Fasano, O., Bruns, W., Crechet, J. B., Sander, G., & Parmeggiani, A. (1978) *Eur. J. Biochem. 89*, 557–565.
Fischer, E., Wolf, H., Hantke, K., & Parmeggiani, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4341–4345.
Goody, R. S., & Lebermann, R. (1979) *FEBS Lett. 102*, 269–272.
Hamel, E., & Nakamoto, T. (1972) *Biochemistry 11*, 3933–3938.
Kaltschmidt, E., & Wittmann, H. G. (1970) *Anal. Biochem. 36*, 401–412.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Lucas-Lenard, J., & Beres, L. (1974) *Enzymes, 3rd Ed. 10*, 53–86.
Maurel, P., & Douzou, P. (1978) in *Frontiers in Physicochemical Biology* (Pullman, B., Ed.) pp 421–457, Academic Press, New York.
Miller, D. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 752–755.
Miller, D. L., & Weissbach, H. (1970) *Arch. Biochem. Biophys. 141*, 26–37.
Miller, D. L., & Weissbach, H. (1977) in *Molecular Mechanisms of Protein Biosynthesis* (Weissbach, H., & Pestka, S., Eds.) pp 323–373, Academic Press, New York.
Parmeggiani, A., & Sander, G. (1980) *Top. Antibiot. Chem. 5*, 159–221.
Quigley, G. J., Teeter, M. M., & Rich, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 64–68.
Sander, G. (1977) *Eur. J. Biochem. 75*, 523–531.
Sander, G., & Crechet, J. B. (1978) in *Ribosomes and Nucleic Acid Metabolism* (Zelinka, J., & Balan, J., Eds.) Vol. 3, pp 357–364, Publishing House of the Slovak Academy of Sciences, Bratislava.
Sander, G., Marsh, R. C., Voigt, J., & Parmeggiani, A. (1975) *Biochemistry 14*, 1805–1814.
Sander, G., Okonek, M., Crechet, J. B., Ivell, R., Bocchini, V., & Parmeggiani, A. (1979) *FEBS Lett. 98*, 111–114.
Setlow, B., & Lowenstein, J. M. (1967) *J. Biol. Chem. 342*, 607–615.
Sillén, L. G. (1971) in *Stability Constants of Metal-Ion Complexes*, Special Publication, part I, the Chemical Sociaty, Alden & Mowbray Ltd., Alden Press, Oxford.
Spiro, T. G. (1973) in *Inorganic Biochemistry* (Eichhorn, G. L., Ed.) Vol. 1, pp 549–581, Elsevier, Amsterdam.
Spitnik-Elson, P., & Elson, D. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 17*, 77–98.
Suelter, C. H. (1974) in *Metals in Biological Systems* (Sigel, H., Ed.) Vol. 3, pp 201–251, Marcel Dekker, New York.
Thompson, R. C., & Stone, P. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 198–202.
Williams, R. J. P. (1971) in *Bioinorganic Chemistry* (Gould, R. F., Ed.) Adv. Chem. Ser. No. 100, pp 155–173, American Chemical Society, Washington, D.C.
Wilson, G. E., & Cohn, M. (1977) *J. Biol. Chem. 252*, 2004–2009.
Wittinghofer, A., & Lebermann, R. (1979) *Eur. J. Biochem. 93*, 95–101.
Wolf, H., Chinali, G., & Parmeggiani, A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4910–4914.
Wolf, H., Chinali, G., & Parmeggiani, A. (1977) *Eur. J. Biochem. 75*, 67–75.

pH Dependence of Adenosine 5′-Triphosphate Synthesis and Hydrolysis Catalyzed by Reconstituted Chloroplast Coupling Factor†

Teruhiro Takabe and Gordon G. Hammes*

ABSTRACT: The purified ATP-synthesizing complex from chloroplasts has been reconstituted into phospholipid vesicles with bacteriorhodopsin by use of octyl glucoside. Phosphorylation rates up to 90 nmol of ATP (mg of protein)$^{-1}$ min^{-1} have been achieved. The dependence of the steady-state kinetic parameters on external and internal pH for both synthesis and hydrolysis was determined. The Michaelis constants are independent of the magnitude of the pH gradient at external pH values of 6.6 and 8.0. The dependence of the maximum velocity for ATP synthesis on the external pH is bell shaped at a constant pH gradient with a maximum at about pH 6.7. The variation of the maximum velocity with external pH is not dependent on the magnitude of the pH gradient. At external pH values of 6.6 and 8.0, the maximum velocity for ATP synthesis varies with approximately the 2.3 power of the internal hydrogen ion concentration. The maximum velocity for ATP hydrolysis also is dependent on the external pH, with a maximum at about pH 8.4; however, most of the ATPase activity is not coupled to the proton flux. Both Mg^{2+} and Mn^{2+} are good cofactors for ATP synthesis and hydrolysis whereas Ca^{2+} is completely ineffective for synthesis and only about 10% as effective as Mg^{2+} and Mn^{2+} for hydrolysis. The results obtained suggest that ATP synthesis or hydrolysis may be coupled to proton pumping indirectly, as, for example, by conformational changes.

