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VAT-1 from *Torpedo* is a membranous homologue of zeta crystallin

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VAT-1 is a major protein from *Torpedo* synaptic vesicles. A protein data-base search revealed a striking homology to ζ crystallin from guinea pig lens. The overall amino-acid identity is 27%, and 58% similarity is reached by including conserved substitutions. The highest similarity (60% to 85%) between the two proteins is observed in five discrete domains, which are also conserved in zinc-dependent dehydrogenases, particularly in the alcohol dehydrogenase family. The cofactor-binding domain of oxidoreductases is conserved in VAT-1 and in ζ crystallin. VAT-1 preferably binds NADPH in the presence of zinc. In contrast with its homologous proteins, VAT-1 is an integral membrane protein of synaptic vesicles.

Electric organ; Lens crystallin; Oxidoreductase; Protein evolution; Synaptic vesicle

1. INTRODUCTION

VAT-1 is a protein which was identified and cloned from an electric lobe cDNA library from *Torpedo californica*. It is a major protein of synaptic vesicles from the electric organ. A direct microsequencing of a 41-kDa protein band from this preparation confirmed its localization to the vesicular membrane [1]. As reported, the highest homology observed was with members of the alcohol dehydrogenase family [1]. Search over a more recent version of the databank raised an evolutionary relationship between VAT-1 and a taxon-type crystallin.

Taxon-specific crystallins are major structural proteins in the lens of phylogenetically restricted groups. All taxon-specific crystallins analyzed to date (about 15) exhibit a significant homology to various enzymes. However, the enzymes in this group share no common substrate specificity or catalytic activity [2]. Moreover, while some crystallins fully retain the enzymatic activity of their respective homologues, for others the activity is virtually undetected [3]. A major component of the water-soluble proteins in the guinea pig (*Cavia porcellus*) lens, is a 38-kDa protein, named ζ crystallin [4]. The protein sequence revealed a significant homology to the alcohol/sorbitol dehydrogenases [5]. The surprising similarity of taxon-type crystallins to their related enzymes raises intriguing evolutionary and functional speculations [2,3,6–8].

In this report we present the highly homologous relation between a synaptic-vesicle *Torpedo* protein and ζ crystallin. This homology extends to the zinc-dependent dehydrogenase gene family. Despite the overall similarity between VAT-1 and its homologous proteins, it is an integral membrane protein of the vesicular membrane, while its known homologues are cytosolic. Possible implications of the similarity between VAT-1 and its homologues are discussed.

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ADH, alcohol dehydrogenase; FAS, fatty acid synthase; aa, amino acid

2. MATERIALS AND METHODS

2.1. *Sequence analysis and data-base search*

The sequence was determined by the standard method of dideoxynucleotide-chain termination using Sequenase II (USB) [9]. An additional C at position 1078 changed the reading frame for the last 24 aa (correction to the published sequence [1]). The database was searched by FASTA and PROFILE programs (GCG, Wisconsin DNA Analysis Package [10]). Reference data banks are Swissprot, release 22, and Genbank, release 72.

2.2. *Expression of VAT-1 fusion protein in E coli*

The VAT-1 clone from nucleotide 34 to 1195 [1] encoding a full-length protein, was subcloned in gluthatione *S*-transferase fusion vector (pGEX-KG) [11], and introduced to *E. coli* (LonA$^-$). Cells were induced for 2–3 h and harvested by centrifugation (6000 × *g*, 10 min). Cell lysis and fractionation into soluble and insoluble fractions were performed as described [11] and analyzed by 10% SDS-PAGE [12]. Almost all of VAT-1 fusion protein (90%), was recovered with the insoluble fraction, and further purified on gluthatione-agarose beads (Sigma). Cleavage by thrombin (Sigma) was performed as in [11], except that cleavage conditions were slightly modified (3 μg/ml, 1 h, 25°C). The purity and the yield of VAT-1 were determined by protein staining and Western analysis.

