SYNTHESIS OF ATP BY AN ARTIFICIALLY IMPOSED ELECTROCHEMICAL PROTON GRADIENT IN CHROMAFFIN GRANULE GHOSTS

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Received 28 March 1980

1. Introduction

Chromaffin granules, the catecholamine storage organelle of adrenal medulla accumulate noradrenaline by an active ATP-dependent process [1,2]. A Mg^{2+}-dependent ATPase of the bovine chromaffin granule membrane has been shown to be an inward H^+-translocase (H^+-pump) generating a transmembrane potential $\Delta\psi$ (inside positive) and/or a pH gradient ΔpH (inside acidic) [3–6] (D. S., J. P. H., submitted). Catecholamine uptake is driven by the proton electrochemical gradient ($\Delta\mu_{H^+}$) created by the H^+-pump [6–8]. In view of the similarities both functional and structural which exist between chromaffin granule and mitochondrial ATPase [9,10], the reversibility of the H^+-pump from the former source has been examined. Both ATP synthesis by an artificially generated $\Delta\mu_{H^+}$ and $^{32}P_i$–ATP exchange have been shown to occur in ghosts derived from chromaffin granules.

2. Materials and methods

2.1. *Materials*

Carrier-free $^{32}P_i$ from CEA, Saclay, was heated at 100°C for 60 min in 1 N HCl before use. Hexokinase, glucose 6-phosphate dehydrogenase were from Boehringer, firefly tails and valinomycin from Sigma. Nigericin was a gift of Dr A. Stempel, Hoffman La Roche.

Abbreviations: Mes, 2-(*N*-morpholino) ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid; EPPS, *N*-2 hydroxyethylpiperazine propane sulfonic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; MAO, monoamine oxidase; DBH, dopamine-β-hydroxylase

2.2. *Chromaffin granule ghosts*

Ghosts were prepared by osmotic lysis in K^+-free media [7] of granules purified as in [10]. The preparations were frozen in liquid nitrogen and were kept at −80°C. They were thawed at 37°C for 7 min before use. All pH adjustments were done with NaOH.

2.3. *Estimation of ATP*

ATP formation was followed by esterification of $^{32}P_i$. The reaction was stopped by addition of 10% trichloroacetic acid (0.2 ml) to the incubation mixture (0.1 ml). After centrifugation at 6000 × *g* for 10 min the supernatant (0.25 ml) was mixed with 0.3 ml 2,5% ammonium molybdate in 2.5 N H_2SO_4. Unesterified $^{32}P_i$ was extracted 3 times with 1.5 ml isobutanol–benzene (1:1) saturated with H_2O. An aliquot (0.4 ml) of the aqueous phase was counted in 5 ml 50% ethanol, using Cerenkov emission. ATP was also assayed by the firefly bioluminescence technique. The sample (0.1 ml) was injected in 2 ml of a mixture of purified luciferase and luciferin [11] in 5 mM $MgSO_4$, 25 mM Tris–HCl at pH 7.5. Alternatively, ATP was converted to glucose 6-phosphate by hexokinase (7 units) in presence of glucose (50 mM). Glucose 6-phosphate was then oxidized by NADP (0.4 mM) and glucose 6-phosphate dehydrogenase (1 unit) in 5 mM $MgSO_4$, 100 mM Hepes (pH 7.5) and the NADPH formed was assayed fluorimetrically.

2.4. *Analytical techniques*

Density gradient analysis of membranes was performed on 0.45–1.45 M sucrose linear gradients centrifuged at 270 000 × *g* for 3 h in a SW-41 rotor, according to [12]. Noradrenaline uptake was measured as in [13], with noradrenaline at 10 μM. ATPase activity was followed at pH 7.5 by the release of P_i assayed as in [14]. Dopamine β-hydroxylase and

monoamine oxidase were assayed as in [15] and [16], respectively. Proteins were estimated by the Folin technique.

