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LABELING OF INTRAMEMBRANE SEGMENTS OF THE α-SUBUNIT AND β-SUBUNIT OF PURE MEMBRANE-BOUND ($Na^+ + K^+$)-ATPase WITH 3-TRIFLUOROMETHYL-3-(*m*-[^{125}I]IODOPHENYL)DIAZIRINE

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The photoactivatable carbene precursor 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) was tested as a probe for labeling lipid-embedded segments of the proteins of pure membrane bound ($Na^+ + K^+$)-ATPase. The probe labeled the α-subunit (100 kDa), its major tryptic and chymotryptic fragments of 78 kDa, 58 kDa, and 46 kDa, and the β-subunit (38 kDa) from within the lipid bilayer to nearly the same specific activity. The labeling was resistant to extensive proteolysis and the distribution of label among the proteolytic fragments and the two subunits was independent of a 47-fold variation in concentration of [^{125}I]TID. The data show that several transmembrane segments are distributed along the sequence of the α-subunit and that also the β-subunit traverses the bilayer. [^{125}I]TID provides a more uniform labeling of the transmembrane segments of the α-subunit and β-subunit than that obtained with other hydrophobic reagents. This will facilitate further studies of the primary structure and folding pattern of the Na^+,K^+-pump proteins in the membrane.

Introduction

Information about the structure of the intramembrane portion of the α-subunit and β-subunit of ($Na^+ + K^+$)-ATPase is of particular importance for understanding the mechanism of active transport of Na^+ and K^+ across the membrane. To characterize the lipid-associated domains, the intramembrane segments must be identified and localized in the protein sequences. In combination with labeling from the membrane surfaces and selective cleavage of the peptides this may lead to a description of the path of the polypeptide chains in the membrane.

Photoactivable hydrophobic reagents are introduced into the bilayer in the dark with the purpose of identifying lipid-embedded protein segments. Upon activation by light, they label lipids and lipid-embedded domains of membrane proteins in a manner largely determined by the chemical properties of the reactive intermediate(s) [1–3]. [^{125}I]Iodonaphthylazide is a nitrene precursor with a partition coefficient of 163,000 and thus a large preference for the lipid phase relative to water [3]. At low concentration [^{125}I]iodonaphthylazide inserts preferentially in the 46 kDa fragment of the α-subunit of ($Na^+ + K^+$)-ATPase [4]. At higher concentration, [^{125}I]iodonaphthylazide labels both the 46 kDa and the 58 kDa segment of the α-subunit and the β-subunit [5]. It was concluded that [^{125}I]iodonaphthylazide identified lipid-embedded segments of the proteins because reduced gluta-

Abbreviations: TID, 3-trifluoromethyl-3-(*m*-iodophenyl)diazirine; SDS, sodium dodecyl sulfate.

thione did not affect the extent or the nature of [^{125}I]iodonaphthylazide labeling and extensive proteolysis removed a major fraction of protein from the membrane surface without reducing the content of [^{125}I]iodonaphthylazide associated with peptides in the membrane [5].

Aryl nitrenes show low reactivity for C-H bonds and they may rearrange into electrophilic compounds. Attempts were therefore made to synthesize hydrophobic carbene precursors. [^{3}H]Adamantane diazirine [6] with a partition coefficient of only 1750, exclusively labels the 58 kDa fragment of the α-subunit in renal ($Na^+ + K^+$)-ATPase and [^{3}H]adamantane diazirine does not insert into the 46 kDa fragment or into the β-subunit [7]. The reason why [^{3}H]adamantane diazirine appears to be more selective than [^{125}I]iodonaphthylazide could be that it photoisomerizes to diazoadamantane, which is a powerful electrophile that may react preferentially with carboxyl groups [2]. The problem with the reactive diazoisomers can be eliminated by introducing a trifluoromethyl group adjacent to the diazirine function [8–10]. Thus, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) does not generate a reactive diazoisomer in the course of its photolysis. [^{125}I]TID has a partition coefficient of 24000, and hydrophobic labeling of erythrocyte and small-intestinal brush-border membranes confirmed the usefulness of [^{125}I]TID as a reagent for the labeling of intramembrane portions of protein [9,10].

