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Identification and characterization of membrane-associated polypeptides in *Torpedo* nicotinic acetylcholine receptor-rich membranes by hydrophobic photolabeling

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Abstract

To identify membrane-associated polypeptides present in *Torpedo* nicotinic acetylcholine receptor (AChR)-rich membranes, we used hydrophobic photolabeling with [^{3}H]diazofluorene ([^{3}H]DAF) and 1-azidopyrene (1-AP) to tag the membrane proteins which were then identified by amino-terminal sequence analysis of labeled fragments isolated from proteolytic digests by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by reverse-phase high-performance liquid chromatography. In addition to AChR subunits, identified polypeptides include the 95 kDa α-subunit of the (Na^++K^+)-ATPase, the 89 kDa voltage-gated chloride channel (CLC-0), the 105 kDa SITS-binding protein, and 32 and 34 kDa polypeptides identified as *Torpedo* homologues of the mitochondrial membrane ATP/ADP carrier protein and the voltage-dependent anion channel (VDAC), respectively. Further, individual amino acids that reacted with [^{3}H]DAF and therefore likely to be in contact with lipid were identified in the transmembrane segment M3 of the α-subunit of the (Na^++K^+)-ATPase and in a putative transmembrane β-strand in VDAC. Collectively these results demonstrate that [^{3}H]DAF/1-AP photolabeling provides an effective method for tagging the membrane-associated segments of polypeptides in a way that makes it easy to isolate the labeled polypeptide or polypeptide fragments by fluorescence and then to identify amino acids at the lipid-protein interface by ^{3}H release.

Keywords: [^{3}H]Diazofluorene; Nicotinic acetylcholine receptor; (Na^++K^+)-ATPase; Voltage-dependent anion channel; Photoaffinity labeling; *Torpedo californica*

1. Introduction

The electric organ of the marine elasmobranch *Torpedo californica* provides an extremely rich source of nicotinic acetylcholine receptor (AChR) protein. For nearly 30 years *Torpedo* electric organ postsy-

Abbreviations: AChR, nicotinic acetylcholine receptor; VDAC, voltage-dependent anion channel; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; 1-AP, 1-azidopyrene; [^{3}H]DAF, [^{3}H]diazofluorene; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; V8 protease, *Staphylococcus aureus* V8 protease; PTH, phenylthiohydantoin; TPS, *Torpedo* physiological saline (250 mM NaCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0)

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naptic membrane preparations have been instrumental to studies of the AChR [1,2]. While these membrane preparations are highly enriched in AChR protein, even in the most highly enriched preparations AChRs comprise only about 25% of the membrane protein, with polypeptides other than the AChR subunits present either as constituents of the nicotinic postsynaptic membrane or in contaminating membrane fragments. Over the years a number of non-AChR proteins have been identified, including the 43 kDa AChR-associated protein rapsyn [3], as well as integral and peripheral membrane proteins of the dystrophin complex (dystrophin (approx. 400 kDa [4]), syntrophin (58 kDa [5,6]), dystrobrevin (87 kDa [7,8]), and α-, and β-dystroglycan (190, 50 kDa [9])) that are also enriched in the nicotinic postsynaptic membrane. In addition, a 95 kDa polypeptide has been identified as the α-subunit of the $(Na^+ + K^+)$-ATPase [10], the major polypeptide component present in membrane fragments originating from the non-innervated surface of the electrocyte. Given the importance of the *Torpedo* AChR-rich membranes as a source of components of the nicotinic postsynaptic membrane, we thought it useful to further identify other membrane proteins abundant in the preparation. To do this we used a method developed in studies of the AChR [11] that involves tagging the lipid-exposed segments of membrane polypeptides with both fluorescent and radioactive photoactivatable hydrophobic probes. By fluorescently tagging the membrane-associated segments of polypeptides, the intact protein or proteolytic fragments can be directly visualized when resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or by reverse-phase high-performance liquid chromatography (HPLC). Inclusion of the radiolabeled hydrophobic probe provides the sensitivity needed to identify individual labeled amino acids using amino-terminal sequence analysis.

The goals of the work presented here are two-fold: to identify membrane-associated polypeptides present in *T. californica* AChR-rich postsynaptic membrane preparations and to determine whether this hydrophobic labeling strategy can be used to identify amino acids at the lipid interface in proteins other than the AChR. *T. californica* AChR-rich membranes were photolabeled with 1-azidopyrene (1-AP), which is fluorescent, and with 2-[^{3}H]diazofluorene ([^{3}H]DAF), photoactivatable hydrophobic probes that have been used to label the lipid-exposed regions of a number of different membrane proteins [12,13] including the AChR [11]. Amino-terminal sequence analysis of purified [^{3}H]DAF/1-AP-labeled proteolytic fragments was used to either reveal or confirm the presence of the following polypeptides in AChR-rich membrane preparations: the α-subunit of the $(Na^+ + K^+)$-ATPase (approx. 95 kDa), *Torpedo* homologues of the mitochondrial membrane ATP/ADP carrier protein (32 kDa) and the voltage-dependent anion channel (VDAC; 34 kDa) respectively, as well as the 89 kDa voltage-gated chloride channel (CLC-0), the 105 kDa 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS)-binding protein, and the 37 kDa calelectrin protein. Further, individual amino acids that reacted with [^{3}H]DAF and therefore are believed to be in contact with lipid were identified in the transmembrane segment M3 of the α-subunit of the $(Na^+ + K^+)$-ATPase and in a putative transmembrane β-strand in VDAC.

