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AFFINITY MODIFICATION OF CREATINE KINASE AND ATP-ADP TRANSLOCASE IN HEART MITOCHONDRIA: DETERMINATION OF THEIR MOLAR STOICHIOMETRY

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Oxidized dialdehyde analogs of ADP or ATP (oADP and oATP) were shown to inhibit irreversibly adenine nucleotide translocator (T) and creatine kinase (CK) in heart mitochondria. Inactivation of T and CK was parallel with carboxyatractyloside - sensitive and (ADP + phosphocreatine) - sensitive incorporation of o[^{3}H]ADP into mitochondria, respectively. o[^{3}H]ADP incorporation sensitive to CAT or ADP+phosphocreatine was used to determine T and CK contents in mitochondria. T content in cardiac mitochondria from rat, rabbit, dog, and chicken was calculated to be 2.6 - 2.9 moles/mole cyt.aa$_3$. The same value of T/cyt.aa$_3$ ratio was found in liver mitochondria with lower cytochrome aa$_3$ content. In all types of cardiac mitochondria CK content was found to be 2.4 - 2.6 moles/mole cyt.aa$_3$. The data show that T and CK are present in molar ratio 1:1 in all types of cardiac mitochondria.

ATP-ADP translocase and creatine kinase are functionally coupled in heart mitochondria and form the initial cycle of the phosphocreatine shuttle (1,2). Their effective interaction is most easy to explain if one supposes the presence of both proteins in molar ratio 1:1 in heart mitochondria (3). However, there are only very few data on determination of creatine kinase content in heart mitochondria, and the content of adenine nucleotide translocase is usually determined with labelled inhibitors such as [^{35}S]carboxyatractyloside which is a rare reagent (4). In recent years, on the other hand, an increasing number of nucleotide binding enzymes have been investigated by using 2,3-dialdehyde derivatives of ATP or ADP (oATP and oATP, correspondingly) (5-6). In the present work we have shown that oADP and oATP are irreversible inhibitors of creatine kinase and

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ATP-ADP translocase , and these compounds have been successfully used to determine molar content of the proteins in question in cardiac mitochondria.

MATERIALS AND METHODS

Mitochondria were isolated from the heart muscle as described earlier (2) . Liver mitochondria were isolated according to ref. (7). Mitoplasts were obtained by a method of Schnaitman and Greenwalt (8). The intactness of the inner mitochondrial membrane was tested by inhibition of the respiratory rate in state 3 more than 85% by carboxyatractyloside (CAT) , 50 μM. Creatine kinase activity was determined as indicated earlier (2). Oxygen consumption rate was determined polarographically using a Yellow Spring Clarck electrode (2). Cytochrome aa_3 content was determined spectrophotometrically at wavelength pair 605-630 nm using an extinction coefficient value 24 mM^{-1} cm^{-1} (9). oATP and oADP were synthesized by a periodate method (10) and purified on a Sephadex G-10 column. The final concentration of oATP and oADP was determined at 258 nm, E=14.9 mM^{-1} cm^{-1} (11).

Activity of ATP-ADP translocase was determined by a method described by Chan and Barbour (12). Inactivation of ATP-ADP translocase in the presence of 5-200 μM oADP or oATP was assessed by determination of changes in the state 3 oxygen consumption rate or directly by changes in the rate of [^{3}H]ADP uptake. Inactivation of creatine kinase by oATP or oADP was detected by determining changes in the enzyme activity after incubation of mitochondria (1mg/ml) in a medium containing 20 mM HEPES-Na, pH 5.5-7.5 , 20 mM Tris-HCl, pH 7.5-9.0 , 3 mM Mg acetate, 0.05-1.5 mM inhibitor. After definite time intervals of incubation (0-60 min) samples were withdrawn, diluted 100-200 times by activity assay medium and enzyme activity was determined.

Binding of o[^{3}H]ADP to translocase and creatine kinase in heart mitochondria was determined in a medium containing 20 mM HEPES-Na, pH 7.0, 3 mM Mg acetate, 10 mkg/ml oligomycin, 0.3 mg (1.2 mg/ml) mitochondria and 0.7 mM o[^{3}H]ADP. After incubation for 0-40 min. the mixture was filtered through Millipore filters (0.45 mkm), filters were washed three times by a cold incubation buffer, dried and dissolved in 10 ml of dioxane scintillator for radioactivity counting in "LKB Rack Beta 1215" counter. For determination of specific binding of o[^{3}H]ADP with adenine nucleotide translocase the experiments were repeated in presence of 50 μM CAT and the difference in radioactivity was taken to show the amount of o[^{3}H]ADP bound to ATP-ADP translocase (see the "Results" section). Similarly , to determine the specific binding with creatine kinase in mitochondria a difference in total o[^{3}H]ADP binding and that in the presence of 0.5 mM MgADP and 15 mM phosphocreatine was determined. o[^{3}H]ADP binding with isolated and purified creatine kinase was determined after incubation of enzyme (0.5 mg/ml) with 1 mM of reagent for 35 min at 30° C and after removal of an excess of the reagent by gel-filtration on Sephadex-G25 fine .

