Biochimica et Biophysica Acta, 503 (1978) 135—154

BBA 47520

AFFINITY CHROMATOGRAPHY OF H^+-TRANSLOCATING ADENOSINE TRIPHOSPHATASE ISOLATED BY CHLOROFORM EXTRACTION OF *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES

MODIFICATION OF BINDING AFFINITY BY DIVALENT CATIONS AND ACTIVATING ANIONS

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(Received November 14th, 1977)

Summary

1. ATPase isolated from *Rhodospirillum rubrum* by chloroform extraction and purified by gel filtration or affinity chromatography shows three bands (α, β and γ) upon electrophoresis in sodium dodecyl sulphate.

2. Ca^{2+}-ATPase activity of the preparation is inhibited by aurovertin and efrapeptin but not by oligomycin. Activity may be inhibited by treatment with 4-chloro-7-nitrobenzofurazan and subsequently restored by dithiothreitol.

3. The enzyme fails to reconstitute photophosphorylation in chromatophores depleted of ATPase by sonic irradiation.

4. Most of the active protein from the crude chloroform extract binds to an affinity chromatography column bearing an immobilised ADP analogue but not to a column bearing immobilised pyrophosphate.

5. In the absence of divalent cations, a component with a very high specific activity for Ca^{2+}-ATPase is eluted from the column by 1.6 mM ATP. This protein migrates as a single band on 5% polyacrylamide gel electrophoresis and only possesses three subunits. At 12 mM ATP an inactive protein is eluted which does not run on acid or alkali polyacrylamide gels and shows a complex subunit structure.

6. ATPase preparations prepared by acetone extraction or by sonic irradiation of chromatophores may also be purified 10-fold by affinity chromatography.

7. The inclusion of 5 mM $MgCl_2$ or $CaCl_2$ during affinity chromatography

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Abbreviations: FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; Bchl, bacteriochlorophyll.

of chloroform ATPase increases the capacity of the column for the enzyme and demands a higher eluting concentration of ATP.

8. When the enzyme is more than 90% inhibited by efrapeptin or 4-chloro-7-nitrobenzofurazan, the binding characteristics of the enzyme are not affected.

9. 10 mM Na_2SO_3, which greatly stimulates the Ca^{2+}- and Mg^{2+}-dependent ATPase activity of the enzyme and increases K_i (ADP) for Ca^{2+}-ATPase from 50 to 850 μM, prevents binding to the affinity column. Binding may be restored by the addition of divalent cations.

10. Na_2SO_3 increases the rate of ATP hydrolysis, ATP-driven H^+ translocation and ATP-driven transhydrogenase in chromatophores.

11. It is proposed that anions such as sulphite convert the chromatophore ATPase into a form which is a more efficient energy transducer.

Introduction

A five-subunit ATPase which functions as a coupling factor for photophosphorylation may be dislocated from chromatophore membranes of several species of photosynthetic bacteria [1—4]. The structure and enzymic properties of chromatophore ATPase are strikingly similar to the equivalent proteins isolated from mitochondria, chloroplasts and other bacteria. The mechanism by which these ATPases may utilise the free energy made available by the electron transport reactions in the synthesis of ATP is still a matter for speculation, although a common, underlying mechanism for different membrane systems is generally acknowledged. Two problems which have recently received attention and which are pertinent to the data presented in this paper are (a) the functional significance of the individual subunits and (b) the nucleotide binding affinities of the enzyme.

The primary aim of these experiments was to investigate the nucleotide binding properties of *Rhodospirillum rubrum* ATPase by affinity chromatography. The usual preparatory methods for this enzyme involve either a sonication/pressure cell step or extraction of an acetone powder made from isolated chromatophores [1—4]. In both cases, uncontaminated enzyme may only be obtained after lengthy purification procedures. We have therefore adopted the chloroform extraction technique developed by Beechey et al. [5], for mitochondrial ATPase. With only minor modifications a high yield of a rather pure chromatophore ATPase may be easily isolated. This method has been reported to give rise to either a five-subunit [6] or a three-subunit [5] ATPase from beef heart mitochondria and a four- or five-subunit enzyme from chloroplasts [7]. With *Rhs. rubrum* the analogous protein possesses only the α, β and γ subunits when isolated by chloroform extraction.

As a method for studying the nucleotide binding properties of this kind of ATPase, affinity chromatography using immobilised nucleotide analogues appeared as an attractive possibility. Hulla et al. [8] have used an ATP analogue to purify F_1 ATPase from *Micrococcus* Sp ATCC 398E. We have used the carefully characterised ADP and pyrophosphate derivatives described by Trayer et al. [9] with the chloroform ATPase preparation from *Rhs. rubrum*. The

advantage of these ligands is their quite similar structure to ADP and pyrophosphate and the simplicity of the six-carbon spacer arm. The versatility of this method is such that we may study nucleotide affinities under conditions in which the enzyme activity is modified by divalent cations and by specific inhibitors and activators. In particular we have concentrated our efforts on those anions which stimulate ATP hydrolysis in the enzyme isolated from *Rhs. rubrum* [10] (and the analogous enzyme from mitochondria [11—15], chloroplasts [16] and yeast [17]). The anions are especially interesting since they appear to restore the properties of the solubilised enzyme to those which are normally associated with the membrane-bound system [10].

Materials and Methods

Preparation techniques. Wild-type *Rhs. rubrum* and *Rhodopseudomonas capsulata* were grown in the light and chromatophores were prepared by mechanical disruption with glass beads in 50 mM KCl/50 mM tricine/8 mM $MgCl_2$/10% sucrose at pH 7.4 as previously described [18]. If no further preparatory work was required the final chromatophore pellet was resuspended in 50 mM tricine/10% sucrose, pH 7.4.

For preparation of the chloroform ATPase from *Rhs. rubrum*, the chromatophore fraction was washed once in 10 mM Tris/sulphate/1 mM EDTA, pH 7.6, and the procedure of Beechey et al. [5] as described by Webster et al. [10] was followed without modification. The protein solution, after dialysis and the addition of 10% glycerol, is henceforth called the crude chloroform ATPase. In some cases the enzyme solution was concentrated up to 5 mg/ml without appreciable loss of activity by removal of water with Aquacide before the glycerol step. Storage properties were greatly improved in the presence of glycerol. The enzyme stored at 4°C was used for periods of up to 2 weeks with no significant loss of activity. In the absence of glycerol the enzyme lost 70% of its initial activity over a 2 week period.

For preparation of acetone-powder ATPase, the *Rhs. rubrum* chromatophores were washed once in 50 mM tricine/10% sucrose, pH 7.4. The method of Johansson et al. [3] was followed up to and including the ammonium sulphate fractionation step.

Assays. Bacteriochlorophyll concentrations in the *Rhs. rubrum* chromatophores were measured using the in vivo extinction coefficient $\epsilon_{880} = 140\ mM^{-1} \cdot cm^{-1}$ given by Clayton [19]. The bacteriochlorophyll from *Rps. capsulata* was estimated from the absorption ($\epsilon_{772} = 75\ mM^{-1} \cdot cm^{-1}$) of a methanol/acetone extract. Protein was determined by a turbidometric assay [20]. ATPase was most conveniently assayed by the colorimetric determination of P_i release as described by Edwards and Jackson [18]. The reaction was quenched after 3—5 min with trichloroacetic acid and phosphate concentration was measured with ammonium molybdate/ferrous sulphate. In some cases the ATPase rate was assayed by measuring H^+ production by the glass electrode technique [21].

