Biochimica et Biophysica Acta 810 (1985) 84–93
Elsevier

BBA 41866

Structural and ATP-hydrolyzing properties of the ATP synthase isolated from *Wolinella succinogenes*

M. Bokranz, E. Mörschel and A. Kröger *

Fachbereich Biologie, Philipps-Universität, Karl von Frischstrasse, D-3550 Marburg (F.R.G.)

(Received May 2nd, 1985)
(Revised manuscript received July 29th, 1985)

Key words: ATP synthase; Furmarate respiration; ATPase; Electron microscopy; (*W. succinogenes*)

The ATP synthase was isolated from the cytoplasmic membrane of the anaerobic bacterium *Wolinella* (formerly *Vibrio*) *succinogenes*, using a non-ionic detergent. After 20-fold purification the enzyme was homogeneous. The M_r was determined to be 410000. Gel electrophoresis in the presence of dodecylsulfate separated eight different peptides, seven of which appeared to be subunits of the enzyme (M_r 56000, 50000, 36000, 19000, 13000, 11000 and 8000). Dicyclohexylcarbodiimide (0.6 mol per mol enzyme) was specifically bound to the M_r 8000 subunit. In electron micrographs, after negative staining, the enzyme appeared as a dumb-bell having a globular portion of 10.0–10.8 nm diameter on one end. The K_i for ADP as a competitive inhibitor of ATP hydrolysis was about 10-times smaller than the K_M for ATP. Incorporation of the enzyme into liposomes caused the K_i and K_M to decrease to values that approached those measured with the bacterial membrane. Treatment of the membrane with $CHCl_3$ was the only procedure found that could split the ATPase from the ATP synthase. The soluble enzyme isolated after this treatment exhibited a 15-times greater specific activity of ATP hydrolysis than ATP synthase. The ATPase was made up of three different subunits (M_r 56000, 50000 and 36000). The M_r was determined to be 340000. In electron micrographs, after negative staining, the ATPase appeared as spherical particles which were similar to the globular part of the ATP synthase. The particles showed a hexagonal fine structure with a seventh element in the centre of the hexagon, suggesting an $\alpha_3\beta_3\gamma$ composition of the enzyme.

Introduction

Wolinella (formerly *Vibrio*) *succinogenes* is an anaerobic bacterium that can grow at the expense of reaction (a) [1,2]. This reaction

$$H_2 + \text{fumarate} \rightarrow \text{succinate} \qquad \text{(a)}$$

is catalyzed by an electron transport chain [3–6] and is coupled to the phosphorylation of ADP by phosphate [7,8]. Earlier it was proposed that the free energy of reaction (a) was transferred to the phosphorylation reaction via the electrochemical proton potential ($\Delta\tilde{\mu}_H$), the latter having been generated by transmembrane electron transport [3,9,10].

This hypothesis requires that the cytoplasmic membrane of the bacterium contains an ATP synthase which can be driven by the $\Delta\tilde{\mu}_H$ across the membrane [11]. To test the hypothesis we have isolated and characterized the ATP hydrolyzing enzyme of the membrane. In a subsequent pub-

* Present address: Institut für Mikrobiologie, Fachbereich Biologie, Theodor-Stern-Kai 7, Haus 75A, 6000 Frankfurt am Main, F.R.G.

Abbreviations: DCCD, *N*,*N'*-dicyclohexylcarbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; Mes, 4-morpholineethanesulphonic acid.

lication the function of this enzyme in ATP synthesis will be described [45].

In the following, the enzyme isolated by detergent extraction is termed ATP synthase. The enzyme obtained after treatment of the membrane with $CHCl_3$ is termed ATPase.

Methods

Preparation procedures

Purification of ATP synthase. W. succinogenes was grown with formate and fumarate in the presence of yeast extract as described [2]. The cells were harvested by centrifugation and suspended in 50 mM Tris and 0.2 mM phenylmethylsulfonylfluoride (pH 8), 0°C (buffer A) and stored in liquid N_2.

Cell homogenate. The thawed cells (9 g protein) were suspended in 0.8 l of buffer A containing 10 mM EDTA and stirred for 15 min. Lysozyme (0.5 g/l) was added and after an additional 30 min 25 mM $MgCl_2$ and 10 mg DNAse/l were also added. Stirring was continued for 30 min.

Membrane fraction. The cell homogenate was centrifuged for 20 min at $12000 \times g$. The resulting sediment was suspended in 0.3 l of buffer A, containing 1 mM $MgCl_2$ and 1% Tween 80, and centrifuged for 20 min at $12000 \times g$.

Membrane extract. The membrane fraction (3.5 g protein) was homogenized in 0.3 l of buffer A containing 1 mM $MgCl_2$, 2% (w/v) Aminoxid WS 35 (acyl (C_{11}–C_{17}) amidopropyldimethylaminoxide (purchased by Goldschmidt, Essen, F.R.G.) and 0.1 M NaCl. This homogenate was stirred for 30 min at 0°C and then centrifuged for 20 min at $30000 \times g$.

