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THE EFFECT OF COENZYME A ON ENERGY-LINKED REACTIONS OF MAIZE MITOCHONDRIA*

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Abstract—Mitochondria isolated from seedlings of inbred maize (*Zea mays* L.) parents were found to exhibit enhanced respiratory control and oxidative phosphorylation in the presence of physiological concentrations of coenzyme A. Significant differences in the response to the coenzyme of mitochondria of parental maize varieties, their F_1 hybrid, and a 1:1 mixture of parental mitochondria were observed. Coenzyme A selectively enhances oxidative phosphorylation of mitochondria of inbred maize but does not significantly affect mitochondria of the F_1 hybrid of these inbreds. Treatment of maize mitochondria with bovine serum albumin, cysteine, or glutathione does not enhance oxidative phosphorylation as does coenzyme A. The coenzyme A is most effective in enhancing oxidative phosphorylation when the mitochondria are in the contracted steady state, State 3.

INTRODUCTION

MITOCHONDRIA of hybrids of maize (*Zea mays* L.) were found to have greater phosphorylative and oxidative activities than mitochondria of parents of those hybrids. In attempting to describe the mechanism(s) of this obvious manifestation of heterosis (hybrid vigor), we postulated that hybrids contain mitochondria normally present in both parents of these hybrids and that this mixture of mitochondria *in vivo* led to some type of interaction. This hypothesis was supported by our observations that mitochondria of two parental strains of maize, Wf9 and Oh 45, have different sedimentation properties and other characteristics which showed that mitochondrial polymorphism exists.[1] *In vitro* studies showed that mixtures of mitochondria from both parents had greater respiratory activity (μM O_2/mg N/min) than mitochondria from either one of the parents alone. The respiratory rate was nearly the same as that obtained using mitochondria from the hybrid. This complementation in mitochondrial function did not occur when mitochondria from parents which did not produce heterotic hybrids were mixed.[2] It has been shown that mitochondrial heterosis and complementation occur at specific steps of the Krebs cycle.[3] While this knowledge was of great interest in that it could account for heterosis on a molecular level, it did not enable us to fully understand mitochondrial polymorphism and its relationship to heterosis.

Earlier studies in this laboratory implicated coenzyme A as a necessary cofactor in the response of mitochondria to indoleacetic acid (IAA).[4] When coenzyme A was included in

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[1] I. V. SARKISSIAN and R. G. MCDANIEL, *Proc. Nat. Acad. Sci.* **57**, 1262 (1967).
[2] R. G. MCDANIEL and I. V. SARKISSIAN, *Science* **152**, 1640 (1966).
[3] R. G. MCDANIEL and I. V. SARKISSIAN, *Genetics* **59**, 465 (1968).
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the reaction medium mitochondria isolated from inbred maize scutella exhibited enhanced oxygen uptake and phosphorylation in the presence of physiological concentrations of IAA. Without coenzyme A, no such response was observed. The addition of coenzyme A and IAA was found to have little or no effect on mitochondria of the hybrid. Thus, we had a lead which we could follow in our efforts to understand the relationship of mitochondrial differences to their activities. Specifically, it was of interest to learn more about involvement of coenzyme A in mitochondrial heterosis and complementation and especially about the differential responses of maize inbreds and their hybrid to coenzyme A.

Effect of Coenzyme A on Mitochondrial Activity

The results of the polarographic study shown in Table 1 demonstrate striking differences in response to coenzyme A. In the absence of this cofactor, mitochondria from either of the inbred parents yielded lower ADP:O and respiratory control ratios as well as lower rates of

TABLE 1. THE DIFFERENTIAL RESPONSE OF CORN MITOCHONDRIA TO COENZYME A

Source of mitochondria	CoA*	μM O_2	RC ratio	ADP:O ratio	% increase in ADP:O on addn. of CoA
Wf9/Oh 45 (hybrid)	–	56·8	2·19	2·13	108
	+	55·0	2·54	2·30	
Wf9 + Oh 45 (mixture)	–	48·8	2·14	2·04	108
	+	47·3	1·80	2·20	
Wf9	–	42·3	1·44	1·36	154
	+	40·8	1·60	2·10	
Oh 45	–	25·8	1·66	1·58	139
	+	26·5	1·75	2·20	

* 0·15 mg coenzyme A per reaction was added immediately after substrate (α-ketoglutarate), and immediately before ADP-induced rapid respiration. Values for non-CoA experiments are averages of from four to ten replicated experiments. CoA values are averages of four observations within a single experiment. Oxidative activity (State 3) expressed as μM O_2/mg N/min.

respiratory activity than mitochondria from the hybrid (Wf9/Oh 45) or the mixture (Wf9 + Oh 45).