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out as described in ref. 5 in 5% (w/v) gels but in the absence of ATP and EDTA. For determination of subunit composition and molecular weight,

protein was denatured in sodium dodecyl sulphate in the presence of mercaptoethanol [22] and run on 10% (w/v) gels containing 0.1% sodium dodecyl sulphate. The gels were calibrated with bovine serum albumin, trypsin, pepsin and cytochrome *c* and stained with Coomassie Blue.

ATPase activity was detected in 5% acrylamide gels run as described above and using the staining method of Wachstein and Meisel [23]. ATPase was assayed at 30°C after 10 min in a medium containing 50 mM Tris/sulphate/5 mM Na_2SO_3/5 mM $CaCl_2$/2.5 mM ATP, pH 7.5.

Affinity chromatography. The synthesis of 8-(6-aminohexyl)amino-ADP and 6-aminohexan-1-ol pyrophosphate and coupling of the ligands to cyanogen bromide-activated Sepharose 4B was carried out as described by Trayer et al. [9]. The amount of ligand coupled to the gel appeared to be rather critical. The columns used in the present experiments contained 2.2 μmol 8-(6-aminohexyl)amino-ADP/g of packed wet weight of Sepharose. At ligand concentrations less than 1 μmol/g, the ATPase preparations failed to bind under the conditions we employed. The ligand-bearing Sepharose was packed into a 0.8 × 8 cm column and equilibrated with the appropriate buffer (see figure legends). The ATPase preparations usually containing up to about 0.5 mg protein in a volume of about 1.0 ml were applied directly to the column at room temperature and eluted with buffer at a flow rate of 0.2 ml/min. At least three void volumes of the column were eluted before applying step or gradient eluant. Nucleotides were applied either as a single linear gradient (0—20 mM) or as a double, linear gradient (0—6 and 6—20 mM).

At the end of an experiment the column was washed with 2 M KCl and 6 M deionised urea and stored at 4°C in 0.02% NaN_3. The column material aged after 10—20 experiments (judged by elution of ATPase activity within the void volume) and was discarded.

Spectroscopic studies. Spectral changes of the endogenous carotenoid pigments of *Rhs. rubrum* chromatophores were monitored with an optically chopped dual wavelength spectrophotometer at 530—508 nm. Flash-induced changes in *Rps. capsulata* were measured with a two-photomultiplier dual wavelength spectrophotometer at 528—512 nm and signal averaged over 16 flashes at 0.033 Hz.

The fluorescence quenching of 9-aminoacridine was measured at 90°C using the filter combination described by Melandri et al. [24].

Inhibitors. Efrapeptin was a generous gift from Dr. D.E. Griffiths, University of Warwick and aurovertin from Dr. R.B. Beechey, Shell Research, Sittingbourne. 4-chloro-7-nitrobenzofurazan was purchased from Aldrich, Gillingham, Dorset, oligomycin and FCCP from Sigma, London.

Chemicals. ADP was purchased from Boehringer, Mannheim, ATP from Kyowa Hakko Kogyo Ltd., Tokyo, Sepharose 4B and 6B from Pharmacia. Other reagents were of analytical grade.

Results

Subunit structure of the chloroform ATPase

When subjected to electrophoresis on a 5% polyacrylamide gel, crude aqueous extract of ATPase (specific activity routinely 3 μmol ATP/mg protein

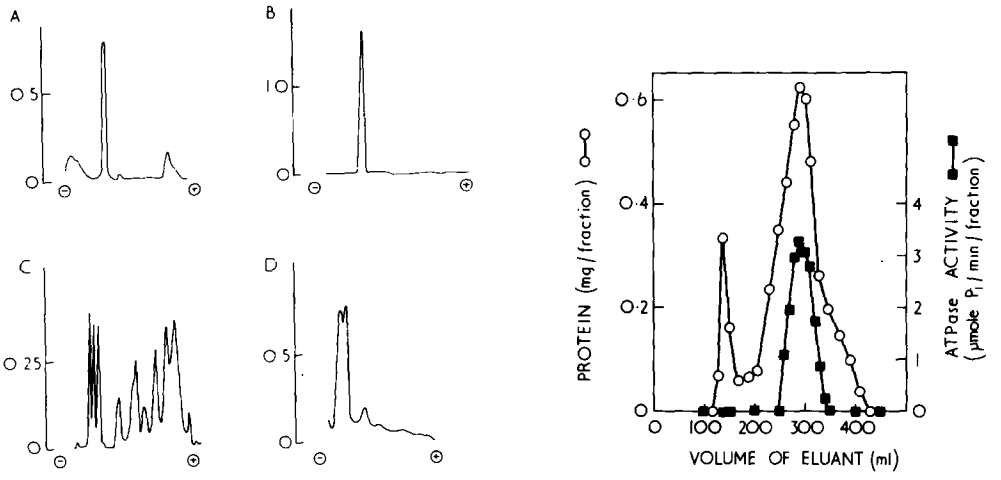

Fig. 1. Gel electrophoresis of chloroform ATPase from *Rhs. rubrum*. A and B, 5% polyacrylamide gel electrophoresis of crude enzyme (A) and enzyme purified by affinity chromatography (B). The crude sample contained 50 μg protein and the purified sample 70 μg. C and D, 10% polyacrylamide gel electrophoresis of 50 μg crude enzyme (C) and 70 μg of enzyme purified by affinity chromatography (D) in the presence of sodium dodecyl sulphate.

Fig. 2. Purification of chloroform ATPase from *Rhs. rubrum* by gel filtration on Sepharose 6B. Crude chloroform ATPase, containing 8.7 mg protein (specific activity 2.1 μmol ATP/mg protein per min) was applied to a 95 X 2.5 cm column of Sepharose 6B, pre-equilibrated with 10 mM Tris/sulphate/1 mM EDTA, pH 7.6. The column was run in the same buffer at 56 ml/h and 10-ml fractions were collected. 0.15-ml aliquots were assayed for ATPase activity by P_i release after 6 min at 37°C in a 2.0 ml medium containing 10 mM Tris sulphate/1 mM EDTA/5 mM $CaCl_2$/2.5 mM ATP, pH 7.6.

per min) released after chloroform treatment shows a single major band after staining with Coomassie Blue (Fig. 1). Some protein does not run into the gel. Following a chromatography step on Sepharose 6B (Fig. 2) the specific activity of the enzyme is increased 2—3 fold. No increase in total activity is observed. This protein shows a similar 5% polyacrylamide gel electrophoresis pattern to the crude enzyme but with less protein in the minor bands. A further 2-fold purification, judged by the increasing specific activity may be achieved by affinity chromatography (see later section) but only a small change in the gel electrophoresis profile is observed. The purification procedures predominantly remove the protein component(s) which in the crude enzyme are immobile during gel electrophoresis. Small samples of crude enzyme may be purified to high specific activity with the affinity chromatography step alone (routinely 15—30 μmol ATP/mg protein per min). Gels of the crude chloroform ATPase stained for ATPase activity showed a single band coincident with the Coomassie Blue stain.

If the enzyme, purified by affinity chromatography, is denatured by sodium dodecyl sulphate and fractionated by electrophoresis, three bands of molecular weight 55 000, 50 000 and 32 000 may be distinguished (Fig. 1). These molecular weights correspond to the α, β and γ subunits of mitochondrial ATPase and acetone powder ATPase from *Rhs. rubrum* [25]. The proportion of γ subunits, estimated by stain intensity is rather less than that described for the purified, five-subunit enzyme [25]. Sodium dodecyl sulphate-polyacryl-

amide electrophoresis of the crude chloroform enzyme showed numerous other bands in addition to those described for the purified enzyme. These components must be derived from that fraction of the crude enzyme which does not migrate on the 5% polyacrylamide gel (see also the section on affinity chromatography of the crude chloroform enzyme).

