*Biochimica et Biophysica Acta*, 975 (1989) 377–383
Elsevier



BBABIO 43030

# Inhibition of the bovine-heart mitochondrial $F_1$-ATPase by cationic dyes and amphipathic peptides

David A. Bullough, Eduardo A. Ceccarelli, David Roise and William S. Allison

*Department of Chemistry, University of California at San Diego, La Jolla, CA (U.S.A.)*

(Received 6 February 1989)

Key words: ATPase, $F_1$-; Amphiphilic peptide; Cationic dye; Inhibitory site

**The bovine heart mitochondrial $F_1$-ATPase is inhibited by a number of amphiphilic cations. The order of effectiveness of non-peptidyl inhibitors examined as assessed by the concentration estimated to produce 50% inhibition ($I_{0.5}$) of the enzyme at pH 8.0 is: dequalinium (8 $\mu$M), rhodamine 6G (10 $\mu$M), malachite green (14 $\mu$M), rosaniline (15 $\mu$M) > acridine orange (180 $\mu$M) > rhodamine 123 (270 $\mu$M) > rhodamine B (475 $\mu$M), coriphosphine (480 $\mu$M) > safranin O (1140 $\mu$M) > pyronin Y (1650 $\mu$M) > Nile blue A (> 2000 $\mu$M). The ATPase activity was also inhibited by the following cationic, amphiphilic peptides: the bee venom peptide, melittin; a synthetic peptide corresponding to the presence of yeast cytochrome oxidase subunit IV (WT), and amphiphilic, synthetic peptides which have been shown (Roise, D., Franziska, T., Horvath, S.J., Tomich, J.M., Richards, J.H., Allison, D.S. and Schatz, G. (1988) EMBO J. 7, 649–653) to function in mitochondrial import when attached to dihydrofolate reductase ($\Delta$11.12, Syn-A2, and Syn-C). The order of effectiveness of the peptide inhibitors as assessed by $I_{0.5}$ values is: Syn-A2 (40 nM), Syn-C (54 nM) > melittin (5 $\mu$M) > WT (16 $\mu$M) > $\Delta$11,12 (29 $\mu$M). Rhodamines B and 123, dequalinium, melittin, and Syn-A2 showed noncompetitive inhibition, whereas each of the other inhibitors examined (rhodamine 6G, rosaniline, malachite green, coriphosphine, acridine orange, and-Syn-C) showed mixed inhibition. Replots of slopes and intercepts from Lineweaver-Burk plots obtained for dequalinium were hyperbolic indicating partial inhibition. With the exception of Syn-C, for which the slope replot was hyperbolic and the intercept replot was parabolic, steady-state kinetic analyses indicated that inhibition by the other inhibitors was complete. The inhibition constants obtained by steady-state kinetic analyses were in agreement with the $I_{0.5}$ values estimated for each inhibitor examined. Rhodamine 6G, rosaniline, dequalinium, melittin, Syn-A2, and Syn-C were observed to protect $F_1$ against inactivation by the aziridinium of quinacrine mustard in accord with their experimentally determined $I_{0.5}$ values. Since derivatization of one or more of the carboxylic acid side chains of the $_{394}$DELSEED$_{400}$ segment of the $\beta$ subunit has been shown to be associated with inactivation of $F_1$ by quinacrine mustard (D.A. Bullough, E.A. Ceccarelli, J.G. Verburg and W.S. Allison (1989) J. Biol. Chem. 264, 9155–9163), it is possible that this segment also comprises part of the site that binds inhibitory, amphiphilic cations.**

## Introduction

The $F_0F_1$-ATP synthases couple proton translocation across energy-transducing membranes to the condensation of ADP with $P_i$. The $F_0$ component is an integral membrane protein complex which mediates proton translocation. $F_1$, the component that contains the active site for ATP synthesis, is a peripheral membrane protein complex. When removed from the membrane by chemical or mechanical menas, $F_1$ catalyzes ATP hydrolysis. The $F_1$-ATPases are composed of five different polypeptide chains designated $\alpha$–$\varepsilon$ in order of decreasing molecular mass with the stoichiometry $\alpha_2\beta_3\gamma\delta\varepsilon$ [2,1]. The molecular weight of the bovine heart mitochondrial $F_1$-ATPase ($F_1$), as deduced from the amino acid sequences of its polypeptide components, is 371 000 [3].

The $F_1$-ATPases have been shown to be inhibited by a variety of amphiphilic cations that include chlorpromazine, certain local anesthetics, and positively charged, substituted xanthenes [4–9]. In an earlier study from this laboratory [10], the capacity of a number of lipophilic, cationic dyes to inhibit an oligomycin-sensitive $F_0F_1$-ATPase, prepared from bovine heart submitochondrial particles, was compared. Since the $F_0F_1$-ATPase examined in the earlier study was insensitive to

Abbreviations: $P_i$, inorganic phosphate; Mops, 4-morpholineethanesulfonic acid.

