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Localization of TCA cycle dehydrogenases in the mitochondria

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Abstract

The site of localization of TCA cycle dehydrogenases in mitochondria has been investigated by observing the dehydrogenase activities and fine structure of the fractionated samples after freezing and thawing or sonication of beef heart and rat liver mitochondria. 1. In the sonicated mitochondria, activities of malic and isocitric dehydrogenases were highest in the supernatant fraction centrifuged at 198,000 x g for 60 minutes, while the specific activity of a-ketoglutaric dehydrogenase was higher in the fluffy or residue fraction. The distribution of the activity of pyruvic dehydrogenase was similar to that of a-ketoglutaric dehydrogenase. 2. In a sucrose density gradient fractionation of the fluffy fraction obtained by centifugation of sonicated mitochondria at 198, 000 x g for 60 minutes, the activities of malic and pyruvic dehydrogenase were observed in the top (or low density) layer in the form of fine particles, while that of a-ketoglutaric dehydrogenase was observed in the middle (or medium density) layers in the form of aggregates of fine particles and membranous fragments. 3. In the samples fractionated after freezing and thawing of mitochondria, which were considered to be a relatively mild disruption, the specific activity of a-ketoglutaric dehydrogenase was higher in the residue (submitochondria) fraction than that in the supernatant fraction (centrifuged at 144,000 x g, 30 minutes), and the activity of malic dehydrogenase still remained significantly high in the residue fraction. 4. It was deduced that the TCA cycle dehydrogenases could be localized in the matrix of the mitochondria by a loose binding to the inner membrane.

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LOCALIZATION OF TCA CYCLE DEHYDROGENASES IN THE MITOCHONDRIA

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It has already been clarified by many investigators[1,2] that TCA cycle dehydrogenases are mainly located in mitochondria. However, it remains still obscure as to the exact sites of localization of these enzymes in the mitochondria. It has been generally considered that the TCA cycle dehydrogenases, except succinic dehydrogenase (EC 1. 3. 99. 1) may be localized in the matrix of mitochondria as they are easily solubilized by mechanical disruption of mitochondria[2,3]. GREEN and his collaborators reported, however, that these enzymes may be localized on the outer membrane of mitochondria[4].

In the present paper, the sites of localization of TCA cycle dehydrogenases in mitochondria were investigated by observing the dehydrogenase activities and structures of the samples fractionated by differential centrifugation and sucrose density gradient after severe or mild disruption of mitochondria.

MATERIALS AND METHODS

Preparation of mitochondria : Mitochondria were isolated from rat liver or beef heart by the modification of the method of HOGEBOOM[5] or CRANE *et al*[6]., respectively.

Sonication of mitochondria : Mitochondria (70 mg protein) were suspended in 10ml of 0.25 M sucrose solution containing 0.01 M Tris-HCl buffer (pH 7.4) and 0.7mg of vitamin E, and frozen at −25°C for 24 hours before sonication. The mitochondria were thawed under tap water and sonicated immediately for 5 minutes at maximum intensity with a sonicator (Kaijo Electric Co., 20 KC, 7 mm tip at 150 W in air).

Freezing-thawing of mitochondria : Mitochondria (100 mg protein) were suspended in 10 ml of 0.05 M sucrose solution containing 0.01 M Tris-

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HCl buffer, pH 7.4, and frozen in dry ice-acetone and thawed under tap water. The freezing and thawing were repeated three times.

Centrifugal fractionation of disrupted mitochondria : Mitochondria disrupted with sonication (So) or the supernatant of frozen-thawed mitochondria centrifuged at 11,000 x g for 15 minutes (S_0) were centrifuged at 33,000 x g for 15 minutes, and separated into supernatant (S_1) and residue (R_1). The S_1 was centrifuged at 144, 000 x g for 30 minutes, and separated into supernatant (S_2) and residue (R_2). The supernatant (S_2) was finally centrifuged at 198, 000 x g for 60 minutes and separated into supernatant (S_3), fluffy layer (F_3) and residue (R_3). The fluffy layer (F_3) was further fractionated on a 0.1 to 0.6 M sucrose density gradient at 160, 000 x g for 60 min.

Assay of dehydrogenase activities in the fraction : The activity of ferricyanide linked α-ketoglutaric dehydrogenase (EC 1. 2. 4. 2) in the fractions was determined by the method of MASSAY[7].

