Biochimica et Biophysica Acta 891 (1987) 138–144
Elsevier

BBA 42506

Specific inhibition of ATP–ADP translocase in cardiac mitoplasts by antibodies against mitochondrial creatine kinase

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(Received 19 August 1986)

Key words: Creatine kinase antibody; Translocase, ATP-ADP; Enzyme inhibition; Immunochemistry; (Heart)

Mitochondrial creatine kinase was purified from rat hearts and used to produce antibodies in chicken and rabbits. Antibodies were purified to a high degree of homogeneity by an affinity chromatography method. Chicken antibodies against mitochondrial creatine kinase inhibited this enzyme in rat-heart mitochondrial inner membrane and matrix preparation, and simultaneously blocked oxidative phosphorylation. Under these conditions respiratory chain activities remained unchanged, but adenine nucleotide translocase was inhibited. Removal of mitochondrial creatine kinase from the membrane by pretreatment with 0.15 M KCl and 20 mM ADP completely abolished the effect of antibodies against mitochondrial creatine kinase on oxidative phosphorylation. Noninhibitory antibodies from rabbit with high affinity to rat mitochondrial creatine kinase inhibited neither creatine kinase activity nor oxidative phosphorylation. These data show close and specific spatial arrangement of mitochondrial creatine kinase and adenine nucleotide translocase in mitochondria. It is supposed that there is a fixed orientation of these proteins in the cardiolipin domain in the membrane and that their interaction may occur by a frequent collision due to their lateral movement.

Introduction

High activity of mitochondrial creatine kinase is found in heart, skeletal muscle and brain [1]. This specific creatine kinase isoenzyme is localized on the outer side of the inner mitochondrial membrane and is thought to be an initial part of the phosphocreatine shuttle for intracellular energy transport [2–7]. The functional cooperation between mitochondrial creatine kinase and adenine nucleotide translocase is a well-documented phenomenon: creatine kinase uses ATP produced in mitochondrial matrix for efficient phosphorylation of cytoplasmic creatine and by direct delivery of ADP controls both translocase activity and mitochondrial oxidative phosphorylation in these types of cell [4–10]. However, the precise structural relationship between creatine kinase and ADP–ATP translocase is not known. This problem was studied in the present work by using purified antibodies against mitochondrial creatine kinase.

Preliminary report of this work has been published [11].

Materials and Methods

Mitochondria were isolated from rat heart by using a trypsin digestion method [8]. Mitoplasts (inner membrane + matrix preparation) were ob-

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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tained according to Schnaitman and Greenawalt [12]. Antibodies against rat-heart mitochondrial creatine kinase were produced in chicken or rabbits. Purified mitochondrial creatine kinase was obtained by the method of Blum et al. [13] and injected into animals subcutaneously in amounts of 100 μg three times with an interval of 10 days. After 6 days following the last injection blood was taken to isolate purified antibodies by an affinity chromatography method using Sepharose columns with immobilized mitochondrial creatine kinase.

Binding of purified antibodies with creatine kinase was determined by the ELISA method [13]. Creatine kinase activity was assayed spectrophotometrically at 340 nm in a medium containing 0.25 M sucrose/25 mM HEPES-Na (pH 7.4)/5 mM $MgCl_2$/0.5 mM dithiothreitol/1 mM ADP/10 mM phosphocreatine/20 mM glucose/0.6 mM NADP/7 mM AMP/2 IU per ml hexokinase/2 IU glucose-6-phosphate dehydrogenase. Temperature was 30 °C. The velocity of oxidative phosphorylation was determined spectrophotometrically by recording the rates of ATP production at 340 nm in a medium containing 0.25 M sucrose/10 mM borate-Na/10 mM Tris-HCl (pH 7.4)/20 mM succinate/3 mM KH_2PO_4/0.7 mM NADP/0.1 mM ADP/20 mM glucose/0.2 mM dithiothreitol/5 mM $MgCl_2$/2 IU per ml hexokinase/2 IU per ml glucose-6-phosphate dehydrogenase. Temperature was 30 °C.

