DODECYLSULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS AT ACIDIC pH SUITABLE FOR DETECTION OF LABILE PROTEIN BOUND FUNCTIONAL GROUPS. (1982)

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Running title: **Gel electrophoresis at acidic pH**

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Abstract

The SDS-po1yacrylamide gel electrophoresis of Weber and Osborn (1969, J.Biol.Chem. 244, 4406-4412) has been modified in order to make it suitable for detection of labile protein-bound functional groups, which include sulfenyl-phosphate, acyl-phosphate, tyrosine-phosphate, as well as acetyl-tyrosine. The introduced acetate-buffer, pH 5.0, for electrophoresis and protein solubilization, gave the best results. The electrophoretic mobility as plotted against the logarithm of the known polypeptide chain molecular weights results in a straight line between Mr = 120 kDa and 7.5 kDa. Since the protein samples have to be solubilized without the use of nucleophilic compounds, which include disulfide reducing agents, the method is also suitable for detection of intrinsic disulfide crosslinked subunits in multienzyme complexes. Herein, beef heart mitochondrial F_1- ATPase has been studied, where thiol/disulfide interchange may play an important role in the control of activity. Some reagents, modifying activity, change also the pattern of appearance of crosslinked subunits. If no crosslinking across other functional groups is present, the method may generally be used to look under various conditions also at thiol/disulfide interchange in enzyme complexes.

Introduction

During the course of studying the interaction of the high energy compound picrylacetate with the mitochondrial energy transfer and utilization system, it was necessary to develop a suitable method for detection of proteins acety1ated by 3H-picrylacetate, since the label incorporated as ^{3}H-acety1 proved to be sensitive toward nucleophiles and pH changes (2). No suitable method is described in the literature. ^{32}P-Phosphoprotein has been demonstrated on a 5 % SDS-po1yacrylamide gel system with phosphate-buffer, pH 6.0, but the conditions used are not described. Convincing proof that $^{32}P_i$ is covalent1y attached to an acyl-group has also not been shown (3). Since the ^{3}H-acetyl label introduced in the protein sample is sensitive toward phosphate ions, phosphate buffer could not been used. Protein precipitation with trichloroacetic acid needed addition of 1 N NAOH for dissolving of the pelleted protein sample, therefore another milder treatment of the modified protein sample was necessary. This has been achieved by precipitation with $AmSO_4$ and resolubilization in acidic SDS-sample buffer, without the use of dithiol reducing agents. The absence of dithiol reducing agents may pose problems in assaying the monomeric subunit structure of enzyme complexes. Thiol/disulfide interchange may play an important role in control of mitochondrial (BF_1)and chloroplast ATPase (CF_1) (2,4,5,6). The ATPase was therefore the ideal system to look at this problem. It is possible to demonstrate that the new gel system is not on1y useful to detect labile protein-bound functional groups, but also to assay changes in appearance of intrinsic disulfide crosslinked subunits. The method may also be

used to look at intrinsic crosslinking across other functional groups, e.g. structures like P-$X^{\delta+}$~$Y^{\delta-}$-P`, which may be stable at acidic pH.

Material and Methods

Chemicals: Acrylamide, N,N'-methy1enebis(acrylamide), N,N,N',N`-tetramethy1ethy1ene-diamine, and ammonium persulfate used to make the po1yacrvlamide gels were electrophoretic grade and obtained from Serva, as was Coomassie brilliant blue (R-250) or sodium dodecylsulfate. Bromphenol blue was obtained from Merck, all other chemicals used were of the highest purity available. ^{3}H-picrylacetate (^{3}H-PA), picry1acetate (PA) and N-(N'-n-nonyl-4-sul-famoy1phenyl)maleimide (NSPM) were synthesized as described elsewhere.

Proteins: Beef liver catalase, horse heart cytochrome c were from Boehringer Mannheim, rabbit skeletal muscle phosphorylase kinase and rabbit skeletal muscle sarcoplasmic reticulum (SR)-ATPase were Gifts from Dr. M. Varsanyi, complex V was prepared from beef heart mitochondria as described (7), BF 1 was purified according to(8,9).