2. Material and methods

2.1. Materials

[^{3}H]DAF of specific activities ranging from 0.67 to 1.4 Ci/mmol was prepared from [2-^{3}H]fluorenone according to the procedure described in [14] and stored at −20°C in ethanol. 1-Azidopyrene was purchased from Molecular Probes. *Staphylococcus aureus* V8 protease was purchased from ICN and Genapol C-100 (10%) from Calbiochem. Prestained low molecular weight gel standards were purchased from Life Technologies.

2.2. Photolabeling AChR-rich membranes with [^{3}H]DAF and 1-azidopyrene

Torpedo AChR-rich membranes [11] in *Torpedo* physiological saline (TPS, 250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0) were incubated at 2 mg protein/ml (12–15 mg protein) with [^{3}H]DAF at a final concentration of approx. 5 μM. After 30 min incubation, suspensions were irradiated for 5 min at a distance of less than 1 cm with a 365 nm lamp (EN-Spectroline).

Following irradiation, each sample was pelleted (15 000×*g*) and then resuspended at 2 mg protein/ml in TPS, and 1-AP was added to a final concentration of 350 μM. After 30 min incubation, membranes were once again irradiated (10 min) with a 365 nm lamp, and each sample was pelleted. Samples were then solubilized in sample loading buffer and submitted to preparative scale SDS-PAGE.

2.3. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed as described in [15] using either 1.0 mm (analytical) or 3.0 mm thick (preparative scale) 8% polyacrylamide gels with 0.33% bis-acrylamide. For preparative scale gels, polypeptides incorporating 1-AP were visualized from their associated fluorescence when the gels were illuminated at 365 nm on a UV light box. Bands containing fluorescence were excised from the gel, and [^{3}H]DAF/1-AP-labeled polypeptides were isolated by passive elution [11]. The eluate was filtered (Whatman No. 1), and the protein concentrated using a Centriprep-10 (Amicon). Excess SDS was removed by acetone precipitation (overnight at −20°C).

2.4. Purification of proteolytic digests of [^{3}H]DAF/1-AP-labeled Torpedo membrane polypeptides

For *S. aureus* V8 protease digestion, *Torpedo* membrane polypeptides were resuspended in 100 mM NH_4HCO_3, 0.1% SDS, pH 7.8 at 1–2 mg/ml protein. V8 protease was added to a total of 1:10 (w/w) enzyme to substrate and incubated at room temperature for 3–4 days. Digests were separated on a Tricine SDS-PAGE gel system [16], composed of a 10 cm long (1.5 mm thick) small pore separating gel (16.5% T (total acrylamide concentration), 6% C (bis-acrylamide cross-linker concentration)), a 2 cm spacer gel (10% T, 3% C), and a 2 cm stacking gel (4% T, 3% C). 1-AP-labeled bands were visualized from the associated fluorescence when gels were placed on a 365 nm UV light box.

[^{3}H]DAF/1-AP-labeled fragments were isolated from excised gel pieces and further purified by reverse-phase HPLC using a Brownlee Aquapore C_4 column (100×2.1 mm). Solvent A was 0.08% trifluoroacetic acid in water, and solvent B was 0.05% trifluoroacetic acid in 60% acetonitrile/40% 2-propanol. The flow rate was maintained at 0.2 ml/min and 0.5 ml fractions collected. Peptides were eluted with a non-linear gradient (Waters Model 680 gradient controller, curve No. 7) from 25% to 100% solvent B in 80 min. The elution of peptides was monitored by the absorbance at 210 nm and by fluorescence emission (357 nm excitation, 432 nm emission). The elution of [^{3}H]DAF was monitored by scintillation counting of an aliquot (25 μl) of each fraction.

2.5. Sequence analysis

Amino-terminal sequence analysis was performed on a Applied Biosystems (ABI) Model 477A protein sequencer using gas phase cycles. Pooled HPLC samples were dried by vacuum centrifugation, resuspended in a small volume of 0.05% SDS (approx. 20 μl) and immobilized on chemically modified glass fiber disks (Beckman Instruments) [11]. Approx. 30% of the released phenylthiohydantoin (PTH)-amino acids were separated by an on-line Model 120A PTH-amino acid analyzer, and approx. 60% was collected for determination of released ^{3}H by scintillation counting of each sample for three 10 min intervals.

