ATP Synthesis and Generation of Electrochemical Gradients of Protons in the Catecholamine Storage Organelle of the Adrenal Medulla

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The Authors Wish to Dedicate this Work to W. Hasselbach on the Occasion of His 60th Birthday

Catecholamine Storage Organelles, Membrane Ghosts, Mg-ADP Evoked ΔµH⁺, ATP Synthesis, ATP-Pi Exchange

The reaction rates of ATP formation from ADP and inorganic phosphate (Pi) by the catecholamine storage organelles isolated from bovine adrenal medulla accelerated 5–6 fold, when Pi was added at various times after addition of Mg-ADP, as compared to the rates observed when the reaction partners were added simultaneously. The increase of the rates of ATP-Pi exchange upon subsequent addition of Pi to Mg-ATP was less prominent. Mg-ADP induced a Δψ (60–70 mV, positive inside), almost equal that induced by Mg-ATP. In both cases Δψ was significantly higher in the absence of Pi than in its presence. At pH 7.4 of the medium the ΔµH⁺ was 1.4 units indicating an internal pH of 6. It was stable during the reaction time in the presence of Mg-ATP, while in the presence of Mg-ADP the internal pH increased gradually by 0.2 units. Both the ATP forming reactions as well as the nucleotide induced Δψ were uncouplersensitive (CCCP). Though inhibiting ATP formation from ADP + Pi, by 50%, 0.1 mM AP5A (an inhibitor of adenylate kinase) did neither affect Δψ nor ΔµH⁺, hence ruling out the possibility that ΔµH⁺ induced by Mg-ADP would be actually due to ATP formed from ADP. Membrane ghosts were not able of ATP synthesis (in the absence of valinomycin), the ATP-Pi exchange was only 10–20% of that of intact organelles, due to the low ΔµH⁺ in the absence of intravesicular soluble constituents. It is proposed that the energy for ATP synthesis is furnished by the high gradients of protonated soluble constituents in the intact organelles.

Introduction

Recently we have shown that the H⁺ translocating ATPase of the catecholamine storage organelle is “reversible”, i.e. capable of ATP formation from ADP + Pi. This function is confined to the intact storage organelle suspended in media of low ionic strength [1]. Membrane ghosts containing the complete ATPase apparatus are in principle likewise able to synthetize ATP, provided they are supplied with an energy source, which was achieved by a valinomycin induced K⁺ gradient dependent diffusion potential [2].

Unlike the H⁺ transferring systems of chloroplasts, bacteria and mitochondria, the catecholamine storage organelle does not dispose of an external energy source to support the energy requiring reaction of ATP formation from ADP + Pi. Under the conditions of ATP formation the energetic state of the storage organelle, represented in ΔµH⁺, was studied using the methods of methylamine and thiocyanate distribution [3, 4] as measures of ΔpH and Δψ across the membrane.

Methods

Catecholamine storage vesicles from bovine adrenal medulla were isolated in 0.3 m unbuffered sucrose at 4 °C [5]. The catecholamine content of the purified preparation was 2.56 ± 0.07 mg protein⁻¹. Membrane ghosts were formed from storage vesicles sedimanted in 1.6 m sucrose according to [6] (KCl-ghosts). Alternatively, sodium isethionate was used to form membrane ghosts in a KCl-free medium (IE-ghosts). Resealing of the membrane fragments to closed vesicles was tested by the ATP-dependent accumulation of catecholamines [6].

The standard conditions were 0.3 m sucrose, buffered to pH 7.4 with 10 m Tris maleale, 1 mM

Abbreviations: [¹⁴C]SCN, Potassium [¹⁴C]thiocyanate; [¹⁴C]-CH₃NH₂, Methylamine hydrochloride; TCA, Trichloro acetic acid; CCCP, Carboxylicyamid-m-chlorophenyl hydrazone; DCCD, Dicyclohexylcarbodiimide; ATP₆, P₆, P₆di(adenosine-5')-pentaphosphate.

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[32P]orthophosphate (3-5 μCi × mmol⁻¹), adjusted to pH 7.4, ATP-Mg 5 mM or ADP-Mg 1 mM, 31 °C and constant shaking. All experiments were started by the addition of the vesicular suspension. The protein concentrations varied in the different series of experiments and are given in the legends.

