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The "γ Subunit" of Na,K-ATPase: A Small, Amphiphilic Protein with a Unique Amino Acid Sequence†

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ABSTRACT: The "γ subunit", or "proteolipid", of Na,K-ATPase is a small, membrane-bound protein that copurifies with the α and β subunits of this enzyme. The importance of γ in the function of Na,K-ATPase remains to be established, but some evidence indicates that it may be involved in forming a receptor site for cardiac glycosides. We have previously communicated [Reeves, A. S., Collins, J. H., & Schwartz, A. (1980) *Biochem. Biophys. Res. Commun. 95*, 1591–1598] the purification and amino acid composition of sheep kidney γ, and in this paper we present the first available sequence information on this protein. Although the amino terminus of γ seems to be blocked and it is resistant to proteolytic cleavage, we have determined approximately half of its amino acid sequence. Our results indicate that γ contains a total of 68 amino acid residues, with a calculated M_r of 7675. The sequenced portion appears to be at the carboxyl terminus of the polypeptide chain. The γ sequence is unique, providing strong evidence for its homogeneity and establishing for the first time that it is not a breakdown product of the α or β subunits. γ is not a true proteolipid, but rather it is an amphiphilic protein with two distinct structural domains. The amino-terminal domain (residues 1–49) is very hydrophilic, with many charged amino acid side chains, and must be extracellular. This domain includes a concentrated segment of four aromatic residues which may be involved in glycoside binding. The carboxyl-terminal domain (residues 50–68) is hydrophobic and probably spans the cell membrane.

Na,K-ATPase is the enzyme responsible for the active transport of Na^+ and K^+ across animal cell membranes. The resulting electrochemical gradients are essential for a variety of vital physiological functions [for reviews, see Schwartz and Collins (1982), Jorgensen (1982), Kaplan (1985), Stahl and Harris (1986), and Stahl (1986)]. Na,K-ATPase contains two major protein subunits: the α, or catalytic subunit (M_r ~ 112 000), and the β, or glycoprotein subunit (M_r ~55 000).

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The amino acid sequences of α and β subunits of Na,K-ATPases from sheep kidney (Shull et al., 1985, 1986a) and several other sources (Kawakami et al., 1985, 1986a,b; Noguchi et al., 1986; Mercer et al., 1986; Shull et al., 1986b; Ovchinnikov et al., 1985, 1986a,b) have recently been determined and models of their transmembrane arrangements have been developed. Much less attention has been paid to a small protein that may represent a third subunit of the Na,K-ATPase. This protein was first noticed by Rivas et al. (1972), who suggested that Na,K-ATPase may contain a small proteolipid (a very hydrophobic protein) that takes part in forming an extracellular receptor site for ouabain and other cardiac glycosides. Later, Racker (1976) hypothesized that a proteolipid of Na,K-ATPase may form an ion channel through the cell membrane. More compelling evidence for the existence of a third subunit in Na,K-ATPase was provided by Forbush et al. (1978), who found that a photoactive ouabain derivative labeled both the α subunit and a protein of M_r ~12 000, indicating that both proteins may be involved in formation of the glycoside receptor site. This finding was confirmed and extended in other studies (Forbush & Hoffman, 1979; Rogers & Lazdunski, 1979a,b; Hall & Ruoho, 1980; Rossi et al., 1980; Forbush, 1983).

As part of our investigation of the structure of sheep kidney Na,K-ATPase (Collins et al., 1983; Collins & Zot, 1983), we developed a procedure for the large-scale purification of the small protein, which we designated the "γ component" of Na,K-ATPase (Reeves et al., 1980). In this paper we shall refer to this protein simply as γ. We determined the amino acid composition of γ and showed that it was present in approximately equimolar amount to the α and β subunits. We also showed (Collins et al., 1982) that γ was identical with the small protein that is labeled (Forbush et al., 1978) with a photoactive ouabain derivative. In the meantime, Hardwicke and Freytag (1981) reported a similar amino acid composition and stoichiometry for shark rectal gland γ and suggested that γ is not essential for Na,K-ATPase activity. To further investigate the possibility that this protein may be an important component of Na,K-ATPase, we have studied the primary structure of sheep kidney γ. In this paper we present the first available sequence information on γ from any source.