2.3. *Blue-Sepharose column chromatography*

40 μg of VAT-1 protein (0.8 mg/ml, 90% purity) were loaded onto a 0.5 ml Blue-Sepharose CL-6B column (Pharmacia). Equilibration

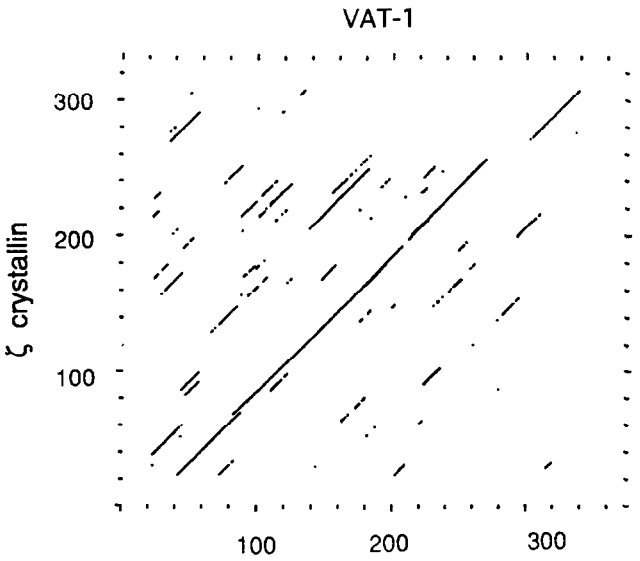

Fig. 1. Comparison of the amino acid sequence of VAT-1 [1] with the sequence of ζ crystallin [5] using a diagonal plot. DOTPLOT program (GCG, [10]). Window and stringency are set for 21 and 14 residues, respectively. Numbers in X and Y axes stand for aa position for VAT-1 and ζ crystallin, respectively.

was done in buffer A (20 mM imidazole, pH 7.0, 0.2 M KCl, 1 mM DTT, 0.1 mM EDTA and 0.25% Tween-20). The column was washed (3 vol. of buffer A) and eluted by stepwise increments of 0.2 M KCl (0.2 M to 1.2 M) either with or without $MgCl_2$ (2 mM), or $ZnCl_2$ (0.2, 2 mM). Each fraction was divided (1/3 and 2/3) and analyzed by slot blot. The nitrocellulose was stained with 0.1% Ponceau-S (Sigma) and photographed for densitometry. A known amount of pure VAT-1 was blotted directly (in 2-fold dilution series) as an internal standard.

2.4. *Extraction of VAT-1 from synaptic vesicles*

A synaptic vesicle preparation (70 μg/ml) from *Torpedo oscellata* was prepared as described [1]. The vesicles (10 μg) were subjected to various treatments (30 min, 4°C). Soluble and membranous fractions were separated by TLA.100 Centrifuge (380,000 × *g*, 15 min, Beckman), and analyzed by 10% SDS-PAGE. The gel was blotted onto nicrocellulose for Western analysis.

2.5. *Miscellaneous methods*

Protein assays were performed according to the method of Bradford [13]. Western blot analysis and antibody specifications, were as described [1].

3. RESULTS

VAT-1 is specifically expressed in the electric lobe of the marine-ray fish, while the protein accumulates in nerve terminals of the electric organ, and mostly co-purifies with synaptic vesicles.

By comparing the aa sequence of VAT-1 with the available protein data bank, a striking homology to the ζ-crystallin gene from guinea-pig lens is obtained. The similarity between VAT-1 and ζ crystallin using FASTA and PROFILE search is at the level of 70–90 S.D.s above the mean. DOTPLOT analysis of the two sequences is shown in Fig. 1. An almost perfect diagonal line reflects the homology which extends over their entire length. No internal repeats exist in VAT-1 or in ζ crystallin. *Torpedo* VAT-1 contains 371 aa while ζ crystallin's length is only 328 aa [5]. The best alignment is achieved by inserting 4 small gaps (3 to 9 aa each) at the carboxy-terminal domain. The two sequences are 27% identical and 58% similar (according to Dayhoff substitution matrix [14]).

ζ crystallin was reported as a taxon-specific crystallin resembling ADH of mouse and yeast [5]. Five domains of high similarity are evident when comparing VAT-1 with ζ crystallin. The similarity in those domains extends to the ADH and FAS families as shown in Fig. 2. Only identical aa between VAT-1 and each of the other representative proteins are highlighted. Moreover, the similarity reaches a level of 69–93% in all domains for VAT-1 and ζ crystallin and stays as high as 45%–78% for FAS and ADH (not including the second domain of FAS, which is very weak). The sequential appearance of the 5 domains along the proteins' length as well as their spacing is conserved.