Correspondence: William S. Allison, Department of Chemistry, M-001, University of California at San Diego, La Jolla, CA 92093, U.S.A.

inhibition by oligomycin and the dyes in the absence of phospholipid vesicles, it was thought that the cationic dyes caused inhibition by interacting with $F_0$. However, contrary to this view, it was reported recently that two rhodamines [8,9], which were earlier reported to inhibit the $F_0F_1$-ATPase preparation only in the presence of phospholipid vesicles, are also inhibitors of isolated $F_1$-ATPases in the absence of phospholipid vesicles. Emaus et al. [8] reported that rhodamine 123 inhibited partially purified $MF_1$ with a $K_i$ of 177 $\mu$M at pH 7.4 and Wieker et al. [9] reported that the yeast $F_1$-ATPase is inhibited by rhodamine 6G with a $K_i$ of 2.4 $\mu$M at pH 8.0, whereas rhodamine 123 inhibited the yeast enzyme with a lower affinity. The $K_i$ values determined for the inhibition of $F_1$-ATPases by these rhodamines are in the same range as the $I_{0.5}$ values estimated for the inhibition of the $F_0F_1$-ATPase preparation in the presence of phospholipid vesicles [10]. This prompted an examination of the capacity of representative dyes, that were shown earlier to inhibit the $F_0F_1$-ATPase in the presence of phospholipid vesicles, to inhibit $F_1$ in the absence of phospholipid vesicles. Weiss et al. [11] and Bodden et al. [12] have recently reported that dequalinium (1,1′-(10-decanediyl)bis-[4-amino-2-methylquinolinium]), another amphiphilic cation, mimics the anticarcinoma activity of rhodamine 123 [13], and also inhibits activation of cAMP phosphodiesterase by calmodulin [12]. Since most of the lipophilic cations which have been hown to inhibit $F_1$ also bind to calmodulin [14], we have examined the effects of dequalinium and melittin on the activity of $F_1$. When melittin, a bee venom peptide that folds into a cationic, amphiphilic helix, was found to be a strong inhibitor of the ATPase, this study was extended to include a synthetic peptide corresponding to the presequence of yeast cytochrome oxidase subunit IV as well as synthetic analogs of the presequence peptide which are known to fold into amphiphilic structures [15,16]. The findings and implications of this investigation are reported and discussed here.

## Materials and Methods

*Materials.* Rhodamine 6G, rhodamine 123, safranin O, rosaniline, and malachite green were from Eastman Kodak. Pyronine Y was from Roboz Surgical Instrument Co. Coriphosphine was from Polysciences. Rhodamine B and Nile blue A were from Aldrich Chemical Co. Dequalinium, melittin, acridine orange, and the reagents used in the ATPase assay were from Sigma Chemical Co. The preparation and determination of concentrations of synthetic peptides was performed as described in detail previously [16].

$F_1$ was prepared from bovine heart mitochondria as described previously [17], and was stored at 4°C as a suspension in 55% saturated $(NH_4)_2SO_4$ (pH 7.0), containing 4 mM ATP and 2 mM EDTA. Stock solutions of $F_1$ for inhibition experiments were prepared by removing the enzyme from storage suspension by centrifugation, decanting mother liquor, and then dissolving the pellet at a concentration of about 1 mg per ml in 50 mM triethanolamine-$H_2SO_4$ (pH 8.0). Enzyme activities were determined spectrophotometrically at 30°C and pH 8.0 by coupling ATP regeneration catalyzed by pyruvate kinase to the oxidation of NADH by lactate dehydrogenase [18]. To ensure that the ATPase was rate limiting, 9 units of pyruvate kinase and 6 units of lactate dehydrogenase were used in the assays. For determining $I_{0.5}$ values, 2.0 mM ATP was used as substrate in the presence of 2.5 mM $Mg^{2+}$. To determine $K_i$ values from steady-state kinetic analyses, initial velocities, expressed in the figures as mmol ATP hydrolyzed per min per mg protein, were determined in the presence of various concentrations of the lipophilic cations using ATP concentrations ranging from 0.2 to 2.0 mM in the presence of 2.5 mM $Mg^{2+}$. Lineweaver-Burk plots were linear in this limited range of ATP concentration. Three ATPase preparations were used in these studies. The specific activities varied from 90–120 $\mu$mol ATP hydrolyzed per min per mg of protein, with the activity of each preparation diminishing in this range as it aged.

## Results

*Comparison of lipophilic, cationic, dyes as inhibitors of $F_1$*

The $F_1$-ATPase was assayed in the presence of a range of concentrations of each dye examined. From these analyses it was possible to estimate $I_{0.5}$, the concentration that produced 50% inhibition of ATPase activity. Table I compares $I_{0.5}$ values determined for the inhibition of the soluble $F_1$-ATPase by several dyes with those previously obtained for the inhibition of a detergent solubilized $F_0F_1$-ATPase preparation by the same dyes in the presence of phospholipid vesicles [10].