3. Results

When AChR-rich membranes are fractionated on an 8% polyacrylamide gel and stained by Coomassie blue (Fig. 1, lane A), the most prominently stained polypeptides are the subunits of the AChR (α, β, γ, δ) and rapsyn, a 43 kDa peripheral protein that associates with the cytoplasmic aspect of the AChR, as well as the (Na^++K^+)-ATPase α-subunit (α_{NK}) that originates from contaminating membrane fragments [7,10]. As is evident in the fluorogram of the dried acrylamide gel (Fig. 1, lane B), there was prominent photoincorporation of [^{3}H]DAF into the AChR subunits, the (Na^++K^+)-ATPase α-subunit, and polypeptides of 34 and 32 kDa, but not into rapsyn. These same polypeptides were also labeled by the fluorescent hydrophobic probe 1-AP (Fig. 1, lane C), and in addition there were readily identifiable fluorescent bands of 37 kDa, 89 kDa and 105 kDa

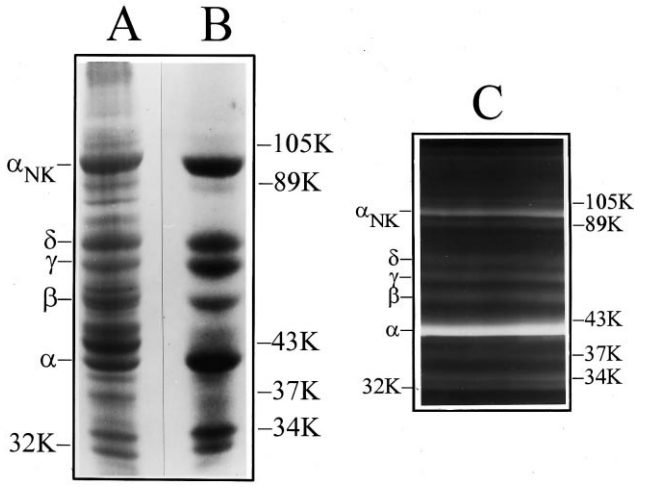


Fig. 1. Photoincorporation of [^{3}H]DAF and 1-azidopyrene into AChR-rich membranes. AChR-rich membranes were equilibrated with [^{3}H]DAF (5 μM) or 1-azidopyrene (350 μM) for 1 h and irradiated at 365 nm for 5 min. Polypeptides were resolved by SDS-PAGE, visualized by Coomassie blue stain (lane A) and processed for fluorography (4 week exposure; lane B). For 1-azidopyrene (lane C), labeled polypeptides were visualized by placing the unstained gel on a UV light box (365 nm excitation). Labeled lipid and free photolysis products were electrophoresed from the gel with the tracking dye. The AChR subunits, the (Na^++K^+)-ATPase α-subunit (α_{NK}), and bands of 105 (105K), 89 (89K), 37 (37K), 34 (34K), and 32 (32K) kDa are indicated. The AChR-associated 43 kDa protein (rapsyn) is also indicated, migrating with a slightly slower mobility than the AChR α-subunit.

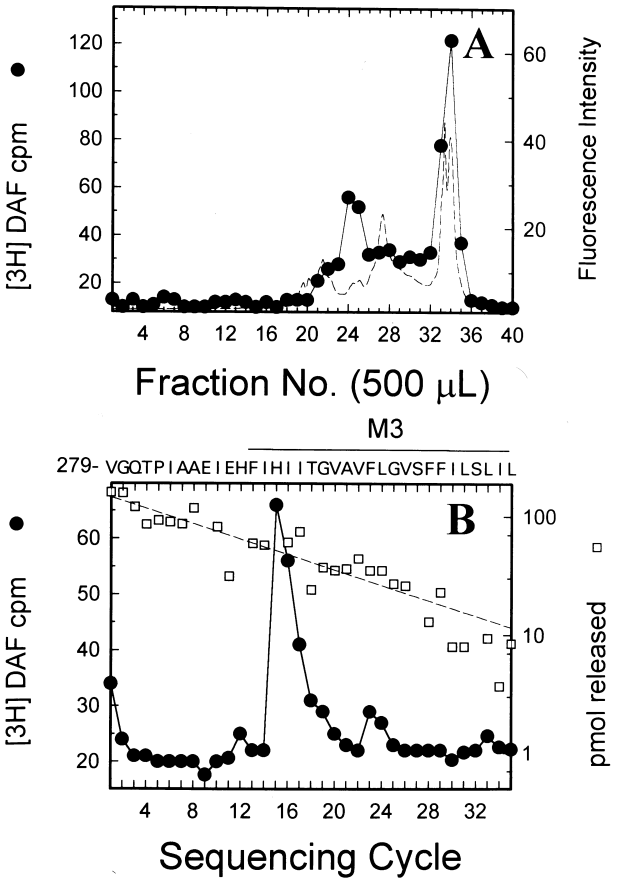


Fig. 2. Proteolytic mapping the sites of [^{3}H]DAF/1-AP photoincorporation in the *Torpedo* (Na^++K^+)-ATPase α-subunit. The [^{3}H]DAF/1-AP-labeled α-subunit of the (Na^++K^+)-ATPase polypeptide band (Fig. 1, α_{NK}) was isolated when *Torpedo* AChR-rich membranes (approx. 15 mg) were resolved on a 3.0 mm 8% polyacrylamide gel. The α_{NK} band was digested in solution with V8 protease (see Section 2) and several fluorescent bands were apparent when the digest was fractionated on a 1.5 mm Tricine SDS-PAGE gel (not shown). A fluorescent band of 8.5 kDa (V8-8.5K) was excised from the gel, and the eluted material was further purified by reverse-phase HPLC. The elution of the [^{3}H]DAF/1-AP-labeled peptide (A) was determined by monitoring the fluorescence emission intensity (dashed line; excitation, 357 nm; emission, 432 nm) and by liquid scintillation counting of aliquots (5%) of the collected fractions (●). HPLC fractions 33–35 were pooled and sequenced. A single sequence was evident (B; initial yield, 145 pmol; repetitive yield, 93%; 4140 cpm loaded, 1081 cpm remaining on the filter after 35 sequencing cycles). In each cycle of Edman degradation approx. 60% of the material was analyzed for released ^{3}H (●) and 30% for PTH-amino acids (□) with the dashed line corresponding to the exponential decay fit of the amount of detected PTH-amino acids.