Preparation of protein solutions: The proteins were incubated at 20^{0}C for 30 minutes, with occasional shaking, in 0.2 M sodium acetate buffer, pH 5.0, 3 % in SDS, and 5 mM in EDTA. Hardpacked pelleted protein samples were allowed to stand overnight at 20^{0}C in this sample buffer.

Preparation of gels: Gel buffer, pH 5.0, contained 296 ml 0.2 M acetic acid, 704 ml 0.2 M sodium acetate, 10 g of SDS per liter. For the 10 % acrylamide solution, 22.2 % (w/v) of acrylamide and 0.6 % (w/v) of methylenebisacrylamide were dissolved in gel buffer. The solution was kept at 4^{0}C in a dark bottle. The glass gel tubes were 12 cm long with an inner diameter of 7 mm. Before use they were soaked in cleaning solution, boiled in 10 % nitric acid, rinsed, and oven-dried. For a typical run of 12 gels 20 ml of gel buffer were mixed with 18 ml of acrylamide solution. After deaeration, 2 ml of freshly made ammonium persulfate solution (25 mg per ml) and 40 µ1 of N,N,N',N'-tetramethy1ethylenediamine were added. After mixing, each tube was filled 10 cm high. Before the top of the gel hardened. a few drops of water were layered above the gel solution. The gel had to stand about 2 hrs before it had solidified totally. After that, the water layer was removed and the gels were electrophoresed in gel buffer for about two hours at room temperature (about 20^{0}C). Applied was a constant current of 4 ma per gel with the positive electrode in the lower chamber.

Preparation of samples: The gel buffer was removed from the upper compartment of the electrophoresis apparatus and from the top of the gels. 10 to 50 µ1 protein solution, 10 µl tracking dye (0-05 % Bromphenol blue in water) and 1 drop of glycerol were layered on the top of the gel. After mixing by shaking the gel tube, the gel buffer from the prerun was carefully layered on top of each sample to fill the tubes. Then the upper compartment of the electrophoresis apparatus was filled with the gel buffer from the prerun. Electrophoresis was performed at a constant current of 3 ma per gel with the positive electrode in the lower chamber, overnight at room temperature until the marker dye was about 1 cm from the end of the gel tubes (about 14 hrs). Runs at a higher current, up to 8 ma per gel, are possible in about 3 to 4 hrs. After electrophoresis, the gels were removed from the tubes by squirting water from a syringe which had been fixed with a high pressure tubing to a water supp1y in order to exert pressure. The distance moved by the dye was marked with a needle.

Determination of radioactivity labeled protein bands: The ^{3}H-radioactivity labeled samples were electrophoresed on duplicate gels. One gel was stained for protein 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, if two radioactivity labeled protein bands were running close together, as in the case of the α- and ß-subunits of BF_1- ATPase. The second unstained duplicate gel was cut into 1 mm thick slices or frozen at -20^0C 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 18 to 20 %.

Staining and destaining: The gels were fixed and washed 3 times 1 hr in 25 % isopropanol, 10 % acetic acid (v/v) to remove SDS, at this step scanning for protein in the UV-region is possible. Gels were stained 5 hrs with 0.035 % Coomassie blue (w/v) in 25 % isopropanol and 10 % acetic acid, followed by additional 5 hrs with 0.0035 % Coomassie blue in 10 % isopropanol, 10 % acetic acid (at this stage the bands are already visible) and destained with 7 % acetic acid, 5 % methanol. The gels were kept at 7 % acetic acid solution.

Amount of protein: Protein was determined by the method of Lowry et al (16). Usua11y 5 µg protein per protein-band was applied per gel, but also a lower amount could be detected. Higher amounts up to 20 µg were used if the radioactivity to be counted was expected to be low. 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 gels had been treated exactly the same way. The method was especially useful in calculating ^{3}H-acetyl group binding to BF_1-ATPase subunits.

