Differential Effects on Energy Transduction Processes by Fluorescamine Derivatives in Rat Liver Mitochondria†

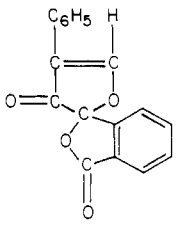
Fausto Ramirez,* David Shiuan, Shu-I Tu,* and James F. Marecek

ABSTRACT: Intact rat liver mitochondria were treated with compounds derived from the reaction of fluorescamine with various types of primary amines, including the mycosamine-containing antibiotics amphotericin B and nystatin. The effect of varying amounts of these compounds on ATPase-linked inorganic phosphate (P_i) formation, on oxygen consumption, and on MgATP-linked and succinate-linked proton movements was examined. The antibiotic–fluorescamine compounds did not affect the P_i formation rate but strongly inhibited both the ATPase-linked and the succinate-linked H^+ extrusion rates to approximately the same extent. The antibiotic derivatives decreased the oxygen consumption rate, but this effect was much smaller than the decrease in the respiration-dependent proton extrusion rate. The benzylamine–fluorescamine compound significantly increased the P_i formation rate, in contrast to the antibiotic analogues. The benzylamine derivative, like the antibiotic derivatives, inhibited both types of proton extrusion rates. The slight decrease in the oxygen consumption rate caused by the benzylamine derivative was significantly smaller than the corresponding decrease observed with the antibiotic derivatives. These studies, in which fluorescamine derivatives bind reversibly to mitochondria, are compared with previous studies in which fluorescamine itself binds irreversibly to mitochondria and results in a P_i formation rate increase and MgATP- and succinate-linked proton extrusion rate inhibition but has no effect on the oxygen consumption rate.

The coupling between electron transport and vectorial proton movement in mitochondria is a controversial subject. It has been proposed that respiration and proton translocation are directly linked by redox "loops" in the inner membrane (Mitchell, 1966, 1977; Mitchell & Moyle, 1970). However, the H^+/site ratio of 2.0 required by this hypothesis has been challenged by the work of Lehninger and co-workers (Brand et al., 1976a,b; Reynafarje et al., 1976). Additional evidence against a loop arrangement in coupling site III has been presented recently (Wikstrom & Krab, 1978; Artzatbanov et al., 1978; Casey et al., 1979a) but has been subsequently criticized (Moyle & Mitchell, 1978; Lorusso et al., 1979). The recent finding of Casey et al. (1979b) that DCCD[1] inhibits the proton extrusion but not the electron transport of cytochrome oxidase supports an indirect link type of mechanism, such as the vectorial "Bohr effect" (Papa, 1976; Chance, 1977) or the conformational coupling model (Boyer, 1975).

The proton extrusion associated with mitochondrial MgATPase is thought to be tightly linked to ATP hydrolysis (Mitchell & Moyle, 1970). A constant value of 3.0 has been observed for the H^+ /hydrolyzed ATP ratio (Reynafarje & Lehninger, 1978; van Dam et al., 1978). However, it is not clear at the present time which step in the pathway of ATP hydrolysis is responsible for the activation of the proton pump (Boyer et al., 1977; Kayalar et al., 1977; Rosing et al., 1977).

A recent paper from this laboratory (Tu, 1979) reported that the modification of intact mitochondria by the primary amine specific reagent fluorescamine (Udenfriend et al., 1972; Weigele et al., 1972) causes an increase in the P_i formation rate and a decrease in the related H^+ extrusion rate. Subsequently, it was observed that fluorescamine does not alter the rate of oxygen consumption in succinate oxidation, although it inhibits the related H^+ extrusion (Lam & Tu, 1979). Fluorescamine does not affect the permeability of membrane to protons or the kinetics of the H^+ influx induced by uncouplers, e.g., DNP. On the other hand, fluorescamine inhibits the ability of the uncouplers to stimulate respiration. The main conclusion reached from these observations was that electron transport and its associated proton movement are only indirectly linked (Tu, 1979; Lam & Tu, 1979). These and other studies on fluorescamine modification of mitochondria (Shiuan & Tu, 1978) do not resolve the question of whether the observed effects are due to the modification of surface-exposed amino groups or are the result of implantation of a modified fluorescamine moiety in the membrane. To obtain further evidence on the origin of these effects, we have modified the fluorescamine molecule, **1**, by a preliminary reaction with