3. Results

3.1. *Synthesis of ATP driven by an artificially imposed* $\Delta\mu_{H^+}$

To generate large $\Delta\mu_{H^+}$ ghosts were first incubated with valinomycin at pH 5.0 in K^+-free malonate buffer (acid stage) and to this medium was then added a basic buffer containing K^+ associated with the impermeant sulfate anion, to give a final pH of 8.3 and a K^+ external conc. 60 mM (base stage). This generated a transient pH gradient (inside acidic) and transmembrane potential (inside positive). When Mg^{2+}, ADP and $^{32}P_i$ were added to the base stage solution, $^{32}P_i$ was esterified (fig.1). The reaction was completed in

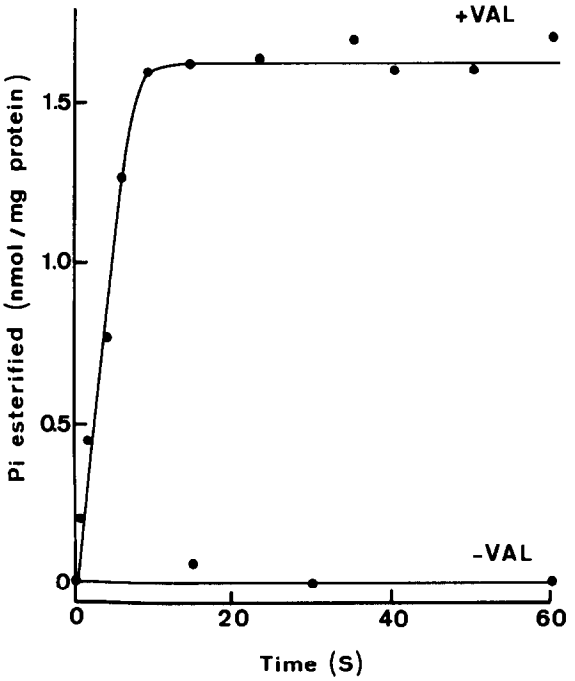

Fig.1. Time course of $^{32}P_i$ esterification driven by an imposed $\Delta\mu_{H^+}$. Ghosts (200 μg protein) were first incubated at 37°C for 10 min with Na^+-malonate (2.5 μmol) pH 5.0 and, where indicated, with valinomycin (0.4 nmol) in 0.05 ml final vol. A base stage solution (0.05 ml) containing K_2SO_4 (3.0 μmol), Na-ADP (0.2 μmol), $MgCl_2$ (0.2 μmol), sucrose (5 μmol), Na-EPPS (5 μmol) and $^{32}P_i$, Na^+ salt (0.5 μmol, 10^6 cpm) was then added. The reaction mixture was final pH 8.3. The reaction was terminated at the indicated time and was processed as in section 2.

Table 1
Identification of the esterified P_i

$^{32}P_i$ esterified (nmol/mg protein)	ATP formed[a] (nmol/mg protein)	
	Val-sensitive	Val-independent
2.45	2.54	0.64

[a] Assayed fluorimetrically as in section 2: the ATP formed in absence of valinomycin (Val-independent) was attributed to adenylate kinase and was substracted from the value obtained in presence of valinomycin to derive the ATP formed by the H^+-pump (Val-sensitive)

$^{32}P_i$ esterification and ATP formation were measured after a 10 s reaction time in the incubation mixture described in fig.1

~10 s and 1–4 nmol $^{32}P_i$ were esterified/mg protein. Addition of glucose and hexokinase to the base stage did not modify the time course of the reaction. Omission of valinomycin in the acid stage solution (which suppressed K^+ entry and imposed $\Delta\psi$) resulted in complete inhibition. Valinomycin at 2 μM (0.11 μg/mg protein) gave maximal esterification. ADP was required for the reaction with maximal synthesis for 2 mM ADP. With 10 mM P_i the amount of esterified $^{32}P_i$ was the same as with routine 5 mM.

The esterified P_i product was identified as ATP since it supported the production of glucose 6-phosphate by hexokinase (table 1). This experiment was complicated by the presence of a low adenylate kinase activity in the membrane preparation (3.6 nmol ATP formed . mg protein^{-1} . min^{-1}). This activity was distinguished from ATP synthesis since it required

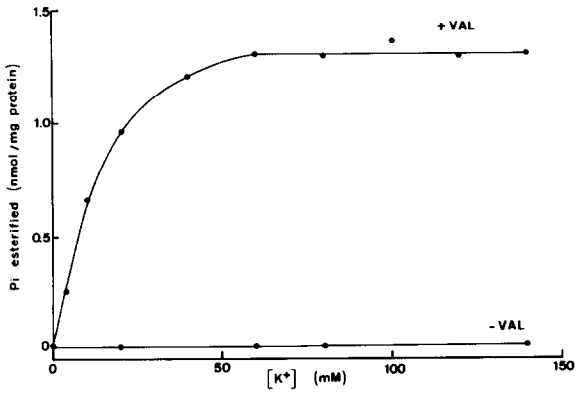

Fig.2. Effect of K^+ on ATP synthesis. The experiment was performed as in fig.1, except that the K^+ concentration of the base stage solution was varied to give the final indicated concentration. The reaction was stopped after 10 s of base stage.

