# Relationship of Cysteine and Tyrosine Residues to Adenosine Triphosphate Hydrolysis by Mitochondrial Adenosine Triphosphatase†

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ABSTRACT: The soluble beef-heart mitochondrial ATPase prepared by the method of A. E. Senior and J. C. Brooks (*Arch. Biochem. Biophys. 140*, 257 (1970)) contains a total of eight sulfhydryl groups and two disulfide bonds per molecule. Two of the sulfhydryl groups are freely accessible to iodoacetate or Ellman's reagent. When the enzyme is heated these two sulfhydryl groups become buried inside the molecule. This conformational change is associated with an increase in adenosine triphosphatase (ATPase) activity. The ATPase activity was remarkably resistant to reagents which normally react rapidly with sulfhydryl groups in proteins, such as *N*-ethylmaleimide, mercurials, and Ellman's reagent. Tetranitromethane and iodoacetate inactivated the ATPase rapidly, and the inactivation was fully or partly prevented by ATP and/or ADP. The evidence suggested that this inactivation was not due to modification of sulfhydryl groups and it seems that sulfhydryl groups are not present at the ATP binding site. When inactivation by tetranitromethane was almost complete and if ATP was absent, a total of nine–ten tyrosine residues were nitrated. ATP reduced the amount of inactivation by half and reduced the number of tyrosines nitrated to six–seven. Thus tyrosine residues may be involved in the binding of ATP and in its hydrolysis. An artifact was noted, which showed that caution should be used when using sodium dodecyl sulfate to solubilize proteins to which pH-sensitive chromophoric groups have been attached for experimental purposes.

The oligomycin-insensitive ATPase from beef-heart mitochondria is a soluble enzyme (also called "$F_1$") which is believed to carry the catalytic site at which ATP is synthesized or hydrolyzed in mitochondria (Pullman *et al.*, 1960; Penefsky *et al.*, 1960; Racker, 1970). The soluble enzyme, which is prepared by disrupting mitochondrial membranes by sonication or mechanical agitation, will readily rebind to depleted mitochondrial membranes, with restoration of normal morphology and function of the membranes (Racker and Horstman, 1967).

The general architecture of the molecule is now reasonably well defined. This is a large spherical enzyme complex of mol wt ~360,000 (Lambeth *et al.*, 1971; Knowles and Penefsky, 1972a) containing five different types of tightly bound subunits. An additional loosely bound sixth component, called the "specific inhibitor protein," is present in some preparations (Brooks and Senior, 1971). Each subunit has been purified and partly characterized (Brooks and Senior, 1971, 1972; Knowles and Penefsky, 1972b) and a tentative overall subunit structure has been proposed (Senior and Brooks, 1971).

Hilborn and Hammes (1973) reported that the soluble beef-heart ATPase has two nucleotide binding sites. One site binds a variety of nucleoside diphosphates and triphosphates quite loosely and is the catalytic site, and the other site binds ADP "tightly" and specifically. It may be a regulatory site (Nelson *et al.*, 1972a). It is not known where these sites are on the molecule, or which amino acids form the sites, and in order to understand the catalysis and its regulation the two nucleotide binding sites must be located and examined. One approach to this problem is to apply techniques developed by protein chemists to modify and locate specific amino acids in proteins.

There are a few reports in which specific chemical modifications of the catalytic site of the soluble ATPase were attempted but no inhibition of ATP hydrolysis was achieved (*e.g.*, Pullman *et al.*, 1960). All of these previous experiments are inconclusive for the reasons that (i) the incubations were carried out for short times (usually 15 min or less) and (ii) the incubations were carried out in the presence of saturating concentrations of ATP whereas it is now well established that saturating concentrations of substrates will often protect active-site residues from modification. Penefsky (1967) has shown that iodine, in the presence of 4 mM ATP, inactivates the ATPase rapidly and suggested that cysteine and tyrosine groups might be intimately involved in the hydrolytic activity.

Since reagents are available which are capable of relatively selective modification of cysteine and tyrosine in proteins it seemed worthwhile to commence a study of the nucleotide binding sites of the ATPase by examining the effects of these reagents. In this paper conditions under which the enzyme can be studied in the presence or absence of nucleotides are described, with the results of experiments carried out with several sulfhydryl reagents, tetranitromethane, and iodoacetate. The two latter reagents rapidly inactivate the enzyme and there is significant protection from inactivation by substrate.

## Materials and Methods

*Preparation and Assay of ATPase.* The enzyme was prepared from beef heart mitochondria as described previously (Senior and Brooks, 1970) with the modification described (Brooks and Senior, 1972). The eluate at step 4 was used directly. The specific activity of the enzyme, assayed as described (Senior and Brooks, 1970), was around 100 μmol of ATP hydrolyzed/min per mg.

