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Mechanism of local anesthetic effect on mitochondrial ATP synthase as deduced from photolabelling and inhibition studies with phenothiazine derivatives

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The mode of interaction between mitochondrial ATP synthase and two phenothiazine derivatives, chlorpromazine (CPZ) and trifluoperazine (TFP), was studied as a model for the interaction of local anesthetic drugs with membrane proteins. Photolabelling experiments demonstrated that CPZ and TFP interact with various subunits of either the peripheral F_1 moiety or the membrane-embedded F_0 sector. Both drugs, however, labelled the membrane sector much more heavily. Qualitative differences in labelling were observed between CPZ and TFP, indicating non-identical sites of interaction. These diversities appeared related to the different hydrophobicities of the two drugs since: (a) TFP, which has a higher lipid/water partition coefficient, labelled the more hydrophobic subunits more markedly than CPZ; (b) reduced glutathione, a hydrophilic free radical scavenger that does not penetrate the membrane continuum, had a negligible effect on the labelling by TFP, whereas it reduced the labelling of various subunits by CPZ; (c) the labelling by [^{3}H]TFP was poorly antagonized by cold CPZ, whereas it was almost totally prevented by fluphenazine, a phenothiazine similar to TFP in hydrophobic character. Consistently, double-inhibition experiments showed that TFP and fluphenazine are mutually exclusive inhibitors of mitochondrial ATP synthase, whereas TFP and CPZ are mutually nonexclusive. The nature of the phospholipid bilayer influenced neither the labelling nor the inhibition patterns. The complex of these data indicate that tertiary amine local anesthetics affect the activity of membrane proteins by interacting with a multiplicity of relatively aspecific hydrophobic sites located preferentially, but not exclusively, on the membrane-embedded domains. It is suggested that at least two phenothiazine derivatives of different hydrophobicities be used in photolabelling experiments, before any generalization is made, since the molecular targets of these drugs vary according to their hydrophobic character.

Introduction

There is now general consensus that the ultimate effect of local anesthetic agents is on membrane proteins [1–9] However, the molecular mechanism underlying this effect is still a matter of considerable debate The local anesthetic action has been in turn ascribed to a direct interaction with either the peripheral [1,2] or the lipid-embedded moiety [3–7] of proteins, or to an indirect effect through a general perturbation of the phospholipid bilayer [8,9] The mitochondrial ATP synthase complex has become a widely used model system for such studies (see Ref 10 for a review) It consists of two structurally distinct components, a sector embedded in the membrane, termed F_0, which functions as a proton channel, and a peripheral sector, termed F_1, which catalyzes ATP synthesis/hydrolysis The latter component can be isolated from the membrane sector in a water-soluble form that retains ATPase activity [11,12] It has been reasoned that if local anesthetic drugs interact with the membrane sector, no inhibitory effect should be exerted on the isolated F_1 component On the contrary, similar inhibitory patterns should be observed with either the isolated F_1 or the whole enzyme complex if the drug–protein interaction is at the level of F_1 [1] On these grounds, the finding that the isolated F_1 component and the whole, membrane-bound enzyme complex were similary affected by phenothiazines and other tertiary amine local anesthetics was considered an indi-

Abbreviations TFP, trifluoperazine, CPZ, chlorpromazine, Tris, tris(hydroxymethyl)aminomethane, Mes, 2-(*N*-morpholino)ethanesulfonic acid, SDS, sodium dodecyl sulfate, PC, phosphatidylcholine, PS, phosphatidylserine, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, GSH, reduced glutathione

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cation of a selective drug interaction with the peripheral sector of the enzyme complex [1,2] However, other observations such as, (1) the greater sensitivity of the membrane-bound enzyme to the inhibitory effects and (2) the decreased sensitivity to the phenothiazines in conditions that also decrease oligomycin sensitivity suggested an effective drug interaction also with the membrane-bound F_0 sector [13–15] Covalent labelling of the binding sites can be of help in resolving this question Such a labelling can be easily accomplished with phenothiazines which are naturally photolabile compounds Upon ultraviolet illumination, these drugs convert to free cation radicals that react covalently with nucleophilic groups present at their binding sites [16,17] Therefore, the use of radioactive phenothiazines allows an identification of the phenothiazine binding sites by determining the distribution of the protein-associated radioactivity after SDS-PAGE The possibilities of this technique have been fully explored by Changeux and co-workers in a series of studies on the interaction of chlorpromazine with the nicotinic acetylcholine receptor (reviewed in Refs 18 and 19) Such studies have shown that this technique, albeit not strictly quantitative, offers sufficient selectivity for identifying phenothiazine-binding domains on macromolecules

Recently, we obtained evidence that the same binding sites are involved in the inhibition of mitochondrial ATP synthase by either phenothiazines or their photogenerated radicals [15] In particular, the same order of potency was observed among photoactivated and unmodified drugs The inhibitory efficacy was directly proportional to the drug hydrophobicity and inversely related to the radical lifetime This implies that the photoinduced inactivation of the enzyme did not occur by reaction with free radicals generated in free solution, but was the result of an interaction with drug molecules photoactivated at their binding sites On the basis of these results, we here apply the photolabelling technique to mitochondrial ATP synthase Using two phenothiazine derivatives, trifluoperazine (TFP) and chlorpromazine (CPZ) of high and low hydrophobicity, respectively, and the same enzyme preparations used for the inhibition studies [15], we provide direct evidence that phenothiazines interact preferentially with the membrane sector of the enzyme and identify the affected subunits Because of the unexpected results of the photolabelling experiments, i e , mutually non-exclusive binding of TFP and CPZ, we also performed double-inhibition experiments and confirmed that phenothiazines of different hydrophobicity actually interact with non-identical inhibitory sites

