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Characterization of the ATP Synthase of *Propionigenium modestum* as a Primary Sodium Pump[†]

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ABSTRACT: The ATP synthase (F_1F_0) of *Propionigenium modestum* has been purified to a specific ATPase activity of 5.5 units/mg of protein, which is about 6 times higher than that of the bacterial membranes. Analysis by SDS gel electrophoresis indicated that in addition to the five subunits of the F_1 ATPase, subunits of M_r 26 000 (a), 23 000 (b), and 7500 (c) have been purified. The ATPase activity of F_1F_0 was specifically activated about 10-fold by Na^+ ions. The enzyme was strongly inhibited by dicyclohexylcarbodiimide, venturicidin, tributyltin chloride, and azide. After incubation with [^{14}C]dicyclohexylcarbodiimide, about 3-4 mol of the inhibitor was bound per 500 000 g of the enzyme. The radioactive label was specifically bound to subunit c. These subunits form stable aggregates which resist dissociation by SDS at 100 °C. The monomer is formed upon heating with SDS to 121 °C or by extraction of the membranes with chloroform/methanol. The ATP synthase was incorporated into liposomes by a freeze–thaw–sonication procedure. The reconstituted proteoliposomes catalyzed the transport of Na^+ ions upon ATP hydrolysis. The transport was completely abolished by dicyclohexylcarbodiimide. Whereas monensin prevented the accumulation of Na^+ ions, the uptake rate was stimulated 4–5-fold in the presence of valinomycin or carbonyl cyanide *m*-chlorophenylhydrazone. These results indicate an electrogenic Na^+ transport and also that it is a primary event and not accomplished by a H^+-translocating ATP synthase in combination with a Na^+/H^+ antiporter.

The strictly anaerobic bacterium *Propionigenium modestum* grows from the fermentation of succinate to propionate and CO_2 (Schink & Pfennig, 1982). The pathway of succinate fermentation involves the intermediates succinyl-CoA, (*R*)- and (*S*)-methylmalonyl-CoA, and propionyl-CoA. The mechanism of ATP synthesis in this organism deserves special attention (Hilpert et al., 1984): the small free energy change of the decarboxylation of succinate to propionate ($\Delta G^{o'} = -20.6$ kJ/mol) does not allow a substrate-linked phosphorylation mechanism, and no redox reactions occur which could drive electron-transport phosphorylation. The only step of the fermentation pathway that is sufficiently exergonic for energy conservation is the decarboxylation of (*S*)-methylmalonyl-CoA to propionyl-CoA. The free energy of the decarboxylation reaction is conserved by conversion into an electrochemical Na^+ gradient, which, therefore, provides the only energy source for ATP synthesis.

Preliminary experiments with bacterial membrane vesicles indicated that the synthesis of ATP was coupled directly to the Na^+ gradient (Hilpert et al., 1984): the vesicles contained an ATPase that was specifically activated by Na^+ ions and catalyzed Na^+ uptake upon ATP hydrolysis that was not sensitive to the uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone. In order to firmly establish the ATP synthesis mechanism in *P. modestum*, however, the energy-converting enzymes have to be purified and their functions have to be demonstrated with a reconstituted proteoliposomal system.

We have recently shown (Laubinger & Dimroth, 1987) that the Na^+-stimulated ATPase of *P. modestum* is of the F_1F_0 type. The F_1 portion of the enzyme has a very similar subunit pattern as, e.g., the F_1 ATPase of *Escherichia coli*. Upon dissociation of the F_1 moiety from the membrane-bound subunits (F_0), the specific activation of ATP hydrolysis by Na^+ ions was lost; this activation was restored by reconstitution of the enzyme complex from the membrane-bound F_0 part and purified F_1 ATPase. In this paper, we describe the purification

of the ATP synthase (F_1F_0) and its functional reconstitution into liposomes. The effect of ionophores on the transport demonstrates the function of the ATP synthase as the primary Na^+ pump.

EXPERIMENTAL PROCEDURES

Growth of Propionigenium modestum. *Propionigenium modestum* was grown on succinate under strictly anaerobic conditions as described (Hilpert et al., 1984), but with the following modifications. Na_2S was replaced by sodium thioglycolate (0.75 g/L); the following reagents were added in the concentrations indicated in parentheses: biotin (0.1 mg/L); sodium succinate (80 mM); $CoSO_4 \cdot 7H_2O$ (1.12 mg/L); $MgCl_2 \cdot 6H_2O$ (0.4 g/L). Bacteria collected by continuous centrifugation were washed with 20 mM potassium phosphate buffer, pH 7.0, containing 5 mM $MgCl_2$, 0.35 M NaCl, and 0.1 mM dithioerythritol and stored in liquid N_2.

Purification of ATP Synthase. The bacteria (10 g wet weight) were disintegrated with a French press and the membranes isolated by fractional centrifugation, as described (Laubinger & Dimroth, 1987).

