logical Systems (King, T. E., & Klingenberg, M., Eds) Vol. 1, Marcel Dekker, New York.
Kamen, M. D., Kakuno, T., Bartsch, R. G., & Hannon, S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1851–1854.
Kassner, R. J., Rubinow, S. C., & Cusanovich, M. A. (1983) *Biochim. Biophys. Acta 743*, 195–199.
Kastner, M. E., Scheidt, W. R., Mashiko, T., & Reed, C. A. (1978) *J. Am. Chem. Soc. 100*, 666–667.
Kitagawa, T., Ozaki, Y., Kyogoku, Y., & Horio, T. (1977) *Biohim. Biophys. Acta 495*, 1–11.
LaMar, G. N., Jackson, J. T., & Bartsch, R. G. (1981) *J. Am. Chem. Soc. 103*, 4405–4410.
Lundrum, J. T., Hatano, K., Scheidt, W. R., & Reed, C. A. (1980) *J. Am. Chem. Soc. 102*, 6729–6735.
Maltempo, M., Moss, T. H., & Cusanovich, M. A. (1974) *Biochim. Biophys. Acta 342*, 290–305.
Maltempo, M. M. (1976) *Biochim. Biophys. Acta 434*, 513–518.
Maltempo, M. M., & Moss, T. H. (1976) *Q. Rev. Biophys. 9*, 181–215.
Maltempo, M. M., Moss, T. H., & Spartalian, K. (1980) *J. Chem. Phys. 73*, 2100–2106.
Meyer, T. E., & Kamen, M. D. (1982) *Adv. Protein Chem. 35*, 105–212.
Moore, G. R., McClune, G. J., Clayden, N. J., Williams, R. J. P., Alsaadi, B. M., Angstrom, J., Ambler, R. P., Van Beeumen, J., Tempst, P., Bartsch, R. G., Meyer, T. E., & Kamen, M. D. (1982) *Eur. J. Biochem. 123*, 73–80.
Moss, T. H., Bearden, A. J., Bartsch, R. G., & Cusanovich, M. A. (1968) *Biochemistry 7*, 1583–1590.
Ogoshi, H., Sugimoto, H., & Yosida, Z. (1980) *Biochim. Biophys. Acta 621*, 19–28.
Olson, J. S., McKinnie, R. E., Mims, M. P., & White, D. K. (1983) *J. Am. Chem. Soc.* 105, 1522–1527.
Peisach, J. (1975) *Ann. N.Y. Acad. Sci. 244*, 187–202.
Rawlings, J., Stephens, P. J., Nafie, L. A., & Kamen, M. D. (1977) *Biochemistry 16*, 1725–1729.
Rebane, K. K., & Avarmaa, R. A. (1982) *Chem. Phys. 68*, 191–200.
Reed, C. A., Mashiko, T., Bently, S. P., Kastner, M. E., Scheidt, W. E., Spartalian, K., & Lang, G. (1979) *J. Am. Chem. Soc. 100*, 2948–2958.
Reisberg, P. I., & Olson, J. S. (1980) *J. Biol. Chem. 255*, 4144–4150.
Rougee, M., & Brault, D. (1975) *Biochemistry 14*, 4100–4106.
Scheidt, R. W., & Reed, C. A. (1981) *Chem. Rev. 81*, 543–555.
Scheidt, W. R., Haller, K. J., Fons, M., Mashiko, T., & Reed, C. A. (1981) *Biochemistry 20*, 3653–3657.
Sponholtz, D. K., Brautigan, D. L., Loach, P. A., & Margoliash, E. (1976) *Anal. Biochem. 75*, 255–260.
Stetzkowski, F., Cassoly, R., & Banerjee, R. (1979) *J. Biol. Chem. 254*, 11351–11356.
Strekas, T. C., & Spiro, T. G. (1974) *Biochim. Biophys. Acta 351*, 237–245.
Taniguchi, S., & Kamen, M. D. (1963) *Biochim. Biophys. Acta 74*, 438–455.
Teraoka, J., & Kitagawa, T. (1980) *J. Phys. Chem. 84*, 1928–1935.
Traylor, T. G., Campbell, D., Tsuchiya, S., Mitchell, M., & Stynos, D. V. (1980) *J. Am. Chem. Soc. 102*, 5939–5941.
Treidel, P. M. (1973) *Adv. Organomet. Chem. 11*, 21–86.
Valentine, J. S., Sheridan, R. P., Allen, L. C., & Kahn, P. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1009–1013.
Weber, P. C. (1982) *Biochemistry 21*, 5116–5119.
Weber, P. C., Bartsch, R. G., Cusanovich, M. A., Hamlin, R. C., Howard, A., Jordan, S. R., Kamen, M. D., Meyer, T. E., Weatherford, D. W., Xuong, N. H., & Salemme, F. R. (1980) *Nature (London) 286*, 302–304.
Weber, P. C., Howard, A., Xuong, N. H., & Salemme, F. R. (1981) *J. Mol. Biol. 153*, 399–424.