In the present study, we labeled the pure membrane-bound ($Na^+ + K^+$)-ATPase with [^{125}I]TID in order to compare its insertion into the α-subunit, β-subunit and lipids with the previous conflicting results obtained with [^{125}I]iodonaphthylazide and [^{3}H]adamantane diazirine.

Methods

Purified ($Na^+ + K^+$)-ATPase with specific activity 39 μmol/min per mg protein was obtained from the outer medulla of pig kidney by incubation of a membrane fraction for 30 min at 20°C with SDS in the presence of ATP followed by isopycnic centrifugation in the Ti-14 Beckman zonal rotor [11]. The preparation was suspended in 10% sucrose/1 mM EDTA/25 mM imidazole (pH 7.5) and stored frozen at −80°C. Assays of ($Na^+ + K^+$)-ATPase and potassium phosphatase were carried out as before [5,12].

Prior to labeling with [^{125}I]TID, aliquots containing 1 mg protein were dialyzed for 24 h against 150 mM KCl/25 mM imidazole/1 mM EDTA (pH 7.5). The samples were flushed with argon for 30 min. For labeling at a low concentration of [^{125}I]TID, samples of 1 ml containing 1 mg protein were supplemented with 3 μl containing 1.5 nmol [^{125}I]TID and 4 μl ethanol. For labeling at a high concentration of [^{125}I]TID, samples of 1 ml containing 1 mg ($Na^+ + K^+$)-ATPase protein were supplemented with 3 μl containing 1.5 nmol [^{125}I]TID and with 4 μl ethanol containing 70 nmol nonradioactive TID. The samples were transferred into a stirred quartz cuvette and photolyzed for 45 s with a 350 W medium pressure mercury lamp as a light source (Illumination Industries, Inc. Type 350-1008). The beam was directed through a 30 mm filter of circulating cold water and a 20 mm filter consisting of a saturated solution of copper sulfate to cut off radiation shorter than 315 nm [9,10].

After photolysis, the samples were dialyzed for 24 h against 150 mM KCl/25 mM imidazole/1 mM EDTA (pH 7.5) containing 0.3% bovine serum albumin and then for another 24 h in the same buffer without albumin.

For graded tryptic digestion, aliquots containing 80 μg of labeled membrane-bound ($Na^+ + K^+$)-ATPase were incubated with trypsin for 80 min or 120 min at 20°C in 800 μl containing 0.16 μg of trypsin, 2 mM KCl or 10 mM NaCl, 1 mM ADP in 25 mM Tris-HCl/0.2 mM EDTA (pH 7.5). Digestion was stopped by mixing with 1.25 μg soybean trypsin-inhibitor (Sigma type 1-S) in 20 μl. The mixture was centrifuged for 90 min at 40000 rpm in the Beckman type 65 rotor. The pellets were resuspended in 100 μl 2% SDS/1% mercaptoethanol and heated for 10 min at 55°C. Urea was added to 6 M and aliquots containing 25–40 μg protein were applied on polyacrylamide gels. Gels were formed in 25 mM phosphate buffer (pH 7.0)/6 M urea as 12-cm separating gels, 9% in acrylamide and 1.8% crosslinker, with 1.5-cm stacking gels, 6% in acrylamide and 10% crosslinker. The gels were maintained at 20°C during electrophoresis at a current of 2–3 mA per gel for

15–20 h. After electrophoresis, one gel was cut into slices 1.5 or 3 mm thick and counted for radioactivity. The other gel was stained with Coomassie blue. Molecular weights of the polypeptide chains were determined by calibration against crosslinked samples of pyruvate kinase and lactate dehydrogenase as described before [12].

For extensive tryptic digestion, 70 μg of [^{125}I]TID-labeled ($Na^+ + K^+$)-ATPase protein was incubated for 80 min at 37°C in 800 μl 150 mM KCl/1 mM $CaCl_2$/25 mM Tris-HCl/0.1 mM EDTA (pH 7.5) with 7 μg trypsin per 100 μg ($Na^+ + K^+$)-ATPase protein. In a second protocol, the labeled ($Na^+ + K^+$)-ATPase was incubated for two consecutive periods of 60 min at 37°C with 20 μg trypsin per 100 μg protein. In a third protocol, labeled ($Na^+ + K^+$)-ATPase was incubated for one period of 60 min at 37°C with 20 μg trypsin per 100 μg protein followed by a second period of 60 min at 37°C with 20 μg chymotrypsin per 100 μg protein. Digestion was stopped with 3–4-fold excess of trypsin inhibitor. The digested membranes were sedimented by centrifugation in the Beckman type 65 rotor and prepared for gel electrophoresis as above.