The mechanism of ATP synthesis remains an unresolved question, despite the large amount of published work concerned with this problem (Boyer et al., 1977; Harris, 1978; Penefsky, 1979; Shavit, 1980; Fillingame, 1980). The chemiosmotic hypothesis (Boyer et al., 1977) forms a base for mechanistic considerations, but considerable controversy exists with regard to other aspects of the mechanism. We have embarked on a long-range study of the physical and catalytic properties of the purified dicyclohexylcarbodiimide-sensitive ATPase (DSA)[1] from chloroplasts (Pick & Racker, 1979; Baird et al.,

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[1] Abbreviations used: DSA, dicyclohexylcarbodiimide-sensitive ATP-synthesizing complex from chloroplasts; EDTA, ethylenediaminetetraacetic acid.

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1979; Cerione & Hammes, 1981; Dewey & Hammes, 1981). A completely defined, easily modulated ATP-synthesizing system can be made by the incorporation of DSA and bacteriorhodopsin into phospholipid vesicles (Racker & Stoeckenius, 1974). The internal pH can be maintained at a desired value by varying the light intensity used to activate the bacteriorhodopsin proton pump, and the external pH and substrate concentrations also can be easily controlled. The number of protons pumped per ATP hydrolyzed or synthesized in the reconstituted system has been determined to be 3, and a general mechanism for the catalysis and proton pumping has been proposed (Dewey & Hammes, 1981).

In the work reported here, the dependence of ATP synthesis and hydrolysis on both the internal and external pH has been determined for DSA and bacteriorhodopsin reconstituted into phospholipid vesicles. The steady-state kinetic parameters have been determined, and the metal ion specificity also has been examined.

Materials and Methods

Chemicals. Cholic acid was purchased from Sigma Chemical Co. and recrystallized prior to use (Kagawa & Racker, 1971). Octyl glucoside was purchased from Calbiochem-Behring Corp. Hexokinase, ATP, ADP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone, 9-aminoacridine, and valinomycin were obtained from Sigma Chemical Co. Asolectin (95% purified soybean phospholipids) was from Associated Concentrates Inc. Dicyclohexylcarbodiimide was from Aldrich Chemical Co. [γ-^{32}P]ATP from New England Nuclear was purified on a Bio-Rad AG-1-4x column with Bio-Rad AG-50-W-8x Li^+ resin (Bruist & Hammes, 1981). The [^{32}P]phosphate from I.N.C. was heated to 90–100 °C in 2 N HCl for 1–2 h to hydrolyze any pyrophosphate present. All other chemicals used were analytical grade, and solutions were made with deionized distilled water.

Protein Preparation. Cell suspensions of *Halobacterium halobium* S-9 were a gift from Professor Russell McDonald. Bacteriorhodopsin was purified from these suspensions on sucrose density gradients as previously described (Cerione & Hammes, 1981). The concentration of bacteriorhodopsin was determined by the absorbance at 560 nm, assuming an extinction coefficient of 54000 M^{-1} cm^{-1} and a molecular weight of 26000 (Oesterhelt & Stoeckenius, 1971). The DSA complex was prepared from market spinach by a modification of the procedure of Pick & Racker (1979) (Cerione & Hammes, 1981). The concentration of DSA was determined by a modified Lowry procedure (Bensadoun & Weinstein, 1976).

Reconstitution. Phospholipid vesicles were made by sonicating asolectin (40 mg/mL) to clarity in 0.15 M KCl, 10 mM *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (pH 7.3), and 0.5 mM EDTA. Bacteriorhodopsin (0.5 mg/mL) was reconstituted into the vesicles (20 mg of asolectin/mL) by incubation on ice for 5 min in the presence of 1.25% octyl glucoside. After centrifugation for 30 min at 40000 rpm in a Beckman Ti60 rotor, the pellet was taken up in the buffer mixture to give asolectin and bacteriorhodopsin concentrations of 40 and 1.0 mg/mL, respectively. The DSA (0.35 mg/mL) was reconstituted into these preformed vesicles by incubation on ice for 5 min in the presence of 0.7% octyl glucoside followed by dilution into the reaction mixture (10–100-fold). Freeze–thaw (Dewey & Hammes, 1981), cholate dilution (Racker et al., 1975), or direct dilution (Hauska et al., 1980) reconstitutions also were utilized.

