxidation by mitochondria from fresh spinach leaves as a function of malate concentration. (A) Without ADP (incubation time 4 min). (B) With 1.5 mM ADP (incubation time 3 min). The standard assay solution (see Material and Methods) was used with 1.36 mg (state 4) and 0.8 mg (state 3) mitochondrial protein and 3 mM sodium arsenite. Final volume of the reaction mixture was 1 ml. The reaction was stopped directly in the electrode cell by adding 0.3 ml 20% $HClO_4$.

remained constant after the first minute in all metabolic situation studied (see Fig. 1) and amounted to about 2 μM.

In addition, we have verified that spinach leaf mitochondria has no appreciable pyruvate carboxylase and no appreciable oxaloacetate decarboxylase activities.

Consequently these results together strongly support the presence of two enzymes (NAD^+-linked malic enzyme and malate dehydrogenase) oxidizing malate in spinach leaf mitochondria.

In order to understand the way in which the balance of oxidation by these two enzymes is regulated we have investigated the effects of bicarbonate and oxaloacetate on mitochondrial malate oxidation. It is very likely that the accumulation of not only oxaloacetate but also bicarbonate in the incubation medium or matrix space could explain the fact that at pH values above 7.0 the rate of O_2 consumption supported by malate oxidation decreases as the oxidation proceeds [26].

Effect of bicarbonate on malate oxidation

CO_2 is a reaction product of malate oxidation catalysed by the NAD^+-linked malic enzyme localized in the matrix space. Consequently, during the course of malate oxidation, an accumulation of bicarbonate could occur inside the matrix space, especially at alkaline pH, shifting the equilibrium of the reaction catalysed by the malic enzyme towards malate [27–30].

The effect of pH and bicarbonate on the activity of isolated malic enzyme from spinach leaf mitochondria were first examined. The assays showed that, with 15 mM malate and 1 mM NAD^+ (see Material and Methods), the optimum

pH for the reaction was between pH 7.0 and 7.2, a value slightly higher than that reported for the cauliflower bud enzyme [28] and for the tubers of Jerusalem artichoke [5]. Fig. 2 shows that bicarbonate inhibits malate oxidation by the NAD^+-linked malic enzyme. However, the concentration of bicarbonate which brings about half-maximal inhibition of NAD^+-linked malic enzyme activity is higher at pH 7.2 (30 mM) that at pH 7.5 (5 mM). Whereas at pH 6.5, bicarbonate up to 40 mM is practically without effect on the NAD^+-linked malic enzyme activity. The inhibition was relieved by higher malate or NAD^+ levels probably by shifting the reaction towards pyruvate production. This inhibition is also relieved by addition of 20 μM CoASH. It is interesting to note that CoASH which has practically no effect on the assay system at pH 6.5 is found to activate considerably NAD^+-linked malic enzyme at pH 7.5 (Fig. 2). Furthermore, the NAD^+-linked malic enzyme reaction, activated by CoASH, showed a rather broad pH optimum between 7.3 and 7.5. In addition, and in good agreement with Dittrich [31], CoASH increased V and decreased the substrate