It should be noted that although the presence of coenzyme A raised the RC and ADP:O ratios of parental mitochondria nearly to the level of the parental mixture, the mixture exhibited complementation of oxidative activity regardless of whether coenzyme A was present or not.

Coenzyme A and Respiratory Lag

In early studies, the enhanced RC and ADP:O ratios following the addition of coenzyme A to mitochondria were not observed consistently. The response elicited by coenzyme A was found to vary with the manner in which coenzyme A was added to mitochondrial preparations. When coenzyme A was added to mitochondria in the absence of substrate, a long (10 min) lag in oxidative and phosphorylative activity was observed after initial additions of substrate and ADP (Fig. 1). This lag was only observed with mitochondria of inbreds. After an initial lag period, the inbred mitochondria exhibited enhanced RC and

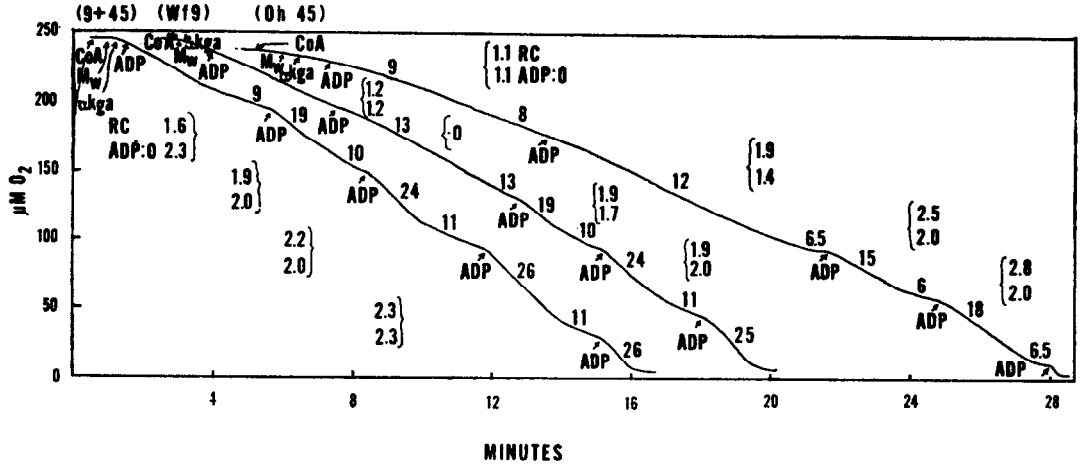

FIG. 1. POLAROGRAPHIC TRACES SHOWING THE EFFECT OF COENZYME A ON MAIZE MITOCHONDRIA.
Numbers along the traces represent μM O_2 uptake per minute. Reaction mixture as given in materials and methods. 0·15 mg coenzyme A added to each reaction at the indicated point. M denotes point of addition of mitochondria.

ADP:O ratios just as they had when coenzyme A was added after substrate (Table 1). It is important to note that this lag was not observed with the mitochondrial mixture (Fig. 1) or with mitochondria of the hybrid. This lag effect had been noted previously in manometric studies of inbred maize mitochondria using AMP.[3] Figure 2 shows the response of mitochondria from inbred Wf9 and the hybrid to coenzyme A added after two cycles of respiratory

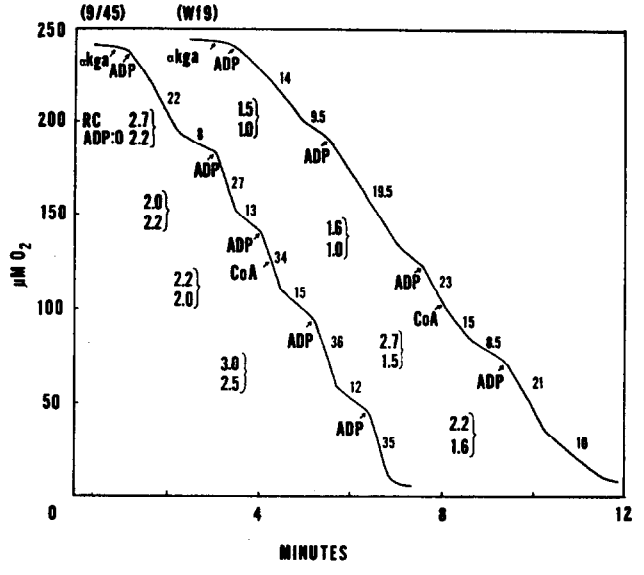

FIG. 2. POLAROGRAPHIC TRACES SHOWING THE EFFECT OF COENZYME A ADDED TO MAIZE MITOCHONDRIA FOLLOWING TWO CYCLES OF RESPIRATORY CONTROL.
Reaction mixture as given in Materials and Methods. 0·15 mg coenzyme A added to each reaction at the indicated point. Numbers along the traces denote μM O_2 uptake per minute.