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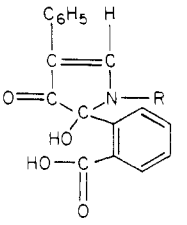

2, R = $C_6H_5CH_2$
3, R = $CH(COOH)CH_2C_6H_5$
4, R = antibiotic residue

simple primary amines (De Bernardo et al., 1974) or with α-amino acids. These modifications produce fluorescent compounds of types **2** and **3** (e.g., from benzylamine and phenylalanine, respectively). We have also modified fluorescamine by reaction with the mycosamine moiety of the macrolide polyene antibiotics amphotericin B and nystatin (Kasumov et al., 1979; Pandey & Rinehart, 1976), which are known to have a strong affinity for biomembranes. The antibiotic–fluorescamine compounds are formulated as shown in formula **4**.

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[1] Abbreviations used: DCCD, dicyclohexylcarbodiimide; ATP, adenosine 5′-triphosphate; P_i, inorganic phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; NEM, *N*-ethylmaleimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*, *N*′-tetraacetic acid; Me_2SO, dimethyl sulfoxide; DNP, 2,4-dinitrophenol; FCCP, carbonyl dicyanide *p*-(trifluoromethoxy)phenylhydrazone.

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From studies of the effects of amine–fluorescamine compounds on the ATPase- and respiration-dependent activities in rat liver mitochondria we have obtained evidence that in both types of activities the proton movement and its respective energy-producing step are only indirectly linked in the overall biochemical pathway.

Materials and Methods

Amphotericin B, nystatin, valinomycin, rotenone, oligomycin, Hepes, sucrose, sodium succinate, EGTA, and malachite green were purchased from Sigma. NEM (Gold Label) was from Aldrich. Fluorescamine (Roche), flaminox solution (10%), and DNP (freshly recrystallized from benzene) were obtained from Fisher. FCCP was purchased from Pierce Chemicals. All other reagents were of analytical grade.

Rat liver mitochondria were isolated in 0.25 M sucrose containing 3 mM Hepes at pH 7.4 as previously described (Shiuan & Tu, 1978). The protein concentrations were determined by the biuret method. The respiration and its dependent proton movement were measured simultaneously in a modified Gilson Medical oxgyen chamber. The mitochondrial suspension containing 3–5 mg of protein, 40 nmol of NEM per mg of protein, 1 μg of oligomycin per mg of protein, 500 ng of valinomycin, and 3 μM rotenone was incubated at 22 °C for 2 min and was added to a solution prepared by the addition of various amounts of antibiotic or the amine–fluorescamine compound to a 2.1-mL aliquot of a solution containing 120 mM LiCl, 10 mM KCl, 3 mM EGTA, and 3 mM Hepes, at pH 7.4. The reaction was started by addition of 1 mM succinate (final concentration). Proton extrusion was followed by a Markson Model J-445 combination electrode connected to a Corning Model 112 pH meter; the change in H^+ concentration was recorded by a Varian A-25 recorder. The buffering capacity of the mitochondrial suspension was determined by injecting a series of 2-μL aliquots of standard HCl before and after the experiment. Respiration rate was followed polarographically with a Yellow Spring Model 53 oxygen monitor. It was established that the small amounts of organic solvents employed (less than 15 μL of acetonitrile, acetone, or Me_2SO) had no significant effect on either proton movement, respiration, or any other biochemical activity measured.

The proton extrusion associated with MgATPase action was determined in a similar manner, except that oligomycin and NEM were omitted and the reaction was started by the addition of 5 mM ATP and $MgCl_2$. The P_i formation was measured in a separate aliquot with the same composition as that of the solution used for the H^+ extrusion measurement. The P_i formed over a period of 10 min was determined as previously described (Tu, 1979).