ATP Synthesis Catalyzed by the Purified Erythrocyte Ca-ATPase in the Absence of Calcium Gradients†

Michele Chiesi, Mauro Zurini, and Ernesto Carafoli*

ABSTRACT: The Ca^{2+}-transporting ATPase of erythrocytes was isolated by calmodulin affinity chromatography. The backward reaction of the ATPase was investigated. The phosphorylation of the solubilized enzyme by P_i required Mg and was inhibited by Ca and vanadate in the micromolar concentration range. Significant amounts of phosphoenzyme could be obtained only in a medium containing high dimethyl sulfoxide concentrations (>25%) in order to diminish water activity at the phosphorylation site. The phosphoenzyme formed in this way could not phosphorylate ADP. However, upon addition of Ca^{2+} ions and dilution of dimethyl sulfoxide in the phosphorylated preparation (water activity jump), a highly reactive phosphoenzyme species was obtained which could transfer phosphate in nearly stoichiometric amounts to ADP to form ATP.

Most plasma membranes have been found to contain a Ca^{2+}-transporting ATPase activity which contributes to the maintenance of the low Ca^{2+} concentration in the cytosol. A very interesting property of this ATPase is its ability to interact in a Ca^{2+}-dependent way with the cytosolic regulator protein calmodulin. The interaction stimulates the ATPase activity (Jarret & Penniston, 1977; Gopinath & Vincenzi, 1977) and has made it possible to purify the enzyme from various plasma membrane sources by calmodulin affinity chromatography (Niggli et al., 1979; Caroni & Carafoli, 1981; Wuytack et al., 1980). Analysis of the reaction mechanism of the ATPase has

† From the Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), CH-8092 Zurich, Switzerland. *Received October 3, 1983.* This investigation was supported by the Swiss Nationalfonds (Grant 3.634-0.80).

0006-2960/84/0423-2595$01.50/0

been carried out both on the intact membranes and on the isolated enzyme. One of the limitations to studies of this enzyme is the extremely small amount of ATPase available for investigation. It has nevertheless been possible to demonstrate that hydrolysis of ATP occurs via a multistep reaction cycle [for a review, see Rega et al. (1979) and Schatzmann (1982)]. As observed for other ion-motive ATPases (classified as E_1E_2 ATPases), such a process involves the formation of sequential phosphorylated intermediate forms of the enzyme with different reactivity of the acyl phosphate group incorporated. A useful approach to understand how an enzyme can utilize chemical energy to perform vectorial translocation of ions against their chemical gradient is to investigate the requirements needed to induce reversal of the transport cycle. Preliminary attempts, performed on closed membrane structures containing the Ca-ATPase (i.e., red cells, inside-out ghosts), have indicated that a Ca^{2+} gradient can be utilized to synthesize ATP (Ferreira & Lew, 1975; Rossi et al., 1978; Wüthrich et al., 1980), as has clearly been shown for the Ca^{2+}-ATPase of sarcoplasmic reticulum (Makinose & Hasselbach, 1971). Recent work on the latter enzyme has shown that ATP can be synthesized in the absence of a Ca^{2+} gradient (Knowles & Racker, 1975). In this paper, the reversibility of erythrocyte Ca-ATPase has been further investigated. It has been found that also the purified Ca^{2+}-ATPase of plasma membrane can synthesize ATP in the absence of a Ca^{2+} gradient. The results emphasize the importance of changes in water activity in the catalytic pocket of the enzyme during the hydrolytic reaction cycle.

Materials and Methods

Materials

[γ-^{32}P]ATP was obtained from Amersham. [^{32}P]P_i was obtained as an acidic solution from New England Nuclear and was centrifuged in a Beckman airfuge for 30 min at 30 psi. The upper half of the solution was withdrawn after centrifugation and used. All other reagents were of the best quality available. Calmodulin was isolated from bovine brain as previously described (Watterson et al., 1976).

The erythrocyte Ca-ATPase was isolated to purity from erythrocyte ghosts by calmodulin affinity chromatography as described by Niggli et al. (1979). The most active fraction eluted from the column was used for the experiments (about 1 mL in a typical preparation). Usually the protein concentration in this fraction was 200–300 μg/mL, and its activity, in the presence of saturating amounts of calmodulin, was 5–10 μmol of ATP hydrolyzed mg^{-1} min^{-1}. The medium in which the ATPase was stored contained 50 mM tris(hydroxymethyl)aminomethane (Tris),[1] pH 7.4, 60 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.05% Triton X-100, and 0.5 mg/mL phosphatidylcholine and was supplemented with 1 mM $CaCl_2$ and 1 mM dithiothreitol to preserve activity. Sarcoplasmic reticulum membranes were isolated from the hind legs of rabbit as described by Eletr & Inesi (1972).

Methods

Phosphoenzyme Formation with [γ-^{32}P]ATP. Aliquots of erythrocyte Ca-ATPase (10 μg) were supplemented with 2 μg of calmodulin and directly phosphorylated at 0 °C by the addition of 20 μM [γ-^{32}P]ATP (specific radioactivity, ca. 8 Ci/mmol). The reaction was stopped in acid after sequential intervals of time, and the Ca^{2+}-dependent phosphorylation was determined after six to seven washes of the denatured protein in acid as described (deMeis & Inesi, 1982). The radioactivity incorporated was measured by Cerenkov counting and corrected for the content of residual protein present, which was subsequently determined according to Lowry et al. (1951).