Assays. ATP hydrolysis and synthesis were assayed as previously described (Dewey & Hammes, 1981). The reaction mixture was illuminated by a GE EHL 300-W lamp, with the light being filtered by a Corning CS 3-69 filter and 6 cm of water. The pH gradient across the vesicles was monitored by the quenching of the fluorescence of 9-aminoacridine, which usually was present at a concentration of 12.5 μM. The fluorescence was excited at 380 nm with a Hanovia 200-W Xenon arc lamp coupled to a 1/4 m Bausch and Lomb monochromator. The fluorescence was detected with a Ditric 480-nm narrow band-pass filter and an EMI 9635 QB photomultiplier. The rate of reaction was determined by taking aliquots at 3 or more times (usually 0, 5, and 10 min); the slope of the linear plot of [^{32}P]P_i (hydrolysis) or [^{32}P]glucose 6-phosphate (synthesis) vs. time was taken as the initial velocity. The assay mixture contained reconstituted DSA, the desired concentrations of substrates and Mg^{2+}, 5 mM dithiothreitol, 0.3 μM valinomycin, 0.15 M KCl, and 50 mM buffer. For the assay of ATP synthesis, 10 units/mL hexokinase and 5 mM glucose also were present. The results on different days or preparations were normalized to give the same activity under a given set of experimental conditions. All experiments were carried out at room temperature (22 ± 2 °C). The free metal concentration was calculated by using known binding constants for metal association with ATP, ADP, and P_i (Dawson et al., 1969). The internal volume of the octyl glucoside reconstituted vesicles containing DSA and bacteriorhodopsin was estimated by measuring the amount of 0.2 M ferricyanide per milligram of lipid trapped in the liposomes during reconstitution after passage through a Sephadex G-25 column (1.0 × 25 cm) (Orlich & Hauska, 1980). After corrections for light scattering and absorption on the outer surface of liposomes, the internal volume was found to be 1.1 μL/mg of phospholipid following reconstitution in the presence of octyl glucoside.

Table I: Activity from Various Reconstitution Procedures[a]

method	ATP hydrolysis (nmol mg^{-1} min^{-1})	ATP synthesis (nmol mg^{-1} min^{-1})	fluorescence quenching (%)
freeze–thaw (without bacteriorhodopsin)	400		
freeze–thaw	82	5.1	43
dilution	370	6.1	52
1.25%–0.7% octyl glucoside	460	50.0	51

[a] ATP hydrolysis assay: 4 mg of phospholipid, 35 μg of DSA, 0.1 mg of bacteriorhodopsin/mL 3 mM ATP, 8 mM Mg^{2+}, 5 mM dithiothreitol, 0.3 μM valinomycin, 0.15 M KCl, and 50 mM Tricine, pH 8.4, at 22 °C. ATP synthesis assay: phospholipid, DSA, and bacteriorhodopsin as in hydrolysis assay, 30 μM ADP, 2 mM P_i, 5.4 mM Mg^{2+}, 5 mM dithiothreitol, 12.5 μM 9-aminoacridine, 10 units/mL hexokinase, 5 mM glucose, 0.3 μM valinomycin, 0.15 M KCl, 50 mM 2-*N*-morpholinoethanesulfonic acid, pH 6.6, at 22 °C. The procedure used for freeze–thaw reconstitution is given in Dewey & Hammes (1981), and that for octyl glucoside reconstitution is given under Materials and Methods. In the dilution reconstitution, bacteriorhodopsin was reconstituted into phospholipid vesicles as described under Materials and Methods, DSA (0.35 mg/mL) was added, and the mixture was diluted to the desired concentration in the assay medium.

Results

The rates of ATP hydrolysis and synthesis obtained by different reconstitution procedures are presented in Table I. The procedure utilizing octyl glucoside gives the highest synthetic rates and has been used to obtain the results presented here. Specific activities for ATP synthesis as high as

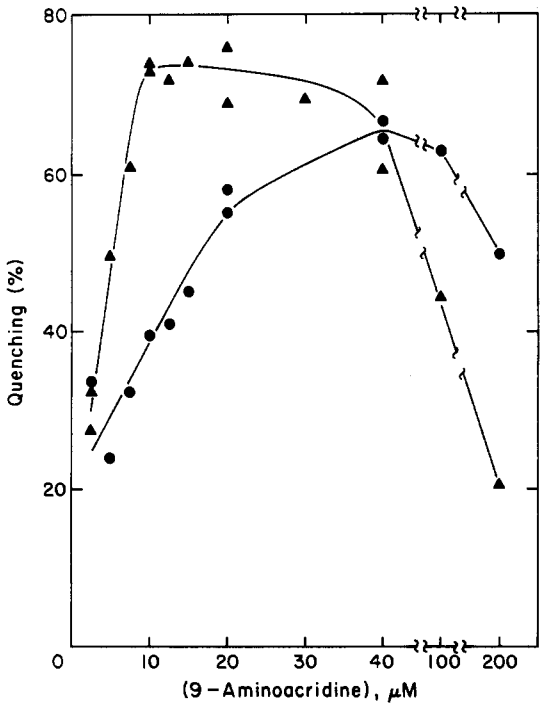

FIGURE 1: Plot of the observed fluorescence quenching of 9-aminoacridine in bacteriorhodopsin-DSA phospholipid vesicles at constant light intensity vs. the concentration of 9-aminoacridine. The fluorescence was excited at 380 nm and observed at 480 nm. The vesicles were prepared by octyl glucoside reconstitution as described under Materials and Methods and were in 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (pH 7.3), 5 mM $MgCl_2$, 0.15 M KCl, 0.3 μM valinomycin, and 5 mM dithiothreitol: (▲) V = 0.00128; (●) V = 0.00428.