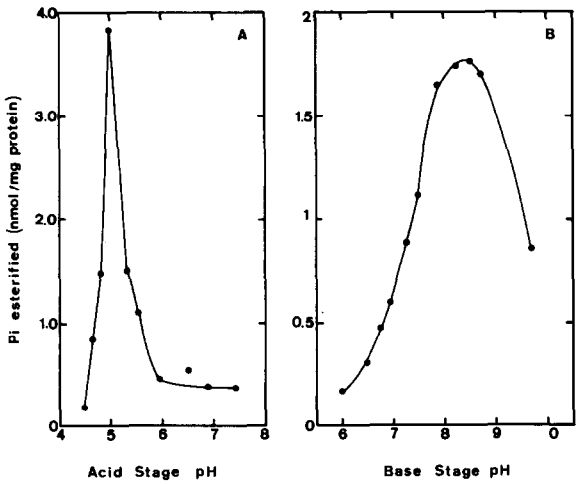

Fig.3. Effect of the pH of the acid and base stage solutions on ATP synthesis. The experiment was carried out as in fig.1, with 250 μg protein and a 10 s reaction time. (A) Effect of the acid stage pH. The pH of the malonate buffer was adjusted to give the indicated final pH. The final pH after mixing with the base stage solution was 8.3. (B) Effect of the base stage pH. The buffer of the base stage was: MES (pH 6.0–6.5), Hepes (pH 7.0–8.3), EPPS (pH 8.0–10.0). The indicated pH are final values. The acid stage pH was 5.0.

neither P_i nor valinomycin. The same results were obtained by the firefly bioluminescence technique (using purified luciferase devoid of adenylate kinase activity [11]) and 2.1 (SEM ± 0.4) nmol ATP . mg protein^{-1} . min^{-1} and 5.0 (SEM ± 0.5) nmol ATP . mg protein^{-1} . min^{-1} were derived for ATP synthesis and adenylate kinase, respectively.

Fig.2 shows the effect of the imposed $\Delta\psi$ magnitude on the reaction. ATP synthesis measured after a pH shift of 3.3 units (corresponding to $Z\,\Delta pH$ = 200 mV) increased with K^+ up to 60 mM and at zero external K^+ conc. no reaction was observed. K^+ in the internal space was estimated to be 2.8 mM from measurement of the null point of K^+ diffusion potentials induced by valinomycin and monitored with the fluorescent dye oxonol-V (D. S., J. P. H., submitted) and it was calculated that under these conditions maximal ATP synthesis occured with a $\Delta\psi$ of ~80 mV and thus a $\Delta\mu_{H^+}$ of 280 mV. In absence of valinomycin no reaction was observed, whatever the external K^+ concentration. On the other hand, in the absence of a previous acid stage, valinomycin (4 μM) and K^+ (60 mM) did not induce $^{32}P_i$ esterification.

The effect of the size of the pH gradient was studied by varying either the pH of the acid stage at fixed pH of the base stage (fig.3A) or varying the pH of the base stage at fixed pH of the acid solution (fig.3B). In the first type of experiment (fig.3A) and with a base stage at pH 8.3, maximal synthesis was observed for an acid stage at pH 5.0. More acidic pH probably inactivated the H^+-pump since its ATPase activity decreased dramatically at this value. When the pH of the base stage was varied and that of the acid stage maintained at pH 5.0 (fig.3B), an optimum

Table 2
Effect of inhibitors

	$^{32}P_i$ esterification (%)	$^{32}P_i$-exchange (%)
Control[a]	100	100
Nigericin (5 μg/ml)[b]	3.4	37.5[c]
NH_4Cl (30 mM)	0	74.2
$(NH_4)_2SO_4$ (30 mM)	–[d]	78.0
CCCP (2 μM)[b]	13.0	20
DCCD (80 μM)[b]	74.0	11.1
(400 μM)[b]	6.6	–[d]
Tributyltin chloride (50 μM)[b]	0	4.5
Triphenyltin chloride (100 μM)[b]	41.0	11.2

[a] $^{32}P_i$ esterification was measured as in fig.1 with a 10 s reaction time. The control was 1.1 nmol ATP/mg protein. $^{32}P_i$–ATP exchange was as in fig.4 with a 20 min reaction time. Control was 1.6 nmol ATP . mg protein^{-1} . min^{-1}. Values are the mean of at least 3 experiments

