Book-Chapter2: **Interaction of Picrylacetate with the mitochondrial F_1 – ATPase** (1982-3)

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Summary

0-acetyl picric acid (picrylacetate, PA) inhibits very effectively mitochondrial F_1 - ATPase activity. The inhibition is biphasic, the first phase to about 50 % ATPase inhibition being reversible by the treatment with dithioerythritol but not by treatment with bovine serum albumin. At this 50 % inhibiting concentrations, ^{3}H-acetyl group incorporation from ^{3}H-picrylacetate into the α-subunit being about 0.9-1.4 moles, into the ß-subunit about 0.65-0.7 moles and into the γ-subunit about 0.7-0.9 moles per mol F_1 - ATPase. The incorporation of these acetyl groups into the ß-subunit decreases in the presence of phosphate, arsenate or 4-chloro-7-nitrobenzofurazan. It is concluded, on these reasons, that a ß-subunit tyrosin at the phosphate binding site is acetylated. However, removal of these acetyl groups from the ß-subunit alone is not accompanied by higher ATPase activity. Reactivation of PA inhibited ATPase activity by dithioerythritol treatment requires removing of acetyl groups from all 3 subunits.

Picrate and 2,4-dinitrophenol stimulated ATPase activities are inhibited by PA.

In the presence of aurovertin or by cold treatment, acetylation of the α- and γ- subunits are raised concomitantly PA inhibited ATPase activity lowered. By contrast, in the presence of adenylylimidodiphosphate or by short exposure to low pH, acetylation of the α-subunit alone is increased but PA inhibited ATPase activity remains unchanged.

Abbreviations: PA, 0-acetyl-picric acid or picrylacetate; EDTA, ethylenediaminetetraacetate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NSPM, N`-(N``-n-nonyl-4-sulfamoylphenyl) maleimide; DNP, 2,4-dinitrophenol; FDNB, 1-fluoro-2,4nitrobenzene; FSBA, p-fluorosulfonyl-benzoyl-5`-adenosine; SDS, sodium dodecylsulfate; DCCD, dicyclohexylcarbodiimide; F_1-bovine mitochondrial F_1- ATPase.

Introduction

Picrylacetate (PA) has been described as uncoupling agent for rat liver mitochondrial preparations via released picric acid into the mitochondrial matrix [1]. Subsequent studies [2] showed that picric acid was a potent uncoupling agent for submitochondrial particles but not for mitochondria. Further studies on submitochondrial particles showed that picrate abolished very effectively energy-dependent spectral response of oxonol VI [3], suggesting that abolishment of membrane potential is responsible for uncoupling of submitochondrial particles by picrate. Recent results obtained with picrylacetate on mitochondria, submitochondrial particles and F_1 - ATPase [4, 5] show that this reagent is not only reacting via released picrate but also by its property as acetylating agent. Acetylation affects the energy transfer and utilization system of mitochondria and submitochondrial particles, while the primary action of released picrate seems to be on the ATPase molecule itself [6].

Earlier experiments on the F_1-ATPase, using ^{3}H-acetic anhydride acetylation, demonstrated incorporation of 5.2 moles acetyl groups per mole of F_1 - ATPase [8] without abolishing ATPase activity and the ability to bind to the mitochondrial membrane. Only extensive acetylation of the enzyme resulted in loss of ATPase activity and of its capacity to bind to F_0. Acetic anhydride is a powerful acetylating reagent for lysine residues [9] and thus may decrease salt bridges in enzymes by acetylation of these residues [10]. A more specific lysine reagent, 4-chloro-7-

nitrobenzofurazan (NBD-C1) has been explored [11] which inactivates F_1 - ATPase by reaction with a single ß-subunit lysine residue.
NBD-Cl first binds to a tyrosine residue with concomitant loss of ATPase activity, then it undergoes an intramolecular transfer to an adjacent lysine residue [12]. 1-Fluro-2,4-nitrobenzene (FDNB), a compound structurally similar to picrylacetate, seems to modify the same residues as NBD-Cl [13]. The adenine nucleotide analogue, p-fluorosulfonyl-benzoyl-5`-adenosine (FSBA) reacts with a different tyrosine residue on the ß-subunit[14]. The first indication for an essential amino group on the γ-subunit involved in ATPase activity has been obtained by use of glutaraldehyde modification [15].
The salicylate sensitive transfer of an acetyl group from acetylsalicylic acid to the ε-amino group of a lysine residue of human serum albumin (transacetylation) at a drug binding site has been demonstrated [16]. Because of a possible charge transfer interaction between the aromatic systems, nitrophenylesters are expected to modify tyrosine residues as well. Indeed, tyrosin acetylation at the primary binding site for small apolar anions in human serum albumin could be shown by use of p-nitrophenylacetate [17]. Picrylacetate can be expected to engage in stronger charge transfer interaction with other aromatic systems because of the three nitro groups present in the molecule. It has therefore been used in labeled and unlabeled form to specifically modify the F_1 - ATPase at lysine and tyrosine residues. Picrate, which can also interact with lysine residues, is released during the reaction with picrylacetate and therefore always present in the experiments. This introduces complications for elucidating the specific effects obtained by acetylation with picrylacetate. In these studies, therefore, experiments with picrylacetate were accompanied by those using picrate for comparison. In the present work, labeling of F_1 - ATPase with picryl-[3 H]-acetate (^{3}H-PA) and its sensitivity to ligand binding and conformational changes were studied.

