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Oxidative Phosphorylation계에 의한 Phosphocreatine의 생성 기작

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Evidences for Functionally Direct Coupling between Mitochondrial Phosphocreatine Formation and Oxidative Phosphorylation

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ADP 농도를 증가시킴에 따라 미토콘드리아에 의한 Phosphocreatine의 생성속도는 포화 경향을 보이면서 증진되었다. 이 조건에서 Phosphocreatne 생성 속도를 측정하여 가해준 ADP에 대한 Km 값을 구해본 결과 0.0185 mM 임을 알았고, 이 Km값은 미토콘드리아의 Oxidative Phosphorylation계와 연계되지 않은 (추출한) 미토콘드리아 Creatine Kinase의 ATP에 대한 Km 값인 0.21 mM보다 훨씬 낮음을 알 수 있었다. 이 외에도 ADP 존재하에 Oxidative Phosphorylation에의한 ATP 생성에 대한 Creatine Kinase의 활성의 영향을 살펴본 결과 이 조건에서 Phosphocreatine은 반응시간에 정비례하게 생성 되었으나, 생성된 ATP는 반응시간에 무관함을 보였다. 또한 Oxidative Phosphorylation에의해 미토콘드리아 외부 Respiration 용액 내에 이 ATP가 축적되는 속도도 미토콘드리아 Creatine Kinase의 Phosphocreatine 생성과 무관함을 알수 있었다.

이상의 결과들은 Mitochondrial Creatine Kinase가 Oxidative Phosphorylation계와 기능적으로 밀접하게 연계되어 ATP가 아닌 Phophocreatine이 에너지 전달 물질로 직접 이용될 가능성을 시사해준다.

As concentration of ADP was increased, the rate of phosphocreatine formation by respiring heart **mitochondria** was increased. The value of apparent Km of the phosphocreatine-forming mitochondria for **ADP** was estimated to be 0.0185 mM. This value was much lower than that of Km for ATP (0.31 mM) **which** was determined from the reaction of the soluble form of mitochondrial creatine kinase. The **concentration** of ATP remained constant during the respiring in the presence of ADP. The rate of **accumulation** of oxidative-phosphorylated ATP in the mitochondrial respiration medium was continuously **monitored** as a function of ADP concentration with the firefly luciferase-coupled assay. In that case, **exogenous** creaine did not affect the rate of accumulation of ATP, indicating that phosphocreatine-**forming** (i.e.,respiring) mitochondria in the presence of ADP did not use the ATP in the medium as a **substrate**.

These results suggest that the heart mitochondrial creatine kinase bound to the inner membrane functionally tight-coupled to the oxidative phosphorylating system with respect to the respired ATP.

Keywords : Oxidative, Phospholylation, Mitochondrial, Creatime, Kinase

Introduction

Since the discovery of a new form of mitochondrial creatine kinase (EC 2.7.3.2) bound to the inner membrane of rat heart mitochondria[1], it has been suggested by a number of research groups that the mitochondrial creatine kinase may have a specific function in the muscle energy metabolism. They reported that the activity of the bound form of mitochondrial creatine kinase might be coupled directly to the mitochondrial oxidative phosphorylation including ATP/ADP translocation between inner mitochondrial membrane[1-5]. Fractional extractions of heart muscle have revealed that about 30 % of the cellular creatine kinase activity is located in the mitochodria[6-10]. Based on the its molecular structure and property, Hall et al.[11] suggested that mitochondrial creatine kinase is quite different from cytoplasmic isoenzyme, and thus these two isoenzymes are expecting to play differently in the energy metabolism. In contrast to the kinetically reverse direction (i.e., phospho- creaine + ADP → creatine + ATP)-favorable nature of cytoplasmic creaine kinase reaction(12, 13), the mitochondrial creatine kinase reaction might be adapted to phosphocreatine-forming direction so that these isoenzymes could play a major role in the intracellular transport in form of phosphocreatine from oxidative phosphorylating system to energy-utilizing system lopcated in cytoplam[14,15].

In this communication, we report a series of experimental results supporting the coupling between production of phosphocreatine and oxidative phosphorylation of ADP to ATP.