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*Modification Reagents.* $Nbs_2$[1] was made up weekly as a 10 mM solution in 10 mM potassium phosphate (pH 7.0) and stored at −30°. Sodium iodoacetate was dissolved as a 1 M stock solution and stored at −30° in the dark. Tetranitromethane was diluted freshly for each experiment. A portion of stock solution (8.4 M) was diluted to 8.4 mM in absolute ethanol. This diluted solution was kept on ice and used within 30 min. Additions of these and other reagents in modification reactions were made with Hamilton microliter syringes.

*Equilibration of Enzyme with Buffer Solutions.* The enzyme suspension in ammonium sulfate solution was centrifuged in an MSE Model GT-2 bench centrifuge at 2000*g* for 10 min, and the pellet was dissolved in required buffer. After mixing and standing for 5 min at room temperature, the solution (which was normally slightly turbid) was centrifuged at 2000*g* for 15 min. The clear supernatant was then equilibrated with buffer by gel filtration on Sephadex G-25 (column measurements were 26 cm × 0.9 cm).

*Calculation of Sulfhydryl Groups, Nitrotyrosine Residues, and Protein Concentration.* A molar extinction coefficient of $13.6 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 412 nm and pH 8.0 was assumed for 3-thio-6-nitrobenzoate (Ellman, 1959). Experiments in which β-mercaptoethanol, diluted freshly in degassed distilled water, was used as a standard confirmed that value. A molar extinction coefficient of $4.1 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 428 nm and pH 8.0 was assumed for the nitrotyrosine residue (Sokolovsky *et al.*, 1966). Protein was estimated by Miller's method (1959), using dry bovine serum albumin as a standard. The values obtained for ATPase concentrations by this method correlated with those obtained by drying and weighing when multiplied by a factor of 0.96. The presence of 1% sodium dodecyl sulfate in the protein sample did not affect the final absorption values. For approximate estimations of protein concentration, a rapid spectrophotometric assay was used. At 280 nm, pH 7–8, a solution of 1 mg of ATPase/ml had an absorption of 2.05.

*Routine Procedures and Chemicals.* "ASU" and "SU" particles were prepared as described by Racker and Horstman (1967). These submitochondrial particles are preparations of inner membrane which are fully depleted of ATPase and which rebind soluble ATPase readily. Sodium dodecyl sulfate (95%) was obtained from Mallinckrodt and used directly. Sodium iodoacetate (iodine-free) was obtained from Calbiochem. $Nbs_2$ and *N*-ethylmaleimide were obtained from Eastman. *p*-Chloromercuribenzoate and *p*-chloromercuriphenylsulfonate were obtained from Sigma. Tetranitromethane was obtained from Aldrich. All other chemicals used were the best available commercial grades.

## Results

*Stability of the Enzyme in Solution.* When the enzyme was equilibrated by Sephadex G-25 gel filtration with buffers containing Tris-$SO_4$ (50 mM)–EDTA (1 mM) (TE buffer) or Tris-$SO_4$ (50 mM)–EDTA (1 mM)–$K_2SO_4$ (60 mM) (TEK buffer),[1] at pH 7.5–9.0 and 1–4 mg/ml, full ATPase activity was retained for several hours at 25, 30, or 37°. Below pH 7.0 activity was lost rapidly and the solution became turbid. When ATP was present, the enzyme retained activity for 2 days at pH 7.5–9.0. It has been reported that ATP binds to the catalytic site and ADP binds to the "tight," ADP-binding site in buffers containing EDTA (Hilborn and Hammes, 1973). No hydrolysis of ATP occurs when EDTA is present in excess of $Mg^{2+}$ (Pullman *et al.*, 1960). Thus, under these conditions both the catalytic and "tight" ADP-binding sites may be examined by chemical modification experiments.

*No Effect of "Sulfhydryl" Reagents.* When the enzyme was incubated with the following reagents at the final concentrations stated in the absence of ATP or ADP, no loss of ATPase activity was seen: sodium iodoacetate, 5 mM; $Nbs_2$, 0.1–1 mM; *p*-chloromercuribenzoate, 0.83 mM; *p*-chloromercuriphenylsulfonate, 2–3 mM; *N*-ethylmaleimide, 1–2 mM. (Conditions were, enzyme concentration 1–2 mg/ml, in TE or TEK buffer, at pH 7.5 or 8.0, at 30°, for 20 min to 3 hr.) The enzyme is therefore remarkably resistant to inhibition by high concentrations of these "sulfhydryl" reagents.