Methods

Beef heart mitochondria were isolated by the method of Smith [18]. Beef heart mitochondrial F_1 - ATPase was prepared according to [19] as modified in [20]. The F_1 - ATPase has been stored at 2^0C as suspension in 50 mM Tris-sulfate, 1 mM EDTA, 2 mM ATP, pH 7.4, 60 % ammonium sulfate at a concentration of 3 to 6 mg protein/ml. Molar concentrations of purified F_1 - ATPase are based on a molecular weight of 380 000 [21]. The F_1-suspension was used as such without desalting procedure, since this procedure changed the activity of the F_1 preparation. Only F_1-preparations with ATPase activities above 50 μmol/min/mg of protein, detected in the spectrophotometric assay system, were used.
The protein concentrations of mitochondria and submitochondrial particles were determined as described [22]. F_1 - ATPase protein concentrations were measured by the method of Bradford [23] using bovine serum albumin as a standard.
Spectrophotometric measurement of initial ATPase activities were carried out at 20^0C by use of the ATP regenerating system described in [24]. The reaction medium contained 0.25 mM saccharose, 50 mM Tris-acetate, pH 7.4, 5 mM $MgCl_2$, 0.5 mM NADH, 2 mM phosphoenolpyruvate, 1.5 units/ml each of pyruvate kinase and lactate dehydrogenase, and an aliquot fraction of the F_1 - ATPase suspension (1 μ1). The reaction was started after two minutes with 5 mM ATP. The ATPase activities varied between 50 and 60 μmol/min/mg of protein.
Inorganic phosphorus released was determined at 20^0C as described in [25]. The reading has been made at 800 nm. The reaction medium contained 0.3 M saccharose, 25 mM Tris-sulfate

pH 7.4, 12.5 mM $MgCl_2$ and an aliquot of F_1- ATPase suspension (0.5 - 2 µl). The reaction was started after two minutes with 5 mM ATP. Reaction continued during a five minute period. The ATPase activities varied between 15 and 20 µmol/min/mg of protein in this assay system. The phosphate release measurement has been used because of the interference of picrate with the spectrophotometric assay.

Assays for ATPase activity inhibition or stimulation were carried out as follows. In the phosphate release measurements F_1- ATPase has been incubated in the assay mixture with the modifier for 2 or 30 minutes, if employed additional 2 minutes with a second modifier, before start of the ATPase reaction with 5 mM ATP.

^{3}H-Acetyl group labeling of F_1- ATPase has been performed as follows. 100 µg F_1- ATPase were treated in 1 ml phosphate assay mixture for 2 or 30 minutes with the modifiers before 2 minutes acetylation with ^{3}H-PA followed, then ATPase reaction was started with 5 mM ATP. ATPase reaction continued during a five minute period. A two minute dithioerythritol treatment has been carried out after acetylation reaction and before start with ATP. The modified enzyme has been precipitated by addition of solid $(NH_4)_2 SO_4$ to 60 % saturation. The protein gel pattern of the F_1 - ATPase treated with the various compounds did not change significantly. In some cases various amounts of intrinsic crosslinked products appeared, but the amount of the products was in most cases far below 1 % of the total protein put on the gel. The presence of NSPM gave rise to appearance of a 92 kDa product. A relatively high amount of crosslinked product was formed by treatment of F_1 with high PA concentrations at low pH or by 0.4 mM DCCD at pH 7.5-DCCD had no effect on ^{3}H-acetyl group incorporation into the F-ATPase under these conditions.

The overall radioactivity incorporation into the F_1- ATPase by 3H-PA treatment found in the gel-system according to [26] was lower as found in the acidic gel system used in this study.

The acidic SDS acrylamide gel electrophoresis was performed in analogy to [26] by using 1 % SDS in 0.2 M sodium acetate buffer, pH 5.0, and 10 % acrylamide. The gels had to stand about 2 hrs before they had solidified totally, then a prerun of 2 hrs was carried out. The electrophoretic mobility of protein standards was similar to the mobility as obtained in the gel system according to [26]. For electrophoresis in the acidic gel system, F_1 was solubilized in 0.2 M sodium acetate buffer, pH 5.0, 3 % SDS, 5 mM EDTA. The ^{3}H-radioactivity labeled samples were electrophoresed on duplicate gels. One gel was stained for protein with Coomassie blue and then scanned in a Zeiss gel scanner. The stained bands were cut out from the gels with a razor blade and dissolved for radioactivity counting. This method was especially useful, when two radioactivity labeled protein bands were running close together, as in the case of the α- and ß-subunits of F_1- ATPase. The second unstained duplicate gel was cut into 1 mm thick slices or frozen at $-2O^0C$ until cutting. The individual 1 mm slices were each dissolved in 0.5 ml of fresh 28 % H_20_2 by heating at 60^0C overnight in Zinsser-Polyvials. The radioactivity in each solution was measured in a Beckmann LS-250 scintillation counter after addition of 5 ml of dioxane containing 0.5 % 2.5-diphenyloxazole (PPO) and 10 % naphthalene.

For solubilization and counting of the stained bands 1 ml H_20_2 and 10 ml scintillation liquid has been used. Counting efficiency was 12 to 25 %. The exact amount of protein on individual gels could be measured with the following procedure. A calibration curve with a series of known protein amounts was made by cutting and weighing peaks from the protein gel patterns obtained by scanning on paper. The unknown amount of protein was then found by comparing peak weights. The method was especially useful in calculating the stoichiometry of 3 H-acetyl group binding to the F_1 - ATPase subunits.