Materials and Methods

ATP, phospho*enol*pyruvate, chlorpromazine HCl, trifluoperazine 2 HCl and all buffers were obtained from Sigma, acrylamide and bisacrylamide from Bio-Rad, fluphenazine 2 HCl was a kind gift from Squibb [^{3}H]CPZ (spec act 26 7 Ci/mmol) and [^{3}H]TFP (spec act 12 8 Ci/mmol) were obtained from New England Nuclear, Boston, MA and Rotem, Israel, respectively ATP synthase complexes were prepared from bovine heart Mg-ATP submitochondrial particles as originally described [20,21] The delipidated ATP synthase complexes obtained by these methods were reconstituted with phospholipids according to the cholate-dialysis method [22] In summary, 0 2–1 mg of the delipidated complexes were reconstituted with 3 μmol/mg protein of sonicated liposomes of either egg phosphatidylcholine (prepared as previously described [23]) or egg phosphatidylcholine plus 10% HPLC-purified bovine brain phosphatidylserine (FIDIA, Abano Terme, Italy) in the presence of 2 mg/ml of potassium cholate Cholate was then removed by overnight dialysis against 1000 ml of Tris-Mes 25 mM (pH 7 4) at 0 °C The dialysis medium was changed twice, after 1 and 4 h from the start of the dialysis The ATPase activity of the reconstituted complexes varied from 1 2 to 3 0 μmol/mg per min for Complex V [21] and from 4 1 to 9 5 μmol/mg per min for the preparation of Serrano et al [20] Protein determination was performed according to Lowry et al [24]

Photolabelling procedure

100–200 μg of enzyme complexes were incubated 10 min under red safety light with [^{3}H]TFP or [^{3}H]CPZ (1 000 000 cpm corresponding to 0 8 and 0 4 μM drug, respectively) in a final volume of 100 μl Tris-Mes (pH 7 4) Lipid-protein samples were then irradiated at 256 nm for 15 min with an ultraviolet, UVSL-56 Mineralight lamp (S Gabriel, CA) This relatively long illumination time was made necessary by the low yield of the photolabelling reaction [16] Decreasing the time of illumination while increasing phenothiazine concentrations had no effect on the labelling patterns At the end of the illumination period, 5 mM dithiothreitol and 1 5% defatted bovine serum albumin were added to prevent possible postphotolytic labelling [25] The labelled proteins were recovered by centrifugation on a 20% sucrose solution buffered with 25 mM Tris-Mes (pH 7 4), in the presence of 1% CHAPS (3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate) to eliminate the bound phospholipids, which would otherwise alter the electrophoretic pattern of low molecular weight ATP synthase subunits Among the various ionic and non-ionic detergents tested (SDS, potassium cholate, potassium deoxycholate, Lauryl diamine oxide, Triton X-100) only CHAPS provided 100% protein recovery The pellets were dissolved in 4% SDS, 3% β-mercaptoethanol, 10 mM Tris-acetate (pH 8 2) (sample buffer) and subjected to electrophoresis on 20 cm long polyacrylamide linear gradient (13–19%) gels as described

before [26] After staining with Coomassie blue, the gel patterns were recorded by scanning with a Shimadzu Dual Chromato Scanner CS-930 The gels were then treated for fluorography with Amplify (Amersham) and the dried gels were exposed to X-O-Mat 5AR Kodak films at −80 °C [27] The intensity of the spots on the developed films was determined by scanning as above

Double-inhibition studies

Inhibition experiments were performed in the dark to prevent the possible formation of phenothiazine free radicals [28] ATPase activity was determined at 37 °C by monitoring spectrophotometrically at 340 nm the oxidation of NADH in a coupled lactate dehydrogenase-pyruvate kinase ATP regenerating system [21] Initial rates of hydrolysis were used in all calculations The results presented are the means of three separate experiments performed in duplicate

Results

Photolabelling of F_0–F_1 ATP synthase complexes

The preparation of Serrano et al [20] and Complex V of Stiggall et al [21], previously employed for the inhibition experiments with photoactivated phenothiazines [15], were also used for the photolabelling experiments These enzyme preparations appeared to be particularly suited for such experiments, since they are highly purified and fully characterized in their subunit compositions They are obtained as lipid-free inactive complexes, that can be reactivated by reconstitution with phospholipid liposomes Therefore, the possible influence can be evaluated of the composition of the lipid matrix on the drug-protein interaction In addition to the F_0–F_1 complex, the preparation of Serrano et al [20] contains the adenine nucleotide carrier which is likely to interact directly with ATP synthase [29] and