Materials and Methods

Materials

Latate dehydrtogenase, pyruvate kinase, hexokinase and glucose-6-phosphate were obtained from Calbiochem-Behring Corp.; firefly luciferase and bovine serum albumin from Sigma Chemical Co.; trypsin and soybean trypsin inhibitor from Miles Lab. Biochemicals including luciferin, NADH, NADP, ATP, ADP, AMP, creatine, phosphocreatine, phosphoenolpyruvate, glucose, oligomycin, antimycin, atratyloside, FCCP, NEM, DTT, deoxycholate, glutamate, malate, Tris and Hepes were from Sigma. Pic reagent(tetrabutylammonium phosphate) was supplied from Waters Associates, and other chemicals were of the highest grade locally available.

Preparation of Mitochondria

The pig heart muscle tissue (100 g) was minced and washed three times with the isolation medium containing 0.3 M sucrose and 0.2 mM EDTA in 10 mM Tris-HCl buffer, pH 7.2. The well minced heart tissue was then suspended in 100 ml of the isolation medium, and homogenized with a Waring blender at low speed for few seconds. After this mild homogenation, the homogenate was incubated at 0 °C for 10 min with 5 mg of trypsin[16], and then mixed with 200 ml of the isolation medium containing 30 mg of soybean trypsin inhibitor. The trypsinized tissue suspension was homogenized five more times with a Waring blender each for 15 seconds at low speed. The final tissue homogenate was filtered through a cheese cloth, and then centrifuged at 1,500×g for 10 min. The resultant supernatant was recentrifuged at 10,000×g for 20 min to collect tissue mitochondrial pellet. The

mitochodrial pellet was washed twice in the isolation medium containing 1mg/ml of bovine serum albumin and 0.3 mM of DTT at pH 7.4 by centrifugation 10,000 ×g for 20 min[17,18,19]. The washed mitochondrial pellet was then suspended in the same isolation medium resulting in a mitochondrial protein concentration at 20 mg per ml. The concentration of mitochondrial protein was determined by a biuret method in the presence of 0.2 % deoxycholate[20].

Preparation of Soluble Form of Mitochondrial Creatine Kinase

The mitochodrial pellet was suspended in100 mM of sodium phosphate, pH 8.0 containing 1 mM EDTA, and 10 mM DTT to make up 1 ml of suspension per mg of wet weight of mitochondria. The suspension was stirred solwly at 4 °C. After stirring overnight, the mitochodrial pellet was removed by the centrifugation at 10,000×g for 20 min. The supernatant showing creatine kinase activity was further fractionated with ammonium sulfate. Solid ammonium sulfate was added to give first 30 % saturation, followed by increasing cuts at 5 % intervals to 55 % saturation. Each of the precipitates was suspended in 25 ml of Hepes buffer solution containing 10 mM DTT. After dialysis, the final preparation of the soluble form of mitochodrial creatine kinase showed its specific activity of 8.5 units per ml for the direction of phosphocreatine formation in the standard reaction mixture at 30 °C, pH 7.4. The activity of solublelized mitochondrial creatine kinase was determined by the coupled enzyme assay with pyruvate kinase and lactate dehydrogenase for the direction of phosphocreatine formation. For the determination creatine-forming activity, hexokinase and glucose-6-phosphate dehydrogenase were used as coupled enzymes. The reactions were carried out under standard reaction mixtures[21].

Measurement of Rate of Mitochondrial Respiration

The rate of mitochondrial oxygen consumption was determined by a polarographic technique using a clark oxygen electrode(Yellow Spring Ins.). The changes in electrode potential were recorded with a Varian A-25 strip chart recorder. The composition of respiration medium was same as that of the reaction mixture for the bound form of mitochondrial creatine kinase. In this experiment, the rate of oxygen consumption was calibrated with the standard solubility data of oxygen at 30 °C(400 ng-atoms of oxygen per ml of medium)[17]. The respiratory control ratio(the ratio of respiratory rate after adding FCCP to that before adding FCCP) was between 5 and 10 for heart mitochondria.