Anion exchange chromatography. Protein from the membrane extract was absorbed onto a DEAE Sepharose CL-6B column (165 ml, 4 cm diameter) that had been equilibrated with buffer A, containing 0.1 M NaCl, 3 mM (average molecular weight 342 given by the manufacturer) Aminoxid WS 35, 1 mM $MgCl_2$ and 60 μM soybean phospholipids (Sigma Nr. P 5638) (buffer B). After rinsing the column with 400 ml of buffer B, the enzyme was eluted with 0.4 l of a linear NaCl gradient (0.1–0.3 M) in this same buffer. The fractions containing ATPase activity, at sufficiently high specific activity, were pooled and concentrated 10-fold by pressure dialysis.

Density gradient centrifugation. Three milliliter aliquots (45 mg protein) of the concentrated solution containing ATPase activity were layered onto 40 ml of buffer B, containing a linear sucrose gradient (10–20%), in centrifuge tubes. After centrifugation for 3 h, in a VTi 50 rotor (Beckman Instruments) at $200000 \times g$, the contents of the tubes were fractionated. The fractions containing the greatest amount of ATPase activity were pooled and concentrated by pressure dialysis.

Gel filtration. The concentrated enzyme preparation (4 ml containing 76 mg protein) was subjected to chromatography on Sephacryl S 300. The column (300 ml, 2 cm diameter), equilibrated with buffer B, was eluted with the same buffer at a flow velocity of 5 ml/h. The fractions containing the ATPase activity were pooled, concentrated by pressure dialysis and stored in liquid N_2.

Purification of ATPase

The membrane fraction (see above) was suspended in buffer A, containing 1 mM $MgCl_2$, such that the final concentration of protein was 20 g/l. An equal volume of $CHCl_3$ was added and the suspension was shaken vigorously for 30 s at room temperature [12]. Phase separation was accomplished by centrifugation of the suspension for 30 min at $2000 \times g$. The soluble ATPase was purified by anion exchange chromatography, gel filtration and density gradient centrifugation as described above. Aminoxid WS 35 and phospholipids were, however, omitted from all buffers used.

Analytical procedures

ATP-hydrolyzing activity. The activity of ATP hydrolysis was measured at 37°C, using one of the three methods described below. One unit of activity (U) was equivalent to the hydrolysis of 1 μmol ATP/min. The turnover number was calculated from the specific activity and the molar mass of ATP synthase ($4.1 \cdot 10^5$ g/mol) or ATPase ($3.4 \cdot 10^5$ g/mol).

Phosphate assay. The reaction mixture contained 50 mM Hepes/3 mM Aminoxid WS 35/5 mM ATP/2.5 mM $MgCl_2$ (pH 8). The reaction was started by addition of the enzyme. At various time intervals (1–10 min) 50 μl samples were removed and mixed with 0.65 ml trichloroacetic

acid (0.5 M). Phosphate was determined in this mixture using the procedure described previously [13].

pH-electrode recording [14]. The reaction mixture contained 10 mM Hepes/0.1 M KCl/3 mM Aminoxid WS 35/5 mM ATP/2.5 mM $MgCl_2$ (pH 8). The reaction was started by addition of the enzyme, and the pH of the mixture was recorded using a glass-electrode. The amount of H^+ released, which was equivalent to that of phosphate liberated, was determined using an HCl standard.

Photometric recording [15]. NADH oxidation was recorded photometrically in a reaction mixture containing 50 mM Hepes/3 mM Aminoxid WS 35/5 mM ATP/5 mM $MgCl_2$/1.5 mM phosphoenolpyruvate/1 mM NADH, 20 U lactate dehydrogenase and 5 U pyruvate kinase (pH 8). The reaction was started by addition of the enzyme. The velocity of NADH oxidation was equivalent to that of the ATP hydrolysis.

Protein. Protein was determined photometrically using the Biuret method with KCN [16]. Bovine serum albumin was used as standard. Alternatively, protein was also estimated photometrically using the protein stain with Amidoblack on nitrocellulose sheets [17], or by determining the amount of radioactivity incorporated by the bactèria growing in the presence of [^{3}H] leucine [18].

Electron microscopy. The negative staining procedure was similar to that described previously [19]. Sodium phosphotungstate (1–2%, w/v, pH 7.0) was used as negative stain. The stain was mixed directly with the protein suspensions before absorbed onto carbon films (4–5 nm), or the stain was added after the absorption of the protein to the films. The preparations were examined in a Philips 301 G electron microscope fitted with a liquid nitrogen cooled finger and operated at 80 kV, with calibrated primary magnifications.

Results

Isolation of ATP synthase

The enzyme purification was monitored by measuring the ATP hydrolyzing activity. Thawed cells of *W. succinogenes* were lysed in the presence of lysozyme and EDTA, and the membrane fraction was isolated by centrifugation. The membrane fraction was solubilized using Aminoxid WS 35, and the extract obtained was subjected to anion exchange chromatography. Further purification was achieved using density gradient centrifugation, while subsequent gel filtration resulted in only a slight increase in specific activity. The specific activity of the resulting isolated enzyme was 20-fold greater than that of the membrane fraction, and the yield was about 30% (Table I). With the various separation methods employed there was no indication that that more than one enzyme species was present in the bacterial membrane.

TABLE I

PURIFICATION OF ATP SYNTHASE

The activity of ATP hydrolysis at 37°C was measured using the pH-electrode [14]. Protein was determined using the Biuret method [16].