The content of cytochrome aa_3 was determined at the wavelength pair 630–605 nm in an Aminco DW ultraviolet spectrophotometer taking $\varepsilon = 24$ $mM^{-1} \cdot cm^{-1}$ [15].

Activity of succinate-cytochrome *c* oxidoreductase (respiratory chain segment II–III) was determined in a medium containing 50 mM K_2HPO_4 (pH 7.4)/20 mM succinate/1 mM NaCN/13 μM oxidized cytochrome *c* at 550 nm ($\varepsilon = 18.5$ $mM^{-1} \cdot cm^{-1}$) [15]. Cytochrome *c* oxidase activity was recorded at the same wavelength according to Ref. 15.

The activity of the adenine nucleotide translocase was determined by measuring [^{3}H]ADP uptake as described earlier [9,16]. These determinations were carried out at 0°C in the medium containing 0.25 M sucrose/10 mM sodium borate/0.5 mM dithiothreitol/50 μM diadenosinepentaphosphate/2 μg per mg oligomycin/10 μM [^{3}H]ADP/25 mM Hepes-Na (pH 7.4). The mitoplasts concentration was 60 μg/ml. The reaction was started with [^{3}H]ADP and was terminated after 5, 10 or 15 s by addition of an equal volume of the 50 μM solution of carboxyatractyloside. A reaction mixture was filtered through Millipore filters (0.45 μM). Filters were washed with 10 ml of solution containing 0.25 M sucrose/10 mM Tris-HCl (pH 7.4) and counted in a RackBeta 1215 counter (LKB, Sweden). Nonspecific binding of [^{3}H]ADP was accounted for by addition of carboxyatractyloside before [^{3}H]ADP. The rate of time-dependent [^{3}H]ADP incorporation into the mitoplast pellet was calculated as the rate of ADP translocation. All enzymes, adenine nucleotides and NADP used were purchased from Sigma, U.S.A. [^{3}H]ADP was obtained from Amersham, U.K.

Results

Fig. 1 shows the results of electrophoretic analyses and of isoelectric focusing of the purified mitochondrial creatine kinase and antibodies against rat cardiac mitochondrial creatine kinase. Both preparations are homogenous and free of contamination. The p*I* for mitochondrial creatine kinase is 9.4 (Fig. 1G), showing that the protein is positively charged at neutral pH; subunit molecular masses are 42 kDa (Fig. 1A). Chicken antibodies against mitochondrial creatine kinase consisted of 50 kDa and 25 kDa subunits (Fig. 1E), total molecular mass was 150 kDa, according to the results of gel-filtration on a Ultragel A22 column (not shown). Thus, antibodies against mitochondrial creatine kinase are of IgG group of immunoglobulins.

Fig. 2 shows that rat heart mitochondria contain only one immunoreactive polypeptide with respect to the chicken antibodies against mitochondrial creatine kinase. The position of this polypeptide coincides with position of creatine kinase on the electrophoregrams.

Fig. 3A shows that chicken antibodies against mitochondrial creatine kinase effectively inhibit creatine kinase activity in the rat heart mitoplasts. This is completely consistent with a view of localization of creatine kinase on the outer side of the inner mitochondrial membrane [2,3]. Fig. 3B shows