Experimental Procedures

Preparation of Protein. γ was prepared by the method of Reeves et al. (1980), as modified by Collins et al. (1982), using two steps of size-exclusion chromatography. Sheep kidney Na,K-ATPase was dissolved in a solution of 1% SDS,[1] then the α, β, and γ subunits were separated on a Sepharose CL-6B column equilibrated with a buffer containing 0.1% SDS. The γ fraction was lyophilized, redissolved in 88% formic acid, and separated from excess SDS, membrane lipids, and other low molecular weight material on a column of Sephadex LH-60 equilibrated with a 3:1 (v/v) mixture of 95% ethanol and 88% formic acid. Two interchangeable fractions, γ-1 and γ-2, were recovered. γ-1, which elutes at the void volume of the LH-60 column, is an aggregated, and possibly contaminated (Collins et al., 1982), form of γ-2. The γ-2 fraction was used as the starting material for the present studies.

Carboxymethylation. The alkylation procedure is similar to one we described previously (Collins et al., 1986). Approximately 60 nmol of γ was dissolved in 1 mL of alkylation buffer (8 M urea, 1% SDS, 1 mM EDTA, 5 mM Tris-HCl, 0.01% NaN_3, pH 8.0). SDS was necessary for solubilization of the protein. Bubbling with N_2 was begun and continued through the entire procedure, maintaining the pH at 7.9–8.1. After a few minutes, 15 μL of 2-mercaptoethanol (a 3000-fold molar excess over protein thiol groups, assuming one per molecule) was added. Thirty minutes later, 37 mg of iodoacetic acid (3300-fold molar excess) was added over a 15-min period. After the pH had stabilized, another 15 μL of 2-mercaptoethanol was added. For desalting, the sample (~2 mL) was centrifugally filtered through a YM-10 membrane, on an Amicon Centricon-10 microconcentrator, to a volume of 0.2 mL. Then, 2 mL of 95% ethanol–88% formic acid (3:1 v/v) was added, and the sample was concentrated to 0.5 mL; this procedure was repeated 3 times. A large portion of protein had precipitated during the course of the desalting and had become embedded in the membrane. The retentate was collected, the membrane was washed with 200 μL of 88% formic acid, and the washings were combined with the retentate. No protein was found in the filtrate. The recovery of protein (CM-γ) was 73%, as determined by amino acid analysis.

Tryptic Digestion. A 30-nmol sample of CM-γ was dissolved in 500 μL of digestion buffer (20 mM TES, 1 mM $CaCl_2$, pH 8.0). Initially the sample appeared as large, undissolved particles, but after being heated (55 °C) and vortexed several times a fine, a colloidal suspension was obtained. Then, 50 μL of TPCK-trypsin (freshly prepared 1 mg/mL aqueous solution) was added. Digestion took place at 37 °C, with stirring, for 20 h. The digest was filtered through a Centricon-10, concentrating it to a volume of ~50 μL. The retentate was then diluted with 250 μL of digestion buffer and concentrated again to a volume of ~50 μL; this procedure was repeated. The retentate was diluted with 200 μL of 88% formic acid and collected, and the membrane was washed with another 100 μL of 88% formic acid to dissolve any insoluble peptides. The filtrate was applied directly to reverse-phase HPLC (Collins et al., 1986) for purification of the soluble tryptic peptides.

Procedures used for amino acid analysis and gas-phase sequencer analysis have been described previously (Collins et al., 1986).

Results and Discussion

Sequencer analysis of whole CM-γ revealed a low-yield sequence (about 5% of the amount of protein applied to the instrument), which started with a Pro residue and continued to a Leu at cycle 29, after which no further sequence could be detected. The low yield of this sequence suggested that intact γ actually has a blocked amino terminus and that a small amount of unexpected peptide bond cleavage had produced a fragment with a free amino terminus for sequencing. Since Pro was the amino-terminal residue, we suspected that the fragment was produced as a result of specific acid cleavage of an Asp–Pro bond (Landon, 1977) arising from prolonged exposure of γ to concentrated formic acid during its preparation. It is likely that the fragment represents the carboxyl terminus of γ, although this conclusion must be regarded as somewhat tentative until the complete protein sequence is available. We attempted to confirm the carboxyl-terminal sequence of γ by digesting it with carboxypeptidase Y, but the results were inconclusive due to low yields of released amino acids.