The fourth domain (Fig. 2, aa 168 in VAT-1) is conserved in many oxidoreductases and contains the nicotin-amide cofactor binding domain. The extended consensus was identified as $GXG(X)_2G/A(X)_3A/G(X)_6G$. The importance of the different aa in creating an appropriate nucleotide binding fold was analyzed [15]. Purified VAT-1 obtained from expression in *E. coli* was assayed for its ability to bind nicotinic nucleotide com-

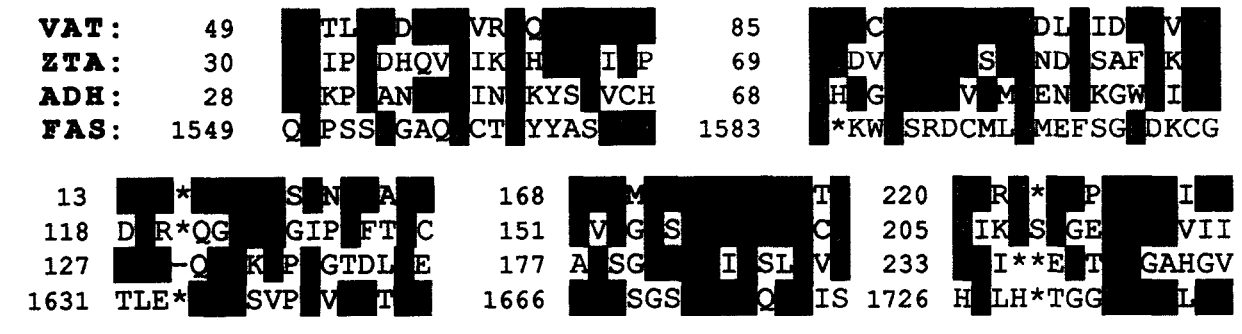

Fig. 2. Sequence alignment in discrete domains of VAT-1, ζ crystallin (P11415), ADH (P20368) and FAS (P12785). The data bank access number is in parentheses. First amino acid in each domain is marked. Only identical amino acids are framed in black. For better alignment a gap of one nucleotide (*) or an insertion (−) were included.

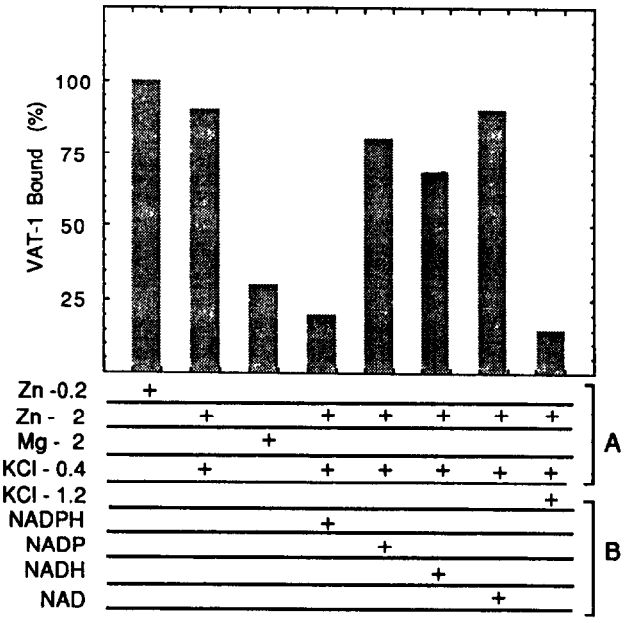

Fig. 3. Binding capacity of purified VAT-1 protein on Blue-Sepharose column. 40 μg purified VAT-1 preparation were loaded and subjected to various loading and elution conditions as in Section 2. Each histogram reflects 3 different experiments. The variations between assays were less than 10%, using the same protein preparation. The values given for Zn^{2+} and Mg^{2+} are in mM and in M for KCl. Nicotinamide compounds (10 mM) were added to the appropriate elution buffer. A and B represent the loading and elution condition, respectively.

pounds. The results are summarized in Fig. 3. Two main observations could be drawn from the quantitative analysis of the binding and elution profile of VAT-1. The binding is strongly dependent on addition of divalent ions. Zn^{2+} is most efficient while Mg^{2+} only poorly supports the binding. Addition of 10 mM of the oxidized forms of NAD and NADP changes the elution profile only slightly. In contrast, an addition of 10 mM NADPH (and to a lesser extent, NADH), was sufficient to reduce the salt concentration needed for eluting VAT-1 from the column, from 1.2 M to 0.4 M.