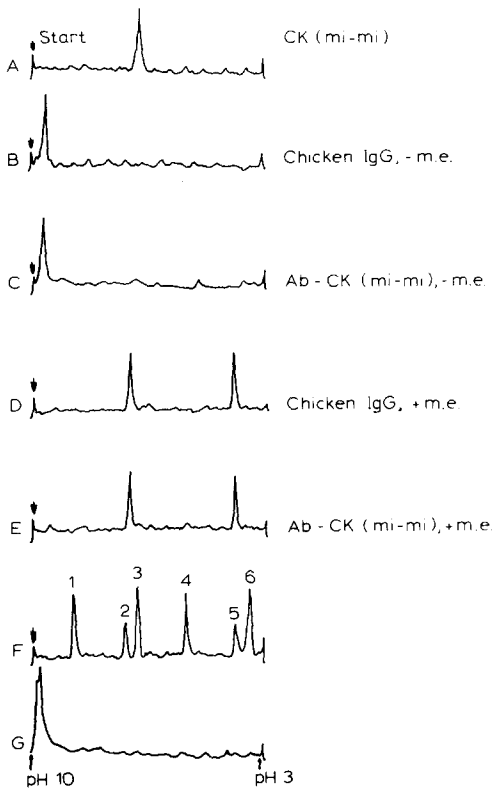

Fig. 1. Analysis of the purified preparations of rat-heart mitochondrial creatine kinase, CK(mi–mi), and chicken antibodies against this enzyme, Ab–CK(mi–mi). Mitochondrial creatine kinase was isolated according to Blum et al. [13] and analysed by a method of SDS electrophoresis [27] (trace A). SDS electrophoresis of antibodies against mitochondrial creatine kinase (traces C and E) and standard chicken IgG (traces B and D). m.e., mercaptoethanol, 25 mM. Marker proteins (1–100 kDa; 2–64 kD; 3–45 kD; 4–27 kDa; 5–21 kDa; 6–14 kD.) (trace F). Isoelectric focusing of mitochondrial creatine kinase in pH range 3–10 (trace G). All densitograms of gels were obtained on an LKB 2202 ULTROSCAN Lasser Densitometer.

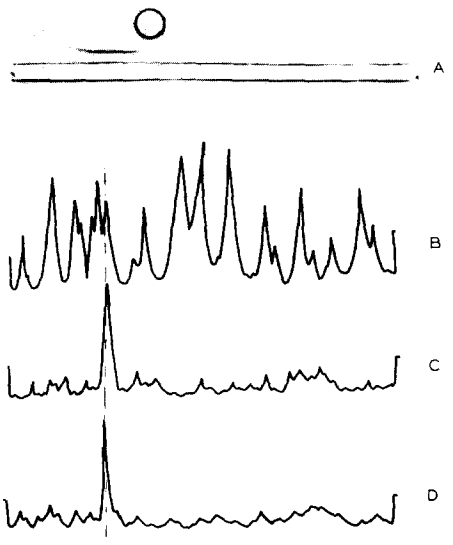

Fig. 2. (A) Immunophoresis of rat-heart homogenate. Heart homogenate was obtained in 5 vols. of 100 mM phosphate buffer (pH 7.4), and centrifuged at $10000 \times g$ for 15 min. Supernatant was used for electrophoresis in 1% agarose gel, 90 min, 20 V/cm gel. In the second step, double diffusion analysis was performed by using purified chicken antibodies (in longitudinal basin) against rat-heart mitochondrial creatine kinase. (B–D) Densitograms of the electrophoregrams of cardiac mitochondria (B, C) and purified mitochondrial creatine kinase (D). Electrophoresis was carried out in 10% polyacrylamide gels in the presence of sodium dodecylsulphate according to Laemmli [27] and proteins were transferred in the electric field onto nitrocellulose plates. B and D, staining of proteins with Amido-Schwarts, C, staining for peroxidase after incubation with antibodies against mitochondrial creatine kinase conjugated with horseradish peroxidase. Densitograms were recorded on the Opton densitometer (F.R.G.).

