Biochimica et Biophysica Acta, 462 (1977) 171–176

BBA 47371

STUDIES ON THE INNER MITOCHONDRIAL MEMBRANE LOCALIZATION OF PROLINE DEHYDROGENASE

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(Received January 28th, 1977)

SUMMARY

1. The site of proline dehydrogenase (EC 1.5.99.-) activity in blowfly (*Phormia regina*) flight muscle mitochondria has been investigated employing the inner membrane-impermeable $Fe(CN)_6^{3-}$ as electron acceptor. Antimycin had no inhibitory effect on ferricyanide reduction due to proline dehydrogenase activity. Ferricyanide reductase activity due to inside localized dehydrogenase activity was antimycin sensitive. These results indicate that the interaction between proline dehydrogenase and ferricyanide was direct and not dependent on respiratory chain activity.

2. The stimulatory action of the effector, ADP, on proline dehydrogenase activity was insensitive to atractyloside, an indication that the site of dehydrogenase interaction with ADP was external to the atractyloside barrier.

3. Swelling studies revealed that proline does not readily penetrate the matrix space.

4. An outside localization for proline dehydrogenase is discussed in terms of the role of proline in insect flight muscle metabolism.

INTRODUCTION

Evidence has been obtained to indicate that proline oxidation may serve as a means of replenishing tricarboxylic acid cycle intermediates and thus facilitate the rapid utilization of pyruvate which tends to accumulate during the initial phase of insect flight [1, 2]. Production of tricarboxylic acid cycle intermediates from proline oxidation would be via glutamate, whose synthesis in flight muscle [3] is via the two-step sequence seen in mammalian liver [4, 5] and microorganisms [6]. The sequence is as follows:

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid; ClCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

$$\text{L-proline} + \tfrac{1}{2}\,O_2 \xrightarrow[\text{proline dehydrogenase}]{} \Delta^1\text{-pyrroline 5-carboxylate} + H_2O$$

$$\Delta^1\text{-pyrroline 5-carboxylate} + NAD^+ + 2H_2O$$

$$\downarrow \quad \Delta^1\text{-pyrroline 5-carboxylate dehydrogenase}$$

$$\text{glutamate} + NADH + 2H^+$$

In addition to a role for proline oxidation in the supply of tricarboxylic acid cycle intermediates, recent studies in this laboratory (Hecht, R. and Balboni, E., unpublished) indicate the existence of a proline cycle [7] that functions in the transfer of reducing equivalents from extramitochondrial NADH to the respiratory chain (see Results and Discussion below). The existence of a proline cycle in turn would impart to proline oxidation a role in extramitochondrial events in addition to its role in the intramitochondrial supply of tricarboxylic acid cycle intermediates for the priming of pyruvate oxidation.

An essential prerequisite to any considerations concerning the participation of proline in the intra-extramitochondrial reactions discussed above would be enhanced if information concerning the mitochondrial site of proline oxidation were available. In this regard, all that has been established to date is that proline dehydrogenase activity is associated with the inner mitochondrial membrane fraction [3, 8].

It was the purpose of this study to attempt a more precise localization of the dehydrogenase to the extent that it could be localized either on the outer or inner phase of the inner membrane.

METHODS

Blowflies (*Phormia regina*), 7–21 days old, were immobilized by chilling. The thoraces (100–150) were isolated and placed in an ice-cold mortar containing 15 ml isolation medium consisting of 0.25 M sucrose, 0.5 mM HEPES, 10 mM EDTA, 0.5 % bovine serum albumin, pH 7.4. The thoraces were gently pounded and the resultant brei filtered through four layers of cheesecloth previously moistened with isolation medium. The filtrate was centrifuged at $120 \times g$ for 3 min, the supernatant drawn off and the sediment discarded. The supernatant was centrifuged at $8700 \times g$ for 15 min. The reddish-brown pellet was resuspended in 3.0 ml isolation medium (minus EDTA).

Dehydrogenase activity was followed employing the ferricyanide assay [9]. Measuring wavelength, 420 nm; reference wavelength, 460 nm. Sonicated submitochondrial particles were prepared according to Gregg [10]. Mitochondrial protein was estimated employing the biuret reagent [11].

RESULTS AND DISCUSSION

In Fig. 1 are reported the results of studies of the effects of antimycin on ferricyanide reduction accompanying the oxidations of proline, pyruvate, and glycerol 1-phosphate.