Phosphoenzyme Formation from [^{32}P]P_i. To compensate for the small amount of protein available for each experiment, high specific radioactivity [^{32}P]P_i had to be used. Typically, ca. 10 μg of ATPase in 100 μL was phosphorylated with 2 mM [^{32}P]P_i (50–100 μCi) for 1 min at room temperature. The basic reaction medium contained 50 mM Tris (at varying pH), 30 mM NaCl, 0.5 mM dithiothreitol, 0.05% Triton X-100, and 0.25 mg/mL phosphatidylcholine. The final concentration of EGTA, Ca, and Mg was as indicated in the figures, and Me_2SO, when required, was added. Phosphorylation reactions were quenched in acid, and the phosphoenzyme was measured as described above. Alternatively (see Figures 1 and 2), acid-quenched samples were applied on acidic gels with lithium dodecyl sulfate at pH 5.5, and, after electrophoresis, radioactivity incorporated into the Ca-ATPase was visualized by autoradiography as previously described (Chiesi & Wen, 1983).

ATP Synthesis. A 50–60-μg sample of ATPase was first phosphorylated with 1 mM [^{32}P]P_i (300 μCi) as described above, in the presence of 20 mM $MgCl_2$, 5 mM EGTA, and 40% Me_2SO at pH 8. The phosphorylated enzyme mixture was then diluted 4-fold in a medium containing 50 mM Tris, pH 8, 20 mM $MgCl_2$, 0.05% Triton X-100, and, when required, one or more of the following: Me_2SO (40%), EGTA or $CaCl_2$ (2 mM), and/or ADP (1 mM). After 20 s, the reaction was stopped in 7% Cl_3CCOOH. After centrifugation, the residual phosphoenzyme was determined in the denatured protein pellet as described above, while the supernatant was used to determine the synthesized [γ-^{32}P]ATP. [^{32}P]P_i was extensively extracted in cold isobutyl alcohol–benzene as previously described (de Meis & Tume, 1977). After each extraction cycle, the radioactivity left in the water phase (containing [γ-^{32}P]ATP) was determined by Cerenkov counting to establish that a limiting value had been reached (usually seven extractions were required). Because of the high amount of radioactivity used in each experiment, a high level of nonextractable background radioactivity (which was not related to [γ-^{32}P]ATP) was left in the water phase. This radioactivity was constant for a given batch of [^{32}P]P_i and was carefully determined in control experiments and subtracted from all experimental values. Known amounts of [γ-^{32}P]ATP were also subjected to an identical extraction sequence to correct for possible ATP losses (usually 25–35%) during the procedure.

Results

Since the Ca-ATPase of erythrocytes has the characteristics of an E_1E_2 ATPase, some requirements for the phosphorylation by P_i can be predicted. First, the equilibrium between the two conformational states E_1 and E_2 must be shifted in favor of the second species. This can be achieved experimentally by chelating Ca^{2+} ions, which would otherwise bind to the E_1 form of the enzyme. In addition, the P_i concentration should be as high as possible to saturate the binding sites on the E_2 form. This was a limiting factor in the present experiments. Indeed, due to the small amounts of enzyme available and to its dilution in the reaction mixture (usually about 100 μg/mL), high amounts of radioactivity ([^{32}P]P_i) had to be used. Moreover, Mg^{2+} and the pH of the reaction mixture were possible factors

[1] Abbreviations: SR, sarcoplasmic reticulum; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Me_2SO (DMSO in figures), dimethyl sulfoxide; Tris, tris(hydroxymethyl)aminomethane; Cl_3CCOOH, trichloroacetic acid.

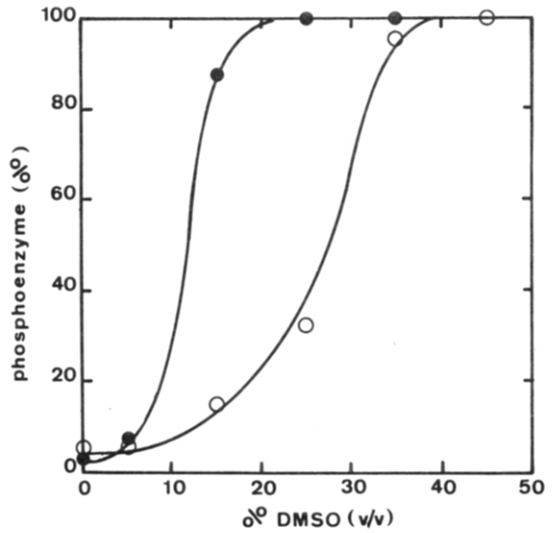


FIGURE 1: Stimulation by Me_2SO of the phosphorylation by P_i of the erythrocyte and sarcoplasmic reticulum Ca-ATPases. Freshly prepared erythrocyte Ca-ATPase was diluted to a concentration of 50 μg/mL. The final composition of the medium was 50 mM Tris-HCl, pH 8, 30 mM NaCl, 0.5 mM $CaCl_2$, 5 mM EGTA ($[Ca]_{free}$ $<10^{-8}$ M), 22 mM $MgCl_2$, 2 mM EDTA, 0.05% Triton X-100, 0.25 mg of phosphatidylcholine/mL, and Me_2SO as indicated. Phosphorylation reactions were carried out with 5–7 μg of ATPase at room temperature by adding 2 mM $[^{32}P]P_i$ and were stopped after 1 min in 7% Cl_3CCOOH. Quenched samples were analyzed by gel electrophoresis and autoradiography as described (Methods). Sarcoplasmic reticulum membranes were phosphorylated exactly as described for the erythrocyte ATPase. (●) SR Ca-ATPase; (○) erythrocyte Ca-ATPase.