0-acetyl-picric acid (PA) was prepared according to [1]. 0-[^{3}H]-acetyl-picric acid (3H-PA) was sythesized from ^{3}H-acetic anhydride (Amersham, 500 mCi/mmol) as follows. 64.6 mg picric acid (0.282 mmol) were warmed up to 56^{0}C, then a mixture of 25 µl acetic anhydride and 40 µl ^{3}H-acetic anhydride was added and stirred with a glass rod. After further addition of 1 µl 70 % perchloric acid, the mixture was kept for 5 minutes at 56^{0}C, then cooled to 0^{0}C and the product precipitated by addition of 1 ml ice-cold water. The product was purified by washing 3 times with 1 ml of 4 % ice-cold acetic acid and dried over anhydrous calcium chloride. The yield of ^{3}H-PA was 55 mg and the specific radioactivity was 22900 dpm/0.1nmol.PA is hydrolysed (Fig.1a) in buffered aqueous solutions above pH 7 in several minutes ($t_{1/2}$ ~ 6 minutes) to picrate and acetic acid. A 20 mM solution of PA in acetone was made shortly before performing experiments.

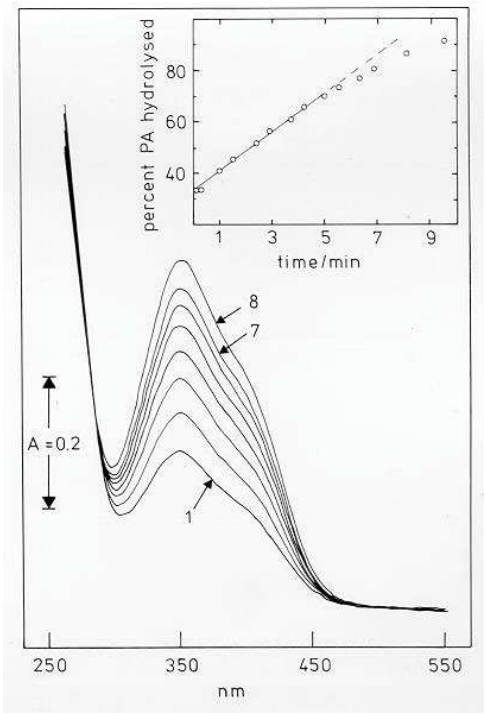


Fig.1. Kinetics of PA hydrolysis. 20 mM PA in acetone diluted to 40 µM with 0.25 M saccharose, 50 mM Tris-acetate, pH 7.4, 5 mM $MgCl_2$.pectra taken at 20^{0}C every 80 seconds (1) to (7), plus 10 mM KOH (8)..

Materials

De-fatted bovine serum albumin and NBD-CI were from Serva; nucleotides and AMP-PNP from Sigma; lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, ATP from Boehringer; picric acid from Merck; DNP from Eastman; aurovertin was a gift from R.B. Beechey; NSPM was synthesized essentially according to [27]. All other chemicals were reagent grade quality.

Results

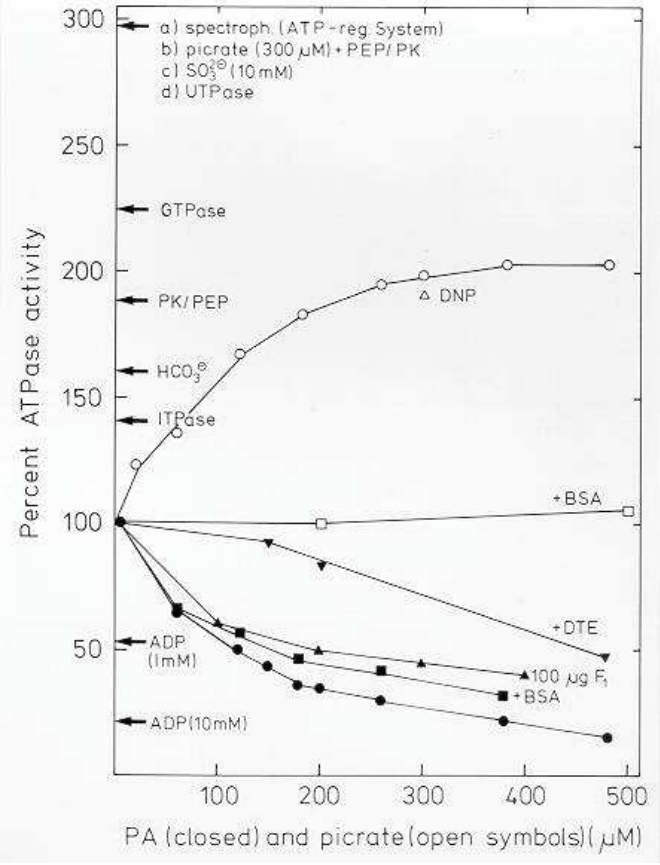
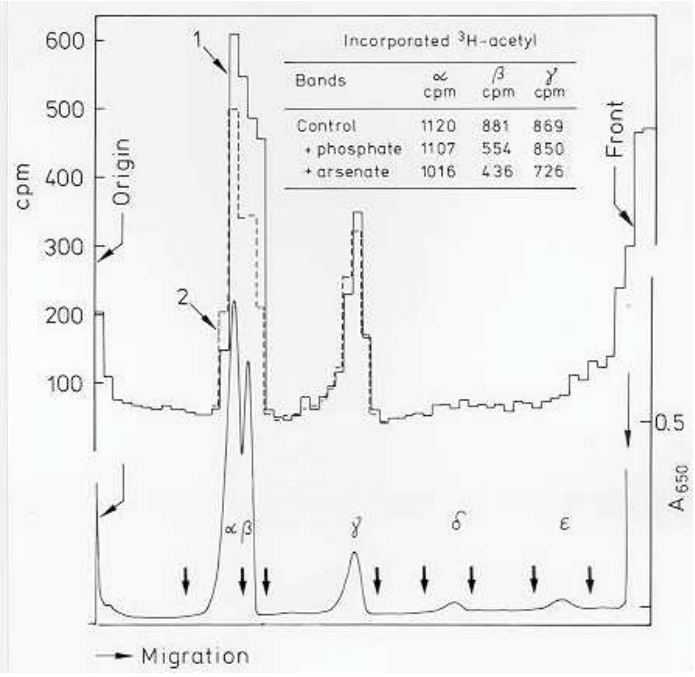