(*A*) *Fractionated Precipitation with Poly(ethylene glycol).* The ATP synthase was solubilized from the membranes with 11 mL (final volume) of 50 mM Mops/KOH buffer, pH 7.0, containing 1% Triton X-100 as described (Laubinger & Dimroth, 1987). To each 1 mL of the Triton extract were added 0.05 mL of 1 M $MgCl_2$ and 0.06 mL of a solution of poly(ethylene glycol) 6000 in H_2O (50% w/w). The mixture was stirred for 20 min, and the precipitate was removed by centrifugation (39000g, 15 min). The enzyme was precipitated by slowly adding with stirring 0.106 mL of the poly(ethylene glycol) solution per milliliter of the supernatant and collected after 20 min by centrifugation as above. The enzyme was dissolved in 2.5 mL of 5 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithioerythritol, 0.1 mM diisopropyl fluorophosphate, and 0.05% Triton X-100. Insoluble material was removed by centrifugation at 39000g for 15 min, and the ATP synthase was stored under liquid nitrogen until use.

(*B*) *Purification on Hydroxyapatite.* The ATP synthase was extracted from the membranes of 1 g of bacteria with 1.1 mL (final volume) of 50 mM potassium phosphate buffer, pH 8.0, containing 1% Triton X-100, 1 mM dithioerythritol, 0.1 mM diisopropyl fluorophosphate, and 0.2 M ammonium acetate. After 15 min at 4 °C with occasional shaking, the suspension was centrifuged at 200000g for 30 min.

The clear supernatant was applied to a hydroxyapatite column (1 × 6 cm) which was equilibrated with 50 mM potassium phosphate buffer, pH 8.0, containing 0.2 M ammonium acetate, 30% (v/v) glycerol, 0.1 mM diisopropyl fluorophosphate, 1 mM dithioerythritol, and 0.1% Triton X-100 (equilibration buffer). The column was washed at 0.5 mL/min with 8 mL of equilibration buffer, and the enzyme was eluted with 100 mM potassium phosphate buffer, pH 8.0, containing 0.2 M ammonium acetate, 30% (v/v) glycerol, 1 mM dithioerythritol, 0.1 mM diisopropyl fluorophosphate, and 0.5% Triton X-100. The ATP synthase containing fractions were pooled and concentrated to about 1 mL by ultrafiltration on a PM 10 membrane (Amicon).

Isolation of Subunit c (*Dicyclohexylcarbodiimide Binding Polypeptide*). Subunit c was extracted from the bacterial membranes with chloroform/methanol as described (Sebald et al., 1979) and stored as a solution in chloroform/methanol (2:1) under liquid N_2.

Labeling of ATP Synthase with [^{14}C]*Dicyclohexylcarbodiimide.* The incubation mixtures contained the following in 0.6 mL at 0 °C: 5 mM potassium phosphate buffer, pH 7.0,

0.05% Triton X-100, the purified ATP synthase (3.6–4.6 mg of protein), and 50 μM [^{14}C]dicyclohexylcarbodiimide (54 μCi/μmol). After 15 h, the solution was diluted with buffer to 0.72 mL to yield final concentrations as follows: 50 mM potassium phosphate, pH 8.0, 10% glycerol, 100 mM ammonium acetate, 0.1% Triton X-100, 6 μM $CaCl_2$, and 0.1 mM diisopropyl fluorophosphate (column buffer); a fraction of the diluted solution (0.6 mL) was applied to a HPLC column of hydroxyapatite (100 × 7.8 mm), equilibrated with column buffer. The ATP synthase was adsorbed, and unbound carbodiimide was eluted within 20 min at 0.3 mL/min. Subsequently, the labeled ATP synthase was eluted with a linear gradient of column buffer to 150 mM potassium phosphate buffer, pH 8.0, containing 20% glycerol, 2 μM $CaCl_2$, 0.5% Triton X-100, and 0.1 mM diisopropyl fluorophosphate (20 min). Fractions with the highest radioactivity (~2 mL) were placed into a dry dialysis bag and concentrated to about 0.4 mL by adding solid poly(ethylene glycol) 6000 to the outside.

Reconstitution of Proteoliposomes. A suspension of 60 mg of phosphatidylcholine (Sigma, type II-S) in 1.8–1.96 mL of buffer A (5 mM potassium phosphate, pH 7.0, containing 5 mM $MgCl_2$, 100 mM K_2SO_4, 2 mM NaCl, and 1 mM dithioerythritol) was sonicated for 2 × 1 min at 80 W under a nitrogen atmosphere, while cooling with ice. The purified ATP synthase (0.3–1.5 mg of protein in 0.04–0.2 mL of 5 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithioerythritol, 0.1 mM diisopropyl fluorophosphate, and 0.05% Triton X-100) was mixed with the suspension of the liposomes (final volume 2.0 mL) and incubated for 10 min at 25 °C with occasional shaking. The mixture was frozen in liquid nitrogen and thawed after 5 min at 25 °C. Freezing and thawing were repeated twice. The proteoliposomes were collected by centrifugation at 15 °C (220000g, 45 min), suspended in 0.6 mL of buffer A (final volume 1.1 mL), and sonicated 2 × 5 s at 40 W.

Orientation of the ATP Synthase in the Proteoliposomes. The percentage of ATP synthase that was incorporated into the proteoliposomes with the substrate binding site facing outward was determined by comparing the ATPase activity of the proteoliposomes with the total ATPase activity of F_1F_0 applied for reconstitution. Both measurements were performed in the absence of Triton X-100 to exclude activation of the enzyme by the detergent (Laubinger & Dimroth, 1987). Control experiments were performed in the presence of 0.1% Triton X-100 to determine the percentage of ATP synthase incorporated into the proteoliposomes (in either direction) of the total ATP synthase applied during reconstitution; the value was close to 100%.