in the backward phosphorylation by P_i, as already observed with the sarcoplasmic reticulum Ca-ATPase (Punzengruber et al., 1978; Beil et al., 1977). Therefore, the first attempts to phosphorylate the purified erythrocyte ATPase with P_i were made in the presence of EGTA (5 mM), high Mg^{2+} concentration (ca. 20 mM), and 2 mM $[^{32}P]P_i$ at various pH values (6–8). Under these conditions, however, no significant amounts of phosphoenzyme were detected. Only when the water activity of the medium was reduced by the addition of Me_2SO did radioactivity become incorporated in the Ca-ATPase. As seen in Figure 1, Me_2SO concentrations of up to 30% were required to obtain maximal levels of phosphoenzyme. Such a stimulatory effect of Me_2SO on the backward phosphorylation has already been observed with the Ca-ATPase of sarcoplasmic reticulum (deMeis et al., 1980, 1982; deMeis & Inesi, 1982; Figure 1) where the hypothesis has been advanced that the low-energy acyl phosphate formed by P_i might be located in a hydrophobic pocket of the catalytic site. The results presented here show that the hypothesis may be valid for the erythrocyte Ca-ATPase as well.

The backward phosphorylation of the erythrocyte Ca-ATPase in the presence of 40% Me_2SO was characterized. Maximal phosphoenzyme levels obtained in the presence of 2 mM P_i with typical ATPase preparations ranged between 0.7 and 1.0 nmol/mg (Table I). This corresponds to only a fraction of the ATPase available (about 7 nmol/mg). Similar or lower amounts of phosphoenzyme were obtained when the same ATPase preparation was phosphorylated with $[\gamma\text{-}^{32}P]$-ATP (Table I). The latter experiments were carried out at 0 °C in the presence of calmodulin, and the phosphoenzyme steady state was maximal 5–10 s after addition of ATP (20 μM). After this time, the phosphoenzyme level started to decline, probably because of ATP depletion. It has recently been observed (Luterbacher & Schatzmann, 1983) that La^{3+} can increase the steady-state level of phosphoenzyme during

Table I: Comparison between the Phosphorylation Levels of the Plasma Membrane Ca^{2+}-ATPase in the Presence of ATP or P_i

addition	phosphoenzyme (nmol/mg of protein)
$[\gamma\text{-}^{32}P]ATP$ [a]	0.6–0.8 (5) [c]
$[^{32}P]P_i$ [b]	0.7–1.0 (5)

[a] Fifty-microliter aliquots containing 10 μg of Ca-ATPase, 2 μg of calmodulin, 60 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM $CaCl_2$, 0.05% Triton X-100, and 0.5 mg of phosphatidylcholine/mL were phosphorylated at 0 °C for 5 s by the addition of 20 μM $[\gamma\text{-}^{32}P]ATP$. The phosphoenzyme was determined as described (see Methods) after acid quenching of the reaction. [b] The erythrocyte Ca-ATPase was phosphorylated with 2 mM $[^{32}P]P_i$ as described in the legend for Figure 2, in the presence of 40% Me_2SO, a high Mg concentration (18 mM), and low Ca concentrations ($<10^{-8}$ M) at pH 8. The phosphoenzyme level was determined as described in footnote *a*. [c] Values in parentheses are the number of experiments performed.

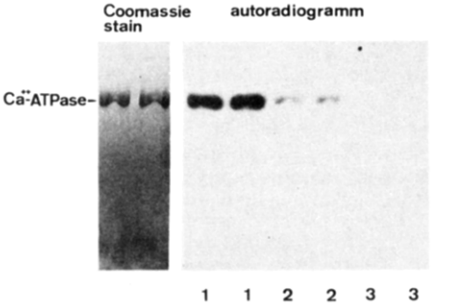


FIGURE 2: Effect of Mg^{2+} and Ca^{2+} ions on the formation of phosphoenzyme from P_i. Erythrocyte Ca-ATPase was phosphorylated and analyzed as described in the legend to Figure 1 in the presence of 40% Me_2SO. The final concentrations of Ca and Mg were modified as follows: (1) 0.5 mM $CaCl_2$, 5 mM EGTA, 22 mM $MgCl_2$, and 2 mM EDTA ($[Ca]_{free}$ $<10^{-8}$ M and $[Mg]_{free}$ $\simeq$17 mM); (2) 2 mM $CaCl_2$, 0.5 mM EGTA, 22 mM $MgCl_2$, and 2 mM EDTA ($[Ca]_{free}$ $\simeq$1.5 mM and $[Mg]_{free}$ $\simeq$20 mM); (3) 0.5 mM $CaCl_2$, 5 mM EGTA, 1 mM $MgCl_2$, and 20 mM EDTA ($[Ca]_{free}$ $<10^{-8}$ M and $[Mg]_{free}$ $<10^{-6}$ M).

ATP hydrolysis by erythrocyte ghosts. Under the present experimental conditions, however, no significant increase of the phosphoenzyme level was obtained after inclusion of 200 μM $LaCl_3$ in the reaction mixture. These observations indicate that the substoichiometric phosphorylation levels of the erythrocyte Ca-ATPase by P_i are not due to suboptimal phosphorylation conditions but to an intrinsic property of the enzyme preparation used. The possibility of partial inactivation of the Ca-ATPase cannot be ruled out. Figure 2 shows that the phosphorylation of the erythrocyte Ca-ATPase by P_i was strongly inhibited by Ca^{2+} ions and absolutely dependent on Mg^{2+} ions. This indicates the requirement for the formation of a ternary complex, $E_2 \cdot Mg \cdot P_i$, as demonstrated for sarcoplasmic reticulum ATPase (Punzengruber et al., 1978). Under optimal phosphorylating conditions (40% Me_2SO, high Mg concentration, 2 mM P_i), the pH of the medium had little effect on the reaction when it was varied between 6 and 8 (Table II). On the other hand, micromolar amounts of vanadate drastically inhibited the phosphorylation (Table II). This is expected of an E_1E_2 ATPase, since it is accepted that vanadate interacts with high affinity with the enzyme conformation that binds P_i (i.e., E_2) and thus acts as an inhibitor of P_i binding (Pick, 1982).