control. When coenzyme A was added while the mitochondria were in the ADP-induced state of rapid respiration, RC and ADP:O ratios of the inbred were enhanced with no lag period. Only slight, non-significant responses to coenzyme A were observed in any case with mitochondria from the hybrid.

The polarographic determinations revealed the specific character of oxidation of α-ketoglutarate by these mitochondria. Previous manometric studies had shown an enhancement of oxidative rate of mitochondria from inbreds in the presence of coenzyme A.[2] Table 1 shows no significant enhancement of respiratory rate of inbreds with added coenzyme A. These differences are easily explainable when one considers the time length of manometric experiments (60 min) compared to polarographic experiments (6–12 min); and, when one considers the "lag" effect inherent to the respiratory rate of inbred mitochondria assayed manometrically.[2]

The Role of Coenzyme A in Oxidative Phosphorylation

McMurray and Lardy[5] observed responses of animal mitochondria to coenzyme A which they believed implicated it as a coupling factor in eliciting phosphorylation in these particles. Lehninger and Remmert[6] later attributed the property of coenzyme A of enhancing mitochondrial phosphorylation to its ability to esterify fatty acids which were being liberated from rat liver mitochondria upon aging or sonication. As fatty acids are known uncouplers of oxidative phosphorylation and esterification is a proven method of detoxification of these compounds, this would seem to be a reasonable explanation for the effect of coenzyme A on animal mitochondria.

A question may be raised regarding the integrity of mitochondria from maize inbreds, especially since these mitochondria do not exhibit high RC and ADP:O ratios. It may be that mitochondria from inbreds contain associated proteins or other substances, such as fatty acids, which inhibit their activity, and that coenzyme A by some means can inactivate these inhibitors.

TABLE 2. THE EFFECT OF BOVINE SERUM ALBUMIN ON MAIZE MITOCHONDRIA

Source of mitochondria	BSA*	μM O_2	RC ratio	ADP:O ratio
Wf9/Oh 45 (hybrid)	–	61	2·56	2·43
	+	63	2·61	2·47
Wf9 + Oh 45 (mixture)	–	52	2·12	1·91
	+	52	2·22	1·96
Wf9	–	39	1·20	1·54
	+	42	1·29	1·50
Oh 45	–	28	1·56	1·62
	+	33	1·71	1·65

* Three mg BSA per reaction. Oxidative activity expressed in μM O_2/mg N/min. Reaction mixture as in Materials and Methods. No coenzyme A was present.

[5] W. C. MCMURRAY and H. A. LARDY, *J. Am. Chem. Soc.* **79**, 6563 (1957).
[6] A. L. LEHNINGER and F. REMMERT, *J. Biol. Chem.* **234**, 2459 (1959).

Fatty acids are known to cause animal mitochondria to swell and lose respiratory control.[7] Bovine serum albumin (BSA) has been shown to bind inhibitory proteins and fatty acids, and to restore respiratory control to aged plant and animal mitochondria.[8,9] As maize scutellum is rich in lipid, one might expect mitochondria from this tissue which respond to coenzyme A to respond similarly to BSA. Our data indicated that this was not the case, and no significant response to BSA by corn mitochondria at pH 7·0 was observed (Table 2). These mitochondria thus did not possess inhibitory substances removable by BSA. It appears then that the action of coenzyme A in its effect(s) on regulation of respiration is not one of removal or deactivation of those types of inhibitors which is accomplished by BSA.