Determination of Na^+ Transport. The incubation mixtures contained the following at 25 °C in 0.7 mL: 5 mM potassium phosphate buffer, pH 7.0, 5 mM $MgCl_2$, 100 mM K_2SO_4, 20 units of pyruvate kinase, 6 mM phosphoenolpyruvate, 2 mM $^{22}NaCl$ (380–420 cpm^{-1} nmol^{-1}), and the reconstituted proteoliposomes (6.7 mg of phospholipid). The transport was initiated after 5 min by adding 2.5 mM K-ATP. Samples (0.09 mL) were taken at various times, and external Na^+ was separated from that entrapped within the liposomes by passage over a small column of Dowex 50, K^+, as described (Dimroth, 1982). The radioactivity of the internal $^{22}Na^+$ was subsequently determined by liquid scintillation counting. Ionophores or dicyclohexylcarbodiimide was added as an ethanolic solution to yield final ethanol concentrations of 1% and 0.5%, respectively. Controls were performed containing the same amount of ethanol.

Table I: Purification of ATP Synthase of *P. modestum*[a]

step	protein (mg)	activity units	activity units/mg	yield (%)
(I) Purification with Poly(ethylene glycol)				
washed membranes	20	18	0.9	100
Triton extract	6.5	17	2.6	94
poly(ethylene glycol) fractionation	1.7	9	5.3	50
(II) Purification with Hydroxyapatite				
washed membrane	20	18	0.9	100
Triton extract	6.3	17	2.7	94
eluate from hydroxyapatite	1.8	10	5.7	56

[a] Starting material, 1 g of bacteria (wet weight). ATPase activities are recorded; all measurements were performed in the presence of 0.1% Triton X-100, as described under Experimental Procedures.

Determination of ATP Synthase. The ATP synthase was determined by its ATPase activity as described (Laubinger & Dimroth, 1987), in the presence of 0.1% Triton X-100. The ATPase activity of reconstituted proteoliposomes was measured at 5 mM potassium phosphate and 100 mM K_2SO_4 and in the absence of Triton X-100, unless indicated otherwise.

Amino Acid Analysis. Amino acid analyses were performed as described (Moore & Stein, 1963). Samples were hydrolyzed in 6 M HCl in sealed evacuated tubes for 24, 48, or 72 h at 110 °C, and for 20 h after performic acid oxidation (for the determination of cysteine and methionine). The data from the amino acid analyzer (Biotronic) were corrected for destruction and incomplete hydrolysis (Glazer et al., 1975). Tryptophan was determined by ultraviolet spectroscopy (Beaven & Holiday, 1952).

SDS Gel Electrophoresis. SDS gel electrophoresis was performed as described (Schägger et al., 1985). The protein samples were either placed into a boiling water bath or treated at 121 °C for 5 min in an autoclave.

The radioactivity of subunits labeled with [14C]dicyclohexylcarbodiimide was determined by cutting the unstained gel into strips of 2-mm length and placing them into 0.5 mL of cathode buffer (0.1 M Tris, 0.1 M Tricine, and 0.1% SDS). After 15 h at 60 °C, the scintillation cocktail was added, and the radioactivity was measured. A sample electrophoresed in parallel was stained with Coomassie brilliant blue.

Protein was determined according to Bradford (1976), with the modifications described (Laubinger & Dimroth, 1987).

RESULTS

Purification of ATP Synthase of P. modestum and Catalytic Properties. The purification of the ATP synthase was monitored by measuring the ATP hydrolyzing activity. The enzyme was extracted from the bacterial membranes with Triton X-100 in a yield of about 94% (Table I). Its further purification was accomplished either by fractional precipitation with poly(ethylene glycol) or by chromatography on a hydroxyapatite column. The specific ATPase activity of the final product obtained with either purification procedure was about 5.5 units/mg of protein which is approximately 6 times higher than that of the bacterial membranes. For its characterization, ATP synthase purified by poly(ethylene glycol) fractionation was used because this preparation contained less Triton X-100.

The results of Figure 1 indicate strong activation of ATPase activity of the purified ATP synthase by Na+ ions. The ATP hydrolysis rate increased as the Na+ concentration increased until saturation was reached at about 3 mM NaCl. The K_m for Na+ was about 0.8 mM. Extrapolation of the activation curve to zero Na+ concentration indicated that a certain amount of the ATPase activity (about 10% of maximum) was Na+ independent. These results are in accord with the notion that stimulation of the *P. modestum* ATPase activity by Na+