The ATP forming reactions were terminated by adding 10% (w/v) TCA. P_i was separated from the nucleotides according to [7], with the modification that isobutanol:toluene (1:1) was used for the extraction of P_i. ATPase activity in the presence of P_i was determined with [32P-γ]ATP (specific activity 0.1-0.4 μCi × mmol⁻¹). The 32Pi was separated from [32P]ATP by the charcoal method [1].

Δψ and ΔpH were measured by the distribution of [14C]SCN⁻ and [14C]CH₃NH⁺ between the medium and vesicular interior (v_i), which was determined as difference between H₂O- and [14C]dextran spaces [3, 4]. The reactions were stopped by sedimentation of the vesicles in a cooled Beckman-airfuge at 160000 × g for 2 min. H and C radioactivities were extracted from the sediment with 10% (w/v) TCA.

Protein was determined by the Kjeldahl method, catecholamines according to [8].

**Materials**

ATP and ADP (Pharma Waldhof, Mannheim, Bundesrepublik Deutschland); [32P]orthophosphate, Potassium [14C]thiocyanate, Tritium (Hydrogen-3) H₂O (The Radiochemical Centre, Amersham, GB); Methylamine hydrochloride [14C], Dextran-Carboxyl [carboxyl-14C] (NEN Chemicals GmbH, Dreieichenhain, Bundesrepublik Deutschland); 32P-γ-ATP was synthesized according to [16]; CCCP, DCCD (Serva, Heidelberg, Bundesrepublik Deutschland); AP₅A (Boehringer, Mannheim, Bundesrepublik Deutschland); All chemicals were of reagent purity.

**Results**

Intact catecholamine storage organelles, suspended in iso-osmotic medium of low ionic strength are capable — in a reverse reaction of ATP hydrolysis — to form ATP from ADP + P_i, and to exchange the γ-phosphate group of ATP with P_i. As shown by the time courses, both the ATP forming reactions accelerated with time, while the velocity of ATP hydrolysis slowed down concomitantly (Fig. 1).

Accumulation of ADP as cause for the increasing velocity of the ATP-P_i exchange had been excluded [1]; the opposite changes in the velocities of the ATP-forming reactions on the one hand, and of ATP hydrolysis on the other hand, ought to indicate an altered state of the system itself, induced by ATP-Mg and also by ADP-Mg.

Addition of P_i, after the storage organelles had reacted for 3-30 min with ATP-Mg resulted in about a two fold acceleration of the reaction rates compared to that observed when ATP and P_i were added simultaneously (Fig. 2a). A dramatic acceleration of the velocity of ATP synthesis occurred, increasing progressively with time when P_i was added to the assays subsequent to ADP-Mg (Fig. 2b). The reaction rates obtained when P_i was added 3 to 30 min after ADP-Mg were about 5-6 times higher as those with the simultaneous addition of P_i. The rates even exceeded the rates of the ATP-P_i exchange obtained under similar conditions. In contrast, with simultaneous addition of P_i and the nucleotide, the ATP-P_i exchange reaction was 2-3 times faster than ATP synthesis from ADP + P_i (Fig. 2a and b). The rates of ATP hydrolysis were not affected by the subsequent addition of P_i (data not shown).

The protonophore, CCCP, added before or together with the nucleotide and P_i abolished both
ATP-forming reactions completely (Table I) and it enhanced the ATPase activity 2–2.5 fold.

When the inhibitor of energy transduction, DCCD, was added 10 min before starting the reaction with the nucleotide, the ATP-P_i exchange reaction was inhibited by 60%, ATP synthesis from ADP + P_i by 75% (Table I), and ATPase activity was diminished by 25%. Added simultaneously with the nucleotides, DCCD displayed only minor effects.

Since the catecholamine storage vesicle preparation contains adenylate kinase activity [1, 9] AP_5A, a potent inhibitor of this enzyme [10] was used at a high concentration (0.1 mM). As shown in Table I, AP_5A inhibited ATP synthesis from ADP + P_i by 50%, whereas the ATP-P_i exchange reaction as well as the ATPase activity were only insignificantly affected.