Ferricyanide linked pyruvic dehydrogenase (EC 1, 2. 4. 1) activity in the fractions was measured by a modification of the method of MASSAY[8] in which pyruvate was replaced with α-ketoglutarate as substrate and thiamine pyrophosphate (2.5×10^{-4} M) was used as supplement.

The activity of NADP-linked isocitric dehydrogenase (EC 1. 1. 1. 42) in the fraction was estimated by the method of PLAUT and SUNG[8] in the presence of potassium cyanide (10^{-3} M).

The activity of malic dehydrogenase (EC 1. 1. 1. 37) in the fraction was measured by the method of OCHOA[9] in the presence of 10^{-3}M potassium cyanide.

Determination of protein : Protein was estimated by the method of LOWRY *et al*[10]. or the method of GORNALL *et al.* [11].

Electron microscopy of the fractionated samples : Electron microscope observation was made on the samples negatively stained with 1 % potassium phosphotungstate, pH 7.0[12].

RESULTS

Distribution of TCA cycle dehydrogenase activities in sonicated mitochondrial fractions : Distributions of TCA cycle dehydrogenase activities in sonicated beef heart mitochondria and rat liver mitochondria are shown in Table 1 and Table 2, respectively. The protein recovery was 62.5 or 74.5 % in the supernatant fraction (S_1) of the sonicated beef heart or rat liver mitochondria centrifuged at 33, 000 x g for 15 minutes, respectively. The majority of activities of TCA cycle dehydrogenases was recovered in the S_1 fraction except pyruvic dehydrogenase which was considerably inactivated by the centrifugal

Table 1 TCA cycle dehydrogenase activities in the fractions from sonicated beef heart mitochondria

Fraction	Protein recovery %	Fe(CN)$^{=}_{6}$-Pyruvic DH.		Fe (CN)$^{=}_{6}$-α-KG DH.		NADP-Isocit. DH.		Malic DH.	
		S. A. *	T. A. **	S. A. *	T. A. **	S. A. *	T. A. **	S. A. *	T. A. **
Sonicated Mt S_0	100.0	40	9, 280	106	24, 600	420	97, 400	31	7, 200
33, 000xg, 15′ S_1	62.5	11	1, 600	150	21, 800	320	46, 500	48	6. 950
R_1	41.0	10	950	80	7, 600	96	9, 100	0	
144, 000xg, 30′ S_2	25.3	25	1, 460	160	9, 450	705	41, 600	115	6, 800
R_2	26.7	3	186	50	3, 100	41	2, 500	0	
198, 000xg, 60′ S_3	15.6	21	762	96	3, 500	1000	36, 300	110	4, 000
F_3	3.9	16	118	240	1, 800	568	4, 200	371	2, 745
R_3	4.9	37	418	360	4, 100	158	1, 900	0	

* : S. A., specific activity, mμmole/min/mg protein. ** : T. A., total activity, mμmole/min.

Table 2 TCA cycle dehydrogenase activities in the fractions from sonicated rat liver mitochondria

Fraction	Protein recovery %	Fe(CN)$^{=}_{6}$-Pyruvic DH.		Fe (CN)$^{=}_{6}$-α-KG DH.		NADP-Isocit. DH.		Malic DH.	
		S. A. *	T. A. **	S. A. *	T. A. **	S. A. *	T. A. **	S. A. *	T. A. **
Sonicated Mt S_0	100.0	43	12, 900	37	11, 100	45	13, 500	4.0	1, 200
33, 000xg, 15′ S_1	74.5	20	4, 480	31	6, 950	50	11, 200	3.2	720
R_1	14.2	0		29	1, 230	22	936	0	
144, 000xg, 30′ S_2	52.2	32	5, 030	41	6, 450	60	9, 430	4.2	660
R_2	12.4	0		11	410	10	372	0	
198, 000xg, 60′ S_3	38.5	15	1, 740	36	4, 180	114	13, 200	4.7	545
F_3	7.7	6	121	64	1, 480	60	1, 390	4.6	106
R_3	7.2	21	454	22	475	20	432	4.6	99

* : S. A., specific activity, mμmole/min/mg protein. ** : T. A., total activity, mμmole/min.