that were labeled less prominently by [^{3}H]DAF. [^{3}H]DAF photoincorporation into each of the AChR subunits was extensively characterized in an earlier report [11]. Based on liquid scintillation counting of excised gel bands as well as densitometric analysis of fluorograms, photoincorporation of [^{3}H]DAF into the AChR subunits accounts for approx. 60% of the total labeling in polypeptides present in the membrane preparation. To identify the sites of incorporation of [^{3}H]DAF within the (Na^++K^+)-ATPase α-subunit and also to identify the other labeled polypeptides, the corresponding polyacrylamide gel bands were excised and the materials eluted from those bands were digested with V8 protease (10%, w/w, enzyme to substrate) for 3–4 days. The digests were then resolved on preparative scale (1.5 mm) Tricine SDS-PAGE gels (16.5% T, 6% C), and the major fluorescent (1-AP-labeled) bands were excised. Material eluted from each of the bands was then further purified by reverse-phase HPLC,

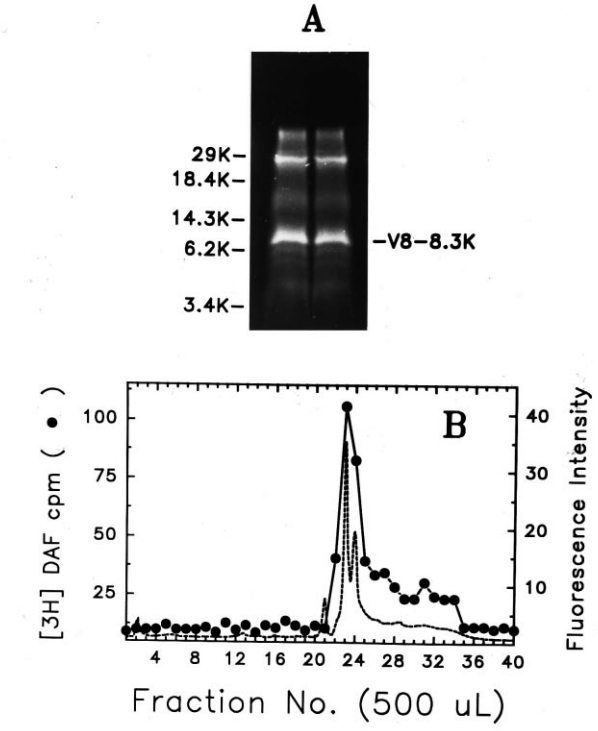
and the fractions containing peaks of fluorescence and ^3H were pooled for amino-terminal amino acid sequence analysis.

3.1. Sites of [^{3}H]DAF/1-AP photoincorporation in the α-subunit of the (Na^++K^+)-ATPase

When the V8 protease digest of the [^{3}H]DAF/1-AP-labeled α-subunit of the (Na^++K^+)-ATPase (approx. 95 kDa) was fractionated on a Tricine SDS-PAGE gel, several fluorescent bands were apparent when the gel was placed on a UV light box. A particularly intense fluorescent band migrating with an apparent molecular mass of 8.5 kDa (V8-8.5K), which corresponded to a band of ^3H on a fluorogram of an analytical Tricine gel, was excised from the gel, and the eluted material was further purified by reverse-phase HPLC. The HPLC elution profile (Fig. 2A) exhibited a peak of fluorescence and ^3H eluting at approx. 90% solvent B (fraction 34). Amino-terminal sequence analysis of the pooled fractions (33–35) revealed the presence of a single sequence beginning at Val-279 of the α-subunit of the *T. californica* (Na^++K^+)-ATPase [17] (Fig. 2B). Based on its apparent molecular weight, the fragment would include the hydrophobic segments M3 and M4 and have a predicted carboxy terminus at Glu-362. The ^3H release profile (Fig. 2B) exhibited a major peak of release in cycle 15 and a minor peak of release in cycle 23. Comparison of the pattern of release with the corresponding identified amino acids indicates that the labeled amino acids include His-293 and Phe-301, both of which are contained within the hydrophobic segment M3.

3.2. Identification of the 32 kDa polypeptide as the mitochondrial ADP/ATP carrier protein

When material from the V8 protease digest of the [^{3}H]DAF/1-AP-labeled 32 kDa band was resolved on a Tricine SDS-PAGE gel, two major fluorescent bands were evident with apparent molecular masses of 26 and 8.3 kDa (Fig. 3A). Bands of ^3H migrating with identical mass were also evident in the fluorogram of a Tricine SDS-PAGE gel (data not shown). Material contained within the 8.3 kDa fragment (V8-8.3K) was further purified by reverse-phase HPLC,



