Independent Inhibitions of Mitochondrial Complex V by the Adenosinetriphosphatase Inhibitor Protein and Active-Site Modifiers†

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ABSTRACT: The methyl 4-azidobenzimidate derivative of the naturally occurring ATPase inhibitor protein (IF_1) of mitochondria binds to the β subunit of soluble F_1-ATPase upon photoactivation [Klein, G., Satre, M., Dianoux, A.-C., & Vignais, P. V. (1981) *Biochemistry 20*, 1339–1344]. A number of specific ATPase inhibitors, namely, 4-chloro-7-nitrobenzofurazan (NBF-Cl), efrapeptin, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA), phenylglyoxal, aurovertin, tridentate ferrous bathophenanthroline, and octylguanidine (referred to hereafter as "artificial" inhibitors), are also considered to bind to the β subunit, and there is strong evidence that the first three bind at the active site. Since the inhibition by IF_1 of complex V ATPase activity can be reversed by incubation of the inhibited complex at pH 8.0, this system was used to investigate whether the inhibitions brought about by IF_1 and the artificial inhibitors were independent, mutually interfering, or mutually exclusive. The experiments were carried out in two ways. (a) Complex V was first maximally inhibited by IF_1. Then an artificial inhibitor was added and allowed to react. Excess artificial inhibitor was removed by precipitation of the doubly inhibited complex V with ammonium sulfate and resuspension in inhibitor-free buffer at pH 8.0. Incubation at pH 8.0 released the inhibition due to IF_1. However, it was found that the factor that controlled reemergence of ATPase activity was the degree of inhibition exerted by the artificial inhibitor. When the artificial inhibitor was removed first (which was done by addition of dithiothreitol when the artificial inhibitor was NBF-Cl), then reemergence of activity depended on incubation at pH 8.0 to reverse the inhibition due to IF_1. These results indicated that IF_1-inhibited complex V could be independently inhibited by various artificial inhibitors. The artificial inhibitors used in this type of study were NBF-Cl, efrapeptin, aurovertin, FSBA, and phenylglyoxal. (b) Complex V was first treated with the artificial inhibitor (ferrous bathophenanthroline or octylguanidine) and then with IF_1. Results showed that prior treatment of complex V with these inhibitors did not interfere with IF_1 subsequently exerting maximal and reversible inhibition. The above results have been discussed in view of the recent finding that F_1-ATPase contains two functional and interacting hydrolytic sites [Grubmeyer, C., & Penefsky, H. S. (1981) *J. Biol. Chem. 256*, 3718–3727].

The ATPase inhibitor protein (IF_1)[1] of beef heart mitochondria is a polypeptide of M_r ~10 500 [Pullman & Monroy, 1963; Brooks & Senior, 1971; for recent reviews, see Ernster et al. (1979) and Pedersen et al. (1981)]. It combines with the ATPase complex and inhibits ATP hydrolysis (>90%) and the initial, but not the steady-state, rate of ATP synthesis (Harris et al., 1979; Gomez-Puyou et al., 1979). Inhibition of ATPase activity by added IF_1 requires the presence of a hydrolyzable nucleoside triphosphate plus Mg^{2+}, and an active ATPase (Horstman & Racker, 1970; Gomez-Fernandez & Harris, 1978; Galante et al., 1981). Recent studies by Klein et al. (1980, 1981) have suggested that IF_1 binds to the β subunit of the soluble F_1-ATPase, and there is considerable evidence that the β subunit of ATPase bears the hydrolytic site (Wagenvoord et al., 1977; Esch & Allison, 1978; Slater et al., 1979; Ting & Wang, 1980; Grubmeyer & Penefsky, 1981).

Our previous studies showed that IF_1 inhibits the ATPase activity of complex V (purified mitochondrial ATP synthetase complex) in a reversible manner (Galante et al., 1981). Inhibition required the presence of NTP·Mg^{2+} and an active enzyme, and was promoted at pH <7.0. Reversal of inhibition was brought about by incubation of the inhibited complex V in the absence of NTP·Mg^{2+} at pH >7.0. The present studies show that a number of specific inhibitors of F_1-ATPase, such as NBF-Cl, efrapeptin, aurovertin, phenylglyoxal, FSBA, tridentate ferrous bathophenanthroline, and octylguanidine, appear to inhibit ATPase activity in a manner which is independent from the inhibition brought about by IF_1.

Materials and Methods

Complex V was prepared as described before from mitochondria or submitochondrial particles (Stiggall et al., 1978, 1979). IF_1 was purified essentially by the method of Horstman & Racker (1970) up to step 4, the heat treatment (step 5) was omitted, and IF_1 was purified to homogeneity by DEAE-cellulose chromatography as detailed previously (Galante et al., 1981). ATPase activity was measured either by spectrophotometry in the presence of a regenerating system or by direct determination of the orthophosphate liberated (Stiggall et al., 1978). ATPase inhibitor activity was determined essentially by the procedure of Horstman & Racker (1970) in 10 mM Tes–Tris or 10 mM potassium phosphate, both at pH 6.7, as specified in the figure legends. The order of additions to the preincubation mixture was buffer, complex V, IF_1, and ATP·Mg^{2+}. Other inhibitors were added either before addition of IF_1 or following the addition of IF_1 and ATP·Mg^{2+} after maximal IF_1-induced inhibition had been reached. Other details are given in the text and the figure legends. The extent of bound [^{14}C]phenylglyoxal or [^{14}C]FSBA was determined as follows. Complex V was incubated at 30 °C with the radioactive inhibitor under the conditions specified in the figure legends. At the intervals indicated, either aliquots were withdrawn and filtered through a Sephadex G-25 medium

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[1] Abbreviations: IF_1, ATPase inhibitor protein; NTP, nucleoside triphosphate; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; NBF-Cl, 4-chloro-7-nitrobenzofurazan; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone; AMP-P(NH)P, adenyl-5′-yl imidodiphosphate; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; PPO, 2,5-diphenyloxazole.

