lar-mass fragments and the appearance of additional (spurious) proteolytic fragments (see Fig. 1, lanes 2–5).

Since the level of aggregation normally increases with temperature and time of heating (see, for example, (3)), one approach is to add SDS/β-mercaptoethanol followed by heating and cooling as quickly as is consistent with full denaturation of the protease. However, this is difficult to achieve consistently and some degree of aggregation is always apparent. An alternative strategy is to use centrifugation to separate the protein (in the membrane pellet) from the protease (in the supernatant). In practice, we find that many proteases (e.g., proteinase K, trypsin) cannot be removed completely; even after repeated spin washes and use of high or low salt conditions, a proportion of the protease appears to remain bound to the membrane.

We have found recently that the presence of a high concentration of urea (approx 8 M) in the SDS–sample allows us to heat the proteinase K-treated Ca^{2+}-ATPase from sarcoplasmic reticulum at 100°C for 70 s without inducing aggregation (Fig. 1, lanes 10–13). We add crystals of solid urea to each aliquot thereby minimizing the dilution of the sample (which is about 1.5-fold). The method has also been used successfully to decrease aggregation with the H^+,K^+-ATPase (le Maire, Raussens, and Goormaghtigh, unpublished results) and there are previous reports indicating that urea may be useful in reducing the aggregation state of other membrane proteins (see e.g., (4)). In addition, Fig. 1 suggests that this method helps in overcoming the selective loss of certain proteolytic products through aggregation: two peptides from the C-terminal part of Ca^{2+}-ATPase, p27C and p19 (see upper and lower arrows, respectively, in Fig. 1), appear to have a higher relative abundance in both unheated samples (Fig. 1, lanes 3–5) and in heated samples containing urea (Fig. 1, lanes 11–13) than in the heated samples which contain no urea (lanes 7–9). Our results suggest that this simple method may be effective in overcoming the selective loss of certain proteolytic products through aggregation while permitting instantaneous protease inactivation by heating. This may therefore improve the quantitative reliability of these proteolysis experiments.

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Electroblotting of Proteins to Teflon Tape and Membranes for N- and C-Terminal Sequence Analysis

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Since the introduction of electroblotting from polyacrylamide gels, various membranes have been evaluated for their usefulness in electroblotting and the further characterization of the blotted protein (1). One of the critical properties determining a particular membrane's usefulness has been its chemical inertness. Proteins electroblotted to nitrocellulose, for example, can be subjected to *in situ* proteolytic digestion, but not Edman sequencing. Being inert to the Edman degradation chemistry has made PVDF[2] membranes the most widely used for N-terminal sequencing applications.

Since PVDF membranes are not inert to the chemistry used on the Hewlett–Packard G1009A C-terminal sequencer, we initiated a study to evaluate electroblotting of proteins to Teflon tape and membranes. We now report the optimized conditions for electroblotting to low-density Teflon tape and GORE-TEX expanded PTFE membranes which were found to give similar performance as when blotting to PVDF. In a previous study, Teflon was shown to be a suitable Edman sequencing support (2). We show sequence data from the Hewlett–Packard G1009A C-terminal sequencer for proteins electroblotted to Teflon. In addition, Teflon blots can be subjected to amino acid analysis, *in situ* proteolytic digestion, and a combination of N-terminal sequencing followed by C-terminal sequencing.

Materials and methods. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed us-

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[2] Abbreviations used: BSA, bovine serum albumin; HSA, human serum albumin; PTFE, polytetrafluoroethylene; PVDF, polyvinylidene difluoride.

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TABLE 1

Comparison of Recoveries of Electroblotted Proteins

	Yield (pmols)			
	Teflon tape		GORE-TEX	
Protein	2-in.	8-in.	0.1 μm	0.2 μm
HSA	200.4 (80%)	208.3 (83%)	193.0 (77%)	178.6 (71%)
PDE	210.2 (84%)	215.7 (86%)	182.9 (73%)	180.6 (72%)
BSA	160.8 (64%)	140.5 (56%)	147.4 (59%)	141.3 (57%)
Myoglobin	133.1 (53%)	ND	130.5 (52%)	108.4 (43%)
GST	190.1 (76%)	187.8 (75%)	168.6 (67%)	145.2 (58%)

Note. Total of 250 pmol loaded on gel in five lanes for each protein; percentage recovery is shown in parentheses.