The phosphoenzyme intermediate of the erythrocyte Ca-ATPase formed with P_i in the presence of Me_2SO was tested for its ability to phosphorylate ADP. Dilution of phosphorylated protein samples into a medium containing high concentrations of Me_2SO (40%), Ca^{2+}, and ADP yielded no ATP

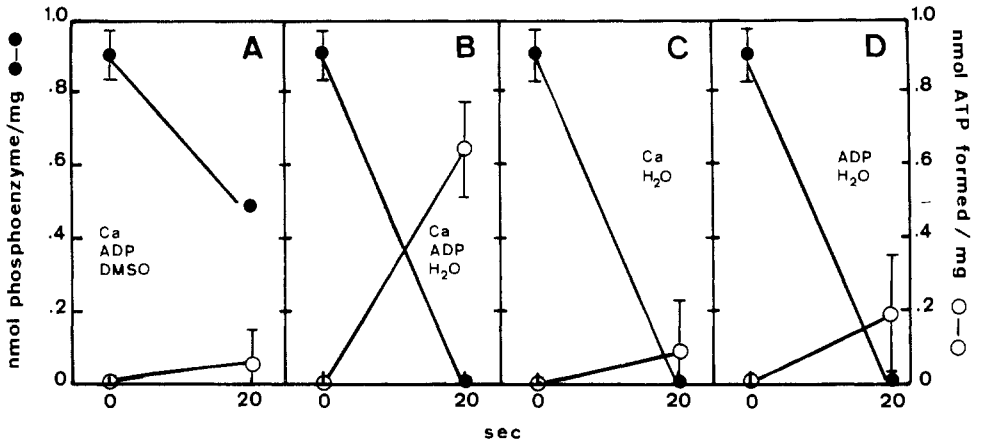


FIGURE 3: ATP synthesis catalyzed by the solubilized erythrocyte Ca-ATPase. 50–60-μg aliquots of erythrocyte Ca-ATPase were phosphorylated at room temperature by the addition of 1 mM [^{32}P]P_i in the presence of high Mg concentration (20 mM) and low Ca concentration ($<10^{-8}$ M) at pH 8 in a medium containing 40% Me_2SO. After 1 min, a small aliquot was quenched in acid (see Materials and Methods) and the phosphoenzyme level determined. The remaining phosphorylated mixture was diluted 4-fold into a medium containing 50 mM Tris-HCl, pH 8, 20 mM $MgCl_2$, 0.05% Triton X-100, and (A) 2 mM $CaCl_2$, 1 mM ADP, and 40% Me_2SO, (B) 2 mM $CaCl_2$ and 1 mM ADP, (C) 2 mM $CaCl_2$, or (D) 1 mM ADP and 2 mM EGTA. After 20 s, the reaction was blocked with Cl_3CCOOH. The level of remaining phosphoenzyme and the [γ-^{32}P]ATP synthesized were determined. Details of the procedure are described under Materials and Methods. The data are given as $\bar{X}$ = SEM (n = 8).

Table II: Effect of Vanadate and pH on Ca-ATPase Phosphorylation by P_i in the Presence of 40% Me_2SO[a]

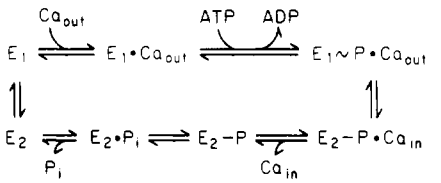
pH dependency		vanadate inhibition (at pH 8)	
pH	phosphoenzyme level (nmol/mg of protein)	[vanadate] (μM)	phosphoenzyme level (nmol/mg of protein)
6	0.9–1.1 (3)[b]	0	0.7–0.9 (4)
6.5	0.85 (1)	1	0.4–0.6 (3)
7	1.0–1.2 (3)	5	0.2–0.4 (3)
7.5	0.8 (1)	10	0 (3)
8	0.7–0.9 (3)	20	0 (3)

[a] The backward phosphorylation of the erythrocyte Ca-ATPase with 2 mM [^{32}P]P_i was carried out in the presence of 40% Me_2SO, 18 mM $MgCl_2$, and low Ca concentrations ($<10^{-8}$ M) at various pHs essentially as described in the legend for Figure 2. The effect of vanadate at pH 8 was investigated by adding varying amounts to the incubation mixture. [b] Values in parentheses are the number of experiments performed.

synthesis (Figure 3A). On the other hand, P_i transfer to ADP could be achieved when the water activity of the medium was increased by omitting Me_2SO in the dilution medium (Figure 3B). In addition to ADP, Ca^{2+} was required for ATP synthesis (Figure 3B–D). Figure 3B shows that the phosphoenzyme level decreased to zero concomitant to ATP synthesis. The amount of ATP formed corresponded to 60–70% of the amount of phosphoenzyme present previous to the addition of ADP and Ca^{2+}, indicating that during dilution most of the phosphoenzyme was converted to a highly reactive species able to phosphorylate ADP. After dilution, the phosphoenzyme level also decreased when no ATP synthesis occurred (Figure 3A,C,D). In this case, however, the disappearance of the phosphoenzyme represents its hydrolysis and not the reversal of the transport cycle. Interestingly, Me_2SO in the dilution medium drastically inhibited the hydrolysis rate (Figure 3A).