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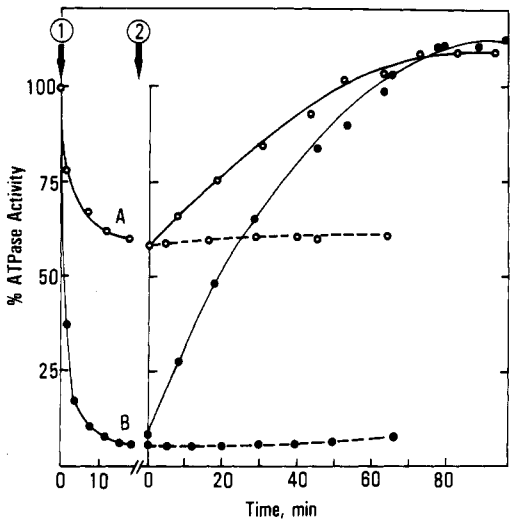

FIGURE 1: Reversible inhibition of the ATPase activity of complex V by IF_1. (Left panel, inhibition phase) Complex V was suspended at 1.2 mg of protein/mL in a medium at pH 6.7 containing 0.25 M sucrose, 10 mM potassium phosphate, 1 mM ATP, and 1 mM $MgSO_4$. The mixture was divided into two equal parts, A and B, and incubated at 30 °C. At the time marked by arrow 1, 3.0 μg of IF_1 per unit of complex V ATPase activity was added to B (●) and an equal volume of buffer to A (○). They were then sampled for ATPase activity assay at the intervals shown. At arrow 2, saturated ammonium sulfate (saturated at room temperature) was added to A and B to half-saturation, and the tubes were centrifugated at 105000*g* for 10 min. (Right panel, reactivation phase) After centrifugation, the supernatants were discarded, and the pellets were suspended at 8 mg of protein/mL in 0.25 M sucrose containing 50 mM potassium phosphate, pH 8.0, in the absence (solid lines) and presence (dashed lines) of 1 mM $ATP{\cdot}Mg^{2+}$. They were then incubated at 30 °C and sampled for ATPase activity assay at the intervals shown. 100% ATPase activity of complex V was 8 μmol of ATP hydrolyzed per min per mg of protein. One unit of ATPase is that amount of enzyme which causes the hydrolysis of 1 μmole of ATP per min at 30 °C. In Figures 2, 3, , 6, and 7, inhibition and reactivation buffers (always in the absence of $ATP{\cdot}Mg^{2+}$ in the reactivation buffer), treatments, incubation conditions, and activity assays were the same. Other changes are specified in the respective legends.

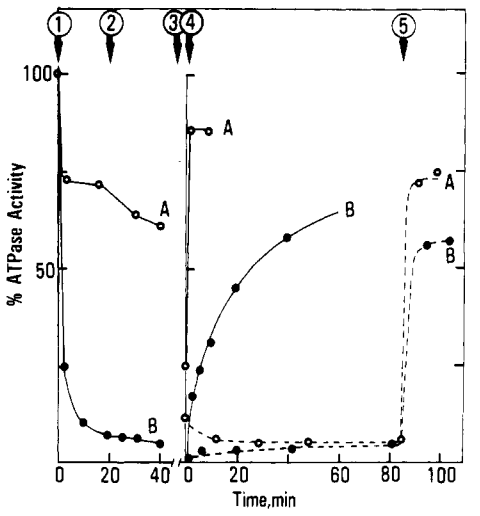

FIGURE 2: Inhibition of complex V ATPase activity by IF_1 and NBF-Cl. (Left panel) Conditions were the same as in Figure 1, except that at arrow 1 3.8 μg of IF_1 per unit of complex V ATPase activity was added to B (●) and at arrow 2 200 μM NBF-Cl was added to both A (○) and B (●). Arrow 3 indicates the addition of ammonium sulfate and centrifugation. (Right panel) Arrows 4 and 5 indicate addition of 10 mM dithiothreitol to mixtures A and B, respectively, at zero time (solid lines) and after 82 min of incubation at pH 8.0 and 30 °C (dashed lines). 100% ATPase activity was 7.7 μmol min^{-1} mg^{-1}.

column (6 × 1.5 cm) to eliminate free [^{14}C]phenylglyoxal or the protein was precipitated with 90% ethanol to eliminate free [^{14}C]FSBA. Protein-bound radioactivity was measured in a Triton X-100–toluene–PPO cocktail (300 mL of Triton X-100, 700 mL of toluene, and 5 g of PPO) in a Beckman LS 250 liquid scintillation counter.

Phenylglyoxal was a product of Aldrich, and phenyl[2-^{14}C]glyoxal was purchased from Research Products International. Bathophenanthroline was from Sigma Chemical Co. and NBF-Cl from Pierce. Other inhibitors of F_1-ATPase were kindly supplied by the following investigators: efrapeptin by Dr. L. Hamill, Eli Lilly; aurovertin by Dr. B. Beechey, Shell Research Laboratories; octylguanidine by Dr. A. Gomez-Puyou, Instituto Politecnico Nacional, Mexico City; and FSBA and [^{14}C]FSBA by Dr. A. di Pietro, Universite Claude Bernard de Lyon. All other chemicals were reagent grade.

Results

As shown elsewhere (Galante et al., 1981), preparations of complex V contain low levels of bound IF_1. When complex V is incubated at pH 6.7 and 30 °C in the presence of $ATP{\cdot}Mg^{2+}$, the endogenous IF_1 of the preparation results in partial (25–50%) inhibition of the ATPase activity of the preparation. Added IF_1 under these conditions increases the degree of inhibition to the maximal level of ≥90%. The maximally inhibited complex V can then be precipitated by addition of saturated ammonium sulfate to half-saturation, sedimented by centrifugation, separated from the soluble material, and resuspended in buffer. These treatments do not result in the removal of IF_1 and release of inhibition. However, when the resuspended complex V is incubated at 30 °C and pH >7.0 in the absence of $ATP{\cdot}Mg^{2+}$, the ATPase activity of the complex reemerges in a time-dependent manner. An example of this reversible inhibition of complex V is shown in Figure 1. The left panel in this figure (and in Figures 2, 3, 4, 6, and 7) shows the inhibition phase for the endogenous (trace A) and added (trace B) IF_1 at pH 6.7 in the presence of $ATP{\cdot}Mg^{2+}$. The right panel shows the reversal of IF_1 inhibition at pH 8.0 in the absence of $ATP{\cdot}Mg^{2+}$. It is seen in the left panel that at pH 6.7 the endogenous and the added IF_1 caused, respectively, 40% and 95% inhibition of ATPase activity. After precipitation of complex V with ammonium sulfate and resuspension at pH 8.0 in the absence of $ATP{\cdot}Mg^{2+}$, both complex V samples slowly recovered ≥100% of the original activity of complex V (Figure 1, right panel). The dashed lines in the right panel show that incubation at pH 8.0 in the *presence* of $ATP{\cdot}Mg^{2+}$ does not result in release of inhibition. In the studies described below, this system was used to see whether IF_1-inhibited complex V was protected against attack by several active-site (and/or regulatory-site) modifiers of ATPase.