We have consistently failed to reconstitute chloroform ATPase with ATPase-depleted chromatophore membranes [1,26]. The latter were prepared by sonication in the presence of 1 mM EDTA and routinely manifested photophosphorylation rates between 20 and 30% of the native chromatophores. Incubation of resolved chromatophores in the presence of $MgCl_2$ with either sonic supernatant or with ATPase prepared from an acetone extract led to complete restoration of photophosphorylation. Under similar circumstances however, chloroform ATPase, either the crude preparation or after Sepharose or affinity chromatography, did not give rise to any increase in the rate of photophosphorylation in the resolved system, even with amounts of protein considerably in excess of that removed by sonication.

Solution kinetics of the chloroform ATPase

The ATPase activity of the chloroform preparation is Ca^{2+} dependent and is competitively inhibited by free Mg^{2+} [10]. In this respect it is similar to the five-subunit (F_1) ATPase isolated from acetone powders of *Rhs. rubrum* chromatophores [3]. Mg^{2+}-ATP may in fact serve as a substrate, for the enzyme (see also ref. 4) but owing to extreme sensitivity to inhibition by free Mg^{2+} [10,18] only low rates may be measured and only when ATP is in excess over Mg^{2+}. The addition of activating anions such as sulphite leads to an increased

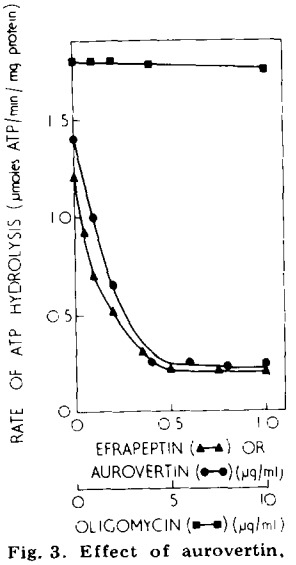

Fig. 3. Effect of aurovertin, efrapeptin and oligomycin on ATPase activity in the crude, chloroform enzyme from *Rhs. rubrum*. ATPase activity was assayed by P_i release in a 2.0 ml medium containing 10 mM Tris/sulphate/1mM EDTA/ 5 mM $CaCl_2$/2.5 mM ATP, pH 7.6, for 5 min at 37°C. In the aurovertin experiment, the enzyme contained 74 μg protein, in the efrapeptin experiment, 50 μg and in the oligomycin experiment 85 μg.

rate of Ca^{2+}-ATPase and an apparent unmasking of Mg^{2+}-ATPase due to a decreased inhibition by free Mg^{2+} [10]. The crude chloroform preparation does not hydrolyse inorganic pyrophosphate, even though the parent chromatophore suspension did show high pyrophosphatase activity.

Fig. 3 shows that the Ca^{2+}-ATPase activity is inhibited by the usual F_1 inhibitors such as aurovertin (50% inhibition of 75 μg enzyme by 0.33 μg/ml) and efrapeptin (50% inhibition of 50 μg enzyme by 0.14 μg/ml) but not by oligomycin (up to 10 μg/ml). Methanol and not ethanol was used as a solvent for these inhibitors since in contrast to the chromatophore system ethanol significantly inhibits ATPase activity even at concentrations less than 1%. Interestingly, chloroform is also a potent but reversible inhibitor, hence the dialysis step in the preparation procedure [10].

Treatment of the ATPase with 4-chloro-7-nitrobenzofurazan for several hours leads to an almost complete loss of ATPase activity which may be restored by the addition of dithiothreitol (Table I). This agent also inhibits mitochondrial [27], bacterial [28] and chloroplast [29] F_1-type ATPases and the case has been argued that the reaction involves an especially nucleophilic tyrosine residue in one of the β subunits [27,29].

Affinity chromatography of the chloroform ATPase

The elution profile of crude chloroform ATPase on 8-(6-aminohexyl)amino-ADP-Sepharose 4B in the absence of divalent cation is shown in Fig. 4. A low (less than 20%) and variable amount of protein passes straight through the column in the void volume. Unless the column capacity is saturated (applied protein more than 1 mg) then no ATPase is detectable in these protein fractions. When buffer is continuously applied, then further protein drifts off the column at only a very low rate, no ATPase activity was detected after elution by 20 times the void volume of the column. Following the application of a concentration gradient of ATP to the column, two sharp protein peaks may be detected in the eluate. The first, at approx. 1.6 mM ATP, manifests Ca^{2+}-

TABLE I

INHIBITION OF CHLOROFORM ATPase FROM *Rhs. rubrum* BY 4-CHLORO-7-NITROBENZOFURAZAN

Two incubations (20°C) were set up containing, in 1.0 ml, 10 mM Tris/sulphate, 1 mM EDTA, 10% glycerol, pH 7.6, and chloroform ATPase containing 1.43 mg protein. The contents of one sample were treated with 0.25 mM 4-chloro-7-nitrobenzofurazan. Samples (38 μg protein) were periodically removed and assayed for ATPase activity for 6 min at 37°C in a medium containing 10 mM Tris/sulphate, 1 mM EDTA, 5 mM $CaCl_2$, 2.5 mM ATP, ±1 mM dithiothreitol, pH 7.6.

Incubation time (min)	Specific activity (μmol ATP/mg protein per min)			
	Untreated	Treated	Untreated plus dithiothreitol	Treated plus dithiothreitol
15	1.48	0.87	1.48	1.48
30	1.48	0.37	1.44	1.44
60	1.48	0	1.48	1.48
150	1.39	0	1.54	1.26
300	1.48	0	1.48	1.15

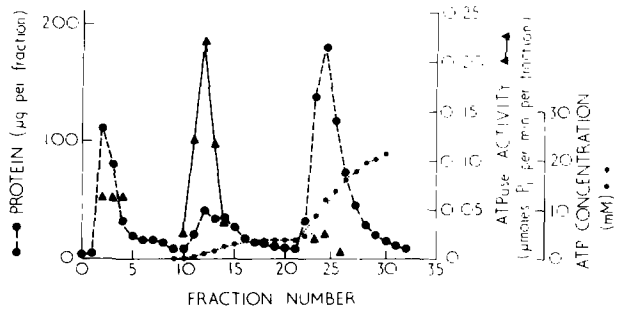

Fig. 4. Affinity chromatography of the chloroform ATPase from *Rhs. rubrum*. Elution by ATP. Crude chloroform ATPase, containing 1.4 mg protein (specific activity 3 μmol ATP/mg protein per min) was applied to a 8 × 0.8 cm column of 8-(6-aminohexyl)amino-ADP-Sepharose 4B pre-equilibrated with 10 mM Tris/sulphate/1 mM EDTA, pH 7.6, at room temperature (20°C). The column was then washed with 20 ml of the same buffer at 16.5 ml/h. At fraction 10, 20 ml of a linear concentration gradient (0—5 mM) of ATP and at fraction 16, 50 ml of a similar gradient (4—30 mM) were applied keeping a constant flow rate and constant buffer concentration. The ATP concentration in the 3.3-ml eluted fractions was estimated from the A_{259nm}. ATPase activity was measured as described in Fig. 2, using a 0.2 ml aliquot of each fraction. Zero time blanks were run for each fraction. The ATPase activity of fraction 12 was 30 μmol ATP/mg protein per min.

dependent ATPase (about 30 μmol ATP/mg protein per min in the peak fractions). All the applied Ca^{2+}-ATPase activity may be recovered in this protein peak. The second protein peak, eluted by 12 mM ATP, does not possess either Ca^{2+}- or Mg^{2+}-ATPase activity. It does not migrate on either acid or alkaline polyacrylamide gels and shows complex structure on sodium dodecyl sulphate-polyacrylamide gels (not shown). Subunits with equivalent mobilities to the α, β and γ bands and about six other bands may be detected.