*Arrangement of Sulfhydryl Groups and Disulfide Bonds in the Molecule.* When $Nbs_2$ was added to the enzyme (above) the solution turned yellow indicating that some sulfhydryl groups were reacting. When the progress of the reaction was followed spectrophotometrically at 412 nm and 30°, it was found that the absorption rose rapidly on addition of $Nbs_2$ and reached a plateau after 15 min, and thereafter a very slow increase in absorption followed which continued for several hours. The same result was found when ATP ($\leqslant$10 mM), ADP ($\leqslant$10 mM), or potassium phosphate (5 mM) was present. If sodium dodecyl sulfate were added first to unfold the protein, a much larger change in absorption at 412 nm occurred which was complete in 15 min. Calculation of the numbers of –SH groups reacting (Table I) showed that two sulfhydryl groups per molecule were freely accessible to $Nbs_2$ and to 5 mM iodoacetate and about eight sulfhydryl groups per molecule reacted with $Nbs_2$ in the presence of sodium dodecyl sulfate. (The same result was obtained using 6 M guanidine hydrochloride as denaturant.) When the enzyme was depolymerized in sodium dodecyl sulfate and incubated with dithiothreitol to cleave disulfide bonds and then separated from the dithiothreitol by gel filtration in sodium dodecyl sulfate containing buffer, the total number of reactive SH groups was now about 12, indicating that two disulfide bonds had been broken. (These disulfide bonds could not be broken by dithiothreitol in the absence of a depolymerizing agent.)

This enzyme is remarkably heat stable and activation of ATPase activity is observed on heating. In a typical experiment the specific activity rose from 100 to 140. It was found that the free sulfhydryl groups became buried after heating and no longer reacted with $Nbs_2$. The total number of –SH groups in the depolymerized heated enzyme remained unchanged (Table I). Since this enzyme preparation contains no specific inhibitor protein (Brooks and Senior, 1971) it is concluded that activation of ATPase activity seen on heating was associated with the conformational change which occurred.

The enzyme also binds readily to depleted mitochondrial membranes (ASU or SU particles) with restoration of membrane morphology and function (Racker and Horstman, 1967). It was interesting therefore to find whether the labeling of the two free –SH groups with the carboxymethyl side chain or the more bulky 3-thio-6-nitrobenzoic acid side chain interfered in any way with the binding of the enzyme to the membrane. The results (not shown) indicated that this binding was unaffected by modification of the two free –SH groups with $Nbs_2$ or iodoacetate (5 mM) and the bound, modified enzyme was fully oligomycin sensitive. In the conditions of these experiments maximum binding of the ATPase was achieved by incubating 300 μg of ATPase with 1 mg of particles and

[1] Abbreviations used are: $Nbs_2$, 3,3′-dithiobis(6-nitrobenzoic acid) ("Ellman's reagent"); TE buffer, Tris-$SO_4$ (50 mM)–EDTA (1 mM); TEK buffer, Tris-$SO_4$ (50 mM)–EDTA (1 mM)–$K_2SO_4$ (60 mM).

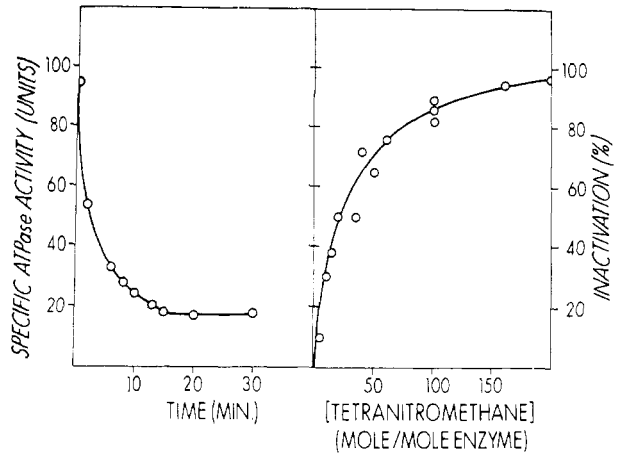


FIGURE 1: Inactivation of ATPase by tetranitromethane. The enzyme solution (1 mg/ml in TEK buffer, pH 8.0) was incubated at 30°, with tetranitromethane. Samples (5 μl) were withdrawn for ATPase assay at intervals: left, time course, tetranitromethane concentration was 100 mol/mol of enzyme; right, degree of inactivation after 15 min with various concentrations of tetranitromethane.

where subsaturating amounts of ATPase were used (20–150 μg/mg of particle) the modified enzyme bound to the same extent as the native enzyme.