TABLE I

*Concentrations of dyes that produce 50% inhibition of $F_1$-ATPase activity at pH 8.0*

| Inhibitor | $F_1$-ATPase $I_{0.5}$ ($\mu$M) | $F_0F_1$-ATPase $I_{0.5}$ ($\mu$M) |
|---|---|---|
| Rhodamine 6G | 10 | 2 |
| Rhodamine B | 475 | 125 |
| Rhodamine 123 | 270 | 141 |
| Safranin O | 1140 | 175 |
| Rosaniline | 15 | 16 |
| Malachite Green | 14 | 7 |
| Acridine orange | 180 | 1 |
| Coriphosphine | 480 | 16 |
| Nile blue A | > 2000 | 16 |
| Pyronin Y | 1650 | 10 |
| Dequalinium | 8 | |

TABLE II

*Parameters obtained from replots of slopes and intercepts of Lineweaver-Burk plots obtained for the inhibition of the $F_1$-ATPase by amphipathic cations*

n.d., not determined.

| Inhibitor | $K_{is}$ ($\mu$M) | $K_{ii}$ ($\mu$M) | Curvature |
|---|---|---|---|
| Rhodamine 6G | 46 | 12 | s-parabolic [a], i-parabolic [a] |
| Rhodamine B | 280 | 280 | s-parabolic [a], i-parabolic [a] |
| Rhodamine 123 | n.d. | n.d. | s-parabolic, i-parabolic |
| Rosaniline | 50 | 12 | s-parabolic [a], i-parabolic [a] |
| Malachite green | 68 | 18 | s-parabolic [a], i-parabolic [a] |
| Coriphosphine | 63 | 388 | s-parabolic [a], i-parabolic [a] |
| Acridine orange | n.d. | n.d. | s-parabolic, i-parabolic |
| Dequalinium | 7.5 | 7.5 | s-hyperbolic, i-hyperbolic |
| Melittin | 4.8 | 4.8 | s-linear, i-linear |
| Syn-A2 | 0.054 | 0.054 | s-parabolic [a], i-parabolic [a] |
| Syn-C | n.d. | 0.040 | s-hyperbolic, i-parabolic [a] |

[a] The replots were linear except for the highest inhibitor concentration examined.

As assessed from the experimentally determined $I_{0.5}$ values, rhodamine 6G, rosaniline, and malachite green were the most effective of the dyes that were found to inhibit soluble $F_1$. To evaluate these inhibitors in more detail, the steady-state kinetics of the ATPase were examined with ATP varying from 0.2–2.0 mM at fixed dye concentrations. Lineweaver-Burk plots obtained for inhibition of $F_1$ in the presence of increasing concentrations of rhodamine 6G, rosaniline, or malachite green displayed the same pattern, with the lines intersecting at a common point below the abscissa. This represents a form of mixed inhibition [19]. The $K_{is}$ and $K_{ii}$ values determined from the slopes and intercepts, respectively, of the Lineweaver-Burk plots are given in Table II. The replots deviated from linearity at the highest concentration of each of these dyes with a concave-upward curvature. This indicates parabolic, mixed inhibition and suggests at least two binding sites for high concentrations of these inhibitors [20]. The $K_{ii}$ value determined for each dye corresponded closely to its $I_{0.5}$ value shown in Table I.

Mixed inhibition of another type was observed in the presence of acridine oragne or coriphosphine. The double reciprocal plots obtained for increasing concentrations of these dyes intersected at a common point above the abscissa. Replots of neither slopes nor intercepts of the Linewaver-Burk plots obtained in the presence of acridine orange were sufficiently linear to permit estimation of $K_{is}$ or $K_{ii}$ values. However, for inhibition by coriphosphine, replots of slopes and intercepts were linear at low dye concentrations. This permitted estimation of the $K_{is}$ and $K_{ii}$ values shown in Table II. Owing to its extinction at 340 nm, the wavelength at which the oxidation of NADH is monitored with the coupled assay system, inhibitory characteristics of coriphosphine were evaluated only below 500 $\mu$M.

In contrast to rhodamine 6G, which exhibited mixed inhibition with lines intersecting at a common point below the abscissa, Lineweaver-Burk plots for inhibition by different concentrations of rhodamine B or rhodamine 123 intersected at a common point on the abscissa. This is characteristic of noncompetitive inhibition. Whereas replots of both slopes and intercepts of data obtained from Lineweaver-Burk plots for inhibition by rhodamine B deviated from linearity at the highest concentration examined (500 $\mu$M), replots of the data obtained for inhibition by rhodamine 123 were non-linear at all dye concentrations. In both cases, the curvature was concave upward. This is parabolic, noncompetitive inhibition and suggests more than one binding site on the enzyme for the inhibitor [19,20].