	Specific activity (U/mg protein)	Total activity (U)
Cell homogenate	0.43	3680
Membrane fraction	1.03	2670
Membrane extract	1.47	2304
Anion exchange chromatography	6.73	1480
Density gradient centrifugation	17.4	1325
Gel filtration	19.5	1070

Isolation of ATPase

Treatment of the membrane fraction with either 0.5 mM EDTA or 1.5 M LiCl did not bring about

TABLE II

PURIFICATION OF ATPASE

The activity of ATP hydrolysis was measured at 37°C using photometric recording (see Methods section).

	Specific activity (U/mg protein)	Total activity (U)
Membrane fraction	0.64	1713
Aqueous phase after $CHCl_3$ extraction	34.3	2420
Anion exchange chromatography	122	2190
Gel filtration	188	1560
Density gradient centrifugation	281	1449

the solubilization of the ATPase. Treatment with $CHCl_3$ did, however, cause the appearance of the ATPase in the aqueous phase. This step caused a 50-fold increase in specific activity (Table II). The enzyme was purified in the absence of detergent using anion exchange chromatography, gel filtration and density gradient centrifugation. This approach yielded a preparation, with a specific activity that was 15-times greater than that of the ATP synthase (Table I). Assuming that the increase in specific activity was brought about by the $CHCl_3$ treatment (Table II), the yield of this step was calculated as 10%. The recovery of the last three purification steps was 60%.

Molecular properties

Hydrodynamic properties. When subjected to gel filtration or density gradient centrifugation, the ATP synthase, as well as the ATPase, reacted as homogeneous enzyme proteins. The protein was distributed in single symmetrical bands, which coincided with enzymic activity (not shown). The sedimentation coefficients of the enzymes were determined using sucrose density gradient centrifugation [20], with urease, catalase and alcohol dehydrogenase as reference proteins (Fig. 1). The ATP hydrolyzing activities were found as bands in the gradients, with a shape and width similar to those of the reference enzymes. The ATP synthase band nearly coincided with that of catalase (Fig. 1B), while that of the ATPase migrated faster (Fig. 1A). The slowness of ATP synthase migration was probably due to the detergent and phospholipid content of the ATP synthase. The sedimentation coefficients, 11.7 S (ATP synthase) and 12.9 S (ATPase), were obtained from plots of the migration distances as compared to the reference values [20]. The Stokes radii were determined via FPLC gel filtration using reference proteins as a comparison (Fig. 2). The values obtained were confirmed using classical gel filtration with Sepharose CL-6B (not shown). The Stokes radius of the ATPase (6.4

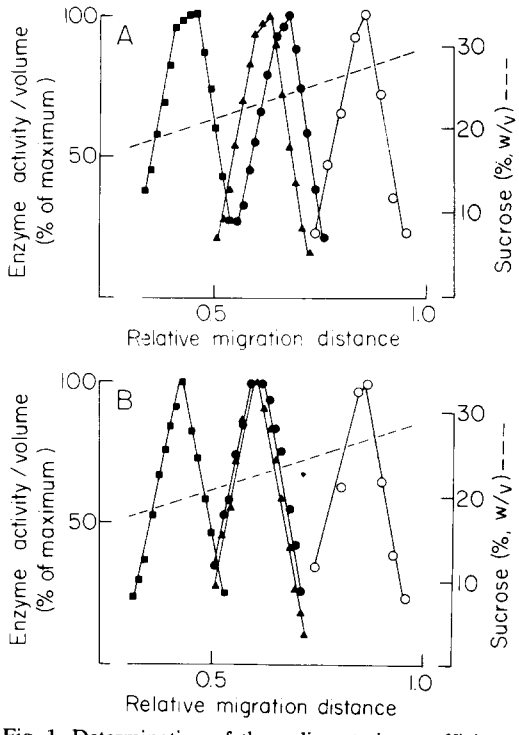

Fig. 1. Determination of the sedimentation coefficients of the ATPase (A) and ATP synthase (B). A solution (0.1 ml) containing 0.5 mg alcohol dehydrogenase from yeast (7.4 S) (■), 0.2 mg catalase from bovine liver (11.3 S) (▲), 0.5 mg urease from *Canavalia* (18.6 S) (○) and either 0.5 mg ATPase or 0.5 mg ATP synthase (●) was layered onto 5 ml of a Tris buffer (50 mM) containing a linear sucrose gradient (10–30%) and 3 mM Aminoxid WS 35 at pH 8 and 5°C. The gradients were centrifuged for 1 h in a VTi 65 rotor (Beckman Instruments) at 65000 rpm. The enzymes were assayed using their activities.