The amine–fluorescamine compounds were prepared in situ prior to their utilization. Fluorescamine (1.1 molar equiv) and the amine (benzylamine or *n*-butylamine) or the antibiotic (amphotericin B or nystatin) were mixed in acetonitrile or Me_2SO solutions at 25 °C and allowed to react for 5 min to yield compounds **2** and **4**. Any excess fluorescamine that may be present at this point was destroyed by addition of water. The same procedure was utilized for the α-amino acid–fluorescamine compounds, **3** (with isoleucine or phenylalanine), except that the reaction was carried out in aqueous acetone containing triethylamine.

Fluorescamine (0.1 mmol) was dissolved in 0.5 mL of CD_3CN, and the ^1H NMR spectrum of the solution was examined. Benzylamine (0.1 mmol) was added, and the yellow solution was kept at 25 °C for 36 h. The ^1H NMR spectrum of the solution was examined at various time intervals. The observed changes corresponded to the following reaction sequence, where the first step, **1** → **2**, was completed within 1 min:

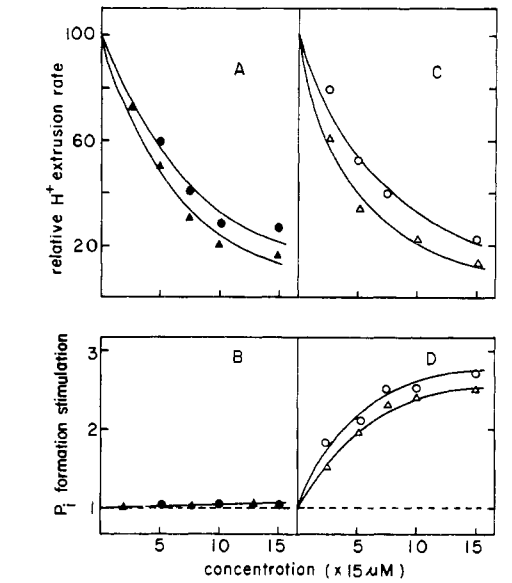
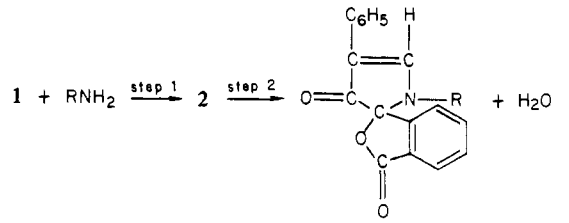


FIGURE 1: Effect of primary amine–fluorescamine compounds on MgATPase-dependent activities in rat liver mitochondria. (A and B) Antibiotic compounds; (C and D) simple amine compounds. (●) Amphotericin–fluorescamine; (▲) nystatin–fluorescamine; (△) benzylamine–fluorescamine; (○) phenylalanine–fluorescamine. The intitial H^+ extrusion rate and the average P_i formation rate for mitochrondria in the presence of increasing concentrations of modifier compound were determined as described under Materials and Methods; the normal values for unmodified mitochondria are 100 = 92 nmol of H^+ per min per mg and 1 = 22 nmol of P_i per min per mg, respectively.



5, R = $C_6H_5NH_2$

The cyclization to the lactone **5** required several hours. Evaporation of the solvent under vacuum gave the nonfluorescent lactone **5**, which was dried over P_2O_5 at 25 °C (0.1 torr): mp 147–149 °C; λ 383 (ε 3200) and 270 nm (ε 10 000) in CH_3CN. Anal. Calcd for $C_{24}H_{17}O_3N$: C, 78.5; H, 4.5; N, 3.8. Found: C, 78.6, H, 4.8; N, 3.8.

The cyclization **2** → **5** was complete in a few minutes upon addition of DCCD.

N-Acetylated amphotericin B and nystatin were prepared as described by Pandey & Rinehart (1976).

Results

Effect of Amine–Fluorescamine Compounds on Mitochondrial MgATPase-Dependent Activities. Figure 1A shows that the antibiotic–fluorescamine compounds ("class I modifiers") inhibit the rate of generation of the proton gradient in the presence of K^+, valinomycin, EGTA, and respiration inhibitors. On the other hand, in the same concentration range, these class I modifiers have no effect on the P_i formation rate associated with MgATP hydrolysis (Figure 1B).