*Inhibition of $F_1$ by dequalinium and melittin*

Lineweaver-Burk plots of kinetic data obtained in the presence of dequalinium or melittin showed that these amphiphilic cations are also noncompetitive inhibitors of $F_1$, with lines intersecting at a common point on the abscissa. Replots of slopes and intercepts obtained for inhibition by dequalinium gave a value of 7.5 $\mu$M for both $K_{is}$ and $K_{ii}$. This is equivalent to the $I_{0.5}$ value estimated for this reagent shown in Table I. The slope and intercept replots for dequalinium deviated from linearity at the highest concentration examined. The curvature was convex downward. This is characteristic of hyperbolic, noncompetitive inhibition and indicates partial inhibition in which product is formed more slowly from the ESI complex than it is from the ES complex [19,20]. On the other hand, replots of the slopes and intercepts of the Lineweaver-Burk plots for inhibition by melittin were linear for the entire range of concentrations examined. Thus, melittin is a linear, noncompetitive inhibitor in which the ESI complex does not form product. Replots of slopes and intercepts of the Lineweaver-Burk plots for inhibition by melittin gave a value of 4.8 $\mu$M for $K_{is}$ and $K_{ii}$. These are the same as the $I_{0.5}$ value estimated for this inhibitor shown in Table III.

TABLE III

*Concentrations of cationic, amphiphilic peptides that produce 50% inhibition of the $F_1$-ATPase activity at pH 8.0*

| Peptide | Sequence | $I_{0.5}$ ($\mu$M) |
|---|---|---|
| Melittin | GIGAVLKVLTTGLPALISWIKRKRQQ-$NH_2$ | 5 |
| WT | MLSLRQSIRFFKPATRTLCSSRYLL-$NH_2$ | 16 |
| Δ11,12 | MLSLRQSIRFPATRTLCSSRYLL-$NH_2$ | 29 |
| Syn-A2 | MLSRLSLRLLSRLSLRLLSRYLL-$NH_2$ | 0.042 |
| Syn-B2 | MLSRQQSQRQSRQQSQRQSRYLL-$NH_2$ | $\gg$ 50 |
| Syn-C | MLSSLLRLRSLSLLRLRLSRYLL-$NH_2$ | 0.058 |

*Inhibition of $F_1$ by amphiphilic presequence peptides*

Evidence has accumulated that import of nascent proteins into the inner membrane and matrix of the mitochondrion is associated with amphiphilicity of the presequences of the transported proteins [15,16]. Melittin folds into an amphiphilic helical structure [21] and is an effective inhibitor of $F_1$. Since synthetic peptides, which were designed to study the importance of cationic amphiphilicity in the presequence of cytochrome oxidase subunit IV for mitochondrial import were available, inhibition of $F_1$ by these peptides was examined. The primary sequences of the peptides examined [16] and their corresponding $I_{05}$ values at pH 8.0 are presented in Table III. The synthetic peptides, Syn-A2 and Syn-C, with $I_{0.5}$ values of about 50 nM, were the most effective inhibitors. These findings provoked a more detailed analysis of the inhibitory properties of the two peptides by steady-state kinetics. From examination of Lineweaver-Burk plots for inhibition of $F_1$ by Syn-A2 and Syn-C, it was shown that Syn-A2 produced non-competitive inhibition, whereas inhibition in the presence of Syn-C was mixed. Lines generated at 0, 48 and 80 nM Syn-C intersected at a common point below the abscissa. The line generated at 160 nM Syn-C intersected the others to the left of their common point of intersection. For Syn-A2, both slope and intercept replots deviated from linearity, convex upward, at the highest concentration of inhibitor examined. This is characteristic of parabolic inhibition. The replot of intercepts of Lineweaver-Burk plots in the presence of Syn-C also deviated from linearity at the highest concentration with convex curvature at the highest concentration examined, thus indicating parabolic, mixed inhibition. However, the replot of the slopes of Lineweaver-Burk plots obtained in the presence of Syn-C deviated from linearity in the opposite direction at the highest concentration examined, indicating hyperbolic, mixed inhibition. These results are difficult to interpret.

A value of 54 nM was determined for $K_{is}$ and $K_{ii}$ from the replots for Syn-A2. The value of $K_{ii}$ for Syn-C was determined to be 40 nM from the intercept replot. These values are consistent with the $I_{0.5}$ values for the peptides presented in Table III.