[1] Abbreviations: CNBr, cyanogen bromide; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; CM-γ, carboxymethylated γ subunit of sheep kidney Na,K-ATPase; PLB, phospholamban; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; TPCK, *N*-tosylphenylalanyl chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

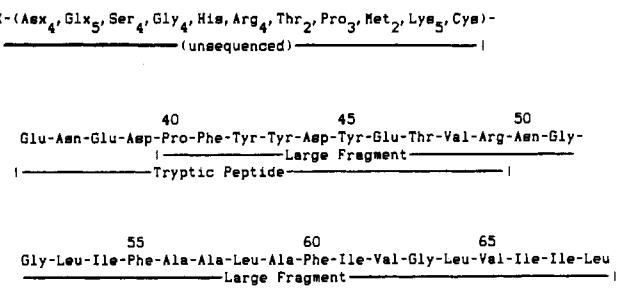

FIGURE 1: Partial sequence of the γ component of sheep kidney Na,K-ATPase.

HPLC (not shown) of the filtrate from the tryptic digest of γ yielded one large peptide peak (detected by absorbance at 220 nm). Many minor HPLC peaks, probably arising as a result of partial cleavage at the estimated five Arg and five Lys residues, were also obtained, but these were not characterized. The yield of the major tryptic peptide was only ~0.07 mol/mol, indicating that γ was cleaved by trypsin to only a minor extent. Amino acid analysis and sequencing of the major tryptic peptide showed it to contain 14 amino acid residues, with the sequence shown in Figure 1. This peptide apparently contains all three Tyr residues of γ, accounting for its relatively high absorbance on HPLC. The tryptic peptide obviously overlaps the large fragment described above, confirming the existence of the Asp–Pro bond and extending our sequence by four residues.

Sequencer analysis of the retentate of the tryptic digest gave complex results, but we were able to detect the simultaneous appearance of two major sequences: one was that of the 14-residue soluble tryptic peptide already described, and the other was the expected 19-residue carboxyl-terminal sequence beginning with Asn and ending with Leu (see Figure 1). Several other cleavage methods were used, without success, in attempts to extend our sequence determination of the amino-terminal portion of γ. These included digestion with cyanogen bromide, pepsin, and *Staphylococcus aureus* V8 protease.

γ is a small protein that one might expect could be easily sequenced by standard protein chemistry techniques. Indeed, if its amino terminus were not blocked, it is conceivable that the entire sequence could be obtained directly from a single sequencer run. Since this is not the case, we tried to cleave γ into smaller peptides whose sequences could be used to assemble the sequence of the whole protein. Numerous previous attempts (Collins and Zot, unpublished) to obtain sequence information by this strategy have been unsuccessful, due to the protein's insolubility and resistance to fragmentation. Although the results of the present study have been somewhat encouraging, it is clear that completion of the sequence of γ will be more easily achieved by the techniques of cDNA cloning and sequencing.

The amino acid composition of CM-γ was essentially identical with that which we determined previously (Collins et al., 1982). By assuming that we had accounted for all of the Tyr, Phe, Val, Ile, and Leu in the sequenced portion, we estimated a minimum polypeptide length of 68 amino acid residues for γ, with a calculated M_r of 7675. This compares favorably with a maximum value of 9500 estimated by SDS–urea–polyacrylamide gel electrophoresis (Forbush, 1983). Figure 1 shows a proposed partial sequence of γ in which the unsequenced portion is presumed to be the amino-terminal 35 residues of a 68-residue protein. The composition of the unsequenced portion was obtained by subtracting the sequenced residues from the composition of whole CM-γ.