Results and discussion

Figure 1 shows examples of the separation of various protein samples. It may be seen that there is the same separation on these acid gels as normally obtained in neutral or slightly basic gels. In each case a single protein band with an electrophoretic mobility similar to the one in Weber-Osborn SDS-gels (W/O-gels) was observed. In beef heart ATPsynthetase (CV) part of the 30 kDa hydrophobic protein (HP) (17) is obviously polymerized at the top of the gel, the relative amount of the 30 kDa proteins is lowered as compared to the W/O-gels (not shown) and the R_f -value of residual HP becomes identical to the value for the γ-subunit, as can be seen. This polymerization behavior does not appear

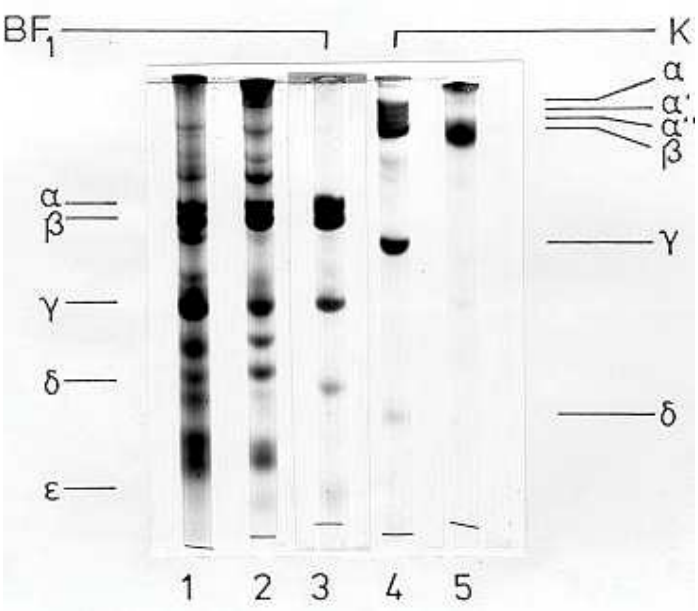


Figure 1. Electrophoretic separation of various protein samples. 1)90 µg beef heart submitochondrial particles, 2)40 µg OS-ATPase (complex V), 3) 30 µg -beef heart

mitochondrial F_1-ATPase (BF_1). 4) 20 µg rabbit skeletal muscle phosphorylase kinase (K), 5)10 µg rabbit skeletal muscle sarcoplasmic reticulum (SR)-ATPase.

in submitochondrial particles (SMP) and may be due to the changed surrounding of this protein and the low pH used. As in the case of the Laemmli gels (14,18) the membrane proteins (M_1 and M_2) are split into two separate peaks and the mobility of M_2 becomes identical with OSCP. This abnormal behavior of M_2 in the various gel systems is also seen sometimes in W/0-gels if the pH is only slightly changed (17). HP, M_2 and proteolipid are all integral membrane proteins which show high degree of hydrophobicity and low content of charged groups. The folding in this proteins is expected to be totally different from the normal soluble globular protein foldings. From this property it is obvious that the lipophilic proteins are forming different complexes with SDS and that the molecular weights estimated from the gels have to be judged with caution.

The physiological mixture of white and red muscle phosphorylase kinase shows in this gel system a splitting of one of the α- or α`-subunits into 2 distinct subunits, a phenomenon which has been discussed also for the ß and γ-subunits (19). A similar splitting could be observed occasionally for the α-subunit of BF_1 - ATPase.

Table I. Proteins studied by SDS-electrophoresis:

Protein	Mol wt. of po1ypeptide chain	Reference
Phosphorylase Kinase:		10, 11
K-α	118 000/145 000	
K-ß	108 000/128 000	
K-γ	43 000/ 45 000	
K-δ	17 000	12
SR-ATPase	100 000	13
Catalase	60 000	
BF_0F_1 (BF_1):		14, 15
α (BF_1)	58 000	
β (BF_1)	52 000	
γ (BF_1)	31 000	
HP	31 000	
M_1	23 000	
M_2	19 000	
OSCP	19 000	
δ(BF_1)	15 000	
DCCD-binding peptide	10 000	
ε(BF_1)	7 500	
Cytochrome c	12 500	