C

```
32K(V8-8.3K)-   EXQYXGIIDXVVRIPKEQGFLSFWRG     -26
                ¦ ¦¦¦¦¦¦¦ ¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦
ADT1_BOVIN   -SAEKQYXGIIDCVVRIPKEQGFLSFWRGNLA -75
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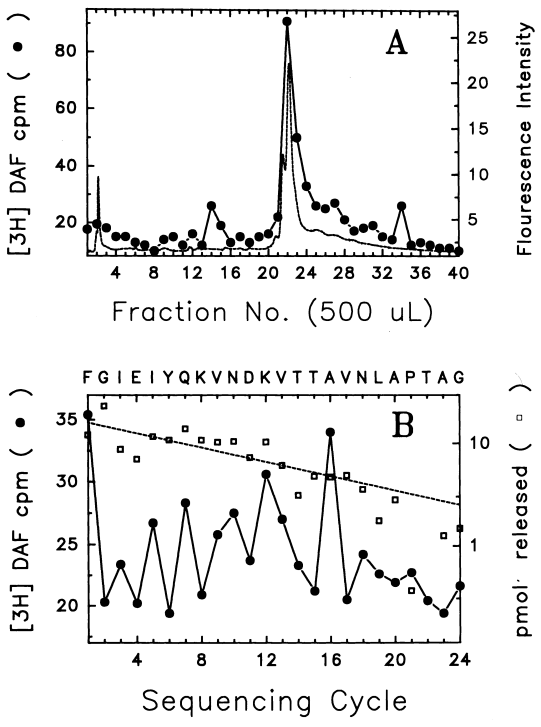
Fig. 3. Proteolytic mapping of the sites of [^{3}H]DAF/1-AP photoincorporation in the 32 kDa polypeptide present in *Torpedo* AChR-rich membranes. A [^{3}H]DAF/1-AP-labeled 32 kDa polypeptide band (Fig. 1, 32K) was isolated when *Torpedo* AChR-rich membranes (approx. 15 mg) were resolved on a 3.0 mm 8% polyacrylamide gel. The 32 kDa band was digested in solution with V8 protease and the digest fractionated on a 1.5 mm Tricine SDS-PAGE gel. 1-AP-labeled fragments were visualized by illumination at 365 nm (A), and the molecular masses were estimated from the migration of prestained molecular weight standards (Life Technologies) which are indicated on the left. One of two principal fluorescent bands, indicated on the right as V8-8.3K, was excised from the gel, and the eluted material was further purified by reverse-phase HPLC. The elution of the [^{3}H]DAF/1-AP-labeled peptide (B) was determined by monitoring the fluorescence emission intensity (- - -) and by liquid scintillation counting of aliquots (5%) of the collected fractions (●). HPLC fractions (22–23) were pooled and sequenced. A single sequence was evident (C; initial yield, 17 pmoles; repetitive yield, 92%; 2800 cpm loaded, 860 cpm remaining on the filter after 25 sequencing cycles) which in a search of the protein database was a nearly perfect match to residues 47–72 of the deduced sequence of the bovine inner mitochondrial membrane ADP/ATP carrier protein (C; ADT1_BOVIN [18]).



C

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34K(V8-7.9K)-    EFGIEIYQKVNDKVTTAVNLAPTAG     -25
                 ||| |||||| | ||||||| |||
PORI_HUMAN   -DGTEFGGSIYQKVNKKLETAVNLAWTAGNSN -215
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Fig. 4. Proteolytic mapping of the sites of [^{3}H]DAF/1-AP photoincorporation in the 34 kDa polypeptide present in *Torpedo* AChR-rich membranes. A [^{3}H]DAF/1-AP-labeled 34 kDa polypeptide band (Fig. 1, 34K) was isolated when *Torpedo* AChR-rich membranes (approx. 15 mg) were resolved on a 3.0 mm 8% polyacrylamide gel. When a V8 protease digest of the 34 kDa band was fractionated on a 1.5 mm Tricine SDS-PAGE gel, a ladder of fluorescent bands was seen that extended from approx. 5 to 8 kDa. Fluorescent bands of 5 kDa (V8-5K) and 7.9 kDa (V8-7.9K) were excised from the gel and further purified by reverse-phase HPLC. The elution of the [^{3}H]DAF/1-AP-labeled V8-7.9K fragment (A) was determined by monitoring the fluorescence emission intensity (- - -) and ^{3}H (●) as described in the legend to Fig. 2. HPLC fractions 22–23 were pooled and sequenced. A single sequence was evident (B; initial yield, 16 pmol; repetitive yield, 92.2%; 3400 cpm loaded, 760 cpm remaining on the filter after 24 sequencing cycles). In each cycle of Edman degradation approx. 60% of the material was analyzed for released ^{3}H (●) and 30% for PTH-amino acids (□), with the dashed line corresponding to the exponential decay fit of the amount of detected PTH-amino acids. (C) Alignment of the observed amino-terminal sequence of the fragment of the *Torpedo* 34 kDa polypeptide with an internal sequence of human B lymphocyte porin [20].

resulting in peaks of fluorescence and of ^{3}H eluting at approx. 51% solvent B (fraction 23, Fig. 3B). Sequence analysis of the pooled fractions (22–23) revealed the presence of a primary sequence (Fig. 3C) which in a search of the protein database was nearly a perfect match to residues 47–72 of the deduced sequence of the bovine inner mitochondrial membrane ADP/ATP carrier protein [18]. No significant release of ^{3}H above background (approx. 18 cpm) was detected in any of the sequencing cycles.

3.3. Identification of the 34 kDa polypeptide as a mitochondrial VDAC

When the V8 protease digest of the [^{3}H]DAF/1-AP-labeled 34 kDa band was resolved on a Tricine SDS-PAGE gel, a ladder of fluorescent bands (and corresponding bands of ^{3}H in a fluorogram of the gel) was evident with apparent molecular masses extending from approx. 5 to 8 kDa (data not shown). Material contained within a 7.9 and a 5 kDa fragment were further purified by reverse-phase HPLC. The HPLC profile of the 7.9 kDa fragment (V8-7.9K) exhibited a sharp peak of fluorescence and of ^{3}H eluting at approx. 