The crude chloroform ATPase does not bind to a column of hexylamine-Sepharose 4B suggesting that interaction is specific for the ADP moiety. A column composed of 6-aminohexan-1-ol pyrophosphate-Sepharose 4B failed to bind the active ATPase component which was detected in the void volume but did retard the inactive protein fraction until the ATP gradient was applied (eluting concentration 12 mM). Chromatography on such columns bearing immobilised pyrophosphate groups does give rise to considerable purification owing to the retention of inactive material, the specific activity of the ATPase fraction is increased to around 25 μmol CaATP/mg protein per min.

The protein from the crude chloroform preparation bound to a 8-(6-aminohexyl)amino-ADP-Sepharose 4B column may also be eluted by an ADP concentration gradient (Table II). Active ATPase and inactive protein are eluted by similar concentrations to those of ATP. After elution by ADP, which competitively inhibits ATPase activity, assays were performed after dilution of the column fractions and in the presence of 10 mM Na_2SO_3 in order to optimise the rates [10]. A gradient of potassium phosphate also elutes bound protein from 8-(6-aminohexyl)amino-ADP-Sepharose 4B but in this case the elution profile resembles that due to elution by salt. High concentrations of phosphate or KCl are required and no clear protein peaks are detectable in the eluted fractions. Rather the protein (inactivated by high salt) leaches from the column across a wide concentration of eluant (see Fig. 5).

TABLE II

AFFINITY CHROMATOGRAPHY OF CHLOROFORM ATPase FROM *Rhs. rubrum* ; ELUTION BY ADP

Conditions as Fig. 4, except that the flow rate was 13.8 ml/h and 2.3-ml fractions were collected. ATPase activities in 0.1-ml aliquots were measured in the medium described in Fig. 2 except that 10 mM Na_2SO_3 was added to minimise inhibition by ADP. The specific activity of the applied enzyme in the absence of sulphite was 2.5 μmol CaATP/mg protein per mg.

	Applied enzyme	Protein eluted during column washing	Peak 1	Peak 2	Peak 3
Eluting conditions	—	—	1.9 mM ADP	11 mM ADP	2 M KCl
Protein (mg)	1.3 (total)	0.33 (total)	0.133 (peak fraction)	0.422 (peak fraction)	0.15 (peak fraction)
Specific activity Ca^{2+} ATPase (μmol/mg protein per min)	5.65	7.0	38	0	0

We have investigated the effects of divalent cations on the binding affinity of the chloroform enzyme for 8-(6-aminohexyl)amino-ADP-Sepharose 4B. Note that since Mg^{2+} normally inhibits the ATPase activity, the assay solutions (not the column buffer) were supplemented with 10 mM Na_2SO_3 [10]. Since CaATP is rapidly hydrolysed by the enzyme preparation, the fractions collected in the presence of $CaCl_2$ were quenched in 10 mM EDTA after elution from the column. Two effects of divalent cations on the elution profile may be distinguished. First, the amount of active enzyme which passes through the column in the washings is lower in the presence of either $CaCl_2$ or $MgCl_2$. This effect is very much more dramatic in "aged" columns (see Materials and Methods). For instance in some experiments with such columns only 30% of the applied enzyme binds in the absence of divalent cations compared with virtually complete binding on the same column in the presence of either Ca^{2+} or Mg^{2+}. Second, the concentration of ATP which is required to elute active ATPase is significantly higher in the presence of these divalent cations (4.5 mM ATP with 5 mM column $CaCl_2$ and 4.0 mM ATP with 5 mM $MgCl_2$) than in their absence (routinely 1.5—1.8 mM ATP).

This technique clearly has application to the study of nucleotide binding to ATPase under conditions in which the catalytic properties of the enzyme are modified by specific inhibitors or general protein reagents. As an example, the data of Fig. 6 show that when the chloroform enzyme from *Rhs. rubrum* is completely inactivated by incubation with 4-chloro-7-nitrobenzofurazan, the binding affinity for 8-(6-aminohexyl)amino-ADP-Sepharose 4B is unchanged. The protein elution profile by an ATP gradient is similar to that observed with the untreated enzyme but no activity was detected in any of the fractions. The ATPase activity of the protein fraction eluted by 1.6 mM ATP may be restored by including 1 mM dithiothreitol in the assay solution.

The addition of efrapeptin to the eluting buffer at a concentration sufficient to completely inhibit ATPase activity failed to dislodge the enzyme from a 8-(6-aminohexyl)amino-ADP-Sepharose column (results not shown). Moreover,

when chloroform ATPase, completely inhibited by efrapeptin, was applied to the affinity column the binding was quite normal and ATP at 1.6 mM released a protein with about 50% of the usual activity. The partial recovery of activity is probably due to the fact that efrapeptin does not bind tightly to chromatophore ATPase in the absence of nucleotide (Webster, G.D. and Edwards, P.A., unpublished observations).

Affinity chromatography of the five-subunit ATPase of Rhs. rubrum

The five-subunit ATPase prepared by extraction from acetone powders of *Rhs. rubrum* chromatophores and purified as far as the ammonium sulphate fractionation step also binds to a column of 8-(6-aminohexyl)amino-ADP-Sepharose 4B (Fig. 7). Less than 20% of the applied protein is eluted in the column washing. Upon application of a linear concentration gradient of ATP, protein is eluted from the column. As with the chloroform enzyme, the elution profile is modified by the presence of divalent cation in the eluant. In the absence of divalent cation, an inactive protein is eluted by 1 mM ATP. ATPase is eluted as a protein shoulder at 2 mM ATP. The specific activity of the most active fractions using CaATP as substrate was 4 μmol/mg protein per min. When 5 mM $CaCl_2$ is included in the column buffer both protein and ATPase activity are eluted as a single peak by 5.5 mM ATP. Typical data are shown in Fig. 7. Inclusion of $MgCl_2$ in the column buffer also leads to a single protein peak eluting at about 5 mM ATP (not shown).