Removal of creatine kinase from the membrane of mitoplasts was performed by incubation of mitoplasts (5 mg/ml) for 30 min in a medium containing 0.125 M KCl, 20 mM HEPES-Na, pH 7.4 , 0.3 mM dithiothreitol, 1 mg/ml bovine serum albumin , 20 mM ADP, 4° C. The mixture after incubation was diluted by solution containing

0.3 M sucrose, 10 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, 1 mg/ml bovine serum albumin and centrifuged for 10 min at 10 000 g. The procedure was repeated twice and the pellet obtained was resuspended to a concentration of 50-80 mg/ml. Creatine kinase was isolated and purified from rat heart mitochondria according to a procedure discribed by (13).

Materials. Enzymes used in the coupling enzyme assays, creatine, phosphocreatine, nucleotides, HEPES,bovine serum albumine, sucrose, dithiothreitol, malate, EGTA, trypsin, trypsin inhibitor, were purchased from "Sigma",USA. Digitonin, EDTA, Tris, glutamate were from "Serva", FRG and [^{3}H]ADP from "Amersham",England.

RESULTS AND DISCUSSION

Incubation of mitochondria with 2,3-dialdehyde derivates of ADP or ATP resulted in rapid inactivation of both adenine nucleotide translocase and creatine kinase. After incubation of rat heart mitochondria with 200 μM of oADP for 10 min at 30° C the uptake of [^{3}H]ADP was less than 1% of control. This inhibition could be easily seen from inhibition of state 3 respiration (Fig.1). Fig.1 shows in double reciprocal plots the dependence of the state 3 respiration rate on ADP concentration. The character of these dependences shows a competitive type of interaction between ADP and oADP that may be taken to indicate

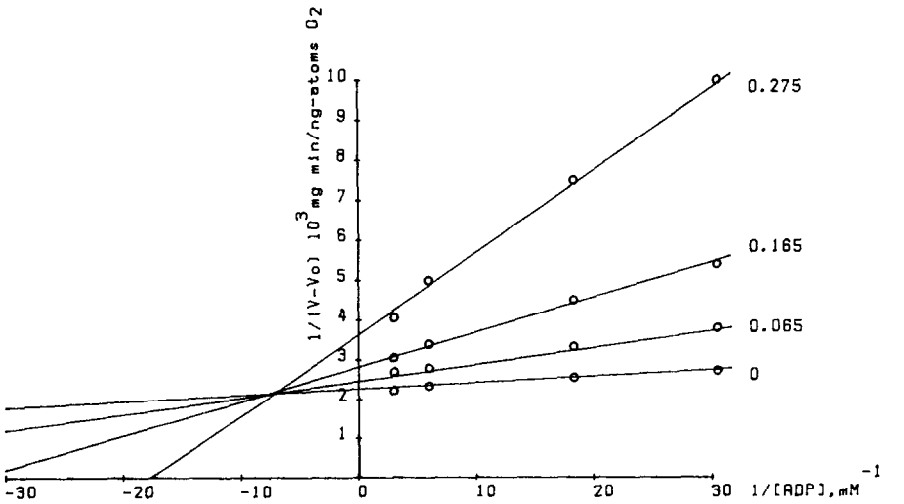

Fig.1. The double reciprocal presentation of the dependence of the rate of state 3 respiration upon ADP concentration at different oADP concentration in the medium (indicated in mM at straight lines). The rate of respiration in the presence of ADP was corrected for uncoupled respiration.

binding of oADP to the nucleotide binding sites of adenine nucleotide translocase. When $K_m^{app.}$ values for ADP obtained from Fig.1 were plotted against inhibitor (oADP) concentration, a straight line was obtained giving $Ki_{(oADP)}$ = 0.065 mM. The value of this parameter for oATP was found in similar experiments to be 0.14 mM.