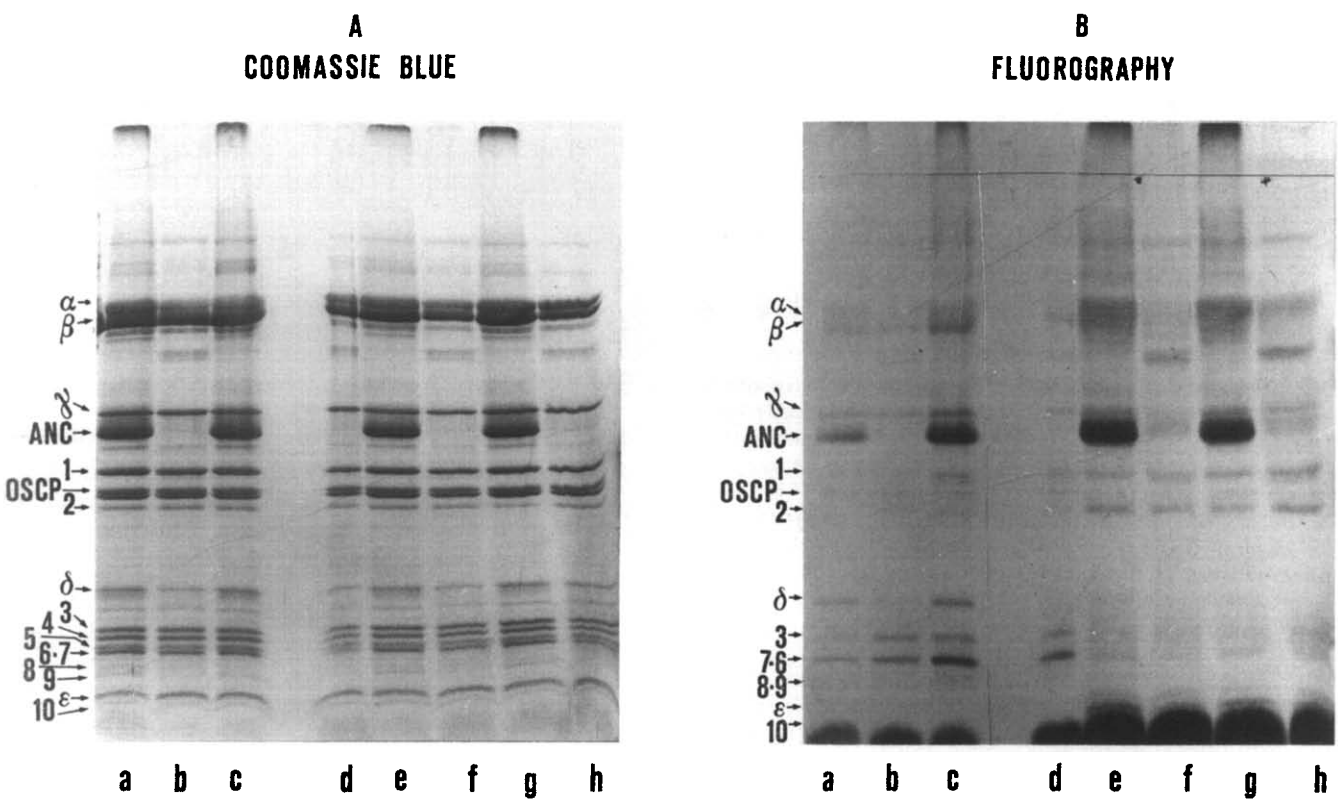

Fig 1 SDS-PAGE and fluorography of ATP synthase Samples of phospholipid-reconstituted enzymes (about 100 μg protein) were incubated 10 min under red safety light with [^{3}H]TFP or [^{3}H]CPZ and then irradiated at 256 nm for 15 min with an ultraviolet lamp as described in Materials and Methods The labelled proteins were recovered by centrifugation on 20% sucrose solution buffered with 25 mM Tris-Mes (pH 7 4) in the presence of 1% CHAPS The pellets were dissolved in the sample buffer and subjected to electrophoresis on a 20 cm long polyacrylamide linear gradient (13–19%) Greek letters indicate the F_1 subunits Numbers denote the bands found associated with F_0, according to Montecucco et al [26] ANC and OSCP denote adenine nucleotide carrier and oligomycin sensitivity conferring protein, respectively Subunits 4, 5, 6 and 8 are also referred to as ATPase inhibitor, F_6, A6L and DCCD binding protein, respectively (A) SDS-PAGE of Coomassie blue-stained egg-PC/Serrano et al [20] ATP synthase (lane a), egg-PC/complex V (lane b), egg-PC/10% PS/Serrano et al [20] ATP synthase (lane c), egg-PC/10% PS/complex V (lane d) These patterns were obtained from enzyme preparations labelled with [^{3}H]TFP Lanes e, f, g and h represent the Coomassie blue-stained electrophoretic patterns of the same enzyme preparations, in the same order, after labelling with [^{3}H]CPZ (B) Fluorograms of [^{3}H]TFP labelled egg-PC/Serrano et al [20] ATP synthase (lane a), egg-PC/complex V (lane b), egg-PC/10% PS/Serrano et al [20] ATP synthase (lane c), egg-PC/10% PS/complex (lane d) Lanes e, f, g and h represent the fluorographic patterns of the same enzyme preparations, in the same order, after labelling with [^{3}H]CPZ