The series of experiments discussed above emphasizes the similarity of biochemical properties between VAT-1 and its homologous enzymes. Yet, in contrast to ζ crystallin and the dehydrogenases which are water soluble proteins, VAT-1 was purified from an enriched fraction of pure synaptic vesicles. Although no classical transmembrane domain is evident in the sequence, the membranous characteristics of VAT-1 are indicated by the following observations: a major part of the protein is protected from cleavage by various proteases only in its native state, within the synaptic vesicle membrane [1]. Moreover, following treatment with high salt (1.0 M NaCl) or hypotonic shock of the synaptic vesicles, no change in VAT-1 partition to the soluble fraction was observed. Only extreme conditions such as high pH (11.2) and the presence of detergents (0.8% Triton X-100), were efficient in releasing VAT-1 from the membrane (Fig. 4). Consequently, VAT-1 is an integral protein of the synaptic vesicle membrane.

4. DISCUSSION

A search for VAT-1 homologues in the protein data bank reveals a high score for the proteins discussed above. The significance of the score is above any statistical doubt (even comparison of VAT-1 with more distant homologues within the ADH family exhibits a level of 15–25 S.D. above the mean). Proteins which share common motifs but differ in their function and localization are believed to result from divergent evolution [16]. The high homology between VAT-1 and ζ crystallin and their differences in other aspects (e.g. tissue specificity, cellular localization), lead to our search for a biological significance. We defined short domains of high similarity and searched the data base for each domain independently. Only proteins belonging to the protein families we have discussed, display high levels of homology in the independent domains. Moreover, the position of the domains with respect to one another is conserved. An automated search program was recently developed using a similar approach [17].

The different cellular localization of ζ crystallin, VAT-1, and their related enzymes suggests a divergent evolution. These proteins diverged in such a way as to accommodate different targeting properties, while preserving other major biochemical properties (e.g. protein length, nucleotide binding domain).

The pyridine nucleotide level in guinea-pig lens is extremely high and is directly correlated to the ζ crystallin concentration [18]. It has been suggested that crystallin/ enzyme proteins in other lenses play a similar role [7]. That VAT-1 remains a very close homologue to ζ crystallin despite over 400 millions years of evolution is surprising. It raises the possibility that VAT-1 plays a non-classical role in the electroplaque of *Torpedo*. The binding of nucleotides to VAT-1 could be directly re-

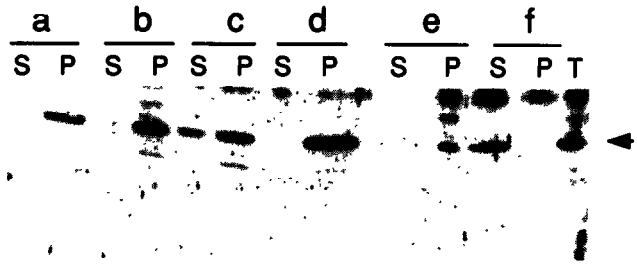

Fig. 4. VAT-1 is an integral protein of synaptic vesicle membranes. 10 μg protein of pure synaptic vesicles were treated (30 min, 4°C) before separating the soluble and the membranous fractions (as in Section 2). a, hypotonic shock; b, 100 mM acetate buffer (pH 5.0); c, 50 mM borate buffer; d, 1.0 M NaCl; e, 20 s sonication; f, 0.8% Triton X-100; t, total, untreated. S and P are soluble and membranous fractions, respectively. The partition of VAT-1 was monitored by Western blot analysis.

lated to oxidation–reduction level, or by itself, provide a nucleotide buffering mechanism. Alternatively, this binding may support an indirect effect on VAT-1 structural properties such as its thermodynamic stability. The notion that nucleotides provide a higher stability and a better compaction, and thereby could have been a selective force in crystallin evolution, was previously discussed [2]. The biological relevance of binding to nucleotides in the nerve terminal is under study.

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