*Inhibition of the ATPase Activity by Reaction with Tetranitromethane.* In a typical experiment such as shown in Figure 1 inhibition of the ATPase activity proceeded rapidly for about 15 min and thereafter the reaction proceeded very slowly or ceased. Reactions were therefore run routinely for 15 min. ATP gave protection from inactivation, but even at high concentrations of ATP, only 50% protection was achieved (Figures 2 and 3). Kinetic analysis showed the reaction to be rather complex and it was not fully analyzed. Tetranitromethane was itself rapidly hydrolyzed during the incubation, as was shown by the rapid increase in absorbance at 350 nm that occurred due to formation of the nitroformate ion, and this hydrolysis was virtually complete within 15 min. ATP did not react with tetranitromethane. The rate of nitroformate formation in the presence of ATP and enzyme was about two-

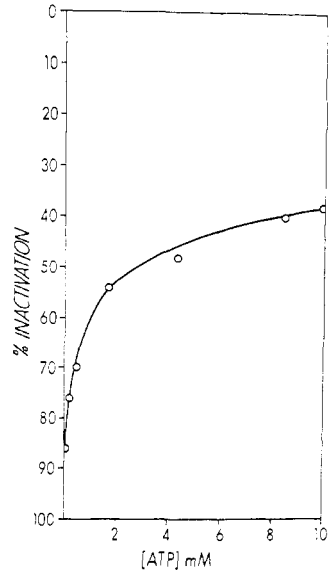


FIGURE 2: Protection of ATPase from inactivation by tetranitromethane. Experiments performed as in Figure 1. The tetranitromethane concentration was 100 mol/mol of enzyme.

TABLE I: Sulfhydryl Groups and Disulfide Bonds in the ATPase.

| ATPase | Additions to ATPase | SH Groups/mol of Enzyme[e] |
|---|---|---|
| Not heated[a] | None | 1.96 (1.50–2.52; 31) |
| Not heated[a] | Sodium dodecyl sulfate (1%) | 8.05 (6.9–9.15; 18) |
| Not heated[a] | Sodium dodecyl sulfate (1%) and dithiothreitol (0.05 M)[b] | 12.1 (11.3–13.1; 10) |
| Not heated[a] | Iodoacetate (5 mM), then sodium dodecyl sulfate (1%)[c] | 6.20 (6.15–6.25; 2) |
| Heated[d] | None | 0.37 (0.15–0.62; 7) |
| Heated[d] | Sodium dodecyl sulfate (1%) | 7.6 (7.2–7.8; 4) |

[a] The enzyme was equilibrated in TE buffer, pH 8.0, at 1–2 mg/ml as described under Materials and Methods. Samples (0.5 ml) of enzyme were warmed to 30° in a water-jacketed cell compartment of a Gilford 240 spectrophotometer, and the absorption at 412 nm was noted. $Nbs_2$ (10 mM) (10 μl) was added and the change in absorption at 412 nm after 20 min was noted. Correction was made for a slight change in absorption in a control buffer sample. Where indicated, 5 μl of a 10% sodium dodecyl sulfate solution was added before the $Nbs_2$ (final sodium dodecyl sulfate concentration, 1%). [b] To the enzyme solution was added sodium dodecyl sulfate and dithiothreitol, and the solution was allowed to stand for 3 hr at 25°. The solution was then passed through a Sephadex G-25 column to remove the dithiothreitol. The column buffer was Tris-$SO_4$ (50 mM)–EDTA (1 mM)–sodium dodecyl sulfate (1%) (pH 8.0). –SH groups were estimated in 0.5-ml samples of the effluent protein peak as above. [c] The enzyme was equilibrated in TE buffer as above and sodium iodoacetate (final concentration = 5 mM) was added. The enzyme stood for 1 hr at 25°. The iodoacetate was separated from the protein by Sephadex G-25 gel filtration. The effluent protein peak was collected, sodium dodecyl sulfate (1% final concentration) was added, and the –SH groups in 0.5-ml samples were estimated as above. [d] A sample of ATPase in ammonium sulfate was centrifuged, dissolved in TE buffer, and clarified by centrifugation as described under Materials and Methods. ATP was added to 4 mM concentration. The solution was heated at 65° for 4 min, cooled to 25°, and centrifuged (2000*g* × 15 min). –SH groups were assayed using $Nbs_2$ as described above. [e] Results are expressed as mean values, with range found and number of experiments done in parentheses.