*Effect of amphiphilic inhibitors on inactivation of $F_1$ by quinacrine mustard*

The aziridinium of quinacrine mustard is a potent, irreversible inactivator of $F_1$ [22,23]. It is also a structural analog of several of the dyes which are shown here to be reversible inhibitors of the $F_1$-ATPase. Therefore, it was of interest to examine the capacity of selected amphiphilic, reversible inhibitors surveyed in this study to protect $F_1$ against inactivation by quinacrine mustard. The results of this comparison are shown in Table IV. In these experiments, $F_1$ at 1 mg per ml was incubated in the presence and absence of the indicated concentrations of the reversible inhibitors and 50 $\mu$M purified quinacrine mustard in 50 mM Mops (pH 7.0). After addition of quinacrine mustard, which was added last, samples were removed with time and assayed for residual ATPase activity. Inhibition by the amphiphilic substances is relieved by dilution. The logarithm of residual activity was plotted against time according to Guggenheim [24] to obtain the pseudo-first order rate constants given in Table IV. The relative effectiveness of the amphiphilic substances in decreasing the rate of inactivation of the ATPase by quinacrine mustard, is approximately equivalent to the order of their affinities for $F_1$ as assessed by the kinetic analyses summarized in Tables I–III. The synthetic peptides, Syn-A2 and Syn-C, which are artificial mitochondrial import sequences [15,16], were the most effective protectors of the ATPase against inactivation by quinacrine mustard and also the most potent of the reversible inhibitors of $F_1$ examined in this study.

TABLE IV

*The effect of cationic amphiphilic inhibitors on inactivation of the $F_1$-ATPase by quinicrine mustard*

Inactivation mixtures at 23° C contained 0.4 mg of $F_1$ in 400 $\mu$l (2.7 $\mu$M) of 50 mM MOPS[2] (pH 7.0). After addition of the inhibitor at the concentration stated, each sample was incubated for 3 min prior to addition of 50 $\mu$M of purified quinacrine mustard which initiated inactivation. Samples of the reaction mixtures, 2 $\mu$l each, were removed at 3 min intervals and assayed for ATPase as described in Experimental Procedures. The first order rate constants for inactivation, $k'$, were obtained according to Guggenheim [24]. Controls not containing quinacrine mustard retained complete activity during the course of all inactivations examined.

| Additions | Concentration ($\mu$M) | $10^2 \times k'$ ($min^{-1}$) |
|---|---|---|
| None | | 6.92 |
| Rhodamine 6G | 50 | 5.32 |
| Rosaniline | 50 | 4.77 |
| Dequalinium | 50 | 2.71 |
| Melittin | 50 | 2.31 |
| Syn-A2 | 5 | 1.76 |
| Syn-C | 5 | 2.27 |

## Discussion

The results presented clearly show that a variety of cationic dyes, which were previously thought to inhibit a detergent solubilized preparation of the $F_0F_1$-ATPase by interacting with the $F_0$ moiety [10], also inhibit the soluble $F_1$-ATPase. Similar results have been reported by Emaus et al. [8] for the inhibition of the rat liver mitochondrial $F_1$-ATPase by rhodamine 123 and by Wieker et al. [9] for the inhibition of yeast mitochondrial $F_1$-ATPase by rhodamine 6G, other rhodamines, and ethidium bromide. The previous conclusion that the dyes inhibit the $F_0F_1$ preparation by interacting with $F_0$

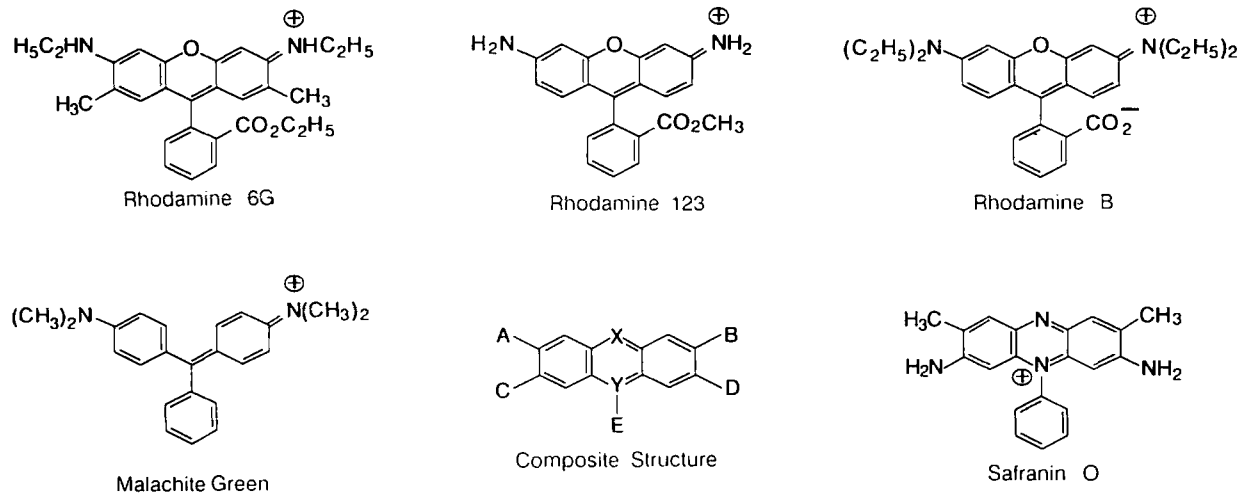
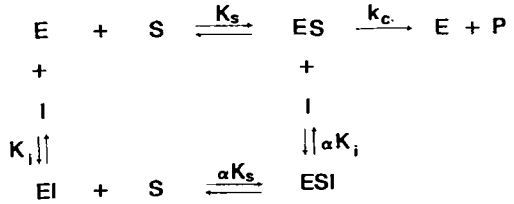



Fig. 1. Structures of representative inhibitory dyes.