separation of sonicated mitochondria. The majority of activities of TCA cycle dehydrogenases in the S_1 fraction was also recovered in the supernatant fraction (S_2) on the centrifugation at 144, 000 x g for 30 minutes. The S_2 fraction was further centrifuged at 198, 000 x g for 60 minutes, and separated into residue (R_3), fluffy layer (F_3) and supernatant (S_3). The recovery of total activity of all TCA cycle dehydrogenases in both heart and liver mitochondria, except α-ketoglutaric dehydrogenase in beef heart mitochondria, was highest in the supernatant fraction (S_3). The specific activities of pyruvic and α-ketoglutaric dehydrogenases were highest in R_3 or F_3 fraction.

As shown in Fig. 1, F_3 and R_3 fractions were composed of particles and membranous structures. The F_3 fraction was further fractionated on a 0.1 M to 0.6 M sucrose density gradient into five layer fractions which were designated as F_3-d_1 (top or 0.1 M sucrose layer) to F_3-d_5 (bottom or 0.6 M sucrose layer). Activities of pyruvic, α-ketoglutaric, and malic dehydrogenases were determined on each of these fractions (Table 3) and electron microscope observation was made (Figs. 2 to 6).

Table 3 Malic, pyruvic and α-ketoglutaric dehydrogenase activities in the density gradient fractions of the fluffy layer* from sonicated beef heart mitochondria

Fraction	Protein recovery %	Malic DH.		$Fe(CN)^{=}_{6}$-Pyruvic DH.		$Fe(CN)^{=}_{6}$-α-KG DH.	
		S. A. **	T. A. ***	S. A. **	T. A. ***	S. A. **	T. A. ***
F_3	100	190	3,990	60	1,260	230	4,830
F_3-d1	47	139	1,390	44	440	65	650
F_3-d2	33	63	442	0		73	520
F_3-d3	18	34	129	0		227	865
F_3-d4	7	0		0		210	328
F_3-d5	2	0		0		0	

*: Fluffy layer of 198,000 xg, for 60 min. **: S. A., specific activity, mμmole/min/mg protein. ***: T. A., total activity, mμmole/min.

The activity of malic dehydrogenase was mainly recovered in the F_3-d_1 fraction, while that of α-ketoglutaric dehydrogenase was mainly recovered in the F_3-d_3 fraction. Although the activity of pyruvic dehydrogenase was considerably inactivated with this procedure, the activity observed was mainly recovered in the F_3-d_1 fraction.

The F_3-d_1 fraction contained mainly of small particles measuring approximately 40 to 100 Å in diameter and the F_3-d_3 fraction contained fairly large particles or aggregates of particles (200 to 500 Å) and small membranous fragments (300 to 600 Å in diameter).

Distribution of α-ketoglutaric and malic dehydrogenase activities in the frozen-thawed mitochondrial fraction : A further attempt has been made to obtain some clues about the binding of TCA cycle dehydrogenases to the mitochondrial inner membrane. After freezing-thawing of mitochondria, which was considered as a relatively mild disruption, the mitochondria were centrifuged at 11,000 x g for 15 minutes, and the supernatant (S_0) was further centrifuged at 33,000 x g for 15 minutes, and separated into supernatant (S_1) and residue (R_1). The supernatant (S_1) was then centrifuged at 144,000 x g for 30 minutes, and separated into the supernatant (S_2) and residue (R_2).

Table 4 shows α-ketoglutaric and malic dehydrogenase activities in these

Table 4 Malic and α-ketoglutaric dehydrogenase activities in the fraction from frozen-thawed rat liver mitochondria

Fraction	Protein recovery %	Malic dehydrogenase		$Fe(CN)^{=}_{6}$-α-KG dehydrogenase	
		S. A. *	T. A. **	S. A. *	T. A. **
11, 000xg, 15′ S_0	100	20	3, 940	122	24, 020
33, 000xg, 15′ S_1	61	20	2, 400	187	22, 430
R_1	20	14	545	121	4, 720
144, 000xg, 30′ S_2	56	21	2, 310	166	18, 250
R_2	8	7	105	335	5, 330

* : S. A', specific activity, mμmole/min/mg protein ** : T. A., total activity, mμmole/min

fractions. In this relatively mild disruption of mitochondria, the specific activity of α-ketoglutaric dehydrogenase was somewhat higher in the R_2 fraction, which was regarded as inner membrane fraction, than in the S_2 fraction. The α-ketoglutaric dehydrogenase in R_2 fraction was solubilized in supernatant fraction by repeated washing of the R_2 fraction. On the other hand, the majority of malic dehydrogenase activity was recovered in S_2 fraction.