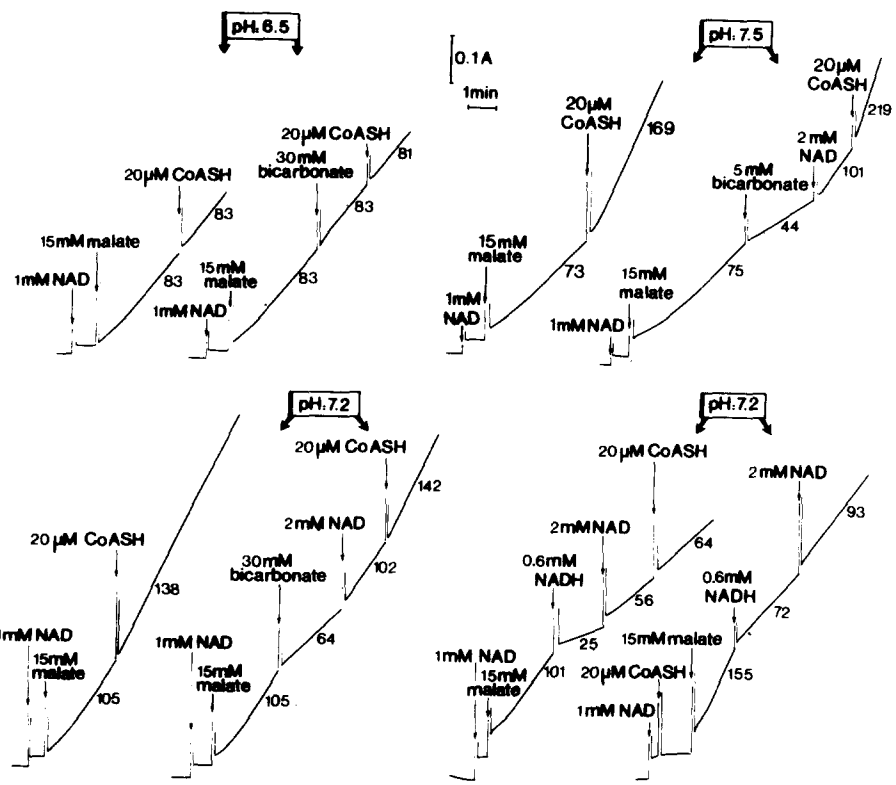

Fig. 2. Inhibition of NAD^+-linked malic enzyme by bicarbonate and NADH. NAD^+-linked malic enzyme activity was measured spectrophotometrically by following the reduction of NAD^+ at 340 nm in the presence of 15 mM malate, 0.43 mg mitochondrial protein and 0.02% Triton X-100 (see Material and Methods). Final volume was 3 ml; incubation temperature 23°C. Numbers on traces refer to nmol NAD^+ reduced/min per mg mitochondrial protein. As indicated NADH was added in the sample and reference cuvettes. At the end of each assay 30 units of lactate dehydrogenase were added. The spectrophotometric control indicated an instantaneous and complete consumption of the produced NADH. The reaction was considered to be sufficient proof for the production of NADH and pyruvate by NAD^+-linked malic enzyme.

concentration required for half-maximal velocity (result not shown). In agreement with Macrae [27] we have also observed that NADH (0.6 mM) is a potent inhibitor of the NAD^+-linked malic enzyme (Fig. 2). This inhibition is also partially released by NAD^+ or by CoASH. Identical results were obtained with NAD^+-linked malic enzyme from mung bean hypocotyl mitochondria. However, the malic enzyme from potato tuber mitochondria was inhibited by concentrations of bicarbonate (half-maximal inhibition: 5 mM at pH 7.2) far below than those required for an effect on the activity of the malic enzyme found in spinach leave mitochondria or mung bean hypocotyl mitochondria (see also Ref. 30).

These results point out the complex regulation of the NAD^+-linked malic enzyme (see also Dittrich [31] and Hatch and Kagawa [32]), and demonstrate that bicarbonate or carbonate, especially at alkaline pH, inhibits the decarboxylation of malate by the malic enzyme. In contrast, at all the pH values tested (6.5—7.5), bicarbonate was without effect on malate dehydrogenase activity.

Before investigating the effect of bicarbonate on malate oxidation by intact plant mitochondria it is necessary to establish that the bicarbonate added in the incubation medium passes through the inner mitochondrial membrane. Consequently, labeled bicarbonate (15 mM) was added to a suspension of mung bean hypocotyl mitochondria oxidizing NADH. We chose NADH instead of malate to prevent the direct formation of bicarbonate inside the matrix space. The uptake of bicarbonate was calculated from the total radioactivity in the NaOH solution (see Material and Methods). We have shown that at pH 7.2, in state 4 (pH of the matrix space: 7.6), the concentration of HCO_3^- in the matrix space was 2.4 times higher than in the medium. In state 3 (pH of the matrix space: 7.3) it was only 1.3 times. Consequently, bicarbonate appeared to equilibrate across the mitochondrial membrane according to the transmembrane pH difference. This may be attained either through free diffusion of dissolved CO_2 or by means of carrier-mediated direct or indirect exchange diffusion of anionic species (HCO_3^-) with OH^-.