[10] was based on the observation that the $F_0F_1$ preparation, but not the soluble $F_1$-ATPase, was inhibited by the dyes in the presence of phospholipid vesicles. In the previous study, the activity of controls containing soluble $F_1$ and the dyes was not examined in the absence of phospholipid vesicles. When the dyes were added to the mixture of $F_1$ and phospholipid vesicles, sequestration of the dyes by the vesicles presumably lowered aqueous concentration to levels where no inhibition of the ATPase was observed. However, it remains unclear why the $F_0F_1$ preparation examined previously is inhibited by the dyes only in the presence of phospholipid vesicles.

With the exception of dequalinium, the non-peptidyl, amphiphilic substances examined that were shown to inhibit soluble $F_1$ in this study have a common core illustrated by the Composite Structure of Fig. 1. Acridine orange, coriphosphine, Nile blue A, and pyronin Y, dyes containing the fused tricyclic ring of the Composite Structure of Fig. 1, but lacking a substituent in position E, show comparatively low affinity for the soluble $F_1$-ATPase, but are rather potent inhibitors of the $F_0F_1$-ATPase in the presence of phospholipid vesicles. It was shown previously that low concentrations of each of these dyes stimulate State 4 respiration and latent ATPase activity of intact mitochondria, whereas rhodamine 6G, rosaniline, and safranin O do not stimulate respiration and inhibit ATPase activity stimulated by dinitrophenol [10]. This suggests that the interaction of the membrane-bound ATPases with dyes lacking a substituent in position E differs from that of the membrane-bound enzyme with dyes containing a phenyl group in position E.

Each of the amphiphilic inhibitors examined by steady-state kinetic analysis exhibited mixed or non-competitive inhibition. From the characteristics of replots of slopes and intercepts of Lineweaver-Burk plots summarized in Table II, dequalinium is the only inhibitor of those tested that showed definite partial inhibition. This is consistent with previously reported results [26] which showed maximal inhibition of $F_1$ by dequalinium is about 85%. With the exception of the synthetic amphiphilic peptide, Syn-C, which exhibits hyperbolic slope and parabolic intercept replots, slope and intercept replots for the other inhibitors examined were either linear or parabolic. In these cases, inhibition appeared to be complete rather than partial. This is in contrast to results obtained by Adade et al. [27] who reported the kinetic characteristics of inhibition of $F_1$ by the local anesthetics, benzocaine, lidocaine, tetracaine, and dibucaine and the tricyclic antipsychotic, chlorpromazine. From the results reported by Adade et al. [27] and other observations on the effects of these compounds on physical properties of $F_1$ [28], it was concluded that local anesthetics and chlorpromazine affect the activity of $F_1$ by interacting with multiple, weak binding sites, thus causing conformational changes which attenuate activity. With the exception of chlorpromazine, which contains a phenothiazine ring (X of the Composite Structure of Fig. 1 is sulfur and Y is nitrogen) substituted with a dimethylaminopropyl group in position E, none of the compounds examined by Adade et al. [27] is closely related in structure to the inhibitors examined in this study. The cationic, amphiphilic compounds examined in the present study, with the exception of safranin O, which contain a fused, tricyclic ring substituted in position E with an aryl group, have higher affinity for $F_1$ than any of the local anesthetics and also cause complete inhibition. These




Scheme I. Scheme for mixed inhibition.

differences suggest that the mechanism of inhibition of $F_1$ by weakly binding local anesthetics and other amphiphilic cations studied by Vanderkooi and his colleagues [27,28] might be different from that of the high affinity dyes examined in this study, which resemble the Composite Structure of Fig. 1, with position E occupied by an aryl group.