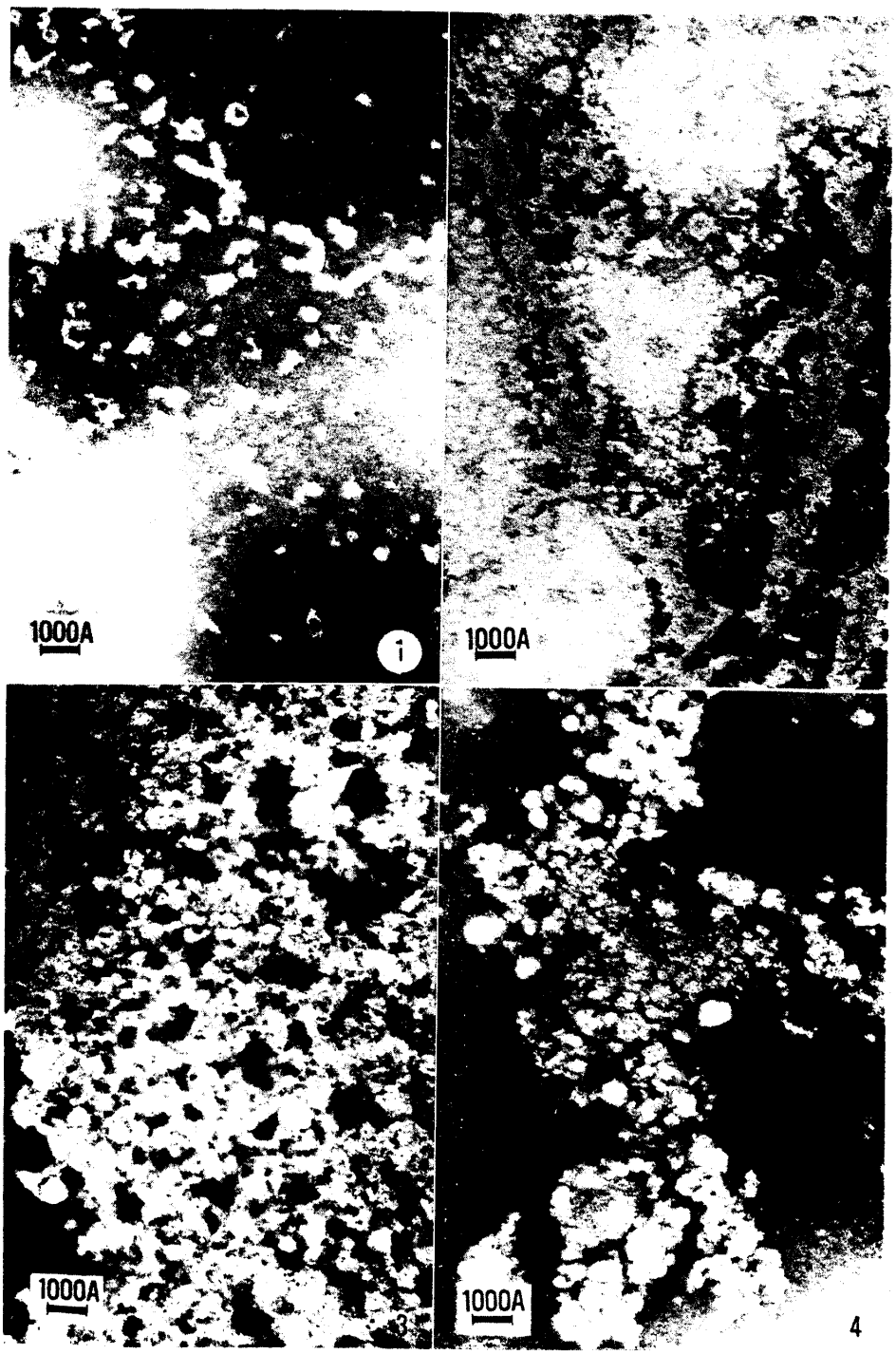
DISCUSSION

The analytical data summarized in Table 5 and Fig. 7 indicate that the degree of the release of TCA cycle dehydrogenases is consistent with that of the rupture of the membranous envelopes; however, there seems to exist a difference in the mode of binding of these dehydrogenases to the mitochondrial membrane.