Fig. 3 illustrates the effect of 15 mM bicarbonate upon the respiratory rates with malate as substrate and at pH 7.2 in spinach leaf mitochondria. When bicarbonate is added to state 4 the respiratory rate decreases rapidly until the reaction stops completely (Fig. 3B). When bicarbonate is added to state 3 the respiratory rates decrease more slowly. Analysis of the reaction products showed that with 15 mM bicarbonate, spinach leaf mitochondria excreted almost exclusively oxaloacetate (Fig. 3B). This is in contrast with mitochondria oxidizing malate in the absence of bicarbonate (Fig. 3A). The bicarbonate-inhibited mitochondria behave like mitochondria maintained at pH 7.5 in the absence of bicarbonate (results not shown). In these mitochondria the inhibition by bicarbonate of the rate of malate oxidation can be attributed to the inhibition of NAD^+-linked malic enzyme present in the matrix space (see Fig. 2) and to the accumulation of oxaloacetate in the medium. Thus, addition of 0.2 mM oxaloacetate to mitochondria oxidizing malate in the presence of bicarbonate stopped almost immediately the rate of O_2 consumption and this inhibition was not relieved with time. Moreover we have demonstrated that when the ratio of mitochondrial volume to suspending medium volume was

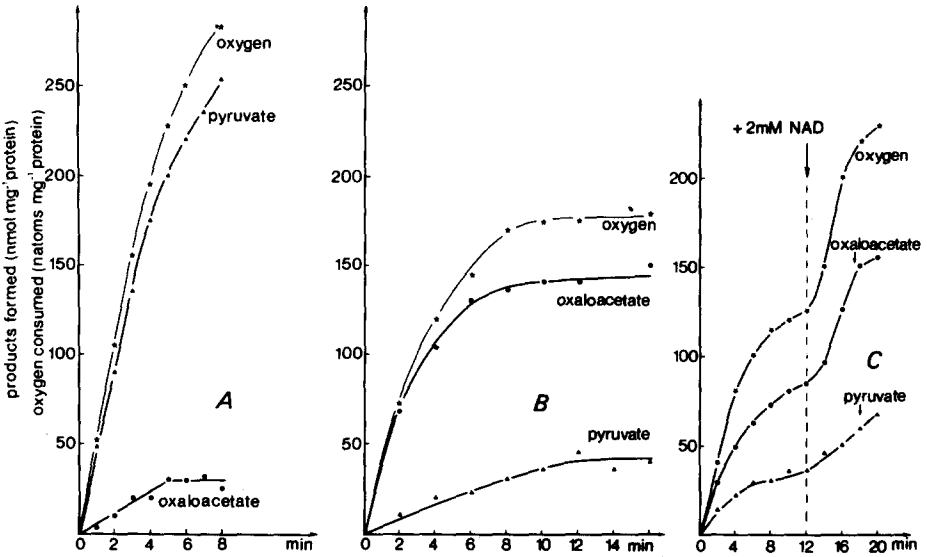

Fig. 3. Production of pyruvate (▲) and oxaloacetate (○) and O_2 consumption (*) during malate oxidation (state 4) by mitochondria from fresh spinach leaves as a function of time. (A) Without bicarbonate. (B and C) With 15 mM bicarbonate. The standard assay solution (see Material and Methods) was used with 1.3 mg (A and B) and 0.7 mg (C) mitochondrial protein/ml, 15 mM malate and 3 mM sodium arsenite. As indicated (C) 2 mM NAD^+ was added. Final pH was 7.2. The final volume of the reaction mixture was 11 ml.