If relative migration is plotted against the log of the molecular weight (table I), a straight line can be fitted extending from molecular weights of 7.5 kDa to at least 100 kDa (Fig 2). If one takes the molecular weight for phosphorylase kinase as measured in (10), then a straight line can be drawn up to 120 kDa. Otherwise, since it is not likely, that a break in the curve appears at 100 kDa it might still be possible to extend the line to 120 kDa. The δ-subunit of the phosphorylase

kinase, calmodulin (12) does not fit into the curve. This may be due to proteolytic degradation at the low pH (19) or due to bound Ca^{2+} , which may change mobility behavior by a Ca^{2+} induced structure followed by different SDS binding (20).

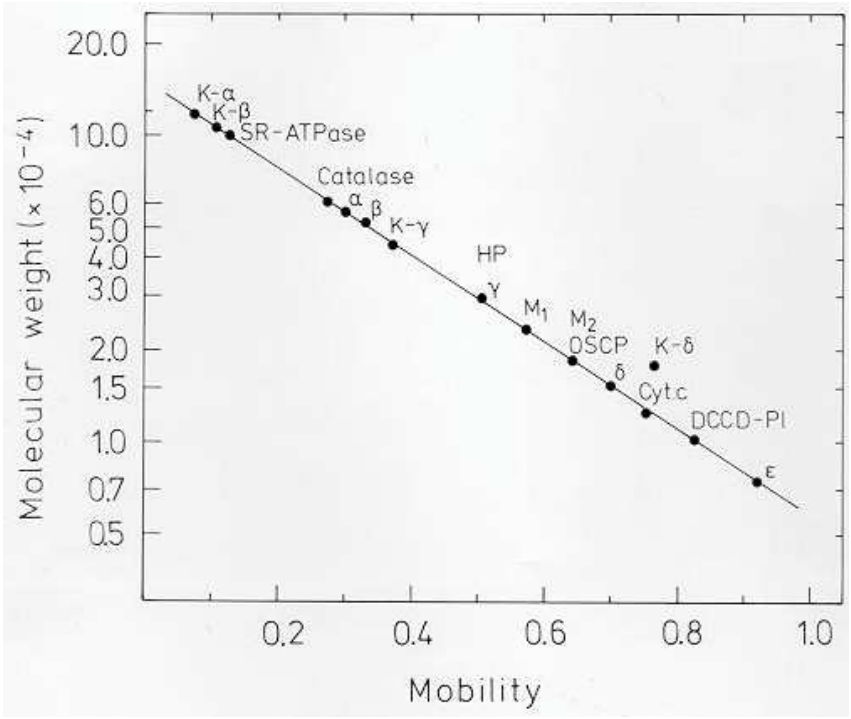


Figure 2. Logarithmic plot of the polypeptide molecular weights against electrophoretic mobility. Protein molecular weights are taken from Table I. K-α to K-δ = subunits of the phosphory1ase kinase, α to ε = subunits of the BF_1-ATPase, HP = hydrophobic protein, M_1 and M_2= membrane proteins, cyt. c= cytochrome c, DCCD-PL = DCCD-binding peptide.

Figures 3 and 4 show patterns of beef heart F_1-ATPase (BF_1) changed under the influence of various modifiers of ATPase activity. BF_1 has been labeled with ^{3}H-acetyl groups using ^{3}H-PA to determine the conformation of BF_1 under the influence of activity modifiers (2). As can be seen, there is appearance of various amounts of intrinsic crosslinked products. The amount of the products is in most cases far below 1 % of the total protein put on the gel, but nevertheless the products as induced by the modifiers can be differentiated. Sulfite apparently lowers the amount of the crosslinked products, which is to be expected from its reaction with disulfide bridges. In contrast, the presence of azide gives rise to high amounts of a 126 kDa products. With both compounds a 40 kDa product is obtained. Sulfite is able to open up dithiols and modify free thiols. The