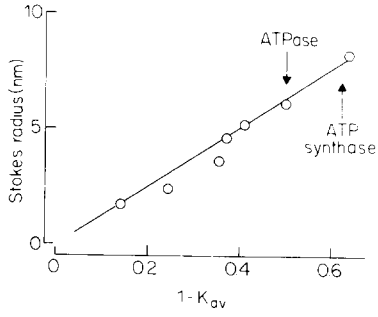

Fig. 2. Determination of the Stokes radii of the ATPase and ATP synthase. The FPLC gel filtration column (Superose 6, bed size 24 ml, 1 cm diameter) was equilibrated with 20 mM Tris, 100 mM NaCl, 3 mM Aminoxid WS 35 (pH 8) at room temperature and a flow rate of 0.2 ml/min. The dead volume (V_0) and the total volume (V_t) of the column were determined using dextran blue and acetone. The elution of the proteins was recorded using their absorbance at 280 nm. The K_{av} values were calculated from the elution volumes (V_e) according to the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$ [18,21]. The following proteins were used as standards: cytochrome *c* from horse heart (1.8 nm), carbonic anhydrase from bovine erythrocytes (2.4 nm), bovine serum albumin (3.6 nm), aldolase from rabbit muscle (4.6 nm), catalase from bovine liver (5.2 nm), ferritin from horse spleen (6.0 nm) and thyroglobuline from pig (8.2 nm).

nm) was close to that of ferritin, yet smaller than that of the ATP synthase (8.0 nm) which was similar to that of thyroglobulin. From the sedimentation coefficients and the Stokes radii, the molar masses of the enzymes were calculated (Table III). In the case of the ATPase, the molar mass was equivalent to the M_r of the enzyme protein (340000). The M_r of the ATP synthase protein was found to be 410000, after correction for the phospholipid and detergent content of the preparation. While the binding capacity of the ATP synthase for Triton X 100 was assayed (Table III), the hydrodynamic properties of the enzymes were determined in the detergent Aminoxid WS 35. It was assumed that the binding capacity of the enzyme for the two detergents (in g per g protein) was nearly the same. The mitochondrial adenine nucleotide carrier, which has a greater detergent binding capacity than the ATP synthase, was found to bind 25% more Triton X-100 than Aminoxid WS 35 [25]. Such difference in detergent binding capacity of the *Wolinella* ATP synthase would not significantly affect the resulting M_r of the enzyme protein.

TABLE III

HYDRODYNAMIC PROPERTIES OF THE ATP SYNTHASE AND ATPase FROM *W. SUCCINOGENES*

The Stokes radii and the sedimentation coefficients were taken from Figs. 2 and 1, respectively. The phospholipid content was calculated from the phosphate content [22] of the preparation, assuming an average molecular weight of 800 for the phospholipids. The content of Triton X-100 was determined by measuring the [^{3}H]-labeled detergent (gift from Rohm and Haas Company, Philadelphia, PA, U.S.A.) that eluted with the enzyme from a DEAE Sepharose CL-6B column, after equilibration with labeled detergent [23]. The molar masses of the enzymes were calculated from the Stokes radii and the sedimentation coefficients, according to the Svedberg equation [18,24]. The M_r of the ATP synthase protein was obtained from the molar mass of the enzyme particle after correction for its phospholipid and detergent content [24].

	ATP synthase	ATPase
Stokes radius (nm)	8.0	6.4
Sedimentation coefficient (S)	11.7	12.9
Phospholipid content (g/g protein)	0.10	–
Triton X-100 content (g/g protein)	0.22	–
Molar mass of enzyme particle (g/mol)	540000	340000
M_r of enzyme protein	410000	340000

Subunit composition. Gel electrophoresis of the ATPase, in the presence of dodecylsulfate, resolved three different polypeptides with the M_r of 56000 (α), 50000 (β) and 36000 (γ) (Fig. 3). Additional subunits were not detected even with gels run with a 5-times greater amount of ATPase. The M_r values were based on those of the *E. coli* ATP synthase subunits [27], which were used as references [28]. From photometric recording of the Coomassie blue stain the relative molar amounts of the subunits ($\alpha:\beta:\gamma$) were estimated to be 3 : 3 : 1.

Gradient gels [29] run with the ATP synthase resolved eight major bands (Fig. 4A). In addition to the α, β and γ subunits of the ATPase, five bands were detected at M_r 19000 (a), 13000 (b), 11000 (c), 8000 (d) and about 5000. Comparison to the electrophoresis pattern of the ATP synthase from *E. coli* [28] (Fig. 4B) showed pronounced differences in the subunit composition of the two enzymes.

From the Coomassie blue stain the molar contents of subunit *a*, *b* and *c* were estimated to be close to that of the γ peptide. The content of *d* was estimated to be smaller (0.2 mol/molγ) and that of the M_r 5000 peptide varied from one preparation to the other (0.5–4 mol/molγ). The variable content suggested that this peptide represented an impurity of the preparation. A M_r 5000 peptide which could not be attributed to one of the known subunits was also found in a prepara-

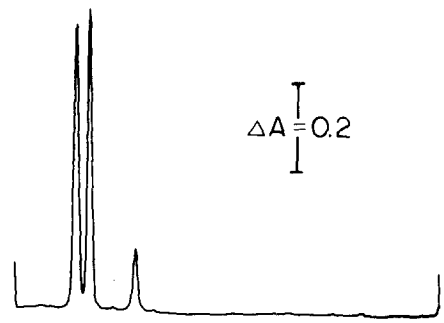

Fig. 3. Polyacrylamide gel electrophoresis of ATPase from *W. succinogenes* in the presence of dodecylsulfate. Photometric scan at 578 nm of a 15% gel [26] run with 25 μg ATPase after staining with Coomassie blue R-250. Before electrophoresis the sample was heated for 5 min at 100°C in a solution containing 60 mM Tris, 4% dodecylsulfate, 10% glycerol and 0.1 M dithiothreitol (pH 6.8).