NBF-Cl, Aurovertin, and Efrapeptin. Figure 2 gives the results of such an experiment with NBF-Cl, which has been shown to bind to a tyrosyl residue apparently at the active site on the β subunit (Ferguson et al., 1975; Lunardi & Vignais, 1979; Ting & Wang, 1980). In Figure 2, at the point shown by arrow 1, $ATP{\cdot}Mg^{2+}$ was added to two incubation mixtures A and B containing complex V at pH 6.7, and purified IF_1 was added to mixture B. The amount of added IF_1 in this and subsequent experiments was always predetermined to result in maximal inhibition without excess IF_1 being added. Incubation of mixtures A and B at 30 °C resulted in 25% inhibition of the ATPase activity of A due to the endogenous IF_1 of the complex V preparation, and ≥93% inhibition of B due to the added IF_1. When maximal inhibition was reached in both A and B, NBF-Cl was added to both incubation mixtures at the time point shown by arrow 2. Since binding of NBF-Cl is pH

dependent and favored under alkaline conditions (Ferguson et al., 1975), addition of this reagent caused little additional inhibition at pH 6.7. At the point shown by arrow 3, neutralized saturated ammonium sulfate was added to mixtures A and B to half-saturation, and the precipitated complex V samples were sedimented by centrifugation. The supernatants were discarded, and the residues were suspended in pH 8.0 buffer in the absence of added IF_1, $ATP{\cdot}Mg^{2+}$, and NBF-Cl. They were then incubated at 30 °C and assayed for activity. It is seen that at the onset of incubation (right-hand panel), the ATPase activities of A and B were, respectively, 90% and 99% inhibited. Addition of dithiothreitol at arrow 4 to incubation mixtures A and B resulted in displacement of enzyme-bound NBF-Cl and immediate reversal of the inhibition of A and recovery of 86% of the original activity of complex V, while mixture B remained inhibited. However, when mixture B was incubated at 30 °C and pH 8.0, its ATPase activity slowly emerged as in mixture B of Figure 1 which had not been treated with NBF-Cl. By contrast, when mixtures A and B were incubated at 30 °C and pH 8.0 in the absence of added dithiothreitol (dashed traces), then their activities remained inhibited. After about 80 min of incubation, dithiothreitol was added to both mixtures at the point shown by arrow 5, and as seen in Figure 2, both A and B showed immediate recovery of ATPase activity. These results indicated, therefore, that (a) inhibition of complex V with IF_1 does not protect the enzyme from independent inhibition by subsequent addition of NBF-Cl, and (b) IF_1 inhibition can be released by incubation at pH 8.0 of complex V treated with IF_1 as well as NBF-Cl both prior and after removal of NBF-Cl by added dithiothreitol. The reason that full activity was not recovered upon addition of dithiothreitol might be due partly to incomplete release of IF_1 inhibition and partly to the fact that NBF-Cl inhibition of ATPase is often not completely reversible, especially after prolonged treatment (Guillory, 1979; Y. M. Galante et al., unpublished observations). When aurovertin or efrapeptin was added to IF_1-inhibited complex V, the results were essentially the same, i.e., to the extent that subsequent incubation of the preparation at pH 8.0 did not result in the release of ATPase activity. However, since aurovertin and efrapeptin bind very tightly to the ATPase with dissociation constants on the order of 10^{-8} (Chang & Penefsky, 1973; Cross & Kohlbrenner, 1978), it was not possible to remove these inhibitors and recover activity by incubation of complex V at pH 8.0. Nevertheless, it is reasonable to assume that, as in the experiment with NBF-Cl, the IF_1-inhibited complex V was not protected against inhibition by efrapeptin or aurovertin. Were this not so, then pH 8.0 incubation of the IF_1-treated complex V (after removal of free efrapeptin and aurovertin from the medium by precipitation and resuspension of complex V) should have resulted in activity recovery due to the release of IF_1 inhibition.

FSBA. Esch & Allison (1978, 1979) and Di Pietro et al. (1979) have studied the inhibition of soluble F_1-ATPase by FSBA. The former authors reported that the enzyme was irreversibly inhibited by FSBA and that an essential tyrosyl residue was sulfonylated by the inhibitor, presumably at the active site on the β subunit. Di Pietro et al. (1979) concluded from kinetic studies that the inactivation of F_1-ATPase by FSBA is biphasic. The first, high-affinity phase reflects the binding of FSBA to the ADP regulatory sites, while the second, low-affinity phase corresponds to binding of FSBA to the ATPase catalytic site. Figure 3 shows in the left panel the effect of FSBA added to complex V which had been previously inhibited partially by endogenous (trace A) IF_1 and ≥93% by

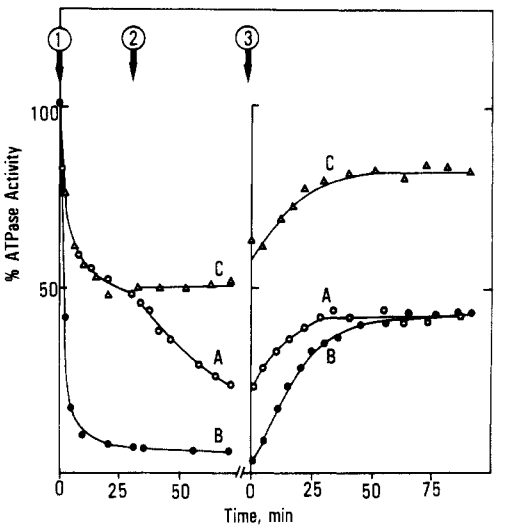

FIGURE 3: Inhibition of complex V ATPase activity by IF_1 and FSBA. (Left panel) Complex V was suspended at 1 mg of protein/mL in 10 mM Tes–Tris, pH 7.0, containing 1 mM ATP and 1 mM $MgSO_4$. At arrow 1, 4.7 μg of IF_1 per unit of complex V ATPase activity was added to B (●), and at arrow 2, 0.5 mM FSBA in dimethyl sulfoxide was added to both A (○) and B (●). Tube C (△) received an equivalent volume of dimethyl sulfoxide only. At arrow 3, all samples were filtered through a Sephadex G-25 column (1.5 × 6 cm) equilibrated in 10 mM Tes–Tris, pH 7.0, in order to remove excess FSBA. The protein peaks were collected and precipitated with ammonium sulfate as described in the legend to Figure 1. (Right panel) The pellets were suspended at 5.5 mg of protein/mL in 50 mM Tris–sulfate, pH 8.0, and incubated and assayed as in Figure 1. 100% ATPase activity was 7.5 μmol min^{-1} mg^{-1}.