Discussion

The investigation of the reaction mechanism of cation-motive ATPases of the E_1E_2 type has provided important information on the mechanism of interconversion between chemical and potential energy (ion gradients) in biological systems. The Ca-ATPase of sarcoplasmic reticulum membranes is among the most intensively studied examples of E_1E_2 ATPases. A simplified mechanism of Ca^{2+} transport for this ATPase is shown in the following scheme:



where the most critical steps are represented by the interconversion between the two phosphorylated forms of the enzyme, $E_1 \sim P$ and E_2–P. These two intermediate forms represent two different phosphorylated conformational states, one containing a highly reactive acyl phosphate ($E_1 \sim P$), which can phosphorylate ADP, and the other a low-energy acyl phosphate (E_2–P), which is hydrolyzed. The early observation that the SR ATPase can synthesize ATP in parallel with Ca^{2+} efflux from Ca^{2+}-loaded vesicles (Makinose & Hasselbach, 1971) has unequivocally shown that all the steps of the transport cycle are reversible. It was generally accepted in the early work that the energy for ATP synthesis was provided by the Ca^{2+} gradient, in line with the chemiosmotic hypothesis which accounts for ATP synthesis by mitochondria and other energy-coupling membranes (Mitchell, 1966). However, later experiments with solubilized sarcoplasmic reticulum Ca-ATPase preparations showed that also in the absence of a membrane, and hence of a Ca^{2+} gradient, it was possible to phosphorylate the enzyme with P_i and to use it to synthesize ATP (Knowles & Racker, 1975). The energy source needed to achieve ATP synthesis in this case was less obvious, and it was postulated that it could be represented by the binding energy of specific ions (like Ca in the scheme), which forces the enzyme to assume a highly energetic conformational state (Knowles & Racker, 1975). It was recently observed that in the presence of Me_2SO the phosphorylation of the solubilized sarcoplasmic reticulum enzyme by P_i to form E_2–P is greatly facilitated (deMeis et al., 1980, 1982; deMeis & Inesi, 1982). This observation led to the concept that the solvation energy of the reactants (George et al., 1970) might be directly involved in the process of energy transduction. Accordingly, the free energy of hydrolysis of the acyl phosphate present in E_2–P

would be much lower than that of an acyl phosphate in water, since the enzyme can provide a hydrophobic microenvironment within the catalytic site. This hypothesis would explain why relatively low concentrations of P_i are sufficient to form E_2–P and also why a reduction of the water activity (achieved experimentally by including high concentrations of Me_2SO) greatly facilitates the phosphorylation reaction. E_2–P, buried within the hydrophobic core of the ATPase, cannot transfer P_i to ADP. To transform this low-energy acyl phosphate into a high-energy species able to phosphorylate ADP ($E_1\sim P$), rehydration of the catalytic pocket and occupancy of internal low-affinity Ca^{2+} binding sites are required. Indeed, experiments have shown that the transition from E_2–P to $E_1\sim P$ is drastically stimulated by increasing the water activity of the reaction mixture (i.e., by a decrease of the Me_2SO concentration of the medium) (deMeis & Inesi, 1982). The role of Ca in the final steps of ATP synthesis is not clearly established. It could be that the ion is necessary to couple the P_i transfer from the high-energy $E_1\sim P$ to ADP (Du Pont, 1983). Indeed, it is known from work on model systems and enzymes that Ca^{2+} ions are highly effective as catalysts of phosphoryl transfer reactions (Knowles, 1980). Once Ca^{2+} and H_2O have gained access to the active site, $E_1\sim P$ and newly formed ATP should in theory be highly unstable. However, the situation is evidently favorable for the transfer of P_i from $E_1\sim P$ to ADP, as documented by experiments of ATPase reversal upon imposition of a Ca^{2+} gradient.

The hypothesis that explains the energy difference between $E_1\sim P$ and E_2–P by a different level of water activity within the catalytic site in the two enzyme conformations was recently confirmed by an elegant experiment, which could demonstrate that a drastic change of the polarity of the catalytic site (from high to low polarity) occurs during the transition $E_1\sim P$ to E_2–P (Dupont & Pougeois, 1983).

The Ca-ATPase of plasma membranes differs in many respects from that of sarcoplasmic reticulum membranes. Among its distinctive properties are the different molecular weights (137 000 vs. 110 000), the completly different pattern of proteolytic digestion, the capability to interact directly with calmodulin and to become stimulated by it, the substrate specificity, and the lack of immunological cross-reactivity. However, the reaction mechanism of this Ca-ATPase also involves the formation of two sequential phosphorylated intermediates with different energy levels. In this work, the problem of the analogy in transport mechanisms between the plasma membrane and the SR Ca-ATPases was tackled in a crucial step, that is, whether the reaction cycle of the plasma membrane Ca-ATPase is also completely reversible and capable of ATP synthesis in the absence of a Ca^{2+} gradient. The requirements for the formation of the high-energy acyl phosphate ($E_1\sim P$) starting from P_i have also been compared. The experiments have shown that the plasma membrane ATPase can catalyze ATP synthesis in a solubilized state and that the basic requirement for this is the change of the hydration level of the catalytic site containing the acyl phosphate group. The results corroborate the concept that the mode of energy transduction accomplished by E_1E_2 ATPases is basically identical and that complete understanding of the energy balance during the hydrolytic cycle must take into account the solvation energy of reactants and products in the catalytic site (deMeis, 1981).