thirds the rate in the presence of enzyme alone. Examination of the absorption spectrum of the inactivated enzyme *vs.* unreacted enzyme showed new peaks at 296 and 428 nm, indicating the presence of nitrotyrosine residues in the inactivated enzyme (Sokolovsky *et al.*, 1966; Christen *et al.*, 1971). The numbers of nitrotyrosine residues formed during the reaction were measured and are shown in Figure 3. It was found that modification of nine–ten tyrosine residues accompanied complete inactivation in the absence of ATP, but in the presence of ATP, when only 50% inactivation was achieved, only six–seven tyrosine residues were modified. No precipitation of the enzyme occurred during the reactions. Tetranitro-

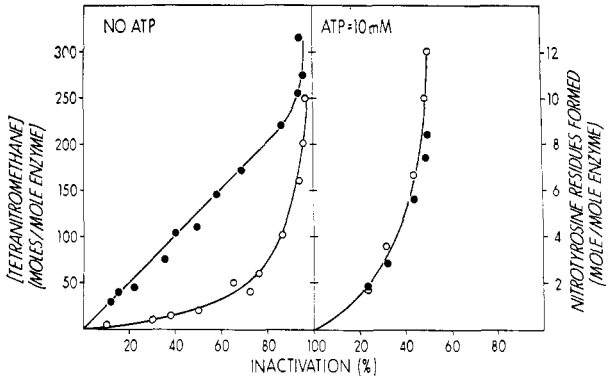


FIGURE 3: Nitrotyrosine residues in the ATPase after reaction with tetranitromethane. Enzyme (0.7 ml) was allowed to react with tetranitromethane as described in Figure 1. After 15 min of incubation the solution was passed through a Sephadex G-25 column in TEK buffer to remove nitroformate and any residual tetranitromethane. The effluent protein was collected and solid ammonium sulfate was added to give 70% saturation. After standing 10 min at 0° the suspension was centrifuged (32.000$g$ × 10 min). The precipitate was finally redissolved in 0.4 ml of buffer containing Tris-$SO_4$ (0.1 M)–urea (6 M) (pH 8.0). The nitrotyrosine content of the final solution was calculated from the absorption at 428 nm: (●) nitrotyrosine residues formed *vs.* inactivation; (○) tetranitromethane concentration *vs.* inactivation.

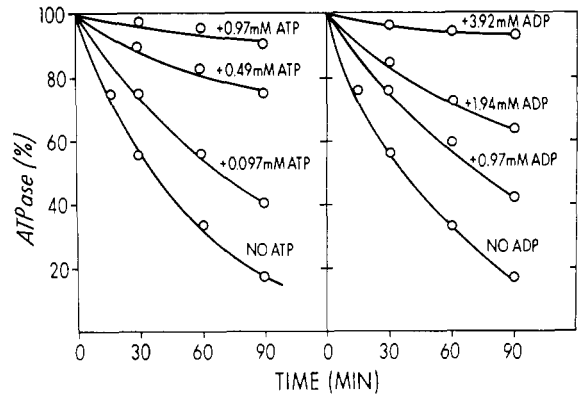


FIGURE 4: Inactivation of ATPase by iodoacetate (111 mM) and protection by ATP and ADP. ATPase was equilibrated with either TE or TEK buffer at pH 7.5 and incubated at 30° and 1 mg/ml in the dark with sodium iodoacetate (111 mM). Samples (5 μl) were withdrawn for ATPase assay at intervals. Control samples (no iodoacetate) retained 100% activity after 90 min of incubation.

methane is known to oxidize sulfhydryl groups readily (Sokolovsky *et al.*, 1969) and it was interesting to find out whether any of the sulfhydryl groups which were not accessible to other reagents (above) were accessible to oxidation by tetranitromethane. The results shown in Table II indicate that even when the enzyme was almost totally inactivated by tetranitromethane only two sulfhydryl groups/mol were modified. This same result was also obtained when ATP (10 mM) was present during the incubation. It seems reasonable to assume that these are the same two sulfhydryl groups which are normally accessible to $Nbs_2$ and iodoacetate, so that none of the buried sulfhydryl groups reacted with tetranitromethane.

*An Artifact.* If 1% sodium dodecyl sulfate–0.1 M Tris-$SO_4$ (pH 8.0) were substituted for urea-containing buffer to clarify the final solution of nitrated ATPase (see Figure 3, legend), the absorption spectrum of the final solution resembled that of nitrotyrosine at low pH (*i.e.* peak at 360 nm, no peak at 428 nm, isosbestic point at 381 nm; Riordan *et al.*, 1967). So it appeared that sodium dodecyl sulfate had the effect of altering the degree of ionization of the nitrotyrosine residues. Failure in the present work to realize this initially caused underestimation of the degree of nitration of the enzyme. It seems likely that other chromophoric groups whose absorption spectra are pH dependent may be perturbed in this fashion by sodium dodecyl sulfate.