Figure 1 shows the inhibition of F_1- ATPase activity by treatment with various amounts of PA. The inhibition can be resolved into two phases: 45 to 55 % of the ATPase activity are lost at a concentration of 150 µM PA, while lowering of the residual ATPase activity requires much higher PA concentrations. The two phases are more clearly seen when dithioerythritol was added. The initial phase of the PA inhibited ATPase activity can be reactivated by dithioerythritol treatment to

Fig.1. Effect of PA and picrate on F_1 - ATPase activity. Released phosphorus was measured as described in Methods. F_1- ATPase, was treated for 2 minutes with various amounts of PA or picrate, and than 2 minutes with 5 mM dithioerythritol (DTE) after acetylation and before start of the reaction with ATP; 36 mg bovine serum albumin (BSA)was added together with ATP; treatment of 5 µg F_1 - ATPase protein, if not indicated otherwise. UTPase, GTPase or ITPase with 5 mM nucleotide, respectively; 2 minutes treatment with 20 mM HCO_3^-.

about 100 % ATPase activity, but not to the higher values expected from the presence of picrate. Treatment of modified ATPase with dithioerythritol has been shown to remove NBD-CL [12] or FDNB[13] from the tyrosine residues. The effect of dithioerythritol treatment on the acetylated ATPase confirms the expectation that tyrosine is also modified by PA. Picrate alone increased ATPase activity up to twofold in a pattern directly opposite to PA inhibition. The stimulating effect by picrate is lost upon bovine serum albumin addition. However, addition of bovine serum albumin to remove picrate, released from PA after acetylation, only slightly stimulates ATPase activity. These results demonstrate that covalent modification by PA takes place and that this modification is responsible for ATPase inhibition.



As shown in Figure 2, when ^{3}H-PA treated F_1 is electrophoresed on 10 % SDS-gels at acidic pH, incorporation of radioactivity can be found in the α, ß and γ- subunits. The radioactivity incorporation in the catalytic ß-subunit is phosphate and arsenate sensitive. At 200 µM ^{3}H-PA and 50 % ATPase activity, there is incorporation of 2.4 to 3.5 nmol ^{3}H-acetyl/mg F_1 - protein into the α-subunits, 1.7 to 1.9 nmol ^{3}H-acetyl/mg F_1-protein into the ß-subunits and 1.9 to 2.3 nmol ^{3}H-acetyl/mg F_1- protein into the γ-subunits (Fig. 3). This low amount of ^{3}H-acetyl group incorporated shows the specific acetylating power of PA in this system.

Fig.2. Incorporation of ^{3}H-acetyl group into the F_1-ATPase. 2 minutes incubation of F_1-ATPase protein with 200 µM ^{3}H-PA. 1)control 2)in the presence of 20 mM phosphate. The arrows indicate razor blade cuttings. The inset shows the amount of radioactivity determined in cut stained gel bands. Pretreatment with 20 mM arsenate or phosphate.

In Fig. 3 are different F_1 - ATPase samples labeled. F_1 stored for three months at 4^0C in$(NH_4)_2SO_4$ showed some lowered ATPase activity, and higher incorporation of radioactivity appeared on ^{3}H-PA treatment with 50 % inhibiting concentration (200 µM) into the α- and γ-subunits, a result which was obtained also with fresh ATPase preparations having lower specific ATPase activity. However, label incorporation into the ß-subunit did not change significantly. Treatment of the ATPase with 50 % inhibiting concentration of ^{3}H-PA at pH 6 for two minutes changes label incorporation into the α-but not into the ß-and γ- subunits (Fig. 3).

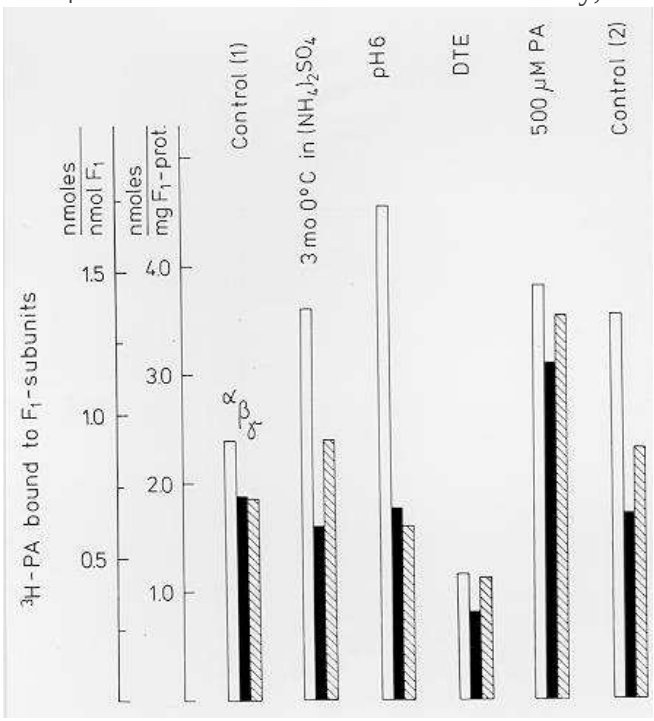


Fig. 3. Incorporation of ^{3}H-acetyl group into the α, ß and γ-subunits of F_1- ATPase. 2 minutes incubation of F_1- ATPase protein with 200 µM ^{3}H-PA, if not indicated otherwise; treatment with 5 mM DTE after ^{3}H-PA incubation; labeling for 2 minutes at pH 6.0. Control (1) ATPase activity 20 µmol phosphorus released/min/mg, 14 µmol phosphorus

released/min/ mg after storage for 3 months at O^0C in $AmSO_4$; control (2) ATPase activity 17 µmol phosphorus released/min/mg at 20 ^0C.