Results

The distribution of covalently attached [^{125}I]TID after gel electrophoresis of the pure ($Na^+ + K^+$)-ATPase in SDS is shown in Fig. 1. The lipids running in front of the marker dye contained 94–95% of the total radioactivity in the membrane-bound ($Na^+ + K^+$)-ATPase, but the high specific activity of [^{125}I]TID allowed separation of the fraction of labeling, 5–6%, that was attached to the protein components. The distributions in a series of four consecutive experiments are shown in Table I. It is seen that a variation in concentration of nonradioactive TID over a 47-fold range only had a moderate influence on the distribution of covalently attached [^{125}I]TID on the protein and lipid components. On average, labeling of the α-subunit was reduced by 15% when the concentration of label was increased from 1.5 μM to 71 μM by adding nonradioactive TID. At the low [^{125}I]TID concentration, there was a slight preference for the α-subunit over the β-subunit, with an α/β ratio for the insertion of 4.1. At the

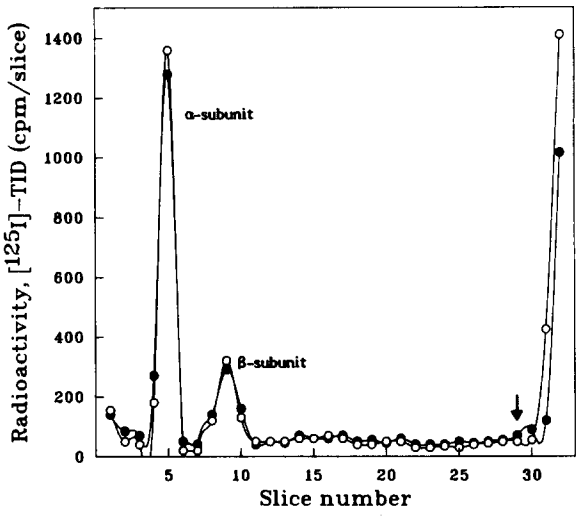

Fig. 1. Labeling of α-subunit and β-subunit with [^{125}I]TID at a low, 1.5 μM (○), and a high, 71 μM (●), concentration. Conditions for labeling as in Table I. Electrophoresis, slicing of gels and counting as described in Methods. Arrow indicates the position of the front of the marker, Bromphenol blue.

TABLE I

CONDITIONS FOR LABELING WITH [^{125}I]TID AND DISTRIBUTION OF [^{125}I]TID on protein subunits and lipid in pure membrane-bound ($Na^+ + K^+$)-ATPase.

It is assumed that TID partitions to more than 99% into the lipid phase [9] and that the lipid phase forms 1% of the incubation volume, since 1 mg protein is associated with 1 mg lipid in the pure ($Na^+ + K^+$)-ATPase [11]. The content of α-subunit is assumed to be 6.8 nmol/mg protein [11].

	Low TID	High TID
Concentration of TID (μM)	1.49	71.0
Total radioactivity (cpm)	$41.5 \cdot 10^6$	$42.5 \cdot 10^6$
Radioactivity remaining after photolysis and dialysis (cpm)	$22 \cdot 10^6$	$23 \cdot 10^6$
Estimated concentration of TID in membrane lipid phase (mM)	1.5	71
Estimated concentration of α-subunit in membrane lipid phase (mM)	6.8	6.8
Distribution of TID (%)		
α-subunit	4.6 ± 1.3(4)	3.9 ± 0.2(4)
β-subunit	1.1 ± 0.1(4)	1.4 ± 0.3(4)
lipid	94.3 ± 1.3(4)	95.0 ± 0.4(4)

