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THE AMINO ACID SEQUENCE OF THE PHOSPHORYLATION DOMAIN OF THE ERYTHROCYTE Ca^{2+} ATPase

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The amino acid sequence of a peptide isolated from a CNBr digest of the erythrocyte Ca^{2+} ATPase has been determined. It contains a highly conserved phosphorylation site sequence common to all aspartyl-phosphate forming ion motive ATPases which have been sequenced so far.

A distinctive feature of ion motive membrane ATPases of the $P(E_1-E_2)$-type (1) is the formation of a phosphorylated intermediate during the reaction cycle. Amino acid sequence analysis of a number of these ATPases (2-9) has shown this intermediate to be an aspartyl-phosphate located in a highly conserved stretch of amino acids. The primary structure of the calmodulin sensitive Ca^{2+} ATPase of plasma membranes, which is larger than all other ATPases of its class (10) and which is present in plasma membranes in extremely low concentrations, has not yet been elucidated. Partial sequencing work, however, has obtained the structure of its fluorescein isothiocyanate (ATP) binding site (11), and that of its calmodulin binding domain (12). This report describes the primary structure of the phosphorylation domain of the erythrocyte Ca^{2+} ATPase. A large scale preparation of the ATPase produced about 40 mg of the purified protein. Sequence analysis of a CNBr peptide corresponding to the domain has shown it to contain the predicted aspartic acid as the phosphate group acceptor, flanked on both sides by the amino acid sequences common to all other ATPases of the group.

MATERIALS AND METHODS

The erythrocyte Ca^{2+} ATPase was isolated from recently outdated human blood obtained from the Red Cross Blood Bank of Zurich, Switzerland,

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according to the procedures described in references 10 and 11. The stabilising phospholipid was phosphatidylcholine and the extracting detergent Triton X100. A longer Ca^{2+} washing step (24 hours) was used to remove minor co-purifying contaminants, sometimes seen in the standard preparations. High pressure liquid chromatography was carried out using LKB equipment (Uppsala, Sweden) using a self packed reverse phase C18 column of 300 Å pore size (Tokyo Soda, Tokyo, Japan) followed by an Applied Biosystems Brownlee C8 column (Foster City, CA, USA). Sequencing was carried out using an Applied Biosystems 470A sequencer with on line PTH detection. All chemicals used for the sequencing work were from Applied Biosystems. The purified ATPase (25 mg) in 6M guanidinium chloride was carboxymethylated with iodo-acetic acid after incubation with dithiothreitol according to reference 13. The guanidinium chloride was removed by extensive dialysis at 40 C against 10 mM phosphate buffer, pH 7.2, with 0.01% Triton X100. The protein was precipitated by adding 150% (w/v) trichloroacetic acid to 7.5% final concentration and the precipitate was centrifuged down at 5000 g for 10 minutes. It was then washed, first with acetone (100 ml + 0.1 ml 34% HCl) and then with cyclohexane. The pellet was solubilised completely by sonifying it in 70% formic acid. CNBr was then added in 10 fold excess over the methionine content of the ATPase (14) and the digestion was carried out in the dark for 24 hours under nitrogen. The reagents were removed by blowing a continuous stream of dry nitrogen over the solution at 40^{0} C until almost dry, then adding 5 ml water and repeating the operation 3 times. The solution was then centrifuged to remove any particulate material and injected onto the HPLC system. Solvent A was 0.1% trifluoroacetic acid in water, solvent B 0.1% trifluoroacetic acid in acetonitrile, and solvent C a 60/40 acetonitrile/water mixture with 0.1% trifluoroacetic acid. Amino acid analysis was carried out according to reference 15.