Figure 1C discloses that compounds derived from the reaction of simple amines, e.g., benzylamine or *n*-butylamine,

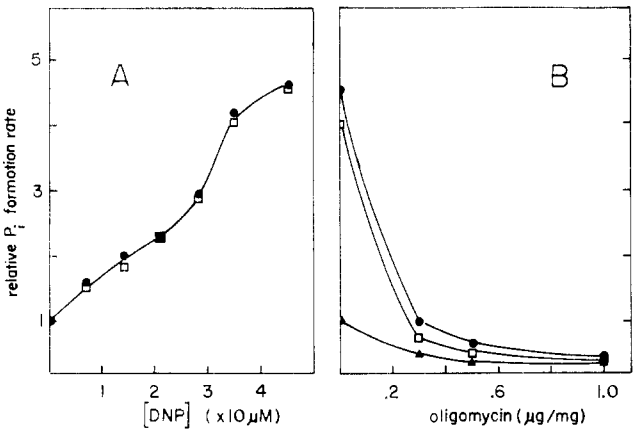


FIGURE 2: (A) Stimulation of P_i formation in mitochondria by increasing concentrations of the uncoupler DNP alone (●) and in the presence of 150 μM nystatin–fluorescamine compound (□) (see Figure 1B). Similar effects were observed with other uncouplers, e.g., FCCP, and with amphotericin–fluorescamine compound. (B) Inhibition of P_i formation in mitochondria by increasing concentrations of oligomycin alone (▲) and in the presence of 30 μM of the uncoupler DNP (□) or of 150 μM benzylamine–fluorescamine compound (●) (see Figure 1D). Similar results were obtained with the butylamine derivative or the α-amino acid derivatives. The normal mitochondrial ATPase P_i formation rate is 1.0 = 22 nmol of P_i per min per mg.

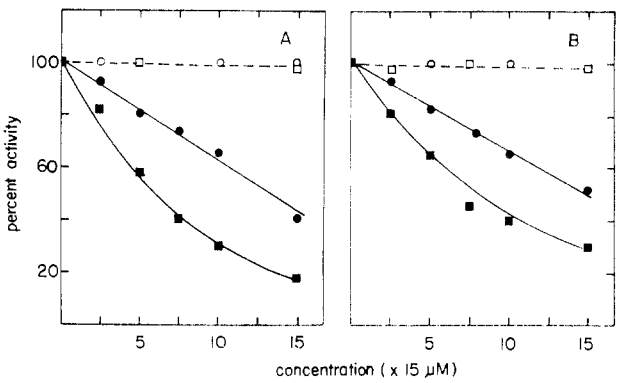


FIGURE 3: Effect of antibiotic–fluorescamine compounds on respiration-dependent activities. (A) Nystatin compound; (B) amphotericin compound. (●) Initial oxgyen consumption rate; (■) initial H^+ extrusion rate. The unmodified antibiotics had no effect (○ and □). Succinate oxidation and the related H^+ extrusion in mitochondria in the presence of increasing concentrations of modifier compound were determined as described under Materials and Methods; the normal values for unmodified mitochondria are 100% = 20.5 ng-atoms of O min per mg and 100% = 180 nmol of H^+ per min per mg, respectively. The N-acetylated antibiotics had no effect on these activities.

or of α-amino acids, e.g., phenylalanine or isoleucine, with fluorescamine ("class II modifiers") also decrease the ATPase-dependent H^+ extrusion rate. The class II modifiers, in contrast to the class I modifiers, significantly increase the P_i formation rate (Figure 1D).

These effects on ATPase-dependent activities are associated with both moieties present in the modifiers. Thus, neither the unmodified antibiotics, amines, or α-amino acids nor the N-acetylated antibiotics have any appreciable effect on ATPase-dependent activities. On the other hand, fluorescamine itself resembles class II modifiers in decreasing the H^+ extrusion rate and increasing the P_i formation rate (Tu, 1979).