What can be seen is that ferricyanide reduction accompanying proline oxida-

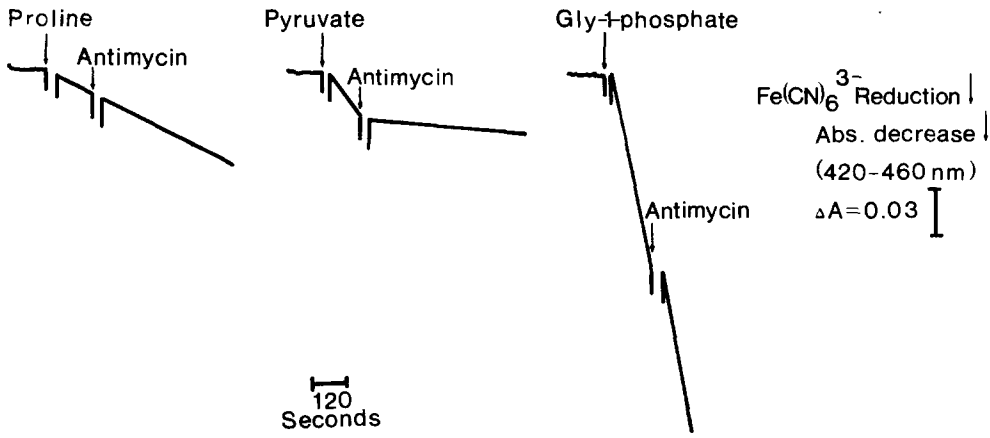

Fig. 1. Effect of antimycin on $Fe(CN)_6^{3-}$ reduction due to proline, pyruvate and glycerol 1-phosphate oxidation. The reaction mixture contained 0.25 M sucrose, 5 mM HEPES, 0.5 % bovine serum albumin, 40 mM sodium phosphate, 3.7 μM ClCCP, 3.4 μM rotenone (absent in pyruvate studies), 1 mM $K_3Fe(CN)_6$, 1 mM KCN, 10 mM KCl, pH 7.34. At the points indicated, 35 μmol proline, 26 μmol pyruvate, and 36 μmol glycerol 1-phosphate were added. Antimycin added, 10 μg; mitochondrial protein, 6.95 mg; volume, 3.25 ml; temperature, 27 °C.

tion or the outside-localized glycerol 1-phosphate oxidation [9] was antimycin insensitive. Ferricyanide reduction due to the inside-localized pyruvate oxidation [12] was antimycin-sensitive. These results suggest that the interaction between proline dehydrogenase and ferricyanide was direct as opposed to indirect, which in turn favors an outside localization for proline dehydrogenase activity.

In the case of blowfly flight muscle mitochondria, both proline [13] and pyruvate [14] oxidation are stimulated by ADP in the presence of oligomycin and uncouplers. The suggested locus of ADP action is dehydrogenase activity.

In Fig. 2 are reported the results of studies on the effect of atractyloside on the ADP-stimulated oxidations of proline and pyruvate, respectively. What can be seen is that in the presence of atractyloside, added ADP was still effective in eliciting stimulation of proline oxidation. Stimulation of pyruvate oxidation by ADP was inhibited by atractyloside. These results are in accord with the results in the Fig. 1 studies which suggest an outside localization for proline dehydrogenase activity.

In Fig. 3 are presented the results of studies on the permeability of flight muscle mitochondria to proline. What is seen is that when isoosmotic solutions of proline were added to suspensions of mitochondria, no significant changes in mitochondrial volume were observed as indicated by the absorbance changes. For comparative purposes, absorbance changes were also followed in the case of the addition of isoosmotic solutions of β-alanine to suspensions of mitochondria. This amino acid penetrates very readily the inner membrane of liver and heart mitochondria [15]. In contrast to proline, β-alanine fostered rapid and large-scale increases in mitochondrial volume, indicating that it readily penetrated the inner membrane. The conclusion to be drawn from these data is that if proline dehydrogenase was localized within the inner membrane, it would be expected that its substrate would be capable of penetrating the inner membrane. This does not appear to be the case.

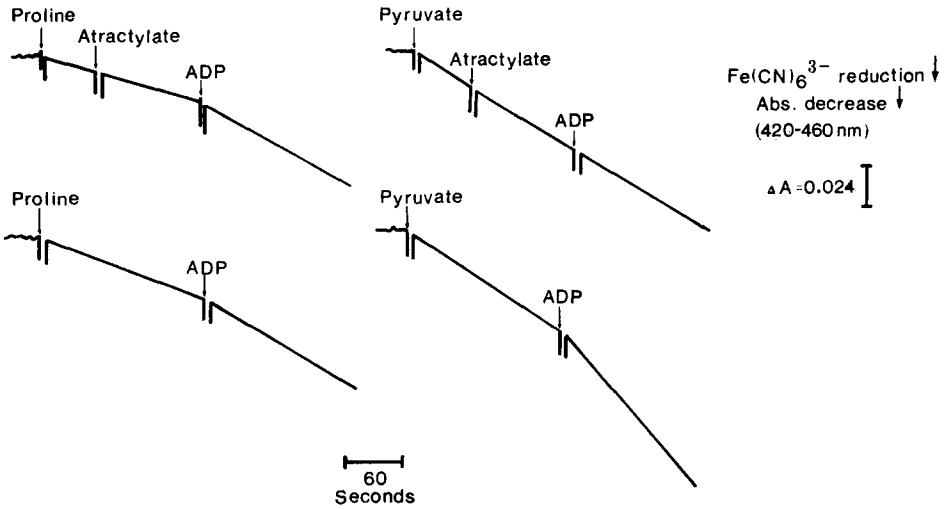

Fig. 2. Effect of atractylate on ADP-stimulated proline and pyruvate oxidations. Reaction conditions as in Fig. 1 with exception that sodium phosphate was 24 mM and rotenone excluded in pyruvate studies. At the points indicated, 3.5 μmol proline, 12 μmol pyruvate, 0.3 μmol atractylate, 9.0 μmol ADP were added. Mitochondrial protein, 7.2 mg.