Although the structures and charge of rhodamines 6G and 123 are similar, the dyes inhibit bovine heart $F_1$ with different characteristics. Rhodamine 6G inhibits with a 27-fold greater affinity and exhibits mixed inhibition with lines intersecting below the abscissa on Lineweaver-Burk plots. In contrast, rhodamine 123 exhibits noncompetitive inhibition. In both cases, the inhibitor binds to both free enzyme and the ES complex as illustrated in Scheme I. In noncompetitive inhibition, $\alpha = 1$ and the inhibitor binds with equal affinity to free enzyme and the ES complex, whereas in mixed inhibition with lines intersecting below the abscissa, $\alpha < 1$ and the inhibitor binds with greater affinity to the ES complex than it does to the free enzyme. Rhodamine 6G differs in structure from rhodamine 123 only in that it contains aliphatic substituents in positions A, B, C, and D of the Composite Structure of Fig. 1. This suggests that the two rhodamines might bind to a common, weakly inhibitory site on the free enzyme, but only rhodamine 6G, owing to its greater amphiphilic character, binds with high affinity to a transient site that is induced by substrate binding. Using a different method of kinetic analysis, Wieker et al. [9] have concluded that rhodamine 6G is an uncompetitive inhibitor of the yeast $F_1$-ATPase, whereas it is a noncompetitive inhibitor of a solubilized preparation of the yeast $F_0F_1$-ATPase and the ATPase of yeast submitochondrial particles. A $K_i$ of 2.4 $\mu$m was observed for all three forms of the enzyme. From this observation, Wieker et al. [9] suggested that binding of $F_1$ to $F_0$ induces the appearance of the same site for rhodamine that is induced by the binding of substrate to the free enzyme.

Ethidium bromide, an amphiphilic cation with a substituted, tricyclic, fused ring structure of the phenathrene-type rather than the anthracene-type, might be considered as a distorted analog of the Composite Structure of Fig. 1. Since ethidium bromide inhibits yeast $F_1$ noncompetitively, whereas rhodamine 6G inhibits the soluble enzyme uncompetitively, Wieker et al. [9] have concluded that the two positively charged inhibitors bind to separate sites. That rhodamine 6G inhibits yeast $F_1$ with pure uncompetitive inhibition and not by mixed inhibition, in which the inhibitor binds to the ES complex with much higher affinity than it does to the free enzyme, cannot be discerned from the results reported by Wieker et al. [9]. If rhodamine 6G is also a mixed inhibitor of yeast $F_1$, it could be argued that ethidium bromide might bind to the same site that binds rhodamine 6G on the free enzyme, but might not interact with a transient amphiphilic surface that is exposed on substrate binding, which has high affinity for rhodamine 6G. This argument is supported by the earlier observation that tetramethylethidium has approx. a 4-fold greater affinity than ethidium for a detergent solubilized preparation of bovine $F_0F_1$ in the presence of phospholipid vesicles [10].

Since most of the non-peptidyl amphipathic cations studied that inhibit $F_1$ also inhibit interaction of calmodulin with target enzymes [14], the effect of melittin on the $F_1$-ATPase was examined. Melittin, a 26 residue bee venom peptide, inhibits calmodulin activation competitively in a $Ca^{2+}$-dependent manner [29]. Displaying a $K_i$ of about 5 $\mu$M, melittin is a relatively potent noncompetitive inhibitor of $F_1$. Therefore, it was not surprising to find that the synthetic peptide corresponding to the presequence of yeast cytochrome oxidase subunit IV, which is also a positively charged, amphiphilic, $\alpha$-helical structure [16], inhibited $F_1$, but with lower affinity ($I_{0.5} = 16$ $\mu$M) than is observed with melittin. Also as expected, the $\Delta$11,12 deletion analog of the presequence peptide, which has lower amphiphilicity than the wild-type peptide on the basis of several criteria [16] inhibits $F_1$ with lower affinity, whereas the synthetic peptide, Syn-B2, which lacks amphiphilicity [16], shows no inhibition of $F_1$ at concentrations up to 50 $\mu$M. What is surprising is the observation that the synthetic peptides, Syn-A2 and Syn-C, inhibit $F_1$ with about a 300-fold greater affinity than the wild-type presequence peptide. The factors responsible for the high affinity of these peptides for the enzyme are not obvious. In the presence or absence of detergent micelles, the Syn-A2 peptide exists primarily as $\beta$ pleated sheet. In buffer alone, Syn-C is partly helical. Addition of detergent micelles induces an increase in secondary structure, but the distribution between $\alpha$- and $\beta$-structure is not clear. Both Syn-A2 and Syn-C exhibit higher capacity to insert into phospholipid monolayers than the wild-type presequence peptide, whereas the order of effectiveness of the three peptides in disrupting phospholipid vesicles is: Syn-A2 > wild-type > Syn-C [16]. Comparison of the properties of the synthetic peptides suggest that they must be amphiphilic to inhibit $F_1$ with high affinity, but that they need not be helical for inhibition with high affinity to occur. It is unclear whether inhibition of $F_1$ by the presequence peptide is of any physiological significance.