The data presented in Figure 2A show that the ability of conventional uncouplers, e.g., DNP, to enhance the P_i formation rate is the same in the absence and in the presence of class I modifiers. Moreover, Figure 2B demonstrates that the ability of oligomycin to arrest the normal P_i formation rate of mitochondria is retained when the P_i formation rate is measured in the presence of increasing amounts of oligomycin and a fixed amount of either the uncoupler DNP or class II modifiers. It is apparent from these data that at least the step in which P_i from MgATP hydrolysis is released to the medium and probably but not necessarily the hydrolysis step as well are not being affected by the class I modifiers in the mitochondrial MgATPase pathway. Secondly, the data in Figure 2 confirm that the P_i formation rate enhancement noted in Figure 1D is indeed due to interactions between class II modifiers and mitochondrial ATPase, just as is the case for the conventional uncouplers, e.g., DNP. However, it should be emphasized that this apparent analogy between class II modifiers and uncouplers is misleading since these two classes of compounds have quite different effects on respiration-dependent activities (see below).

Another inference emerging from a comparison between Figures 1 and 2 is that uncoupler is acting at a mitochondrial site that is not affected by class I modifiers (Figure 2A vs. Figure 1B). Finally, we conclude from Figure 2B vs. Figures 2A and 1D that (a) the site of oligomycin action is different from that of either uncoupler or class II modifiers, with the oligomycin effect preventing the effects of uncoupler and modifiers, or (b) the three types of compounds act on the same site, but the oligomycin effect takes precedence over the uncoupler and class II modifier effects. This last alternative, (b), is rejected on the grounds that it is not likely that the three types of compounds act on the same mitochondrial site, with oligomycin resulting in P_i formation inhibition, and uncouplers and class II modifiers producing P_i formation stimulation.

The main thrust of the data so far presented here is that P_i formation and H^+ extrusion in ATPase activity are not directly linked processes, since class I modifiers can inhibit one activity (H^+ extrusion) without affecting the other while class II modifiers can inhibit one activity and stimulate the other. This in turn suggests that, of the various steps involved in mitochondrial MgATPase, hydrolysis and/or P_i release regulate the subsequent H^+ extrusion. A hypothesis to interpret these observations is advanced under Discussion.

Effect of Amine–Fluorescamine Compounds on Respiration-Dependent Activities. The rate of oxygen consumption during succinate oxidation and the concomitant rate of extrusion of protons from mitochondria to the medium can be measured simultaneously. Figure 3 shows that both activities are inhibited in the presence of class II modifiers, with K^+, valinomycin, and inhibitors of secondary ion movement (oligomycin, NEM, and EGTA) also present in the medium.

The class II modifiers also inhibit the initial rates of oxygen consumption and proton extrusion (Figure 4). With both class I and II modifiers, the H^+ extrusion rate decreases more rapidly than the oxygen consumption rate as modifier concentration is increased. The class I modifiers inhibit oxygen consumption to a greater extent than the class II modifiers at a given level of H^+ extrusion inhibition, in particular at higher modifier concentration. The concentration of modifier needed to cause the same inhibition of the initial H^+ extrusion rate is smaller for class I than for class II modifiers.

Direct fluorescamine treatment has no appreciable effect on the oxygen consumption rate, although it decreases the concomitant H^+ extrusion rate, and this observation led to the conclusion that proton extrusion is only indirectly linked to respiration in the sense that at least one intermediate step, either of a chemical or of a conformational nature, is required to link these two events (Lam & Tu, 1979). The present data, showing also a preferential inhibition of the H^+ extrusion rate vs. the oxygen consumption rate by class I and II modifiers, support this indirect link conclusion for respiration. In ad-