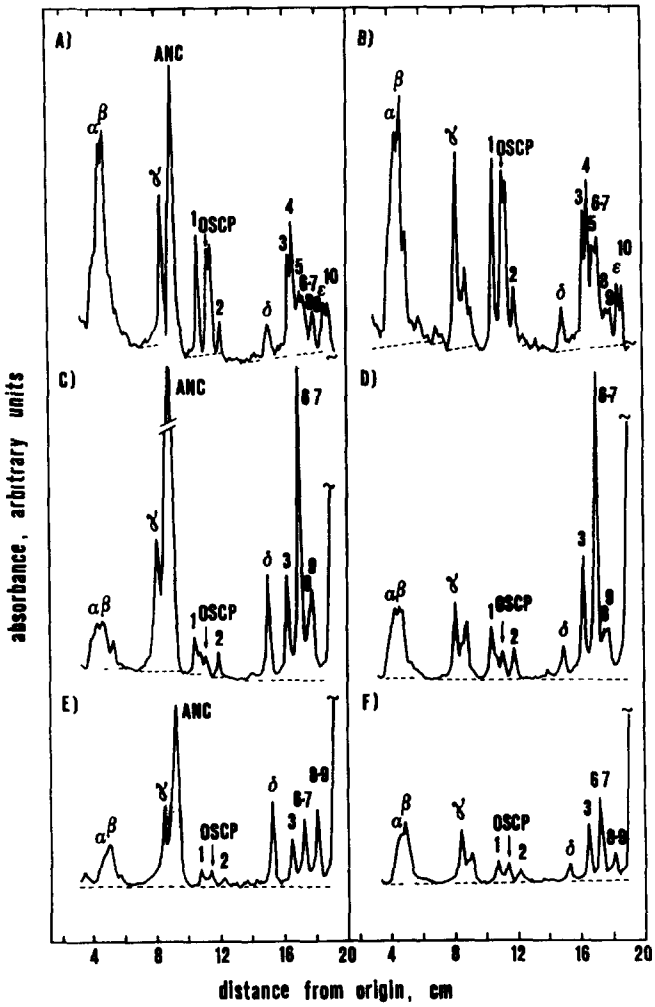

Fig 2 Photolabelling with [^{3}H]TFP of phospholipid-reconstituted ATP synthase complexes Densitometric traces of Coomassie blue-stained egg-PC/Serrano et al [20] ATP synthase (A) and egg-PC/complex V (B) Densitometric traces of fluorograms of egg-PC/Serrano et al [20] ATP synthase (C), egg-PC/complex V (D), egg-PC/10% PS/Serrano et al [20] ATP synthase (E) and egg-PC/10% PS/complex V (F) The densitometric traces of Coomassie blue-stained SDS-PAGE and of fluorograms were obtained from the gels and fluorograms shown in Fig 1, recorded by scanning with a Shimadzu Dual Chromato scanner CS-930 at 570 nm For symbols see legend to Fig 1

has been shown to influence its interaction with either drugs [29] or phospholipids [23,26] Fig 1 shows the electrophoretic and fluorographic patterns obtained from the Serrano et al [20] preparation and complex V after reconstitution with isoelectric or acidic phospholipids and ultraviolet irradiation in the presence of 0 8 μM [^{3}H]TFP or 0 4 μM [^{3}H]CPZ Isotopical dilution of [^{3}H]TFP or [^{3}H]CPZ with unlabelled TFP or CPZ up to their I_{50} values (7 and 20 μM, respectively, see below) had no effect on the labelling patterns The corresponding densitometric traces are shown in Fig 2 for [^{3}H]TFP and Fig 3 for [^{3}H]CPZ From these figures it is evident that either drug labelled both the F_1 and the F_0 components of the two ATP synthase complexes However, if the relative molecular weights of F_1 and F_0 are considered, the latter component appears much more heavily labelled For example, with complex V and TFP (Fig 2B and D) the ratio of fluorographic to Coomassie blue area was 0 41 for F_1 and 1 55 for F_0 Similar values were obtained with CPZ or the enzyme preparation of Serrano et al [20] Unexpectedly, as is apparent from a comparison of Figs 2 and 3, the two drugs exhibited somewhat different labelling patterns As far as F_1 was concerned, TFP labelled more heavily the γ subunit than did CPZ In addition, TFP did not label subunit ϵ, whereas CPZ did not label subunit δ Neither drug labelled heavily the α and β subunits, if one considers

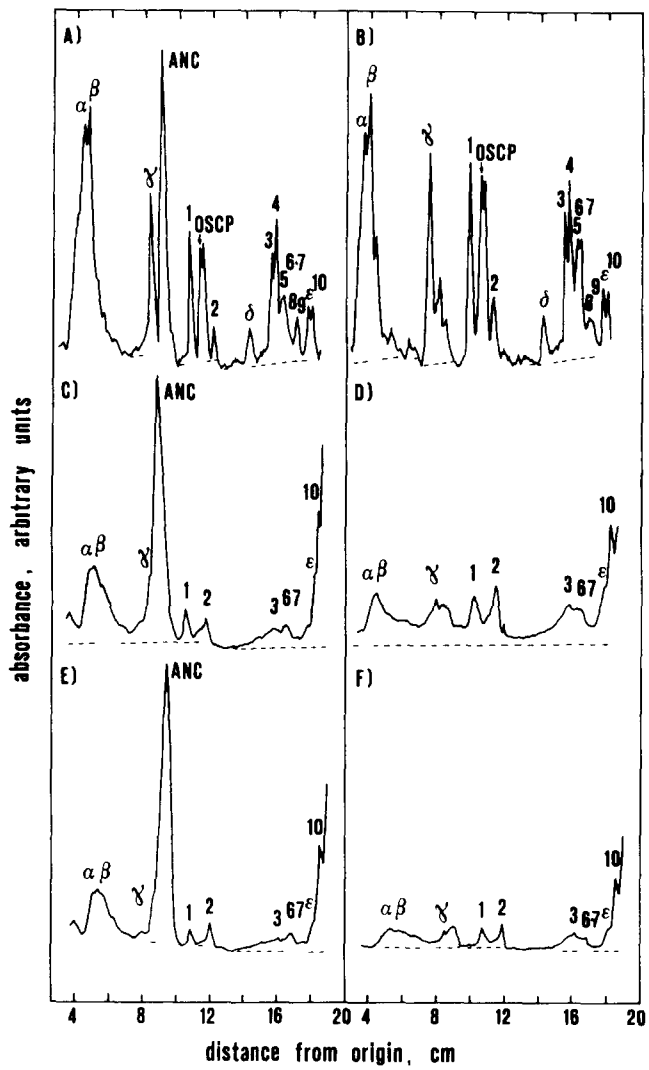

Fig 3 Photolabelling with [^{3}H]CPZ of phospholipid-reconstituted mitochondrial ATP synthase Densitometric traces of Coomassie blue-stained egg-PC/Serrano et al [20] ATP synthase (A) and egg-PC/complex V (B) Densitometric traces of fluorograms of egg-PC/Serrano et al [20] ATP synthase (C), egg-PC/complex V (D), egg-PC/10% PS/Serrano et al [20] ATP synthase (E) and egg-PC/10% PS/complex V (F) The densitometric traces of Coomassie blue-stained SDS-PAGE and of fluorograms were obtained as in Fig 2 Symbols as in Fig 1