The other class of ion-motive ATPases, the F_1F_0 class, has a different reaction scheme. Its most distinctive features are the tight ADP and P_i binding to the catalytic site, the direct synthesis of ATP without the intermediate formation of phosphorylated enzyme states, and the release of the synthesized ATP to the aqueous medium. A proton gradient is the driving force for the reaction: however, it has not been conclusively established which of the steps of the ATP synthesis sequence utilize the energy provided by the electrochemical potential difference, $\Delta\mu_{H^+}$. Current hypotheses postulate that energy is used either in the steps in which ATP is synthesized from ADP and P_i (Mitchell, 1977) or, alternatively, for the release of the newly synthesized ATP from the catalytic site (Rosen et al., 1979). Strong support for the latter hypothesis has recently been provided by a study in which ATP synthesis in the absence of $\Delta\mu_{H^+}$ was achieved in a solubilized F_1-ATPase preparation (Sakamoto & Tonomura, 1983). However, the ATP synthesized was still associated with the ATPase protein, and the reaction was dependent on the presence of high Me_2SO concentrations. This last observation is of high interest, in that it suggests that the two types of ATPases (i.e., F_0F_1 and E_1E_2) might share basic features. It is attractive to imagine that also during ATP synthesis by the F_0F_1-ATPase an essential change occurs in the solvation level of the catalytic site. In particular, a hydrophobic environment at the site where P_i and ADP are bound would shift the equilibrium in favor of ATP synthesis (this would correspond to the formation of E_2–P). A subsequent conformational change inducing solvation of the catalytic site (corresponding to the formation of $E_1\sim P$) would then induce release of the newly synthesized ATP to the medium. In the membrane-associated state, it could be proposed that the electrochemical potential, $\Delta\mu_{H^+}$, would be required to induce and coordinate these conformational changes, as the Ca^{2+} gradient presumably does in the Ca-ATPase of sarcoplasmic reticulum and erythrocytes.

Registry No. ATP, 56-65-5; ATPase, 9000-83-3; Me_2SO, 67-68-5; Ca, 7440-70-2; Mg, 7439-95-4; P_i, 14265-44-2; vanadate, 37353-31-4.

References

Beil, F. U., Chak, D., & Hasselbach, W. (1977) *Eur. J. Biochem. 81*, 151–164.

Caroni, P., & Carafoli, E. (1981) *J. Biol. Chem. 256*, 3263–3270.

Chiesi, M., & Wen, Y. S. (1983) *J. Biol. Chem. 258*, 6078–6085.

deMeis, L. (1981) in *The Sarcoplasmic Reticulum* (Bittar, E. E., Ed.) Wiley, New York.

deMeis, L., & Tume, R. K. (1977) *Biochemistry 16*, 4455–4463.

deMeis, L., & Inesi, G. (1982) *J. Biol. Chem. 257*, 1289–1294.

deMeis, L., Martins, O. B., & Alves, E. W. (1980) *Biochemistry 19*, 4252–4261.

deMeis, L., Souza Otero, A., Martins, O. B., Alves, E. W., Inesi, G., & Nakamoto, R. (1982) *J. Biol. Chem. 257*, 4993–4998.

Dupont, Y. (1983) *FEBS Lett. 161*, 14–20.

Dupont, Y., & Pougeois, R. (1983) *FEBS Lett. 156*, 93–98.

Eletr, S., & Inesi, G. (1972) *Biochim. Biophys. Acta 282*, 174–179.

Ferreira, H. G., & Lew, V. L. (1975) *J. Physiol. (London) 252*, 86P.

George, P., Witomsky, R. J., Trachtman, M., Wu, C., Dorwart, W., Richman, L., Richman, W., Shurayh, F., & Lentz, B. (1970) *Biochim. Biophys. Acta 223*, 1–15.

Gopinath, R. M., & Vincenzi, F. F. (1977) *Biochem. Biophys. Res. Commun. 77*, 1203–1209.

Jarrett, H. W., & Penniston, J. T. (1977) *Biochem. Biophys. Res. Commun. 77*, 1210–1216.

Knowles, A. F., & Racker, E. (1975) *J. Biol. Chem. 250*, 1949–1951.

Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877-919.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 5150-5157.
Luterbacher, S., & Schatzmann, H. J. (1983) *Experientia 39*, 311-312.
Makinose, M., & Hasselbach, W. (1971) *FEBS Lett. 12*, 271-272.
Mitchell, P. (1966) *Biol. Rev. Cambridge Philos. Soc. 41*, 445.
Mitchell, P. (1977) *FEBS Lett. 78*, 1-20.
Niggli, V., Penniston, J. T., & Carafoli, E. (1979) *J. Biol. Chem. 254*, 9955-9958.
Pick, U. (1982) *J. Biol. Chem. 257*, 6111-6119.
Punzengruber, C., Prager, R., Kolassa, N., Winkler, F., & Suko, J. (1978) *Eur. J. Biochem. 92*, 349-359.
Rega, A. F., Garrahan, P. J., Barrabin, H., Horenstein, A., & Rossi, J. P. (1979) in *Cation Flux Across Biomembranes* (Mukohata, Y., & Packer, L., Eds.) p 67, Academic Press, New York.
Rosen, G., Gresser, M., Vinkler, C., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 10654-10661.
Rossi, J. P., Garrahan, P. J., & Rega, A. F. (1978) *J. Membr. Biol. 44*, 37-46.
Sakamoto, J., & Tonomura, Y. (1983) *J. Biochem. (Tokyo) 93*, 1601-1614.
Schatzmann, H. J. (1982) in *Membrane Transport of Calcium* (Carafoli, E., Ed.) p 41, Academic Press, London.
Watterson, D. M., Van Eldik, L. J., Msith, R. E., & Vanaman, T. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2711-2715.
Wüthrich, A., Schatzmann, H. J., & Romero, P. (1979) *Experientia 35*, 1589-1590.

In Vitro Methylation and Demethylation of Methyl-Accepting Chemotaxis Proteins in *Bacillus subtilis*†

Daniel J. Goldman and George W. Ordal*

ABSTRACT: *Bacillus subtilis* responds to attractants by demethylating a group of integral membrane proteins referred to as methyl-accepting chemotaxis proteins (MCPs). We have studied the methylation and demethylation of these proteins in an in vitro system, consisting of membrane vesicles, and purified methyltransferase and methylesterase. The chemoattractant aspartate was found to inhibit methylation and stimulate demethylation of MCPs. *Escherichia coli* radiolabeled membranes in the presence of *B. subtilis* enzyme do not respond to aspartate by an increase demethylation rate. We also report that *B. subtilis* MCPs are multiply methylated, demethylation resulting in slower migrating proteins on sodium dodecyl sulfate-polyacrylamide gels.

Posttranslational modification of proteins is involved in a number of regulatory processes. In bacterial chemotaxis, the level of methylation of certain integral membrane proteins, referred to as methyl-accepting chemotaxis proteins (MCPs), has been correlated with the adaptation of bacteria to various chemoeffectors (Goy et al., 1977; Goldman et al., 1982).

We have been involved in studying the response of *Bacillus subtilis* toward the amino acid attractants. We have found that all the amino acids function as attractants for *B. subtilis*. The response of the bacteria is proportional to the number of receptors occupied by the amino acid (Goldman & Ordal, 1981). In vivo methylation experiments indicate the *B. subtilis* responds to amino acid attractants by demethylating its MCPs, and it is during this increased rate of demethylation that the bacteria swim smoothly (Goldman et al., 1982). This is a unique finding in bacterial chemotaxis. The Gram-negative bacteria like *Escherichia coli* respond to amino acid attractants by an increased rate of MCP methylation (Goy et al., 1977) and a decreased rate of MCP demethylation (Toews et al., 1979).

S-Adenosylmethionine serves as the methyl donor for MCPs (Rollins & Dahlquist, 1980). A methyltransferase enzyme responsible for catalyzing this methylation has been purified from *B. subtilis* (Burgess-Cassler et al., 1982). Recently, we have purified the methylesterase responsible for catalyzing removal of these methyl groups (Goldman et al., 1984).

Utilizing an in vitro system consisting of purified enzymes and membrane vesicles, we have examined the methylation/demethylation reactions individually. We also investigated the influence of chemoattractant on these reactions. We report here that the chemoeffector aspartate stimulated MCP demethylation in vitro and also inhibited MCP methylation. Using a heterologous sytem consisting of *E. coli* membranes and *B. subtilis* methylesterase, we present evidence that the effect of aspartate on MCP methylation is mediated through the membranes. Furthermore, we present evidence that *B. subtilis* MCPs are multiply methylated.

Materials and Methods

Chemicals. *S*-Adenosyl[*methyl*-^{3}H]methionine (15 Ci/mmol) and L-[*methyl*-^{3}H]methionine (78-93 Ci/mmol) were obtained from Amersham Corp. or ICN. L-Aspartic acid potassium salt was purchased from Sigma. All other chemicals were reagent grade.

Bacterial Strains. Strain 0I 1085 is a chemotactically wild-type strain of *Bacillus subtilis*. Strain 0I 1100 was obtained from 0I 1085 by mutagenesis and is a chemotaxis mutant (Ullah & Ordal, 1981; Burgess-Cassler et al., 1982). *Escherichia coli* strain RP4612 (RP437 *cheR*) is a chemotaxis methyltransferase mutant obtained from J. S. Parkinson.

Media. Tryptone broth contains 1% tryptone and 0.5% NaCl. L broth contains 1% tryptone, 0.5% NaCl, and 0.5% yeast extract. FP buffer and MT buffer have been previously described (Burgess-Cassler et al., 1982).

Membrane Vesicle Isolation and Enzyme Purification. *B. subtilis* and *E. coli* were grown in tryptone broth overnight, then subcultured and grown in L broth to 180 Klett units (*B. subtilis*) or to A_{595} of 1-1.7 (*E. coli*) ~(2-8 × 10^8 cells/mL) at 37 °C. Cells were chilled to about 4 °C and all subsequent

† From the School of Basic Medical Sciences and the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received October 10, 1983.*

0006-2960/84/0423-2600$01.50/0