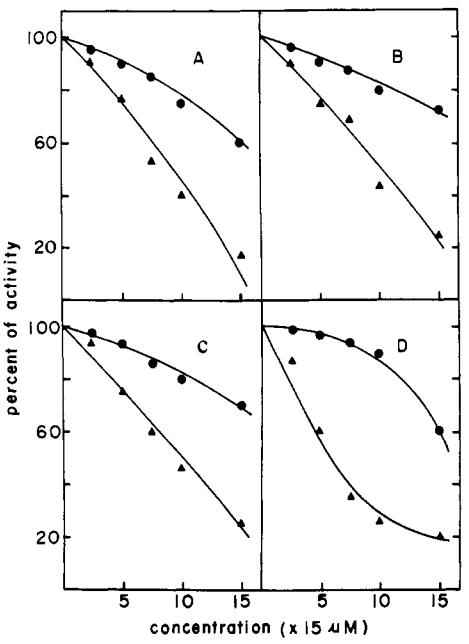


FIGURE 4: Effect of simple amine compounds on respiration-dependent activities. (A) Benzylamine compound; (B) *n*-butylamine compound; (C) isoleucine compound; (D) phenylalanine compound. (●) Initial oxygen consumption rate; (▲) initial H^+ extrusion rate. Succinate oxidation and the related H^+ extrusion in the presence of modifiers were determined as indicated in Figure 3.

dition, the results with class I and II modifiers suggest that destruction of surface-exposed amino groups may not be the determinant factor in the fluorescamine effects in mitochondria, as was originally envisioned (Tu, 1979; Lam & Tu, 1979), since class I and II modifiers (formulas **2**–**4**) lack the chemical reactvity toward primary amino groups which is characteristic of fluorescamine (**1**), as demonstrated by the following results.

Mitochondria were removed by centrifugation from media which contained class I or II modifiers. The recovered mitochondria were resuspended in media free from modifier. This treatment restored normal activities to the mitochondria comparable to those observed with fresh mitochondria washed in a similar manner but not exposed to modifiers. However, the activity of mitochondria treated with fluorescamine cannot be restored by a comparable treatment (Tu, 1979; Lam & Tu, 1979). The reversibility of the effects of class I and II modifiers is understandable in terms of formulas **2**–**4** since these molecules lack the ability to form covalent bonds with the functional groups present in the phospholipids and proteins of the membrane, under conditions which lead to extremely rapid covalent bond formation between fluorescamine and primary amines. Therefore, the effects of modifiers of classes I and II are reasonably attributed to noncovalent binding by the molecule to some important sites of the membrane components. Note in particular the carboxy, hydroxy, and keto groups as possible binding functions in the modifiers.

The kinetic studies of Stein et al. (1974) suggest that intact fluorescamine (**1**) reaches the mitochondria where it can react rapidly with the primary amine function of phosphatidylethanolamine and/or lysine residues to give fluorescent compounds analogous to **2** and **3**. Evidently the preformed amine–fluorescamine compounds are no longer able to bind covalently to mitochondria by the same mechanism. The present work shows that these modifiers react only at an extremely slow rate with additional primary amines or with water (pH 7 to 8). The cyclization of the hydroxy acid function of modifiers (**2**–**4**) to the corresponding lactone (**5**) occurs only at a very slow rate (except in the presence of the activator DCCD). The nonfluorescent lactone (**5**) is relatively stable toward primary amines or water (pH 7 to 8). In contrast, the reaction of fluorescamine with primary amines is virtually instantaneous. It is noteworthy that fluorescamine reacts only at a relatively very slow rate with imidazole and hence with the histidine residue of proteins.

As in the case of ATPase-dependent activities, neither the unmodified antibiotics, amines, or α-amino acids nor the N-acetylated antibiotics have any apprecialbe effect on respiration-dependent activities (data now shown). This observation is particularly relevant to the antibiotics, since Kasumov et al. (1979) have reported that amphotericin and nystatin create H^+ channels by interacting with cholesterol in synthetic membranes. Cholesterol is not present in mitochondrial membranes.

Proton Leak Studies. We have measured the H^+ leak rate by the oxygen-pulse method as previously described (Lam & Tu, 1979) in the presence and absence of class I and II modifiers (0–150 μM). The invariance of the H^+ leak rate (data not shown) indicates that the observed inhibition of proton extrusion is not due to an enhancement of proton leak through the inner membrane.

