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Primary Structure of the cAMP-Dependent Phosphorylation Site of the Plasma Membrane Calcium Pump†

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ABSTRACT: The primary structure of a region of the erythrocyte plasma membrane calcium pump which is phosphorylated by the cAMP-dependent protein kinase has been determined. The sequence is A-P-T-K-R-N-S-S(P)-P-P-P-S-P-D. The site is located between the calmodulin binding domain and the C-terminus of the ATPase. The ATPase is phosphorylated only at this site by the cAMP-dependent protein kinase, and the phosphorylation is inhibited by calmodulin. The effect of the phosphorylation is to decrease the K_m for Ca^{2+} of the purified ATPase from about 10 μM to about 1.4 μM and to increase the V_{max} of ATP hydrolysis about 2-fold.

It has become increasingly evident during the last few years that protein kinases modulate the activity of several, possibly most, membrane transport proteins, particularly in the plasma membrane. The calcium pump of plasma membranes has been shown to be activated by a cAMP-dependent protein kinase (Caroni & Carafoli, 1981; Neyses et al., 1985). This is analogous to the case of the (Na^+–K^+)-ATPase (Lin & Cantley, 1984) but is at variance to that of the sarcoplasmic reticulum calcium ATPase which is only activated indirectly by both cAMP- and calmodulin-dependent phosphorylation of the low molecular weight acidic proteolipid phospholamban (Tada & Katz, 1982). The phosphorylation of several other calmodulin-dependent enzymes has been demonstrated [e.g., myosin light chain kinase and phosphofructokinase (Lukas et al., 1986; Buschmeier et al., 1987)], and the sites of phosphorylation have been shown to be located near the calmodulin binding domains of these enzymes or within them. In these cases, the phosphorylation was shown to increase the K_m of the proteins for calmodulin, thus preventing activation. The site of phosphorylation in these enzymes is a serine-rich region immediately C-terminal to the calmodulin binding domain.

The sequences of the plasma membrane Ca^{2+} pumps from rat brain and human teratoma cells have recently been deduced from the corresponding cDNAs (Shull & Greeb, 1988; Verma et al., 1988). The sequence of the calmodulin binding domain has been determined by photoaffinity labeling and Edman degradation of the cross-linking peptides (James et al., 1988). The domain was found to be located near the C-terminus of the enzyme and to contain a serine-rich region homologous to the phosphorylation domains of the above-mentioned enzymes. The expectation was thus that a similar phosphorylation mechanism may occur in the plasma membrane Ca^{2+}-ATPase. The work presented here locates the site of cAMP-dependent phosphorylation in the ATPase: Contrary to expectation, it occurs at a site approximately 5 kDa C-terminal to the serine-rich region mentioned above. Its phosphorylation produces an activation of the purified ATPase and not an inhibition as seen with phosphofructokinase and myosin light chain kinase.

Materials and Methods

Staphylococcus aureus V8 protease was purchased from ICN Immunobiochemicals (Lisle, IL), ethanethiol (purissimum) was from Fluka AG (Buchs, Switzerland), and all HPLC solvents were from May and Baker (Dagenham, U.K.). The catalytic subunit of the cAMP-dependent protein kinase

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from bovine heart was P2645 from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was purchased from Amersham Corp. (Amsterdam, Holland). The chemicals for the sequencing work were purchased from Applied Biosystems (Foster City, CA). Phosphatidylcholine was purchased from Lipid Products (Nuffield, U.K.). All other reagents were of the highest purity grade commercially available.

Preparation of the ATPase. The ATPase was isolated from human erythrocyte ghosts as previously described (Niggli et al., 1979), using an extended (24 h) Ca^{2+} washing step of the calmodulin (CaM)[1] column to ensure high purity of the preparation. Triton X-100 was used as the detergent and phosphatidylcholine as the stabilizing phospholipid.

Measurement of ATPase Activity and K_m Determination. The Ca^{2+}-ATPase activity of the purified enzyme was continuously monitored spectrophotometrically by using the coupled enzyme assay described by Niggli et al. (1981). The concentrations of free Ca^{2+} in the media were calculated with the help of a computer program assuming a K_d of the Ca^{2+}-EGTA complex of 0.21 μM at pH 7.0 (Martell & Smith, 1974; Itarafugi & Ozawa, 1980).