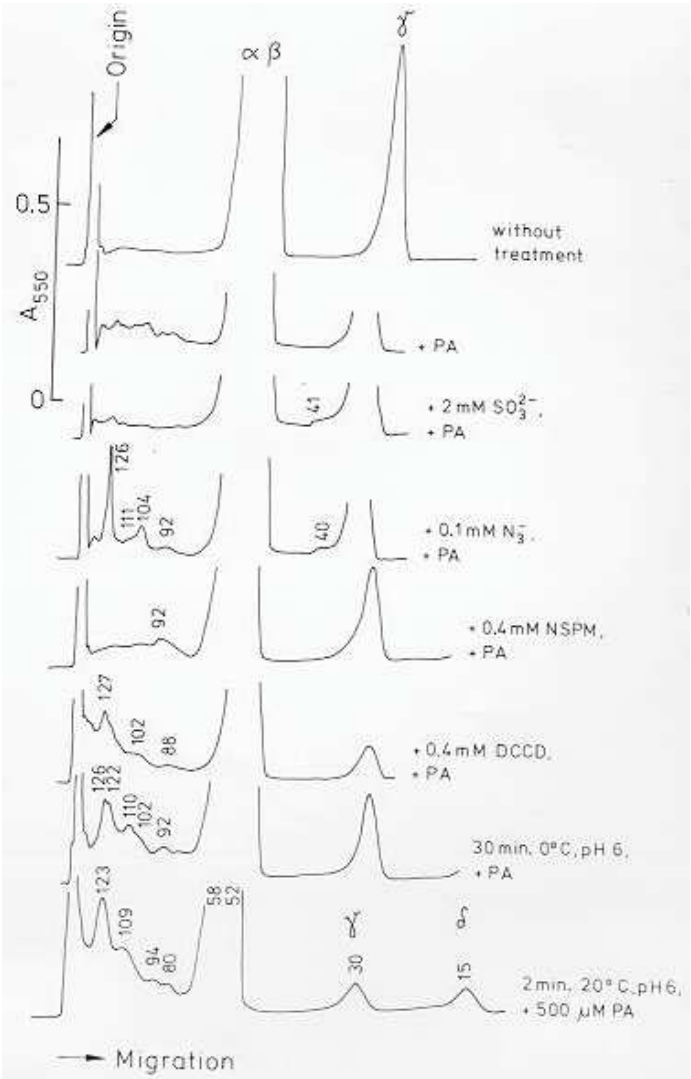


Figure 3. Pattern of beef heart mitochondrial F_1- ATPase as influenced by various modifiers of ATPase activity. 100 µg BF_1-ATPase in 1 ml of 0.3 M sucrose, 25 mM Tris-sulfate, 12.5 mM $MgCl_2$, pH 7.5, has been treated for 2 minutes with SO_3^{2-}, N_3^-, 30 minutes with DCCD, NSPM at 20^0C and as indicated, then for additional 2 minutes with 200 µM PA, if not marked otherwise and 5 minutes with 5 mM ATP, except in the case of the NSPM and cold treated enzyme. The enzyme precipitated by 0.453 g $AmS0_4$/ml was resolubilized in 100 µl acidic SDS-buffer, 30 µl of this solution was applied to the gel. The amount of protein on

individual gels (18 to 20 μg) has been determined as described in methods. The numbers above the peaks represent the molecular weights in kDa

modified enzyme complex as normally controlled by dithiol/thiol interchange (2,4,5,6) is then rapidly turning over, as can be seen on the sulfite activated BF_1-ATPase activity(2). The crosslinking as induced by the nucleophile azide is not easy to explain, but the fact alone that it changes the intrinsic crosslinking may be a hint to its action on ATPase activity. The thiol reagent NSPM, which changes structure and modifies the α-subunit of BF_1 (2,5), gives rise to appearance of a 92 kDa product. A relatively high amount of crosslinked products is formed by treatment of the complex with PA at low pH or by dicyclohexylcarbodiamide (DCCD) (Fig.3).