The observed decrease of the H^+ extrusion rate could also be due to the enhancement of succinate uptake and/or the interference on the inhibitory effect of NEM on phosphate transport by the modifiers. These two possibilities were checked by examining the uptake rates of succinate and phosphate by using the osmotic swelling method as described by Mitchell & Moyle (1969). It was found that the succinate uptake rate in isotonic media containing sucrose and 1–50 mM succinate is not affected by the presence of modifiers. Furthermore, the phosphate uptake in the isotonic medium of potassium phosphate (pH 7.4), induced by valinomycin and FCCP, is completely blocked by the presence of 40 nmol of NEM per mg of protein in the absence or presence of modifiers (data not shown).

Relative Effects of Amine–Fluorescamine Compounds on ATPase- and Respiration-Dependent H^+ Extrusion. A semiquantitative comparison between the efficiences of a given amine–fluorescamine compound in inhibiting ATPase-dependent and respiration-dependent H^+ extrusions is presented in Figure 5. It is apparent that the two effects are either very similar (parts A and B of Figure 5) or virtually identical (parts C and D of Figure 5) among both class I and II modifiers.

These data, it seems to us, complement the recent finding by Casey et al. (1979b) that DCCD inhibits the proton extrusion associated with both ATPase action and the activity of coupling site III by interactions with specific H^+ channels. In the case of our class I and II modifiers, the data for the inhibition of ATPase- and respiration-dependent H^+ extrusions favor a pump mechanism for both types of proton movements, although both processes are associated with quite different types of molecular events. As stated above, the linking of P_i formation to its H^+ extrusion and of oxygen consumption to its H^+ extrusion is only an indirect one in both cases. Hence, one must now consider a picture whereby events needed to energize the mitochondrial membrane are indirectly transmitted to events needed to activate the proton pump. From this picture we infer that the mechanisms for activating the ATPase-dependent proton pump and the electron transport dependent proton pump may basically be identical or at least very similar. A hypothesis to account for this proton pump basic mechanism will be presented under Discussion.

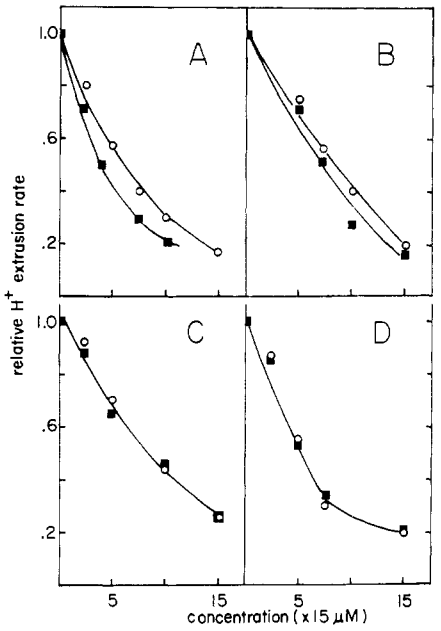


FIGURE 5: Comparison of ATPase-dependent and respiration-dependent H^+ extrusion effects induced by amine–fluorescamine compounds. (A) Nystatin compound; (B) *n*-butylamine compound; (C) isoleucine compound; (D) phenylalanine compound. (■) and (○) represent the initial H^+ extrusion rates associated with ATPase action and respiration, respectively; typical values for unmodified mitochondria are 1 = 92 and 180 nmol of H^+ per min per mg, respectively.

Discussion

In a general sense, the differential effects of fluorescamine and of amine–fluorescamine compounds on the two energy-yielding steps and their respective proton movements in rat liver mitochondria are consistent with the "proton pump" (Chance, 1977; Papa, 1976) or the "conformational coupling" (Boyer, 1975) hypotheses. However, a more specific interpretation of our results can be given in terms of the "protein–product complex" hypothesis recently suggested for energy transduction in actomyosin MgATPase coupled to muscle contraction (Ramirez et al., 1979; Shukla et al., 1979; Levy et al., 1979). In this hypothesis, energy from the hydrolysis of MgATP tightly bound to the myosin active site is transferred directly to conformational strain in a flexible segment of the myosin head and the result of the hydrolysis step is the generation of a protein–product complex formulated as myosin·ADP·Mg·P_i. The interaction of actin with this complex (or "energy trap") results in a conformational energy release which generates the sliding motions of the actin and myosin filaments.