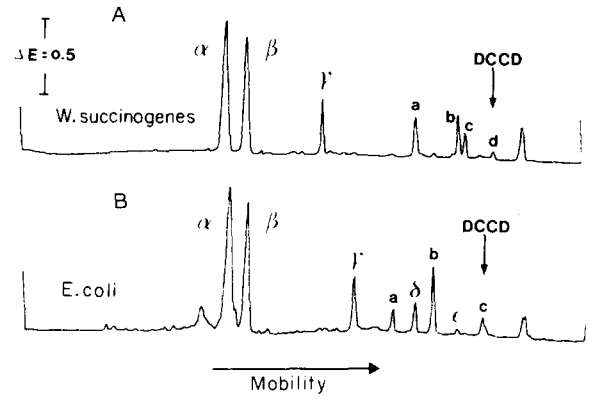

Fig. 4. Gradient gel electrophoresis of ATP synthase from *W. succinogenes* (A) and *E. coli* (B) in the presence of dodecylsulfate. Photometric scans at 578 nm. The gels prepared with 5–20% acrylamide contained a linear sucrose gradient (0–15%) [29]. The samples (20 μg protein) were treated as described in the legend of Fig. 3.

tion [28] of the ATP synthase from *E. coli* (Fig. 4B).

The ATP synthase from *W. succinogenes* was incubated for 8 h at 0°C with a 10-fold molar excess of labeled DCCD. After precipitation with acetone (final proportion, 90% v/v) and rinsing with ether, the protein contained 0.8 mol DCCD/mol ATP synthase. Using gel electrophoresis it was found that 75% of the label was associated with subunit *d*. This subunit, therefore, represents the equivalent to subunit *c* of the *E. coli* enzyme (Fig. 4).

The subunit composition of the ATP synthase was investigated using an enzyme preparation which was obtained from bacteria grown with [^{3}H]leucine. After electrophoresis and protein staining, the gel was sliced and the tritium contents in the individual bands were determined (Table IV). The specific label contents which were calculated using the M_r of the subunits suggested the molar composition of the ATPase part to be $\alpha_3\beta_3\gamma$, on the basis that the proportion of leucine in the three subunits was identical. This was found to be valid with the *E. coli* ATPase [27]. Thus the composition of the ATPase as deduced from the protein stain was confirmed.

The label present in the mebraneous peptides exceeded that of γ by a factor of 1.6–2.8. This was in contrast to the composition suggested by the Coomassie stain ($\alpha_3\beta_3\gamma$ a b c). The discrepancy was probably due to variations of both staining and leucine content of the individual peptides. The DCCD-binding subunit (*d*) contained nearly 3-times the label of γ. The corresponding peptide (*c*) of the *E. coli* enzyme was found to contain 1.5-times more leucine than the ATPase subunits [27]. Assuming that this would hold also with the *Wolinella* enzyme, subunit *d* would amount to two copies per molecule ATPase.

Electron microscopy. Visible in electron micrographs of the negatively stained ATP synthase preparation (Fig. 5A and C) were particles with the typical asymmetrical dumb-bell structure, consisting of a more globular portion, a stalk and a base piece. In addition, aggregates were seen that consisted of dumb-bells connected at the base pieces (Fig. 5A). The globular part of the protein (i.e., the ATPase) had a diameter of 10.4–10.8 nm, while the stalk was 4.0–4.4 nm long and 2.4–2.8 nm thick. The height of the base piece was 8.0 nm, and its length varied between 10 and 16 nm dependent on the amounts of lipid and detergent bound. The minimal length was similar to the diameter (10.0 nm) of the enzyme portion associated with the membrane, as observed in freeze

TABLE IV

TRITIUM CONTENTS OF THE SUBUNITS OF THE ATP SYNTHASE ISOLATED FROM *W. SUCCINOGENES* GROWN IN THE PRESENCE OF [^{3}H]LEUCINE [18]

The isolated ATP synthase was subjected to gel electrophoresis with dodecylsulfate, and the gel was stained (see legend of Fig. 4). The gel was sliced and the slices treated with H_2O_2. The specific label content was calculated using the M_r values which were taken from gel electrophoresis. The enzyme contained 140 dpm tritium/μg protein. 70% of the radioactivity was associated with the peptides indicated.

Peptide	M_r	Tritium content (dpm/slice)	Specific label content (10^{-3} dpm/mol)
α	56000	2366	44
β	50000	2226	45
γ	36000	563	16
a	19000	653	34
b	13000	328	25
c	11000	329	30
d	8000	358	45
–	5000	419	84

fracture micrographs after incorporation of the enzyme into liposomes (not shown). As judged from the dimensions, the base piece appeared about as big as the ATPase part of the particle. In contrast, the hydrodynamic properties of the enzymes suggested that the M_r of ATP synthase exceeded that of the ATPase ($M_r = 340\,000$) by only 70 000 (Table III). The reason for this discrepancy is not clear.