increased in the presence of bicarbonate, the inhibition of malate oxidation occurred more rapidly and vice-versa. In fact, the practical consequence of a large suspending medium volume is that oxaloacetate lost from isolated mitochondria (0.9 $\mu l \cdot mg^{-1}$ protein) will not accumulate in the medium very rapidly: the medium acts as a buffer volume. As long as oxaloacetate is excreted we have shown that O_2 consumption keeps going. Above a threshold of oxaloacetate concentration in the incubation medium, oxaloacetate is no longer excreted and the equilibrium of the reaction catalysed by the malate dehydrogenase is shifted towards the formation of malate. Further addition of malate (30 mM) in the medium temporarily relieves the O_2 consumption and enhances the accumulation of oxaloacetate until a new threshold of oxaloacetate is attained in the medium. In fact, we have observed that the higher the malate concentration in the external medium the higher the malate concentration in the matrix space. Consequently it is more likely that adding more malate push the malate dehydrogenase equilibrium further towards oxaloacetate production. Likewise, addition of 2 mM NAD^+ in the medium enhances temporarily the accumulation of oxaloacetate (Fig. 3C) because we have verified that NAD^+ is transported into the matrix space [33] and shifts slightly the reaction catalysed by the malate dehydrogenase towards oxaloacetate production.

Finally, addition of a known amount of HCl which causes a rapid fall of the pH from 7.2 to 6.5 immediately triggers pyruvate formation (Fig. 4) because the inhibition by bicarbonate of the NAD^+-linked malic enzyme is released at acidic pH (Fig. 2). The NADH produced by the non-inhibited malic enzyme is probably diverted from the respiratory chain to reduce the excess of oxalo-

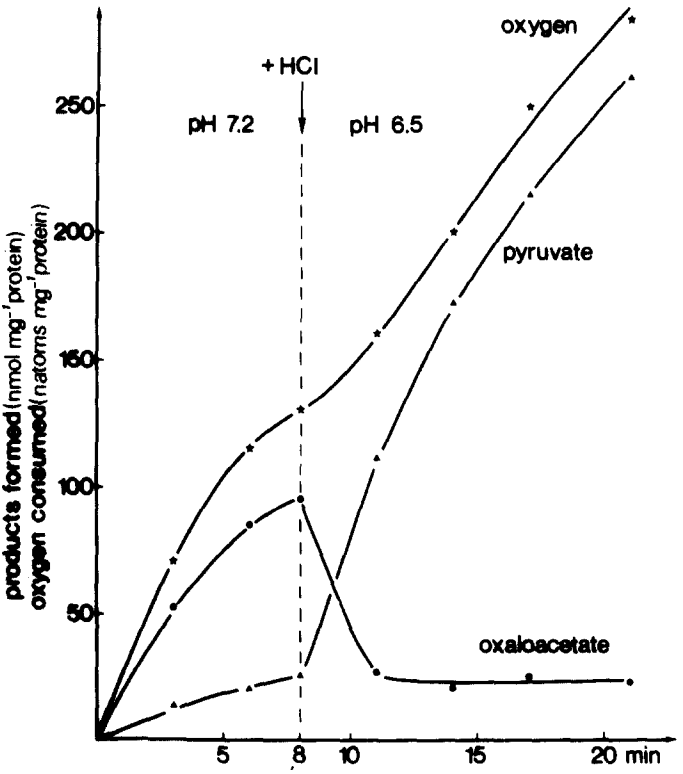

Fig. 4. Production of pyruvate (▲) and oxaloacetate (●) and O_2 consumption (*) during malate oxidation (state 4) by mitochondria from fresh spinach leaves as a function of time. As indicated 3.2 µl 1 N HCl was added. The standard assay solution (see Material and Methods) was used with 0.8 mg mitochondrial protein/ml, 15 mM malate, 3 mM sodium arsenite and 15 mM bicarbonate. Initial pH was 7.2. Final pH was 6.5. The final volume of the reaction mixture was 8 ml.

acetate [34]. This last result prompted us to examine the effect of oxaloacetate on malate oxidation by spinach leaf mitochondria.