In accordance with this hypothesis, we assume that in oxidative phosphorylation the protein–product energy trap created by mitochondrial or F1 MgATPase is relatively close in the primary structure to the protein segment whose conformational changes result in H^+ extrusion (the "protogenic protein"). Changes in the protogenic protein conformation could be associated with key enzymatic activities leading eventually to the synthesis of ATP from ADP and P_i. Changes in ATPase protogenic protein conformation may also be linked to changes in respiration-associated activities (see below). The essential feature of this hypothesis is that the H^+ extrusion rate, and hence operation of the ATPase proton pump, is separated from the MgATP hydrolysis step by the step which releases P_i to the medium. The formation of F1·ADP·Mg·P_i is a protein conformation change which leads to operation of the pump and to concomitant enzymatic activities.

The class I modifier effects are understandable on the assumption that the antibiotic moiety of the modifier has membrane sector recognition capabilities while the fluorescamine moiety binds noncovalently to the protein which is involved in the ATPase H^+ pump (protogenic protein). The class I modifiers do not affect the ATPase active site or the integrity of the protein–product complex F1·ADP·Mg·P_i, since there is no appreciable effect on the P_i formation rate. However, the binding of modifiers to the protogenic protein affects the rate of protein conformational changes and hence the rate of the H^+ extrusion. The effects of class I modifiers on respiration can be achieved by alterations of the control of respiration by ATPase activity. Thus, if modification of the ATPase protogenic protein is transmitted to the respiration-associated protogenic protein, the operation of the respiration pump is also inhibited and its rate of H^+ extrusion is reduced. The oxgyen consumption rate may decrease as a result of effects on the electron transport chain and/or the succinate dehydrogenase domain. A related interpretation would involve direct binding of class I modifiers to both the ATPase and the respiration protogenic proteins, with the consequent inhibition of their respective proton extrusion rates. These ideas are consistent with the domain hypothesis in oxidative phosphorylation (Lee et al., 1969; Ernster et al., 1973).

The class II modifier effects are understood in this hypothesis if the membrane sector recognition ability is lost upon replacement of the antibiotic moiety by the simple amine moiety. The class II fluorescamine moiety binds to the ATPase protogenic protein as before; however, since class II modifiers also increase the P_i formation rate, we must assume that the integrity of the protein–product complex F1·ADP·Mg·P_i is also affected by the class II modifiers. This difference between the two classes of modifiers, which we attribute to their differences in membrane sector recognition abilities, is one of the signficant results of this work. Note that in this interpretation, as the modifier becomes less selective toward the membrane sector, the modifier effect on ATPase activity becomes more drastic (P_i formation rate is affected). On the other hand, the effect on respiration becomes less noticeable, since the oxgyen consumption rate is inhibited to a lesser extent by class II modifiers than by class I modifiers. The respiration-associated H^+ extrusion is affected by both types of modifiers.

The effect on ATPase activities is qualitatively similar with fluorescamine and class II modifiers, which is reasonable if the main effect is on the ATPase protogenic protein and the protein–product complex in both cases. Fluorescamine and class II modifiers also inhibit the respiration-dependent H^+ extrusion, but the oxgyen consumption rate is not affected by fluorescamine. In other words, the irreversible binding of fluorescamine has less of an effect on respiration than the reversible binding of class I and II modifiers, suggesting a more localized effect, presumably only on protein residues, when irreversible covalent bonding can be established between the modifier (fluorescamine) and mitochondria.

In the above hypothesis the step that follows MgATP hydrolysis and operation of its associated proton pump is the release of P_i to the medium. In myosin MgATPase, the P_i release step (not the hydrolysis step) is rate limiting in the absence of actin. One of the functions of actin is, in fact, to activate P_i release. By analogy, there could be some mechanism to regulate the P_i release step in F1 MgATPase.

This work shows that oxgyen consumption and its associated proton pump are also indirectly linked. It follows from the present hypothesis that there should be some step separating the transfer of redox energy to a relatively flexible protein segment in the form of conformational strain energy, on the

one hand, and the operation of its associated proton pump, on the other hand.

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