A Method for Determining the Adenosine Triphosphatase Content of Energy-Transducing Membranes

REACTION OF 4-CHLORO-7-NITROBENZOFURAZAN WITH THE ADENOSINE TRIPHOSPHATASE OF BOVINE HEART SUBMITOCHONDRIAL PARTICLES

By STUART J. FERGUSON, WILLIAM J. LLOYD and GEORGE K. RADDA

*Department of Biochemistry, University of Oxford,
South Parks Road, Oxford OX1 3QU, U.K.*

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1. Modification of a single amino acid residue by introduction of the nitrobenzofurazan group inactivates mitochondrial ATPase (adenosine triphosphatase) when membrane-bound in submitochondrial particles. The similarity between the reactions of both membrane-bound and isolated ATPase with 4-chloro-7-nitrobenzofurazan indicates that the single essential tyrosine residue identified in the isolated enzyme [Ferguson, Lloyd, Lyons & Radda (1975) *Eur. J. Biochem.* **54**, 117-126] is also a feature of the membrane-bound ATPase. 2. A procedure is presented for estimating the ATPase content of the inner mitochondrial membrane. It is based on the specificity of the incorporation of the nitrobenzofurazan group, and the ready removal of this group by compounds that contain a thiol group. This method indicates that 8.5% of the membrane protein is ATPase. The procedure should be applicable to the titration of the energy-transducing ATPases of bacterial plasma membranes and of the thylakoid membranes of chloroplasts. 3. Combination of the data obtained on the ATPase content of the bovine heart inner mitochondrial membrane with a titration of the cytochrome *bc*₁ complex with antimycin indicates that these two components of the membrane are present in approximately equal amounts.

The inner mitochondrial membrane contains an ATPase* which catalyses the synthesis of the ATP that is produced by oxidative phosphorylation. This mitochondrial ATPase can be extracted from the membrane as a water-soluble protein of mol.wt. 360000, which has a complex structure comprising five types of polypeptide chain, α , β , γ , δ , ϵ , in the proportions 3:3:1:1:1 (Senior, 1973). This purified ATPase is not sensitive to oligomycin, the classical inhibitor for the ATPase of the inner mitochondrial membrane.

An intriguing feature of the soluble ATPase is that one of its tyrosine residues is specifically modified by 4-chloro-7-nitrobenzofurazan to give an inactive enzyme (Ferguson *et al.*, 1974a, 1975a). At pH 9.0 the nitrobenzofurazan group of the modified enzyme transfers to a nitrogen atom (Ferguson *et al.*, 1975b). Although the kinetics of the reaction between Nbf-Cl and both the soluble and membrane-bound ATPase are indistinguishable (Ferguson *et al.*, 1975a), the stoichiometry of the reaction with the enzyme when bound to submitochondrial particles is not known. As it is possible that the unusual stoichiometry of reaction between the isolated ATPase and Nbf-Cl

arises from a perturbation of the enzyme structure caused by isolation, it is important to ascertain whether the membrane-bound enzyme also has a single essential tyrosine residue that is reactive towards Nbf-Cl.

A complete understanding of the properties of the inner mitochondrial membrane requires a quantitative knowledge of the various components that are present in the membrane. The ATPase accounts for a large amount of the membrane protein; a common estimate is 10% (Beechey, 1974). This value has been inferred either from electron micrographs or by comparing the ATPase activity of certain types of submitochondrial particle with the activity of the isolated enzyme (Racker & Horstman, 1967). The specific binding of aurovertin to the mitochondrial ATPase, and the accompanying large fluorescence enhancement, has enabled Bertina *et al.* (1973) to titrate the enzyme in mitochondria with this antibiotic. However, as their result was obtained on mitochondria it does not relate directly to the amount of inner-membrane protein that is ATPase.

The ready reversibility and specificity of the reaction between Nbf-Cl and the membrane-bound ATPase provides a direct method for titrating the ATPase in submitochondrial particles. Berden & Slater (1972) have described a procedure, based on

* Abbreviations: ATPase, adenosine triphosphatase (EC 3.6.1.3); Nbf-Cl, 4-chloro-7-nitrobenzofurazan (formerly NBD-Cl).

the quenching of antimycin fluorescence, for estimating the amount of cytochrome *bc*₁ complex in mitochondria. A combination of this technique with a new method for titrating the ATPase provides an estimate of the relative stoichiometries of these components.