the amount of protein mass contributed by these polypeptides. For example, with TFP (Fig 2,) the ratio of the fluorographic to Coomassie blue areas for the α and β subunits was 0.20–0 25, as compared to 0.40–0 60 for the γ subunit and 0.65–0 75 for the δ subunit

Different labelling patterns were observed also for F_0, TFP interacted more extensively than CPZ with subunits 3 and 6-7, whereas CPZ showed greater affinity for subunits 1 and 2 Moreover, TFP did not label subunit 10, whereas CPZ did not label OSCP as well as subunits 8 and 9 Subunits 4 and 5 were labelled by neither drug. The adenine nucleotide carrier, when present, was heavily labelled, but did not alter the profile of labelling of the other polypeptides, so that no qualitative differences in labelling pattern were observed between the two ATP synthase complexes For this reason only one enzyme preparation was used in the subsequent experiments The mode of labelling was also independent of the charge of the phospholipid bilayer The presence of negatively charged phospholipids, that bind cationic amphipatic drugs more strongly [30], merely reduced the amount of protein-bound radioactivity. With TFP, this effect included all polypeptides and was particularly evident for subunit 6-7 of the F_0 component Although with CPZ the labelling of the ATP synthase complexes was even more markedly reduced, the interaction with the adenine nucleotide carrier was virtually unaffected, indicating a much stronger affinity of CPZ for this protein

A different labelling pattern of F_1, i e , much heavier labelling of the β subunit, has been observed by Laikind and Allison [31] with the physicochemically related drug quinacrine The possibility was, therefore, explored that the lack of labelling of certain subunits by the phenothiazines was due to a lack of reactivity of such subunits towards the drug radicals rather then a lack of affinity for the parent drugs To this purpose, we performed labelling experiments also in the presence of the SDS, which causes enzyme dissociation and denaturation Fig 4 shows that, after treatment with SDS, all subunits were labelled by both drugs In particular, subunits α, β, 4, 5 and OSCP, which did not interact significantly with photoactivated drugs when the enzyme was in its native conformation, were markedly labelled after exposure to SDS This indicates that reactive nucleophilic groups are present also on these subunits and the lack of labelling of such subunits in the native enzyme was due to a lack of receptor sites for the parent drugs rather than a lack of reactivity towards the free radicals The differences in labelling patterns between phenothiazines and quinacrine are most likely related to the different mechanisms of interaction of these drugs with F_1 Unlike phenothiazines which are noncompetitive inhibitors [14], quinacrine competes with the substrate [31] and is, therefore, expected to label the β subunit which contains the catalytic site

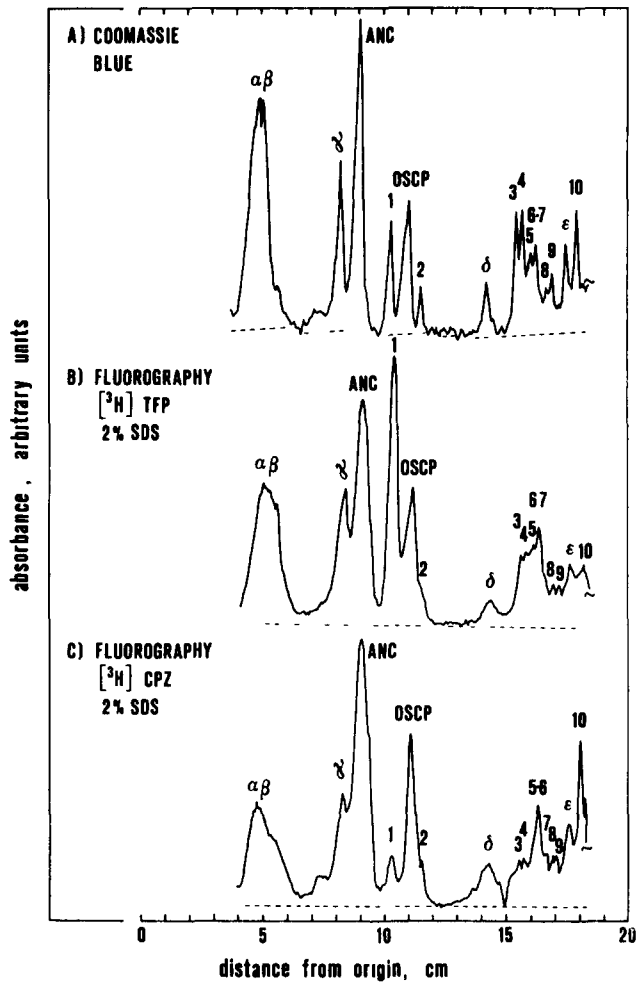

Fig 4 Photolabelling with [^{3}H]TFP and [^{3}H]CPZ of SDS-treated ATP synthase Samples of 100 μg of Serrano et al [20] ATP synthase not reconstituted with phospholipids were preincubated in 20 μl of Tris-Mes 25 mM (pH 7 4) containing 2% SDS and 0 8 μM [^{3}H]TFP or 0 4 μM [^{3}H]CPZ under red safety light for 10 min and then irradiated for 15 min at 256 nm The labelled proteins were immediately dissolved in 20 μl sample buffer and subjected to SDS-PAGE and fluorography as described in Materials and Methods (A) densitometric trace of Coomassie blue-stained enzyme preparation (B) and (C) densitometric traces of fluorograms obtained after labelling with [^{3}H]TFP and [^{3}H]CPZ, respectively Symbols as in Fig 1