Radioactivity incorporation into the α-subunit almost doubles, while no change in ATPase inhibition appears, if assayed at pH 7.5 direct after acidic PA treatment. The above results may be taken as an indication that labeling of the ß-subunit is responsible for ATPase inhibition. However, DTE treatment of labeled ATPase does remove radioactivity from all 3 bands to about 60 %, although, the ß-subunit labeling seems to be most sensitive (Fig. 3). At 500 µM ^{3}H-PA the label incorporation in α, ß and γ is increased by 60, 64 and 90 % respectively (Fig. 3), but the ATPase activity is inhibited only to about 70 % (Fig. 1). This result shows that PA labeling becomes unspecific at higher concentrations.

Because of the apparent specific labeling of the F_1 - ATPase obtained by ^{3}H-PA treatment, detection of ligand induced structural changes and competition experiments for binding sites appeared possible with this compound. A conformational change of the enzyme might expose new ^{3}H-PA sensitive residues, while competing ligands would decrease the number of reactive side chains. Before performing this kind of study the specific interaction of the ligands with the F_1- ATPase has been explored.

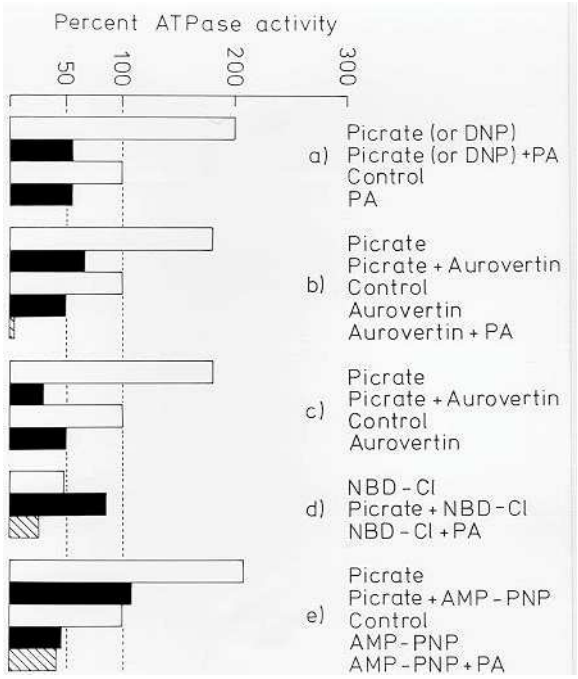


Fig. 4. ATPase activity of F_1- ATPase in the presence of inhibitors and uncouplers obtained by measurement of phosphate released. Treatment for 2 minutes with 300 µM picrate or 300 µM DNP, before addition of 120 µM PA. 2 minutes reaction with PA. Treatment for 30 minutes with 3 µg/ml (b) or 12 µg/ml (c) aurovertin, 0.4 mM NBD-CL or 30 µM AMP-PNP, either before or after addition of 120 µM PA or 300 µM picrate. 2 minutes reaction with PA or picrate. Treatment in the indicated order.

Figure 4 shows the effect of combinations between picrate or PA and different ATPase modifiers on F_1- ATPase activity. The data are obtained under the actual condition of ^{3}H-PA-labeling to facilitate comparisons between ligand modified enzyme activity and ^{3}H-acetyl group incorporation in the F_1-ATPase. PA eliminates the stimulatory effect of picrate and DNP (Fig.4a). There is additive abolishment of ATPase activity by the combinations of PA and aurovertin (Fig.4b), higher abolishment by PA and NBD-CL combinations (Fig.4d), but not by PA and AMP-PNP combinations (Fig.4e). Thus 50 % inhibiting concentrations of each aurovertin and PA result in almost 100 % inhibition of ATPase activity. In contrast, PA seems to act independent from the presence of AMP-PNP, since the ATPase activity obtained by combinations of

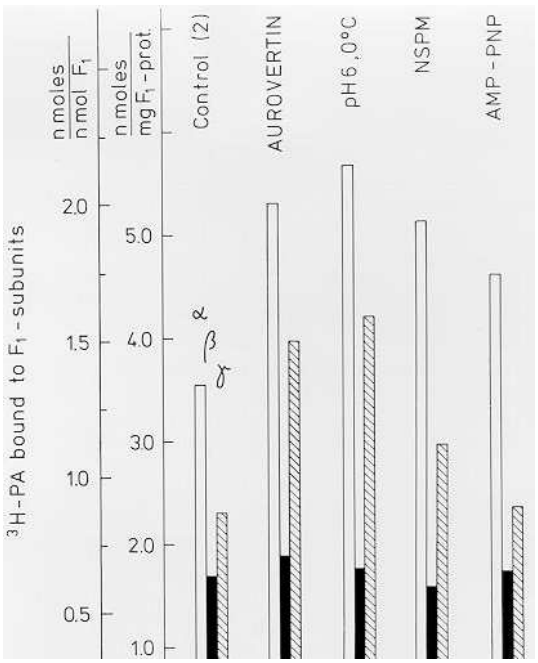


Fig. 5. Incorporation of ^{3}H-acetyl group into the α, ß and γ- subunits of F_1 - ATPase. 2 minutes incubation of F_1- ATPase protein with 200 µM ^{3}H-PA, in the presence of 10 µg aurovertin, 100 µM AMP-PNP, 0.4 mM NSPM. 30 minutes preincubation with aurovertin, AMP-PNP,

NSPM. F_1 for 30 minutes at 0^0C in 50 mM Tris-sulfate, pH 6.0, cold inactivated before PA addition.