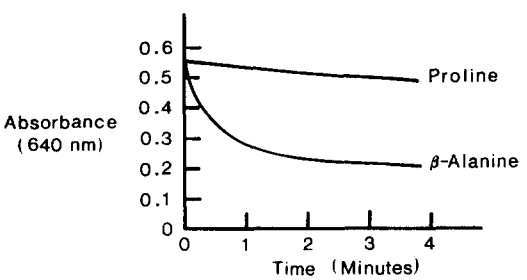

Fig. 3. Effect of isoosmotic solutions of L-proline and β-alanine on mitochondrial volume. Mitochondria (1.3 mg protein) were added to a reaction medium that contained 250 mM L-proline or β-alanine plus 5 mM Tris · HCl, 1 mM EGTA, 5 μg antimycin, 2.8 μM rotenone, pH 7.34. Temperature, 27 °C; volume, 2.6 ml. Light scattering changes were followed at 640 nm. A decrease in absorbance is taken as an increase in mitochondrial volume (swelling).

Sonicated submitochondrial particles exhibit an inverted orientation with respect to the inner membrane of intact mitochondria [16, 17]. In the inverted orientation, it would be expected that proline would have to traverse the inner membrane in order to undergo oxidation. Since the swelling studies indicated that proline does not penetrate the inner membrane, it would be expected that submitochondrial particles would exhibit negligible rates of proline oxidation in comparison to intact mitochondria. This turned out to be the case (Fig. 4). In fact, rates of proline oxidation were so negligible, that it was impossible to discern clear-cut effects of antimycin on the oxidation.

In Fig. 4 are also presented data on glycerol 1-phosphate and succinate oxidations by sonicated particles. These data are in accord with an outside localization for

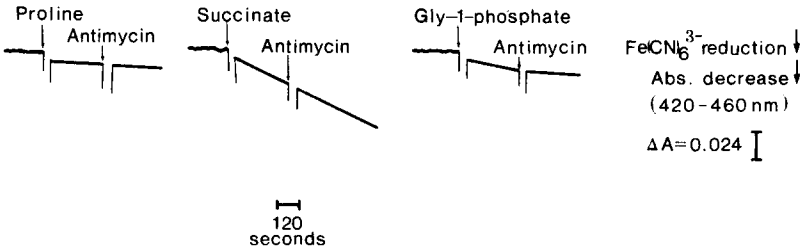

Fig. 4. Ferricyanide reduction by blowfly submitochondrial particles due to proline, succinate and glycerol 1-phosphate oxidations. Reaction conditions as in Fig. 1. Succinate added, 39 μmol; submitochondrial protein, 9.3 mg.

glycerol 1-phosphate oxidation and an inside localization for succinate oxidation in the case of intact mitochondria.

The studies reported here suggest that proline dehydrogenase is localized and operative at, or in close proximity to the outer phase of the inner mitochondrial membrane.

What might be the functional significance of such a localization? Recently, evidence has been obtained in this laboratory of the presence in soluble fractions of flight muscle of pyrroline-5-carboxylate reductase (EC 1.5.1.2) that catalyzes the reduction of pyrroline-5-carboxylate to proline. The enzyme specifically requires NADH and exhibits no activity when NADPH serves in its place as coenzyme. The reaction is also rotenone insensitive. Operation of a soluble pyrroline 5-carboxylate reductase in conjunction with mitochondrial proline dehydrogenase could constitute a proline cycle for the reoxidation of extramitochondrial NADH and the transfer of reducing equivalents to the mitochondrial respiratory chain. In this regard, localization of proline dehydrogenase in the outer phase of the inner membrane could be regarded as an efficient mechanism in the operation of such a cycle since an outside-localized proline dehydrogenase would not require proline to penetrate the inner membrane in order to undergo oxidation. Nor, would the product of proline oxidation pyrroline-5-carboxylate, be required to traverse the inner membrane in order to undergo reduction by the cytoplasmic reductase.

The question arises concerning the feasibility of a proline cycle in blowfly muscle in light of the presence in that tissue of an active glycerol 1-phosphate pathway. In this regard, O'Brien et al. [18] have reported that ring-substituted cinnamic acids are inhibitory to the operation of the glycerol 1-phosphate cycle of housefly flight muscle preparations. Yet, none of the inhibitory compounds were toxic in vivo. It could be that the operation of the glycerol 1-phosphate cycle is not vital in the intact insect because of the existence of alternative mechanisms for the reoxidation of extramitochondrial NADH. A proline cycle could represent such an alternative mechanism.

ACKNOWLEDGEMENT

The authors wish to acknowledge with thanks the support of the Faculty Research Foundation of The City University of New York.

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