Methods

Bovine heart submitochondrial particles were prepared from mitochondria by Step 1 of the procedure of Knowles & Penefsky (1972) for the purification of mitochondrial ATPase. The particles, which could be prepared in batches of 10g, were stored in liquid N₂. Where appropriate the ATPase activity of these particles was maximized by incubation at room temperature (20°C) for 4 or 5 h in 0.2M-sucrose/2mM-EDTA/4mM-ATP at a concentration of 20mg of protein/ml, with the pH adjusted to 9.2 by the addition of conc. NH₃ (sp. gr. 0.88). After this exposure to high pH, the particles were centrifuged in the no. 40 rotor of the Spinco preparative centrifuge for 30 min at 40000 rev./min, and resuspended in 0.2M-sucrose/2mM-EDTA/4mM-ATP/10mM-triethanolamine hydrochloride, pH 7.5, and then passed through Sephadex G-50 as described by Racker & Horstman (1967). For reaction with Nbf-Cl, submitochondrial particles were incubated at a concentration of 20mg of protein/ml at pH 7.5 in the sucrose/EDTA/ATP/triethanolamine hydrochloride buffer; 50 μ M-Nbf-Cl was added and the reaction allowed to proceed at room temperature until the ATPase activity was decreased to the desired extent. Excess of Nbf-Cl that had not reacted was removed by washing the particles three times by centrifugation (40000 rev./min for 30 min) and resuspension in the same buffer. Alternatively, with experiments in which the quantity of ATPase in the membrane was investigated, the excess of Nbf-Cl was removed by dialysis against 20 vol. of a similar particle suspension. The particles outside the sac were able to remove excess of Nbf-Cl, by reaction with their unmasked thiol groups.

Thiol groups in the membrane were blocked by suspending the particles at a concentration of 15mg of protein/ml at pH 7.5 in the sucrose/EDTA/ATP/triethanolamine hydrochloride buffer, and then adding 10 μ mol each of *N*-ethylmaleimide, iodoacetate and iodoacetamide/ml. After 60 min at 20°C the particles were centrifuged at 40000 rev./min in the no. 40 rotor of the Spinco preparative centrifuge for 1 h at 0°C, followed by resuspension in the same buffer and centrifugation (as above) to give a washed, alkylated preparation of particles.

The quantity of nitrobenzofurazan group released from the membrane-bound ATPase was estimated from the E_{475} of *N*-(7-nitrobenzofurazan)-cysteine in a supernatant after removal of particles by centrifugation in the no. 40 rotor of the Spinco preparative

centrifuge at 40000 rev./min for 1 h at 0°C. A molar extinction coefficient at 475 nm of 17400 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ was used for *N*-(7-nitrobenzofurazan)-cysteine. This value was obtained by treating a known amount of Nbf-Cl with a 1.5-fold excess of cysteine. As this reaction goes rapidly to completion, the final E_{475} was used to calculate the molar extinction coefficient. ATPase activity was assayed in 50mM-Tris/maleate/5mM-ATP/5mM-MgCl₂, pH 8.0. Release of P_i was measured continuously in a Technicon autoanalyser by the method of Hurst (1964). The ATPase activity of the particles was always inhibited by 95% or more on addition of 2 nmol of oligomycin/mg of protein.

Protein was determined by the biuret method (Gornall *et al.*, 1949) with crystalline bovine serum albumin as standard.

Fluorescence measurements were made on a Perkin Elmer MPF2A fluorimeter operating in the ratio mode, by using excitation and emission band-widths of 6 nm. Sedimentation coefficients were measured in a Spinco model E analytical ultracentrifuge with schlieren optics. Electrophoresis in 10% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate was carried out as described by Weber & Osborn (1969), in 40mM-sodium phosphate buffer, pH 7.2, which contained 0.1% sodium dodecyl sulphate.