that simultaneously with inhibition of mitochondrial creatine kinase, chicken antibodies against mitochondrial creatine kinase block the oxidative phosphorylation of extramitochondrial ADP — a process which is mediated by adenine nucleotide translocase and is carboxyatractyloside-sensitive (Fig. 3B, recording 1). The data of Table I show that under these conditions the adenine nucleotide translocase is specifically inhibited by antibodies against mitochondrial creatine kinase. The data in Fig. 3D and E and Table I show that the inhibition of oxidative phosphorylation by antibodies against mitochondrial creatine kinase is not a result of an inhibition of the activity of the respiratory chain. Neither the succinate-cytochrome *c* oxidoreductase nor the cytochrome *c* oxidase segment is sensitive to antibodies against mitochondrial creatine kinase. Therefore, antibodies against mitochondrial creatine kinase do not cause overall non-specific changes in the mitochondrial membrane after their binding to mitochondrial creatine kinase. When mitochondrial creatine kinase was removed from the membrane by pretreatment of mitoplasts with 0.15 M KCl and 20 mM ADP, antibodies against mitochondrial creatine kinase did not have any effect on the oxidative phos-

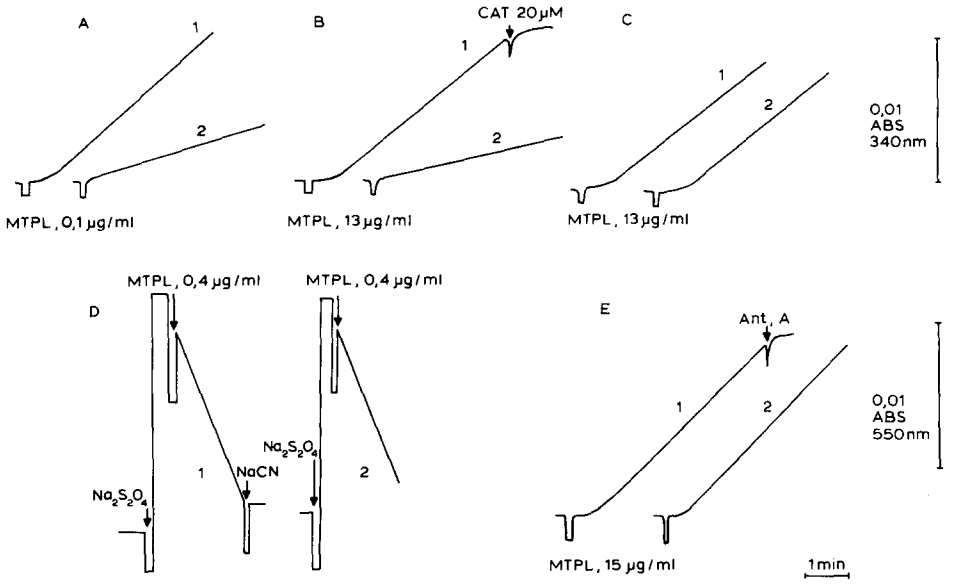

Fig. 3. Recordings of the enzymatic activities of the rat-heart mitochondrial inner membrane plus matrix preparation. The effect of antibodies against mitochondrial creatine kinase. Mitoplasts 0.125 mg/ml, were incubated in a medium contining 200 mM Na-borate (pH 8.0), for 10 min at 30°C without (curve 1) or with (curve 2) chicken Ab-Ck (mi-mi), 5 mg/ml. Samples of 0.8–120 µl were taken for activity determination. A, creatine kinase activity; B, the rate of oxidative phosphorylation (ATP production) in the presence of 0.02 mM ADP (CAT, carboxyatractyloside); C, the same as B, mitoplasts were preteated by incubation for 20 min at 0°C in the presence of 150 mM KCl and 20 mM ADP to remove mitochondrial creatine kinase, centrifuged for 20 min at 10000 × *g*, resuspended and used in experiments. Creatine kinase was decreased by this treatment from 2.62 IU/mg to 0.2 IU/mg; D, cytochrome *c* oxidase activity. 13 µM ferricytochrome *c* was reduced by 20 µM $Na_2S_2O_4$, the buffer was oxidized by bubbling O_2 through the reaction mixture for 3 min and mitoplasts were added to 0.4 mg/ml; E, succinate-cytochrome *c* oxidoreductase activity. Ant. A, antimycin A.