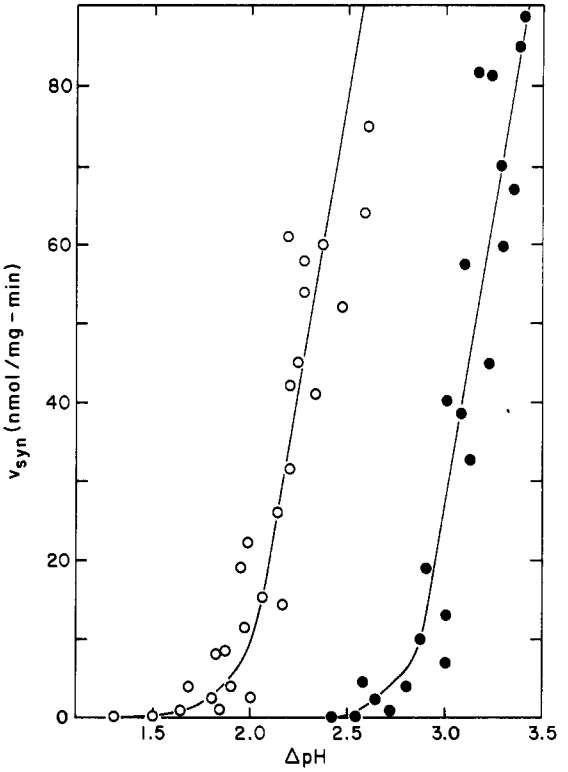

FIGURE 2: Plot of the initial velocity of ATP synthesis, v_{syn}, vs. the apparent ΔpH at two different bacteriorhodopsin–DSA vesicle concentrations: (○) V = 0.00428; (●) V = 0.00128. The assays were carried out with 30 μM ADP, 2.0 mM P_i, 5.4 mM Mg^{2+}, 12.5 μM 9-aminoacridine, 50 mM 2-*N*-morpholinoethanesulfonic acid (pH 6.6), 0.15 M KCl, 0.3 μM valinomycin, 5 mM dithiothreitol, 10 units of hexokinase, and 5 mM glucose.

90 nmol of ATP mg^{-1} min^{-1} have been achieved. The rate of ATP synthesis is linear with time for at least 30 min [pH 7.0, 30 μM ADP, 2.0 mM P_i, 5.5 mM Mg^{2+}, 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, and 0.15 M KCl]. The initial rate of hydrolysis is proportional to the amount of DSA over the range 3.5–35 μg/mL, but some deviation from linearity occurs for ATP synthesis at concentrations greater than 25 μg/mL.

The difference in pH between the inside and outside of the vesicles, ΔpH, can be calculated from the relationship (Schuldiner et al. 1972)

$$\Delta pH = \log Q/[(1 - Q)V] \quad (1)$$

where Q is the fractional fluorescence quenching of 9-aminoacridine and V is the ratio of the internal volume of the vesicles to the external volume. However, this is an empirical relationship, and we have found that the observed quenching (at constant light intensity) depends on both the vesicle and 9-aminoacridine concentrations. Some typical results are shown in Figure 1. The drop in quenching at high concentrations of 9-aminoacridine is due to the uncoupling properties of the fluorescent indicator. For an internal to external volume ratio of 0.00128, a plateau is reached over the concentration range of 10–30 μM 9-aminoacridine. For an internal to external volume ratio of 0.00428, a plateau region is not observed. (These results also may depend on the method of reconstitution.) The dramatic effect this can have on the apparent dependence of the rate of ATP synthesis on ΔpH is shown in Figure 2 where the rate is shown as a function of the calculated ΔpH for two different concentrations of vesicles at constant light intensity and 9-aminoacridine concentration. In this work, we have corrected all values of the calculated ΔpH to correspond to that which would be observed with 12.5 μM 9-aminoacridine and V = 0.00128 (the plateau region of Figure 1). This corresponds, for example, to adding 0.9 to the values of ΔpH for the curve at the higher vesicle concentration in Figure 2. However, most of the results reported were obtained at V = 0.00128. The presence of valinomycin prevented the formation of a membrane potential; this was experimentally verified by measurements of the fluorescence of 8-anilino-1-naphthalenesulfonic acid during the assay (Dewey & Hammes, 1981).