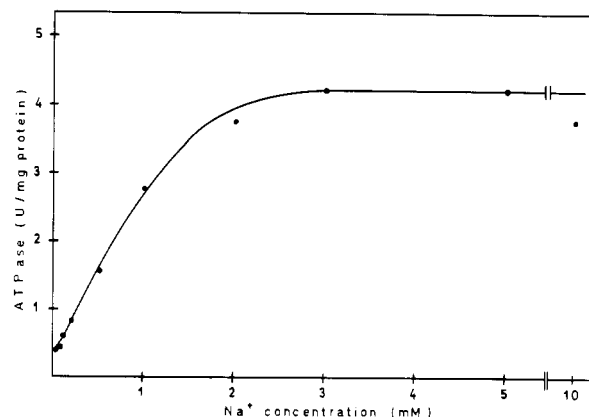

FIGURE 1: Effect of Na+ concentration on the ATPase activity of purified ATP synthase. Activity determinations were performed in the absence of Triton X-100 as described under Experimental Procedures. The usual measures were taken to keep endogenous Na+ concentration at a minimum (20 μM under these conditions).
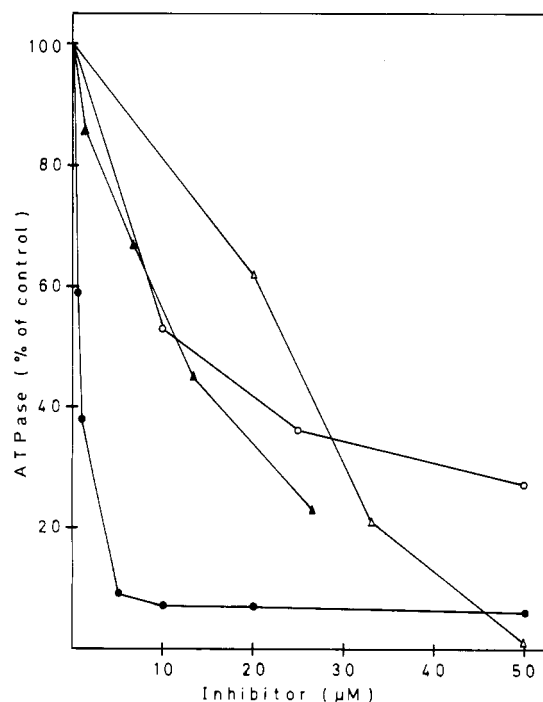

FIGURE 2: Inhibition of ATP synthase by dicyclohexylcarbodiimide (△), venturicidin (▲), tributyltin chloride (●), and azide (O). ATPase activities of purified ATP synthase were determined after a 5-min incubation (10 min in the case of venturicidin) of the enzyme at 25 °C with the inhibitor in the concentration indicated. 100% activity corresponds to 0.1 unit (venturicidin and tributyltin chloride) and 0.2 unit (dicyclohexylcarbodiimide and azide).

requires the intact F_1F_0 complex (Laubinger & Dimroth, 1987) and further indicate that the Na+-stimulated enzyme complex has been purified. As the cells are grown in the presence of 350 mM Na+ salts, the internal Na+ concentration is expected to be sufficiently high to saturate the ATPase.

The ATPase activity of F_1F_0 was strongly inhibited by low concentrations (<50 μM) of dicyclohexylcarbodiimide (Figure 2). This inhibition is a characteristic of all F_1F_0-type ATPases; it is caused by an irreversible and specific reaction of dicyclohexylcarbodiimide with an acidic amino acid residue of subunit c (Fillingame, 1976; Sebald & Hoppe, 1981). The inhibition correlates with the specific covalent labeling of subunit c of the *P. modestum* ATP synthase upon incubation with [14C]dicyclohexylcarbodiimide (cf. Figure 3). Other inhibitors of procaryotic F_1F_0 ATPases that interact with the F_0 moiety are venturicidin and tributyltin chloride (Linnett
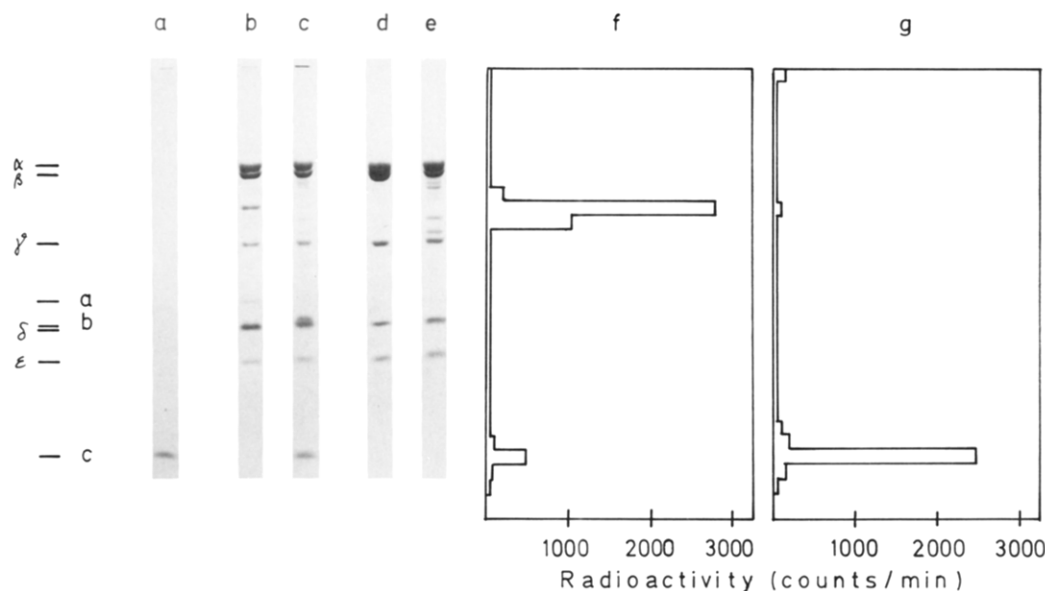
FIGURE 3: SDS electrophoresis of ATP synthase, F_1 ATPase, and the isolated subunit c. Lane a, subunit c extracted from the bacterial membranes with chloroform/methanol; lanes b and c, ATP synthase labeled with [^{14}C]dicyclohexylcarbodiimide; lanes d and e, F_1 ATPase. Samples applied to lanes b and d were denatured by boiling with SDS for 5 min, and the samples of lanes c and e were heated in an autoclave with SDS to 121 °C for 5 min. (f) Radioactivity profile corresponding to lane b; (g) radioactivity profile corresponding to lane c. The amounts of protein applied to the different lanes were (a) 1, (b) 7, (c) 7, (d) 5, (e) 5, (f) 7, and (g) 7 μg. For details, see Experimental Procedures.