Phosphorylation of the ATPase. The catalytic subunit of the cAMP-dependent protein kinase was reconstituted from the supplied powder before every experiment by incubation in 50 mg/mL DTT, pH 7.4 (0.5 mg/mL protein), at room temperature for 10 min. Phosphorylation in the absence of CaM was carried out at 37 °C in a buffer of 20 mM HEPES, 130 mM NaCl, 0.1% Triton X-100, 0.5 mg/mL phosphatidylcholine, 10 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 1 mM EGTA, and 10 nM [γ-^{32}P]ATP (buffer A). The ratio of ATPase to kinase and the incubation times are detailed in the legends to the figures. In the presence of CaM (10:1 molar ratio to ATPase), the same buffer was used, but with the substitution of 50 μM $CaCl_2$ for EGTA and 20 mM $MgCl_2$ for 10 mM $MgCl_2$ (buffer B). Hydroxylamine treatment was performed by adding hydroxylamine/sodium acetate, pH 5.4, to a final concentration of 0.8 M and incubating for 30 min at 37 °C.

Measurement of ^{32}P Incorporation. Samples of the purified ATPase were spotted onto 2 cm^2 squares of Whatman P-81 phosphocellulose paper (Corbin & Reimann, 1974) and air-dried. The samples were then washed separately in 75 mM orthophosphoric acid (5 × 15 min, each time 30 mL). After being air-dried, the phosphocellulose pieces were counted in 30 mL of scintillation cocktail in a Beckman LS180 1 β counter. For activity measurements, the ATPase was loaded onto a CaM column of 0.5-mL bed volume in buffer A and washed with 10 bed volumes of this buffer before elution with buffer B. This was done to avoid any interference by the kinase or excess ATP in the assay.

The synthetic peptides were phosphorylated in the presence of 20 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, and 10 nM[γ-^{32}P]ATP. Kemptide was used as an artificial substrate control to measure kinase activity (Kemp & Clark, 1978).

A second method of determining ^{32}P incorporation was used to confirm that phosphate was indeed incorporated into the ATPase; 500-pmol samples of ATPase from a stock solution of known concentration (as determined by amino acid analysis) were incubated with a known dilution of radioactive ATP. A weight ratio of 25:1 ATPase to cAMP-dependent kinase was used and the reaction terminated by adding Laemmli (1970) sample buffer and boiling for 3 min. The samples were then run on 7% Laemmeli gels and stained with Coomassie blue. The ATPase band was excised, and the crushed gel pieces were counted in a scintillation counter to ensure that only phosphate incorporated into ATPase was measured.

Preparation of Labeled ATPase for Digestion. Twenty nanomoles of ATPase was incubated with 1 nmol of cAMP-dependent protein kinase at 37 °C for 30 min with [γ-^{32}P]-ATP. A 10-fold excess of cold ATP over the ATPase was then added and the mixture incubated for 30 additional min.

The ATPase was then purified on a calmodulin column as described above (Niggli et al., 1979). The protein eluted with EDTA was precipitated by using sodium deoxycholate followed by addition of trichloroacetic acid to a final concentration of 10% at 4 °C. The precipitate was washed by resuspension in 10% trichloroacetic acid, centrifuged, and washed again with acetone hydrochloride (99.9 mL of acetone and 0.1 mL of concentrated HCl) and finally with cyclohexane (Filoteo et al., 1987) to remove lipids, detergents, and nonbound phosphate. The protein was dissolved in 700 μL of 100% formic acid, and 300 μL of water was added. CNBr was added in 20-fold molar excess over the methionine in the ATPase. The digestion was carried out at room temperature for 18 h under nitrogen in the dark. Water was then added in 5-fold excess and the solution concentrated to 100 μL under a stream of dry nitrogen. Water was again added to a volume of 1 mL and the procedure repeated 3 times. The solution was then centrifuged, and the supernatant, containing virtually all (95%) of the radioactivity from the original washed pellet, was injected onto the HPLC system.