higher [^{125}I]TID concentration, the α/β ratio for the labeling was 2.8. This value is identical to the α/β mass ratio in the pure renal $(Na^+ + K^+)$-ATPase [11,13]. The data therefore show that the specific activity of [^{125}I]TID was the same in the two proteins. In agreement with the previous observations with [^{125}I]iodonaphthylazide [4,5], [^{125}I]TID did not identify a γ-subunit which has been observed in some purified preparations of $(Na^+ + K^+)$-ATPase after covalent labeling with ouabain [14]. The incorporation of [^{125}I]TID depressed the $(Na^+ + K^+)$-ATPase activity by 40–60%, but it is obvious from Fig. 2 that the labeling did not prevent the transitions between the E_1Na and the E_2K conformations of the α-subunit as monitored by tryptic digestion. After trypsinolysis in KCl, the labeling was distributed both on the 58 kDa and on the 40–46 kDa fragments, Fig. 2. The specific activity of [^{125}I]TID in the 40–46 kDa fragments was estimated to be close to that in the 58 kDa fragment if allowance is made for distribution of the label on several fragments in the range 40–46 kDa and for comigration of the 58 kDa fragment with the β-subunit. After digestion of the E_1 form of the α-subunit in medium containing NaCl plus ADP, labeling of the 78 kDa fragment was also observed (Fig. 3).

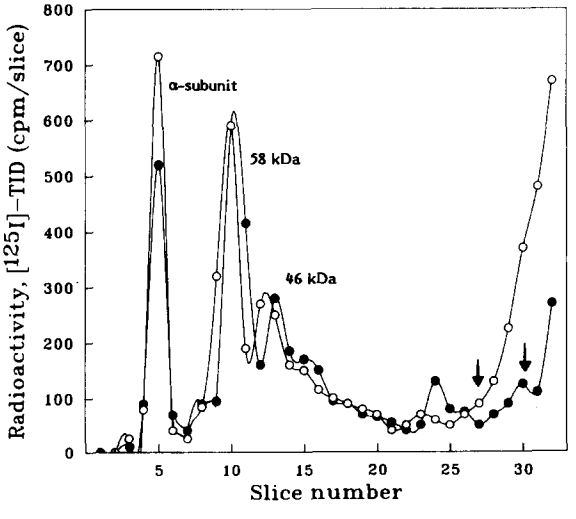

Fig. 2. [^{125}I]TID labeling at a low (○) and a high (●) concentration of $(Na^+ + K^+)$-ATPase protein subunits and the 58 kDa fragment and 40–46 kDa fragments arising by tryptic cleavage of the α-subunit in KCl medium. Incubation with trypsin, sedimentation of the digested membranes and gel electrophoresis as described in Methods. Arrows indicate the position of the front of the marker, Bromphenol blue.

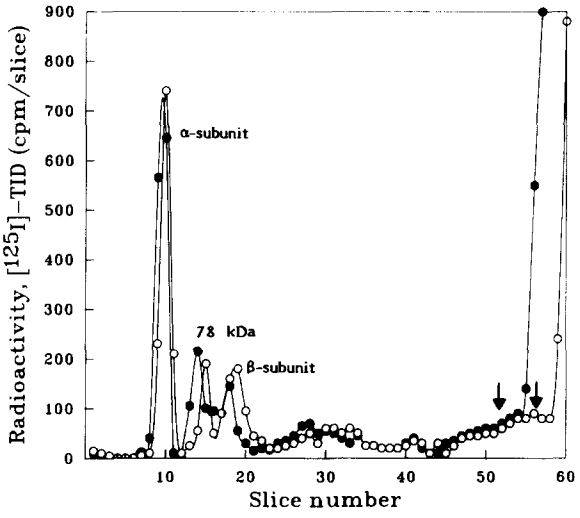

Fig. 3. [^{125}I]TID labeling at a low (○) and a high (●) concentration of $(Na^+ + K^+)$-ATPase protein subunits and the 78 kDa fragment arising by tryptic cleavage of the α-subunit in medium containing 10 mM NaCl and 1 mM ADP. Incubation with trypsin, sedimentation of the digested membranes and gel electrophoresis as described in Methods. Arrows indicate position of the front of the marker, Bromphenol blue.