The "coupling factor" supernatant obtained after sonication of chromatophores in EDTA [26] (specific activity 0.11 μmol CaATP/mg protein per min) may also be purified by affinity chromatography (Fig. 8). A large fraction of the protein with very low specific activity is eluted from the column within the void volume. This of course is to be expected owing to the relative impurity of this preparation. After application of a linear ATP gradient, protein is eluted

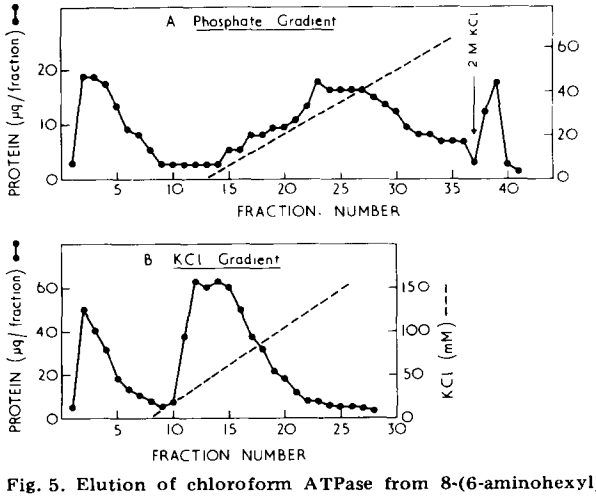

Fig. 5. Elution of chloroform ATPase from 8-(6-aminohexyl)amino-ADP by KCl, and potassium phosphate. Conditions as Fig. 4 with the following modifications: applied protein, 0.7 mg (A and B); flow rate, 12 ml/h (A) and 11.2 ml/h (B); specific activity of applied enzyme, 1.5 μmol ATP/mg protein per min (A) and 1.6 μmol ATP/mg protein per min (B); fraction volume, 2.7 ml (A) and 3.0 ml (B); gradient, 80 ml, linear, 0—100 mM, potassium phosphate, measured with ferrous sulphate/ammonium molybdate (A) and 170 ml, linear, 0—0.5 M KCl, measured conductimetrically (B).

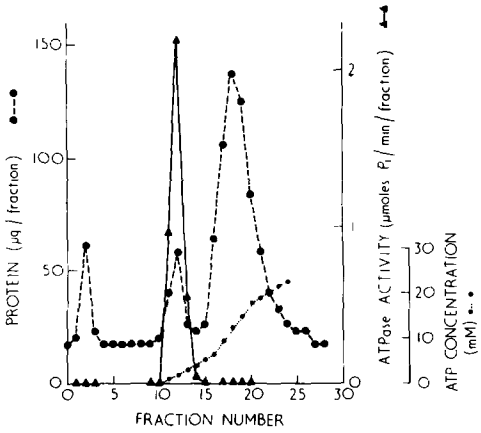

Fig. 6. Affinity chromatography of chloroform ATPase treated with 4-chloro-7-nitrobenzofurazan. Conditions as Fig. 4 except that the applied enzyme (1.7 mg) of original specific activity 2 μmol ATP/mg protein per min had been incubated overnight at 4°C in the presence of 0.6 mM 4-chloro-7-nitrobenzofurazan, thereby reducing ATPase activity to zero. Minor modifications from Fig. 4: flow rate, 13.3 ml/h; fraction volume, 2.2 ml; a linear (0—6 mM) ATP gradient applied at fraction 10 and a linear (6—30 mM) ATP gradient at fraction 22. When ATPase was assayed on 0.1-ml aliquots under the conditions described in Fig. 2, no activity was detected in any of the eluted fractions. When the assays were performed in the presence of 1 mM dithiothreitol, ATPase was detected as shown in the figure.

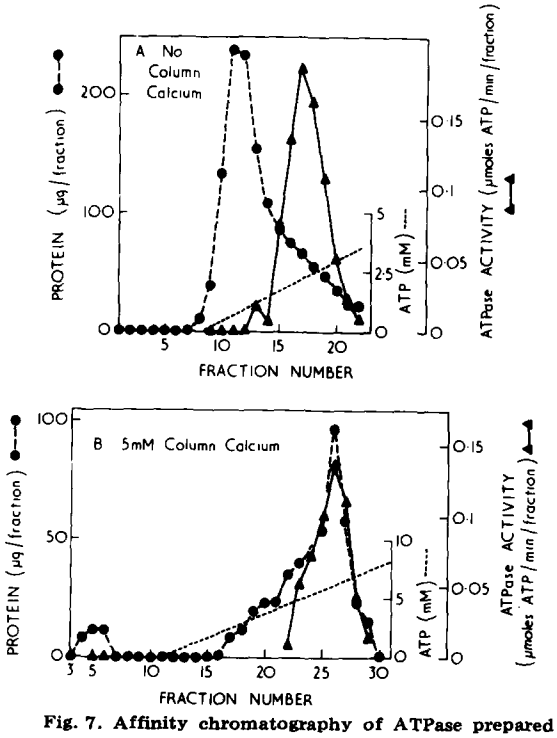

Fig. 7. Affinity chromatography of ATPase prepared from acetone powder extraction of *Rhs. rubrum* chromatophores. (A) Acetone powder ATPase, purified up to and including $(NH_4)_2SO_4$ precipitation [3], containing 1.5 mg protein and of specific activity 0.5 μmol ATP/mg protein per min, was applied to a 8-(6-aminohexyl)amino-ADP affinity chromatography column, pre-equilibrated with 10 mM tricine/1 mM EDTA, pH 7.6. After washing the column with a similar buffer, at 15 ml/h and collecting 1.3-ml fractions, a single, linear concentration gradient (50 ml from 0—10 mM) ATP was applied at fraction 8. ATPase activity in 200-μl aliquots of the eluted fractions was measured as described in Fig. 2 except that tricine replaced Tris/sulphate. (B) As A but using 0.5 mg applied protein and including 5 mM $CaCl_2$ in the column buffer and elution gradient. Each fraction was collected in 10 mM EDTA to quench the ATPase activity. In the ATPase assay, the $CaCl_2$ concentration was increased to 10 mM.

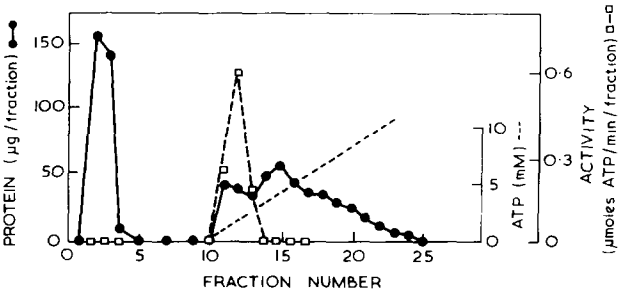

Fig. 8. Affinity chromatography of ATPase prepared by sonic irradiation of *Rhs. rubrum* chromatophores. The enzyme, prepared by 90 s sonication (see ref. 26), had a specific activity of 0.11 μmol CaATP/mg protein per min. An amount containing 1.08 mg was applied to a column as described in Fig. 7 except that the flow rate was 16.5 ml/h and 3.3-ml fractions were collected. At fraction 10 a linear, ATP concentration gradient (50 ml, 0—20 mM) was applied. 1.0-ml aliquots of the eluted fractions were assayed for ATP hydrolysis as decribed in Fig. 7A.

in two broad peaks centred around 1.3 and 8.4 mM ATP and ATPase is detected in a sharp band at 3.25 mM ATP (absence of calcium). The specific activity was 1.0 μmol/mg protein per min. This procedure clearly results in extensive purification but specific activities after affinity chromatography are still much lower than those obtained with purified chloroform or acetone powder ATPase.

Effects of activating anions on nucleotide binding affinity of the chloroform ATPase

It has been shown that the change in properties produced by solubilising the chromatophore ATPase may also be observed in the membrane-bound enzyme in the presence of high concentrations of uncoupling agent [10]. The anions, which have been shown to be effective in stimulating mitochondrial ATPase [11—14] particularly sulphite and selenite, cause a reversal of this change in properties [10], viz. the kinetic parameters characteristic of tightly coupled chromatophore ATPase may be reproduced in solubilised ATPase or in uncoupled chromatophores by including sulphite in the assay medium. Moyle and Mitchell [13] have noted a considerable increase in K_i (ADP) of mitochondrial ATPase in the presence of sulphite. We have now observed a similar change (from 50 to 850 μM ADP) induced by sulphite in Ca^{2+}-dependent chromatophore chloroform ATPase. The data are included in Table III which summarises the properties of the two different kinetic states of the chromatophore ATPase.