ing 4–20% or 8–16% Novex (San Diego, CA) minigels (100 × 100 × 1 mm) in Tris–glycine buffer at 125 V for approximately 2 h. For most trials, 50 pmol of each protein was loaded per lane. Protein concentration was determined by amino acid analysis. Electrophoretically separated proteins were electroblotted onto Teflon supports using a Hoefer TE 22 mini transfer unit. Transfer was carried out for 3 h at 200 mA in 12.5 mM Tris, 96 mM glycine, and 10% methanol, pH 8.3 (3). Low-density Teflon tape (2 × 260 × 0.002 in.) was purchased from Allied Signal Fluorglass (Hoosick, NY) as A-1 commercial thread tape; 8-in.-wide Teflon tape (8 × 3600 × 0.003 in.) was purchased from Saunders (Wood Dale, IL). GORE-TEX expanded PTFE membrane sheets (8.5 × 10.5 in., 0.1- and 0.2-μm pore sizes) were purchased from W. L. Gore & Associates (Elkton, MD). Absolute ethanol was used to thoroughly wet the Teflon tape or GORE-TEX prior to assembling the blotting sandwich. Following transfer, proteins were stained with 0.005% sulforhodamine B (Sigma) in 30% methanol for 10 min (4). Blots were washed several times with distilled water to remove excess stain prior to drying.

For automated sequencing, blotted protein bands were excised from dry blots using dissecting scissors and inserted directly into the reaction cartridge without additional washing. C-terminal sequencing was performed on the Hewlett–Packard G1009A C-terminal sequencing system with on-line thiohydantoin analysis using Routine 2.0 chemistry (5, 6). Blotted protein from one or more gel lanes was sequenced. N-terminal sequencing was performed on the Hewlett–Packard G1005A protein sequencing system utilizing 3.0 chemistry and Routine 3.0 cycles. Amino acid analysis was performed by subjecting blotted protein bands to vapor-phase hydrolysis at 150°C for 1 h. After hydrolysis, 20 μl absolute ethanol was added to rewet the Teflon pieces followed by 80 μl internal standard solution. When performing *in situ* digestions, excised bands were kept wet. Proteolytic digestion using endoproteinase Lys-C (Wako) or trypsin (Promega) was carried out in 0.1 M Tris–HCl, pH 8.5, containing 40% acetonitrile for 24 h at 37°C. Peptides were extracted by washing the membranes with two washes of 20% acetonitrile and two washes of 60% acetonitrile in 0.1% trifluoroacetic acid.

Results and discussion. We have found low-density Teflon tape and GORE-TEX expanded PTFE membranes to be suitable supports for electroblotting and sequencing applications. Due to their low porosity, high-density Teflon tapes did not work for electroblotting and were not evaluated further in this study. Two types of Teflon tape and two types of GORE-TEX PTFE were used. Teflon tape is more like a film while GORE-TEX PTFE is a true membrane with controlled pore size. The handling and performance of these four materials were similar during electroblotting. The wetting properties of the Teflon membranes were quite different from PVDF. Therefore, it was essential to wet the membranes with absolute ethanol or isopropanol, not methanol. In addition, the membranes were kept wet with alcohol and not equilibrated with transfer buffer before being placed next to the gel on the blotting sandwich. Assembling the blotting sandwich in this manner resulted in consistent and reproducible blots by eliminating air bubbles from the sandwich. Although we have performed many successful transfers with the 8-in. Teflon, being thicker made it more prone to having "bubble" problems and uneven transfers.

As determined by amino acid analysis, recoveries on the 2- and 8-in. tapes were not significantly different, while most on the GORE-TEX were slightly lower. Recoveries ranged from 43 to 86% depending on the protein and the membrane used as shown in Table 1. All the membranes evaluated in this study had several characteristics in common. Visualization of the protein bands was accomplished with or without staining. After blotting, proteins appeared as a sheen against a dull background when the blot was placed in water. Coomassie brilliant blue, amido black, and sulforho-