added (trace B) IF_1. Trace C is the control in the absence of added IF_1 or FSBA. Since, unlike NBF-Cl, the FSBA inhibition is irreversible, the experiment was designed such that the amount of added FSBA would cause partial inhibition, and, therefore, partial recovery of activity due to reversal of IF_1 inhibition could subsequently be detected. After transition to pH 8.0, the control mixture C recovered 82% of the original activity, while the FSBA-treated preparations, though originally inhibited to different extents by IF_1, recovered exactly the same extent (42%) of the original ATPase activity. These results indicated, therefore, that (a) FSBA caused the same degree of inhibition when added to two mixtures of complex V previously inhibited by IF_1 to the extent of 50% and ≥93%, and (b) partial inhibition by FSBA did not affect the reversal of inhibition due to IF_1.

Phenylglyoxal. It has been shown that the arginine-modifying reagents phenylglyoxal and butanedione inhibit the ATPase activities of soluble F_1-ATPase and complex V and that ADP, GDP, and IDP protect against these inhibitions (Marcus et al., 1976; Frigeri et al., 1977, 1978). These results have suggested the possible modification of an essential arginyl residue at the ATPase active site. This interpretation is consistent with the fact that a segment of the β subunit bearing covalently bound FSBA, and presumed to represent a portion of the enzyme active site, contains an arginyl group four residues removed from the FSBA binding site (Esch & Allison, 1978). Figure 4 shows an experiment in which 1 mM phenylglyoxal was added to complex V which had been previously inhibited partially by endogenous IF_1 and ≥90% by added IF_1 (traces A and B, respectively, in the left panel of Figure 4). Once again, the amount of added phenylglyoxal was predetermined to cause only partial inhibition under the conditions used [i.e., pH 6.7; see also Takahashi (1968)] so that partial recovery of activity due to reversal of IF_1 inhibition could be detected at pH 8.0. This partial recovery of activity resulting

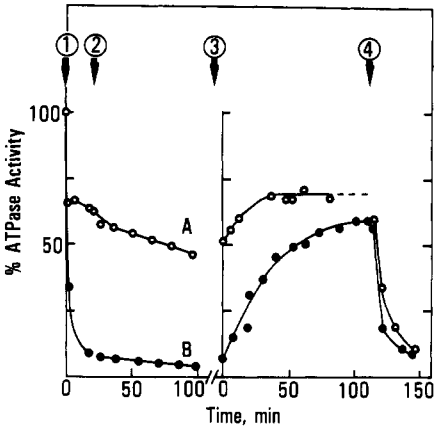

FIGURE 4: Inhibition of complex V ATPase activity by IF_1 and phenylglyoxal. (Left panel) Complex V was suspended at 1 mg of protein/mL in the same buffer as in Figure 1. At arrow 1, 3.5 μg of IF_1 per unit of complex V ATPase activity was added to B (●), and at arrow 2, 1 mM phenylglyoxal was added to both A (○) and B (●). Arrow 3 indicates ammonium sulfate precipitation of complex V and resuspension in sucrose–phosphate, pH 8.0, at 6 mg of protein/mL, and arrow 4 indicates addition of 5 mM phenylglyoxal to both A (○) and B (●). 100% ATPase activity was 7 μmol min^{-1} mg^{-1}.

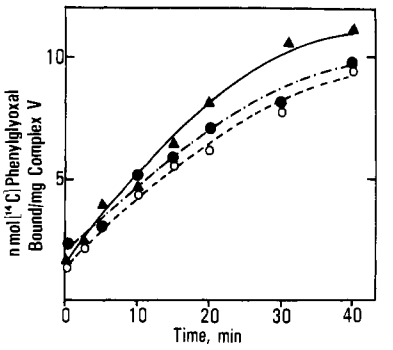

FIGURE 5: Binding of [^{14}C]phenylglyoxal to complex V in the presence and absence of IF_1. Complex V was incubated at a protein concentration of 1.2 mg/mL in 0.25 M sucrose containing 10 mM Tes–Tris, pH 6.7, 1.0 mM ATP, and 1.0 mM $MgSO_4$ at 30 °C. 3.0 μg of IF_1 per unit of complex V ATPase activity was added only to (●). After 20 min, the mixtures were brought to half-saturation with saturated, neutralized ammonium sulfate and centrifuged at 105000g. The pellets were resuspended at a protein concentration of 8 mg/mL in 0.25 M sucrose containing 10 mM potassium phosphate, pH 6.7 [in order to keep (●) inhibited], plus (●, ○) or minus (▲) 0.5 mM ATP, and 0.5 mM $MgSO_4$. 2.9 μg of IF_1 per unit of complex V ATPase activity was added to (▲) only. Then 1 mM [^{14}C]phenylglyoxal (specific radioactivity 804 cpm/nmol) was added to each mixture and the incubation continued at 30 °C. At the intervals indicated, aliquots of 0.4 mL each were withdrawn and filtered through Sephadex G-25 (medium) columns (1.5 × 6 cm) equilibrated in the same buffer as described above. The protein peaks were collected, protein concentrations were determined, and 2.0-mL samples of each peak were counted for radioactivity in a Triton–toluene–PPO cocktail as described under Materials and Methods. (●) Complex V treated with IF_1 + ATP·Mg^{2+} at pH 6.7 and then with [^{14}C]phenylglyoxal; (▲) complex V treated at pH 6.7 with IF_1 in the absence of ATP·Mg^{2+} and then with [^{14}C]phenylglyoxal; (○) complex V treated at pH 6.7 with ATP·Mg^{2+} in the absence of added IF_1 and then with [^{14}C]-phenylglyoxal.

from incubation of the inhibited complex V at pH 8.0 is shown in the right panel of Figure 4. In addition, it is shown that a second addition of a higher concentration of phenylglyoxal (5 mM) after reversal of IF_1 inhibition resulted in >90% inhibition of both samples. The results of Figure 4 show, therefore, that >90% inhibition of complex V by added IF_1 did not protect the enzyme against attack by phenylglyoxal.