Digestion of 50 nmol of the labeled ATPase using *Staphylococcus aureus* V8 protease in 50 mM ammonium acetate buffer, pH 4.0, was carried out for 2 h at 37 °C using an ATPase:V8 protease weight ratio of 40:1. After addition of fresh V8 to a final weight ratio of 20:1, the digestion was carried out for a further 2 h. The single radioactive peptide from this digest was used for derivatization with ethanethiol to establish which serine had been phosphorylated.

HPLC Analysis and Amino Acid Sequencing of Separated ATPase Fragments. The reversed-phase buffers used were as follows: A, 0.1% TFA in water; B, 0.1% TFA/80% acetonitrile in water; C, 50% acetonitrile/0.1% TFA in water. Chromatography was carried out with LKB equipment (Uppsala, Sweden), a Machery and Nagel (Düren, FRG) C-18, 300-Å, 7-μm nucleosil reversed-phase (250 × 10 mm) semipreparative column, and a Brownlee Laboratories (Santa Clara, CA) C-8, 300-Å, 7-μm (250 × 1.0) microbore column. Optical detection was carried out at 206 nm, and the radioactivity was measured by removing aliquots by hand, mixing them with the scintillation cocktail, and counting. Sequencing was performed on an Applied Biosystems 470A sequencer (Foster City, CA) with an on-line 120A PTH analyzer.

Phosphopeptide Modification for Sequencing. The single radioactive peptide isolated from the V8 digest of the ATPase was used to carry out the conversion of phosphoserine into *S*-ethylcysteine as described by Holmes (1987). The peptide was subsequently purified by microbore HPLC, collected directly onto a polybrene-treated glass fiber disk, and sequenced.

Synthesis of Peptides B28 and C28W. Peptide B28 was synthesized according to the *tert*-butyloxycarbonyl (*t*-Boc)-benzyl strategy with *t*-Boc derivatives and peptide C28W according to the N^{α}-fluorenylmethoxycarbonyl/*tert*-butyl

[1] Abbreviations: CaM, calmodulin; MLCK, myosin light chain kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PFK, phosphofructokinase.

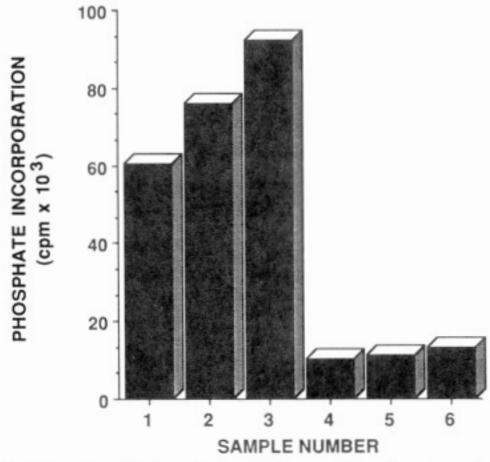

FIGURE 1: Phosphorylation of the ATPase. The phosphorylation was carried out as described under Materials and Methods. 50 pmol of ATPase was used in each sample together with 2 pmol of the cAMP-dependent protein kinase (total reaction volume 100 μL). Columns 1, 2, and 3 represent phosphate incorporation at 37 °C after 15, 30, and 45 min, respectively. Columns 4, 5, and 6 are the same as columns 1, 2, and 3, except that the incubation was performed at 4 °C. The reaction was arrested by removing 50-μL aliquots from the reaction medium and spotting them onto phosphocellulose filters which were then dried. The filters were washed and counted in a scintillation cocktail.