Determination of Activity of Bound Form of mitochondrial Creatine Kinase linked to the Respiration

The activity of mitochondrial creatine kinase bound to the inner membrane was measured by using a paired ion reverse-phase high pressure liquid chromatographic method[23]. All reactions were carried out in the respiration medium containing 0.25 M sucrose, 1 mg/ml of bovine serum albumin, 0.3 mM DTT, 5.0 mM malate, 10 mM glutamate, 4.0 mM M_gCl_2, 5.0 mM K-salt of phosphate and 20 mM creatine in 3.0 ml of 20 mM Hepes buffer, pH 7.4, at 30 °C. The concentration of phosphocreatine formed by the respiring mitochondria was measured by peak to peak analysis of

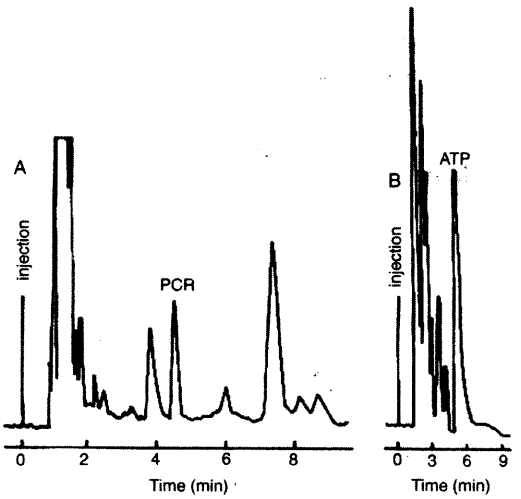

Fig. 1 HPLC Chromatogram for Reaction Mixture to determine Phosphocreatine (PCR) or Adenosine Triphosphate(ATP). The details are in Mertirials and Method.

the chromatogram. A 0.2 ml of the reaction sample was removed from the oxygraph chamber and added to an Eppendorf tube containing 0.06 ml of 20 % $HClO_4$, vortexed for about 15 seconds, and allowed to sit on ice for 2 hrs. The perchloric acid-treated sample was neutralized with 0.02 ml of 5.0 M K_2CO_3. After centrifugation by using a Beckman microfuge, 0.02 ml of the supernatant was injected into the column(micro bondapak C18) for the assay of phosphocreatine formed during the reaction time. The output of UV detector(Waters Model 441) was connected to a Linear recorder, and the optical absorbance was measured at 214 nm. The C-18 column was employed with the aqueous buffer (0.01 % KH_2PO_4, 0.04 % tetrabutylammonium phosphate, pH 3.0) as a effluent at the flow rate, 2.0 ml/min. The retention time of phosphocreatine were 4.3 min (Fig. 1).

Measurement of the mitochondrial ATP Formation during Respiration

For the assay of ATP formed by the respiring mitochondria in the presence of ADP, the treated sample was injected into the C-18 column. The output of UV detector was connected to a recorder, and the absorbance was measured at 254 nm. For the standard HPLC operation, C-18 column was employed with the aqueous buffer(12.5 % acetonitrile, 0.098 % tetrabutylammonium phosphate, 0.37 % KH_2PO_4 , pH 5.8) as a effluent at flow rate, 2.0 ml/min. The retention time of ATP was 5.4 min (Fig. 1)

Measurement of Rate of Extramitochondrial ATP Formation

Firefly luciferase luminescience was used for the measurement of the rate of extramitochondrial ATP formation rate linked to the oxidative phosphorylation in the presense or absence of creatine. After rapidly mixing mitochondria with the luciferase and luciferin in a stopped flow apparatus(Durrum Model 110), the intensity of the emitted luminescence was continuously measured with photomultiplier tube connected to a strip chart recorder. The final concentration of mixed reaction medium was 10 units of luciferase per ml, 8.0 μM luciferin, 0.56 mg of mitochodria, 1.0 mg of BSA/ml, 0.3 mM DTT, 5.0 mM KH_2PO_4, 0.25 M sucrose, 5.0 mM malate, 10 mM glutamate, and 4.0 mM $MgCl_2$ in 20 mM Hepes buffer, pH 7.4 at 30 °C. The initial maximum light intensity after mixing was taken as a measure of the initial velocity of ATP formation[24]. Under the condition that the intensity of luminescence is proportional to ATP concentration, the concentration of ATP was caliblated upon the light intensity[25].