With membrane ghosts formed and suspended in KCl, ATP-P_i exchange and ATP synthesis were very low, amounting to 6–12% of the reaction velocities observed with intact storage organelles (Table II). Isethionate ghosts were only able to an ATP-P_i exchange at rates decreasing from 20 to 10% with proceeding reaction time compared to those obtained with intact storage organelles. The synthesis of ATP from ADP + P_i (i.e. in absence of any added energy source) was negligible, although the preparation contained the complete and unchanged ATPase apparatus [6]. In contrast to the acceleration of the reaction velocities observed with the intact storage organelle, the slow reactions of ghosts proceeded linearly with time. This data confirm the results of [2] who showed that the membrane of the emptied

ATP forming reactions with simultaneous and subsequent addition of P_i. 2a: ATP-P_i exchange, 2b: ATP formation from ADP + P_i.

Concentration of vesicle protein 0.25–0.4 mg x ml⁻¹, pH 7.4 (10 mM Tris maleate), 31 °C, ATP-Mg 5 mM, ADP-Mg 1 mM, P_i 1 mM. Symbols: — • addition of P_i simultaneously with ATP-Mg, □ — □ addition of P_i at time intervals indicated on the figure after ATP-Mg. O—O addition of P_i  simultaneously with ADP-Mg. The results are presented as reaction rates in nmol x mg protein⁻¹ x 3 min⁻¹. Mean of 6 experiments, the SEM were between 10 and 15%.

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Table I. Effect of agents on the ATP forming reactions and the components of ΔμH°. The experiments were performed at pH 7.4 (10 mM Tris maleate) and 31 °C. The reactions were started with the addition of the vesicle suspension giving a final concentration of 1.5–2.0 mg protein x ml⁻¹; ATP-Mg 5 mM, ADP-Mg 1 mM, P_i 1 mM. The results are presented in percent of the untreated controls (mean ± SEM of n experiments).

<table>
<thead>
<tr>
<th>Agent</th>
<th>ATP = P_i</th>
<th>ADP + P_i → ATP</th>
<th>ATP-Mg</th>
<th>ADP-Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol ^32P_i incorporated into nucleotide x mg protein⁻¹ x min⁻¹)</td>
<td>Δψ [mV]</td>
<td>ΔpH [mV]</td>
<td>Δψ [mV]</td>
</tr>
<tr>
<td>control</td>
<td>0.33 ± 0.07 (n = 8)</td>
<td>0.12 ± 0.02 (n = 8)</td>
<td>64.7 ± 1.4 (n = 8)</td>
<td>76.1 ± 1.7 (n = 8)</td>
</tr>
<tr>
<td>percent of activity remaining after treatment with agents</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP_5A</td>
<td>82.0 ± 7.1 (n = 6)</td>
<td>48.4 ± 6.2 (n = 7)</td>
<td>107.1 ± 2.0 (n = 3)</td>
<td>97.1 ± 2.3 (n = 3)</td>
</tr>
<tr>
<td>0.1 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCD</td>
<td>5.2 ± 1.4 (n = 4)</td>
<td>not measurable (n = 4)</td>
<td>11.6 ± 1.3 (n = 3)</td>
<td>76.7 ± 8.0 (n = 3)</td>
</tr>
<tr>
<td>0.015 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCCD</td>
<td>41.5 ± 6.6 (n = 4)</td>
<td>26.2 ± 4.1 (n = 4)</td>
<td>88.1 ± 2.2 (n = 3)</td>
<td>76.6 ± 7.4 (n = 3)</td>
</tr>
<tr>
<td>0.02 mM</td>
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</table>
Fig. 3. Time courses of $\Delta \psi$ and $\Delta \text{pH}$ of intact storage vesicles and of membrane ghosts upon addition of ATP-Mg or ADP-Mg. 1.8–2.5 mg x ml$^{-1}$ of vesicle protein were suspended in 0.3 M sucrose, pH 7.4 (10 mM Tris maleate), 31 °C. 0.2–0.3 mg x ml$^{-1}$ of membrane protein were suspended in 0.16 M solutions of sodium isethionate or KCl. ATP-Mg 5 mM, ADP-Mg 1 mM, when present, 1 mM. $\Delta \psi$ and $\Delta \text{pH}$ were measured as described in "Methods". $\Delta \psi$ and $\Delta \text{pH}$ in presence of ATP-Mg or ADP-Mg were determined in parallel experiments of the same preparations. Symbols: •—• $\Delta \psi$ and $\Delta \text{pH}$ induced by 5 mM ATP-Mg in the presence, ○—○ and in the absence of 1 mM P$_i$. ▲—▲ $\Delta \psi$ and $\Delta \text{pH}$ induced by 1 mM ADP-Mg in the presence, △—△ and in the absence of 1 mM P$_i$. $\Delta \psi$ and $\Delta \text{pH}$ induced by 5 mM ATP-Mg ■—■ or by 1 mM ADP-Mg □—□ across the membrane of ghosts formed in 15 mM KCl and suspended in 0.16 M KCl at pH 7.4. $\Delta \psi$ and $\Delta \text{pH}$ induced by 5 mM ATP-Mg ●—● or 1 mM ADP-Mg ○—○ across the membrane of ghosts formed in 15 mM isethionate and suspended in 0.16 M isethionate at pH 7.4. Ordinates: left ordinate: $\Delta \psi$ (mV); center ordinate: $\Delta \text{pH}$ (pH units); right ordinate: pH in the vesicular interior. The results are means of 7 experiments (vesicle experiments) the SEM were between 2 and 4%. The membrane ghost experiments are means of 5 experiments, the SEM were between 8 and 10%.
catecholamine storage organelle is only capable of ATP synthesis when supplied with an energy source (which was artificially imposed in the form of a KCl concentration gradient induced by valinomycin).