& Beechey, 1979; Perlin et al., 1985). These compounds were also effective inhibitors of the ATP synthase of *P. modestum*; 50% inhibition of the ATPase activity of this enzyme was observed in the presence of 12 μM venturicidin and 0.7 μM tributyltin chloride, respectively (Figure 2). Azide, an inhibitor that interacts with the F_1 moiety of various F_1F_0 ATPases (Linnett & Beechey, 1979), also inhibited the ATPase activity of F_1F_0 of *P. modestum*, 50% inhibition being observed in the presence of 13 μM azide. Vanadate, however, an efficient inhibitor of ATPases of the E_1E_2 type (Pedersen & Carafoli, 1987), was without a significant effect on the ATP synthase of *P. modestum* (not shown). Thus, the effect of various inhibitors on the ATP synthase of *P. modestum* is in accord with its proposed structure as an F_1F_0-type enzyme.

Molecular Properties of the ATP Synthase. Figure 3 shows a comparison by SDS electrophoresis of the ATP synthase and the F_1 ATPase of *P. modestum*. Five of the polypeptides of the ATP synthase were thus easily identified as F_1 subunits; they are termed α, β, γ, δ, and ϵ and have apparent molecular weights of 58 000, 56 000, 37 600, 22 700, and 14 000, respectively (Laubinger & Dimroth, 1987). The pattern of subunits of the ATP synthase revealed in addition three bands that are not seen in the F_1 preparation, corresponding to apparent molecular weights of 45 000, 26 000, and 23 000 (compare lanes b and d of Figure 3). Whereas subunits of M_r 45 000 and 26 000 are seen as distinct bands, the presence of that with M_r 23 000 is indicated by the clearly increased staining intensity and width of the band at the position of the δ chain in ATP synthase (lane b) as compared to F_1 (lane d). The other bands corresponding to each other (Figure 3, lanes b and d), however, are more intensely stained in the F_1 preparation. The F_0 portion of the *P. modestum* ATP synthase, therefore, probably consists of three different subunits. In analogy to other ATP synthases, the polypeptides of M_r 26 000 and 23 000 are termed a and b, respectively.

Subunit c is known as a highly hydrophobic polypeptide of M_r ~8000 (Fillingame, 1976; Sebald et al., 1979). Under the usual conditions of sample preparation, a band of this size is not seen in the *P. modestum* ATP synthase, which instead yields a band corresponding to M_r 45 000. However, after the

enzyme was heated with SDS to 121 °C for 5 min, the band at the position of M_r 45 000 disappeared, and a band at the position of M_r 7500 became clearly visible (Figure 3, lanes b and c). These results could indicate the presence of subunit c and the aggregation of the polypeptide to a supramolecular complex that resists dissociation by SDS even at 100 °C. A comparison of lanes b and c or d and e of Figure 3 also reveals that heating to 121 °C causes some proteolytic degradation. Remarkable is the disappearance of subunit a from F_1F_0 and the appearance of a fragment moving just behind the subunits of M_r 23 000 (compare lanes b and c). In analogy to other ATP synthases (Fillingame, 1976), subunit c of the *P. modestum* enzyme was extracted from the bacterial membranes with chloroform/methanol. The extract apparently consisted of pure subunit c in its monomeric form (Figure 3, lane a). The pattern of subunits of F_1F_0 was identical regardless whether the enzyme was purified by fractionation with poly(ethylene glycol) or by hydroxyapatite column chromatography (cf. Table I). The intensities of the bands seen in Figure 3, lane b, increased in parallel with the increase in specific activity of the ATP synthase; other bands apparent in less pure preparations of the enzyme disappeared during purification.

The presence of subunit c in the ATP synthase and its aggregation to a supramolecular complex of M_r 45 000 were confirmed by labeling with [^{14}C]dicyclohexylcarbodiimide, which is known to bind specifically to subunit c (Fillingame, 1976). Under the experimental conditions applied, about 3–4 mol of the inhibitor became covalently linked to 500 000 g of the enzyme. The radioactivity was specifically bound to fractions containing subunit c; depending on the conditions of sample preparation (see above), either the monomer of subunit c or the aggregate of apparent molecular weight 45 000 became labeled (Figure 3, lanes f and g). In summary, we assume that the F_0 part of the *P. modestum* ATP synthase consists of three subunits, a, b, and c, with apparent molecular weights of 26 000, 23 000, and 7500, respectively.