Effect of oxaloacetate on malate oxidation

Up to pH 7.2 addition of oxaloacetate to spinach leaf mitochondria supplemented with 15 mM malate in the absence of ADP induces a clear inhibition of the O_2 consumption which is gradually reversed (Fig. 5). Enzymic analyses have shown that, during the time of inhibition, oxaloacetate is rapidly metabolized whereas the rate of pyruvate formation is not affected (Fig. 5). As the mitochondria are devoid of oxaloacetate decarboxylase activity it is clear that oxaloacetate is rapidly converted to malate and this conversion is dependent on the reduced pyridine nucleotide generated by the NAD^+-linked malic enzyme localized in the matrix space. When the oxaloacetate concentration becomes very low, the inhibition of O_2 consumption is released. When the same experiment is carried out in the presence of ADP oxaloacetate only causes a partial inhibition of O_2 uptake which shows a slow tendency to recover (Fig. 6). In addition, in state 3, oxaloacetate added at a final concentration below the concentration it attains at equilibrium (see Fig. 1B) is not metabolized. Under

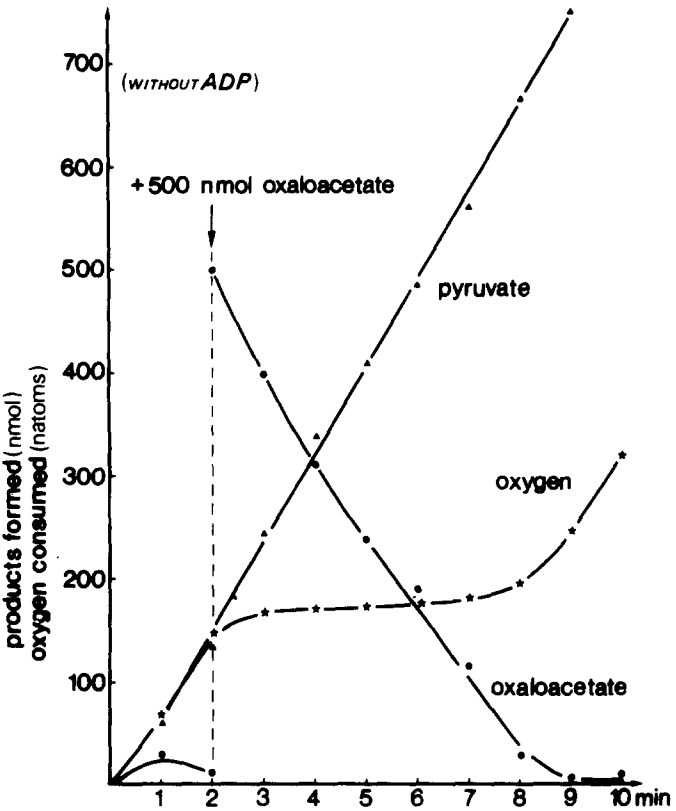

Fig. 5. Disappearance of added oxaloacetate (at the arrow) during malate oxidation (state 4) by mitochondria from fresh spinach leaves. The standard assay solution (see Material and Methods) was used with 0.75 mg mitochondrial protein/ml, 15 mM malate and 3 mM sodium arsenite. Final pH was 7.0. The final volume of the reaction mixture was 11 ml.

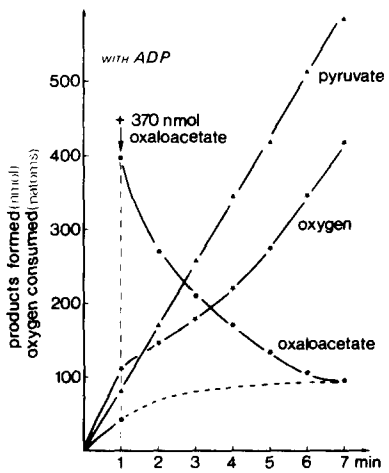

Fig. 6. Disappearance of added oxaloacetate (at arrow) during malate oxidation (state 3) by mitochondria from fresh spinach leaves. The standard assay solution (see Material and Methods) was used with 0.75 mg mitochondrial protein/ml, 15 mM malate, 1 mM ADP and 3 mM sodium arsenite. Final pH was 7.0. ------, oxaloacetate excreted by the mitochondria. The final volume of the reaction mixture was 8 ml.