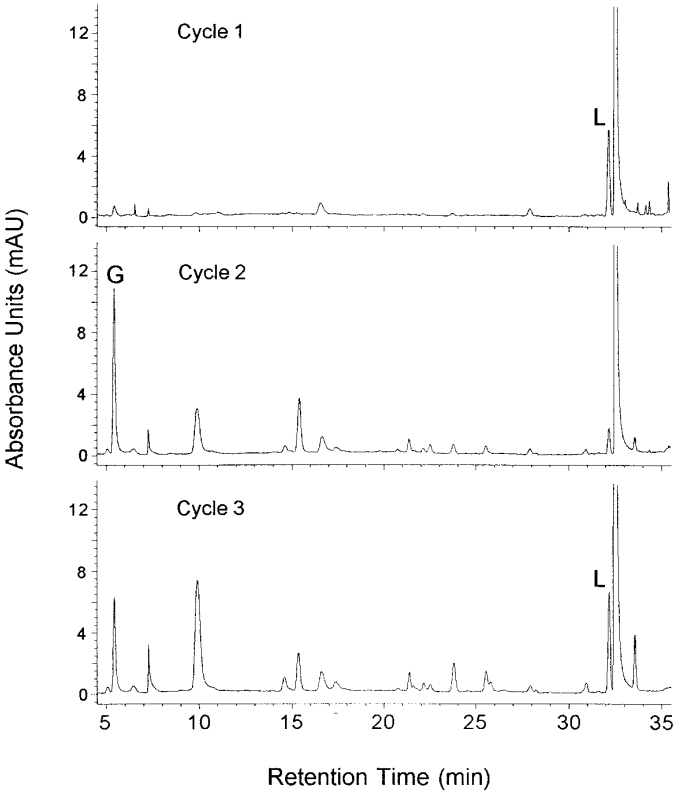

FIG. 1. Chromatograms for three cycles of C-terminal sequencing on HSA (total of 150 pmol loaded on gel in three lanes) blotted to 2-in. Teflon tape. The amino acid sequence from the C-terminal with thiohydantoin amino acid yields in picomoles is Leu (106)-Gly (93)-Leu (125).

doamine B were all used for staining. Sulforhodamine B became our preferred stain since it was rapid and caused no interference in further characterization of the protein. If present in an *in situ* digest, residual Coomassie blue produces a multitude of UV-absorbing peaks that interfere with the identification of peptides during chromatography.

Electroblotting to Teflon provided a facile means of preparing samples for C-terminal sequencing on the HP G1009A sequencer. Figure 1 shows results of sequencing HSA blotted to 2-in. Teflon tape. Similar results were obtained when sequencing an equivalent amount of protein spotted directly onto the Teflon membrane. Although initial yields and sequencing efficiency were found to be very similar regardless of the membrane used, the GORE-TEX runs always had higher background noise. Therefore, we concentrated on evaluating the 2-in. Teflon tape. Figure 2 demonstrates that the same 2-in. Teflon blot can be subjected to several cycles of Edman degradation followed by C-terminal sequencing. Using the HP G1005A, N-terminal sequencing was performed on BSA for five cycles, followed by three cycles of C-terminal sequencing on the HP G1009A. Since exposure to the C-terminal chemistry blocks the N-terminus, this process cannot be reversed.

Proteins electroblotted to Teflon membranes were amenable to both N- and C-terminal sequence analysis. We have found exposure to N-terminal sequencing first to have no effect on the ability to perform C-terminal sequencing on a sample. Although C-terminal sequencing results cannot be compared for PVDF versus Teflon blots, we have seen similar initial and repetitive yields for both types of membranes when performing N-terminal sequencing. Comparable results have also been obtained regardless of the membrane when performing *in situ* digestions. The ease of preparing samples on Teflon blots was similar to handling PVDF as long as the described wetting procedure was followed.