Mitochondrial Steady State and Coenzyme A

Of special interest is the relationship of the steady state of the mitochondria to coenzyme A effects (Table 3). When mitochondria of inbreds are in steady State 1 or 4, addition of

TABLE 3. THE INFLUENCE OF MITOCHONDRIAL STEADY STATE ON THE EFFECT OF COENZYME A ON OXIDATIVE PHOSPHORYLATION OF INBRED MAIZE

Mitochondrial steady state at time of coenzyme A addition*	Additions	Effect of coenzyme A
State 1 →	add coA → add αKGA → add ADP	→ Apparent uncoupling Low RC and ADP:O ratios → Time lag → Coupling regained → Enhanced RC and ADP:O ratios
State 2 →	add αKGA → add coA → add ADP	→ Immediate enhancement of RC and ADP:O ratios
State 3 →	add αKGA → add ADP → add coA	→ Initiation of intermediate steady state: Enhanced RC and ADP:O ratios
State 4 →	add αKGA → add ADP → add coA → add ADP	→ Apparent uncoupling Low RC and ADP:O ratios → Time lag → Anaerobiosis

* Nomenclature according to the literature[15] except that, as used here, State 2 denotes high substrate concentration, low ADP concentration.

coenzyme A apparently causes uncoupling of phosphorylation. After 10 min, respiratory control and oxidative phosphorylation are regained (Fig. 1). If coenzyme A is added when the mitochondria are in State 3, enhanced respiratory control and coupling result immediately (Fig. 2). Of significance in interpreting these observations is the relationship of steady states with the contraction-swelling cycles of the mitochondria. It is known that ADP, and in some cases substrate, cause a contraction of plant and animal mitochondria.[8,9] It is in this ADP-stimulated, contracted steady state that rapid, tightly coupled, oxidative reactions of mitochondria occur. When ADP is exhausted, the mitochondria return to the "relaxed" state (State 4), in which a low rate of oxidative activity is maintained. It appears that when mitochondria of inbreds are in the contracted (ADP-stimulated) steady state, they respond immediately to coenzyme A. When coenzyme A is added to mitochondria in a relaxed state, a lag period following subsequent ADP addition is necessary before the mitochondria can again attain a contracted state and demonstrate respiratory control and coupling.

[7] E. C. WEINBACH and J. GARBUS, *J. Biol. Chem.* **241**, 169 (1966).
[8] J. B. HANSON, C. M. WILSON, M. J. CHRISPEEL, W. A. KRUEGER and H. R. SWANSON, *J. Exp. Botany* **16**, 282 (1965).
[9] E. C. WEINBACH and J. GARBUS, *J. Biol. Chem.* **241**, 3708 (1966).

This ability of coenzyme A to selectively enhance activities of inbred maize mitochondria when they are in the contracted steady state deserves further study. The fact that coenzyme A has an effect on contraction of inbred mitochondria (as judged by restoration of respiratory control) may indicate a direct involvement of coenzyme A in the mechanism of swelling and contraction, or, alternatively, an indirect effect due to the inactivation of an inhibitor which elicits swelling.

SH Groups and Oxidative Phosphorylation

Other workers have shown that the number of free SH groups in mitochondria decreases significantly during the aging of mitochondria at 2° for 1 or 2 days.[10, 11] A direct result of such aging is mitochondrial swelling.[9] Swelling and contraction of animal mitochondria have been found to be dependent on the number of free SH groups in the phospholipid membranes of mitochondria,[11] and preparations of reduced coenzyme A are rich in SH

TABLE 4. THE EFFECT OF REAGENTS CONTAINING SH GROUPS ON OXIDATION AND PHOSPHORYLATION OF INBRED MAIZE MITOCHONDRIA

Reagent	% of control		
	μM O_2	RC ratio	ADP:O ratio
Coenzyme A (0·15 mg)	100	111	142
Bovine serum albumin (3 mg)*	112	108	100
Cysteine (0·15 mg)	100	96	98
Glutathione (0·15 mg)	95	93	105

* α-ketoglutarate utilized as substrate. Each value represents the average of three or more experiments. The values in parentheses are the quantities of reagent present in each 3 ml reaction mixture at pH 7·0. Freshly prepared solutions of coenzyme A (75% purity), L-cysteine, reduced glutathione and bovine serum albumin (fraction V) were used. Reagents were added to respiring mitochondria during the first cycle of ADP-induced rapid respiration (steady State 3). Inbred maize Oh 45 used as source of mitochondria.

bonds. Coenzyme A has been found to affect the physical structure of animal mitochondria, and to act antagonistically to 6-mercaptopurine.[12] The importance of sulphydryl enzymes, and especially structural proteins of mitochondria and nuclei containing SH groups in maintaining membrane integrity has been stressed.[13]

Thus, it was of interest to test other SH compounds in order to determine if the effects of coenzyme A were attributable to its free SH groups. Table 4 presents the relative effectiveness of several sulphydryl compounds in increasing mitochondrial oxidative phosphorylation. None of the compounds tested other than coenzyme A elicited a significant enhancement of oxidative phosphorylation of these mitochondria. These observations would support the idea that coenzyme A has a direct role in some part of the phosphorylative mechanism at the site of the α-ketoglutarate enzyme complex, rather than participating directly in