The steady-state initial rate of ATP synthesis is shown as a function of the external pH at constant illumination in Figure 3. In these experiments, the ADP concentration was 30 μM, the P_i concentration 2.0 mM, and the free Mg^{2+} concentration 5 mM. The buffers (50 mM) used were 2-*N*-morpholinoethanesulfonic acid for pH 5.9–6.6, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid for pH 7.0–7.7, *N*-tris(hydroxymethyl)methylglycine (Tricine) for pH 8.0–8.7, and 0.15 M KCl was present in all cases. Under the conditions employed, essentially the maximum velocity is being measured. The value of ΔpH at constant illumination varies about 0.2 unit over the range of external pH explored and has a broad maximum at pH 7.3. The variation of the maximum velocity with external pH at two different *constant* values of ΔpH, 2.8 and 3.2, is included in Figure 3. The same pH optimum, pH 6.7, is obtained at two different vesicle concentrations, indicating this optimum is not an artifact of using 9-aminoacridine as a measure of ΔpH.

The dependence of the steady-state initial velocity for ATP hydrolysis on the external pH is shown in Figure 4. The concentration of ATP was 3 mM and the free metal and buffer concentrations were the same as those used in the measurement of ATP synthesis. Again essentially the maximum velocity is being measured. The hydrolysis of ATP does not cause the

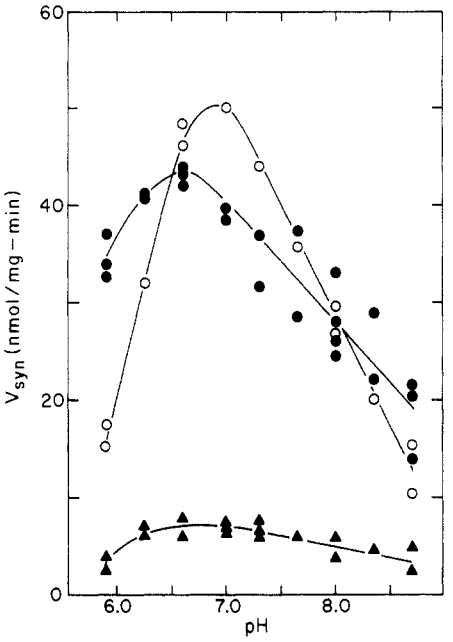

FIGURE 3: Plot of the steady-state maximum velocity for ATP synthesis, V_{syn}, vs. the external pH. The concentrations of reactants and buffer are given in the text: (○) constant illumination (ΔpH varied from 3.1 to 3.3); (●) ΔpH = 3.2; (▲) ΔpH = 2.8.

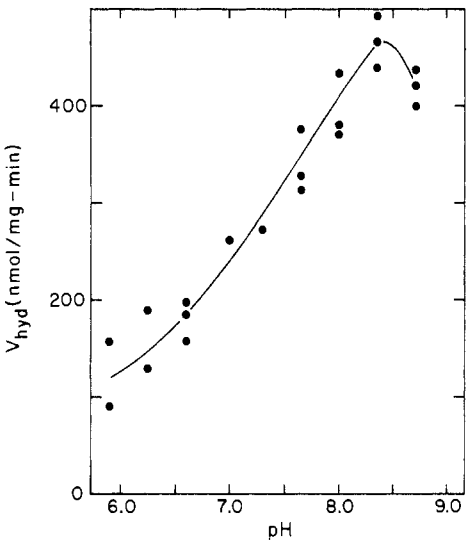

FIGURE 4: Plot of the steady-state maximum velocity for ATP hydrolysis, V_{hyd}, vs. the external pH with no pH gradient present. The concentrations of reactants and buffers are given in the text.

Table II: Kinetic Parameters for ATP Synthesis and Hydrolysis

pH	ΔpH	K_{ATP} (mM)	K_{ADP} (μM)	K_P (mM)	$10^9 K_{ADP-P}$ (M^2)
6.6	0	0.39			
	2.78		0.46	0.110	0.35
	2.98		0.55	0.087	0.32
	3.20		0.65	0.096	0.34
	3.32		0.43	0.119	0.22
8.0	0[a]	0.40			
	2.50[a]		1.5	0.11	1.36
	2.80[a]		2.4	0.15	0.99
8.4	0	0.65			

[a] Results at pH 8 are from Dewey & Hammes (1981); 0.9 has been added to the values of ΔpH reported in that reference.

formation of a significant pH gradient.