phorylation (Fig. 3C). Table II shows that this treatment does not impair oxidative phosphorylation process in the mitochondrial membrane. The residual activity of mitochondrial creatine kinase in mitoplasts after treatment with 0.125 M KCl and 20 mM ADP was not higher than 0.2 IU/mg

TABLE I

THE EFFECT OF ANTIBODIES AGAINST MITOCHONDRIAL CREATINE KINASE, Ab-Ck(mi-mi), ON THE ENZYMATIC PROPERTIES OF THE RAT-HEART MITOCHONDRIAL INNER MEMBRANE PLUS MATRIX PREPARATION

Creatine kinase, cytochrome *c* oxidase, succinate-cytochrome *c* oxidoreductase and the rate of ATP production were determined at 30°C (pH 7.4) (see Materials and Methods). The rate of ATP translocation was determined at 0°C (pH 7.4). Cyt, cytochrome.

Activity	Rate of reaction ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)		
	without Ab-CK(mi-mi)	with Ab-CK(mi-mi) (chicken)	with Ab-CK(mi-mi) (rabbit)
1. Creatine kinase	2.62 ± 0.24	0.42 ± 0.03	2.82 ± 0.31
2. ATP synthesis	0.36 ± 0.05	0.06 ± 0.01	–
3. ATP–ADP translocase	$0.72 \cdot 10^{-3}$ $\pm 0.5 \cdot 10^{-4}$	$0.2 \cdot 10^{-3}$ $\pm 0.1 \cdot 10^{-4}$	$0.72 \cdot 10^{-3}$ $\pm 0.6 \cdot 10^{-4}$
4. Cyt *c* oxidase	0.99 ± 0.01	0.99 ± 0.01	–
5. Succinate- Cyt *c* oxidoreductase	0.036 ± 0.002	0.036 ± 0.003	–

TABLE II

RESPIRATORY PARAMETERS OF RAT CARDIAC MITOCHONDRIA AND MITOPLASTS BEFORE AND AFTER TREATMENT WITH 125 mm KCl + 20 mM ADP

Mitochondria and mitoplasts were isolated as described in Materials and Methods. Mitoplast were incubated with 125 mM KCl + 20 mM ADP for 20 min at 0°C and then centrifuged for 10 min at 6000 × g, resuspended and used for determinations.

Preparation	State 3 rate, (ngatom $\cdot min^{-1} \cdot mg^{-1}$)	Respiratory control index	ADP/O
Mitochondria	370 ± 60	5.5 ± 1.5	2.5 ± 0.5
Mitoplasts	370 ± 60	2.8 ± 0.6	1.5 ± 0.3
Mitoplasts treated with KCl + 20 mM ADP	345 ± 65	2.5 ± 0.7	1.4 ± 0.3

as compared to 2.62 ± 0.24 IU/mg before treatment that shows that more than 90% of mitochondrial creatine kinase was removed by this procedure. It may be concluded that inhibition of oxidative phosphorylation depends completely on the binding of antibody molecules with antigenic determinants on the surface of mitochondrial creatine kinase. Moreover, the results of the Fig. 4 show that for inhibition of oxidative phosphorylation by blocking the activity of adenine nucleotide translocase the binding of mitochondrial creatine kinase with a specific antigen determinant near the mitochondrial creatine kinase active center is necessary. Antibodies against mitochondrial creatine kinase produced in rabbit against rat heart mitochondrial creatine kinase were bound very effectively with antigenic molecules (Fig. 4). However, they did not inhibit mitochondrial creatine kinase activity (Table I). Obviously, the active center area was not affected by antibody binding (only three rabbits gave inhibitory antibodies against rat mitochondrial creatine kinase and those antibodies against mitochondrial creatine kinase were used in the work [8]; others systematically produced noninhibitory antibodies against mitochondrial creatine kinase). These rabbit antibodies against mitochondrial creatine kinase did not inhibit the process of adenine nucleotide translocation (Table I). Therefore, for inhibition of adenine