We had shown previously that ADP and GDP not only protected the ATPase activity of complex V against inhibition by phenylglyoxal but also offered considerable protection (≥50%) against total [^{14}C]phenylglyoxal binding by complex V (Frigeri et al., 1978). It was therefore of interest to see whether the presence of maximally inhibiting levels of IF_1 bound to complex V would alter the binding titer of [^{14}C]-phenylglyoxal. As seen in Figure 5, there was little or no difference in the extent of [^{14}C]phenylglyoxal binding to complex V over a period of 40-min incubation regardless of whether complex V was or was not treated with IF_1 ± ATP·Mg^{2+}. Similar results were obtained when complex V preparations untreated and treated with maximally inhibiting concentrations of IF_1 + ATP·Mg^{2+} were treated subsequently with [^{14}C]FSBA, and the extent of labeling followed over a period of 180 min. These results are, therefore, in full agreement with the dual inhibitions shown in Figures 3 and 4 and are consistent with the interpretation that IF_1 binding by complex V and inhibition of ATPase activity do not alter the binding characteristics and the inhibitory effects of the above active-site modifiers of ATPase.

Ferrous Bathophenanthroline. The tridentate bathophenanthroline chelate of ferrous ion is a potent inhibitor of ATPase and is believed to bind to the β subunit of F_1 (Phelps et al., 1975a; Carlsson et al., 1978). The binding of ferrous bathophenanthroline to F_1 and to complex V is very strong. The fully inhibited enzyme can be passed through Sephadex G-25 without loss of ferrous bathophenanthroline or the degree of inhibition. Most potent uncouplers reverse this inhibition of ATPase activity without releasing ferrous bathophenanthroline from the enzyme. What happens is that uncouplers bind to ferrous bathophenanthroline while the latter is bound to the enzyme (Frigeri et al., 1978). As a result, the uncoupler-complexed ferrous bathophenanthroline no longer inhibits the ATPase activity even though it is still firmly bound to the protein.

Figure 6 shows the effect of ferrous bathophenanthroline on complex V in the absence and presence of IF_1. In this experiment, ferrous bathophenanthroline was first added to two tubes containing complex V at pH 6.7 and 30 °C (Figure 6, arrow 1). The preparations were then assayed for ATPase activity, which showed that both were 75–80% inhibited. At this point, CCCP was added to both tubes in an amount equimolar to ferrous bathophenanthroline (Figure 6, arrow 2). Activity assay showed partial activity recovery up to 68% of the initial activity of complex V. Then at the point shown by arrow 3 in Figure 6, IF_1 was added to one tube (closed circles) followed by ATP·Mg^{2+} to both tubes, and incubation at 30 °C continued with sampling at the intervals shown for the ATPase activity assay. It is seen that in spite of the presence of bound ferrous bathophenanthroline–CCCP complex, both the endogenous (Figure 6, open circles) and the added IF_1 (Figure 6, closed circles) were able to inhibit the enzyme. The right-hand panel of Figure 6 shows the release of inhibition due to IF_1 at pH 8.0. Thus, the results of Figure 6 show that complex V even after binding of the inhibitor ferrous bathophenanthroline could still be reversibly inhibited by added IF_1. Of course, the requirement of an active enzyme for manifestation of IF_1 inhibition necessitated reactivation of the ferrous bathophenanthroline-inhibited enzyme with CCCP before addition of IF_1 + ATP·Mg^{2+}. However, as pointed out above, addition of CCCP does not dislodge ferrous bathophenanthroline from the enzyme, because the ternary complex of enzyme + ferrous bathophenanthroline + CCCP could be precipitated with ammonium sulfate or filtered through Sephadex without any loss of ferrous bathophenanthroline.

In apparent contradiction with the above results, Tuena de

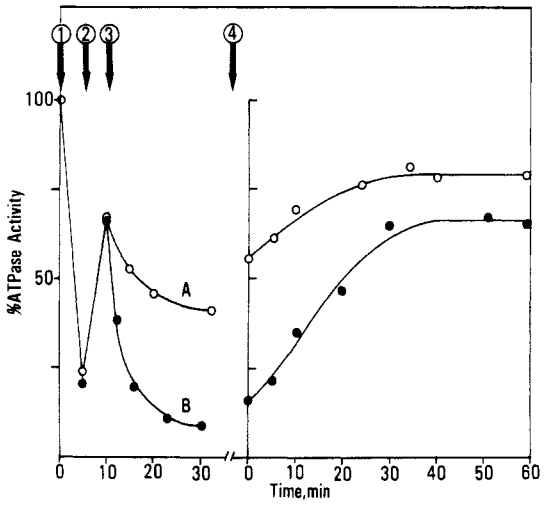

FIGURE 6: Inhibition of complex V by tridentate bathophenanthroline chelate of ferrous ion and by IF_1. (Left panel) Complex V (1.2 mg of protein/mL) in 0.25 M sucrose containing 10 mM potassium phosphate, pH 6.7, was placed into tubes A and B at 0 °C. At arrow 1, 35 nmol of ferrous bathophenanthroline per mg of complex V was added to A and B. ATPase activity was measured, then 36 nmol of CCCP was added at arrow 2 to each tube per mg of complex V, and their activities were measured again. At arrow 3, the tubes were transferred to a 30 °C bath, and 3.5 μg of IF_1 per unit of complex V ATPase activity was added to B (●) followed by 1 mM ATP and 1 mM $MgSO_4$ to both A and B. They were then sampled for ATPase activity measurements at the intervals shown. At arrow 4, both suspensions were precipitated with ammonium sulfate and centrifuged, and the pellets were resuspended at pH 8.0 as described in Figure 1. (Right panel) To each tube was added 40 nmol of CCCP per mg of protein. They were then incubated at 30 °C and sampled for activity measurement at the intervals shown. 100% ATPase activity was 9.2 μmol min^{-1} mg^{-1}.