Time-course of heart mitochondrial creatine kinase inactivation at the different oADP concentrations is shown in Fig.2. The rate of inactivation was dependent on the concentration of oADP; the linear dependence of the logarithm of the activity on time of the reaction allows us to suppose the first order of the reaction. The time of half-inactivation ($\tau_{1/2}$) was found to depend linearly on the 1/[oADP] value with a positive ordinate-intercept. According to (14), these data point to a probability of formation of noncovalent complex enzyme· inhibitor (E·I) before formation of an inactive form of the enzyme - ($E_{inact.}$):

$$E + I \rightleftharpoons E \cdot I \longrightarrow E_{inact.}$$

The rate of creatine kinase inactivation was dependent on pH. Quantitative study of this dependence revealed that some groups with pK - 6.9 may be involved whose protonation leads to acceleration of the enzyme inactivation. Fig.2 shows also that creatine kinase could be protected against inactivation by its substrates, and most effective was a mixture of MgADP (0.5 mM) and phosphocreatine (15 mM) which creates the "working" state of the enzyme, including the transition state complex E·creatine·PO_3^{-2}·MgADP formation (15).

Similar characteristics of inactivation were seen when soluble, purified mitochondrial creatine kinase was used. Inactivation of the enzyme was parallel with incorporation of the radioactive label into the protein, and complete inactivation was

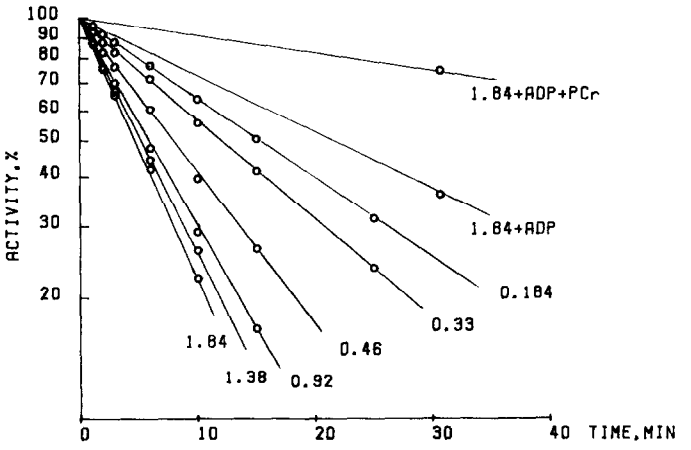

Fig.2. Kinetics of inactivation of creatine kinase in mitochondria by oADP. The numbers show oADP concentration in mM. +ADP - 10 mM ADP was added; +ADP+ PCr - 2mM ADP and 15 mM PCr were added. The reaction mixture is given in "Material and Methods". Mitochondrial concentration was 1.2 mg/ml , 21°C.

observed when 2 moles of o[^{3}H]ADP was bound with 1 mole of dimeric enzyme (M.W.=82 000 (16)).

Figure 3a shows incorporation of o[^{3}H]ADP into mitochondria determined with a Millipore filtration method. Both inactivation of adenine nucleotide translocase and creatine kinase and o[^{3}H]ADP incorporation were irreversible and time dependent. After 30-40 minutes of incubation when translocase and creatine kinase were completely inhibited, about 16 moles of o[^{3}H]ADP per mol of cyt.aa_3 were incorporated. This level of incorporation reflects o[^{3}H]ADP binding to numerous ADP binding proteins in mitochondria. 50 μM carboxyatractyloside decreased label incorporation (Fig.3). Since CAT was shown to inhibit nucleotide binding to translocase (17), the maximal value of the difference, $B_{tot.} - B_{cat} = T$ ($B_{tot.}$ is the total o[^{3}H]ADP binding and B_{cat} in the presence of CAT) was taken to show the content of translocase (T). T was found to be equal to 2.6-2.9 mole/mole cyt.aa_3. This value of translocase content in mitochondria is consistent with all other estimations (18). Table 1 shows T