The amino acid analysis of subunit c of the *P. modestum* ATP synthase is reported in Table II and compared with the amino acid composition of subunits c of other ATP synthases. Subunit c of the *P. modestum* ATP synthase consists of ap-

Table II: Amino Acid Composition of Subunit c of the ATP Synthase of *P. modestum* and of ATP Synthases from *E. coli* (Fillingame, 1976), *Neurospora crassa, Saccharomyces cerevisiae* (Sebald et al., 1979), and Bovine Heart Mitochondria (Graf & Sebald, 1978)[a]

amino acid	amount (no. of residues) in subunit c from				
	P. modestum	*E. coli*	*N. crassa*	*S. cerevisiae*	bovine heart
aspartic acid + asparagine	3.9	5	4	3	3
threonine	2.8	1	2	3	3
serine	4.9	0	5	5	5
glutamic acid + glutamine	5.8	4	5	2	3
proline	2.9	3	1	2	1
glycine	12.8	10	11	10	12
alanine	15.7	14	14	10	14
cysteine	0.0	0	0	1	1
valine	7.2	6	6	6	4
methionine	2.7	8	4	3	3
isoleucine	4.8	8	6	9	7
leucine	6.3	13	11	12	9
tyrosine	1.8	2	2	1	2
phenylalanine	1.0	4	6	6	8
tryptophan	0	0	0	0	0
lysine	3.3	1	2	2	2
histidine	0.0	0	0	0	0
arginine	1.0	2	2	1	1
total residues	(78)	81	81	76	78

[a] The values for subunit c of *P. modestum* were normalized to arginine.

proximately 78 residues yielding a molecular weight of about 7500. Its size and its composition are remarkably similar to subunits c of other ATP synthases. A common feature is the absence of histidine and tryptophan and the low content of polar residues. A characteristic of subunit c of the *P. modestum* enzyme is a comparatively low content of the hydrophobic residues isoleucine, leucine, and phenylalanine (18% versus 28–36% in the ATP synthases listed in Table II).

Na$^+$ Transport into Reconstituted Proteoliposomes. The purified ATP synthase was incorporated into liposomes by a freeze–thaw–sonication procedure, as described under Experimental Procedures. Nearly 100% of the ATP synthase applied was incorporated, and about 40–70% was oriented with the substrate binding site facing outward. The kinetics of Na$^+$ transport into reconstituted proteoliposomes are shown in Figure 4. Almost no Na$^+$ ions were transported into the vesicles in the absence of ATP. Upon its addition, Na$^+$ was rapidly accumulated inside the liposomes. As incubation continued, the rate of Na$^+$ uptake gradually declined, and after about 25 min, the internal Na$^+$ concentration reached a steady state. In the absence of an ATP regenerating system, the initial rate of Na$^+$ uptake was about 40% lower than in its presence. The accumulation of Na$^+$ ions was completely prevented by incubation of the proteoliposomes with 50 μM dicyclohexylcarbodiimide for 20 min (Figure 4). After this treatment, the ATPase activity was completely abolished. The ATPase activity of the untreated proteoliposomes was 58 nmol min^{-1} (mg of phospholipid)$^{-1}$, and the initial rate of Na$^+$ uptake was 2.4 nmol min^{-1} (mg of phospholipid)$^{-1}$. With proteoliposomes containing 4 times less enzyme, the rates for ATP hydrolysis and Na$^+$ uptake were 7 nmol min^{-1} (mg of phospholipid)$^{-1}$ and 1.5 nmol min^{-1} (mg of phospholipid)$^{-1}$, respectively (cf. Figure 5). The system is thus highly uncoupled, and the degree of uncoupling increases as the amount of enzyme incorporated into the liposomes increases. The reason for this effect could be an increasing leakiness of the proteoliposomes to Na$^+$ due to the increasing amount of Triton
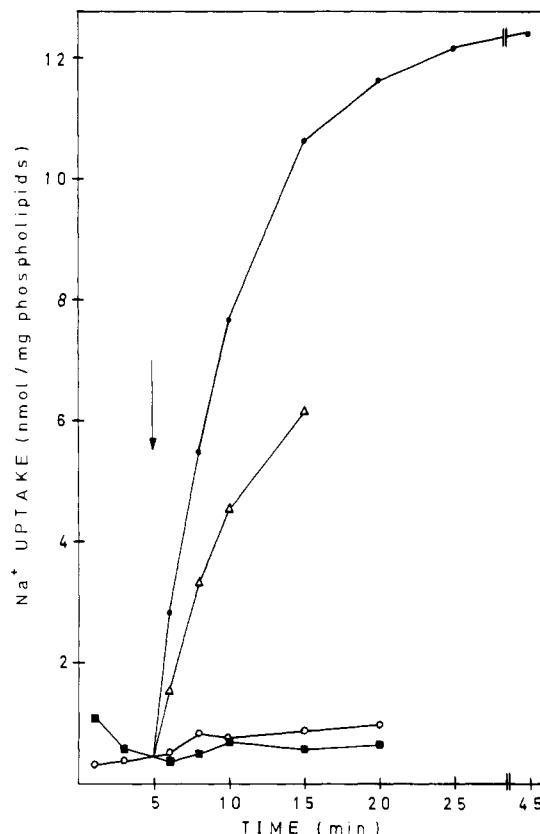

FIGURE 4: Kinetics of Na$^+$ uptake into reconstituted ATP synthase containing proteoliposomes and effect of dicyclohexylcarbodiimide on the transport. The reconstitution of ATP synthase was performed with 20 μg of protein/mg of phospholipid. The uptake of Na$^+$ ions was determined in incubation mixtures containing the proteoliposomes (6.7 mg of phospholipid) in a total volume of 0.7 mL. The addition of ATP is marked by the arrow (●). Parallel experiments were performed with incubation mixtures containing proteoliposomes (3.4 mg of phospholipid) in a total volume of 1.4 mL, preincubated with 50 μM dicyclohexylcarbodiimide for 20 min (O). Control without ATP addition (■). Control with ATP but without the ATP regenerating system (phosphoenolpyruvate and pyruvate kinase) (△). For details, see Experimental Procedures.