Extensive digestion with trypsin alone did not reduce the [^{125}I]TID content in the membrane-bound $(Na^+ + K^+)$-ATPase by more than 10–20%. After incubation with 7 μg trypsin per 100 μg $(Na^+ + K^+)$-ATPase protein for 80 min at 37°C, 92–98% (range for three experiments) of the radioactivity was recovered in the pellet after sedimentation of the membranes. After incubation for two consecutive periods with 20 μg trypsin per 100 μg $(Na^+ + K^+)$-ATPase protein, 82–90% of the radioactivity was recovered in the sediment. Electrophoresis (Fig. 4) showed that [^{125}I]TID remaining in the membranes after this digestion was distributed on numerous broad bands between the 58 kDa fragment and the region of the marker dye. A large fraction of [^{125}I]TID was found in the region with molecular masses 12–30 kDa, but significant proportions remained in bands with masses 58 kDa and 40–46 kDa. After combining tryptic digestion with chymotryptic cleavage of bonds involving hydrophobic side-chains, 30–50% of the radioactivity was released from the membranes, but the distribution of the radioactivity

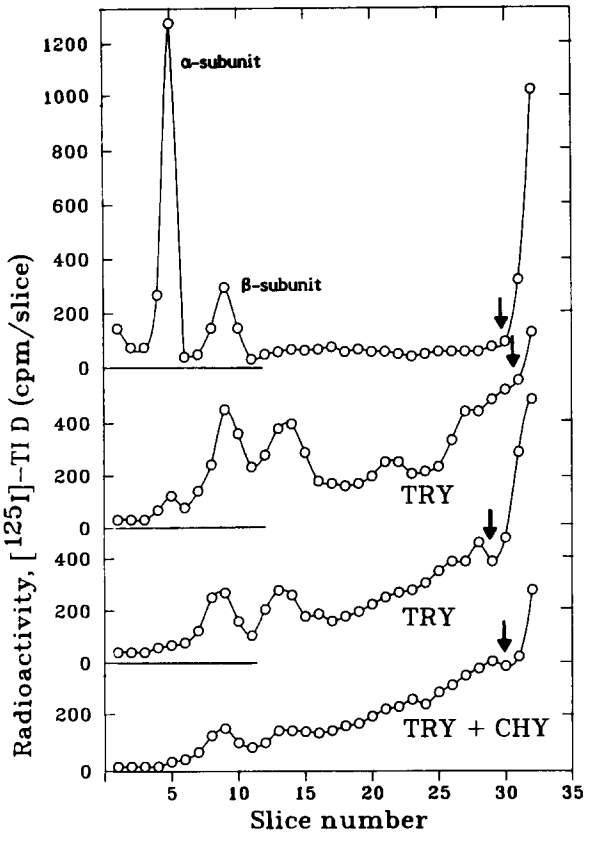

Fig. 4. Distribution of [^{125}I]TID after extensive digestion of ($Na^+ + K^+$)-ATPase protein with trypsin (TRY) or chymotrypsin (CHY). Upper frame: control incubated without trypsin. Second frame: incubation with 7 μg trypsin per 100 μg ($Na^+ + K^+$)-ATPase for 80 min at 37°C. Third frame: incubation for two consecutive period of 60 min at 37°C with 20 μg trypsin per 100 μg protein. Fourth frame: incubation for 60 min at 37°C with 20 μg trypsin and for 60 min at 37°C with 20 μg α-chymotrypsin per 100 μg ($Na^+ + K^+$)-ATPase protein. Arrows indicate the position of the front of the marker, Bromphenol blue.

was about the same as after extensive digestion with trypsin alone.

Discussion

[^{125}I]TID inserted from the hydrophobic bilayer into both the α-subunit and the β-subunit of pure membrane-bound ($Na^+ + K^+$)-ATPase. The two subunits and the major proteolytic fragments of the α-subunit became labeled to nearly the same specific radioactivity. The labeling was resistant to extensive proteolysis and the distribution of label among the two subunits was independent of a 47-fold variation in the concentration of [^{125}I]TID, suggesting that the reagent did not bind to (a) specific site(s) but labeled the lipid exposed sectors of the proteins in a rather unspecific manner. These results confirm the usefulness of [^{125}I]TID as a reagent for identifying membrane-embedded domains of intrinsic proteins.

The labeling data with [^{125}I]TID are in agreement with the previous general conclusions based on labeling with [^{125}I]iodonaphthylazide, ^{32}P and [^{3}H]ouabain [5] that the α-subunit possesses several transmembrane segments that are evenly distributed along its sequence and that also the β-subunit traverses the bilayer. The most promising aspect of the present result is that [^{125}I]TID provides a more uniform distribution of label among the membranous polypeptide segments than is obtained with other photoactivatable reagents. This will facilitate further characterization of these segments in terms of composition and primary structure, and, most important, it provides a basis for studies of the folding pattern and arrangement of polypeptide chains in the membrane.