AMP-PNP and PA remains at the PA inhibited value. Picrate potentiates the inhibition at higher aurovertin concentrations (Fig.4b,c), while picrate and NBD-CL (Fig.4d) or picrate and AMP-PNP (Fig.4e) have no such effect. Aurovertin acts therefore (in contrast to NBD-C1) similarly on the picrate stimulated and the PA inhibited ATPase.

Fig. 5 and 6 show the effect of ATPase modifiers on ^{3}H-acetyl group binding to the F_1 - ATPase. Labeling of the ATPase with ^{3}H-PA shows that acetylation of the α- and γ-subunits are increased in the presence of aurovertin, the lipophilic maleimide NSPM and after cold treatment (Fig. 5). AMP-PNP (Fig.5) and, as shown above, pH change (Fig. 3) increase only the acetylation of the α-subunit. In the presence of aurovertin are 1.7 nmoles ^{3}H-acetyl/mg F_1-protein, after cold treatment about 2 nmoles ^{3}H-acetyl/mg F_1-protein more into each the α-and γ-subunits taken up. In the presence of AMP-PNP on the other hand, are about 1.1 nmoles ^{3}H-acetyl/mg F_1-protein more into the α-subunit alone incorporated by PA labeling. It is interesting that the different effects of aurovertin and AMP-PNP on ^{3}H-acetyl group incorporation into the F_1- ATPase subunits are analog to the ATPase inhibition characteristics obtained by combination of aurovertin or AMP-PNP and PA, described in Fig. 4.

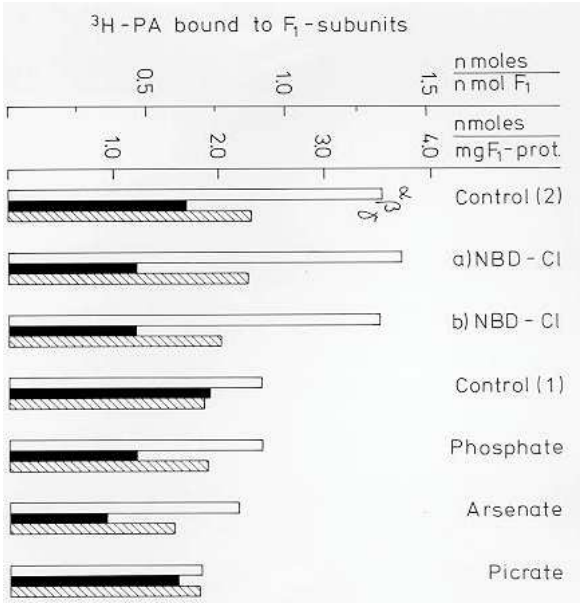


Fig. 6. Incorporation of ^{3}H-acetyl group into the α, ß and γ-subunits of F_1- ATPase. 2 minutes incubation of F_1- ATPase protein with 200 μM ^{3}H-PA in the presence of 50 μM NBD-Cl (a), 500 gM NBD-Cl (b), 20 mM phosphate, 20 mM arsenate or 200 μM picrate. 30 minutes preincubation with NBD-Cl. 2 minutes preincubation with phosphate, arsenate or picrate.

NBD-CL which inhibits ATPase activity by reaction with a ß-subunit tyrosine residue [12] inhibits acetyl group incorporation into the ß-subunit (Fig. 6) by about 30 %. A similar inhibition (40-50 %) could be obtained by use of phosphate or arsenate (Fig. 2, 6). Phosphate decreases also the rate constant for the specific labeling of F_1 - ATPase by NBD-CL [281 and phosphate is suggested to bind at the ß-subunit of F_1 [29]. But in the present study, lowering of ß-subunit labeling by phosphate (or arsenate) is not accompanied with reactivation of ATPase activity as in the case of dithioerythritol treatment.