X-100 added with the enzyme during reconstitution (cf. Experimental Procedures).

The transport of Na$^+$ ions was markedly influenced by the presence of certain ionophores, as shown in Figure 5. Na$^+$ uptake into the proteoliposomes was completely abolished by the Na$^+$-carrying ionophore monensin. On the other hand, valinomycin (in the presence of K$^+$) or the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone stimulated the Na$^+$ transport rate 4–5-fold, without significantly affecting the ATPase activity. Therefore, under these conditions, the Na$^+$:ATP coupling ratio is about 1. The results of Figure 5 are indicative of an electrogenic Na$^+$ transport: a membrane potential which limits the transport rate is released by increasing the conductance of K$^+$ or H$^+$, respectively, across the membrane. These results further demonstrate that a proton gradient is not an intermediate of the Na$^+$ gradient established by the ATP synthase of *P. modestum*. This enzyme is therefore the first clearly defined example of an ATP synthase of the F$_1$F$_0$ type that acts as a primary Na$^+$ pump.

DISCUSSION

In *P. modestum*, ATP synthesis is coupled to the electrochemical Na$^+$ gradient established upon decarboxylation of methylmalonyl-CoA. Two possible coupling mechanisms were conceivable as depicted in Scheme I. Either the Na$^+$ gradient was first converted into a proton gradient by a Na$^+$/H$^+$ an-
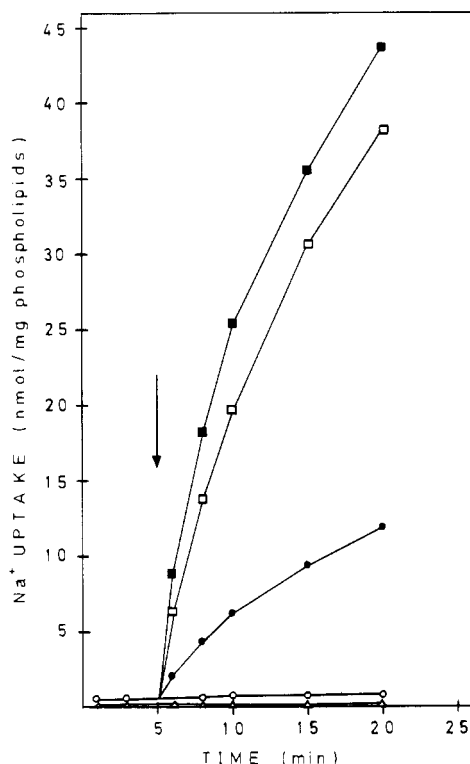
FIGURE 5: Effect of ionophores on the kinetics of Na^+ uptake into reconstituted ATP synthase containing proteoliposomes. The reconstitution was performed at a protein:phospholipid ratio of 5 $\mu g/mg$. The uptake of Na^+ ions was determined in incubation mixtures containing the proteoliposomes (6.7 mg of phospholipid) in a total volume of 0.7 mL. The addition of ATP is marked by the arrow; ionophores were contained as follows: (●) control; (■) 30 μM valinomycin; (□) 50 μM carbonyl cyanide m-chlorophenylhydrazone; (▲) 50 μM monensin; (○) control in the absence of ATP. For details, see Experimental Procedures.

Scheme I: Alternative Models for the Coupling of ATP Synthesis to the Decarboxylation of Methylmalonyl-CoA[a]
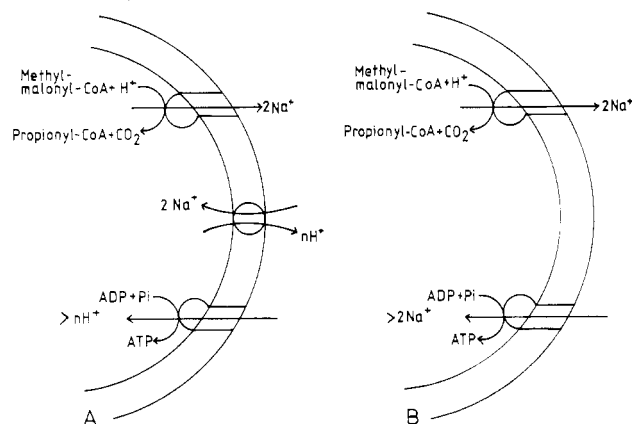
[a] A, indirect coupling by a Na^+ cycle in combination with a H^+ cycle; B, direct coupling by a Na^+ cycle.