The following reagents were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.: antimycin, crystalline bovine serum albumin, oligomycin, ATP, iodoacetate, *N*-ethylmaleimide, triethanolamine hydrochloride, EDTA and Tris. Sucrose was bought from Fisons (U.K.) Ltd., Loughborough, Leics., U.K., Sephadex G-50 from Pharmacia (Uppsala, Sweden) and Nbf-Cl from Serva Feinbiochemica (Heidelberg, West Germany). Iodoacetamide (twice recrystallized from ethanol) and L-cysteine were products of BDH Chemicals Ltd. (Poole, Dorset, U.K.).

Results

Determination of stoichiometry of the reaction between Nbf-Cl and the ATPase of submitochondrial particles

In principle the reaction between Nbf-Cl and the ATPase in submitochondrial particles could be monitored by following modification of one or more tyrosine residues by the increase in E_{385} , as has been described for the purified enzyme (Ferguson *et al.*, 1974a, 1975a). However, this was not possible, because of both the technical problems of observing a small increase in absorbance with a large background from the particles, and the parallel reaction of Nbf-Cl with thiol groups of other proteins in the membrane, which produces an increase in E_{420} (Birkett *et al.*, 1970). Consequently a procedure was adopted in

which the enzyme in the membrane was first labelled with Nbf-Cl, extracted from the particles by the method of Knowles & Penefsky (1972), and then the extent of nitrobenzofurazan incorporation measured. This method of isolation requires prolonged incubation of the enzyme at pH 9.2, and under these conditions it has been shown that on the isolated enzyme-nitrobenzofurazan the group migrates intramolecularly from tyrosine to lysine (Ferguson *et al.*, 1975b). Therefore a prerequisite to isolating the labelled enzyme from the membrane was to establish the effect of incubation at pH 9.0. Table 1 shows that if submitochondrial particles, which had been treated with Nbf-Cl until the ATPase activity was essentially abolished, were incubated at pH 9.0, then the ability of dithiothreitol to restore ATPase activity gradually declined. As dithiothreitol can remove the nitrobenzofurazan group from tyrosine, but not from lysine, this observation can be related to the intramolecular transfer of the nitrobenzofurazan group from tyrosine to lysine (Ferguson *et al.*, 1975b). The rate (half-time 150 min) at which the dithiothreitol-dependent ATPase activity decreased is very similar to that found for the isolated enzyme. Incubation at pH 9.0 caused some re-activation of the ATPase activity of particles that had been treated with Nbf-Cl. This re-activation is attributed to a limited hydrolysis of the nitrobenzofurazan group from the tyrosine residue of the enzyme. Similar behaviour has been observed with the isolated enzyme (Ferguson *et al.*, 1975b).

The ATPase activity of submitochondrial particles is known to increase on incubation at pH 9.0, and this has been explained in terms of a dissociation of the ATPase-inhibitor protein (Racker & Horstman, 1967). It was to avoid this complication that particles used in the experiment reported in Table 1 were first treated with alkali and Sephadex G-50 (see the Methods section) so as to maximize ATPase activity by minimizing the content of ATPase-inhibitor protein.

The data in Table 1 indicate that, by analogy with the isolated enzyme, a transfer of the nitrobenzofurazan group from tyrosine to lysine also occurs when the ATPase is bound to the mitochondrial membrane. Therefore an extraction of the lysine-modified enzyme was attempted to determine the extent of incorporation of the nitrobenzofurazan group into the enzyme. Submitochondrial particles were treated with Nbf-Cl until ATPase activity was 90% inhibited and, after washing of the particles to remove excess of Nbf-Cl, the nitrobenzofurazan group was allowed to transfer fully to lysine by incubating the particles at pH 9.0. The ATPase was then released from the membrane and purified by the method of Knowles & Penefsky (1972). In the last step of this procedure a yellow-coloured protein fraction was eluted from the DEAE-Sephadex A-50

column, and had ATPase activity. Under illumination the characteristic green fluorescence of the *N*-nitrobenzofurazan chromophore could be observed. This protein fraction was homogeneous as judged by observation of a single peak in the analytical ultracentrifuge. A sedimentation coefficient ($s_{20,w}^{0}$) of 13.0S was obtained, which is a typical value for mitochondrial ATPase (Penefsky, 1974). When the protein was examined by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, the standard five-subunit pattern (Senior, 1973) was obtained, with no trace of major contaminating proteins. Illumination of the gel revealed a single green fluorescent band. This band was marked by insertion of a wire strip, and subsequent staining with Coomassie Blue showed that the fluorescence was exclusive to a β subunit of the enzyme, exactly as found for the isolated enzyme (Ferguson *et al.*, 1975b).