[b] Solubilized in ethanol to give a final ethanol concentration of ≤1% which was without effect on the reactions

[c] K_2SO_4 (25 mM) was added to the incubation mixture

[d] Not determined

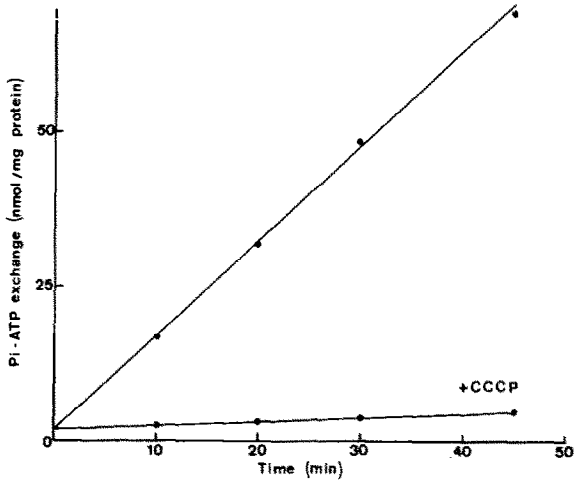

Fig.4. Time course of $^{32}P_i$–ATP exchange. Membranes (200 μg proteins) were incubated at 37°C for various times in a reaction mixture containing ATP (1.5 μmol), $MgSO_4$ (1.5 μmol), $^{32}P_i$, Na^+ salt (1.0 μmol, 10^6 cpm), sucrose (30 μmol) and Hepes (pH 7.0) (0.4 μmol) in 0.1 ml final vol. CCCP (0.2 nmol) was added where indicated. The reaction was terminated at the indicated time and esterified $^{32}P_i$ measured as in section 2. Unextractable $^{32}P_i$ counted for 2 nmol esterified $^{32}P_i$/mg protein.

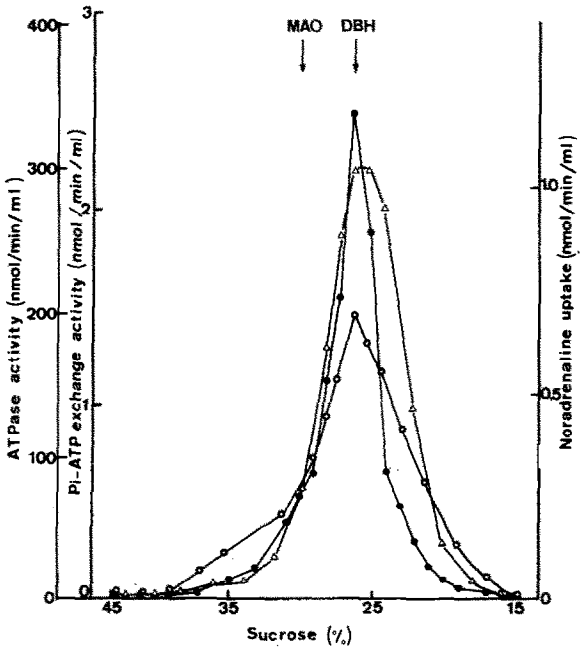

Fig.5. Distribution of $^{32}P_i$–ATP exchange after isopycnic centrifugation on sucrose gradient. Purified chromaffin granule membranes (7.5 mg protein) were centrifuged as in [12]. (•——•) $^{32}P_i$–ATP exchange (60 min incubation); (○——○) ATPase activity; (△——△) noradrenaline uptake.

was observed over pH 8.25–8.75. In these experiments malonate was used as the acid buffer since it gave the largest $^{32}P_i$ esterification. Nevertheless there was no absolute requirement for this anion since succinate, maleate and acetate resulted in reactions which were, respectively, 94, 71 and 29% of that observed with malonate.

The effect on ATP synthesis of compounds altering the $\Delta\mu_{H^+}$ has been investigated (table 2). Addition to the acid stage solution of the ionophore nigericin, which catalyzes the electroneutral H^+–K^+ exchange and thus decreases both the $\Delta\psi$ and the ΔpH blocked the reaction. $^{32}P_i$ esterification was also inhibited by the uncoupler CCCP which produces the same effect as nigericin when added together with valinomycin and by NH_4^+ which collapse the pH gradient. Inhibitors of the H^+-translocase such as DCCD [17] or trisubstituted tin derivatives [9] also blocked ATP synthesis.