Table 5 The dissociability of TCA cycle dehydrogenase in mitochondria

Dissociability	Dehydrogenase
Sonic non-dissociable	Succinic DH., ($NADH_2$ DH.)
dissociable	Pyruvic, Isocitric, α-KG, Malic Dehydrogenases
Freezing-thawing	
dissociable (incompletely)	α-KG DH., Pyruvic DH.
(almost completely)	Malic DH., Isocitric DH.
Hypotonic dissociable	(Cytochrome c in part), (Adenylate Kinase)

This assumption may be supported by the finding that the specific activity of α-ketoglutaric dehydrogenase was highest in the membrane fraction (R_2) obtained from frozen-thawed mitochondria. Electron microscope observation revealed that the submitochondrial fraction (R_2) was composed mainly of membrane fragments derived from the inner membrane containing the elementary particles[13].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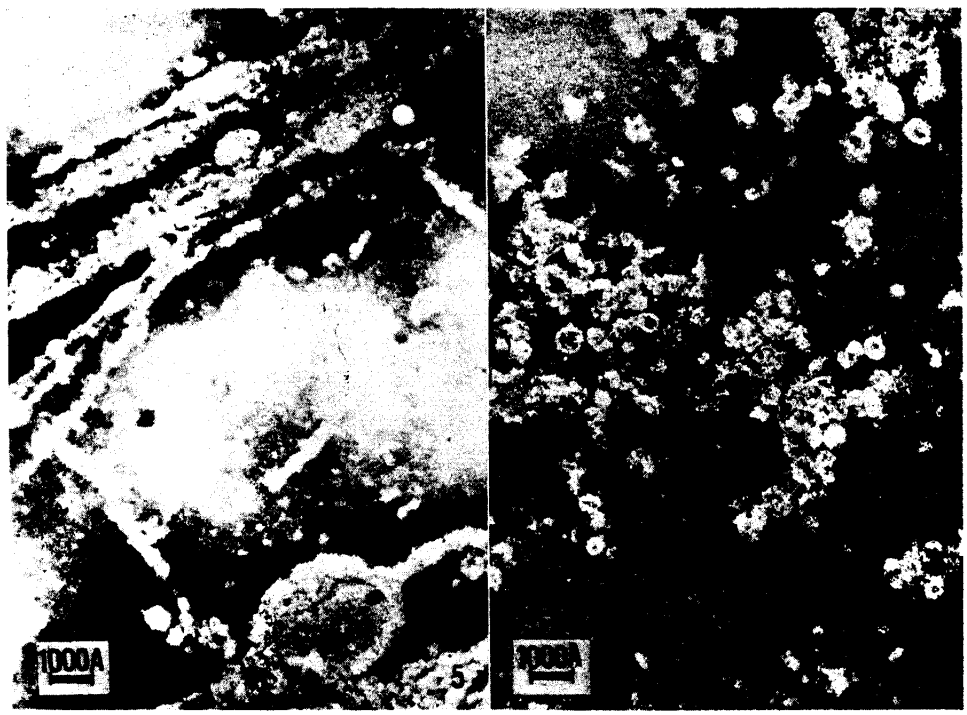

Fig. 1 Electron micrograph of fluffy (F_3) fraction obtained from sonicated beef heart mitochondria (PTA negative staining). (×55,000)

Fig. 2 Electron micrograph of F_3-d_1 fraction obtained from F_3 fraction by sucrose density gradient. (×55,000)

Fig. 3 Electron micrograph of F_3-d_2 fraction obtained from F_3 fraction by sucrose density gradient. (×55,000)

Fig. 4 Electron micrograph of F_3-d_3 fraction obtained from F_3 fraction by sucrose density gradient. (×55,000)

Fig. 5 Electron micrograph of F_3-d_3 fraction. (same specimen as in Fig. 4). (×55,000)

Fig. 6 Electron micrograph of F_3-d_4 fraction obtained from F_3 fraction by sucrose density gradient. (×55,000)

The α-ketoglutaric dehydrogenase in the R_2 fraction can be solubilized by repeated washing of the R_2 fraction. Malic dehydrogenase, on the other hand, is easily solubilized by freezing-thawing of mitochondria. However, hypotonic treatment does not release TCA cycle dehydrogenases in any significant amount, although it does adenylate kinase and secondary phosphate transferases, which are supposed to be localized between the outer and the inner membranes of mitochondria[14].