The fluorescence spectrum of this 'labelled in the membrane' enzyme had an uncorrected emission maximum at 520 nm, which is a shorter wavelength than that found for the model compound *N*^ε-acetyl-*N*^ε-(7-nitrobenzofurazan)-lysine, which has an emission maximum of 536 nm in water (Ferguson *et al.*, 1975b). However, treatment of the enzyme with 6M-guanidinium chloride resulted in a shift of the emission maximum to 534 nm, with an approximately threefold decrease in intensity. The enzyme labelled after isolation had identical properties (Ferguson *et al.*, 1975b).

The extent of incorporation of the nitrobenzofurazan group into the ATPase when bound to sub-

Table 1. *Effect of incubation at pH 9.0 on the extent of re-activation by dithiothreitol of ATPase activity in Nbf-Cl-treated submitochondrial particles*

ATPase activity is measured in μmol of P_i/min per mg. Submitochondrial particles were treated with 50 μM -Nbf-Cl at pH 7.5 in a buffer containing 0.2M-sucrose, 2mM-EDTA, 4mM-ATP and 10mM-triethanolamine hydrochloride. When the reaction was complete the particles were washed and finally resuspended in the same buffer. The pH was raised to 9.0 by the addition of 2M-Tris and the particles were incubated at 30°C. As a control a sample of Nbf-Cl-modified particles was maintained at pH 7.5.

Incubation time (h)	ATPase activity	Dithiothreitol (10mM) added to assay	ATPase activity of control
0	9.5	Yes	9.8
	0.92	No	1.0
2.5	5.1	Yes	9.0
	1.6	No	1.6
5.0	2.9	Yes	9.8
	2.0	No	1.6
8.0	2.2	Yes	9.3
	2.2	No	1.9

mitochondrial particles was estimated in the following way. Measurement of the E_{475} from the 4-amino-7-nitrobenzofurazan chromophore is related to the number of mol of nitrobenzofurazan incorporated by a molar extinction coefficient of $26000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Ferguson *et al.*, 1975b). Determination of the concentration of a sample of isolated 'labelled in the membrane' enzyme showed, when combined with the data on the number of mol of nitrobenzofurazan group present, that 0.7 mol of *N*-nitrobenzofurazan was incorporated per mol of ATPase, assuming that all the protein present was ATPase. The specific activity of this nitrobenzofurazan-modified enzyme was $30 \mu\text{mol}$ of P_i/min per mg of protein, which is 25% of that usually found for unmodified ATPase. As explained above, a certain amount of hydrolytic loss of the nitrobenzofurazan group from the enzyme is expected during incubation of the enzyme modified by nitrobenzofurazan at pH 9.0. Allowing for this, and for the fact that the enzyme in the particles was only 90% inhibited by Nbf-Cl before being incubated at pH 9.0, it is probable that the extracted enzyme is a mixture of 25% fully active ATPase and 75% essentially inhibited *N*-nitrobenzofurazan-labelled enzyme. It follows that the membrane-bound ATPase, like the isolated soluble ATPase, is modified on just one residue by Nbf-Cl, with concomitant loss of activity.

Estimation of the ATPase content of the inner mitochondrial membrane

By knowing the stoichiometry of the reaction between Nbf-Cl and the membrane-bound ATPase, a method could be devised for determining the number

of mol of ATPase per mg of membrane protein. The strategy was to inhibit a defined proportion of the ATPase in submitochondrial particles by reaction with Nbf-Cl, and then add to a nucleophile to remove the nitrobenzofurazan group from the enzyme. After centrifugation of the particles, the nitrobenzofurazan group in the supernatant could be measured spectrophotometrically. The procedure is summarized in Scheme 1.