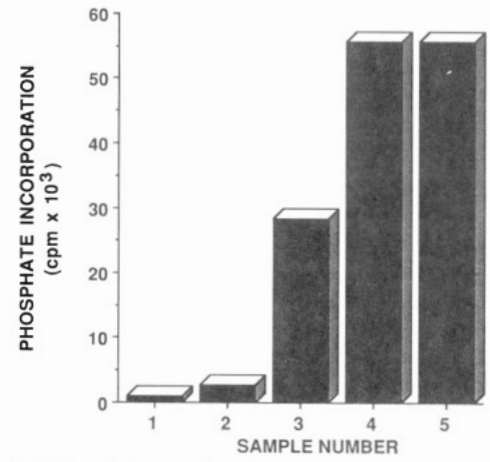

FIGURE 2: Effect of CaM and hydroxylamine upon phosphorylation. 40 pmol of ATPase and 60 pmol of kinase were used in this experiment (total reaction volume 100 μL). In columns 1 and 2 the incubation was carried out in the presence of CaM (10:1 CaM:ATPase ratio M/M) at 37 °C for 30 and 60 min, respectively. Columns 3 and 4 were incubated in the absence of CaM for 30 and 60 min, respectively. For column 5, the incubation was carried out for 60 min in the absence of CaM, and the preparation was subsequently treated with hydroxylamine as described under Materials and Methods.

(Fmoc/*t*-Bu) strategy with chemicals as supplied by Applied Biosystems, using the 430A peptide synthesizer and the standard programs supplied. The peptides were purified to 98% purity by RP-HPLC, as determined by analytical reverse-phase HPLC, sequencing, and amino acid analysis.

Measurement of Protein Concentration. Protein concentrations were determined by the methods of Lowry et al. (1951). The protein was first precipitated with trichloroacetic acid and sodium deoxycholate to avoid interference by Triton X-100 and HEPES (Bensadoun & Weinstein, 1976).

RESULTS

Phosphorylation of the Erythrocyte ATPase. Previous work from this Laboratory (Caroni & Carafoli, 1981) has shown that, at least in the case of heart sarcolemma Ca^{2+}-ATPase, phosphorylation of native sarcolemmal membranes by the cAMP-dependent protein kinase stimulated Ca^{2+} transport. Neyses et al. (1985) have later used the reconstituted erythrocyte Ca^{2+}-ATPase to show that the effect of phosphorylation was due to the phosphorylation of the enzyme proper. However, the technical reasons, a high ratio of ATPase to cAMP-dependent kinase (1:1.5 w/w) was used in the work of Neyses et al. (1985). In order to rule out unspecific phosphorylation reactions and to characterize the activation of the ATPase by the phosphorylation process, the experiments described here were carried out using a much higher ATPase:kinase ratio (25:1 w/w).

Figure 1 shows that phosphate from [γ-^{32}P]ATP was rapidly incorporated into the ATPase at 37 °C while at 4 °C the effect was very slow. The artificial phosphorylation substrate kemptide was always used as a control in these and in the other experiments described here, since the commercially available cAMP-dependent protein kinase preparations showed great variation in their ability to stimulate the activity of the purified Ca^{2+}-ATPase depending on the age and concentration of the ATPase and on the reconstitution procedure. Previous work had shown that the kinase-mediated incorporation of phosphate did not occur into tightly associated phospholipids (Neyses et al., 1985), e.g., phosphatidylinositol. Hydroxylamine treatment of the phosphorylated ATPase (Figure 2) showed that the incorporation of phosphate was not due to the formation of an acyl phosphate intermediate.

In order to further localize the kinase-dependent site of phosphorylation in the ATPase molecule, the effect of calmodulin on the incorporation of phosphate was investigated, since by analogy to MLCK and PFK (Lukas et al., 1986; Buschmeier et al., 1987) the ATPase might have become phosphorylated near the calmodulin binding site. Figure 2 shows the effect of calmodulin addition during the incubation of the ATPase with the kinase. In the presence of CaM (10:1 CaM:ATPase M/M) and Ca^{2+}, an almost complete inhibition of the incorporation of phosphate was seen, even when high concentrations of kinase were used, as in the experiment shown in Figure 2. The figure also shows that the phosphate incorporation was hydroxylamine insensitive.

Control experiments not shown here demonstrated that Ca^{2+} and CaM had no effect on the activity of the kinase, measured with kemptide as an acceptor of phosphate. Finally, it could be shown that calmodulin did not influence phosphorylation by lowering the ATP concentration due to the increased activity of the ATPase, since the inhibition of phosphorylation by calmodulin was seen also in ATPase preparations inactivated by treatment at 100 °C for 5 min.