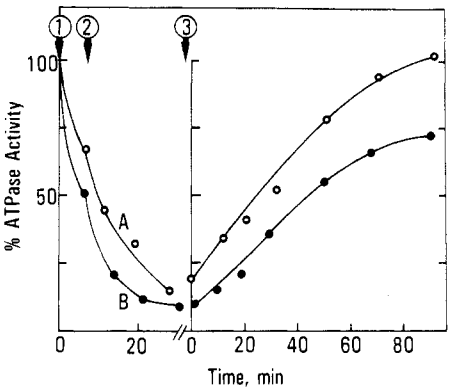

FIGURE 7: Inhibition of complex V by IF_1 in the absence and presence of octylguanidine. (Left panel) Complex V was suspended at 1.5 mg of protein/mL in 10 mM Tes–Tris, pH 6.8, plus 1 mM ATP·Mg^{2+} and divided into two parts. At arrow 1, 250 μM octylguanidine in ethanol was added to B (●) and an equal volume of ethanol to A (○). The tubes were incubated at 30 °C and sampled for activity assay. At this time (arrow 2), 2.0 μg of IF_1 per unit of complex V ATPase activity plus 1 mM ATP·Mg^{2+} was added to both A and B, and incubation and activity assay were continued as shown. At arrow 3, both samples were precipitated with ammonium sulfate, and as described in Figure 1, the pellets were suspended at 5 mg of protein/mL in sucrose–phosphate, pH 8.0. (Right panel) The tubes were incubated at 30 °C and assayed for activity at the intervals shown. 100% ATPase activity was 9 μmol min^{-1} mg^{-1}.

Gomez-Puyou et al. (1980) have concluded in a recent publication that ferrous bathophenanthroline prevents the interaction of IF_1 with soluble F_1-ATPase. Their results, given in Table III of the above reference, show that after F_1 was inhibited by ferrous bathophenanthroline, then added IF_1 had no independent effect, and ferrous bathophenanthroline inhibition could be partially reversed by subsequent addition of the uncoupler FCCP. However, this should not be interpreted to mean that ferrous bathophenanthroline is unique in preventing IF_1 interaction. The results simply confirm the known fact that IF_1 interaction with F_1 requires an active enzyme (Gomez-Fernandez & Harris, 1978). Indeed, in the same table, Tuena de Gomez-Puyou et al. (1980) show that after F_1 was treated with ferrous bathophenanthroline *plus* FCCP, then added IF_1 caused the same degree of inhibition as it did in the absence of ferrous bathophenanthroline.

Octylguanidine. It has been proposed that alkylguanidines inhibit F_1-ATPase activity in a manner and at a site similar to IF_1 (Tuena de Gomez-Puyou et al., 1977). Figure 7 shows the effect of added IF_1 on the ATPase activity of complex V in the absence and presence of octylguanidine. Since the affinity between octylguanidine and enzyme is relatively low, and the inhibition of octylguanidine can be reversed by dilution, the experiment of Figure 7 was done as follows. At the point marked by arrow 1 in the left panel of Figure 7, ATP·Mg^{2+} was added to two tubes A and B containing complex V at 30 °C and pH 6.7. In addition, 250 μM octylguanidine was added to one of the tubes (trace B in Figure 7). The addition of ATP·Mg^{2+} elicited inhibition of ATPase activity due to the endogenous IF_1. Thus, when sampled for the activity assay at the point shown by arrow 2, both mixtures showed partial inhibition, and the mixture containing octylguanidine showed about 15% more inhibition, because octylguanidine was carried over with the enzyme sample into the assay mixture (this point was ascertained in a separate experiment). After being sampled for the activity assay, IF_1 was added to both tubes A and B at the time point marked by arrow 2, and incubation and sampling for the activity assay were continued. It is seen that the enzymes in both A and B were inhibited by the added IF_1 to about the same extent, thus indicating that the presence of a high concentration of octylguanidine in tube B did not protect complex V against inhibition by added IF_1. The reason that complex V in the presence of a high concentration of octylguanidine could still be inhibited by IF_1 might be because at pH 6.7 octylguanidine is not very effective, and the enzyme still exhibited ~30% activity. This activity was presumably sufficient to allow the enzyme to turn over and become inhibited by IF_1. The right panel of Figure 7 shows the reversal of IF_1 inhibition at pH 8.0. The reason that the complex V of tube B did not recover 100% activity might be because of incomplete removal of octylguanidine by ammonium sulfate precipitation of the enzyme. After maximal reactivation, both preparations A and B were assayed in the presence of 50 μM octylguanidine, and both showed complete inhibition of ATPase activity.

Discussion

It has been shown recently that the methyl 4-azidobenzimidate derivative of IF_1 binds to the β subunit of soluble F_1-ATPase upon photoactivation (Klein et al., 1981). Whether such cross-linking is relevant to the mechanism of inhibition of ATPase activity and IF_1 binding to the β subunit involves the active site are issues yet to be clarified. Earlier results of Van de Stadt et al. (1973) and Ernster et al. (1977) showed that IF inhibition of ATPase activity is noncompetitive with respect to ATP. These results suggested that IF_1 does not interact directly with the hydrolytic site. More recently, Krull & Schuster (1981) reported dual inhibitor studies on soluble F_1, using a fixed concentration of IF_1 and varying concentrations of competitive inhibitors of ATP hydrolysis such as AMP-P(NH)P, Cr^{III}ADP, and Cr^{III}ATP. They concluded that both the nucleotide inhibitors and IF_1 reacted with the enzyme at the same time, but at different sites.

Our finding that the IF_1 inhibition of complex V ATPase activity was fully reversible at pH >7.0 made it possible to see whether inhibitions by IF_1 and a group of specific inhibitors believed to act at or near the ATPase active site were independent events or mutually exclusive. The idea was that if IF_1 should bind at the catalytic site, then it might mask the site and prevent the interaction of active-site inhibitors. The inhibitors used in this study in conjunction with IF_1 were NBF-Cl, efrapeptin, aurovertin, FSBA, phenylglyoxal, tridentate bathophenanthroline chelate of ferrous ion, and octylguanidine. As mentioned earlier, NBF-Cl binds to a tyrosyl residue of the β subunit (Ferguson et al., 1975; Lunardi & Vignais, 1979; Ting & Wang, 1980). This binding has been shown to prevent photolabeling of the β subunit by aryl azide derivatives of ADP and ATP (Lunardi & Vignais, 1979). The studies of Ting & Wang (1980) also suggest that the tyrosyl residue modified by NBF-Cl is at the F_1 active site. FSBA is a substrate analogue and is believed to bind at both the active and regulatory sites (Di Pietro et al., 1979). According to Esch & Allison (1979), FSBA binds to a tyrosyl residue at the active site on the β subunit, but this tyrosyl residue is distinct from that which is modified by NBF-Cl. Efrapeptin is also considered to bind at the active site (Grubmeyer & Penefsky, 1981). Kasahara & Penefsky (1978) have shown that efrapeptin inhibits the high-affinity binding of phosphate to F_1, whose location is believed to be on the β subunit (Lanquin et al., 1980).

