STUDIES WITH ISOLATED BUNDLE SHEATH MITOCHONDRIA

Evidence for NAD-malic enzyme-catalyzed decarboxylation of C_4 acids in species representing the three C_4 metabolic subtypes

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1. Introduction

Currently three metabolic subtypes have been identified in species utilizing C_4 pathway of photosynthesis on the basis of differences in the activities of the C_4 acid decarboxylating enzymes localized in bundle sheath cells, namely NADP-ME type, NAD-ME type and PEP-CK type species [1,2]. While NADP-ME and PEP-CK activies (both chloroplastic, [3]) were exclusively confined to species representing the two respective C_4 subtypes [1,3], substantial amounts of NAD-ME activity were detected in all the three C_4 subtypes, the activity being highest in the NAD-ME type species [1,3]. The NAD-ME activity in species classified as NADP-ME type or PEP-CK type was as high as 20–30% of the activity of their major decarboxylase [1,3].

On the basis of intracellular localization of the C_4 acid decarboxylases and related enzymes [3], characteristics of C_4 acid decarboxylation [4–6], and sensitivity to specific inhibitors of the decarboxylases [4,6], three separate mechanisms for C_4 acid decarboxylation in the C_4 bundle sheath cells have been proposed [3,7,8]. These proposals suggest that an NAD-ME catalyzed C_4 acid decarboxylation system, localized in bundle sheath mitochondria [3,9], is common to all species representing each of the three metabolic subtypes, the activity being highest in NAD-ME species. This proposal has been criticized [10], at least for NADP-ME species, on the assumption that the so-called NAD-ME type activity may be due to some NADP-ME activity associated towards NAD. Since NAD-ME is localized in the mitochondria [3,9], the strength of our proposal [3,7,8] is also subject to further criticism as we did not present evidence for NAD-ME dependent mitochondrial decarboxylation of C_4 acids. Therefore, the present study was made in that direction and conclusive evidence is provided for the presence of NAD-ME catalyzed mitochondrial decarboxylation of C_4 acids in all the three C_4 subtypes.

Abbreviations: NAD-ME, NAD-malic enzyme; NADP-ME, NADP-malic enzyme; PEP-CK, phosphoenolpyruvate carboxykinase; α-kg, α-ketoglutarate; 3-MPA, 3-mercaptopicolinic acid; chl., chlorophyll

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2. Materials and methods

C_4 plants used in the present study included *Digitaria sanguinalis*, *Zea mays* (NADP-ME type), *Panicum miliaceum*, *Eleusine indica* (NAD-ME type), *Panicum maximum* and *Eriochloa borumensis* (PEP-CK type) [1–6]. Bundle sheath strands were prepared by pectinase–cellulase digestion of leaf segments [11] and intact bundle sheath mitochondria were isolated as in [3,4,12]. Mitochondria were suspended in 10 mM Na-phosphate buffer containing 0.3 M sorbitol, 5 mM $MnCl_2$, 2 mM $MgCl_2$, 10 mM KCl and 0.1% BSA adjusted to pH 7.1. Decarboxylation experiments were done with isolated mitochondria in sealed ampules [4–6]. The assays were run with the above medium and initiated, unless

otherwise specified, by adding 6 mM each of L-[4-^{14}C]-aspartate ± α-kg or L-[4-^{14}C]malate ± NAD. The released $^{14}CO_2$ was trapped into 0.2 ml 1.0 M hyamine hydroxide placed in a centrally suspended one half of a gelatin capsule [4–6]. Other additions and conditions are described in the text. Stoichiometry of C_4 acid decarboxylation reactions was determined according to [4,9]. Mitochondrial enzyme activities were determined according to established procedures [3,9,13]. Mitochondrial activities are expressed on the basis of total chl. present in the cell homogenate before subjecting to differential centrifugation. See [4,9] for expressing mitochondrial activities on a chl. basis.

The mitochondria used in the present study were judged highly intact as the 10 000 × *g* pellet contained 93–98% of fumarase activity, a marker enzyme for intact mitochondria, and almost all of the cytochrome *c* oxidase activity. There was also no contamination by intact chloroplasts as no ribulose 1.5-bisphosphate carboxylase, a marker enzyme for intact chloroplast, was detected in the mitochondrial fraction. Associated with the 10 000 × *g* mitochondrial pellet, in all the species, was only 4–7% of total chl. present in the cell homogenate, indicating very little contamination by broken chloroplast thylakoids.