In the negatively stained ATPase preparation (Fig. 5B, D and E) could be seen nearly symmetrical disc-shaped particles, with a diameter (10.4 nm) that was similar to that of the globular part of the ATP synthase. Most of the particles were oriented in face view, and showed a hexagonal arrangement of subunits around a central one (Fig. 5B and D). Together with the results of the subunit analysis, these observations suggest that the ATPase has an $\alpha_3\beta_3\gamma$ structure where α and β might represent the peripheral elements and γ the central element. In particles with a side view orientation, a rare occurrence, the central element protruded from the bottom plane (Fig. 5E). This observation suggested that the γ subunit formed a part of the ATP synthase stalk.

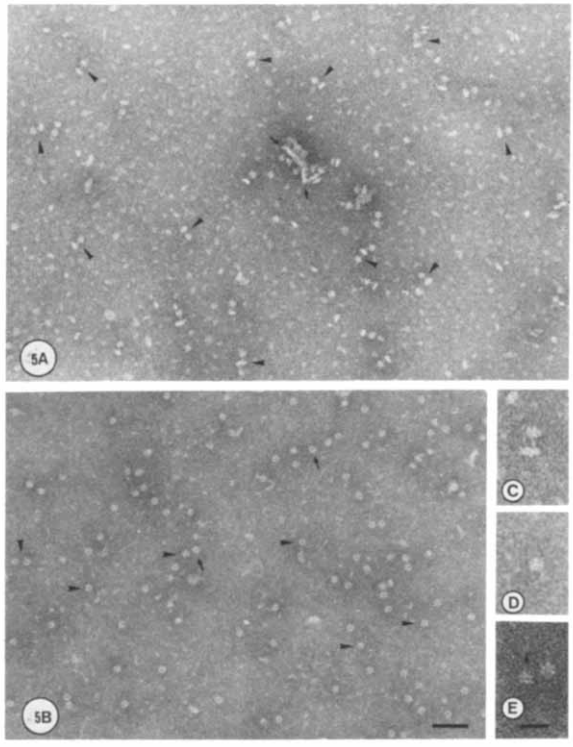

Fig. 5. Electron micrographs of the isolated enzymes after negative staining. The bar represents 50 (A and B) or 20 nm. Magnification, ×95 000 (A and B) or ×190 000. (A) ATP synthase molecules with typical profiles (▶). Some molecules are aggregated with their base pieces (→). (B) The ATPase molecules tend to be oriented such that they are seen in face view (▶); occasionally side views are also seen (→). (C) Single ATP synthase molecule; the three building blocks (sphere, stalk and base piece) are clearly visible. (D) Face view of ATPase molecule revealing the hexagonal structure. (E) ATPase molecule in profile orientation with a small stalk protruding from the sphere (→).

From the observed diameter (5 nm) of the peripheral elements, their M_r was estimated to be 53 000. This value was close to that determined for the α and β subunits as estimated by electrophoresis in the presence of dodecylsulfate. Based upon the assumption that the ATPase consisted of seven subunits of similar M_r (53 000), the M_r of the protein would be 371 000. This value was 10% higher than that determined from the hydrodynamic properties of the enzyme (Table III).

Enzymic properties

The activity of the soluble ATPase, as measured with the pH-electrode, was absolutely dependent upon the presence of divalent metal ions. In the presence of 5 mM ATP and at pH 8 the activity was maximal at a molar ratio ATP/metal ion of 2. Maximum activity was observed with $MgCl_2$ (100%) and less with $MnCl_2$ (82%), $CoCl_2$ (71%), $ZnSO_4$ (67%), $CaCl_2$ (22%). The pH-optimum of the ATPase, measured in the presence of 5 mM ATP and 2.5 mM $MgCl_2$, was between 8 and 9 when 0.1 M Tris, Hepes or glycylglycin were used as buffers. At pH 7 (0.1 M Mops) and 6 (0.1 M Mes), the activities were 50% and 10%, respectively, of the optimum activity. A similar pH-profile was found with the ATP synthase. Under the optimum conditions of ATP hydrolysis (i.e., 100% activity), this preparation catalyzed the liberation of phosphate from GTP (48%), ITP (25%), UTP (5%) and CTP (1%), while ADP was not hydrolyzed. The activity of ATP hydrolysis, for both the ATPase and the ATP synthase, was related to the concentration of ATP according to the Michaelis equation. To determine whether the enzymes were severely altered by the isolation procedures, their Michaelis constants (K_M) for ATP were compared to those measured using membrane vesicles pre-

TABLE V

KINETIC CONSTANTS OF ATP HYDROLYSIS CATALYSED BY VARIOUS ENZYME PREPARATIONS

The activity of ATP hydrolysis (37°C) was measured using the pH-electrode. In the case of liposomes and vesicles, Aminoxid WS 35 was omitted from the test buffer.

Preparation	K_M for ATP (μM)	K_i for ADP (μM)	Maximal turnover number (10^3 min^{-1})
ATPase	175	18	81
ATP synthase	620	190	5.3
ATP synthase in liposomes	270	74	2.2
Membrane vesicles	290	33	2.5

pared from *W. succinogenes* (Table V). The K_M of the purified ATPase was slightly smaller than that measured with membrane vesicles, while that of the isolated ATP synthase was more than twice as large. Incorporation of the ATP synthase into liposomes caused the K_M to decrease to that measured with membrane vesicles.