49% solvent B (fraction 22, Fig. 4A). When fractions 22 and 23 were combined and sequenced, a single sequence was evident (Fig. 4B, upper legend and Fig. 5). A search of the protein database revealed a significant degree of sequence homology with several mammalian members of the outer mitochondrial membrane VDAC family of proteins, also known as 'mitochondrial porin' (Fig. 5) [19–24]. The ^{3}H release profile during sequence analysis of V8-7.9K (Fig. 4B) was low level and complex, with a pattern of alternating cycles of ^{3}H release in the first nine cycles, followed by release in cycles 10 and 12, and the largest ^{3}H release in cycle 16. Comparison of the pattern of ^{3}H release with the corresponding identified amino acids indicates that the labeled amino acids within the V8-7.9K peptide could include Phe-1, Ile-3, Ile-5, Gln-7, Val-9, Asn-10, Lys-12, and Ala-16. Sequence analysis of V8-5K (HPLC fractions 20–23) revealed a single sequence (initial yield, 35 pmol; repetitive yield, 87.9%) which when aligned with the different VDAC sequences (Fig. 5) includes a three amino acid stretch (G-L-K) which is completely conserved.

V8-7.9K

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T. cal.*    E-FGIEIYQKVNDKVTTAVNLAPTA--G   25
Bovine      E-FGGSIYQKVNKKLETAVNLAWTA--G   212
Human       E-FGGSIYQKVNKKLETAVNLAWTA--G   212
Rat         E-FGGSIYQKVCEDFDTSVNLAWTS--G   225
            * **  *****     * **** *   *
N.crassa    SVFSASYYHKVNSQVEAGSKATWNSK-T   213
Yeast       QITTVDFFQNVNAFLQVGAKATMNCKLP   211
DICDI       QILLSTLY-TATSKLSFAGDVTVDLKAS   206
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V8-5K

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T. cal.*    E-DQLKDGLKLFFDITFAPNTTXKK      24
Bovine      E-DQLARGLKLTFDSSFSPNTGRKN      110
Human       E-DQLARGLKLTFDSSFSPNTGKKN      110
Rat         E-DQICQGLKLTFDTTFSPNTGKKS      123
            * **   **** **  * ***  *
N. crassa   A-DNLAKGLKAEGIFSFLPATNARG      110
Yeast       A-N-LTPGLKNELITSLTPGV-AKS      109
DICDI       TIENIIPGLK-----AVANGDSKQN      103
                  ◆◆◆
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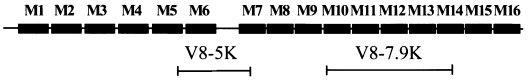

Fig. 5. Alignments of amino acid sequences of the VDAC family and proteolytic fragments of a 34 kDa polypeptide present in *Torpedo* AChR-rich membranes. For the *Torpedo* 34 kDa polypeptide, amino terminal sequences (T. cal.*) were obtained for the fragments of 7.9 kDa (Fig. 4, V8-7.9K) and 5 kDa (V8-5K) isolated from V8 protease digests. The VDAC ('porin') sequences, deduced from the nucleotide sequences, are from the following organisms: *Bos taurus* (Bovine plasmalemmal porin, data submission to NCBI by R. Buettner [19]); *Homo sapiens* (Human B lymphocyte HL 31 [20]); *Rattus norvegicus* (Rat hippocampal porin RB 36 [21]); *N. crassa* (*N. crassa* porin [22]); *Saccharomyces cerevisiae* (Yeast porin [23]); *Dictyostelium discoideum* (DICDI porin [24]). Residue numbers shown are those of each VDAC sequence or in the case of V8-7.9K and V8-5K, the corresponding cycle of Edman degradation. Gaps have been inserted to maximize homology, and the first amino acid residue (Glu) in the sequences of V8-7.9K and V8-5K was inferred from the known cleavage specificity of V8 protease. An asterisk (*) indicates sequence conservation amongst the mammalian VDAC sequences, and diamonds (◆) have been used to denote the three amino acids (G-L-K) which are completely conserved across all species. Indicated at the bottom is a schematic of the primary structure of human VDAC, with the approximate location of the 16 predicted membrane spanning segments indicated as well as the amino acid segments contained within the two sequenced proteolytic fragments of *Torpedo* VDAC (V8-5K and V8-7.9K). The carboxy terminus of each fragment was predicted based on the apparent molecular weight of each fragment and the known substrate specificity of *S. aureus* V8 protease (carboxy-terminal side of glutamic or aspartic acid).

3.4. Identification of the 105 kDa, 89 kDa, and 37 kDa polypeptides

Several *Torpedo* polypeptide bands which incorporated lower amounts of [^{3}H]DAF or 1-AP (Fig. 1) were also identified by use of this same 'internal sequencing' strategy. The 89 kDa band was identified as the *T. californica* voltage-gated chloride channel, CLC-0 [25,26] (Fig. 6). Digestion of the 89 kDa band with V8 protease generated a fluorescent and ^{3}H-

37K

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V8-4K                    EXVILDLLAQRSNAQXQQFIXAYKT  25
V8-4K          EALHAAMKGFGTDEXVILDLLAQR             25
               ¦ ¦  ¦¦¦¦ ¦¦¦¦  ¦¦ ¦¦  ¦¦¦¦¦ ¦¦     ¦¦
ANX5_RAT       EVLRKAMKGLGTDEDSILNLLTARSNAQRQQIAEEFKT  57

TC4                  GFGTDEDVILDLLTQR
                     ¦¦¦¦¦¦ ¦¦¦¦¦¦ ¦¦
V8-4K                    EXVILDLLAQRSNAQXQQFIXAYKT  25
V8-4K          EALHAAMKGFGTDEXVILDLLAQR             25
                     ¦ ¦¦ ¦ ¦    ¦¦ ¦
TC3                  GAGTSENVLIEILASR
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89K

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V8-7.3K          EVSRWRAVKNXLYRHLVKVLGEDWIF         26
                 ¦¦¦¦¦¦¦¦¦¦ ¦¦¦¦¦¦¦¦¦¦¦¦¦
CLCH_T.cal.  VKTEVSRWRAVKNCLYRHLVKVLGEDWIFLL       56
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105K