Controls for aspartate and malate decarboxylation and for assessing the non-enzymic decarboxylation of oxaloacetate, an intermediate formed during aspartate decarboxylation, consisted of reactions identical to the experimental system except that the assay medium containing the mitochondria was boiled for 5 min before the addition of C_4 acid [4–6].

3. Results and discussion

3.1. *Comparison of various C_4 acid decarboxylating and related enzyme activities in isolated bundle sheath mitochondria*

It has been shown that during C_4 photosynthesis in NAD-ME type species, aspartate is decarboxylated in the bundle sheath mitochondria via the following sequence of reactions [3,7,–9]:

This sequence of reactions is catalyzed by:

(i) Aspartate aminotransferase;
(ii) NAD-malate dehydrogenase;
(iii) NAD-ME, respectively [3,7–9], and henceforth referred to as the 'NAD-ME system' collectively. It should be noted that this sequence of reactions depends on the addition of α-kg and a stoichiometric balancing of pyridine nucleotide oxidation–reduction.

The results presented in table 1 clearly demonstrate the presence of the enzymes of the NAD-ME system in bundle sheath mitochondria of species representing the NAD-ME subtype. It should also be noted that species representing NADP-ME and PEP-CK types, in which NADP-ME and PEP-CK are the respective principal decarboxylases [1–3,7,8], are also characterized with substantial activities of the NAD-ME system, the activity of NAD-ME being 40–60% of that observed with the NAD-ME type species. In all the three C_4 subtypes, almost all of the bundle sheath NAD-ME activity was associated with their mitochondria (89–98%), thus confirming the exclusive mitochondrial localization of NAD-ME in all C_4 species, irrespective of their metabolic subdivision. The complete absence of NADP-ME and PEP-CK activity further suggests that the observed NAD-ME activity was not due to any non-specific NADP-ME or PEP-CK activity associated towards NAD.

3.2. *Decarboxylation of C_4 acids by bundle sheath mitochondria*

The results presented in table 2 demonstrate the capacity of intact C_4 bundle sheath mitochondria for decarboxylation of aspartate and malate at the C-4 position. Intact mitochondria isolated from the bundle sheath strands of NAD-ME type species catalyzed the decarboxylation of malate and aspartate with several-fold stimulation by the addition of NAD and α-kg, respectively. The stimulation of malate decarboxylation by NAD suggests that the

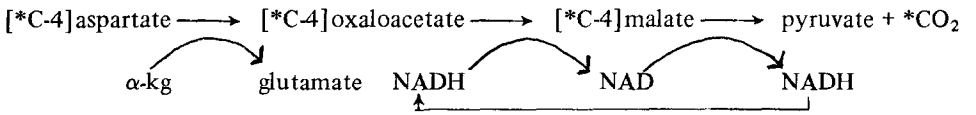

Table 1
Comparison of activities of NAD-malic enzyme and related enzymes in intact bundle sheath mitochondria[a]

Groups and species[b]	Rate (μmol·mg chl.$^{-1}$ min^{-1})					
	NAD-ME	Aspartate aminotransferase	NAD-malate dehydrogenase	NADP-ME	PEP-CK	Fumarase
NAD-ME type						
P. miliaceum	20.5 (98)[c]	60.6(66)	305(55)	0(0)	0(0)	1.86(98)
E. indica	16.1 (95)	49.5(71)	285(58)	0(0)	0(0)	1.94(96)
NADP-ME type						
D. sanguinalis	6.45 (96)	25.7(52)	145(61)	0(0)	0(0)	1.68(96)
Z. mays	7.84 (89)	24.2(49)	126(61)	0(0)	0(0)	2.15(93)
PEP-CK type						
P. maximum	10.5 (91)	74.3(68)	325(35)	0(0)	0(0)	1.75(94)
E. borumensis	11.2 (94)	69.8(64)	341(41)	0(0)	0(0)	1.89(95)

[a] Bundle sheath strands were enzymatically isolated [11] and mitochondria prepared in [3,4,12]. See [3,9,13] for enzyme assay details
[b] See [1,2,6] for the basis of subdividing the C_4 species into three groups
[c] Numbers in parentheses represent percent of total bundle sheath enzyme activity associated with the mitochondrial fractions

Table 2
Decarboxylation of L-[4-^{14}C]aspartate and L-[4-^{14}C]malate by bundle sheath mitochondria[a]