ADP was found to be a competitive inhibitor of ATP hydrolysis activity. The K_i of the ATPase was smaller than that measured with membrane vesicles (Table V), while that of the ATP synthase was distinctly larger. By incorporation into liposomes the K_i of the ATP synthase was decreased to a value (74 μM) approx. 2-fold greater that that measured with membrane vesicles. The maximal turnover number as well as the K_M for ATP or the K_i for ADP of the ATP synthase in liposomes were not affected by presence of a protonophore. In summary, the substrate affinity of the isolated ATP synthase nearly attained its original value after incorporation into liposomes.

Discussion

Function of ATP synthase

Inverted membrane vesicles prepared from the anaerobe *W. succinogenes* were shown earlier [7,8] to catalyze phosphorylation, driven by reaction (a). In that the phosphorylation was blocked by protonophores or DCCD, it was concluded that the mechanism of energy transduction must be similar to that of oxidative phosphorylation. This conclusion is supported by the work reported here describing the isolation, from the cytoplasmic membrane, of an enzyme that resembles the ATP synthases of aerobic or phototrophic bacteria [27,28,30–33], mitochondria [34–36] and chloroplasts [37,38], yet differs from the ATPase of purely fermentative anaerobic bacteria [39].

Consistent with the high phosphorylation activity of the bacterial membrane [8] the ATP synthase contributes to about 5% of the total membrane protein. Its function as an ATP synthase is suggested by the finding that the K_i for ADP, which competitively inhibits the ATP hydrolysis reaction, is about 10-times smaller than the K_M for ATP. In a subsequent publication [45] the K_M for ADP in the ATP synthesis reaction catalyzed by the enzyme, after incorporation into liposomes, will be shown to be close to the K_i value in ATP hydrolysis. The turnover number of the liposomal enzyme in the ATP synthesis reaction can be in the same range as that in the phosphorylation which is driven by reaction (a) in the bacterial membrane.

The well-known ATP synthase inhibitor DCCD [40] is specifically bound to one of the subunits making up the membraneous part of the enzyme. In the following publication it will be shown that the phosphorylation catalyzed by the liposomal enzyme is strongly inhibited by DCCD. In contrast, the ATP hydrolysis activity of the bacterial membrane and of the isolated or liposomal ATP synthase was not affected by incubation with 1000 mol DCCD/mol enzyme.

Structure of ATP synthase

The isolated ATPase consists of only three different subunits and has the composition $\alpha_3\beta_3\gamma$. The corresponding M_r is in close agreement with the M_r obtained from the hydrodynamic properties or from electron microscopy (Table VI). The size of the enzyme is in the range of those observed for ATPases from other sources (325 000–380 000), but appears to be slightly smaller than that of the *E. coli* enzyme [41–43]. Direct measurements with the *E. coli* ATPase gave a M_r of 350 000 [41], while from the subunit composition a value of 382 000 was obtained [43].

The *Wolinella* ATP synthase (Table VI) appears to be considerably smaller than that of *E. coli*. From X-ray scattering in solution an M_r of 450 000

TABLE VI

COMPARISON OF THE M_r VALUES OF THE ATPase AND THE ATP SYNTHASE FROM *W. SUCCINOGENES* AS DETERMINED BY VARIOUS METHODS

The M_r values shown in the first line were taken from Table III, the value in the second line was calculated assuming that the ATPase consists of seven subunits with M_r 53000, and those presented in the last line were calculated on the basis of the subunit composition $\alpha_3\beta_3\gamma$ (ATPase) and $\alpha_3\beta_3\gamma abcd_2$ or $\alpha_3\beta_3\gamma a_2b_2c_2d_3$ (ATP synthase), with the M_r values of the subunits given in Table IV.

Data obtained from	M_r of ATP synthase	M_r of ATPase
Hydrodynamic properties	410000	340000
Electron microscopy	–	371000
Subunit composition	413000–464000	354000

was obtained with the *E. coli* enzyme [44], while the subunit composition gave a value of 530000 [43].

The membraneous part of the *E. coli* ATP synthase consists of three different subunits with the composition ab_2c_{10} [43]. The membraneous part of the *Wolinella* enzyme is composed of four different subunits. As a further difference, the DCCD-binding peptide is not present in a large molar excess. The subunit stoichiometry cannot be determined exactly, since the leucine contents of the subunits are not known. With the assumption that the leucine content of all the ATP synthase subunits is the same, the stoichiometry would be calculated from the data of Table IV to be $a_2b_2c_2d_3$. This corresponds to a maximum M_r of 110000, which is much greater than the value obtained from the hydrodynamic properties of the ATP synthase and ATPase (70000) (Table IV). The leucine contents of the mebraneous subunits of the *E. coli* enzyme are up to 1.8-times greater that that of the ATPase [27]. On the basis that this is also valid for the *Wolinella* enzyme, a minimum composition of $abcd_2$ would be obtained. The corresponding M_r of 59000 is consistent with that calculated from the hydrodynamic properties of the enzymes.

Acknowledgements

The *E. coli* ATP synthase preparation was kindly provided by Dr. P. Friedl and Dr. U. Schairer (GBF Stöckheim). This work was supported by grants from the Deutsche Forschungsgemeinschaft to A.K. and E.M. and from the Fonds der Chemischen Industrie to A.K.