Evidence for binding of phenylglyoxal, ferrous bathophenanthroline, aurovertin, and octylguanidine at or near the ATPase active site is not as strong. However, it was mentioned earlier that ADP, GDP, and IDP protected F_1 and complex V against inhibition of ATPase activity by phenylglyoxal. These substrates also decreased by about 50% the binding of [^{14}C]phenylglyoxal to complex V (Frigeri et al., 1978). In addition, Kohlbrenner & Cross (1978) have shown that efrapeptin protects F_1 against modification by [^{14}C]phenylglyoxal at a high-affinity binding site. Ferrous bathophenanthroline has been suggested to bind to the β subunit (Carlsson et al., 1978), and its inhibition of F_1-ATPase has been shown to be independent of ATP concentration in the range of 1–3 mM (Phelps et al., 1975b). Aurovertin has at least two binding sites on F_1 (Chang & Penefsky, 1973). It was reported to bind to the β subunit of the enzyme (Verschoor et al., 1977) and also that its binding to IF_1-containing submitochondrial particles resulted in a decreased fluorescence yield (Van de Stadt & Van Dam, 1974). It might also be mentioned that according to Van de Stadt & Van Cam (1974), bound aurovertin impedes the association of IF_1 with membrane-bound F_1, whereas Klein et al. (1981) have shown that the covalent binding to F_1 of an aryl azide derivative of IF_1 was not affected by aurovertin. Little is known about the binding site of octylguanidine, but, as mentioned above, it has been suggested that octylguanidine inhibits ATPase activity in a manner similar to that of IF_1 (Tuena de Gomez-Puyou et al., 1977).

Thus, it is clear from the foregoing that, with the possible exception of octylguanidine, all the inhibitors used in this study, including IF_1, are considered to bind to the β subunits of F_1-ATPase. In addition, there is evidence that NBF-Cl, FSBA, efrapeptin, and possibly phenylglyoxal bind at the active site. Nevertheless, our studies suggest independent binding and inhibition of ATPase activity by IF_1 vs. the other inhibitors used. The clearest example was obtained with NBF-Cl, because its inhibition could be reversed by addition of dithiothreitol. Thus, it was shown that IF_1-inhibited complex V could be independently inhibited by NBF-Cl. Covalently bound NBF-Cl could be removed from IF_1-treated complex V by addition of dithiothreitol, but the appearance of ATPase activity required incubation of the IF_1-treated enzyme at pH 8.0, a condition shown in Figure 1 and elsewhere (Galante et al., 1981) to reverse IF_1 inhibition of complex V. The complex V preparation treated with IF_1 and then with NBF-Cl could also be incubated first at pH 8.0 to reverse the inhibition due to IF_1. However, in this case, reappearance of activity depended on the addition of dithiothreitol, which caused the removal of the second inhibitor, namely, NBF-Cl. With aurovertin and efrapeptin, removal of the inhibitor was not possible. However, it was shown that after maximal inhibition of ATPase activity by addition of IF_1 to complex V, aurovertin and efrapeptin appeared to react and exert independent inhibitions, since incubation of the inhibited enzyme at pH 8.0 (after ammonium sulfate precipitation and suspension in buffer not containing added inhibitors) did not result in the reversal of inhibition due to IF_1. This reversal occurred in controls which were treated only with IF_1. FSBA and phenylglyoxal were used at concentrations to cause only partial inhibition, and each was added to a complex V preparation maximally ($\geq$93%) inhibited by added IF_1. Subsequent incubation at pH 8.0 as described above to reverse IF_1 inhibition resulted only in partial recovery of activity, indicating that in the pH 6.7 incubation medium FSBA and phenylglyoxal had caused partial and independent inhibition of the IF_1-treated enzyme. Once again, control experiments in the absence of FSBA or phenylglyoxal showed complete reversal of IF_1 inhibition when the preparations were incubated at pH 8.0.

In the case of ferrous bathophenanthroline and octylguanidine, the experiments were done differently. Complex V was first treated with these inhibitors, and subsequently with IF_1. Since inhibition of ATPase activity by IF_1 requires an active state of the enzyme, the inhibition by ferrous bathophenanthroline was partially reversed by addition of CCCP, which binds to the enzyme–ferrous bathophenanthroline complex to form a ternary complex without causing the release of ferrous bathophenanthroline. After this partial reversal of activity, then IF_1 was added to achieve maximal inhibition. Octylguanidine is a weakly binding inhibitor, and at pH 6.7, the concentration used allowed the enzyme to remain partially active, which appeared to be sufficient for manifestation of inhibition by IF_1 added after octylguanidine. These experiments indicated, therefore, that the presence of enzyme-bound ferrous bathophenanthroline, or of octylguanidine in the medium, did not prevent maximal inhibition of ATPase activity by subsequent addition of IF_1.

Taken together, the above results suggest independent and noninterfering inhibitions by IF_1 and the various other inhibitors used, especially NBF-Cl, aurovertin, efrapeptin, FSBA, and phenylglyoxal. In the case of the latter two inhibitors, our studies have also shown that the extent of [^{14}C]FSBA or [^{14}C]phenylglyoxal binding to complex V is not detectably altered before and after treatment and inhibition of complex V with IF_1. Since there is evidence that the above inhibitors bind to the β subunits, the question arises as to how the results are reconcilable with the possible mode of action of IF_1. One possible explanation might be that IF_1 does not bind to the β subunits, but this explanation would be in disagreement with the photoaffinity binding results of Klein et al. (1981). A second possibility is that IF_1 binds to the β subunits, but not at a site where it would interfere with the binding of the above inhibitors. This explanation would require IF_1 not to mask the active sites, because NBF-Cl, FSBA,