In Table II, the present data are compared with the previous results of labeling membrane-bound ($Na^+ + K^+$)-ATPase from mammalian kidney with [^{125}I]iodonaphthylazide [4,5] and [^{3}H]adamantane diazirine [6,7]. It is seen that the protein/lipid labeling ratio is much higher for [^{125}I]iodonaphthylazide than for [^{125}I]TID. Both compounds label the α-subunit and the β-subunit, but while

TABLE II

COMPARISON OF LABELING OF PURIFIED RENAL ($Na^+ + K^+$)-ATPase WITH [^{125}I]TID IN THE PRESENT STUDY, [^{125}I]IODONAPHTHYLAZIDE (INA) IN REF. 5 AND [^{3}]ADAMANTANE DIAZIRINE IN REF. 7

Values are for distribution of label, in percentage of total. +, presence of label in peptide; 0, absence of label

	[^{125}I]TID		[^{125}I]INA		[^{3}H]-Adamantane diazirine
	low	high	low	high	
Lipid	94	95	18	35	+ [a]
β-subunit	1.1	1.4	7	8	0
α-subunit	4.6	3.9	75	57	+
78 kDa fragment	+	+	0	+	+
58 kDa fragment	+	+	0	+	+
40–46 kDa fragment	+	+	+	+	0

[a] Quantitative data not available.

[^{125}I]TID is evenly distributed on the subunits and on the proteolytic fragments, it is seen that [^{125}I]iodonaphthylazide inserts preferentially into the 46 kDa fragment of the α-subunit, while the concentration of label is lover in the 58 kDa fragment and in the β-subunit [4,5]. The resistance to extensive proteolysis shows that both compounds insert in segments that are embedded in the bilayer, but the distribution of label after extensive proteolysis is different. [^{125}I]iodonaphthylazide is found almost exclusively in fragments with molecular masses around 12 kDa while [^{125}I]TID is distributed on several fragments with masses in the range between 58 kDa and 12 kDa. These data suggest that [^{125}I]TID inserts in a less selective manner in the protein of ($Na^+ + K^+$)-ATPase, while [^{125}I]iodonaphthylazide is more selective. This is consistent with data obtained from photolabeling of the F_0-subunits of ATP-synthase of *Escherichia coli.* The b-subunit was labeled by [^{125}I]iodonaphthylazide at a single residue, cysteine 21, while [^{125}I]TID, in addition to heavily labeling this cysteine residue, reacted also with aliphatic side-chains of most inert amino acids. The proteolipid moiety (subunit c), with its very hydrophobic and inert character, was not detectably labeled by [^{125}I]iodonaphthylazide, but was heavily labeled by the carbene precursor [^{125}I]TID [15].

It is seen from Table II that the pattern of labeling with the carbene precursor [^{3}H]adamantane diazirine is distinctly different from that observed with [^{125}I]iodonaphthylazide and [^{125}I]TID. [^{3}H]Adamantane diazirine labels exclusively the 58 kDa fragment of the α-subunit of renal ($Na^+ + K^+$)-ATPase, while the 46 kDa fragment and the β-subunit are unlabeled [7]. The results with [^{3}H]adamantane diazirine were interpreted to mean that only the 58 kDa segment is inserted in the bilayer, while both the 46 kDa segment of the α-subunit and the β-subunit are positioned on the membrane surfaces [7]. In addition to the data of Table II, experiments that are independent of labeling with hydrophobic reagents show that both the 46 kDa fragment and the β-subunit are indeed embedded in the bilayer. The 46 kDa fragment must traverse the bilayer because it can be labeled with ^{32}P from [γ-^{32}P]ATP from the cytoplasmic surface and with [^{3}H]ouabain from the extracellular surface [5,16]. The β-subunit possesses at least one transmembrane segment, since it can be covalently labeled with diazomalonyldigitoxin from the extracellular surface [17] and it has been shown that antigenic segments of the β-subunit are exposed on the cytoplasmic surface [18]. The selective labeling of the 58 kDa segment by [^{3}H]adamantane diazirine therefore suggests that the specific insertion of [^{3}H]adamantane diazirine into certain groups prevents the compound from identifying several of the transmembrane segments in the proteins of ($Na^+ + K^+$)-ATPase.

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