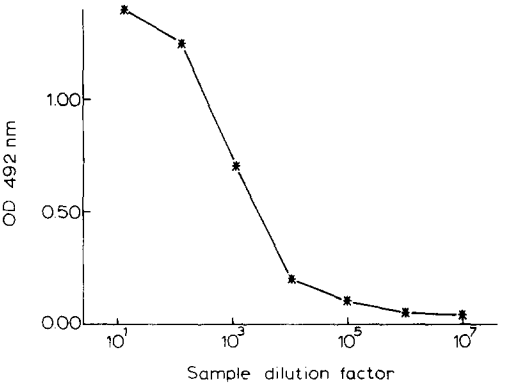

Fig. 4. ELISA analysis of the binding of the rabbit antibodies against rat-heart mitochondrial creatine kinase with purified rat mitochondrial creatine kinase. 2 μg of CK(mi–mi) were carried on a plastic, affinity column rabbit antibodies against mitochondrial creatine kinase were diluted as indicated by a factor of dilution, and 250 μl of solution were added to plastic, an amount of the antibodies against mitochondrial creatine kinase bound was determined by using sheep antibodies against rabbit IgG with immobilized peroxidase [14].

nucleotide translocase the binding of antibodies to or near mitochondrial creatine kinase active centers seems to be required. Comparison of the results described in Fig. 3 and Fig. 4 and Table I suggest that there is a specific orientation of mitochondrial creatine-kinase-active centers and of the nucleotide binding site of the adenine nucleotide

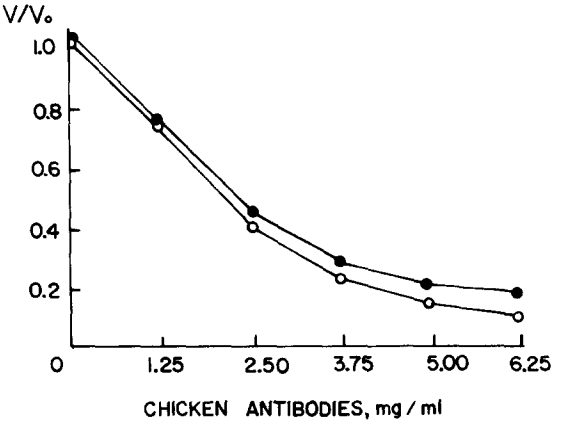

Fig. 5. Titration of creatine kinase activity (open circles) and oxidative phosphorylation (closed circles) with chicken antibodies against rat-heart mitochondrial creatine kinase. Mitoplast concentration in the incubation medium was 0.125 mg/ml. Determinations were performed as described in the legend to Fig. 3.

translocase. This suggestion is supported further by the results shown in Fig. 5 which demonstrates the titration curves for mitochondrial creatine kinase and oxidative phosphorylation when concentration of chicken antibodies was changed. Both titration curves are very close and practically identical to each other.

Discussion

The very specific inhibition of the adenine nucleotide translocase in cardiac mitochondria, only by inhibitory antibodies against mitochondrial creatine kinase from chicken, without any changes in the activity of the other mitochondrial enzymes (respiratory chain segment II, III and IV), shows the specific structural relationship between mitochondrial creatine kinase and adenine nucleotide translocase.

This relationship could be analysed on the basis of several recent important publications. First, Muller et al. [17] have shown that mitochondrial creatine kinase binds mostly to the cardiolipin moiety of the inner mitochondrial membrane. An almost simultaneous publication from Klingenberg's laboratory [18] described the connection between cardiolipin and adenine nucleotide translocase. The latter was found to complex about 6 mol cardiolipin per mol adenine nucleotide translocase.