TABLE I

DEPENDENCY OF THE pH IN THE MATRIX ON THE pH IN THE MEDIUM

The standard assay solution was used with 28 mg mung bean hypocotyl mitochondrial protein/ml. Final pH was as indicated. Substrate 15 mM malate. In order to maintain mitochondria under aerobic condition 400 μM H_2O_2 were added.

pH medium	pH matrix	
	State 3	State 4
6.5	6.7	6.9
7.2	7.3	7.6
7.5	7.65	7.9

these conditions, mitochondria continue to excrete oxaloacetate and its concentration shows a slow adjustment towards the final equilibrium concentration (compare with Ref. 26).

We have also observed that the inhibition by oxaloacetate of malate oxidation by spinach leaf mitochondria is strongly dependent on malate concentration in the reaction medium. At high malate concentration (25 mM) and at pH 7.2 the addition of oxaloacetate results in a discrete inhibition of the rate of O_2 consumption [26] whereas at low malate concentration (5 mM) the addition of oxaloacetate results in a powerful inhibition (results not shown). Again malate probably acts simply to push malate dehydrogenase in the desired direction.

Finally in good agreement with Palmer et al. [26] the inhibition by oxaloacetate of malate oxidation under either state 3 or state 4 was strongly dependent on the pH of the reaction medium. For example, under metabolic state 4 conditions, at pH 6.5, the inhibition was transient and the full rate soon returned [34] whereas above pH 7.3 there appears to be no tendency to recover over the time scale employed. According to our own results the interpretation of this phenomenon is relatively simple. Although the inner mitochondrial membrane constitutes an 'insulating barrier' for the protons we have observed that the pH in the matrix can be varied by changing the pH in the medium. Table I shows the dependency of the pH in the matrix on the pH in the medium as measured at 25°C. Consequently, at low external pH the NAD^+-linked malic enzyme localized in the matrix space is less sensitive to the accumulation of bicarbonate than at alkaline pH (see Fig. 2) and is sufficiently active to provide the NADH necessary to remove rapidly the oxaloacetate. In marked contrast, when the pH of the external medium is slightly alkaline the NAD^+-linked malic enzyme is inhibited (see Fig. 2) and is unable to remove oxaloacetate. Furthermore, by altering the pH of the external medium it is assumed that the levels of bicarbonate within the mitochondria are also changed so that at pH 6.5, bicarbonate concentrations in the mitochondria are low and at pH 7.5, bicarbonate concentrations are high.

Discussion

The data reported in this paper leave little doubt that the spinach leaf mitochondria contain two enzymes: a NAD^+-linked malic enzyme and a malate

dehydrogenase, both able to oxidize malate.

The accumulation of oxaloacetate during the course of malate oxidation is a formidable problem since this anion readily shifts the reaction catalysed by malate dehydrogenase towards malate production. Consequently the results reported in this paper raise the problem of oxaloacetate transport through the inner mitochondrial membrane. The carrier involved which extracts the oxaloacetate from the matrix space could be either the dicarboxylate or the oxoglutarate carrier [35—37] or a specific carrier. The characteristics of this carrier are under investigation. As malate oxidation proceeds, the concentration of oxaloacetate in the medium increases very rapidly up to an equilibrium concentration. When this is achieved, the efflux of oxaloacetate is stopped and the malate dehydrogenase reaction is reversed unless oxaloacetate is utilized directly in the matrix space. The concentration of oxaloacetate attained at the equilibrium is strongly dependent on the malate concentration and on the metabolic state: the excretion of oxaloacetate is very important under state 3 conditions (i.e. when the $NAD^+/NADH$ ratio is high [34]) and in the presence of NAD^+. As a matter of fact, NAD^+ and malate which shift the reaction slightly towards oxaloacetate production, facilitates oxaloacetate excretion.