Whether the inhibitory, amphipathic cations examined, that neither conform to the Composite Structure of Fig. 1, nor exhibit similar inhibitory properties, bind to the same site on $F_1$ as rhodamine 6G cannot be discerned by the methods employed in this study. The observation that rhodamine 6G, rosaniline, dequlinium, melittin, and the synthetic peptides, Syn-$A_2$ and Syn-C, partially protect $F_1$ against inactivation by quinacrine mustard approximately in proportion to their inhibitory

capacities, suggests that the cationic, amphiphilic inhibitors might bind to the common site or sites on the enzyme. The major site of modification of $F_1$ by quinacrine mustard has been localized to the anionic site contained in residues 394–400 of the $\beta$ subunit with the sequence. DELSEED [23]. This sequence resembles the highly negatively charged sequence contained between residues 83–87 of calmodulin that has the sequence, EEEIRE, and is thought to interact with cationic, substituted phenothiazines [30,31] and melittin [32], both of which inhibit activation of cAMP phosphodiesterase by calmodulin [32]. Rhodamine 123 and dequalinium also inhibit activation of cAMP phosphodiesterase by calmodulin [12], presumably by interacting with the same site.

## Acknowledgements

This study was supported by United States Public Health Service Grant GM-16974 and National Science Foundation Grant DMB 84-17723.

## References

1 Hatefi, Y. (1985) Annu. Rev. Biochem. 54, 1015–1069.
2 Senior, A.E. (1988) Physiol. Rev. 68, 177–231.
3 Walker, J.E., Fearnley, I.M., Gay, N.T., Gibson, B.W., Northrop, F.D., Powell, S.J., Runswick, M.J., Saraste, M. and Tybulewicz, V.L.J. (1985) J. Mol. Biol. 184, 677–701.
4 Chazotte, B., Vanderkooi, G. and Chignell, D. (1982) Biochim. Biophys. Acta 680, 310–316.
5 Palatini, P. (1982) Mol. Pharmacol. 21, 415–421.
6 Laikind, P.K., Goldenberg, T.M. and Allison, W.S. (1982) Biochem. Biophys. Res. Commun. 109, 423–427.
7 Bullough, D.A., Kwan, M., Laikind, P.K., Yoshida, M. and Allison, W.S. (1985) Arch. Biochem. Biophys. 236, 567–575.
8 Emaus, R.K., Gruenwald, R. and Lemasters, J.J. (1986) Biochim. Biophys. Acta 850, 436–448.
9 Wieker, H.-J., Kuschmitz, D. and Hess, B. (1987) Biochim. Biophys. Acta 892, 108–117.
10 Mai, M.S. and Allison, W.S. (1983) Arch. Biochem. Biophys. 221, 467–476.
11 Weiss, M.J., Wong, J.R., Ha, C.S., Bleday, R., Salem, R.R., Steele, G.D. and Chen, L.B. (1987) Proc. Natl. Acad. Sci. USA 84, 5444–5448.
12 Bodden, W.L., Palayoor, S.T. and Hait, W.N. (1986) Biochem. Biophys. Res. Commun. 135, 574–582.
13 Nadakavukaren, K.K., Nadakavukaren, J.J. and Chen, L.B. (1985) Cancer Res. 45, 6093–6099.
14 Prozialeck, W.C. and Weiss, B. (1982) J. Pharmacol. Exp. Therap. 222, 509–516.
15 Roise, D., Horvath, S.J., Tomich, J.M., Richards, J.H. and Schatz, G. (1986) EMBO J. 5, 1327–1334.
16 Roise, D., Theiler, F., Horvath, S.J., Tomich, J.M., Richards, J.H., Allison, D.S. and Schatz, G. (1988) EMBO 7, 649–653.
17 Esch, F.S. and Allison, W.S. (1978) J. Biol. Chem. 253, 6100–6106.
18 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329.
19 Segel, I.H. (1975) Enzyme Kinetics, Wiley Interscience, New York.
20 Cleland, W.W. (1970) in The Enzymes, Vol. II (Boyer, P.D., ed.), pp. 1–65, Academic Press, New York.
21 Terwilliger, T.C. and Eisenberg, D. (1982) J. Biol. Chem. 257, 6016–6022.
22 Laikind, P.K. and Allison, W.S. (1983) J. Biol. Chem. 258, 11700–11704.
23 Bullough, D.A., Ceccarelli, E.A., Verburg, J.G. and Allison, W.S. (1989) J. Biol. Chem. 264, 9155–9163.
24 Guggenheim, E.A. (1926) Phil. Mag. 2, 538–543.
25 Berden, J.A. and Voorn-Brouwer, M.M. (1978) Biochim. Biophys. Acta 501, 424–439.
26 Zhuo, S. and Allison, W.S. (1988) Biochim. Biophys. Res. Commun. 152, 968–972.
27 Adade, A.B., Chignell, D. and Vanderkooi, G. (1984) J. Bioenerg. Biomemb. 16, 353–363.
28 Vanderkooi, G. and Adade, A.B. (1986) Biochemistry 25, 7118–7114.
29 Maulet, Y. and Cox, J.A. (1983) Biochemistry 22, 5680–5686.
30 Strynada, N.C.J. and James, M.N.G. (1988) Proteins 3, 1–17.
31 Gresh, N. and Pullman, B. (1986) Mol. Pharm. 29, 355–362.
32 Malencik, D.A. and Anderson, S.R. (1985) Biochemistry 23, 2420–2428.