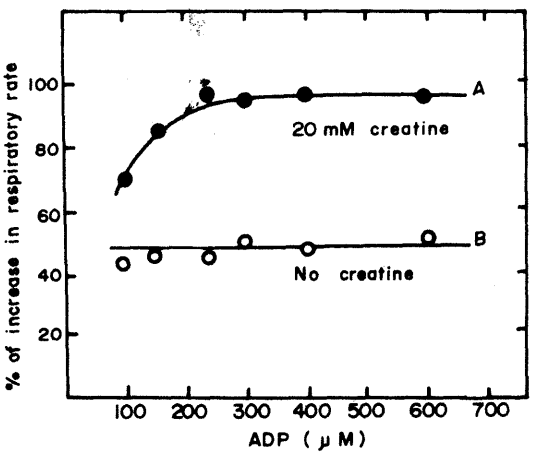

Fig. 2 Effect of ADP on the Respiratory Stimulation given by 20 mM Creatine. The respiration condition are in Materials and Methods. The respiration was initiated by the addition of varying amount of ADP to the medium. 100 % as a maximum value indicated in the ordinate of Figure corresponds to the maximum increase in the respiratory rate immediately after the addition of ADP in the presence of 20 mM creatine.

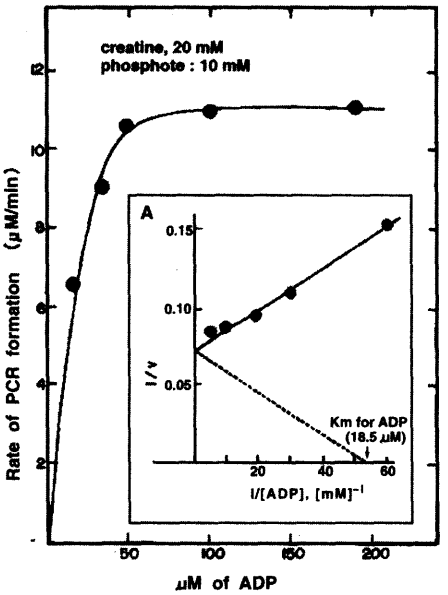

Fig. 3 ADP Concentration Dependency of the Rate of Phosphocreatine Formation by Respiring Heart Mitochondria in the Presence of 20 mM Creatine. The initial velocity of phosphocreatine formation by the respiring mitochondria with varying amount of ADP was measured using HPLC. The reaction was initiated by the addition of varying amount of ADP to the respiration medium. At intervals of 2 min during 10 min reaction time, the reaction was terminated with perchloric acid, and the supernatant of perchloric acid extract was subjected to HPLC C-18 columm to determine phosphocreatne formed (see Fig. 1-A).

Results and Discussions

To examine whether or not mitochondrial phosphocreatine formation is functionally tight-coupled to the oxidative phosphorylation of ADP to ATP, we measured the phosphocreatine formed from the newly synthesized ATP by the respiring mitochondria in the presence of creatine (20 mM). After the addition of varying concentration of ADP to the respiring mitochondria, the phosphocreatine formed was directly measured using HPLC technique at a interval of 2 min during 10 min reaction. In Fig. 2 and FIg. 3 are shown the data on the rates of respiration and phosphocreatine formation as function of the externally added ADP ranging from 0.025 to 0.6 mM in the presense of 20 mM creatine and 10 mM phosphate. As the concentration of ADP was increased, the both rates of phosphocreatine formation and respiration increased showing a saturated pattern. By extrapolating the double reciprocal plot for phosphocreatine formation reaction linked to the oxidative phosphorylation of ADP to ATP, the apparent Km value for the added ADP was estimated to be 0.0185 mM. This Km value is far lower than Km value for ATP (0.31 mM) which was determined from the solubilized mitochondrial creatine kinase reaction in the presence of ATP and creatine (Fig. 3). In case of the determination for the Km, the concentration of added ADP was incresed upto 0.2 mM. Considering that the respiratory rate under the concentration of 0.2 mM ADP does not reach