tiporter and the thus-generated H^+ gradient served a H^+-translocating ATP synthase as energy source for ATP synthesis or a novel type of Na^+-translocating ATP synthase could use the Na^+ gradient directly. To meet the bioenergetic requirements, the Na^+ (or H^+)/ATP stoichiometry is expected to exceed that of Na^+/methylmalonyl-CoA, which is 2 (Dimroth & Hilpert, 1984; Hilpert et al., 1984). Although our previous results were in favor of the direct Na^+-coupled ATP synthesis mechanism, its final proof required purification of the ATP synthase and reconstitution of its energy-converting function. This is described in the present paper. We show

here that proteoliposomes reconstituted with the purified ATP synthase performed an ATP-dependent accumulation of Na^+ ions in an electrogenic manner. Most importantly, the transport was not prevented in the presence of the proton conducter carbonyl cyanide m-chlorophenylhydrazone but stimulated to about the same extent as with valinomycin. These results are in accord only with the ATP synthase acting as a primary Na^+ pump and exclude the other possible mechanism which involves the intermediate formation of a proton gradient. $P.$ $modestum$ is thus the first example of an organism in which ATP synthesis by a chemiosmotic mechanism depends upon the circulation of Na^+ ions and is totally independent of H^+ as the coupling ion. Since the Na^+ circuit directly couples ATP synthesis to the decarboxylation of methylmalonyl-CoA, we propose to term this reaction sequence decarboxylation phosphorylation to distinguish it from electron transport and substrate level phosphorylation.

Our finding of an ATP synthase of the F_1F_0 structure that uses Na^+ and not H^+ as the coupling ion has implications on the mechanism of ATP synthesis in general. In Mitchell's view (Mitchell, 1974), the synthesis of ATP is coupled obligatorily to the vectorial movement of protons. These protons move downhill the electrochemical gradient into the catalytic site where they attack an oxygen atom of phosphate, yielding H_2O and an extremely reactive species which spontaneously reacts with ADP, forming ATP. It is obvious that this mechanism is restricted to protons and cannot be adopted for the Na^+-coupled ATP synthesis. Therefore, if the basic mechanism of the Na^+- and H^+-coupled F_1F_0 ATP synthases is the same, a participation of the vectorial protons in the chemical reaction at the catalytic site must be dismissed. An alternative suggestion by Boyer (1975), however, that the energy available from the electrochemical gradient is used to promote substrate binding and product release by a distinct set of conformational changes is in accord with either H^+- or Na^+-coupled ATP synthesis.

It is remarkable that in spite of using different coupling ions, the ATP synthases of $P.$ $modestum$ and $E.$ $coli$ [for a review, see Futai and Kanazawa (1983)] are related. Both enzymes are composed of a soluble part (F_1) responsible for the chemical reaction and a membrane-bound F_0 moiety which acts as a proton channel in $E.$ $coli$ and probably conducts Na^+ ions in $P.$ $modestum$. Like the $E.$ $coli$ enzyme, the ATP synthase of $P.$ $modestum$ was strongly inhibited by dicyclohexylcarbodiimide, venturicidin, tributyltin chloride, and azide. Vanadate, on the other hand, which is an effective inhibitor of ATPases of the E_1E_2 type, was without effect. The subunit patterns observed by SDS electrophoresis of the ATP synthases of $P.$ $modestum$ and $E.$ $coli$ were remarkably similar. The five polypeptides of the F_1 parts corresponded to each other with respect to molecular weights and staining intensities; thus, the weight-homologous polypeptides could be present within each enzyme complex in the same stoichiometry. The three additional polypeptides present in the ATP synthase of $P.$ $modestum$ have apparent molecular weights of 26 000, 23 000, and 7500 and could thus be homologous to the a, b, and c subunits of the F_0 portion of the $E.$ $coli$ ATP synthase (apparent molecular weights determined by SDS gel electrophoresis of 24 000, 19 000, and 8400) (Foster & Fillingame, 1979).

The homology between subunits c of the $P.$ $modestum$ ATP synthase and that from other sources could be clearly identified: (i) analogous to, e.g., the $E.$ $coli$ enzyme (Fillingame, 1976), subunit c of the $P.$ $modestum$ ATP synthase was specifically labeled with [^{14}C]dicyclohexylcarbodiimide; (ii)

the different subunits c were extracted in a pure state from the membranes with chloroform/methanol; (iii) the amino acid composition of subunit c of the *P. modestum* ATP synthase resembled those of subunits c from other sources (cf. Table II). A remarkable property of subunit c from *P. modestum* is its occurrence as a supramolecular complex probably containing six copies of the polypeptide. This complex is so stable that it resists dissociation by SDS even at 100 °C; 121 °C is required for its dissociation. This is the strongest complex of subunits c known. A hexamer has also been observed for subunit c from yeast mitochondria (Tzagoloff & Akai, 1972). The dicyclohexylcarbodiimide binding protein of chloroplast ATP synthase occurs as a supramolecular complex of probably 12 copies of the polypeptide (Fromme et al., 1987). These complexes, however, dissociate by heating with SDS to 100 °C.

The *P. modestum* ATP synthase is the first demonstrated example of a Na^+-translocating ATP synthase having a F_1F_0 structure. The remarkable resemblance to the H^+-translocating ATP synthase of *E. coli* suggests that the two enzymes are phylogenetically related. In addition, it can be expected that Na^+-translocating ATP synthases also occur in other organisms, for which a Na^+-coupled ATP synthesis mechanism would be advantageous. Possible candidates are other anaerobes, marine and halophilic organisms, and especially alkalophilic bacteria.

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