Cysteine was used as nucleophile to remove the nitrobenzofurazan group from the enzyme, since the *S*-nitrobenzofurazan derivative that is first formed rapidly rearranges, to the *N*-nitrobenzofurazan-cysteine (Price & Radda, 1972). Measuring the *N*-nitrobenzofurazan rather than the *S*-nitrobenzofurazan chromophore had two advantages. First the extinction coefficient of the *N*-nitrobenzofurazan species is higher, and secondly centrifugation of submitochondrial particles always released some soluble material which had a much higher absorbance at 425 nm than at 475 nm. Hence the sensitivity of the method was increased. In addition, a most significant difference between the *N*- and *S*-nitrobenzofurazan chromophores is the 30-fold higher fluorescence emission from the former (Birkett *et al.*, 1970). Thus a sensitive fluorescence assay of the nitrobenzofurazan-cysteine released from the membrane is possible. Although the fluorescence method has not been used in the experiments reported here, it was established in trial experiments that a 10 nM solution of *N*-nitrobenzofurazan-cysteine was easily detectable.

To ensure that the only nitrobenzofurazan group removed from the membranes by cysteine is attached to the tyrosine residue of the ATPase, all the reactive

Scheme 1. Procedure for estimating ATPase content of submitochondrial particles

Table 2. *Measurement of the ATPase content of submitochondrial particles*

Alkylated submitochondrial particles were treated with Nbf-Cl until the ATPase activity was inhibited to the extents shown. After removal of Nbf-Cl that had not reacted, the ATPase was re-activated by cysteine and the particles were centrifuged. The amount of *N*-Nbf-cysteine in the supernatant was estimated from the E_{475} . For the calculation of the ATPase content a mol.wt. of 360000 was assumed for the ATPase. Protein was determined by the biuret method.

Expt. no.	Concn. of submitochondrial particles (mg/ml)	Inhibition of ATPase by Nbf-Cl (%)	<i>N</i> -Nbf-cysteine formed by addition of cysteine to Nbf-Cl-treated particles (nmol/ml)	ATPase content (% of total membrane protein)
1	13.6	90	3.0	8.8
2	14.5	68	2.2	8.2
3	12.5	49	1.4	8.4
4	14.8*	58	2.1	8.7

* These particles were not treated with alkali and Sephadex G-50 (see the text).

Table 3. *ATPase activity of submitochondrial particles at each stage of the procedure used for determining the ATPase content of the mitochondrial membrane*

Stages: A, initial activity; B, after multiple alkylation; C, after Nbf-Cl treatment; D, C assayed in presence of dithiothreitol; E, after re-activation of particles with cysteine; F, treated for 3 h at pH 9.2 after stage E. At all stages oligomycin inhibited by 95–97%.

Stage of procedure	ATPase activity (μmol of P_i /min per mg)	
	Alkali- and Sephadex-treated particles	Particles not treated with alkali and Sephadex
A	8.0	1.5
B	8.5	2.5
C	0.85	1.05
D	8.2	2.4
E	7.9	2.4
F	8.5	6.0

thiol groups of submitochondrial particles was first alkylated. In addition, to show that no other 'hyper-reactive' tyrosines were modified by Nbf-Cl the relation between the different amounts of nitrobenzofurazan released from the membrane with different extents of ATPase inhibition was studied. A direct relationship between the two measurements would then indicate that only the tyrosine residue of the ATPase was being measured, unless there was another tyrosine with identical reactivity towards Nbf-Cl.

Table 2 shows the results of experiments (nos. 1–3) in which the ATPase content of submitochondrial particles was estimated as 8.5% of total membrane protein, irrespective of the extent to which the ATPase was initially inhibited by Nbf-Cl. It was found that centrifugation of particles from a solution of *N*-nitrobenzofurazan-cysteine produced no change in the concentration of this compound in the super-

natant; thus *N*-nitrobenzofurazan-cysteine was not appreciably bound the the particles.