The contention that CPZ and TFP do not possess identical binding sites was further tested by means of cross-competition experiments between labelled and unlabelled drugs. For these experiments a lipid-free enzyme preparation had to be used, since the detergent effect of phenothiazine derivatives in the millimolar range [32] prevented a good protein recovery of the phospholipid-reconstituted complexes (see also Methods). Fig 5A shows the profile of labelling of the preparation of Serrano et al [20] by 0 8 μM [^{3}H]TFP In the presence of 0.5 mM cold TFP (Fig. 5B) no significant labelling could be observed of any of the subunits. By contrast, unlabelled CPZ, at a concentration as high as 3 mM (Fig. 5C), could not prevent the

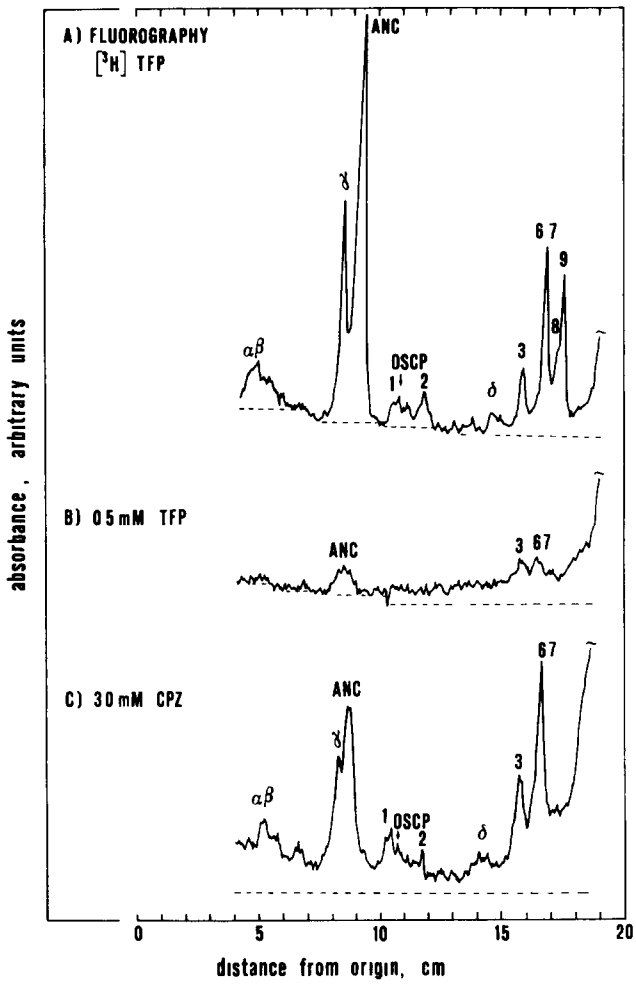

Fig 5 Competition between radioactive and unlabelled drugs Samples of 100 μg of Serrano et al [20] ATP synthase not reconstituted with phospholipids were preincubated with 0 8 μM [^{3}H]TFP in the presence or absence of 0 5 mM unlabelled TFP or 3 mM unlabelled CPZ in 20 μl of Tris-Mes (pH 7 4) for 10 min under red safety light and then, irradiated for 15 min at 256 nm The labelled proteins were immediately dissolved in 20 μl of sample buffer and subjected to SDS-PAGE and fluorography as described in Materials and Methods (A) Densitometric trace of fluorogram obtained after labeling with 0 8 μM [^{3}H]TFP, (B) the same in the presence of 0 5 mM unlabelled TFP (C) the same in the presence of 3 mM unlabelled CPZ Symbols as in Fig 1

incorporation of [^{3}H]TFP into numerous subunits, especially OSCP and subunits 3 and 6-7 of F_0 as well as subunits γ and δ of F_1 This lack of competition appears consistent with the results of Fig 3, showing weak or no labelling of such subunits by [^{3}H]CPZ In experiments not shown, partial cross-competition was also observed between [^{3}H]CPZ and unlabelled TFP, whereas virtually total competition was observed between [^{3}H]TFP and unlabelled fluphenazine, a phenothiazine derivative similar to TFP in hydrophobic character [33] Since the different hydrophobicities of TFP and CPZ appeared to be responsible for their different labelling patterns, we examined the effect of reduced glutathione (GSH) on the labelling reaction This hydrophilic free radical scavenger does not penetrate the membrane continuum and has been used to discriminate between surface and lipid-embedded binding domains [25] With the more hydrophobic TFP molecule, the photolabelling pattern remained qualitatively unchanged in the presence of excess GSH (cf Fig 6A and C), suggesting that the binding domains of TFP are mainly buried within the membrane at the protein/lipid interface This result also excludes that the observed incorporation of radioactivity was the result of a pseudophotoaffinity labelling, since GSH is a highly effective scavenger for phenothiazine radicals [34] With the less hydrophobic CPZ molecule, the labelling of various subunits was significantly reduced (cf Fig 6B and D), suggesting a preferential interaction of this drug with sites accessible from the external medium