Determination of ^{32}P Incorporation. The stoichiometry of phosphorylation was determined by the two distinct methods described under Materials and Methods. In both cases, the amount of [^{32}P]phosphate from [γ-^{32}P]ATP incorporated into the ATPase was found to be 0.71 (SD 0.019) mol of phosphate/mol of ATPase. The reason for the sub-unity value is discussed below.

Effect of Phosphorylation on Activity. For kinetic measurements, 200 pmol of the purified ATPase was incubated for 15 min at room temperature with [γ-^{32}P]ATP using a ATPase to kinase ratio of 1 to 0.04 (w/w) and then for a further 15 min using a 10-fold molar excess of ATP over ATPase. The buffer used was A (see Materials and Methods). The free Ca^{2+} concentration after the incubation was adjusted to 50 μM, and the sample was then loaded onto a mini-calmodulin column which had been previously equilibrated in buffer B (see Materials and Methods). The column was washed with 10 bed volumes of this buffer (until the ^{32}P washout decreased to its basal level), and the ATPase was

Table I: ATPase Activity Measurements[a]

	ATPase act. [μmol of ATP (mg of protein)$^{-1}$ min^{-1}]	
	without CaM	with CaM
control ATPase	0.78	3.77
phosphorylated ATPase	1.42	3.95
stimulation factor	1.80	1.04

[a] The conditions for phosphorylation are described in the legend for Figure 3. The ATP and the kinase were removed by purification of the ATPase on a CaM column (as detailed under Materials and Methods) before assaying the activity. As a control, the ATPase was incubated with the kinase in the absence of ATP before purification.

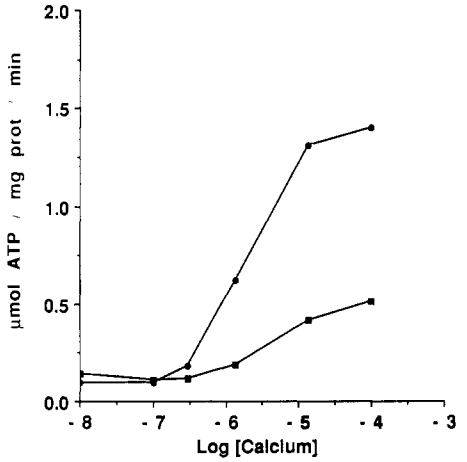

FIGURE 3: Measurement of the K_m of the phosphorylated ATPase. Routinely, 200 pmol of ATPase was incubated with 10 pmol of kinase for 15 min at room temperature with 10 pmol of [γ-^{32}P]ATP, followed by a further 15-min incubation with a 10-fold molar excess of cold ATP (total reaction volume 500 μL). The free Ca^{2+} concentration was adjusted to 50 μM, and the sample was loaded onto a mini-CaM column. The column was washed with 10 bed volumes of buffer A and then eluted with buffer B as described under Materials and Methods. The protein concentration and phosphate incorporation in the active fractions were determined, and the ATPase was assayed in the coupled enzyme medium at various free Ca^{2+} concentrations. The circles represent the activity of the phosphorylated ATPase and the squares that of the control ATPase.

eluted by using the EGTA-containing buffer A. The ATPase-containing fractions were collected, and the ^{32}P incorporation into the ATPase, the ATPase activity, and the protein concentration were determined. Controls were run under identical conditions omitting the ATP or the kinase from the incubation medium containing the ATPase.

Table I presents the results obtained (each figure is the average of four determinations, each one on three different preparations). The effect of the phosphorylation was a 2-fold increase of the basal activity of the nonphosphorylated ATPase. In comparison, calmodulin usually induced a 5-fold increase. The stimulation by CaM and that by phosphorylation were apparently nonadditive, i.e., the maximal ATPase activity in the presence of CaM was the same in the phosphorylated and in the control enzyme.

The effect(s) of the phosphorylation on the kinetics of the ATPase was (were) investigated with respect to the Ca^{2+} requirements (Figure 3). The V_{max} of the enzyme was increased about 2-fold by phosphorylation and the $K_m(Ca^{2+})$ decreased from 10 to 1.4 μM. These results are in line with the general observations by Neyses et al. (1985) on Ca^{2+} uptake by the purified erythrocyte ATPase reconstituted into liposomal vesicles.