*Inhibition of the ATPase Activity by High Concentrations of Sodium Iodoacetate.* Although low levels of iodoacetate (5 mM) did not inhibit the ATPase (see above), high levels (111 mM) produced a rapid inactivation. The inactivation was associated with slight precipitation of the enzyme in the TE buffer, but no turbidity appeared in the TEK buffer. The reaction was first order, $k = 1.8 \times 10^{-4}$ sec$^{-1}$. Iodoacetamide and bromoacetate were less effective inactivating reagents. Both ATP and ADP gave protection against inactivation by iodoacetate (Figure 4). Only two sulfhydryl groups/mol of enzyme were modified by the iodoacetate in these experiments (Table II). In other experiments it has been found that the amino acid compositions of samples of enzyme that had been 90% inactivated by iodoacetate were very similar indeed to that of native enzyme, suggesting that very few groups are modified by iodoacetate. Iodoacetate, therefore, shows promise as a potential catalytic site label. Full details of experiments dealing with subunit labeling by [$^{14}$C]iodoacetate and identification of groups modified, etc., will be presented elsewhere (A. E. Senior, manuscript in preparation).

## Discussion

*Role of Sulfhydryl Groups in the Molecule.* From the evidence presented it seems that none of the buried –SH groups nor either of the free –SH groups are involved in the binding of ATP at the catalytic site. It should be borne in mind, however, that if after the ATP is bound, a large conformational change takes place, which transfers the ATP to a deeply buried region in which hydrolysis actually occurs, then sulfhydryl groups might play a role in hydrolysis but not in binding. The term active or catalytic site should then be distinguished from ATP-binding site. Such deeply buried sulfhydryl groups might be protected from modification by tetranitromethane or iodoacetate (111 mM).

TABLE II: Sulfhydryl Groups in the ATPase after Inactivation by Tetranitromethane and by Iodoacetate.

| Experiment | Total –SH Groups Titratable with $Nbs_2$[c] |
|---|---|
| ATPase plus tetranitromethane, 100 mol/mol of enzyme, for 15 min at 30° [a] | 5.7 (5.2–6.2; 8) |
| ATPase plus sodium iodoacetate, 111 mM, for 60 min at 30° [b] | 5.99 (5.94–6.04; 2) |

[a] The experiment was carried out as in Figure 3, except that the enzyme was dissolved finally in 1 mM EDTA–50 mM Tris-$SO_4$–1% sodium dodecyl sulfate (pH 8.0). The total number of SH groups remaining intact was estimated with $Nbs_2$ as in Table I. [b] The experiment was carried out as above, only the modification reagent and time of incubation were different. [c] Results are means, with range found and number of experiments done in parentheses.

Modification of the two free –SH groups did not inhibit binding of the enzyme to the membrane. Therefore, as yet, no role can be ascribed to the sulfhydryl groups, but some possibilities appear to be eliminated. It is notable that in this respect the mitochondrial ATPase differs sharply from several other ATPase enzymes, *e.g.*, sarcoplasmic reticulum ATPase, myosin and [$Na^+$,$K^+$]-linked ATPases, which have one or more reactive and essential sulfhydryl groups in the catalytic site (Trotta *et al.*, 1968; Panet and Selinger, 1970; Skou, 1965) and which are strongly inhibited by reagents which are shown here to have no effect whatsoever on mitochondrial ATPase ($F_1$) activity.

*Arrangement of Cysteine and Cystine in the Molecule.* Penefsky and Warner (1965) have reported that the soluble ATPase ($F_1$) prepared according to an older procedure (Pullman *et al.*, 1960) contained no free –SH groups, no disulfide bonds, and 11 buried –SH groups/mol of mol wt 284,000. Since that time the molecular weight has been revised upward (see introductory statement). Recalculation of the total –SH groups on the basis of mol wt 360,000 gives 14 buried –SH groups/mol. The results reported here are different (Table I). That Penefsky and Warner found no free –SH groups is consistent with this work and is attributable to the fact that a heating step was the last stage of purification in the older procedure. Penefsky and Warner did not test for the presence of disulfide bonds, but concluded that none was present since the number of total sulfhydryl groups agreed with amino acid analyses of cysteine. More recent amino acid analyses (Knowles and Penefsky, 1972b), however, indicate that a total of 12 cysteine plus half-cystine residues are present per mol wt 360,000 and the present work also supports this value. The presence or absence of disulfide bonds in the molecule may depend on the method of preparation of enzyme but it should be mentioned that other preparative procedures currently in use (Horstman and Racker, 1970; Datta and Penefsky, 1970; Knowles and Penefsky, 1972a) closely resemble the procedure used in this work.

