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THE LOCALIZATION OF MITOCHONDRIAL CREATINE KINASE, AND ITS USE FOR THE DETERMINATION OF THE SIDEDNESS OF SUBMITOCHONDRIAL PARTICLES

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SUMMARY

1. Creatine kinase (EC 2.7.3.2) is extracted from heart mitochondria by suspension in salt-containing media.

2. Digitonin treatment of isolated heart mitochondria causes rupture of the outer membranes and release of adenylate kinase (EC 2.7.4.3), while creatine kinase remains bound to the mitochondrial membranes. Although the outer membranes are ruptured by the action of digitonin, they can not be obtained in reasonable yield by differential centrifugation, indicating that most of the ruptured outer membranes are still connected to the inner membranes, which could be confirmed by electron microscopy.

3. The addition of the detergent Lubrol WX to intact heart mitochondria has no effect upon the activity of the creatine kinase, indicating that this enzyme is not latent.

4. In submitochondrial particles, obtained by ultrasonic treatment of heart mitochondria and removal of intact mitochondria by centrifugation, creatine kinase becomes partially latent. This indicates that in some of the submitochondrial particles the enzyme is localized inside the enveloping inner membrane fragment. Evidence is also presented for the existence of submitochondrial particles with the same orientation of the enveloping membrane as intact mitochondria, as indicated by the latency of aspartate aminotransferase (EC 2.6.1.1), a matrix enzyme that is firmly bound to the inside of the inner membrane.

5. From Paragraphs 2–4 it is concluded that mitochondrial creatine kinase is bound to the outside of the inner mitochondrial membrane, in contrast to mitochondrial adenylate kinase, which is not bound to either of the mitochondrial membranes.

INTRODUCTION

The mitochondrial isoenzyme of creatine kinase was discovered by Jacobs *et al.*[1] in rat heart, skeletal muscle and brain, and in pigeon-breast muscle. Since

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the enzyme could be extracted from rat-heart mitochondria by 0.1 M phosphate buffer (pH 7.2), the enzyme was thought to be localized in the intermembrane[2,3], *i.e.* the enzyme is present in a soluble form between the inner and outer mitochondrial membrane[4]. In this communication, however, evidence is presented that in heart mitochondria creatine kinase is bound to the outer side of the inner membrane, in contrast to adenylate kinase, which is a true intermembrane enzyme, as in rat-liver mitochondria[5–7].

METHODS

The isolation of mitochondria and the preparation of submitochondrial particles

Guinea-pig-heart mitochondria were isolated in 0.25 M sucrose according to Holton *et al.*[8], as described by Wit-Peeters[9]. Rat-heart mitochondria were isolated in 0.25 M sucrose with the aid of a proteinase[10,11]. Two rat hearts (1.4 g) were minced and washed. The mince was taken up in 10 ml 0.25 M sucrose and 0.3 ml of a trypsin solution (2 mg/ml 0.25 M sucrose) was added. After 5 and 10 min at 0 °C the mince was homogenized by one stroke in an electrically driven Potter–Elvehjem homogenizer. 15 min after the trypsin addition the digestion was stopped with 0.4 ml of soybean trypsin inhibitor (Calbiochem, 3 × crystallized 2 mg/ml 0.25 M sucrose), followed by 10 strokes of the homogenizer. The suspension was centrifuged for 3 min at $600 \times g_{max}$ and the supernatant was centrifuged for 10 min at $4500 \times g_{max}$. The sediment was homogenized by hand in a Potter–Elvehjem homogenizer, and again centrifuged for 10 min at $4500 \times g_{max}$. The fluffy layer was discarded. The last centrifugation step was repeated twice. The resulting pellet (the supernatant did not contain mitochondria) was homogenized in 2.5 ml 0.25 M sucrose. 2 ml of this suspension was subjected to ultrasound (0.5 min at 21 kHz, 2 μm from peak to peak) and after sampling the sonicate was centrifuged for 10 min at $15000 \times g_{max}$ to remove intact mitochondria from the supernatant. The latter was used as a preparation of submitochondrial particles.