3.2. *$^{32}P_i$–ATP exchange reaction*

The reversibility of the H^+-translocase was also demonstrated by the $^{32}P_i$–ATP exchange reaction (fig.4). In contrast with $^{32}P_i$ esterification driven by a $\Delta\mu_{H^+}$ transition this reaction was linear with time for >45 min with a rate of exchange of 1.3–1.8 nmol . min^{-1} . mg $protein^{-1}$. It also did not require addition of ADP, which was probably provided by ATP hydrolysis. This reaction involved the H^+-ATPase since it was blocked by inhibitors such as DCCD or trisubstituted tin derivatives (table 2) and was associated with the $\Delta\mu_{H^+}$ generated by the ATPase as shown by the inhibitory effect of CCCP (fig.4 and table 2). Decreasing the ΔpH by nigericin and K^+ also resulted in an inhibition of the reaction. The limited effect of NH_4^+ which collapsed the pH gradient might indicate that the effect on the ΔpH was compensated for by an increased $\Delta\psi$ [6].

3.3. *Cellular origin of the exchange activity*

Since the demonstrated $^{32}P_i$ esterification and $^{32}P_i$–ATP exchange reactions had many characteristics in common with those observed on submitochondrial particles [18,19], the possibility of a contamination of the purified preparation used has been carefully checked by centrifugation to equilibrium in linear sucrose gradients (fig.5). The exchange activity followed the major peak of ATPase activity and was clearly associated with chromaffin granule membranes localized by dopamine β-hydroxylase activity and

catecholamine uptake and not with mitochondrial membranes detected by monoamine oxidase activity [12].

4. Discussion

The evidence presented clearly show the reversibility of the ATP-dependent H^+-pump of chromaffin granule membrane. Applying an external $\Delta\mu_{H^+}$ to the vesicles resulted in ATP synthesis. The transient nature of the imposed $\Delta\mu_{H^+}$ might account for the limited reaction observed as suggested by measurements of the kinetics of decay of the $\Delta\psi$ and the ΔpH. The decrease of the $\Delta\psi$ generated by 60 mM K^+ was measured with the fluorescent probe 8-anilinonaphtalene sulfonate which responds rapidly to such potentials [20]. Biphasic kinetics were observed, 75% of the signal decaying exponentially ($t_{1/2}$ = 15 s at pH 8.3) and 25% being stable for 10 min. Dissipation of the H^+ concentration gradient was investigated by following [^{14}C]methylamine uptake as a function of time after a ΔpH transition. The amount of methylamine decreased exponentially with time ($t_{1/2}$ = 85 s), indicating a ΔpH decrease linear with time (0.3 pH unit/min). Nevertheless a decrease of 0.8 pH units occured during the dead-time of the experiment (~25 s), suggesting the existence of a more rapid H^+ efflux.

The $\Delta\mu_{H^+}$-driven synthesis of ATP by chromaffin granule ghosts is not different from similar reactions by chloroplasts [21], submitochondrial particles [18] or bacterial membranes [22]. These reactions were terminated in ~15 s and the amount of $^{32}P_i$ esterified in the present case compares well with the figure obtained in submitochondrial particles [18]. The characteristics of the reaction (substrate concentration, activity as a function of pH, effect of malonate and inhibitors) were similar to those of the mitochondrial one. ATP synthesis required an imposed $\Delta\mu_{H^+}$ of 160–200 mV similar to the values in [18,22], though it is difficult to correlate the extent of ATP synthesis with the initial value of the transient imposed $\Delta\mu_{H^+}$ as shown by the fact that synthesis occured during the exchange reaction where the H^+-pump generated a $\Delta\mu_{H^+}$ of 120–150 mV [4,6] (D. S., J. P. H., submitted). Optimal synthesis was obtained for a $\Delta\mu_{H^+}$ of ~280 mV, not different from the 260 mV obtained with bacterial membranes [22]. The demonstrated reversibility of the chromaffin granule ATPase is further evidence of the functional resemblance that this molecule bears to mitochondrial ATPase, even though structural differences have been reported between the two enzymes [10].

Acknowledgements

We thank Dr A. M. Michelson in whose laboratory this work was performed for sustained encouragement. We are grateful to the Service Veterinaire (Abattoirs de Mantes) for providing us with the bovine adrenals. This work was supported by contracts from the CNRS (ER 103), the DGRST (contract no. 78.7.2774) and INSERM (contract no. 77.4.0842).

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