Sequence Determination of the Phosphorylation Site Domain. The ^{32}P-labeled ATPase was digested with CNBr as described under Materials and Methods. After centrifugation

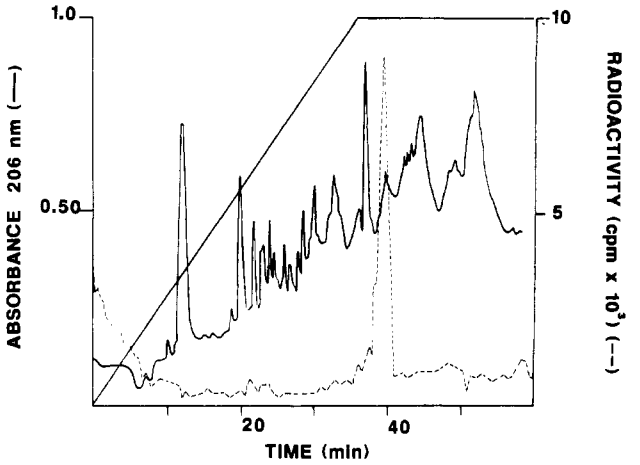

FIGURE 4: HPLC separation of the peptides derived from CNBr cleavage of the labeled ATPase. The digest was separated on a semipreparative reversed-phase column (C-18, 300 Å, 7 μm, 250 × 10 mm, nucleosil). The gradient was run at 100% A for 10 min to wash out free phosphate, and then a gradient was run at 100% A until the base line was reached. A gradient was then developed from 100% A to 100% B in 40 min at 1 mL/min. Detection was at 206 nm (solid line), and 10 μL was withdrawn for scintillation counting (dashed line). The radioactivity eluting at 39 min was pooled and the peptide further purified on the same column using a flatter gradient.

and washing, 98% of the radioactivity was recovered in the supernatant. The supernatant was separated on a semipreparative reversed-phase HPLC column (C-18, 300 Å, 7 μm, nucleosil, 250 × 10 mm) as shown in Figure 4. A gradient was run from 100% A to 100% B in 40 min. A single peak of radioactivity at 39 min was associated with a single peptide which was collected and further purified by repeating the procedure with a flatter gradient in the region where the peptide eluted. The peptide was isolated and used for sequencing and amino acid analysis (10% was used for amino acid analysis, 10% for each sequencing run). An initial yield (as calculated by Applied Biosystems software) of 960 pmol was obtained, and the repetitive yield was 94%. The amino acid analysis indicated that 1.4 nmol of peptide had been loaded onto the sequencer, giving an initial sequencing recovery of 68%. The recovery of radioactivity from that initially incorporated into the ATPase exceeded 70%.

The following peptide sequence was obtained: (M)-T-H-P-E-F-R-I-E-D-S-E-P-H-I-P-L-I-D-D-T-D-A-E-D-D-A-P-T-K-R-N-S-X-P-P-(P)-S-P-D-K-N. The gap labeled X was a cycle in which no new amino acid peak appeared to increase and that of the serine of the previous cycle decreased. The missing peak was tentatively assumed to be phosphoserine or phosphothreonine partly because the preceding sequence appeared to be a canonical substrate site for the cAMP-dependent kinase and partly because phosphoserine and phosphothreonine could not be detected by the analysis system used (Meyer et al., 1986). This seemed probable on the basis of the recently established structure of the human teratoma and rat brain plasma membrane pumps (Shull & Greeb, 1988; Verma et al., 1988). However, work in this laboratory has shown that the erythrocyte ATPase is a different isoform (Verma et al., 1988). It was thus considered essential to verify the assumption that the missing amino acid indeed was a serine. The procedure of Holmes (1987) was used on a ^{32}P-labeled peptide which had been isolated from a V8 digest of the ATPase, treating it with ethanethiol to convert the postulated phosphoserine into *S*-ethylcysteine, which could be identified with the PTH detection system used. After derivatization, the peptide was isolated on the reversed-phase Brownlee Laboratories (Santa Clara, CA) HPLC microbore