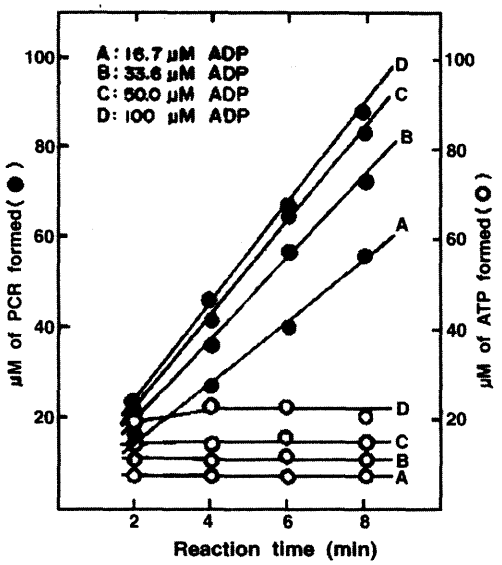

Fig. 4 Changes in the Concentrations of Mitochondrial ATP and phosphocreatine developed by Respiring Mitochondria. The amount of ADP added was indicated in the Figure. ATP and phosphocreatine were determined with HPLC technique (see Fig. 1-A, B).

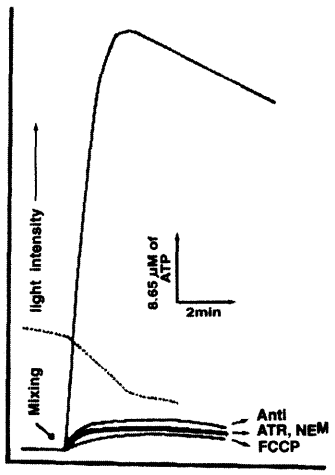

Fig. 5 The Luminescence Response to Mitochondrial ATP Formation. The solid line represents luminescence, and the dotted line shows oxygen concentration by respiring mitochondria. The final concentration of mitochondria after mixing was 0.56 mg of the protein per ml of the medium. Anti, ATR, NEM, and FCCP represent the response in the presence of respective inhibitor of oxidative phosphorylation. Anti : antimycin, 5 μg per mg of mitochondria. ATR: atractyloside, 20 μg/mg. FCCP : 2.5 μg/mg. NEM:N-Ethylmaleimide, 50 μg/mg. The respiratory medium did not contain creatine.

to the maxium rate of respiration, the much lower Km value(0.0185 mM) for this system than Km value for external ATP(0.31 mM) indicates that mitochodrial creatine kinase prefers the newly synthesized ATP by respiring mitochondria to the ATP externally ATP as a substrate for the phosphocreatine-forming reaction.

In addition to the determination of phosphocreatine, we measured the mitochondrial ATP formed by the oxidative phosphorylation. Fig. 4 shows the time-coursed formation of mitochodrial ATP at the same condition as that for phosphocreaine formation. It can be seen that the concentration of phosphocreatine increased linearly with the reaction time, while the concentration of ATP remained constant. This result strengthens our assumption that the ATP formed by oxidative phosphorylation is very effectively used for the phosphorylation of creatine to phosphocrteatine.

To explain the dynamics of this effective coupling between the phosphocreatine formation and the ATP production by respiring mitochodria, we tried to find out a relationship between the both rates of phosphocreatine formation and the accumulation of the extramitochodrial ATP linked to oxidative phosphorylation. By virtue of the great sensitivity of photomultiplier to light, the firefly luciferase assay was employed for determining the rate of the accumulation of oxidative-phosphorylated ATP in the medium surrounding the respiring mitochodria. The control experiment shown in Fig. 5 indicates the response of

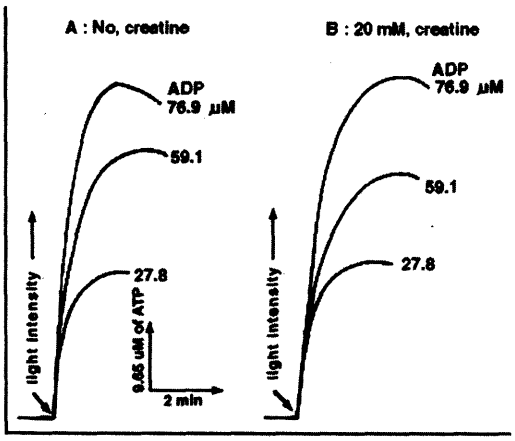

Fig. 6 Effect of Creatine on the Accumulation of Extramitochondrial ATP linked to Oxidative Phosphorylation. Fig. 6-A and -B denote the luminescence response to ATP in the absence or presence of 20 mM creatine, respectively. After mixing the solutions of I and II syringes, ATP formed immediately determined with bioluminescence assay using stopped flow apparatus. The solution I contained mitochondria, and the solution II contained varying amount of ADP, luciferase, luciferin, components of respiration substrate, and creatine in case of B.