The digitonin treatment

Rat-heart mitochondria used for the digitonin treatment were also isolated after trypsin digestion, but in a different medium and with a different centrifugation scheme. The isolation medium was 0.25 M sucrose, 10 mM Tricine–KOH and 1 mM EDTA (pH 7.4 at 0 °C). The 3 min, $600 \times g_{max}$ supernatant was centrifuged for 10 min at $19000 \times g_{max}$. After homogenization of the sediment, the latter centrifugation step was repeated twice. The resulting pellet was homogenized in a few drops of medium and incubated with digitonin[12,13] as indicated. Finally, the suspension was centrifuged and separated into a 10 min, $40000 \times g_{max}$ pellet (Fraction 1), a 1 h, $230000 \times g_{max}$ pellet (Fraction 2) and a 1 h, $230000 \times g_{max}$ supernatant (Fraction 3).

The fractionation of rat heart

A trypsin-treated rat-heart homogenate (see above) was prepared and fractionated in a medium containing 0.25 M sucrose, 10 mM Tricine–KOH and 1 mM EDTA (pH 7.4 at 0 °C). The fractionation was carried out essentially according to Hulsmans[14] as described in earlier work[15], after filtration through fine-mesh nylon cloth. The homogenate was separated into a nuclear fraction (N) and a nuclear-free homo-

genate (E). The latter fraction was further separated into a mitochondrial fraction (M), a microsomal fraction (P) and a particle-free supernatant (S).

Assays

Monoamine oxidase, EC 1.4.3.4 [expressed in units of μmoles tyramine consumed per min at 37 °C and pH 7.95 (ref. 16)]; cytochrome *c* oxidase, EC 1.9.3.1 (μatoms O consumed per min at 25 °C); aspartate aminotransferase (μmoles NADH oxidized per min at 25 °C); creatine kinase (μmoles NADH oxidized per min at 25 °C); adenylate kinase (μmoles $NADP^+$ reduced per min at 25 °C) and pyruvate kinase, EC 2.7.1.40 (μmoles NADH oxidized per min at 25 °C) were determined as described in refs 17, 13, 18, 19, 5 and 20, respectively. Where NADH oxidation was measured, 1.5 μM rotenone was present.

Propionyl-CoA carboxylase, EC 6.4.1.3 (μmoles propionyl-CoA carboxylated per min at 25 °C) was assayed as described in ref. 21 with several modifications. The reaction was carried out in liquid-scintillation vessels placed in a water-bath. The reaction mixture contained, in a final volume of 0.5 ml, 50 mM Tricine–KOH (pH 8.5), 10.4 mM $KH^{14}CO_3$ (226 nCi), 3 mM ATP, 6 mM $MgCl_2$, 0.60 mM propionyl-CoA, 100 mM KCl, 1 mM dithiothreitol, 5 mM tricyclohexylammonium phosphoenolpyruvate, 4 μg pyruvate kinase. The reaction was started by the addition of enzyme. After 15 min the reaction was stopped by the addition of 0.05 ml 2.5 M H_2SO_4. The vessels were gassed with N_2 for 5 s and placed in a vacuum desiccator, which was evacuated by a water pump. After 30 min 10 ml scintillation liquid was added [toluene with fluors–Triton X-100–ethanol (8:4:3, v/v/v)]. After standing at 8 °C, the fluid was mixed and counted in a Packard liquid-scintillation counter, Model 3380, supplied with an absolute activity analyzer, Model 544.

Unless otherwise stated, cytochrome *c* oxidase, aspartate aminotransferase and propionyl-CoA carboxylase were determined after treatment with 0.025% Lubrol WX (ICI) for 12 min at 0 °C (see ref. 6).

The treatment with trypsin (see above) had no marked effect on the activities of the enzymes studied.

Protein was determined according to Lowry *et al.*[22], with egg albumin as a standard.

RESULTS

Extraction of creatine kinase from heart mitochondria

Fig. 1 shows the extraction of creatine kinase from guinea-pig-heart mitochondria by treatment with different concentrations of phosphate. Most creatine kinase activity was released with 40 mM P_i.

Mitochondrial creatine kinase can also be liberated by other salt-containing media. When rat-heart mitochondria were prepared in the medium designed by Chappell and Perry[23] [0.1 M KCl, 50 mM Tris, 5 mM $MgCl_2$, 1 mM ATP and 1 mM EDTA (pH 7.4)] or in 0.1 M KCl the creatine kinase activity of the mitochondria was only 25 and 50%, respectively, of that of mitochondria isolated in 0.25 M sucrose. This may explain why Baskin and Deamer[24] were unable to find creatine kinase activity in rabbit skeletal-muscle mitochondria, isolated in 0.1 M KCl.