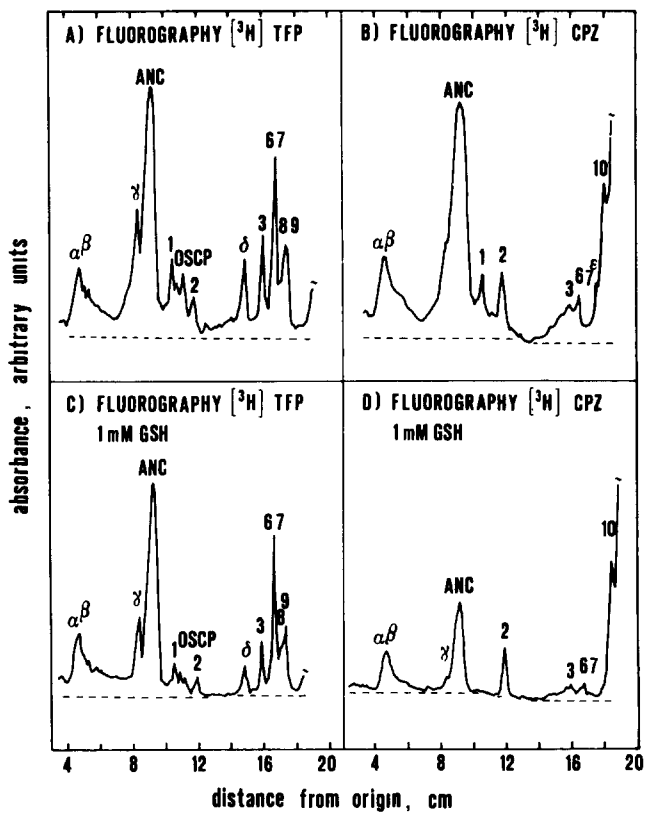

Fig 6 Effect of reduced glutathione on the photolabelling patterns of [^{3}H]TFP and [^{3}H]CPZ Samples of about 100 μg of Serrano et al [20] ATP synthase reconstituted with egg-PC were preincubated with 0 8 μM [^{3}H]TFP or 0 4 μM [^{3}H]CPZ in the presence or absence of 1 mM GSH The samples were then treated as described in the legend to Fig 1 (A) densitometric trace of fluorogram of the enzyme preparation labelled with [^{3}H]TFP, (B) densitometric trace of fluorogram of the enzyme preparation labelled with [^{3}H]CPZ, (C) and (D) the same as in (A) and (B), respectively, in the presence of 1 mM GSH Control experiments demonstrated that 1 mM GSH had no effect on either the enzyme activity or the inhibitory effect of phenothiazines Symbols as in Fig 1

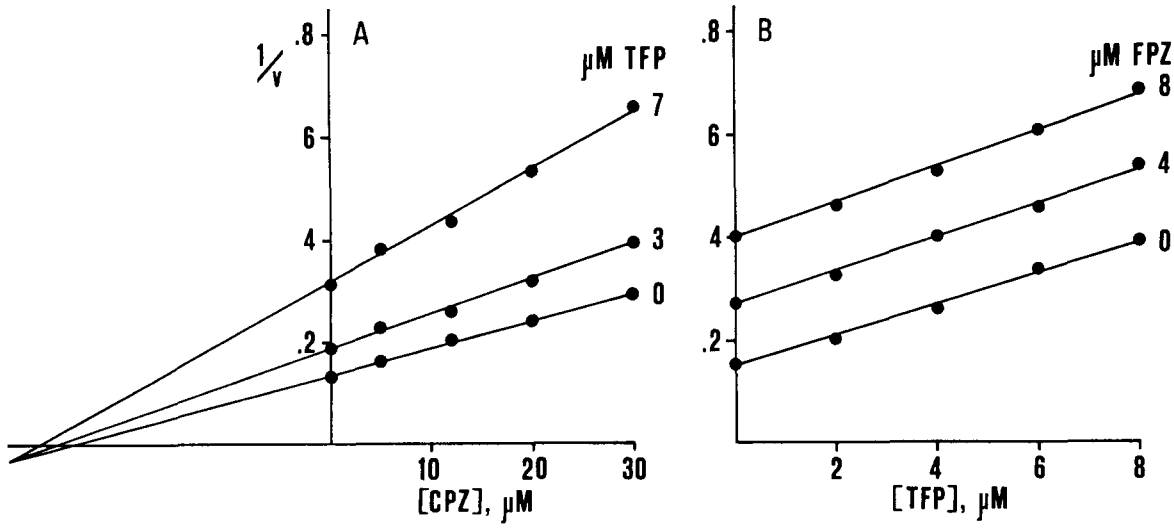

Fig 7 Double-inhibition experiments (A) interaction between TFP and CPZ, (B) interaction between TFP and fluphenazine (FPZ) The enzyme preparation of Serrano et al [20] reconstituted with egg-PC was used for these experiments Identical patterns of interaction were obtained when the enzyme was reconstituted with negatively charged phospholipids, or complex V was used instead of the preparation of Serrano et al [20] Experimental conditions were as described in Materials and Methods