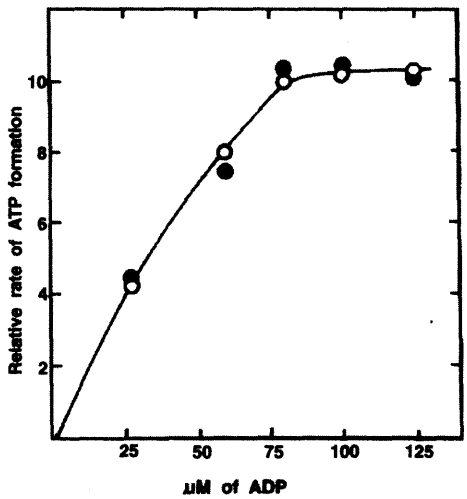

Fig. 7 Effect of Creatine on the Rate of Accumulation of Mitochondrial ATP in the mitochondria-surrounding medium. The value of rate is a relative value which corresponds to the initial maximum light intensity after mixing of solution I and II. The closed circle denotes the velocity as a function of ADP added in the presence of 20 mM creatine, and open circle denotes that in the absence of creatine.

luciferase to the accumulation of the extramitochondrial ATP in the absence of a substrate for creatine kinase, creatine. The ATP accumulation was immediately demonstrated by a steadily rising luminescence after mixing mitochodria with ADP(i.e., after state 3 respiration). Various inhibitors against oxidative phosphorylation such as antimycin, FCCP, N-ethylmaleimide and atractyloside completely blocked the emission of luminescence, indicating that most of the emitted light results from the newly synthesized ATP by oxidative phosphorylation. Fig. 6 shows the trace of luminescence response to the ATP accumulated in the medium as a function of the concentration of added ADP. The relative accumulation rate of oxidative-phosphorylated ATP in the respiration medium was estimated from the initial maximum light intensity shown in Fig. 6. Fig. 7 shows the changes in the relative accumulation velocity in the presence or absence of a excess concentration of creatine(20 mM) as a function of the concentration of added ADP. It is seen that creatine does not affect the velocity of the ATP accumulation, indicating that the ATP in the medium surrounding respiring mitochodria can not be a substrate for the phosphocreatine-forming reaction by respiring mitochodria.

The data presented in this paper confirm the functional tight-coupling between mitochodrial creatine kinase and oxidative phosphorylating system. As it has been shown in Fig. 2, 3 and 4 mitochondrial creatine kinase bound to the exterial surface of the inner

mitochondrial membrane synthesizes phosphocreaine very effectively using the ATP produced by oxidative phosphorylation. Thus the mitochondrial creatine kinase is able to maintain a low and constant steadystate concentration of ATP in the prersence of creatine (Fig. 4). The effective utilization of the newly synthesized ATP by the creatine kinase for the production of phosphocreatine is further confirmed by the much lower Km value determined from the initial rate of phosphocreatine-forming reaction linked to the oxidative phosphorylation of ADP to ATP than that of solubilized enzyme for ATP (Fig. 3). Recently, Erickson-Viitanen et al.[15] suggested that the production of phosphocreatine is effectively coupled to the oxidative phosphorylation through the microenvironment for the creatine kinase compartment which is determined by the outer mitochodrial membrane. In our system, however, the possibility of the coupling between them through such a microenvironment may be excluded by the insensitivity of the accumulation rate of the oxidative-phosphorylated ATP to the activity of creatine kinase (i.e., phosphocreaine-forming activity from ATP and creatine) (Fig. 7). From the result that the accumulation of the ATP followed by the addition of low concentration of ADP is not affected by the presence of a excess concentration of creatine (20 mM) it is concluded that a mitochodrial cycle of conversion of ATP and ADP is directly connected to the external medium via the creatine kinase reaction.

These results may indicate a tight functional coupling between mitochondrial creatine kinase and ATPase via the action of ATP/ADP translocase. In this tightly coupled system the energy for muscle movement can be transferred from mitochondria only in form of phosphocreatine, not in that of ATP molecule[26].

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