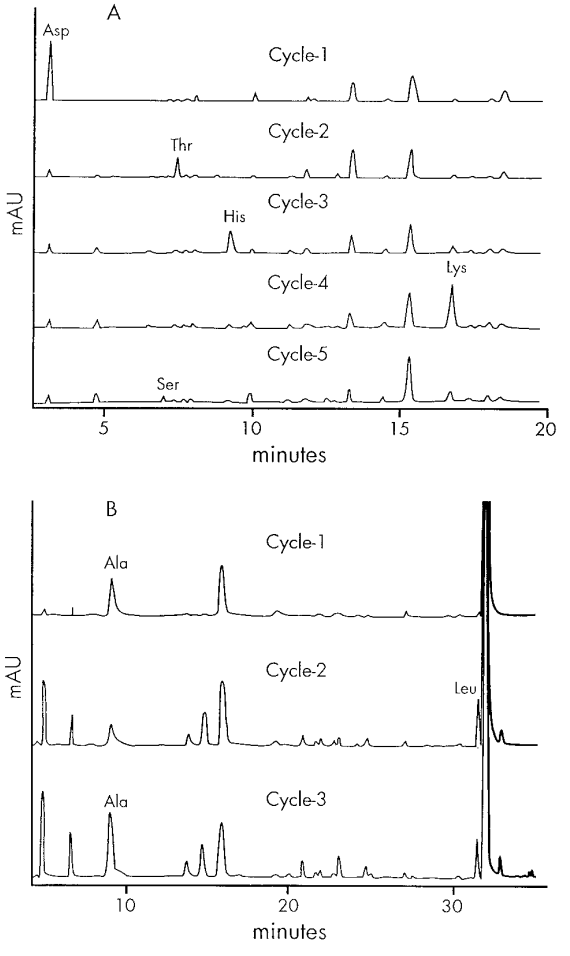

FIG. 2. (A) N-terminal sequence analysis of 250 pmol of BSA applied to gel and electroblotted to 2-in. Teflon tape using the N-terminal sequencer. The amino acid sequence for the N-terminal with PTH–amino acid yields in picomoles is Asp (55)-Thr (26)-His (46)-Lys (59)-Ser (8). (B) C-terminal sequence analysis of a previously N-terminally sequenced sample of BSA. The sequence from the C-terminal with thiohydantoin amino acid yields in picomoles is Ala (90)-Leu (74)-Ala (165).

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A Semiautomated Method for the Assay of Cyclic Adenosine 5′-Monophosphate Phosphodiesterase

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Cyclic nucleotide phosphodiesterases (PDEs)[2] catalyze the hydrolysis of cyclic nucleotides to 5′-nucleotide monophosphates. Currently, there are seven different gene families, each containing one or more isoforms (1). These isoforms have different affinities for substrate (cyclic AMP and cyclic GMP) and different tissue distribution. Within the PDE-4 family, four isoforms have been identified, cloned, and expressed along with several splice variants. Clinical studies suggest that inhibitors of PDE may have useful therapeutic properties in the treatment of such diseases as asthma, allergic rhinitis, and congestive heart failure (2). Due to the possibility that isoform-selective inhibitors may produce fewer adverse side effects than nonselective compounds, several pharmaceutical companies have initiated programs to discover isoform-selective inhibitors (3). Such a search requires a large-scale screening program to evaluate the activity of hundreds of compounds versus a multiplicity of enzymes. Thus, a need has emerged for an accurate, high-throughput assay for cyclic AMP PDE.

Two methods have been published that have the potential to process a large number of compounds. One of these methods is based upon isolation of the reaction product (5′-AMP) by zinc sulfate–barium hydroxide coprecipitation (4). This procedure uses microtiter plates but generates a large amount of radioactive waste. The other method is based upon the separation of 5′-AMP from cyclic AMP using microcolumns of neutral alumina (5). This procedure requires a large number of pipetting steps to isolate the reaction product.

This report describes an alternative, semiautomated procedure for the assay of cyclic AMP PDE that uses microcolumns of acidic alumina to isolate [^{3}H]-adenosine as the final reaction product. It is based upon an established method (6). Recent advances in microtiter plate technology were adopted to achieve a high-throughput assay with a large reduction in radioactive waste.

Materials

Acidic alumina (activity grade 1, Brockmann) was purchased from ICN Biomedicals GmbH (Eschwege, Germany). [2,8-^{3}H]Adenosine 3′,5′-cyclic phosphate, ammonium salt (30–50 Ci/mmol) was purchased from New England Nuclear Co. (Boston, MA). A Tomtec Quadra-96 pipetting robot was purchased from Tomtec, Inc. (Orange, CT). Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Microtiter plates, a multiscreen manifold (MAVM 09601), filter plates (MADVS6510), and a powder dispenser (MACL 09600) were purchased from Millipore, Inc. (Bedford, MA). The PDE inhibitor RP 73401 (3-cyclopentyloxy-*N*-(3,5-dichloro-4-pyridyl)-4-methoxy benzamide) and recombinant human PDE-4A were provided by Dr. Robert Wilhelm and Dr. Earl Shelton, respectively (Roche Bioscience). Pico plates, Microscint 20, and TopCount supplies were purchased from Packard Instruments (Meriden, CT).

Cyclic AMP Phosphodiesterase Assays

High-throughput method. A Tomtec Quadra-96 pipetting device was used to perform all the dilution, pipetting, and mixing procedures described below. Stock solutions of the test compounds were dissolved in 10% DMSO. Aliquots (10 μl) of the inhibitor were added to wells of a standard 96-well microtiter plate. For large-scale screening 88 compounds were tested per plate at a single concentration with four identical plates for each concentration of test compound (0.1 nM–10 μM). Each plate contained four control wells (no test compound) and four blank wells (no enzyme). The inhibitor curve in Fig. 1 represents six concentrations in quadruplicate. After addition of the test compound, 10 μl of concentrated incubation medium was added simultaneously to each of the 96 wells. The enzyme

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[2] Abbreviations used: PDE, phosphodiesterases; DMSO, dimethyl sulfoxide.

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