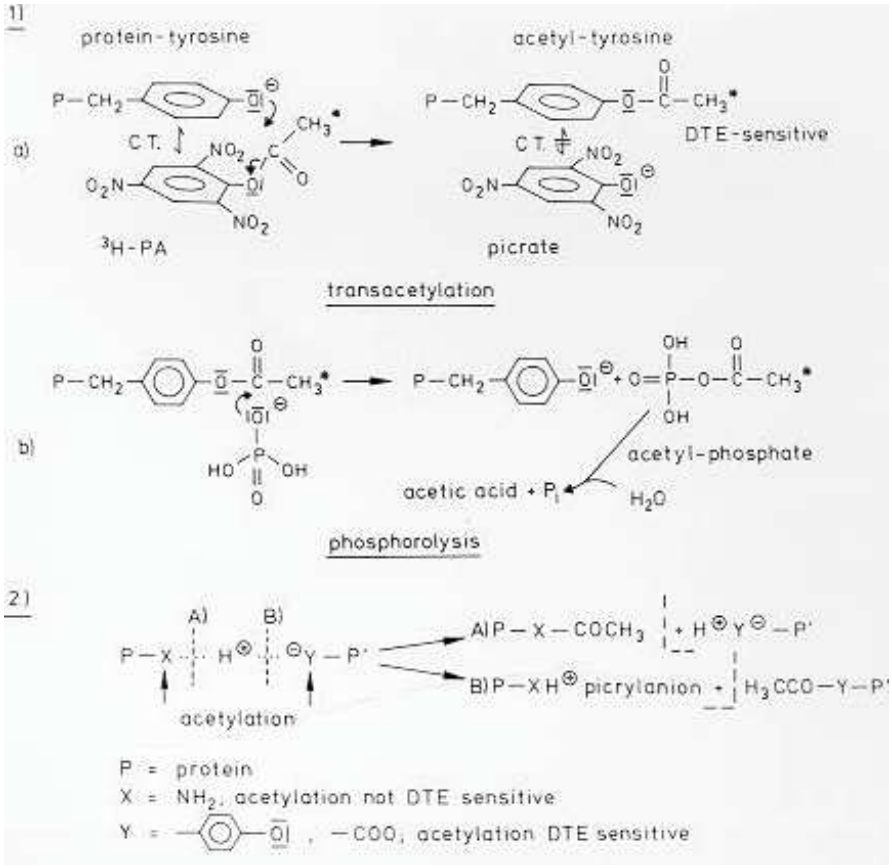
In contrast to NBD-C1, phosphate and arsenate, does picrate not have much effect on ß-subunit acetylation, while acetyl group incorporation into the α-subunit is lowered by about 25 % in the presence of picrate (Fig. 6).

Discussion

As has been discussed for uncoupler interaction with mitochondrial ATP synthesizing machinery [l, 7] the amine reagent picrate (or DNP) binds most likely specifically to a receptor site in the F_1 -

ATPase, such as the ε-amino group of a lysine residue. This binding may result in ATPase activation. Picrate released during the reaction of PA may, of course, also result in ATPase activation. However, the acetylation reaction by PA prevents this effect, when it inhibits ATPase activity and the capability to be stimulated by picrate (Fig. 1). From the structure of PA one can

infer that this compound may be a good acceptor in charge transfer interaction, and therefore able to react with aromatic donor groups such as tyrosine residues with increased specificity (scheme I). A number of other acetylating reagents such as acetic anhydride [8] , acetylsalicylate and acetylimidazole do not inhibit F_1-ATPase activity (not shown). Thus, the charge transfer interaction of PA with other aromatic residues may in fact be an important factor for inducing specific acetylation and inhibition of the ATPase activity. That tyrosine may be acetylated is supported by the fact that ATPase activity is increased by partial removal of acetyl groups with dithioerythritol (Figs. 1, 3 and scheme I), a treatment used to remove NBD-CL [12] and FDNB [13] from the modified tyrosine residue and to reactivate ATPase activity. The sensitivity of acetylation of the ß-subunits against NBD-CL (Fig. 6) is further evidence for the modification of a tyrosine residue. The incorporation of acetyl groups into the ß-subunit is not only sensitive against dithioerythritol or NBD-C1, but also to phosphate and arsenate Figs.2,6) possibly by a mechanism as shown in scheme I. In this scheme, the monoanion of phosphate is assumed to react with the acetyltyrosine group forming acetyl-phosphate, which then becomes hydrolysed by water. The assumption is supported by the finding, that the monoanion of phosphate binds to F_1 [30]. The only partial sensitivity against dithioerythritol, NBD-C1, phosphate or arsenate could be due to modification of lysine adjacent to tyrosine, as has been found in the interaction of NBD-Cl and F_1 - ATPase [11]. 4-azido-2-nitrophenyl-phosphate (ANPP) has been proposed to be a phosphate analog, because it binds at the phosphate binding site on the ß-subunit and leads to F_1 - ATPase inactivation [29]. It is possible that PA binds to the same site as NBD-Cl or ANPP and that this site is identical with the phosphate binding site as shown by the sensitivity against phosphate or arsenate. The sensitivity toward phosphate is also shown for NBD-Cl binding [28]. The acetylation of the ß-subunit by PA may then induce ATPase inhibition as found in the case of NBD-Cl or ANPP modification. However, since PA labels not only the ß-subunit, but modifies the α, ß and γ- subunits to almost equal extent (Fig. 2), it is difficult to ascribe the inhibition of ATPase activity to the labeling of only one F_1 - subunit. Indeed, reactivation of PA inhibited ATPase activity requires removing of acetyl groups from the modified α, ß and γ-subunits.