Digitonin treatment of heart mitochondria

At digitonin concentrations that enable the isolation of outer membranes from liver mitochondria, only a very small amount of impure outer membranes (Fraction 2, see Methods) can be isolated from heart mitochondria, as judged by

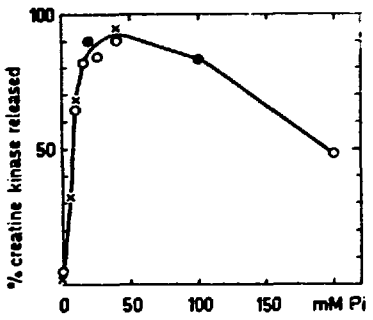

Fig. 1. The extraction of creatine kinase from guinea-pig-heart mitochondria by phosphate. 0.1 ml guinea-pig-heart mitochondria (1.05 mg and 1.07 mg protein in the two experiments shown) in 0.25 M sucrose were suspended for 30 min at 0 °C in 5 ml potassium phosphate buffer (pH 7.2), after which the suspension was centrifuged for 10 min at $26000 \times g_{max}$ and the activity of creatine kinase measured in the supernatant. The activity of the untreated mitochondria was taken as 100%. The specific activity was 1.25 and 0.98 unit/mg. The points ○ and × represent two experiments.

the activity of the outer-membrane marker enzyme monoamine oxidase (Table I and Fig. 2; *cf.* ref. 25). The 1 h, $230000 \times g_{max}$ supernatant (Fraction 3) contains 89% of the adenylate kinase activity, but only 3% of the mitochondrial creatine kinase activity. The corresponding pellet (Fraction 2) contains 4.6% of the monoamine oxidase activity, 3% of the protein and 1.2% of the creatine kinase activity. These data indicate that when the outer membranes are ruptured, as judged by the leakage of adenylate kinase, creatine kinase remains membrane bound, most probably to the inner membranes. Electron microscopy (see Fig. 3) confirms that the outer membranes are ruptured and that the outer-membrane fragments remain in the vicinity of the inner membrane.

TABLE I

THE FRACTIONATION OF RAT-HEART MITOCHONDRIA WITH DIGITONIN

To 21.4 mg of washed rat-heart mitochondria in 0.33 ml 0.25 M sucrose, 10 mM Tricine–KOH and 1 mM EDTA (pH 7.4) was added 0.45 ml of the same buffered sucrose–EDTA solution containing 4.7 mg digitonin (dissolved at 100 °C). After 20 min of stirring at 0 °C 7.5 ml buffered sucrose–EDTA was added, a sample was taken to assess the recoveries, and the remainder was centrifuged as described in Methods. Mit., digitonin-treated mitochondria; Fraction 1: 10 min, $40000 \times g_{max}$ pellet; Fractions 2 and 3: 1 h, $230000 \times g_{max}$ pellet and supernatant, respectively. The corresponding "De Duve-plots" are given in Fig. 2.

	Absolute values	*Percentage values*			*Recovery*
	Mit.	*1*	*2*	*3*	
Protein (mg)	21.4	94.5	3.0	6.5	104.0
Monoamine oxidase (munits)	155	66.8	4.6	15.2	86.5
Creatine kinase (units)	29.4	96.2	1.2	3.0	100.3
Adenylate kinase (units)	2.54	11.2	0.2	89.1	100.5