```
105K             ARRAKKMASNSGDSSPEPGIKEINET         26
                 ¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦¦
SP105_T.cal.     ARRAKKMASNSGDSSPEPGIKEINETWKG      29
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Fig. 6. Alignments of amino acid sequences of *Torpedo* calelectrin, the voltage-gated chloride channel (CLC-0), and the 105 kDa SITS-binding protein with proteolytic fragments purified from the 37, 89, and 105 kDa polypeptides present in *Torpedo* AChR-rich membranes. 37K: for the 37 kDa polypeptide (Fig. 1, 37K), a primary and secondary sequence was revealed by sequence analysis of a 4 kDa fragment (V8-4K) isolated from a V8 protease digest. These two sequences were aligned with the sequences of two tryptic fragments (TC4 and TC3) of *Torpedo marmorata* calelectrin [28] as well as with that of the rat Annexin V sequence (ANX5_RAT) which was deduced from the nucleotide sequence [39]. 89K: for the 89 kDa *Torpedo* membrane polypeptide (Fig. 1, 89K), sequence analysis of a 7.3 kDa fragment (V8-7.3K) isolated from a V8 protease digest revealed the presence of a single sequence (initial yield, 30 pmol; repetitive yield, 88.4%). This sequence was aligned with the deduced sequence of the *T. californica* voltage-gated chloride channel [25], which is a member of the CLC family of channels (CLC-0 [40]). The first residue of the sequence (Glu), inferred from the known cleavage specificity of V8 protease, aligns with Glu-29 of the intact polypeptide. Based on its apparent molecular weight, the fragment would extend through the first hydrophobic segment (D1, Trp-52 to Ile-72) and probably terminate at Glu-88. 105K: sequence analysis of the amino terminus of a 105 kDa *Torpedo* membrane protein (Fig. 1, migrating with slightly slower mobility than α_{NK}) revealed a perfect match to the deduced amino terminus of the *T. californica* SITS-binding protein (SP105_T. cal. [27]).