Picrate competes with PA for the α-subunit (Fig. 6). As mentioned above, picrate is able to form an ionic bond with lysine residues, but interaction with tyrosine residues is not likely. Therefore there is the possibility that PA reacts with a picrate sensitive lysine residue on the α-subunit.
This would be comparable to the salicylate sensitive transfer of an acetyl group from acetylsalicylate to lysine [16] . As in the case of the other subunits (ß and γ-), the incorporation of label into the α-subunit is partly dithioerythritol sensitive. Thus, tyrosine residues or less likely another group adjacent to lysine apear to be modified as well.
The interaction of picrate with the F_1 - ATPase leads to higher ATPase activity (Fig. 1). This picrate stimulated ATPase activity is totally eliminated by PA (Fig. 4). If reaction of PA with the ß- or γ- subunits would be responsible, higher labeling of these subunits by PA (as without picrate) would be obtained, this is not the case. Also, the reaction of PA with the picrate binding site on the α-subunit cannot be responsible, because the incorporation of label is decreased by picrate. This labeling behaviour on the various subunits suggests another mechanism for PA inhibition of picrate stimulated ATPase activity.
Some data obtained in this study can readily be explained with the assumption of different conformational states of the ATPase proposed by others [31-35] .
Fluorescence changes of bound aurovertin were interpreted in terms of changes in conformational state of the ATPase [36] In the presence of this antibiotic, labeling of the F_1-ATPase by ^{3}H-PA results in the incorporation of about one additional ^{3}H-acetyl group into the α- and γ- subunits, respectively (Fig. 5). Under these conditions the enzyme is nearly fully inhibited (Fig.4). The inhibition by aurovertin or PA alone is limited to about 50 %. This kind of additive inhibition implies the presence of two distinct enzymes or sites [37].
The labeling behavior is compatible with two distinct conformations of the enzyme complex. Since aurovertin has been shown to bind to the ß-subunit [38] the induced conformational state may be due to changes in structure of the ß-subunit by aurovertin exposing PA-sensitive residues on the α- and γ- subunits. The aurovertin induced conformational state may also be obtained by limited cold treatment under acidic conditions (Fig. 5) or cold storage in $(NH_4)_2SO_4$ (Fig. 3). Temperature induced conformational changes as reason for the loss of enzymatic activity in cold treated ATPase has already by considered [39] .
The binding of aurovertin to F_1 at a site distinct from the catalytic site [12] has been described to have no effect on the stoichiometry of AMP-PNP binding at the catalytic site [40] . In contrast to aurovertin, AMP-PNP and PA or picrate do not inhibit additively or in an enhanced fashion (Fig. 4). The ATPase activity remains at the PA inhibited value. The differences between aurovertin

and AMP-PNP in combination with picrate or PA is also reflected in different labeling patterns. Only labeling of the α-subunits is sensitive toward AMP-PNP treatment, resulting in about 0.5 more binding sites for PA (Fig. 5). This labeling behavior indicates then another state of the ATPase as the one induced by aurovertin. The AMP-PNP induced conformational state may also be obtained at acidic pH (Fig. 3). In accordance, the ATPase activity remains at the normal PA inhibited value, as in the case of AMP-PNP treatment. Lowering of ATPase activity below the PA inhibited value (by combination with aurovertin) involves also the γ- subunit. The involvement of the γ-subunit in ATPase reaction was already suggested [l 5] .
The results are indication of 3 different conformational states of the F_1 - ATPase detected by PA, whatever the mechanism of inducing these states may be: 1) The normal PA-, NBD-Cl-sensitive state, 2) an AMP-PNP or acidic pH induced state and 3) an aurovertin or low temperature induced state.

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BIOCHEMISTRY

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Manuscript No. → BI830558X 2 ← Reviewer No.

Corresponding Author → Reinhold . Kiehl

Abbreviated Title → Interaction of Picryl Acetate With The...

PLEASE RETURN SIGNED ORIGINAL AND ONE ANONYMOUS COPY

COMMENTS:

In this article, the author reports about (^{3}H)acetyl incorporation into F_1-ATPase, using picryl acetate as donor of acetyl group. The three major subunits (α, β, γ) were labeled. This is not the first attempt to radiolabel F_1. Picrylacetate was used because it was expected to be engaged in strong charge transfer interactions with aromatic rings in the protein. Interestingly the labeling of the β subunit was decreased by P_i, and Nbd chloride. This is the only understandable part of the manuscript. The remaining part is an accumulation of data concerning the effects of mixtures of inhibitors from which it is difficult to draw any sound conclusion. The author, however, speculates on these results and the problems discussed are for remote from the object of the report. As an example, the slight modification of the labeling of the α subunit by picrylacetate by the different ligands tested is meaningless in view of the fact that a shift of 1 unit pH doubles the amount of picrylacetate bound to α.

Other points :

p. 8 assays of ATPase : difficult to understand. How the author proceeds to determine P_i ? Is it on a perchloric extract ?

p.17, line 15 : "steps in inhibition of activity appear with azide and AMPPNP at 50% etc..." Are the steps found systematically or is it fortuitous ?

p.19, line 4 : The inhibition by P_i and arseniate of the labeling of F_1 by picrylacetate is not 60%, but between 30 and 50%.

Legends to the Fig. : in general difficult to understand. As an example in the legend of Fig. 4, three lines from the bottom "ATPase activity 20 µmol, 14 µmol after storage ? (does the author mean µmol/min/mg of F_1 ?).

Figure 1 : from this figure, it is impossible to have a clear idea of the results.

Figures 5 and 6 are without interest.

In conclusion, in the present form, the manuscript cannot be accepted.

COMMENTS:

It is recommended that this manuscript not be accepted for publication.

It is obvious from the amount of work presented that the investigator has expended considerable effort in collecting a number of interesting observations. However, the study suffers seriously from a lack of focus and from the absence of clear unambiguous conclusions. In addition, the manuscript is poorly written with many abrupt transitions in thought.

Biochemistry

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May 23, 1983

Dr. Reinhold Kiehl
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Dear Dr. Kiehl:

Thank you for sending us the manuscript entitled "Interaction of Picryl acetate with the mitochondrial F_1-ATPase". As usual, the manuscript was examined by two independent reviewers who are knowledgeable in this field. Their relevant comments are enclosed.

Both reviewers recommended that publication of the manuscript be declined and we must regretfully concur with their negative recommendation. We are sorry to convey this decision to you, but we should like to thank you for having given us an opportunity to consider your manuscript.

Sincerely yours,

William Parson

William W. Parson
Associate Editor

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Enclosures