efrapeptin, and possibly phenylglyoxal are all considered to bind at the active sites. However, this condition does not agree with two observations: first, the observation of Klein et al. (1981) that quercetin and AMP-P(NH)P inhibit the photoaffinity cross-linking of their aryl azide derivative of IF_1 to the β subunits of F_1;[2] second, our own preliminary findings that IF_1-treated complex V was partially (60%) protected against photoinactivation in the presence of arylazido-β-alanyl-ATP, which according to Cosson & Guillory (1979) is hydrolyzed by F_1 in the dark. A third possibility for independent inhibitions of IF_1 and the other inhibitors used above is suggested by the recent findings of Grubmeyer & Penefsky (1981) and by the results of Klein et al. (1980). Grubmeyer and Penefsky have shown that F_1-ATPase has two functional hydrolytic sites and have concluded that efrapeptin inhibits ATP hydrolysis by preventing nucleotide binding and release at a single catalytic site. Klein et al. found that the phenyl [^{14}C]isothiocyanate derivative of IF_1 became bound to F_1-ATPase and induced maximal inhibition at a ratio of approximately 1 mol of IF_1 per mol of F_1-ATPase. Thus, it is possible that, like efrapeptin, IF_1 binding to only one β subunit is sufficient for maximal inhibition of ATPase activity and that once maximal inhibition is achieved then IF_1 can no longer bind to a second subunit because it requires an active enzyme for binding. However, this latter condition would not be expected to apply to the artificial inhibitors used in this study. Therefore, after IF_1 binding to one β subunit, the artificial inhibitors would still interact with the available β subunit(s). Then when IF_1 inhibition is released at pH 8.0, modification of other β subunit(s) would still keep the enzyme fully inhibited, and only when the second inhibitor is removed (as was demonstrated in the case of NBF-Cl), ATPase activity would emerge. This explanation agrees with the observations of Klein et al. (1981) and is not necessarily in conflict with our binding studies with [^{14}C]phenylglyoxal shown in Figure 5, even if we assume that IF_1 binding involves the active sites. We have shown elsewhere (Frigeri et al., 1978) that although only one essential residue is involved in inhibition of ATPase activity by phenylglyoxal, complete inhibition of activity is associated with the binding of seven to eight molecules of [^{14}C]phenylglyoxal per mole of complex V. Whether such binding studies as shown in Figure 5 can detect possible protection by IF_1 of one residue out of seven to eight residues is doubtful. A similar argument applies to FSBA in view of its reported binding to F_1 at regulatory sites (high affinity) in addition to the active site (low affinity) (Di Pietro et al., 1979).

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[2] This may be related to the fact that an active ATPase is required for IF_1 binding, and once inhibited by quercetin or AMP-P(NH)P—regardless of their site of action—then IF_1 would not bind. This interpretation agrees with our studies on the equilibrium binding of radiolabeled IF_1 to complex V. Our results have shown that the high-affinity binding of IF_1 to complex V is considerably diminished when the latter is treated with oligomycin which acts on the membrane sector of the complex (S.-Y. Wong et al., unpublished experiments).

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Existence of an Adenosine 5′-Triphosphate Dependent Proton Translocase in Bovine Neurosecretory Granule Membrane†

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ABSTRACT: The addition of ATP to bovine neurohypophysial secretory granules suspended in isotonic sucrose medium induces a positive polarization, $\Delta\psi$, of their interior without affecting their internal pH. In KCl-containing media, ATP failed to generate large $\Delta\psi$ but induced a pH gradient (ΔpH; interior acidic). These observations are consistent with the existence in the neurosecretory granule membrane of an ATP-dependent inward electrogenic H^+ translocase (H^+ pump), capable in KCl-containing media of acidifying the granule matrix by H^+–Cl^- cotransport. The $\Delta\psi$ and ΔpH generated by the H^+ pump, defined as the ATP-induced changes sensitive to the H^+ ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), were blocked by *N*,*N*′-dicyclohexylcarbodiimide, an inhibitor of all H^+ pumps, and were insensitive to oligomycin, a mitochondrial ATPase inhibitor. In sucrose medium, measurements were complicated by a Donnan equilibrium reflecting the presence in the granule of peptide hormones and neurophysins which resulted in a CCCP-resistant resting ΔpH. In KCl-containing media, the Donnan equilibrium was destroyed since the membrane is permeable to cations, but under these conditions a CCCP-resistant K^+-diffusion potential was observed. The ATP-induced $\Delta\psi$ was also monitored by the extrinsic fluorescent probe bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol. The hypothesis of a granule H^+ pump is further supported by the presence of an oligomycin-resistant ATPase in the preparation and the ultrastructural localization of such an activity on the granule membrane. The H^+ pump has been found in both newly formed and aged neurosecretory granules. Its possible physiological function is discussed with reference to that of chromaffin granules, with which it has many similarities.

In the neurohypophysis, the peptide hormones ocytocin and vasopressin are localized in specialized organelles, the neurosecretory granules (NSG). These structures play an important role in the maturation, storage, and liberation of the hormones. It has been demonstrated that the hormones are released from the NSG by exocytosis, but little is known concerning the mechanism of this process [for review, see Morris et al. (1978)]. In vitro, ATP induces the release of vasopressin from isolated NSG (Poisner & Douglas, 1968; Russel & Thorn, 1978), and recently Overgaard et al. (1979) have shown that such release is stimulated by addition of chloride ions. Isolated chromaffin granules of adrenal medulla also release the catecholamines of their matrix by an ATP-dependent process which requires the presence of permeant anions such as chloride (Casey et al., 1976; Hoffman et al., 1976). The latter observation has been explained by the existence in the granule membrane of an inward H^+ pump (Casey et al., 1977; Flatmark & Ingebretsen, 1977; Phillips & Allison, 1978). This pump polarizes the vesicle interior, making it positive with reference to the milieu (Pollard et al., 1976; Scherman & Henry, 1980a) or, in the presence of permeant anions, acidifying it by H^+-anion cotransport, resulting under certain conditions in granule lysis. The possible existence of an H^+ pump in NSG was therefore investigated. The present paper provides pieces of evidence for such a pump in NSG.

Two populations of neurohypophysial NSG have been recently characterized: the newly formed (NF-NSG)[1] and the aged (A-NSG) neurosecretory granules. These two populations differ both in their physiological and in their physicochemical properties (Nordmann et al., 1979; Nordmann & Labouesse, 1981). The present data have been obtained with A-NSG, which are the more abundant material, and have been confirmed with NF-NSG. ATP-induced transmembrane potentials ($\Delta\psi$) and pH gradients (ΔpH) have been measured by the well-documented ion partition technique (Rottenberg et al., 1971; Goldman & Rottenberg, 1973; Casey et al., 1977;

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[1] Abbreviations: A-NSG, aged neurosecretory granules; NF-NSG, newly formed neurosecretory granules; OX-V, bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; Tris, tris(hydroxymethyl)aminomethane.

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