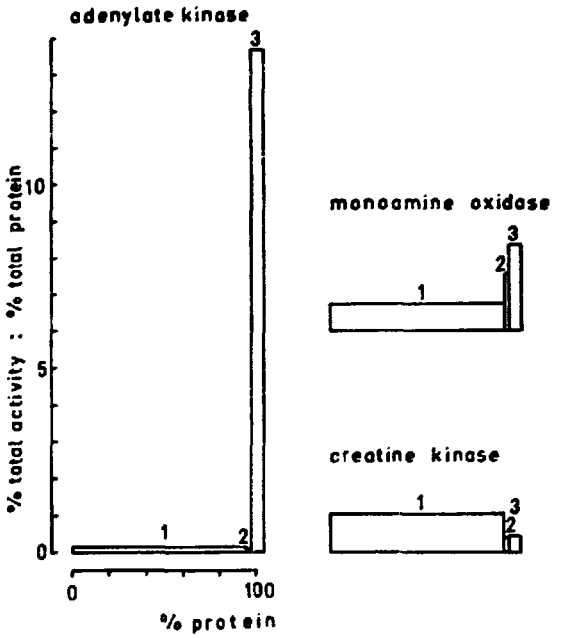

Fig. 2. The fractionation of rat-heart mitochondria with digitonin. For conditions see Table I. 1, 2 and 3 refer to the fractions described in the legend of Table I.

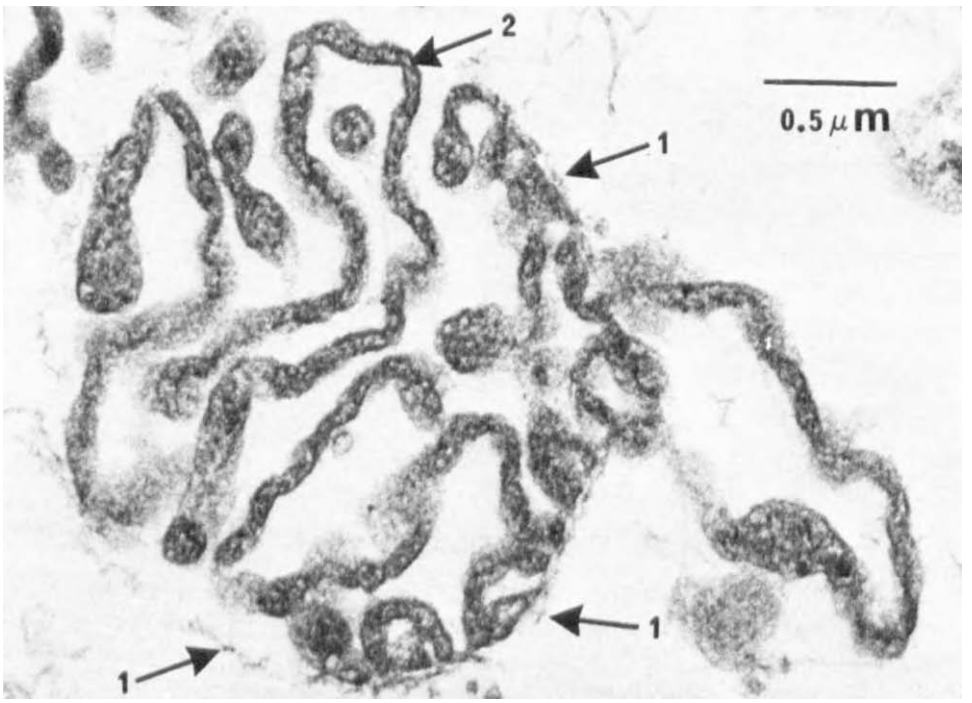

Fig. 3. An electron micrograph of a guinea-pig-heart mitochondrion treated with digitonin. Guinea-pig-heart mitochondria (50 mg per ml 0.25 M sucrose) were stirred with the same volume of digitonin (0.5 mg per ml 0.25 M sucrose, dissolved at 100 °C) for 40 min at 0 °C. The preparation was fixed in 1% glutaraldehyde dissolved in 0.25 M sucrose, for 20 min at 0 °C, followed by 2% OsO_4 in sucrose solution (pH 7.0 tonicity 320 mosM). 1, outer membrane; 2, inner membrane.

On the nature of adenylate kinase activity in heart mitochondria

The question arises concerning the nature of the adenylate kinase activity found in the mitochondria. Since the bulk of the cardiac adenylate kinase is located in the cytosol[26], the possibility has to be considered that the activity in the mito-

TABLE II

THE INTRACELLULAR DISTRIBUTION OF ADENYLATE KINASE IN RAT HEART

As marker enzymes were used cytochrome *c* oxidase for the mitochondria and pyruvate kinase for the cytosol. E is the nuclear-free homogenate, N is the nuclear fraction, M is the mitochondrial fraction, P is the microsomal fraction and S is the final supernatant.