RESULTS

The digest was applied to the C18 reverse phase HPLC column and eluted as shown in Figure 1. The CNBr peptides were eluted using a gradient

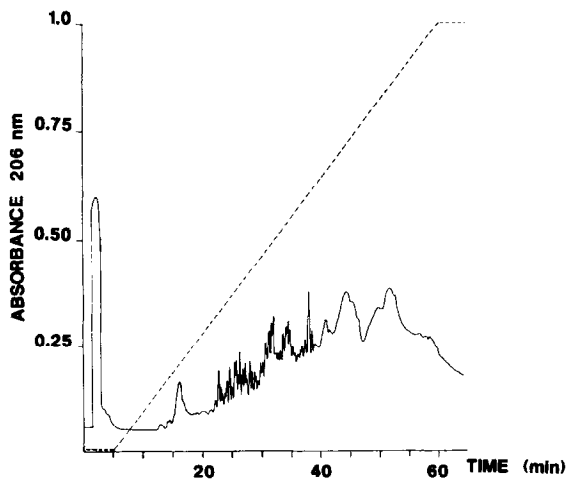

Fig. 1. Preparative separation of the CNBr fragments produced by digestion of the ATPase. The digest was injected onto the C18 300 Å reverse phase column and a gradient run from 100% to 80%B over 60 minutes. Peptides were detected by measuring absorbtion at 206 nm and fractions collected automatically every 30 seconds.

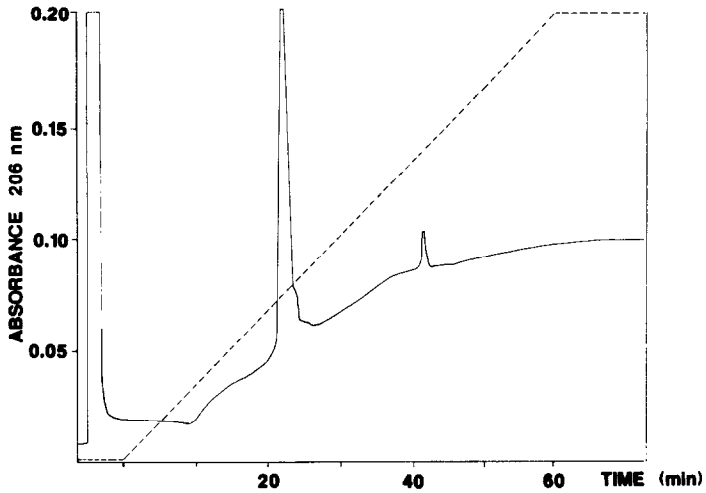

Fig. 2. Purification of the phosphorylation domain-containing peptide. The peak eluting at 36 minutes in Figure 1 was subjected to further purification on a Brownlee 300A C8 reverse phase column. The peak-containing fractions were pooled and reduced in volume by blowing down with dry nitrogen. A gradient was then run between 100% A and 100% C in 60 minutes. The major peak was then used directly for sequencing, without further purification.

running from 100% A to 80% B plus 20% A over 60 minutes (Figure 1) and fractions were collected every 30 seconds. The peak eluting at 36 minutes was subjected to further purification on the Applied Biosystems Brownlee C8 reverse phase column using a gradient running from 100% A to 100% C over 60 minutes (Figure 2). Sequencing of the single peak shown in the figure gave the following peptide structure:

-GLY-ASN-ALA-THR-ALA-ILE-CYS-SER-ASP-LYS-THR-GLY-THR-LEU-THR-

which was confirmed by amino acid analysis. The homology of this peptide sequence to all other ATPases in its class showed clearly that it was the phosphorylation site. Experiments using gamma-^{32}P ATP to label the Ca^{2+} ATPase at the active site and digesting under identical conditions to those described above, produced a single radioactive peak on the first HPLC column which ran in a position close to that of the unlabelled peptide identified here as the phosphorylation site. However, due to the lability of the phosphorylation prodcut produced by this labelling method and the product long digestion times involved, the yield of the phosphorylated material was never high enough to produce clear results from sequencing experiments.