Conditions[b]	Rate of decarboxylation (μmol mg chl.$^{-1}$ h^{-1})					
	NAD-ME type		NADP-ME type		PEP-CK type	
	P. miliaceum	*E. indica*	*D. sanguinalis*	*Z. mays*	*E. borumensis*	*P. maximum*
Malate	15.0	21	4.15	3.18	5.46	5.11
Malate + NAD	346	365	80.7	65.2	91.5	92.6
Aspartate	80	65	18.5	14.5	20.3	18.8
Aspartate + α-kg	365	384	84.3	66.4	97.7	94.4
Aspartate + α-kg + NAD	359	385	87.1	65.9	96.8	95.1
Malate + NAD + HCO_3^-	74	76	18.4	14.8	15.8	19.3
Aspartate + α-kg + HCO_3^-	81	84	19.2	15.1	16.4	20.0
Malate + NAD + oxalate	329	359	81.6	66.3	92.5	89.9
Aspartate + α-kg + oxalate	361	381	85.8	68.9	96.6	91.4
Malate + NAD + 3-MPA	335	369	80.2	65.7	93.1	93.2
Aspartate + α-kg + 3-MPA	358	391	84.8	64.6	95.8	95.6

[a] See [4–6] for assay details
[b] Concentrations of various compounds used were: 6 mM L-[4-^{14}C]malate; 6 mM NAD; 6 mM L-[4-^{14}C]aspartate; 6 mM α-kg; 20 mM HCO_3^-; 0.4 mM oxalate and 0.4 mM 3-MPA

isolated mitochondria may be low in endogenous NAD in supporting the NAD requirement for maximal NAD-ME activity. Stimulation of aspartate decarboxylation by α-kg is expected since aspartate aminotransferase is the initial enzyme for aspartate decarboxylation through the NAD-ME system [3–5]. The absence of NAD stimulation of aspartate decarboxylation supports the contention [7–9] of stoichiometric balancing of pyridine nucleotide oxidation-reduction via NAD malate dehydrogenase and NAD-ME reactions. As there is no net energy consumption, the endogenous levels of NAD would therefore be sufficient in such a system.

From table 2 it is further evident that bundle sheath mitochondria isolated from species representing the NADP-ME and PEP-CK subgroups also resemble those of NAD-ME species in the principal characteristics of their malate and aspartate decarboxylating systems, except that the rates were only 20–26% of those observed with the NAD-ME species.

In all the C_4 species, mitochondrial C_4 acid decarboxylation was inhibited (~80%) by HCO_3^-. Oxalate and 3-MPA, specific inhibitors of NADP-ME and PEP-CK [4,6], respectively, had no effect on the mitochondrial decarboxylation activities.

3.3. *Stoichiometry of C_4 acid decarboxylation*

According to the sequence of reactions proposed for mitochondrial C_4 acid decarboxylation through the NAD-ME system, the release of CO_2 should be accompanied by the stoichiometric production of pyruvate and consumption of aspartate or malate [9]. This stoichiometry was indeed observed during NAD-dependent malate and α-kg stimulated aspartate decarboxylation by bundle sheath mitochondria isolated from several species, irrespective of their metabolic subdivision (table 3).

3.4. *Properties of mitochondrial NAD-ME*

Table 4 summarizes data on the pyridine nucleotide, cation and activator specificities, and also substrate affinities for NAD-ME in the bundle sheath mitochondrial extracts of species representing the three C_4 metabolic subtypes. In all the species, malic enzyme activity was NAD- and Mn^{2+}-dependent, and both CoA and SO_4^{2+} were comparably effective in activating the enzyme. The enzyme was also activated, in all the C_4 species examined, by fructose 1,6-diPO_4, the percent activation being similar to that observed with CoA and SO_4^{2-} (data not shown). The enzyme was virtually inactive with NADP, both in the absence

Table 3
Stoichiometry of mitochondrial C_4 acid decarboxylation[a]

Species and conditions[b]	Rate (μmol/reaction)			Rate (μmol decarboxylated·mg ch.$^{-1}$ h^{-1})
	$^{14}CO_2$ released	Pyruvate formed	C_4 acid consumed	
P. miliaceum (NAD-ME type)				
L-[4-^{14}C]Malate + NAD	4.62	4.58	4.64	315
L-[4-^{14}C]Aspartate + α-kg	5.10	4.97	5.11	331
Z. mays (NADP-ME type)				
L-[4-^{14}C]Malate + NAD	3.44	3.43	3.51	69
L-[4-^{14}C]Aspartate + α-kg	3.69	3.63	3.71	78
E. borumensis (PEP-CK type)				
L-[4-^{14}C]Malate + NAD	3.55	3.53	3.56	84
L-[4-^{14}C]Aspartate + α-kg	3.64	3.61	3.64	93