Double-inhibition experiments

Since TFP and CPZ [15] as well as other phenothiazine derivatives [13] produce qualitatively identical inhibition patterns, these drugs were expected to interact with identical sites The lack of mutual competition observed in the photolabelling experiments led us to analyze the interaction between TFP and CPZ by means of inhibition experiments, using the double-inhibition technique of Yonetani and Theorell [35] Since this type of analysis applies only to linear inhibitors, phenothiazines were kept in a concentration range where they produce essentially linear inhibitory effects According to this method, one inhibitor is varied at different constant concentrations of the other inhibitor and the data are analyzed by means of the Dixon plot Parallel lines are obtained in case of mutually exclusive inhibitor binding, whereas nonexclusive inhibitors yield converging lines It has also been shown that, if at least one of the inhibitors is noncompetitive, the point of convergence lies on the abscissal axis in the absence of inhibitor–inhibitor interaction [36] Fig 7A shows that with TFP and CPZ, both noncompetitive inhibitors of mitochondrial ATP synthase [13–15], the lines converged near the abscissal axis, indicating weak antagonism between the two inhibitors When the interaction between TFP and fluphenazine was analyzed, nearly parallel lines were obtained (Fig 7B), consisent with a virtually exclusive binding of the two drugs The value of the inhibitor interaction factor, determined by fitting the initial velocity data to the appropriate equation [37], was 1 2 for the CPZ-TFP pair, whereas proved to be very large, 12.3, for TFP and fluphenazine Thus, the results of the double-inhibition analysis appear fully consistent with those of the photolabelling experiments and prove that phenothiazines of different hydrophobicities actually interact with different inhibitory sites

Discussion

The reversible interaction of local anesthetic drugs with membrane proteins has been recently characterized in detail by means of various techniques, including inhibition [6,14,15] and equilibrium binding studies [38], differential scanning calorimetry [4,7,39] and fluorescence [7,40] All these studies came to the conclusion that the protein component rather than the lipid matrix is the primary target of membrane-active drugs However, the exact localization of the drug binding domain(s), i e , on the peripheral or the lipid-embedded sector of membrane proteins, could not be unequivocally defined One approach to the identification of drug binding domains involves covalent labelling of the drug binding sites Phenothiazines are well suited for this purpose since, by virtue of their inherent photolability, they do not require introduction into the molecule of a photoreactive moiety which is likely to alter their binding properties and reduce their selectivity The fact that identical binding [18,19,41] and inhibition [15] patterns have been observed with either unmodified phenothiazines or their photogenerated radicals testifies to the selectivity of photoactivated phenothiazines

The photolabelling experiments presented in this report have shown that CPZ and TFP at concentrations lower than, or comparable to, their I_{50} values label numerous enzyme subunits They have also shown that these drugs do not interact exclusively with the peripheral F_1 moiety, but bind preferentially to the

membrane-embedded F_0 sector These data provide an explanation for the results of inhibition studies, which were compatible with the presence of multiple inhibition sites and showed that the intact F_0–F_1 complex was more sensitive than the isolated F_1 to the phenothiazine inhibitory effects [13–15]

Multiple binding sites for local anesthetic drugs have also been detected on the nicotinic acetylcholine receptor, where a population of 10–30 'low affinity' sites for CPZ has been established and tentatively localized at the protein/lipid interface [38] On the basis of fluorescence measurements, approx 60 binding sites on F_1-ATPase have been proposed for tetracaine [40] These observations led to the proposal of models in which the local anesthetic action is viewed as the result of the amphiphile interaction with a multiplicity of relatively aspecific, hydrophobic sites As a consequence of these interactions, small structural perturbations are produced which interfere with the normal functions of proteins [5,42] The present finding that CPZ and TFP produce qualitatively indistinguishable effects by interacting with nonidentical inhibitory sites constitutes a virtually conclusive evidence in favor of these ideas It should be noted that the concept of a relatively nonspecific hydrophobic binding has also emerged regarding the interaction of phenothiazines with calmodulin, which had been initially assumed to be very specific [43,44] With this protein, potentiation rather than competition has been observed between CPZ and TFP, at low drug concentrations (45)

The difference in binding sites observed in this study appears to be related to the different hydrophobicities of the two phenothiazines, as revealed by several observations (a) TFP, the more hydrophobic one, labels much more markedly than CPZ subunits 6-7 and 8-9 of F_0, which are identified as the proteolipid A6L and DCCD-binding protein [26,46,47], two membrane-embedded subunits of high hydrophobicity, (b) the hydrophilic free radical scavenger GSH prevents much more efficiently the labelling by CPZ than by TFP, (c) in either photolabelling or inhibition studies poor antagonism is observed between TFP and CPZ, whereas virtually complete mutual exclusivity is observed between TFP and fluphenazine, a phenothiazine derivative with hydrophobic character very similar to TFP Since various phenothiazine derivatives are used as phototype drugs for investigating the molecular targets of cationic amphiphilic agents, the observed diversities suggest that caution should be exerted in comparing the results of studies employing different phenothiazine derivatives The more hydrophobic TFP molecule appears highly preferable for photolabelling experiments, since it consents a much better resolution of the fluorographic peaks (cf Fig 2 and 3) However, it seems advisable that at least two phenothiazine congeners of different hydrophobicities be tested, before any generalization is made about the mode of binding of these drugs

In summary, this study has presented evidence that phenothiazines do not interact selectively with F_1, but bind to multiple sites located on both the peripheral F_1 moiety and the lipid-embedded F_0 sector of the enzyme It has also shown that the binding domains of such structurally and pharmacologically similar drugs as CPZ and TFP are not overlapping, but are strongly dependent on their hydrophobic characters These observations have been interpreted as indicating that the action of local anesthetic agents is the results of structurally nonspecific interactions with both the peripheral and the integral components of membrane proteins The methodological implications of the differential labelling by the two phenothiazines have also been discussed

Acknowledgements

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