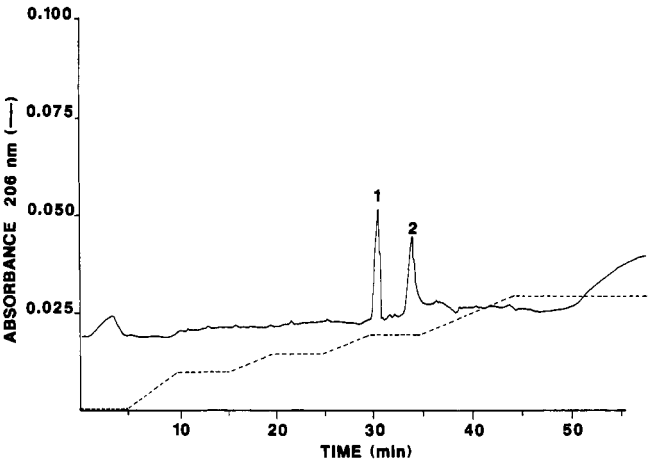

FIGURE 5: Microbore HPLC separation of the labeled peptide from the V8 digestion after derivatization with ethanethiol. The derivatization procedure is described under Materials and Methods. The gradient was run at 100% A for 5 min, developed from 100% A to 10% C in 5 min, held at 10% C for 5 min, and then brought to 20% C in 5 min and held at 20% C for 5 min. It was then brought to 30% C in 5 min, held there 5 min, and finally brought to 50% C in 10 min and held for 20 min. Peak 1 was the phosphorylated peptide and peak 2 the derivatized peptide used for confirmation.

column described under Materials and Methods (C-8, 300 Å, 7 μm, 250 × 1.0 mm). The late eluting peak in Figure 5 was collected onto a polybrene-treated glass fiber disk and loaded onto the sequencer directly. The sequence obtained was A-P-T-K-R-N-S-S-P-P-P-S-P-D where Ser-8 is *S*-ethylcysteine, confirming that the X of the CNBr sequence shown above was indeed phosphoserine. Amino acid analysis agreed with the sequence shown, confirming that even at pH 4.0 the V8 protease had split the enzyme at the expected Asp residue.

Phosphorylation of Synthetic Peptides. A 28 amino acid peptide (B28) containing the phosphorylation site identified above was synthesized on the basis of the sequence of the human teratoma ATPase (Verma et al., 1988): E-D-S-E-P-H-I-P-L-I-D-D-T-D-A-E-D-D-A-P-T-K-R-N-S-S-P-G. The glycine at the C-terminal end of the peptide was substituted for the expected proline for reasons of peptide synthesis. The peptide was incubated under the conditions routinely used for the phosphorylation of the artificial substrate of the cAMP-dependent kinase kemptide. The latter was found to be a very good substrate for it. As an internal control, another peptide (C28W) was synthesized on the basis of the sequence of the erythrocyte pump (James et al., 1988): L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S. The peptide corresponded to the calmodulin binding domain of the pump: when incubated with the cAMP-dependent kinase under conditions which produced phosphorylation of kemptide and peptide B28, no phosphate incorporation was observed.

Discussion

The results presented here confirm that the erythrocyte calcium pump can be directly phosphorylated by the cAMP-dependent protein kinase: the experimental conditions chosen were in principle compatible with a physiological role for the process, but the evidence obtained so far on this point cannot be considered as conclusive. The phosphorylation reaction lowered the $K_m(Ca^{2+})$ of the ATPase from 10 to 1.4 μM. The phosphorylated enzyme could be further activated by calmodulin, but only to the maximal levels seen with CaM alone. Thus, the effects of CaM and of phosphorylation are provisionally considered as not additive (a detailed investigation of this point is currently under way). Although the phosphorylation process was not as potent in activating the ATPase as calmodulin, it could nevertheless provide a second pathway for activation, which would be Ca^{2+} independent. The work has also shown that in the presence of calmodulin, the ATPase could not be phosphorylated by the kinase. The protection of the phosphorylation site was not due to the interaction of calmodulin with the kinase, since control experiments showed that neither calmodulin nor Ca^{2+} inhibited the kinase. This observation supports the idea of a single specific phosphorylation site, since complete inhibition of phosphorylation by CaM was also seen when a large molar excess of the kinase over the ATPase was used, even when CaM was only present in a 1:1 molar amount with the ATPase.