The hydrolysis reaction follows simple Michaelis–Menten kinetics, and the Michaelis constants were found to be 0.39 and 0.65 mM at pH 6.6 and 8.4, respectively (Figure 5). The range of ATP concentrations used was 0.1–5.0 mM. The rate law found for the steady-state initial velocity of ATP synthesis, v_{syn}, is

$$v_{syn} = \frac{V_{syn}}{1 + K_P/(P_i) + K_{ADP}/(ADP) + K_{ADP-P}/[(ADP)(P_i)]} \quad (2)$$

where V_{syn} is the maximum velocity and the K_i's are Michaelis

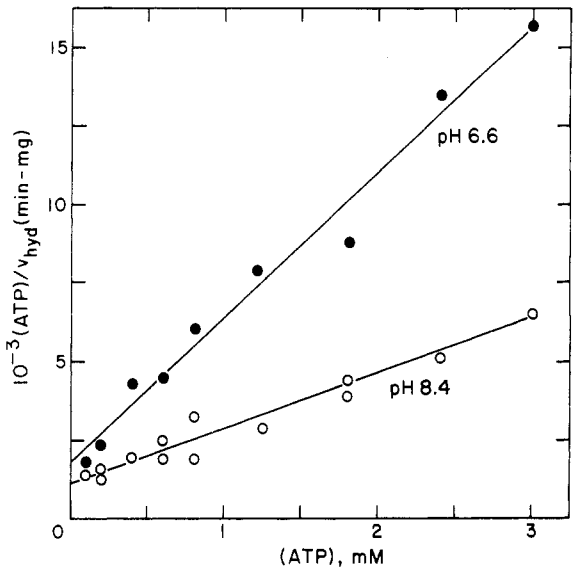

FIGURE 5: Representative plots of the ratio of the ATP concentration to the initial steady-state velocity for ATP hydrolysis, (ATP)/v_{hyd}, vs. the concentration of ATP at enternal pH values of 6.6 (●) and 8.4 (○). The experimental conditions are given in the text.

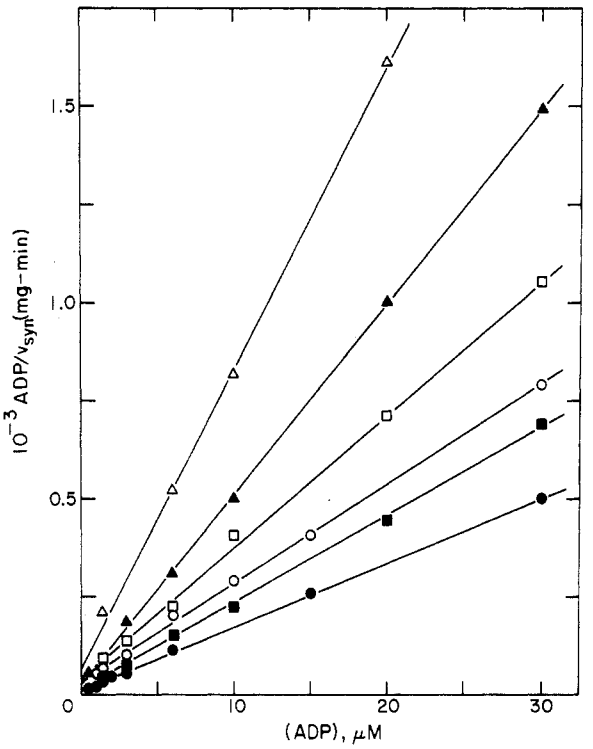

FIGURE 6: Representative plots of the ratio of the ADP concentration to the initial steady-state velocity for ATP synthesis, v_{syn}, vs. the concentration of ADP. (●) ΔpH = 3.32, P_i = 2.0 mM; (○) ΔpH = 3.32, P_i = 0.18 mM; (■) ΔpH = 3.20, P_i = 3.0 mM; (□) ΔpH = 3.20, P_i = 0.18 mM; (▲) ΔpH = 2.98, P_i = 3.0 mM; (△) ΔpH = 2.98, P_i = 0.18 mM. The experimental conditions are given in the text.

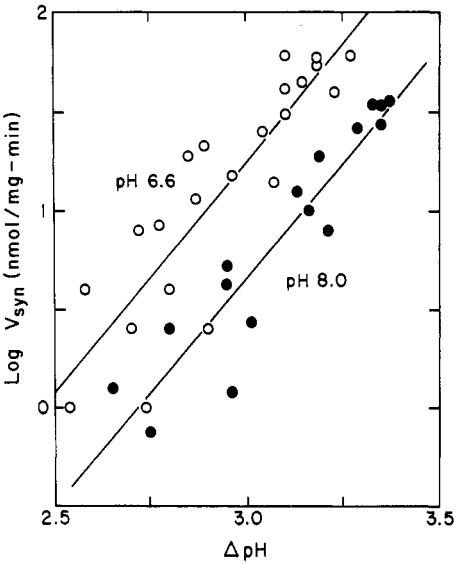

FIGURE 7: Plot of the logarithm of the maximum velocity for ATP synthesis, V_{syn}, vs. ΔpH at an external pH of 6.6 (○) and 8.0 (●). The lines have been calculated by a least-squares analysis. The experimental conditions are given in the text.