The BF_1-sulfhydryl groups and disulfide bonds have been titrated with radioaktive N-ethyl-maleimide (21). The data suggested that disulfide bond formation between the γ- and ε-subunit were present besides a disulfide bond in the α- subunit (20). α_2 or αß-crosslinked products are described in (22). By the method of intrinsic crosslinkage via S-S-bridges γε, α_2 and αß as well as αγ dimers are found (15). The 40, 92, 102,122,126 kDa products found in this study may then be γε, αγ, αγε, α_2, α_2ε across S-S-bridges crosslinked subunits but , as discussed in (22), other combinations for the higher Mr crosslinked products are also possible.

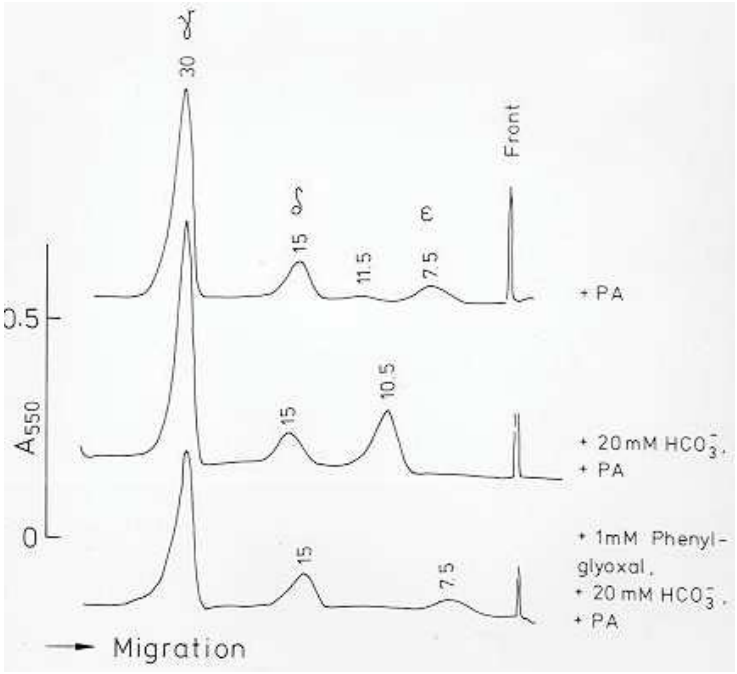


Figure 4. Pattern of beef heart mitochondrial F_1-ATPase as influenced by HCO_3^-. Conditions as in Fig.3. 2 Minutes treatment with HCO_3^- or HCO_3^- plus phenylglyoxal at 20^0C, 2 minutes with 200 μM PA, 5 minutes with 5 mM ATP.
The numbers above the peaks represent the molecular weights in kDa.

A hint to the function of HCO_3 on BF_1-ATPase activity may be the clearly discernible dimerization of the ε-subunit, which cannot be seen in presence of phenylglyoxal (Fig. 4). The molecular weight of ε_2 is not twofold as expected from 7.5 kDa, but 10.5 kDa, suggesting that SDS-micellar weight (23) determines apparent Mr values for included small polypeptides. Also, staining intensity of ε_2 is much higher as of monomeric ε.The molar ratio, (α:β:γ:δ:ε, in chloroform released BF_1-ATPase could be determined out of numerous gel runs by the stain bound per protein band as given in (24). It results in a subunit structure of $\alpha_4\beta_3\gamma_2\delta_1\varepsilon_{1\text{-}2}$ which differs by one additional α and γ subunit from the structure as obtained for BF_1-ATPase (25) isolated with a different procedure (26).

Figure 5 shows the phosphate and arsenate sensitive ^{3}H-acetyl label incorporation into the ß-subunit of BF_1-ATPase. Since the (α-and ß-subunits were not resolved well enough, the stained bands had to be cut out from the gels in order to see which one of the large subunits was sensitive. The radioactivity distribution into each of the subunits under influence of phosphate or arsenate is given in the inset of figure 5.