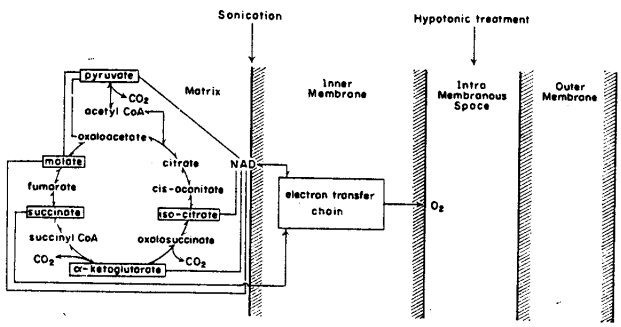

Fig. 7 Localization of TCA cycle dehydrogenases and electron transfer chain in the mitochondria

Recently, some informations have been obtained about the isolation of outer membrane and the localization of enzymes on the outer membrane of mitochondria. GREEN and his collaborators[4,15-17] reported on the isolation and properties of the mitochondrial outer membrane, in which the activities of pyru vic dehydrogenase complex and citric cycle enzymes were observed. Further, it has been reported that rotenone insensitive $NADH_2$-cytochrome c reductase (EC 1. 6. 2. 1) and cytochrome b^5, of which α-band differs somewhat from that in endoplasmic reticulum, are contained in the mitochondrial outer membrane[18,19]. SCHNEITMAN *et al*[20]. demonstrated monoamine oxidase (EC 1. 4. 3. 4) to be a specific enzyme marker for the mitochondrial outer membrane.

The data presented in the present paper suggest that TCA cycle dehydrogenases seem to be localized in the matrix, in which α-ketoglutaric dehydrogenase may be loosely bound to the inner membrane while malic dehydrogenase may be of a soluble form. This assumption is compatible with the fact that externally added $NADH_2$ is scarcely oxidized by intact mitochondria as it cannot enter into the intact mitochondria, while it is most rapidly oxidized by inner membrane fragments, whose matrix side of the membrane is exposed to the reaction medium.

It is suggested that the localization of TCA cycle dehydrogenases in a close relation to the electron transfer chain is rational for the smooth operation of oxi-

dative phosphorylation in the mitochondria.

SUMMARY

The site of localization of TCA cycle dehydrogenases in mitochondria has been investigated by observing the dehydrogenase activities and fine structure of the fractionated samples after freezing and thawing or sonication of beef heart and rat liver mitochondria.

1. In the sonicated mitochondria, activities of malic and isocitric dehydrogenases were highest in the supernatant fraction centrifuged at 198, 000 x g for 60 minutes, while the specific activity of α-ketoglutaric dehydrogenase was higher in the fluffy or residue fraction. The distribution of the activity of pyruvic dehydrogenase was similar to that of α-ketoglutaric dehydrogenase.

2. In a sucrose density gradient fractionation of the fluffy fraction obtained by centifugation of sonicated mitochondria at 198, 000 x g for 60 minutes, the activities of malic and pyruvic dehydrogenase were observed in the top (or low density) layer in the form of fine particles, while that of α-ketoglutaric dehydrogenase was observed in the middle (or medium density) layers in the form of aggregates of fine particles and membranous fragments.

3. In the samples fractionated after freezing and thawing of mitochondria, which were considered to be a relatively mild disruption, the specific activity of α-ketoglutaric dehydrogenase was higher in the residue (submitochondria) fraction than that in the supernatant fraction (centrifuged at 144, 000 x g, 30 minutes), and the activity of malic dehydrogenase still remained significantly high in the residue fraction.

4. It was deduced that the TCA cycle dehydrogenases could be localized in the matrix of the mitochondria by a loose binding to the inner membrane.

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