constants. The Michaelis constants obtained at pH 6.6 for several different values of ΔpH are summarized in Table II, and some representative data are shown in Figure 6. The range of ADP and P_i concentrations used was 0.2–100 μM and 0.04–3.0 mM, respectively. A least-squares analysis of the data was employed as previously described (Dewey & Hammes, 1981). The Michaelis constants for ATP synthesis are essentially independent of ΔpH so that the variation of the rate of ATP synthesis with ΔpH is determined by the dependence of the maximum velocity on ΔpH. The variation of the logarithm of the maximum velocity of ATP synthesis with ΔpH at external pH values of 6.6 and 8.0 is shown in Figure 7. The slopes of the lines are about 2.3, and the same slopes were obtained at two different vesicle concentrations.

The rate of ATP hydrolysis shows very little dependence on ΔpH (<10% decrease in rate up to ΔpH = 3.0). The rate of ATP hydrolysis also is not appreciably altered by dicyclohexylcarbodiimide (50 μM; <10% decrease in rate) and the uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (12.5 μM), whereas the synthetic activity is almost completely inhibited under identical conditions. These results suggest that most of the ATPase activity is not coupled to the proton flux.

The dependence of the steady-state initial rate on the metal ion concentration was examined under conditions where essentially the maximum velocity was being measured. At pH 6.6, the synthesis of ATP proceeds at approximately the same rate with both Mg^{2+} and Mn^{2+} over a range of free metal concentrations from 5 to 15 mM, and Ca^{2+} is completely ineffective. At pH 8.0, ATP hydrolysis occurs approximately equally rapidly with Mg^{2+} and Mn^{2+}, but both metal ions strongly inhibit at total metal concentrations greater than the total concentration of ATP. At a concentration of 10 mM, Ca^{2+} is about 10–15% as effective as the optimum concentrations of Mg^{2+} and Mn^{2+}.

Discussion

The steady-state specific activity for ATP synthesis obtained by reconstitution with octyl glucoside is among the highest reported in reconstituted systems (Sone et al., 1977; Racker et al., 1979; Ryrie et al., 1979; Hauska et al., 1980). This activity has been calculated in terms of the total protein, which is about 30% active DSA (Cerione & Hammes, 1981). However, the specific activity of DSA still is only about 1% of that found in thylakoids (Hauska et al., 1980). Unfortunately the ATPase activity appears to be almost completely uncoupled from the proton flux; this prevents a determination of the proton pumping stoichiometry as done with freeze–thaw reconstituted vesicles (Dewey & Hammes, 1981). The ATPase activity has been activated with dithiothreitol during the preparation of DSA. Further improvement in the specific activity and the coupling between synthesis and hydrolysis will require modifications in both the DSA preparation and reconstitution.

The higher specific activity of the reconstituted system (relative to that obtained by freeze–thaw reconstitution) has permitted a broad range of vesicle concentration, substrate concentration, and pH to be studied. A major problem, however, is the measurement of ΔpH. The complex coupling between 9-aminoacridine and vesicle concentrations in determining the observed fluorescence quenching suggests that the value of ΔpH obtained must be interpreted with considerable caution. In previously published work (Dewey & Hammes, 1981), the lower specific activity of the reconstituted vesicles required the utilization of higher vesicle concentrations, and the values of ΔpH probably were significantly underestimated (by about 0.9 pH unit). If the equilibrium constant for the overall reaction determined in the earlier work is corrected for this underestimation of ΔpH, the value of the equilibrium constant at ΔpH = 0 is similar to that obtained with other methods (Rosing & Slater, 1972).

The results shown in Figure 3 indicate a well-defined maximum at about pH 6.7 for ATP synthesis at constant ΔpH. In chloroplasts (Good et al., 1966) and reconstituted photosystem I DSA (Hauska et al., 1980), the observed optimum for ATP synthesis is at about pH 8.3–8.5. This difference is probably because the dependence of ATP synthesis on external pH generally is obtained at constant illumination rather than at constant ΔpH. Because the maximum velocity depends on approximately the third power of the hydrogen ion concentration, a small variation in ΔpH with external pH can be a major perturbation. The optimum external pH and the substrate Michaelis constants do not show a significant dependence on ΔpH. The Michaelis constants at pH 6.6 all are smaller than those at pH 8 (Table II). The Michaelis constants for ADP and P_i reported here are considerably smaller than those found in steady-state studies with thylakoids at high light intensities (Selman & Selman-Reimer, 1981). The curves at constant ΔpH in Figure 3 suggest a deprotonated ionizable group with an apparent p*K* of about 5 and a protonated ionizable group with an apparent p*K* of about 8.5 are essential for activity. The pH dependence of the maximum velocity for ATP hydrolysis (Figure 4) is quite different from that for ATP synthesis: a maximum occurs around pH 8.4, and the maximum velocity is associated with an apparent p*K* value of about 7. However, this ATPase activity is almost completely uncoupled from the synthetic activity so that the significance of the difference in the activity–pH profile between synthesis and hydrolysis cannot be assessed.