[10] M. RILEY and A. L. LEHNINGER, *J. Biol. Chem.* **239**, 2083 (1964).
[11] P. V. VIGNAIS and P. M. VIGNAIS, in *Regulation of Metabolic Processes in Mitochondria* (edited by J. M. TAGER, S. PAPA, E. QUAGLIARIELLO and E. C. SLATER), p. 368, Elsevier, New York (1966).
[12] J. J. BIESELE, *J. Biophys. Biochem. Cytol.* **1**, 119 (1955).
[13] E. D. WILLS and A. E. WILKINSON, *Intern. J. Radiation Biol.* **13**, 45 (1967).

conformational or other reactions as a SH reagent. The conformational effects observed earlier could result in a decreased permeability of the mitochondrial membrane to coenzyme A, and result in the observed phosphorylative lag.

As shown in these experiments, coenzyme A has been found to selectively enhance the biochemical activity of inbred maize mitochondria. One may question whether mitochondria of maize inbreds age during isolation (usually 60–90 min) comparatively more than do mitochondria from the hybrid. Mixing of parental mitochondria might overcome aging of the individual parental preparations in some manner. Thus, the mitochondrial mixture and the mitochondria of the hybrid could show similar resistance to *in vitro* aging.

In view of these observations, it will be of extreme interest to further assess the role of coenzyme A in respiratory control and coupling observed with mitochondria from lipid-rich tissues. The level of coenzyme A in such tissues could be one control mechanism for the regulation and switching of mitochondrial fatty acid and tricarboxylic acid cycle oxidations in rapidly metabolizing (germinating) lipid-rich plant tissues.

EXPERIMENTAL

Mitochondria were isolated from scutella (diploid cotyledons) of 2- to 3-day-old maize inbreds (Wf9 and Oh 45) and their heterotic hybrid (Wf9/Oh 45)[4,14] with the exception that the final wash was with 0·5 M sucrose instead of sucrose–phosphate–EDTA* grinding buffer. Polarographic determinations of O_2 uptake by mitochondria were made using a Clark-type O_2 electrode (Yellow Springs Instrument Company). Reagents were introduced into the reaction mixture by a syringe through an access slot in the probe holder. Reaction chambers were immersed in a water bath at 27°. Reaction mixtures contained 0·33 mg NAD, 0·5 mg TPP, 0·1 mg cytochrome c, 2·5 μmoles $MgSO_4$, 25 μmoles K phosphate, 0·3 M sucrose, and 0·5 ml mitochondrial suspension. Final volume of the reaction mixture was 3·0 ml with a pH of 7·0. The reaction mixture (2·5 ml) was added to the reaction chamber and aerated for 2 min. Then 0·5 ml of mitochondrial suspension was added, and the suspension was allowed to equilibrate for 1 min. The probe was then inserted in the chamber and measurement of the reaction rate was begun. In a few experiments the mitochondrial suspension was added with a syringe after the probe had been inserted. Additions of substrate (3·3 mM), and ADP were made using a syringe. Each addition of ADP varied between 100 and 200 μM (in increments of 25 μM) depending on the amount of mitochondria in the reaction and on the degree of coupling observed with different mitochondrial preparation. 0·15 mg of coenzyme A (Nutritional Biochemicals Corp.) was added to reactions in which the effect of this coenzyme was being studied. ADP:O ratios were calculated as the ratio of ADP added to O_2 uptake from the point of addition of ADP to the initiation of the subsequent State 4. Respiratory control ratios (RC) were calculated as the ratios of State 3 rate of mitochondrial respiration to State 4 rate of respiration. Nomenclature of respiratory states in this paper is consistent with the literature.[15]

Mitochondrial protein was determined by a modification of the method of Lowry *et al.*,[16] with bovine serum albumin as standard. Mixtures of mitochondria of maize inbreds were made 1:1 with regard to mitochondrial protein in all experiments.

We consider the possibility of bacterial interference very unlikely since all buffers, reaction mixture and mitochondria were kept at 0 to 2° during the 60 min isolation period prior to the measurement of mitochondrial activity at 27°.

* Abbreviations used: EDTA, ethylenediamine-tetraacetate; NAD, oxidized nicotinamide-adenine dinucleotide; TPP, thiamine pyrophosphate; ADP, adenosine diphosphate; αkga, alpha ketoglutarate; BSA, bovine serum albumin; and CoA, coenzyme A.

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