A search of available sequence databases (obtained through Bionet) revealed no known proteins that are identical or homologous with γ. We noted previously (Collins et al., 1982) that the amino acid compositions of γ and the α subunit of Na,K-ATPase are similar. This raised the possibility that γ may actually be a mixture of proteolytic breakdown products of the α (and perhaps also the β) subunit. In support of this idea, it was found that antibodies raised to γ cross-reacted with both the α and β subunits (Ball et al., 1983). However, since we now find that the sequence of γ is different from those of the α and β subunits of sheep kidney Na,K-ATPase (Shull et al., 1985, 1986a), it is obvious that γ cannot be a breakdown product of the larger subunits.

Our results establish that γ is an amphiphilic protein: an examination of the sequence (Figure 1) clearly reveals two structural domains. The amino-terminal portion of γ (residues 1–49) is very hydrophilic, with many charged amino acid side chains. Even though residues 1–35 have not been sequenced, the amino acid content of this region shows that it is too hydrophilic to form a membrane-spanning segment. Since γ can be labeled with photoactive ouabain derivatives [see review by Forbush (1983)], the amino-terminal domain must be located on the extracellular side of the membrane. The carboxyl-terminal domain (residues 50–68), on the other hand, is undoubtedly membrane embedded: it is completely uncharged and includes 15 residues (Ala, Val, Ile, Leu, Phe) that possess hydrocarbon side chains. The picture of γ that emerges, then, is of a small protein whose amino terminus is exposed on the extracellular surface and whose carboxyl terminus spans the cell membrane once.

γ can be completely extracted from intact Na,K-ATPase with chloroform–methanol (Forbush et al., 1978), as would be expected for a classical proteolipid. On the other hand, delipidated γ dissolves poorly in chloroform–methanol, calling into question its proteolipid character (Collins et al., 1982). The sequence of γ reveals that it is not a true proteolipid, although it is conceivable that γ could interact with membrane lipids in some way to form reverse micelles (with the hydrophobic portion exposed to solvent) that would be soluble in chloroform–methanol.

It is of particular interest to try to identify an extracellular region in γ which may be involved in forming a receptor site for ouabain and other cardiac glycosides. The sequence Glu-Asn-Glu-Asp-Pro-Phe-Tyr-Tyr-Asp-Tyr-Glu (residues 36–46 in Figure 1) would seem to be an attractive candidate, since it includes a concentrated stretch of four aromatic Phe and Tyr residues which could form hydrophobic interactions with the glycosides. Furthermore, since the aromatic residues are interspersed with five acidic Asp and Glu residues, this segment probably would be exposed at the extracellular surface, rather than being embedded in the membrane.

During the course of this work, we found it instructive to compare γ with phospholamban (PLB), a protein whose phosphorylation stimulates Ca^{2+} transport and Ca^{2+}-ATPase activity in cardiac sarcoplasmic reticulum [for a review, see Tada and Inui (1983)]. PLB is also a small (52 amino acid residues) membrane-bound protein, and its sequence has recently been determined (Simmerman et al., 1986; Fujii et al., 1986, 1987; Collins et al., 1987). Although the sequences of γ and PLB are not homologous, they are similar in several respects. Each protein has a hydrophilic amino-terminal domain and a hydrophobic carboxyl-terminal domain. Because of its insolubility and tendency to aggregate, PLB, like γ, is a difficult protein to work with. PLB also has a blocked amino terminus (Fujii et al., 1986) but nevertheless yielded some

sequence information due to unexpected peptide bond cleavage. We obtained a low-yield sequence from whole PLB because limited cleavage had removed residues 1–9 from a small fraction of the total protein preparation, exposing Ser-10 as a new amino-terminal residue (Simmerman et al., 1986; Collins et al., 1987). Most of the PLB remained intact (and unavailable for sequencing), as judged by the isolation of peptides corresponding to residues 1–9 from tryptic digests of PLB (Collins, unpublished observations). In sequencing the carboxyl-terminal region of phospholamban, both we (Simmerman et al., 1986) and another group (Fujii et al., 1986) found that sequencer analysis stopped prematurely, and a hydrophobic carboxyl-terminal heptapeptide sequence was not detected. Because of this experience, we are aware of the possibility that part of the unsequenced region of γ may be at its carboxyl terminus.

Supplementary Material Available

Tables I–III containing amino acid compositions and results of sequencer analyses (2 pages). Ordering information is given on any current masthead page.

Registry No. ATPase, 9000-83-3.

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