Although the number of protons pumped per ATP synthesized or hydrolyzed cannot be determined from the kinetic parameters because of the large amount of uncoupled ATPase activity, the dependence of the maximum velocity on ΔpH is similar at an external pH of 6.6 and 8.0 (Figure 7). The number of protons pumped per ATP hydrolyzed or synthesized at pH 8.0 previously was found to be 3.0 (Dewey & Hammes, 1981). The reaction order of the maximum velocity with

respect to protons (~2.3) is consistent with this stoichiometry. Portis & McCarty (1974) found a proton reaction order of 3.0 for ATP synthesis with thylakoids that were not activated by dithiothreitol. The finding that the reaction order is the same at external pHs of 6.6 and 8.0 suggests the proton pumping stoichiometry also may be constant over the range 6.6–8.0. The observed metal ion dependence of this system is similar to that previously reported (Pick & Racker, 1979). The specific role of the metal ion in ATP synthesis merits further study; the binding of metal has been postulated to be an important factor in ATP synthesis (Racker, 1979).

The most interesting result emerging from this work is that the dependence of ATP synthesis on ΔpH is not very dependent on the external pH and vice versa. This suggests the proton pumping and chemical mechanism may not be directly coupled; that is, the regions involved on the enzyme may not be structurally contiguous or identical. An obvious possibility is conformational coupling. This also is consistent with the finding that only the maximum velocities vary appreciably with ΔpH, while both the maximum velocities and Michaelis constants are dependent on the external pH.

References

Baird, B. A., Pick, U., & Hammes, G. G. (1979) *J. Biol. Chem. 254*, 3818–3825.

Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241–250.

Boyer, P. D., Chance, B., Ernster, L., Mitchell, P., Racker, E., & Slater, E. C. (1977) *Annu. Rev. Biochem. 46*, 955–1026.

Bruist, M. F., & Hammes, G. G. (1981) *Biochemistry 20*, 6298–6305.

Cerione, R., & Hammes, G. G. (1981) *Biochemistry 20*, 3359–3365.

Dawson, R. M. C., Elliot, D. C., Elliot, W. H., & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd ed., pp 433–434, Oxford University Press, London and New York.

Dewey, T. G., & Hammes, G. G. (1981) *J. Biol. Chem. 256*, 8941–8946.

Fillingame, R. H. (1980) *Annu. Rev. Biochem. 49*, 1079–1113.

Good, N., Izawa, S., & Hind, G. (1966) *Cur. Top. Bioenerg. 1*, 65–112.

Harris, D. A. (1978) *Biochim. Biophys. Acta 465*, 245–273.

Hauska, G., Samoray, D., Orlich, G., & Nelson, N. (1980) *Eur. J. Biochem. 111*, 535–543.

Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.

Oesterhelt, D., & Stoeckenius, W. (1971) *Nature (London), New Biol. 233*, 149–152.

Orlich, G., & Hauska, G. (1980) *Eur. J. Biochem. 111*, 525–533.

Penefsky, H. S. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 223–280.

Pick, U., & Racker, E. (1979) *J. Biol. Chem. 254*, 2793–2799.

Portis, A. R., Jr., & McCarty, R. E. (1974) *J. Biol. Chem. 249*, 6250–6254.

Racker, E. (1979) *Acc. Chem. Res. 12*, 338–344.

Racker, E., & Stoeckenius, W. (1974) *J. Biol. Chem. 249*, 662–663.

Racker, E., Chien, T.-F., & Kandrach, A. (1975) *FEBS Lett. 57*, 14–18.

Racker, E., Violand, B., O'Neal, S., Alfonzo, M., & Telford, J. (1979) *Arch. Biochem. Biophys. 198*, 470–477.

Rosing, J., & Slater, E. C. (1972) *Biochim. Biophys. Acta 267*, 275–290.

Ryrie, I. J., Critchley, C., & Tillberg, J.-E. (1979) *Arch. Biochem. Biophys. 198*, 182–194.

Schuldiner, S., Rottenberg, H., & Avron, M. (1972) *Eur. J. Biochem. 25*, 64–70.

Selman, B. R., & Selman-Reimer, S. (1981) *J. Biol. Chem. 256*, 1722–1726.

Shavit, N. (1980) *Annu. Rev. Biochem. 49*, 111–138.

Sone, N., Takeuchi, Y., Yoshida, M., & Ohno, K. (1977) *J. Biochem. (Tokyo) 82*, 1751–1758.