### Bioenergetic aspects of the ATP forming reactions

In the absence of added nucleotides, but in the presence of 5 mM Mg²⁺, the resting electrochemical potential of intact catecholamine storage organelles was 25−30 mV; this value was constant over 15 min at 31 °C. Under these conditions, the $\Delta \psi$ was 1.45−1.55 pH units, indicating an internal vesicular pH of 5.9 with the medium at pH 7.4. Thus, under resting conditions, the $\Delta \mu^+$ amounted to 110−120 mV to which the $\Delta \psi$ contributed only 20−25%.

As shown in Fig. 3, ATP-Mg induced a fast enhancement of $\Delta \psi$ to 60−70 mV (positive inside), which was completed within 3−5 min. Upon the addition of ATP-Mg the $\Delta \rho H$ was 1.35−1.4 pH units (Fig. 3). The small differences to that observed in the absence of nucleotides (0.1−0.15 pH units), however, never reached a level of significance. In the presence of ATP-Mg the $\Delta \rho H$ remained constant between 3 and 30 min, indicating that in a sucrose medium no ATP-Mg induced acidification of the vesicular interior took place. These data are in excellent agreement with the results of others [17], with the exception that the internal vesicular pH under our conditions was found to be between 5.8 and 6.1, in contrast to the value of 5.5 as described by [11]. The $\Delta \mu^+$ was 135−145 mV to which the $\Delta \psi$ contributed about 40%.

With the addition of ADP-Mg (in absence of added ATP) an almost as high $\Delta \psi$ arised as with ATP, reaching its maximum between 10−15 min of reaction. As shown in Fig. 3, the $\Delta \rho H$ after the addition of ADP-Mg dropped in a fast initial reaction by 0.1 pH unit and gradually further decreased over 30 min, resulting in an increase of the internal vesicular pH to 6.3. The $\Delta \mu^+$ was about 10 mV lower than that observed in the presence of ATP-Mg, the $\Delta \psi$ contributing to 50% to the $\Delta \mu^+$.

$P_1$, added in order to study its effect on the both components of $\Delta \mu^+$ under the conditions of both ATP forming reactions, significantly decreased the magnitudes of the $\Delta \psi$ compared to those observed in its absence. The $\Delta \rho H$ in the presence of ATP-Mg was not affected by $P_1$, while the ADP-Mg induced fall in $\Delta \rho H$ was slightly diminished by $P_1$ (Fig. 3).