In view of the apparent change in affinity of the enzyme for ADP in the presence of sulphite, we have investigated the effects of this anion on the binding affinity of chloroform ATPase to immobilised ADP by affinity chromatography. The results are shown in Fig. 9. When sulphite was included in the column buffer in the absence of divalent cations (Fig. 9A) almost all of the applied ATPase activity passed straight through the column and was recovered in the washings. Very little protein and activity were eluted by 1—2 mM ATP, the concentration which, in the absence of sulphite, eluted the active ATPase (Fig. 4). The inactive protein, eluting at about 12 mM ATP was unaffected by the inclusion of sulphite.

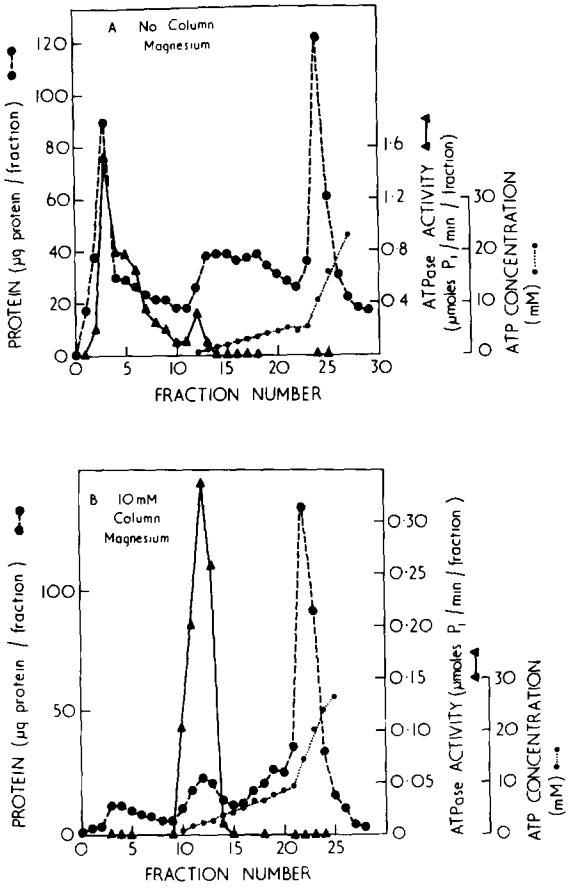

Fig. 9. Affinity chromatography of chloroform ATPase from *Rhs. rubrum* in the presence of sulphite. (A) Conditions as Fig. 4 except that 1.2 mg protein were applied to the column, the running buffer additionally contained 10 mM Na_2SO_3, the flow rate was 13 ml/h and 2.6-ml fractions were collected. ATPase activities in 0.1-ml aliquots of each fractions were measured in the presence of 10 mM Na_2SO_3. (B) Conditions as Fig. 4 except that 0.8 mg protein were applied to the column, the running buffer additionally contained 10 mM Na_2SO_3 and 10 mM $MgCl_2$, the flow rate was 13.2 ml/h and 1.76-ml fractions were collected. ATPase activities in 0.2-ml aliquots of each fraction were measured in the medium described in Fig. 2 but in the presence of 10 mM Na_2SO_3 and with 10 mM $MgCl_2$ instead of the $CaCl_2$.

When the experiment was repeated with 5 mM $MgCl_2$ in the column buffer as well as Na_2SO_3, then the binding of the chloroform ATPase to the immobilised ADP was restored. The active ATPase fraction was recovered in the eluate when the ATP concentration reached 2.5 mM and inactive protein was eluted at 14 mM ATP.

Improved energy transfer efficiency of ATP hydrolysis by sulphite

Membrane energisation by ATP and by light were followed by the quenching of fluorescence of 9-aminoacridine and by the absorption changes associated with the endogenous carotenoid pigments. The addition of ATP to a

TABLE III

PROPERTIES OF THE TWO STATES OF *Rhs. rubrum* ATPase

	Chromatophore membranes no uncoupler (E′) *	Crude chloroform enzyme + 10 mM Na_2SO_3 (E′) *	Chromatophore membranes + 16 μM FCCP (E″) *	Crude chloroform enzyme, no sulphite (E″) *
K_m (CaATP)	0.4 mM **	0.25 mM **	1.3 mM **	1.3 mM **
Inhibition of Ca^{2+}-ATPase by 5 Mg^{2+}	less than 5% ***	less than 5% ***	more than 90% ***	more than 90% †
V (apparent for Mg^{2+}-ATPase	0.5 μmol ATP/ μmol BChl per min ***	4 μmol ATP/ mg protein per min **	0.1 μmol ATP/ μmol BChl/ per min ***	0.2 μmol ATP/ mg protein per min **
Inhibition of Mg^{2+}-ATPase by free Mg^{2+} (5 mM)	less than 5% ***	less than 5% **	more than 90% ***	more than 90% ††
K_i (ATP^{4-}) for Ca^{2+}-ATPase	—	0.4 mM †††	—	1.0 mM †††
K_i (ADP) for Ca^{2+}-ATPase	—	850 μM ‡	—	50 μM

* See Discussion.
** See ref. 10.
*** See ref. 18.
† See refs. 18 and 3.
†† See ref. 4 and this work.
††† See ref. 45.
‡ Ca^{2+}-ATPase was assayed under the conditions outlined in Fig. 2 in the presence of varying concentration of ATP and ADP and the K_i (ADP) values were determined from a Dixon plot of the results.

chromatophore suspension in the presence of 9-aminoacridine gives rise to a fluorescence quenching which has been ascribed to the formation of a pH gradient across the membrane vesicles [24]. The half-time of the reaction is of the order of 25 s. The reaction is accelerated by concentrations of sulphite which increases the rate of ATP hydrolysis under similar conditions (Figs. 10A and B). Sulphite did not affect the fast artifactual change in fluorescence which accompanies the ATP addition, in the presence of efrapeptin. The fluorescence quenching and the ATPase reaction were inhibited by low concentrations of the F_1-inhibitor efrapeptin in the presence and absence of sulphite.

ATP also produces an apparent red shift in the absorption spectrum of the chromatophore carotenoids [30] which is most likely in response to the formation of a membrane potential through the electrogenic H^+ pumping of the ATPase [31]. Fig. 10C shows that the half-time of the reaction is decreased from approx. 30 s to less than 2 s in the presence of mM concentrations of sulphite (experiment in collaboration with Dr. M. Baltscheffsky).

In separate experiments it was shown that these concentrations of sulphite were without effect on the light-induced and pyrophosphate-induced 9-aminoacridine fluorescence quenching and carotenoid shift. This proves that sulphite is without significant effect on the electron transport carriers or the ion-permeability properties of the chromatophore membranes.