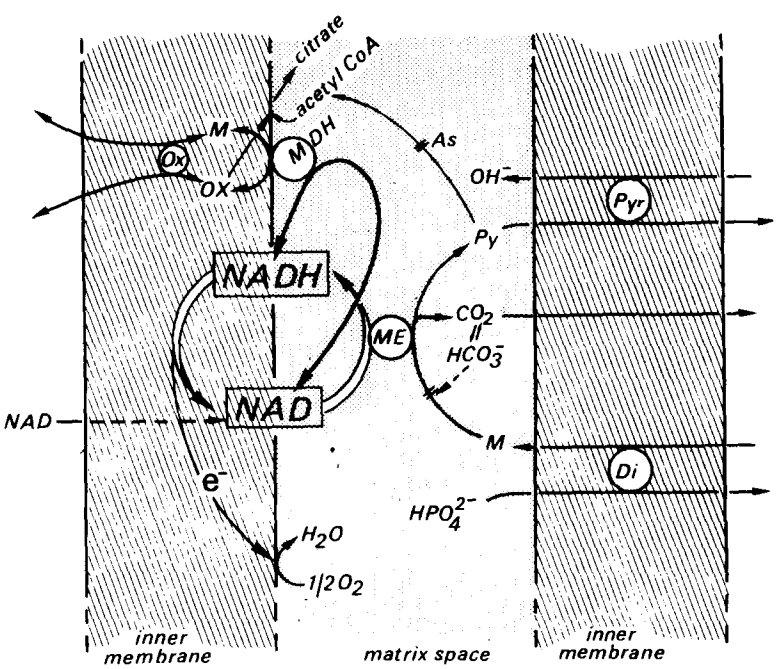

Fig. 7. Pathways of malate oxidation in plant mitochondria. MDH, malate dehydrogenase; ME, NAD^+-linked malic enzyme; ○, oxaloacetate carrier; Pyr, pyruvate carrier (see Refs. 20 and 41); Di, dicarboxylate carrier (see Refs. 42 and 43), Py, pyruvate, M, malate; e^-, respiratory chain; As, sodium arsenite which prevents the convertion of pyruvate to membrane-bound acetyl CoA. This scheme indicates that NAD^+-linked malic enzyme and malate dehydrogenase, located in the matrix space, compete at the level of the pyridine nucleotide pool and that the NAD^+-linked malic enzyme provides NADH for the reversal of the reaction catalyzed by the malate dehydrogenase. Note that, in marked contrast with animal mitochondria, NAD^+ added in the external medium is transported into the matrix space. In addition, 'Ox' is either the dicarboxylate carrier or a specific carrier. Finally bicarbonate produced directly in the matrix space is a potent inhibitor of the NAD^+-linked malic enzyme.

Consequently it is the concentration of oxaloacetate on both sides of the inner mitochondrial membrane which seems to govern the efflux or influx of oxaloacetate. Furthermore, in good agreement with previous suggestions [38,39] the regulation in vivo of malate dehydrogenase can be readily accounted for by equilibrium effects alone.

When the activity of the NAD^+-linked malic enzyme in the matrix space is weakened (high bicarbonate concentration, alkaline pH) oxaloacetate is preferentially excreted and there is a decrease in the rate of malate oxidation as the reaction proceeds. In marked contrast, when the activity of the NAD^+-linked malic enzyme is powerful (low bicarbonate concentration) oxaloacetate concentration is maintained at a low level. In other words, with all the plant mitochondria we have tested whenever the NAD^+-linked malic enzyme activity is weakened the rate of oxaloacetate production is much higher than that of pyruvate. Consequently, in the presence of sodium arsenite, it is clear that both enzymes are competing at the level of the pyridine nucleotide pool and that the NAD^+-linked malic enzyme provides NADH for the reversal of the reaction catalysed by the malate dehydrogenase (Fig. 7).

We have also observed that the rate of malate oxidation by spinach leaf and mung bean hypocotyl mitochondria which contain high endogenous NAD^+ concentration (5—6 nmol/mg mitochondrial protein) is only slightly stimulated by NAD^+ under state 3 condition. This is in contrast with NAD^+-deficient mitochondria isolated from storage tissues such as old potato tubes (0.8 nmol/mg mitochondrial protein) which show a critical dependency on added NAD^+ especially under state 3 condition [40].

Finally, the mitochondria obtained from storage tissues and maintained at pH 7.2 (results not shown) behave exactly like mung bean hypocotyl and spinach leaf mitochondria maintained at pH 7.5, as already noted the NAD^+-linked malic enzyme from storage tissue and spinach leaf mitochondria showed a clearly different sensitivity to bicarbonate.

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