Farron and Racker (1970) have reported that the ATPase enzyme prepared from chloroplasts (called "$CF_1$") has two sulfhydryl groups per molecule which are accessible to $Nbs_2$, a total of eight sulfhydryl groups per molecule and two disulfide bonds per molecule—a remarkably similar arrangement to that shown here for the mitochondial ATPase. The two enzymes are known to have very similar molecular weights and subunit structure (Farron, 1970; Nelson *et al.*, 1972b).

The two "free" sulfhydryl groups that are present in the unheated mitochondrial ATPase, and which react freely with $Nbs_2$ and iodoacetate, should be expected to react rapidly with most modification reagents or with "reporter probes." This should be borne in mind in future investigations. They may be protected by reaction with cystamine (A. E. Senior, unpublished experiments), or they may be "hidden" by heating the enzyme.

*Inactivation by Tetranitromethane and Iodoacetate.* Out of about 85 tyrosine residues in the enzyme, about 9–10 were readily modified by tetranitromethane in the absence of ATP and 6–7 were modified in the presence of ATP. Some protection from inactivation by ATP was noted, and this was shown to be not due to extraneous factors such as faster removal of tetranitromethane in the presence of ATP. These results show that the hydrolytic activity of the enzyme is highly dependent upon the integrity of a relatively small number of tyrosine residues, and suggest (albeit tentatively) that ATP specifically protects a small number of tyrosine residues from nitration. Tetranitromethane may also react with tryptophan in proteins (Sokolovsky *et al.*, 1970). There is some evidence that the mitochondrial ATPase enzyme contains a very small amount of tryptophan (Senior and MacLennan, 1970), and therefore the inactivating effect of tetranitromethane might have been due in some degree to reaction with tryptophan. Further studies to test and apply a suitable radioactive label for tyrosine groups and to test reagents that react more specifically with tryptophan are clearly indicated by these experiments.

The fact that almost complete protection against inactivation by iodoacetate was given by ADP and ATP, and the ready availability of [$^{14}$C]iodoacetate, make this reagent appear a very promising potential label for groups involved in the catalytic site. The nature of the reaction products is currently being scrutinized.

## References


Brooks, J. C., and Senior, A. E. (1971), *Arch. Biochem. Biophys. 147*, 467.

Brooks, J. C., and Senior, A. E. (1972), *Biochemistry 11*, 4675.

Christen, P., Vallee, B. L., and Simpson, R. T. (1971), *Biochemistry 10*, 1377.

Datta, A., and Penefsky, H. S. (1970), *J. Biol. Chem. 245*, 1537.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Farron, F. (1970), *Biochemistry 9*, 3823.

Farron, F., and Racker, E. (1970), *Biochemistry 9*, 3829.

Hilborn, D. A., and Hammes, G. G. (1973), *Biochemistry 12*, 983.

Horstman, L. L., and Racker, E. (1970), *J. Biol. Chem. 245*, 1336.

Knowles, A. F., and Penefsky, H. S. (1972a), *J. Biol. Chem. 247*, 6617.

Knowles, A. F., and Penefsky, H. S. (1972b), *J. Biol. Chem. 247*, 6624.

Lambeth, D. O., Lardy, H. A., Senior, A. E., and Brooks, J. C. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 17*, 330.

Miller, G. L. (1959), *Anal. Chem. 31*, 964.

Nelson, N., Nelson, H., and Racker, E. (1972a), *J. Biol. Chem. 247*, 6506.

Nelson, N., Nelson, H., and Racker, E. (1972b), *J. Biol. Chem. 247*, 7657.

Panet, R., and Selinger, Z. (1970), *Eur. J. Biochem. 14*, 440.

Penefsky, H. S. (1967), *J. Biol. Chem. 242*, 5789.

Penefsky, H. S., Pullman, M. E., Datta, A., and Racker, E. (1960), *J. Biol. Chem. 235*, 3330.

Penefsky, H. S., and Warner, R. C. (1965), *J. Biol. Chem. 240*, 4694.

Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960), *J. Biol. Chem. 235*, 3322.

Racker, E. (1970), *in* Mitochondria and Chloroplasts, Racker, E., Ed., New York, N. Y., Reinhold, pp 127–171.

Racker, E., and Horstman, L. L. (1967), *J. Biol. Chem. 242*, 2547.

Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 358.

Senior, A. E., and Brooks, J. C. (1970), *Arch. Biochem. Biophys. 140*, 257.

Senior, A. E., and Brooks, J. C. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 17*, 327.

Senior, A. E., and MacLennan, D. H. (1970), *J. Biol. Chem. 245*, 5086.

Skou, J. C. (1965), *Phys. Rev. 45*, 596.

Sokolovsky, M., Fuchs, M., and Riordan, J. F. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 167.
Sokolovsky, M., Harrell, D., and Riordan, J. F. (1969), *Biochemistry 8*, 4740.
Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.
Trotta, P. P., Dreizen, P., and Stracher, A. (1968), *Proc. Nat. Acad. Sci. U. S. 69*, 659.

# Gradient Sievorptive Chromatography. A Focusing System for the Separation of Cellular Components†

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ABSTRACT: Chromatographic systems that employ an active combination of molecular sieve and adsorption chromatography are useful for the rapid purification of enzymes. Such systems, called sievorptive chromatography, will separate either strongly interacting macromolecules or similar macromolecules under conditions of maximal resolution. If appropriate gradients are used in sievorptive chromatography, a system is formed that focuses macromolecules within the chromatographic column at positions that depend upon their adsorptive properties. This report introduces focusing chromatography and illustrates its use for the rapid purification of *Escherichia coli* enzymes. Special problems are encountered when salt gradients are used with ion exchange gels due to the formation of inherent gradients. The nature of these gradients and the factors which control them are discussed.

The objective of modern biochemical separation techniques is to fractionate cellular components as rapidly and gently as possible in order to minimize the loss of biological activity. Additionally, it is important to reduce associated manipulations to a minimum. Sievorptive chromatography (Kirkegaard and Agee, 1973), the name for systems that actively combine molecular sieve (gel filtration) and adsorption chromatography, substantially assists in the realization of this objective. This article describes how appropriate gradients expand the usefulness of the two basic mechanisms of sievorptive chromatography. The first mechanism, ion filtration chromatography (Kirkegaard *et al.*, 1972), is a process that is optimized for the separation of macromolecules of similar physical properties. The second, intervent dilution chromatography (Kirkegaard and Agee, 1973), utilizes a solvent boundary to separate strongly interacting macromolecules. These processes, the former using no gradient at all, and the latter a nearly infinite gradient, are the extremes of sievorptive chromatography. Between these extremes is the domain of gradient sievorptive chromatography.

As a brief background to the basic mechanisms of sievorptive chromatography, the process of ion filtration chromatography (Kirkegaard *et al.*, 1972) employs a highly cross-linked ion exchange gel equilibrated at a precisely defined condition of pH and ionic strength such that the enzyme to be purified elutes in the sieving range, *i.e.*, after the peak of noninteracting, totally excluded material but sufficiently before the column liquid volume to permit the removal of low molecular weight substances from the enzyme. The system is adapted for the purification of any desired enzyme by changes in the pH and/or ionic strength of column equilibration or by using an alternate ion exchange gel. A single chromatographic step accomplishes the ion exchange fractionation and dialysis of an enzyme.

Intervent dilution chromatography (Kirkegaard and Agee, 1973) uses the sieving properties of ion exchange gels to repeatedly propel strongly interacting macromolecules across the boundary between a region of concentrated intervents[1] where macromolecules are relatively independent and a region of diluted intervents where macromolecules are separated by adsorptive or ion exchange processes. Under appropriate conditions, this repetitive action will separate ribosomal proteins from ribosomal RNAs, one of the strongest complexes encountered in biochemistry.

Between the extremes of intervent dilution and ion filtration chromatography lie a vast number of chromatographic systems of intermediate properties. A common property of these systems is a gradient of small molecules which extends through the sieving range, hence the description gradient sievorptive chromatography.

When appropriate gradients are used in sievorptive chromatography, a system is formed that focuses macromolecules as they move along the column at positions determined by the chromatographic properties of the molecules. A gradient, composed of small molecules and extending over about one-third of the length of the column, moves along the column at nearly the same rate as the solvent. Macromolecules, on the other hand, are totally excluded and in the absence of adsorption move along the column between two and three times the rate of the solvent. If the direction of the gradient is such that adsorption of a macromolecule is high on the leading edge and absent at the trailing edge, the macromolecule will slow down as it progresses along the gradient. Eventually the macromolecule reaches a level in the gradient that permits it to move along the column at the same rate as the gradient moves.

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[1] Small molecules that reduce or intervene in the attractions between macromolecules are called intervents (Kirkegaard and Agee, 1973).