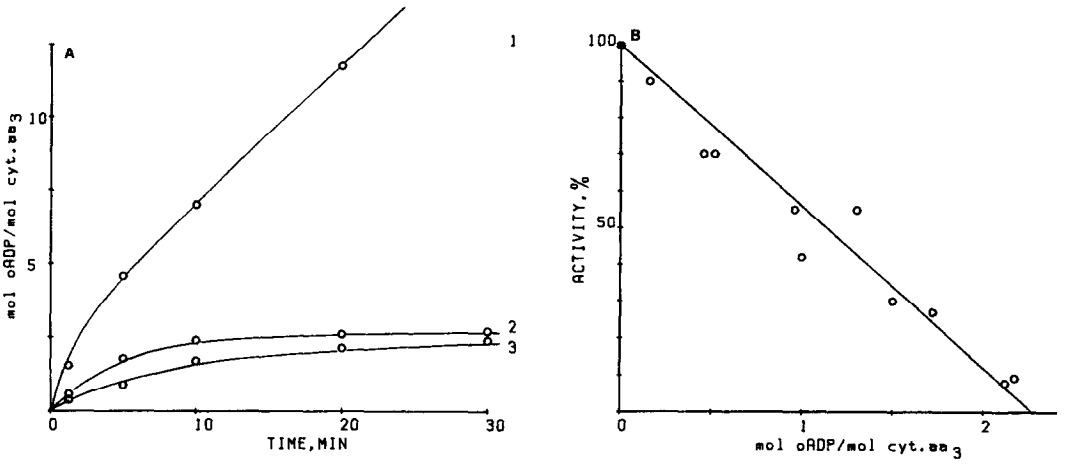

Fig.3. A - Kinetics of incorporation of o[^{3}H]ADP into rat heart mitochondria. 1 - total incorporation; 2 - difference between total incorporation and that in the presence of 50 μM carboxyatractyloside; 3 - difference between total incorporation and that in the presence of 0.5 mM ADP and 15 mM PCr. Inhibitor concentration in all cases was 0.7 mM, 30°C.
B - Linear relationship between creatine kinase activity of rat heart mitochondria and (ADP + PCr) - sensitive incorporation of o[^{3}H]ADP (see curve 3 in Fig.3A).

Table 1. Translocase and creatine kinase contents in mitochondria, isolated from different sources

	Cyt.aa_3 nmoles/mg protein	CK activity, IU/mg	CK, mol/mol cyt.aa_3	T, mol/mol cyt.aa_3
Rat heart mitochondria	0.445±0.09	4.5±0.5	2.35±0.25	2.65±0.25
Rabbit heart mitochondria	0.353±0.07	3.0±0.3	2.43±0.26	2.70±0.2
Dog heart mitochondria	0.440±0.05	2.9±0.3	2.23±0.19	2.80±0.20
Chicken heart mitochondria	0.354±0.06	0.86±0.2	2.60±0.34	2.90±0.25
Rat liver mitochondria	0.246±0.04	0.00	0.00	2.80±0.25
Rat heart mitoplast	0.360±0.06	2.5±0.3	2.02±0.3	2.30±0.30
Rat heart mitoplast incubated in KCl+ADP	0.370±0.05	0.065±0.01	0.20±0.03	2.36±0.30

Mean values and SD are given for four experiments.

for mitochondria from different sources. For all mitochondria the T/cyt.aa_3 ratio was found to be constant.

Fig. 3a shows also that the mixture ADP + phosphocreatine which completely prevents inactivation of creatine kinase (see Fig.2) decreases o[^{3}H]ADP incorporation into mitochondria. The difference, $B_{tot.} - B_{PCr,ADP} = B_{CKmit}$ ($B_{PCr,ADP}$ is o[^{3}H]ADP binding in the presence of ADP and phosphocreatine) was taken to show the binding of the label to creatine kinase , $B_{CKmit.}$ in mitochondria. In the presence of 0.5 mM ADP and 15 mM phosphocreatine, creatine kinase establishes an equilibrium when the ADP/ATP ratio is very low (19). This ADP level (ATP has a lower affinity to T) does not protect translocase against oADP binding. Fig. 3b shows that $B_{CKmit.}$ is linearly related to the decrease in creatine kinase activity, and at complete inactivation $B^{max}_{CKmit.}$ = 2.4-2.5 moles/mole cyt.aa_3. When an ADP + phosphocreatine mixture was used to study CAT-treated mitochondria, the maximal $B_{CKmit.}$ value was also 2.4-2.6 moles/mole cyt.aa_3. The effects of CAT and the ADP + phosphocreatine mixture on o[^{3}H]ADP binding were additive.

The values of CK contents (determined as B_{CKmit}) in different mitochondrial preparations are given in Table 1. Table 1 shows that in liver mitochondria CKmit. = 0; the same value was determined if creatine kinase was removed from mitoplast membrane by KCl treatment. Thus, the method for determination of creatine kinase content in cardiac mitochondria proved to be highly specific. The content of creatine kinase in mitochondria determined in the present work agrees with that determined by using SH-reagents (20). It is interesting to notice that in chicken heart mitochondria creatine kinase content does not differ from that for other types of cardiac mitochondria in spite of its much lower activity (Tabl. 1, see also ref. (21)).The

data of Table 1 show that translocase and creatine kinase are present in almost equimolar amounts in all types of cardiac mitochondria (Table 1). This is consistent with the hypothesis of phosphocreatine shuttle (1,2,3).

In conclusion ,the results show that oADP (or oATP) can be effectively used for determination of the content of ADP binding proteins in mitochondria. This method may be especially important in the cases of different pathology of muscle which may involve alterations in mitochondrial enzymes, including translocase and creatine kinase (21).

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