	Absolute values	*Percentage values*					*Recovery*
	E+N	*E+N*	*N*	*M*	*P*	*S*	
Protein (mg)	207	100	46.0	18.0	4.7	27.4	96.0
Cytochrome *c* oxidase (units)	445	100	22.0	76.2	6.5	2.9	107.5
Pyruvate kinase (units)	75.3	100	3.0	0.3	2.2	91.6	97.0
Adenylate kinase (units)	323	100	4.3	1.0	1.0	92.9	99.2

chondrial fraction is due solely to contamination with the cytosolic enzyme. This, however, is unlikely since only 0.3% of the supernatant enzyme pyruvate kinase is found in the mitochondrial fraction, compared with 1% of the adenylate kinase (ref. 26 and Table II).

A part (11%) of the mitochondrial adenylate kinase activity is not released upon digitonin treatment. Possibly this activity is due to nucleoside monophosphokinase (EC 2.7.4.3), which in liver mitochondria is located in the matrix[27].

The determination of the sidedness of submitochondrial particles by measuring the latency of creatine kinase and aspartate aminotransferase

It is generally assumed that submitochondrial particles obtained by sonication originate by parts of the mitochondrial cristae being pinched off and resealed in such a way that vesicles are formed with the intermembrane space inside, and consequently with outer surfaces that correspond to the original matrix side of the cristae[28–30]. On the basis of electron microscopic evidence Weijers and coworkers[31,32] proposed that submitochondrial particles obtained by sonication may consist of two types, one type with intermembrane space inside, and the other with matrix space inside. In the former type of submitochondrial particles, the so-called "inside-out" particles, enzymes that are bound to the outside of the inner membrane must become latent, whereas matrix enzymes become overt. On the other hand, the latter (or "inside-in") particles behave like intact mitochondria in that enzymes bound to the outside of the inner membrane are overt and matrix enzymes are latent. Of course this holds only if the inner membrane is impermeable to the substrate of the enzyme measured.

Aspartate aminotransferase was chosen as a marker for the matrix side of the inner membrane, to which it is firmly bound[33], and creatine kinase was used as a marker for the outer surface of the inner membrane. The enzyme activities were measured in intact, in briefly sonicated mitochondria and in submitochondrial particles, in the presence or absence of the non-ionic detergent Lubrol WX, to assess the latencies of the enzymes. Latency is expressed as the percentage latent activity, *viz.* 100 × (activity measured with Lubrol *minus* activity measured without Lubrol): (activity measured with Lubrol).

TABLE III

THE LATENCY OF CREATINE KINASE AND ASPARTATE AMINOTRANSFERASE IN INTACT AND SONICATED RAT-HEART MITOCHONDRIA AND IN RAT-HEART SUBMITOCHONDRIAL PARTICLES

Rat-heart mitochondria in 0.25 M sucrose (1.2–5.5 mg/ml) were sonicated as described in Methods. After sampling, the suspension was centrifuged for 10 min at $15000 \times g_{max}$ to remove the intact mitochondria. The supernatant, used as a preparation of submitochondrial particles, contained $36.3 \pm 2.7\%$ of the total mitochondrial protein, $21.4 \pm 2.5\%$ of the mitochondrial creatine kinase activity, and $18.8 \pm 1.5\%$ of the mitochondrial aspartate aminotransferase activity. The averages of 5–8 determinations are given ± the S.E. (standard error of mean). The averages of the latencies were calculated separately. In one experiment monoamine oxidase was measured (at 25 °C). 4.8 mg protein/ml was used. The submitochondrial particles contained 39% of the total mitochondrial activity and 37% of the protein. The monoamine oxidase activity was not latent.

Sample	*Creatine kinase*			*Aspartate aminotransferase*		
	−Lubrol	*+Lubrol*	*Latency*	*−Lubrol*	*+Lubrol*	*Latency*
	(unit/mg)		*(%)*	*(unit/mg)*		*(%)*
Intact mitochondria	1.14±0.06	1.14±0.06	0.3±1.0	0.63±0.08	4.02±0.08	81.1±1.
Sonicated mitochondrial suspension	0.92±0.06	1.02±0.03	9.8±5.2	1.71±0.18	3.63±0.23	53.2±2.
Submitochondrial particles	0.51±0.01	0.61±0.03	22.6±3.3	1.47±0.12	1.88±0.14	21.8±2.