[a] Product concentrations were determined by enzymatic procedures [4,9]. Pyruvate was assayed with lactic dehydrogenase, aspartate with aspartate aminotransferase coupled with malate dehydrogenase, and malate with malate dehydrogenase in a reaction mixture containing hydrazine to trap oxaloacetate. The measurements were made, in each case, by following pyridine nucleotide oxidation or reduction at 340 nm

[b] Assay conditions and substrate concentrations were as in section 2 and table 1, except that 1 mM Na-arsenite, an inhibitor of pyruvate dehydrogenase, was included in the assay medium to prevent entry of pyruvate into TCA cycle

Table 4
Comparison of properties of partially purified NAD-ME from bundle sheath mitochondrial extracts[a]

Groups and species	Rate (μmol·mg chl.$^{-1}$ min^{-1})[b]						K_m (mM)				pH optimum
	NAD Mn^{2+}	NAD Mn^{2+} CoA	NAD Mn^{2+} SO_4^{2-}	NAD Mg^{2+} CoA	NADP Mn^{2+} CoA	NADP Mg^{2+} CoA	NAD	Malate	Mn^{2+}	CoA	
NAD-ME type											
P. miliaceum	5.41	20.5	20.3	0.16	0.51	0.04	0.45	1.34	0.54	0.021	7.4
E. indica	4.36	17.1	16.8	0.18	0.54	0.07	0.39	1.76	0.49	0.023	7.4
NADP-ME type											
D. sanguinalis	2.11	6.62	6.54	0.04	0.43	0.15	0.46	2.15	0.69	0.046	7.3
Z. mays	2.17	7.91	7.87	0.03	0.39	0.18	0.48	2.21	0.76	0.051	7.5
PEP-CK type											
E. borumensis	3.12	10.6	10.1	0.06	0.56	0.08	0.51	1.96	0.66	0.047	7.4
P. maximum	3.24	11.7	11.9	0.08	0.46	0.09	0.49	1.63	0.93	0.084	7.6

[a] Bundle sheath mitochondrial extracts were centrifuged at 15 000 × *g* for 10 min, the protein in the supernatant precipitated by 55% saturated $(NH_4)_2SO_4$, suspended in 25 mM Hepes–KOH (pH 7.5) containing 50 mM mercaptoethanol and 2 mM $MnCl_2$, and eluted through a Sephadex G-200 column. The recovery was ~40–60%. For details, see [13]

[b] NAD-ME was assayed spectrophotometrically by following $NADH_2$ formation at 340 nm in reaction mixtures containing enzyme, 50 mM Hepes–KOH (pH 7.4), 0.2 mM EDTA, 5 mM DTT, 5 mM $MnCl_2$, 5 mM malate and other additions as indicated in a total vol. 1.2 ml [13]. Preincubation of the mitochondrial extracts containing 5 mM DTT under N_2 for 15 min at room temperature preceded prior to the spectrophotometric assay. Concentrations of other compounds used were 5 mM NAD or NADP, 5 mM Mn^{2+} or Mg^{2+}, 75 μM CoA and 7.5 mM SO_4^{2-}

and presence of CoA. The NAD-ME from all the C_4 species was almost inactive when Mg^{2+} replaced Mn^{2+}.

Affinities of various reactants for NAD-ME from various C_4 species, calculated from plots of activity versus reactant concentration, are shown in table 4. The plots were either distinctly sigmoidal or hyperbolic (data not shown). The K_m values are only approximate in some instances as they were derived directly from simple plots of concentration versus activity, except for the few cases where linear double reciprocal plots were obtained. As can be seen from table 4, the K_m values for any given reactant did not vary substantially for the enzymes examined from several C_4 species. The enzymes had more or less similar pH optima (pH 7.3–7.6).

4. Concluding remarks

The present study confirms the operation of NAD-ME catalyzed mitochondrial decarboxylation of aspartate and malate in species representing the NAD-ME subtype. It is further concluded, on the basis of the activities of the enzymes of the NAD-ME system (table 1), characteristics and stoichiometry of mitochondrial C_4 acid decarboxylation (tables 2, 3), and properties of NAD-ME (table 4), that an NAD-ME catalyzed C_4 acid decarboxylation is operative in the bundle sheath mitochondria of species representing the NADP-ME and PEP-CK subtypes also, although the rates were only 20–26% of those observed with the NAD-ME type species. The present study thus provides the first direct and conclusive evidence for the operation of NAD-ME catalyzed C_4 acid decarboxylation in the bundle sheath mitochondria of PEP-CK and NADP-ME subtypes.

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