Expts. 1–3 in Table 2 were done with particles that had been depleted of the naturally occurring ATPase-inhibitor protein. That the inhibitor protein does not impede the reaction with the ATPase is shown by Expt. 4 (Table 2). Although the particles used in this experiment were not inhibitor deficient, they still had the same ATPase content, as shown by the titration with Nbf-Cl. The extent to which these particles were associated with the ATPase inhibitor can be gauged from Table 3. The initial activity (stage A of procedure) of the particles that had been treated with alkali and Sephadex was five times that shown by untreated particles. Therefore, assuming that this increase in ATPase activity represents removal of inhibitor protein, any difference in reactivity of the ATPase-inhibitor protein complex towards Nbf-Cl should have been detected in Expt. 4 of Table 2.

Table 3 shows that the alkylation procedure had little effect on the ATPase activity of particles that had been activated by the alkali and Sephadex treatment. The particles that had not been depleted of inhibitor showed some stimulation of ATPase after alkylation (compare stages A and B in Table 3), which can be attributed to a slight dissociation of the inhibitor protein. However, the much larger stimulation of ATPase by an alkali treatment (stage F) indicated that most of the inhibitor protein remained with the ATPase until this stage.

As the ATPase activity of the inhibitor-depleted particles remained constant throughout the procedure described in Table 3, it may be concluded that very little ATPase was lost from the membrane.

Antimycin titre of submitochondrial particles

Antimycin binds very tightly to the cytochrome bc_1 complex of the inner mitochondrial membrane (Berdn & Slater, 1972; Slater, 1973). Accompanying this binding is a quenching of antimycin fluorescence,

so that the titre of binding sites for this antibiotic can be estimated directly (Berden & Slater, 1972). Application of this method to determine the binding of antimycin to some alkali- and Sephadex-treated particles showed that 4.5 nmol of antimycin saturated the cytochrome bc_1 -binding sites of 15 mg (protein) particles. If the ATPase is 8.5% of the protein in these particles (Table 2), then, assuming mol.wt. 360 000 for this enzyme, there is 3.6 nmol of ATPase in the 15 mg of particles. Thus the Nbf-Cl titration method for determining the ATPase content of the membrane, when combined with the antimycin titration, indicates that there are 1.25 cytochrome bc_1 complexes per molecule of ATPase.

Discussion

The results in the present paper show that introduction of just one nitrobenzofurazan group on to an amino acid residue of the mitochondrial ATPase causes loss of activity. Thus the remarkable result that modification of just one of the three β subunits of the isolated enzyme produces an inactive enzyme (Ferguson *et al.*, 1975a,b) is also true for the membrane-bound enzyme. There is no direct evidence to show that a tyrosine residue of the membrane-bound ATPase is modified, but the identical stoichiometry of reaction, and the very similar kinetics and pH-dependence of the reaction between Nbf-Cl and both the isolated and membrane-bound enzyme (Ferguson *et al.*, 1975a), make it very probable that tyrosine is modified in the membrane-bound ATPase. At pH 9.0 the nitrobenzofurazan group undergoes an intramolecular shift to an amino group in a β subunit in the membrane-bound enzyme as well.

The inhibitor protein of the mitochondrial ATPase (Pullman & Monroy, 1963) does not influence the reaction between the enzyme and Nbf-Cl (see Table 2). This conclusion is in harmony with the finding that the ATPase of phosphorylating membrane fragments from *Paracoccus denitrificans* reacts with Nbf-Cl at a rate identical with that of the mitochondrial ATPase (Ferguson *et al.*, 1974b). The ATPase activity of the enzyme from this bacterium is latent, which may well be a consequence of the binding of a protein inhibitor. It is probable that the putative inhibitor protein has no effect on the reaction between the ATPase of *P. denitrificans* and Nbf-Cl, in agreement with the results obtained for the ATPase of submitochondrial particles.