We have studied the energy-dependent transhydrogenase activity of *Rhs. rubrum* chromatophores first described by Keister and Yike [32], to see whether the increased efficiency of the production of electrochemical H^+ gradient by ATP in the presence of sulphite is effective in promoting the

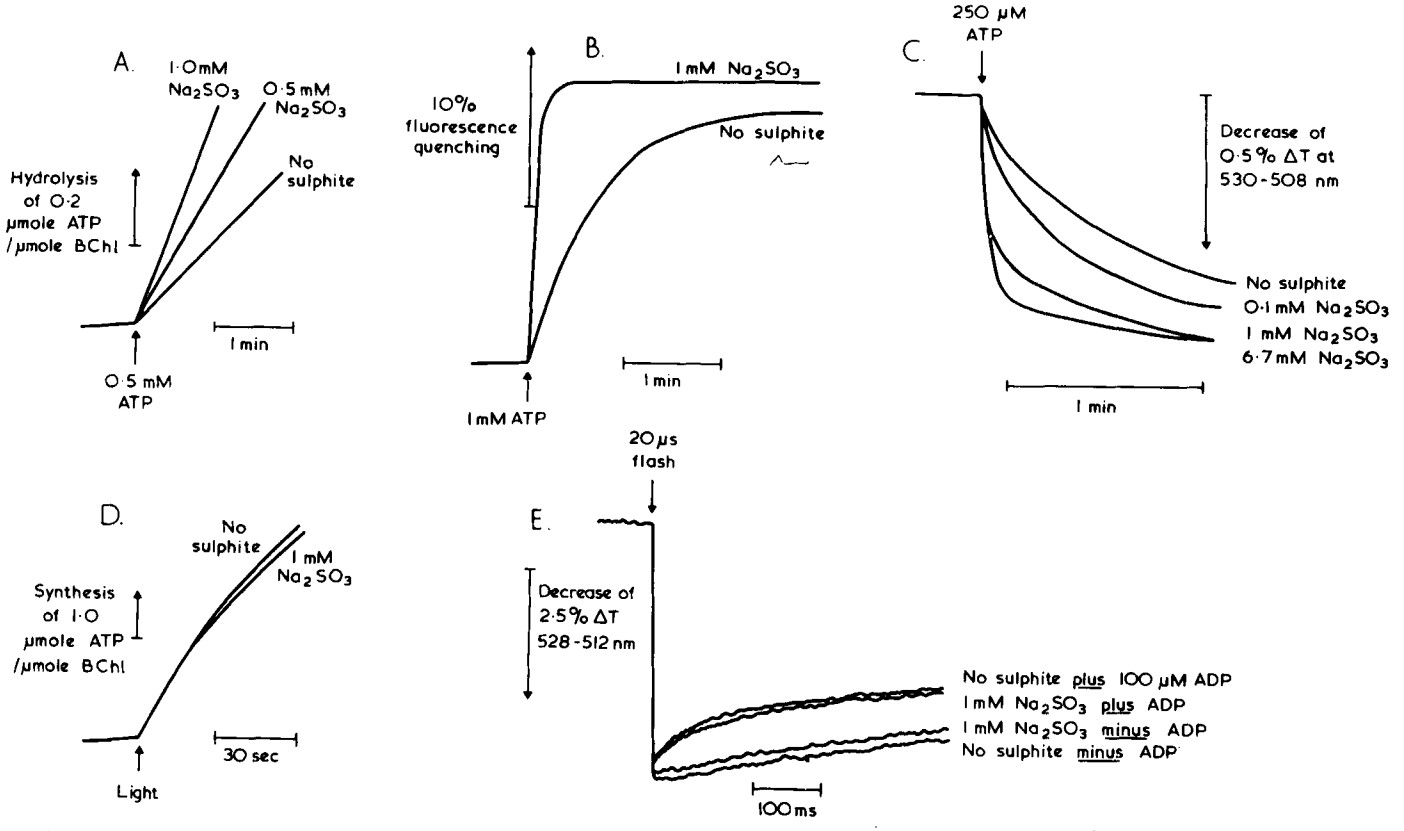

Fig. 10. The effect of sulphite on energy-linked reactions in chromatophores. (A) ATP hydrolysis measured by H^+ release in a 3.0 ml medium containing 50 mM KCl/10 mM $MgCl_2$/0.83 mM EDTA/0.83 mM sodium succinate/1.0 mM tricine/10% sucrose/*Rhs. rubrum* chromatophores (64 μM BChl), final pH 7.4. Sulphite was present at the concentrations shown and the small buffering effect of sulphite is corrected for in the figure. (B) ATP-induced quenching of 9-aminoacridine fluorescence was measured in 2.5 ml of the same medium as in A but additionally containing 50 mM tricine/10 mM $MgCl_2$/*Rhs. rubrum* chromatophores at 12 μM BChl/4 μM 9-aminoacridine/1.6 μg/ml valinomycin, final pH 7.6; The traces only show the efrapeptin-sensitive fluorescence quenching (to eliminate the artifactual change due to the interaction between ATP and 9-aminoacridine). (C) ATP-induced carotenoid band shifts were measured in a 1.5 ml medium containing 50 mM glycylglycine/4 mM $MgCl_2$/*Rhs. rubrum* chromatophores at 20 μM BChl, final pH 8.0. (D) light-induced ATP synthesis was measured in the same medium as in A but containing in addition 1.0 mM ADP and 1.0 mM P_i. Actinic light was saturating and buffering effects of sulphite have been corrected for. (E) The acceleration by ADP of the decay of the carotenoid band shift following a saturating, single turn-over light pulse, was measured in 2.5 ml of a medium containing 50 mM KCl/4.5mM P_i/8 mM $MgCl_2$, 50 mM tricine/10% sucrose/10 μM antimycin A, *Rps. capsulata* chromophores containing 22 μM BChl, final pH 8.0. The traces are averaged over 16 flashed with 35 s between each flash. The rate of photophosphorylation of this preparation was 1.8 μmol ATP/μmol BChl per min in continuous light.

ATP-driven biochemical reactions of the membranes. Indeed, in a typical experiment 0.8 mM Na_2SO_3 accelerated the rate of ATP-driven transhydrogenase activity from 0.36 to 0.77 μmol NADPH/μmol bacteriochlorophyll per min but had no effect on the rate of the pyrophosphate-driven reaction.

The photophosphorylation of ADP in steady-state light is slightly inhibited by sulphite (less than 20%) in the concentration range which gives rise to a dramatic increase in ATPase activity (Figs. 10A and 10D). The stimulation of the carotenoid shift decay of *Rps. capsulata* chromatophores by ADP after a short light pulse [33] is not affected by sulphite (Fig. 10E). This parameter is an indication of the rate of proton flux through the ATPase during flash-induced ATP synthesis [33]. *Rps. capsulata* chromatophores were chosen for this experiment since their carotenoid band shifts are larger and reflect electric field changes more accurately than those of *Rhs. rubrum* (Jackson, J.B. and Packham, N.K., unpublished). The ATPase activity of *Rps. capsulata* is stimulated by sulphite in the same concentration range as was observed with *Rhs. rubrum.*

Discussion

The method of chloroform extraction developed by Beechey et al. [5] provides a useful method for the preparation of quite pure chromatophore ATPase in high yield. The enzyme has similar catalytic properties to that prepared by sonication methods [26], French pressure treatment [4] or by extraction from an acetone powder [3] but after extensive purification it only possesses three of the normal five subunits. Application of this method to beef heart mitochondria gives rise either to a complete five-subunit ATPase [6] or to a three-subunit complex [5] whereas with spinach chloroplasts a five- or four-(δ-deficient) subunit enzyme, depending on chloroform exposure time is produced [7]. Hofman and Bachofen (by personal communication) have also isolated the *Rhs. rubrum* ATPase by chloroform treatment. After purification with ammonium sulphate they find bands on sodium dodecyl sulphate electrophoresis gels which are equivalent to the δ and ε subunits. In view of the results of Younis et al. [7], differences in subunit composition may arise as a consequence of different chloroform exposure times.