Table III shows that in intact mitochondria creatine kinase is overt and that aspartate aminotransferase is largely (81%) latent. In briefly sonicated mitochondria both enzymes show latency. The latency of aspartate aminotransferase can be explained by the presence of intact mitochondria. When these were removed by centrifugation for 10 min at $15000 \times g_{max}$, submitochondrial particles remained in the supernatant in which creatine kinase showed a latency of $23 \pm 3\%$ and aspartate aminotransferase of $22 \pm 3\%$. This indicates that (assuming for the purpose of the comparison that all submitochondrial particles are of the same size) $23 \pm 3\%$ of the submitochondrial particles are perfectly resealed "inside-out", and $22 \pm 3\%$ are perfectly resealed "inside-in" particles. The remaining submitochondrial particles must have been damaged. They are either imperfectly resealed after the sonication or reopened during centrifugation or during the assay. It is unlikely that submitochondrial particles exist in which one of the enzymes has been moved to the opposite side of the membrane, since both enzymes are firmly bound under the conditions of the experiment[33,34].

Latency of aspartate aminotransferase in sonicated mitochondria is not due to the presence of intact mitochondria. This was shown by experiments in which the matrix enzyme propionyl-CoA carboxylase, which is only loosely bound, if at all, to the inner side of the inner membrane[21,33] was used as a marker for the intactness of the mitochondria. This enzyme has a very good chance to escape between the pinching-off of parts of the cristae, and the resealing of the submitochondrial particles. Indeed, it may be concluded from the results given in Table IV that propionyl-CoA carboxylase is not caught by the closing submitochondrial vesicle, since it is not latent in the submitochondrial particles, and it cannot be sedimented by centrifugation for

TABLE IV

THE LATENCY OF CREATINE KINASE, ASPARTATE AMINOTRANSFERASE AND PROPIONYL-CoA CARBOXYLASE IN RAT-HEART SUBMITOCHONDRIAL PARTICLES

Submitochondrial particles were prepared according to Methods by centrifugation of a sonicated mitochondrial suspension for 10 min at $15000 \times g_{max}$. After sampling the suspension was centrifuged for 30 min at $249000 \times g_{max}$, and the sediment was suspended in 0.25 M sucrose with the aid of a loose fitting Potter–Elvehjem homogenizer. In the 30 min, $290000 \times g_{max}$ sediment, 55% of the protein, 73% of the creatine kinase, 79% of the aspartate aminotransferase and 1.0% of the propionyl-CoA carboxylase activity was recovered from the 10 min, $15000 \times g_{max}$ supernatant. The remaining propionyl-CoA carboxylase activity was recovered in the 30 min, $290000 \times g_{max}$ supernatant.

Enzyme	*Submitochondrial particles*					
	10 min, 15000 × g_{max} supernatant			*30 min, 290000 × g_{max} sediment*		
	−Lubrol	*+Lubrol*	*Latency*	*−Lubrol*	*+Lubrol*	*Latency*
	(unit/mg)		*(%)*	*(unit/mg)*		*(%)*
Creatine kinase	0.43	0.71	39.7	0.57	0.93	39.3
Aspartate aminotransferase	0.76	1.07	29.2	1.08	1.53	29.8
Propionyl-CoA carboxylase	0.042	0.041	−1.7	0.00075	0.00073	−2.7

30 min at $249000 \times g_{max}$, this in contrast to the behaviour of aspartate aminotransferase and creatine kinase.

The lack of latency of monoamine oxidase in the submitochondrial particles proves that inclusion of outer-membrane fragments into the submitochondrial particles does not occur (Table III).

Chance *et al.*[35] have also raised the possibility of the existence of a heterogeneous population of vesicles after sonication of mitochondria. However, they prefer to explain their results by the presence of a single particle that is "bisided" with respect to the location of respiratory chains. The present experiments show the existence of both vesicles with matrix space inside and vesicles with intermembrane space inside in a preparation of submitochondrial particles.

When the phosphate extraction method[2,3] is used for the localization of enzymes in heart mitochondria, creatine kinase is a marker enzyme for all enzymes that can be extracted with phosphate, including intermembrane enzymes, and those that can be released from the outer side of the inner membrane and from both sides of the outer membrane, if such enzymes exist.

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