The label incorporated into the α-and γ-subunits was stable against nucleophiles (2), therefore, these subunit labelings are able to function as intrinsic control for changes appearing in the ß-subunit. The label of the ß-subunit is also sensitive against the tyrosine modifying reagent

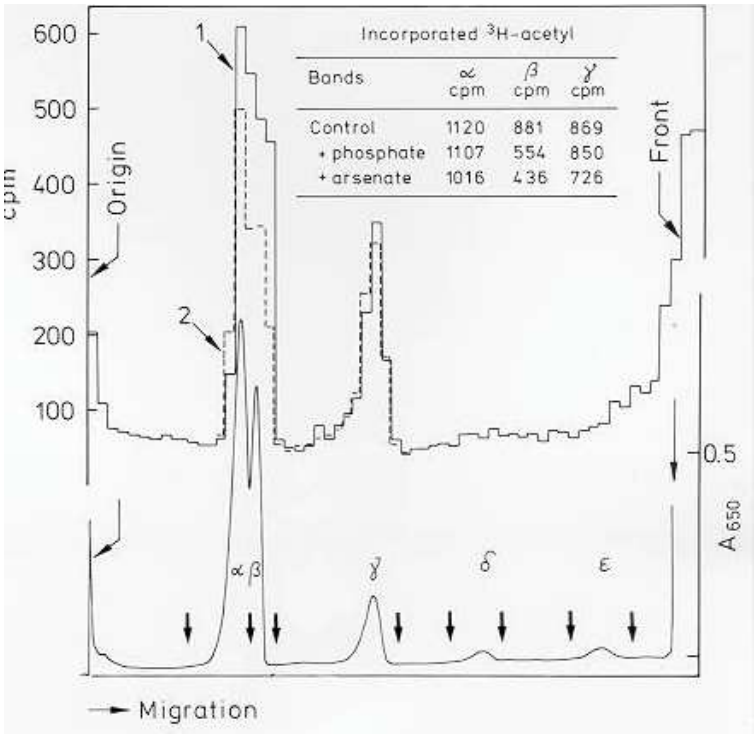
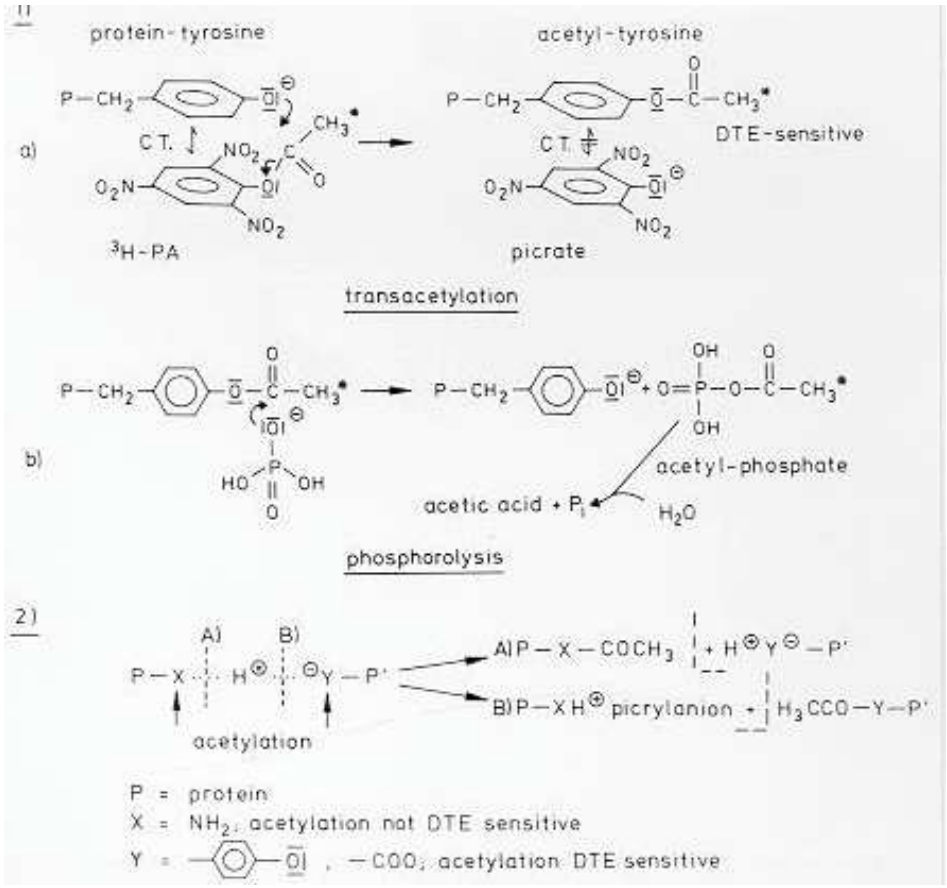