containing 7.3 kDa fragment that was purified by reverse-phase HPLC. When that purified fragment was sequenced for 25 cycles, there was an exact match to the deduced sequence of *Torpedo* CLC-0 beginning at Val-30. Based upon the apparent molecular mass of the fragment, it is predicted to extend to Glu-88 and thus to contain the first predicted hydrophobic segment (D1, Trp-55 to Ile-72). No ^3H release above background was detected in the 25 cycles of sequencing, which extended to Phe-75 near the beginning of the D1 hydrophobic segment. Finally, [^{3}H]DAF/1-AP-labeled polypeptides of apparent molecular masses of 105 and 37 kDa (Fig. 1) were identified respectively as the 105 kDa SITS-binding protein [27] and calelectrin, a member of the annexin family of Ca^{2+}-binding proteins [28] (Fig. 6).

4. Discussion

The hydrophobic photoactivatable probes [^{3}H]DAF and 1-AP photoincorporate into a number of different polypeptides present in *T. californica* AChR-rich membrane preparations (Fig. 1). Aside from the AChR subunits, the major site of [^{3}H]DAF/1-AP photoincorporation was an approx. 95 kDa band previously identified as the α-subunit of the (Na^++K^+)-ATPase [10]. There was also a considerable amount of [^{3}H]DAF and 1-AP photoincorporation into two polypeptides with apparent molecular masses of 32 and 34 kDa which we have now identified as *Torpedo* homologues of the ATP/ADP carrier protein (Fig. 3) and the VDAC (Fig. 5), respectively. These latter two polypeptides are present in the inner and outer mitochondrial membranes, respectively, and their presence presumably indicates that mitochondrial membrane fragments are significant contaminants of the AChR-rich membrane preparation. Along with the α-subunit of the (Na^++K^+)-ATPase, the 89 kDa voltage-gated chloride channel (CLC-0) and the 105 kDa SITS-binding protein are located on the non-innervated electrocyte surface [25,27]. Thus in the AChR-rich membranes at this stage of purity, other than the subunits of the AChR the predominant membrane proteins originate from contaminating membranes from mitochondria and from the non-innervated surface of the electrocyte. Clearly a higher degree of membrane purification will be required to identify integral membrane proteins of the nicotinic postsynaptic membrane such as β-dystroglycan [29] or the agrin receptor, the receptor tyrosine kinase MuSK [30] that are present at much lower abundance than the AChR. It has been shown previously [7,31] that the AChR-rich membranes used in this study, which were purified by centrifugation on discontinuous and continuous sucrose gradients, can be further purified by velocity sedimentation on shallow sucrose gradients to reduce contamination from (Na^++K^+)-ATPase-rich membranes. Alternatively, it may be necessary to use immunoisolation procedures to obtain the purification necessary to characterize membrane proteins of the nicotinic postsynaptic membrane that may be present at only several percent the level of AChR protein.

An initial characterization of the sites of [^{3}H]DAF labeling into each of these polypeptides was performed. In the (Na^++K^+)-ATPase α-subunit, [^{3}H]DAF photoincorporation into His-293 and Phe-301 within the hydrophobic segment M3 was determined by amino-terminal sequence analysis of an 8.5 kDa fragment produced by V8 protease. Photoincorporation into these residues supports the transmembrane nature of the M3 region [32], and, if modeled as an α-helix, the labeled side chains help define the face of the helix in contact with the lipid bilayer (i.e. His-293, Gly-297, Phe-301, Val-304, Ile-308, Leu-311, and Tyr-315). Since [^{3}H]DAF is known to photoincorporate into aliphatic side chains [11], it is likely that the limited number of labeled residues in the M3 segment reflects a preferred orientation of the DAF molecule in the lipid bilayer, rather than an intrinsically greater reactivity of the two labeled amino acids.

No amino acids labeled by [^{3}H]DAF were detected during 25 cycles of Edman degradation of a ^3H-labeled, 8.3 kDa fragment of the 32 kDa ATP/ADP carrier protein (sequence shown in Fig. 3C). However, the 25 cycles of amino-terminal sequencing did not extend into beginning of the hydrophobic, predicted transmembrane segments contained in this fragment [33].

A *Torpedo* homologue of the voltage-dependent anion channel protein (VDAC) was identified by amino-terminal sequence analysis of two V8 protease fragments of a 34 kDa polypeptide present in AChR-rich membranes (Fig. 5). VDAC is a unique integral

membrane protein in that there are no stretches of hydrophobic amino acids of sufficient length to be compatible with membrane spanning α-helices. However, topological models of VDAC have been developed based on the presence of stretches of alternating hydrophobic and hydrophilic residues, which could form β-sheets with hydrophobic residues on one side facing the lipid bilayer and hydrophilic residues on the other forming the lining of the aqueous channel [21,34–36]. By analogy with bacterial porin [37] the VDAC channel, which is also referred to as porin, would have a β-barrel structure comprised of between 12 and 19 β-strands depending on the particular topological model. In the model of human VDAC [36] the majority of the polypeptide is embedded in the lipid bilayer for a total of 16 β-strands (Fig. 5). It is in this context that the complex ^{3}H release profile seen when sequencing the 7.9 kDa V8 protease fragment of *Torpedo* VDAC has important secondary structure implications. While the amount of ^{3}H release is admittedly very low, the pattern of alternating release in the first nine cycles is highly suggestive of a β-sheet type secondary structure. In this respect it is noteworthy that the labeled amino acids (Phe-1, Ile-3, Ile-5, Gln-7, and Val-9) are considerably more hydrophobic than the unlabeled residues ('Glu-0', Gly-2, Glu-4, Tyr-6, and Lys-8), a result expected if the hydrophobic side chains were on the side of a β-strand facing the lipid and the unlabeled, hydrophilic residues were on the other side facing the lumen of the aqueous channel.

In the model of human VDAC [36] the residues homologous to those present in sequencing cycles 0–9 are predicted to be in a β-sheet conformation which spans the length of the bilayer, and the residues homologous to those in cycles 10–13 are in a short loop structure with subsequent residues once again spanning the bilayer in a β-sheet conformation. Consistent with the presence of a change in secondary structure such as the predicted loop structure in the model of human VDAC, the ^{3}H release profile after cycle 9 is considerably more complicated with release in cycles 10, 12, and 16 (Asn, Lys, and Ala respectively). In contrast, the topological model for VDAC from *Neurospora crassa* [38] places this entire segment outside of the lipid bilayer. While further work is certainly warranted, the periodicity of [^{3}H]DAF-labeled residues would appear to be the first direct evidence of a lipid-exposed β-sheet segment within the VDAC polypeptide.

In conclusion, photolabeling of membrane preparations with [^{3}H]DAF/1-AP provides a convenient and effective method for tagging the membrane-associated segments of polypeptides. Further, photolabeling with [^{3}H]DAF/1-AP makes it easy to isolate the labeled polypeptide or polypeptide fragments by 1-AP fluorescence and then to identify amino acids at the lipid-protein interface by ^{3}H release.

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