$AP^+$ inhibiting adenylate kinase which at a high concentration (0.1 mM) inhibited ATP formation from ADP + $P_1$ by 50%, but which allowed an almost unimpaired ATP-$P_1$ exchange reaction (Table I), was without effect upon $\Delta \psi$ and $\Delta \rho H$ under both experimental conditions. This result excludes the possibility that the $\Delta \psi$ induced by ADP-Mg might be due to ATP, produced by adenylate kinase, from ADP.

The protonophore, CCCP, abolished the ATP- as well as the ADP-induced $\Delta \psi$, and was without effect upon the $\Delta \rho H$ (Table I).

DCCD, which strongly inhibited ATP-$P_1$ exchange and ATP formation from ADP + $P_1$, dissipated the ADP-Mg induced $\Delta \psi$ by 50% without affecting the $\Delta \rho H$; however, it affected both the components of $\Delta \mu^+$ occurring in the presence of ATP-Mg slightly (Table I). Membrane ghosts, suspended in iso-osmotic KCl or isethionate, displayed a very low $\Delta \rho H$, indicating an internal pH of 6.6−6.7; which was only slightly acidified by ATP in the Cl− containing medium. The $\Delta \psi$ of 35−40 mV observed under all conditions was almost unchanged with time of reaction (Fig. 3).

### Discussion

The fact that the catecholamine storage organelle is able to form ATP from ADP + $P_1$ without any added energy, suggests that it is equipped with an inherent energy source, which is made available by the addition of ADP. An alternative possibility is
that ATP, formed by adenylate kinase from the added ADP, serves as the energy source, and the product measured in the assays, $^{32}$P-ATP, actually is the result of an ATP-P$_i$ exchange. Both these possibilities were tested in the present study.

Although the membrane and soluble protein moiety of the catecholamine storage organelle contain adenylate kinase [9, 12] and AP$_3$A at high concentration inhibited 50% of ATP formation from ADP + P$_i$, it is highly improbable that the formed ATP is due to ATP-P$_i$ exchange: 1. The ADP-Mg induced energetic state of the organelle is unaffected when ATP formation by adenylate kinase is prevented by AP$_3$A. Thus, the observed inhibition by AP$_3$A must take place at a later step of the ATP formation reaction sequence; it, however, does not interfere with the first fundamental reaction, by which ADP-Mg induces the $\Delta\psi$. 2. Considering that the pH optimum of adenylate kinase is at 6 [13], and the ATP-P$_i$ exchange has a sharp optimum at pH 7 [1], if ATP formation would be due to an ATP-P$_i$ exchange its pH optimum ought to be between 6 and 7. However, ATP formation from ADP + P$_i$ increases with increasing pH of the medium [1].

3. Membrane ghosts (devoid of the large pool of small molecular constituents) which, however, contain adenylate kinase [9] are incapable of ADP + Pi - ATP. With ATP as energy source, only a low ATP-P$_i$ exchange takes place, even though the membrane — supplied with an artificially imposed energy source — is capable of a highly effective ATP synthesis [2].

The difference of $\Delta\psi$ in the presence and absence of P$_i$, which when ADP-Mg was the inducing nucleotide was paralleled by a drop of $\Delta$pH, obviously reflects a withdrawal of energy necessary for ATP formation. The higher magnitude of $\Delta\psi$ occurring in the absence of P$_i$ may explain the increasing acceleration of the ATP forming reactions when P$_i$ was added subsequent to the nucleotides. The low ATP induced $\Delta\mu$H$^+$ observed with membrane ghosts (exactly confirming results of [14]) indicates that the energy from ATP hydrolysis, carrying H$^+$ from the outside to the inside, contributed only a part of the large $\Delta\mu$H$^+$ that is obviously preformed in the intact storage organelle, and which is converted into an useable form by Mg-nucleotides.

Concentration gradients of Na$^+$ and K$^+$ stabilize — as has been shown by Skulachev [15] — the $\Delta\mu$H$^+$ of bacteria and algae. For the catecholamine storage organelle it is proposed that the concentration gradients of small molecular constituents between organelle and cytoplasm are used to establish the $\Delta\mu$H$^+$. Since 0.1 M catecholamine in the medium abolishes ATP synthesis almost completely (unpublished observation) it is most likely that the high catecholamine gradient across the membrane and disequilibration of the Donnan-equilibrium by ADP, that evokes catecholamine efflux, represents the source of energy by which ATP is formed.