Figure 5. Effect of phosphate and arsenate on the ^{3}H-acetyl group incorporation into the BF_1- ATPase subunits. Conditions as in Fig.9. 2 Minutes treatment with 20 mM phosphate or arsenate at 20^0C, 2 minutes with 200 µM ^{3}H-PA, 5 minutes with 5 mM ATP. (1) (solid line) control, (2) (dashed line) plus phosphate. Razor blade cuttings of the stained gels are marked by arrow, the inset shows the amount of radioactivity as obtained from cutted stained gel bands.

4-chloro-7-nitrobenzofurazan (NBD-C1) (27), which may be taken as indication that a ß-subunit tyrosine is acetylated (2). A possible build-up of acetyl-phosphate (acetyl-arsenate) upon phosphate (arsenate) addition may explain its sensitivity against phosphate (arsenate) (scheme 1):

The above results demonstrate that the gel system at the low pH employed behaves like other systems in electrophor- etic mobility, but in addition the low pH inhibits thiol/ disulfide-interchange by protonation of free thiol groups (pK >7) and allows therefore structural studies on enzyme complexes. At acidic pH crosslinkages across other functional groups, e.g. structures like P-$X^{\delta+}$ $Y^{\delta-}$-P`, maybe investigated and studies on acid stabile binding of functional groups to protein amino acids are possible.



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ANALYTICAL BIOCHEMISTRY

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Manuscript by: REINHOLD KIEHL

Ms. No.: E596-A43

Date: 5/11/82

Title: Dodecylsulfate-polyacrylamide gel electrophoresis at acidic pH suitable for detection of labile protein bound functional groups

Comments: (Please continue on additional sheet(s) if necessary, using ordinary paper.)

Unfortunately the principle focus of this manuscript does not appear to be the description and characterization of new methodology. Instead, the author presents a highly specific and poorly substantiated study of the effects of chemical modification on the electrophoretic mobility of F_1 subunits. In addition, parts of the manuscript lack clarity and there is frequent use of unconventional abbreviations. In the opinion of this reviewer the manuscript does not warrant publication in Analytical Biochemistry.

Specific examples to illustrate each of these general criticisms follow:

1. It would be of interest to see how the Weber and Osborn procedure compares with the author's in separating polypeptides without loss of covalently-bound, radioisotope-labeled, chemical reagents. What other procedures have been described to isolate modified proteins and peptides stable at acid pH (i.e., H.V. paper electrophoresis, chromatography, etc.)? How does the author's procedure compare to such alternatives?

2. It is suggested in discussing Fig. 3 that the ability of azide to promote cross-linking of a few percent of the subunits of F_1 may give a hint as to its action on the ATPase activity. How does the amount of cross-linking relate to the

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amount of inhibition obtained at the concentration of azide used? The author reports that the subunit stoichiometry of chloroform released enzyme is $\alpha_4 \beta_3 \gamma_2 \delta_1 \varepsilon_{1\text{-}2}$ based on the relative staining of the subunits on gels. The data are not shown. If the author wishes to propose a new stoichiometry for F_1 subunits, a very thorough documentation would be necessary.

3. The introduction is not well written. Transitions between th^oughts are abrupt.

4. To my knowledge BF_1 is not a common abbreviation for mitochondrial F_1 even if obtained from beef heart. In addition, BF_1 is used by some laboratories to refer to bacterial F_1. I would also question whether W/O gels (Weber and Osborn gels) and K (for phosphorylase kinase) are appropriate abbreviations.

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

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