On average, phosphate incorporation in a number of experiments was always about 70%. This could have been due either to the fact that the ATPase was already partially phosphorylated in vivo or to the presence of more than one ATPase isoform in the preparations, one of which may not be phosphorylated in erythrocytes. Although no decision between these two possibilities can be made at the moment, it may be noted that protein sequencing studies now under way on the purified erythrocyte enzyme (James, unpublished results) indicate at least two isoforms.

The conclusion that only one phosphorylation site exists was further supported by the finding that only a single labeled peptide, containing 72% of the radioactivity incorporated into the ATPase, could be isolated. The sequence obtained contained a canonical substrate sequence for the cAMP-dependent protein kinase. Although not absolute, the kinase shows a preference for sites with the sequences R-R-X-$S/_T$ and K-R-X-X-$S/_T$. That S, rather than T, was the phosphorylated residue was considered likely based on the deduced sequence of the human teratoma clone published (Verma et al., 1988). The finding of PTH-*S*-ethylcysteine after derivatization of the peptide with ethanethiol provided the final demonstration.

The position of the phosphorylation domain in the ATPase in the vicinity of the CaM binding domain is of great interest. Prevention of cAMP-dependent phosphorylation of several CaM-dependent enzymes (and also some CaM binding peptides) by CaM has been previously described (Lukas et al., 1986; Buschmeier et al, 1987; Kemp et al., 1987). Malencik and Anderson (1982) first remarked on the occurrence of cAMP-dependent protein kinase substrate sites within the CaM binding domain of several proteins and peptides, suggesting some form of synergism between CaM and the phosphorylation. Conti and Adelstein (1981) subsequently demonstrated that the cAMP-dependent protein kinase could phosphorylate MLCK at two sites, one of which could be protected by CaM. In contrast to the erythrocyte pump, this phosphorylation produced an inhibition of MLCK activity, i.e., a 20-fold decrease in the affinity of the kinase for CaM.

The exact location of the CaM binding domains and of the cAMP-dependent protein kinase phosphorylation sites has recently been reported for MLCK of smooth and skeletal muscle (Lukas et al., 1986; Kemp et al., 1987). In both cases, a stretch of approximately 30 amino acids was identified. The N-terminal half contains a domain rich in hydrophobic and basic residues having the potential to form an amphiphatic helix; the C-terminal half contains a serine-rich cluster which acts as a substrate for the cAMP-dependent kinase. These observations have led to the hypothesis that the CaM binding domain of smooth muscle MLCK contains a pseudosubstrate sequence (similar to the sequence found around the phosphorylation site in smooth muscle myosin light chains) which may act as a competitive inhibitor which would block the active

site (Kemp et al., 1987). CaM binding to this region would displace the pseudosubstrate sequence from the active site, thus activating (or rather de-repressing) the enzyme. Phosphorylation decreases the affinity of this domain for CaM, thus preventing activation.

The opposite effect was seen in the case of the erythrocyte Ca^{2+}-ATPase, in which phosphorylation by the cAMP-dependent protein kinase. The phosphorylation site described here is further C-terminal to the CaM binding domain. As described previously (Shull & Greeb, 1988; Verma et al., 1988), the CaM binding domain of the erythrocyte pump is located about 18 kDa away from the C-terminus. Very recent work in this laboratory on the effect of calpain on the isolated plasma membrane pump has further defined the domain (James et al., submitted for publication). A fragment of about 124 kDa has been isolated from a calpain digest of the erythrocyte pump, which had thus lost about 14 kDa from its C-terminus. The fragment could still bind CaM but was no longer phosphorylated by the cAMP-dependent kinase.

The mechanism by which the phosphorylation of the pump exerts its effects is currently being investigated with the help of synthetic peptides. A more detailed study of the phosphorylation process in inverted erythrocyte ghosts and in intact erythrocytes should shed light onto the physiological relevance of the observations reported here.

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