On the basis of the ready removal of the nitrobenzofurazan group from the mitochondrial ATPase, we have developed a method for titrating this enzyme in submitochondrial particles. There are few previous measurements of the ATPase content of the inner mitochondrial membrane. From a comparison of the ATPase activity of submitochondrial particles, which were depleted of inhibitor protein, with the activity

of the purified ATPase, Racker & Horstman (1967) concluded that approx. 10% of the membrane protein was ATPase. Bertina *et al.* (1973) used the binding to the ATPase of the antibiotic aurovertin as a method for estimating the quantity of this enzyme in rat liver and rat heart mitochondria. Although the specific fluorescence enhancement of aurovertin on binding to the ATPase provides an attractive method for titration of the enzyme, there is also a drawback in that the number of binding sites for, and quantum yield of, aurovertin on the ATPase may be variable (Chang & Penefsky, 1973). Bertina *et al.* (1973) combined their aurovertin titration with an estimate of the cytochrome bc_1 complex, which was titrated with antimycin. For both rat liver and heart mitochondria, they concluded that equal amounts of the ATPase and cytochrome bc_1 complexes were present in the membrane. An antimycin titration of bovine heart submitochondrial particles that were used for titrating the ATPase with Nbf-Cl indicated that there were 1.25 cytochrome bc_1 complexes for each ATPase molecule. The difference between this result and that obtained by Bertina *et al.* (1973) could be a consequence of an underestimate of the ATPase content, or a difference in the relative stoichiometries of these two components of the inner mitochondrial membrane from bovine heart. Possible errors in the method proposed in this paper for titrating the ATPase will be considered.

The mitochondrial ATPase can be removed from its membrane by sonication procedures (Knowles & Penefsky, 1972). The particles used in the present work were prepared by sonication, and it is possible that some of the ATPase has been lost from the membranes. The cytochrome bc_1 complex is rather more tightly associated with the membrane, and so it may be expected that the ATPase titre could be underestimated relative to that for the cytochrome bc_1 complex. If the submitochondrial particles are a mixture of inverted and 'right-side-out' membranes, the estimated ATPase content again may be too low. Although Nbf-Cl can probably react with ATPase that is inside the particles (in the sense that the ATPase is inside intact mitochondria), it is unlikely that a charged molecule such as cysteine can penetrate the membrane and remove the nitrobenzofurazan group from the ATPase. This could again lead to an underestimate of the ATPase content of the membrane, but is probably not a major difficulty in the present work, in which particles that had been prepared by a relatively harsh procedure were used. We have no way of assessing the leakiness of our membranes, but it is generally accepted that preparations of this type are predominantly orientated with the ATPase facing the medium (Racker, 1970), so that we think that the problem of reagent permeability is unlikely to be significant.

The good correlation between the extent of ATPase

inhibition and amount of *N*-nitrobenzofurazan released on treating the particles with cysteine (Table 2) suggests that all the nitrobenzofurazan groups released from the membrane originate from the modified ATPase. The reliability of the extinction coefficient for *N*-nitrobenzofurazan-cysteine was confirmed by using *N*-acetylcysteine to remove the nitrobenzofurazan group from the ATPase. Measurement of the *S*-nitrobenzofurazan-*N*-acetylcysteine also produced an estimate that 8.5% of the membrane protein was ATPase. A molar extinction coefficient of $13\,000\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ was used for *S*-nitrobenzofurazan-*N*-acetylcysteine (Birkett *et al.*, 1970).

An important question is whether there should be a simple stoichiometric relationship between the ATPase and cytochrome *bc*₁ complex. The mode of energy transfer between these two membrane components may depend on whether there are specific functional relationships between particular cytochrome *bc*₁ and ATPase complexes. Such relationships seem more probable if the two components are present in equal amounts. These relative stoichiometries also have obvious implications for the biosynthesis of the membrane.

The method that we developed for titrating the ATPase can also be applied to other membranes. For instance, the ATPase of the chloroplast thylakoid membrane could be measured in this way along with the ATPase of the plasma membranes of bacteria. Both of these classes of enzymes resemble the mitochondrial ATPase in having a tyrosine residue that is reactive towards Nbf-Cl (Deters *et al.*, 1975; Cantley & Hammes, 1975; Ferguson *et al.*, 1974b). Cox *et al.* (1973) have described a mutant strain of *Escherichia coli*, AN 295, which has a derepressed plasma-membrane ATPase. The Nbf-Cl titration method could be of value in distinguishing between increased synthesis of the ATPase protein and decreased synthesis of the ATPase-inhibitor protein (Smith *et al.*, 1975), both of which would lead to a higher ATPase activity.

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