DISCUSSION

The CNBr fragment purified and analysed in the work reported here contains a sequence which is highly conserved around the phosphorylation site of all other ion motive ATPases of the P-type.

TABLE 1

A comparison of the sequences of the P-type phosphorylation domains. The numbers in parentheses refer to the position of the active aspartic acid in the amino acid sequence of the protein.

ATPase	Sequence	
Erythrocyte, Ca^{2+}	M G N A T A I C S D K T G T L T M	
Sarcoplasmic ret., Ca^{2+}	L G T C S V I C S D K T G T L T T N Q	(348)
Rat stomach, H^+/K^+	L G T C S V I C S D K T G T L T Q N R	(382)
Sheep kidney, Na^+/K^+	L G S T S T I C S D K T G T L T Q N R	(371)
Torpedo calif., Na^+/K^+	L G S T S T I C S D K T G T L T Q N R	(373)
Rat brain, Na^+/K^+	L G S T S T I C S D K T G T L T Q N R	(373)
Neurospora crassa, H^+	L A G V E I L C S D K T G T L T K N K	(375)
E.coli Kdp, K^+	A G D V D V L L L D K T G T I T L G N	(307)
Strept.faecalis, K^+	N N D L D V I M L D K T G T L T Q G K	(275)

Table 1 compares the sequence obtained here to that of the other ATPases. All the eucaryotic enzymes share a stretch of ten identical amino acids around the phosphate acceptor, aspartic acid. However, the cysteine and serine residues contained in this stretch are not conserved in the two procaryotic enzymes analysed so far. In principle then, these two residues must be considered as not essential to the reaction mechanism proper. The function of the cysteine residue adjacent to the aspartate in eucaryotic enzymes is not yet clear, but it is tempting to speculate that it is the cysteine whose blockage inactivates the Ca^{2+} ATPase of sarcoplasmic reticulum (16). The same holds for the lysine residue on the C-terminal side of the aspartate, since binding of pyridoxal phosphate to a single lysine also inactivates the same ATPase (17). Sequencing work now under way in our Laboratories indicates that the phosphorylation domain is one of only a few regions of the erythrocyte membrane Ca^{2+} pump having a high degree of sequence similarity to the other P-type ion motive ATPases.

The P-type ATPases are but one of the classes of ion motive ATPases. Structural and mechanistic differences define at least three other classes: 1) the F-type (F_1-F_0) which function as ATP synthetases in mitochondria, chloroplasts and bacteria; 2) the V-type , found in membrane systems such as chromaffin granules, tonoplasts and the vacuoles of Neurospora and yeasts; and 3) the archaebacterial ATPases. Next to the P-type ATPases, the F-type ATP synthetases are the best known at the molecular level. Their principal mechanistic difference from the enzyme of the P-class is obvious:

they do not form phosphorylated intermediates. They do contain high affinity adenine nucleotide binding sites, as demanded by their reaction mechanism, which requires tight binding of adenine nucleotides. On inspecting the primary structure of the catalytic β-subunit of a number of F-type ATPases a sequence of 8 amino acids is found which is highly homologous to the phosphorylation domain of the P-type ATPases (see Table 1) (18,19):

SER-THR-LYS-THR-GLY-SER-ILE-THR

Alignment of the sequence with the last eight residues of the sequence presented in this report shows that the aspartate residue which is phosphorylated is replaced by a threonine. The lysine in the next position has been shown (in F-type ATPases) to bind photoactivatable analogues of ATP (20). The fact that the phosphorylated sequence of P-type ATPases is highly conserved in a class of ATPases which do not form a phosphorylated intermediate is of considerable interest. Even though the sequence is short and thus presumably inadequate to define the tertiary structure of an active site, it appears permissible to speculate on a possible common evolutionary pathway for F- and P-type ion motive ATPases. Whether the evolutionary branching of the two classes has resulted from the deletion of an aspartic acid, or from its insertion in the place of a threonine is bound to remain a platonic question.

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