Retention of full activity by the α, β and γ subunits of the *Rhs. rubrum* enzyme extends the conclusion reached with chloroplast [29] and bacterial [34] F_1-type ATPase that catalytic activity resides in the α and β subunits. Modification of enzyme activity with divalent cations, activating anions, aurovertin, efrapeptin and 4-chloro-7-nitrobenzofurazan must all be achieved by interaction with either the α, β or γ polypeptide chains. The inability of this preparation to reconstitute photophosphorylation in ATPase-depleted chromatophores is consistent with the conclusion that the δ and ε subunits are necessary for the attachment of the enzyme complex to its intrinsic membrane components [7,29].

The major contaminating protein in this chloroform preparation procedure may be removed by gel filtration on Sepharose 6B or by affinity chromatography. This protein although it possesses polypeptides which co-migrate with

the α, β and γ subunits of the active component and although it binds strongly to the ADP and pyrophosphate affinity absorbents is unable to hydrolyse ATP or pyrophosphate. The electrophoresis pattern of the denatured enzyme in the presence of sodium dodecyl sulphate is rather similar to that shown for the oligomycin-sensitive ATPase from yeast [35]. It may represent inactive aggregates of ATPase although we have not managed to achieve activation of ATPase activity by either heat or trypsin treatment or by incubation in media of high or low salt.

The affinity chromatography procedures, developed by Trayer et al. [9] that we have used in this work provide a convenient purification of *Rhs. rubrum* ATPase. We have achieved higher specific activities for ATP hydrolysis than have been previously reported for this enzyme. The technique may be equally well applied to enzyme prepared by chloroform extraction, acetone powder extraction or by sonication although minor differences in the column elution profile were observed which at present remain unexplained.

The results presented above suggest that the *Rhs. rubrum* ATPase prepared by the chloroform technique binds specifically to the ADP moiety of the 8-(6-aminohexyl)amino-ADP affinity ligand. Failure to bind to a 6-amino-hexan-1-ol pyrophosphate column or a 1,6-diaminohexane-Sepharose 4B column argue for specific involvement of the ligand rather than a hydrophobic interaction or a gel filtration process. Although divalent cation is required for enzyme-catalysed ATP hydrolysis, there is a significant binding to the immobilised ADP even in the absence of Ca^{2+} or Mg^{2+}. Inclusion of either of these ions in the column buffer has two effects: (i) an increase in affinity of ligand for enzyme, as shown by an increased capacity of the column for ATPase, (ii) an increased affinity for the ligand with respect to the eluant ATP. Interestingly, Ca^{2+} and Mg^{2+} are qualitatively similar in these two effects even though, in the isolated enzyme Ca^{2+} is the most effective co-substrate [3]. This is consistent with our earlier observation [18] that Mg^{2+} inhibits Mg^{2+}-ATPase non-competitively at a site distinct from the catalytic site. Hulla et al. [8] found that divalent cation had no effect on the binding of *Micrococcus* ATPase to an affinity ligand 6-[(3-carboxy-4-nitrophenyl)thio]-9-β-D-ribofuranosylpurine 5'-triphosphate. It is not clear whether this reflects a species difference in enzyme properties or a difference in the nature of the affinity ligand.

F_1-type ATPases are known to have multiple nucleotide binding sites. Equilibrium dialysis studies have not revealed common features amongst the multiple nucleotide binding properties of the enzymes from beef-heart mitochondria [36], chloroplasts [37] and *Mycobacterium phei* [38] and unfortunately *Rhs. rubrum* ATPase has not been investigated by this technique. The method of affinity chromatography with immobilised nucleotide analogues would be most valuable in the study of oxidative and photophosphorylation if binding to the enzyme is through the catalytic site. This is difficult to prove but we may cite the increased binding affinity in the presence of divalent cations (Table III) and the equal ability of either ADP or ATP to displace enzyme from the column in support of active site binding. The fact that 4-chloro-7-nitrobenzofurazan inhibited enzyme binds to the affinity adsorbent with apparently unmodified affinity is not inconsistent with active site

interaction. Ferguson et al. [39] have given evidence that this modifier acts subsequently to nucleotide binding in the inhibition of ATP hydrolysis. Efrapeptin-treated ATPase also binds efficiently to 8-(6-aminohexyl)amino-ADP even though the enzymic activity is completely abolished.

In chromatophores we believe that activating anions operate on solubilised enzyme and uncoupled membrane ATPase to restore the conformation which is prevalent in coupled membranes [10] (see Table III):

$$E'_{membrane} \underset{\text{sulphite}\ \Delta\bar{\mu}_{H^+}}{\overset{\text{uncouplers}}{\rightleftharpoons}} E''_{membrane} \equiv E''_{soluble}$$

In the case of partially uncoupled chromatophore membranes, the generation of $\Delta\bar{\mu}_{H^+}$ by light-driven electron transfer may duplicate the effect of sulphite [18] (see also Melandri et al. [40]).

Sulphite stimulates ATP hydrolysis by chromatophores even in the absence of uncoupling agents (Fig. 10) suggesting that even in these conditions the enzyme is not completely in the E′ conformation. Significantly, the increase in rate of ATP hydrolysis is accompanied by an increased rate of useful energy conversion (Fig. 9 and stimulation of ATP-driven transhydrogenase). It would therefore appear that E′ is the more efficient energy-transducing form of the enzyme. During ATP synthesis and coincident outward H^+ translocation through the ATPase (Fig. 9) sulphite has a negligible effect indicating that in the high $\Delta\bar{\mu}_{H^+}$ provided by illumination the enzyme is predominantly E′.

It has been proposed for both the mitochondrial [12] and chloroplast [16] enzymes that activating anions exert their effect by increasing the rate of product release. The substantial change of K_i (ADP) induced by sulphite in mitochondrial [13] and chromatophore Ca^{2+}-ATPase (this work) would support this suggestion. The binding of chromatophore ATPase to 8-(6-aminohexyl)amino-ADP is considerably weakened in the presence of sulphite but is restored by addition of divalent cations. In this respect the ADP binding affinity parallels the change of K_i (ADP), favouring but not proving active site binding to the affinity ligand. It is relevant here to point out that two recent models [41,42] invoke separate sites on the enzyme for substrate binding and catalysis.

The kinetic intermediates and corresponding rate constants of myosin ATPase have been analysed in some detail [43] and a hypothesis for electron transport phosphorylation has been drawn from the muscle system [44]. The model proposed above also shows an interesting analogy with myosin ATPase. In "uncoupled" myosin viz. subfragment 1 or myosin alone, the rate of product release is extremely slow and limits k_{cat} for MgATP hydrolysis (cf. solubilised *Rhs. rubrum* ATPase or uncoupled chromatophore ATPase). In energy-conserving actomyosin the release rate of ADP during MgATP hydrolysis is considerably enhanced (cf. coupled chromatophore ATPase or the simulated, sulphite-treated solubilised enzyme).

Acknowledgements

We are grateful to Dr. I.P. Trayer for the gift of small samples of 8-(6-aminohexyl)amino-ADP and 6-aminohexan-1-ol pyrophosphate during the initial stages of this work. We appreciate the advice given by Dr. Trayer, Mrs. H.R. Trayer and Mr. D.A.P. Small during the preparation of these ligands. We thank Mr. N. Cotton for the preparation of bacterial growth media and the culture of the organisms. Financial support was received from the Science Research Council. G.D.W. acknowledges an award from the Wellcome Foundation.

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