We may suppose that it is that domain of cardiolipin structurally related to adenine nucleotide translocase to which mitochondrial creatine kinase is bound, due to electrostatic interactions between the positive charge of protein molecule and the negative charge of cardiolipin. This hypothetical structural relationship between mitochondrial creatine kinase and adenine nucleotide translocase, where cardiolipin holds together mitochondrial creatine kinase and adenine nucleotide translocase, is shown in Fig. 6. According to the fluid-mosaic model of the biological membranes (developed by Singer and Nicolson [19]) and recent experimental data obtained in Hackenbrock's laboratory [20–22], respiratory chain components interact by means of frequent collisions due to lateral diffusion in the membrane [19–25]. This concept was recently generalized by Slater et al. [26] to involve collisions of respiratory chain components with ATP synthetase complex F_1 as a mechanism of coupling between electron transfer and phosphorylation. According to this general point of view, mitochondrial creatine kinase and adenine nucleotide translocase may interact by frequent collision within a cardiolipin domain in the inner mitochondrial membrane. The frequency of collisions may exceed the turnover of ATP and ADP by adenine nucleotide translocase (see Ref. 26) resulting in an effective functional coupling between adenine nucleotide translocase and mitochondrial creatine kinase experimentally confirmed in numerous investigations [6–11]. We should point out, however, that some additional factor of structural organization not identified by the scheme in Fig. 6 could be involved for precise orientation of mitochondrial creatine-kinase-active center with respect to the adenine nucleotide binding site of translocase. Also, recent studies have shown that mitochondrial creatine kinase and adenine nucleotide translocase are present in the cardiac mitochondrial inner membrane in molar ratio 1 : 1 (about 2.5 mol per mol cytochrome aa_3) [28]. These data may point to the existence of

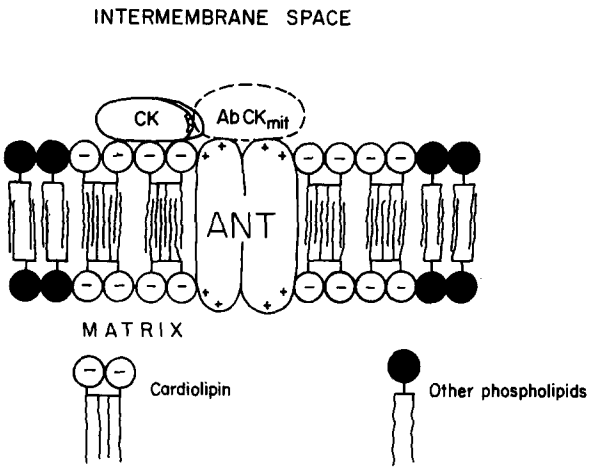

Fig. 6. Schematic presentation of the structure of the inner membrane of heart mitochondria showing possible spatial relationship between adenine nucleotide translocase and mitochondrial creatine kinase. Adenine nucleotide translocase is associated with cardiolipin [18], which in turn, as a glue, binds mitochondrial creatine kinase. Lateral diffusion of adenine nucleotide translocase and mitochondrial creatine kinase result in very frequent collision and in this way in efficient functional coupling between this transport protein and enzyme [8].

some kind of fixed interaction between mitochondrial creatine kinase and adenine nucleotide translocase in the membrane: probably, mitochondrial creatine kinase is bound to the cardiolipin domain in more or less precise orientation to the adenine nucleotide translocase, due to some specific component on the membrane surface still unknown, that may increase the efficiency of interaction between mitochondrial creatine kinase and adenine nucleotide translocase if it occurs by a mechanism of frequent collision. This hypothesis accounts for the data showing that adenine nucleotide translocase can be inhibited without any effect on the activity of mitochondrial creatine kinase [29,30]. Elucidation of exact mechanism of interaction between mitochondrial creatine kinase and adenine nucleotide translocase, however, clearly requires further detailed investigations.

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