References

1 Wolin, M.J., Wolin, E.A. and Jacobs, N.J. (1961) J. Bacteriol. 81, 911–917
2 Bronder, M., Mell, H. and Kröger, A. (1982) Arch. Microbiol. 131, 216–223
3 Kröger, A. (1978) Biochim. Biophys. Acta 505, 129–145
4 Unden, G., Hackenberg, H. and Kröger, A. (1980) Biochim. Biophys. Acta 591, 275–288
5 Unden, G., Böcher, R., Knecht, J. and Kröger, A. (1982) FEBS Lett. 145, 230–234
6 Schröder, I., Roberton, A.M., Bokranz, M., Unden, G., Böcher, R. and Kröger, A. (1985) Arch. Microbiol. 140, 380–386
7 Reddy, C.A. and Peck, H.D. (1978) J. Bacteriol. 134, 982–991
8 Kröger, A. and Winkler, E. (1981) Arch. Microbiol. 129, 100–104
9 Kröger, A. (1977) in Microbial Energetics (Haddock, B.A. and Hamilton, W.A., eds.), pp. 61–93, University Press, Cambridge
10 Kröger, A., Dorrer, E. and Winkler, E. (1980) Biochim. Biophys. Acta 589, 118–136
11 Mitchell, P. (1966) Biol. Rev. 41, 445–502
12 Linnet, P.E., Mitchell, A.D., Partis, M.D. and Beechey, R.B. (1979) Methods Enzymol. 55, 337–343
13 Altmann, H.J. Fürstenau, E., Gielewski, A. and Scholz, L. (1971) Z. Anal. Chem. 256, 274–276
14 Nishimura, M., Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177–182
15 Vogel, G. and Steinhart, R. (1976) Biochem. 15, 208–216
16 Bode, C., Goebell, H. and Stähler, E. (1968) Z. Klin. Chem. Klin. Biochem. 6, 419–422
17 Neuhoff, V. (1979) Z. Physiol. Chem. 360, 1657–1670
18 Kröger, A., Winkler, E., Innerhofer, A., Hackenberg, H. and Schägger, H. (1979) Eur. J. Biochem. 94, 465–475
19 Valentine, R.C., Shapiro, B.M. and Stadtman, E.R. (1968) Biochem. 7, 2143–2152
20 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372–1379
21 Nozaki, Y., Schechter, N.M. Reynolds, J.A. and Tanford, C. (1976) Biochem. 15, 3884–3890
22 Chen, P.S., Toribara, T.Y. and Warnew, H. (1956) Anal. Chem. 28, 1756–1761
23 Von Jagow, G., Schägger, H., Riccio, P., Klingenberg, M. and Kolbe, H.J. (1977) Biochim. Biophys. Acta 462, 549–558
24 Tanford, C., Nozaki, Y., Reynolds, J.A. and Makino, S. (1974) Biochem. 13, 2369–2376
25 Hackenberg, H. (1979) Ph.D. Thesis, University of Munich
26 Lämmli, U.K. (1970) Nature 227, 680–685
27 Futai, M. and Kanazawa, H. (1983) Microbiol. Rev. 47, 285–312

28 Friedl, P., Friedl, C. and Schairer, H.V. (1979) Eur. J. Biochem. 100, 175–180
29 Montecucco, C., Dabbeni-Sala, F., Friedl, P. and Galante, Y.M. (1983) Eur. J. Biochem. 132, 189–194
30 Amzel, L.M. and Pedersen, P.L. (1983) Annu. Rev. Biochem. 52, 801–824
31 Schneider, E., Friedl, P., Schwulera, U. and Dose, K. (1980) Eur. J. Biochem. 108, 331–336
32 Bengis-Garber, C. and Gromet-Elhanan, Z. (1979) Biochemistry 18, 3577–3581
33 Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1975) J. Biol. Chem. 250, 7917–7923
34 Serano, R., Kanner, B.I. and Racker, E. (1976) J. Biol. Chem. 251, 2453–2461
35 Galante, Y.M., Wong, S.-Y. and Hatefi, Y. (1979) J. Biol. Chem. 254, 12372–12378
36 Rott, R. and Nelson, N. (1981) J. Biol. Chem. 256, 9224–9228
37 Shavit, N. (1980) Annu. Rev. Biochem. 49, 111–138
38 Pick, U. and Racker, E. (1979) J. Biol. Chem. 254, 2793–2799
39 Clarke, D.J., Fuller, F.M. and Morris, J.G. (1979) Eur. J. Biochem. 98, 597–612
40 Sebald, W., Graf, T. and Lukins, H.B. (1979) Eur. J. Biochem. 93, 587–599
41 Paradies, H.H. and Schmidt, U.D. (1979) J. Biol. Chem. 254, 5227–5263
42 Yoshida, M., Sone, N., Hirata, H. Kagawa, Y. and Ui, N. (1979) J. Biol. Chem. 254, 9525–9533
43 Foster, D.L. and Fillingame, R.H. (1982) J. Biol. Chem. 257, 2009–2015
44 Paradies, H.H., Mertens, G., Schmid, R., Schneider, E. and Altendorf, K. (1981) Biochem. Biophys. Res. Commun. 98, 595–606
45 Bokranz, M., Mörschel, E. and Kröger, A. (1986) Biochim. Biophys. Acta, Vol. 848, in the press