sensitivity-limiting step in protein sequence analysis.

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REFERENCES

Aebersold, R., Teplow, D., Hood, L. E., & Kent, S. B. H. (1986) *J. Biol. Chem. 261*, 4229–4238.
Aebersold, R., Leavitt, J., Hood, L. E., & Kent, S. B. H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 184*, 6970–6974.
Chang, J. Y. (1977) *Biochem. J. 163*, 517–520.
Chang, J. Y. (1981) *Biochem. J. 199*, 557–564.
Chang, J. Y., Creaser, E. H., & Bentley, K. W. (1976) *Biochem. J. 153*, 607–611.
Ender, B. E., Gassen, H. G., & Machleidt, W. (1984) *Hoppe-Seyler's Z. Physiol. Chem. 365*, 839–845.
Hewick, R. M., Hunkapiller, M. W., Hood, L. E., & Dreyer, W. (1981) *J. Biol. Chem. 256*, 7990–7997.
Horn, M. J., & Laursen, R. A. (1973) *FEBS Lett. 36*, 285–288.
Hunkapiller, M. W., & Hood, L. E. (1983) *Methods Enzymol. 91*, 486–494.
Hunkapiller, M. W., Lujan, E., Ostrander, F., & Hood, L. E. (1983) *Methods Enzymol. 91*, 227–236.
Hunter, W. M., & Greenwood, F. C. (1962) *Nature (London) 194*, 495–496.
Jin, S.-W., Chen, G.-X., Palacz, Z., & Wittmann-Liebold, B. (1986) *FEBS Lett. 198*, 150–154.
Kent, S. B. H., & Aebersold, R. (1984) Oral Presentation at the Fifth International Meeting on Protein Sequence Analysis, Cambridge, U.K., Aug 1984.
Kent, S. B. H., Hood, L. E., Aebersold, R., Teplow, D., Smith, L., Farnsworth, V., Cartier, P., Hines, W., Hughes, P., & Dodd, C. (1987) *Biotechniques 5*, 314–321.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laursen, R. A. (1971) *Eur. J. Biochem. 20*, 89–102.
Laursen, R. A. (1977) *Methods Enzymol. 47*, 277–288.
Machleidt, W., & Wachter, E. (1977) *Methods Enzymol. 47*, 263–277.
Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.
Muramoto, K., Kamiya, H., & Kawauchi, H. (1984) *Anal. Biochem. 141*, 446–450.
Salnikow, J., Lehmann, A., & Wittmann-Liebold, B. (1982) in *Methods in Protein Sequence Analysis* (Elzinga, M., Ed.) pp 181–188, Humana, Clifton, NJ.
Sarin, V. K., Kent, S. B. H., Tam, J. P., & Merrifield, R. B. (1981) *Anal. Biochem. 117*, 147–157.
Silver, J., & Hood, L. E. (1975) *Anal. Biochem. 67*, 392–396.
Ullrich, A., Bell, J. R., Chen, E. Y., Herrea, R., Petruzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfield, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature (London), 313*, 756–762.
Vandekerckhove, J., Bauw, G., Puype, M., Van Damme, J., & Van Montagu, M. (1985) *Eur. J. Biochem. 152*, 9–19.
Wachter, E., Machleidt, W., Hofner, H., & Otto, J. (1973) *FEBS Lett. 35*, 97–102.

Extended N-Terminal Sequencing of Proteins of Archaebacterial Ribosomes Blotted from Two-Dimensional Gels onto Glass Fiber and Poly(vinylidene difluoride) Membrane

Michael J. Walsh, John McDougall, and Brigitte Wittmann-Liebold*
Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, D-1000 Berlin 33, Federal Republic of Germany

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ABSTRACT: Previously uncharacterized proteins from intact ribosomes and ribosomal subunits of the extreme halophile *Halobacterium marismortui* (*Haloarcula marismortui*) were isolated and separated by high-resolution two-dimensional electrophoresis (2DE). N-Terminal amino acid sequences of 14 of these acidic large-subunit proteins were obtained by direct blotting of the separated proteins from two-dimensional electrophoresis gels to sequencer-stable supports followed by excision of the protein spots and sequencing. Furthermore, long internal sequences were obtained by in situ enzymatic cleavage of halobacterial proteins in gel pieces obtained from two-dimensional gels followed by electrophoretic separation of the fragments, blotting, and sequencing. Precautions are outlined for avoidance of N-terminal blockage of proteins, and the preparation and selection of suitable supports for obtaining extended N-terminal sequences are described. The results suggest that when prior fractionation is carried out to enrich for cell organelles, subcellular components of cells, or cell membranes, it is routinely possible to obtain numerous N-terminal sequences from one or a few 2DE gels of such fractions. Our results also indicate that, with appropriate precautions, proteins are routinely obtainable from 2DE gels in a form suitable for both N-terminal and internal sequence determination and show no detectable evidence for N-terminal blockage or destruction or modification of labile amino acid residues.

Immune and image reconstruction electron microscopy, protein–protein, protein–RNA, and RNA–RNA cross-linking, and neutron diffraction have been extensively applied to the study of ribosome topography and have begun to provide a comprehensive view of its higher order structure (Wittmann, 1986). In addition, X-ray analysis of crystals obtained from 70S ribosomes and ribosomal subunits [reviewed by Yonath et al. (1986)] derived from several sources promises to provide a three-dimensional map of the ribosome at high resolution which is a prerequisite for a complete understanding at a

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molecular level of the ways in which proteins are made. Recently, crystals from the 50S subunit of the archaebacterium *Halobacterium marismortui* (Hma),[1] also called *Haloarcula marismortui*, have been obtained which diffract to 6 Å (Makowski et al., 1986). The interpretation of collected data from high-resolution maps of the Hma ribosome will require the sequence determination of all its constituent proteins and RNAs. At this time, the complete amino acid sequences for 11 of the 30S and 50S subunit proteins have been obtained in our institute [see Kimura and Kimura (1987) and references cited therein; also, Hatakeyama and Kimura, unpublished results).

The isolation of the acidic ribosomal proteins (r-proteins) from extreme halophiles such as Hma poses problems not encountered with eubacteria; Hma cells grow more slowly, the yield of ribosomes is considerably lower, more complex methods are required for efficient protein extraction, and these acidic proteins after isolation may be completely insoluble in either low-salt or neutral and acidic buffers. Furthermore, high-performance liquid chromatography (HPLC), which has been widely used in the isolation of basic r-proteins from diverse groups of organisms (Kamp & Wittmann-Liebold, 1988), gives poor resolution and low recoveries when used for separation of the acidic Hma proteins; this also applies to enzymatic and chemical digests of Hma r-proteins (unpublished results).

Recently, it has been reported that N-terminal sequences can be obtained from either previously characterized proteins or proteins with neutral or basic p*I*'s after separation by polyacrylamide gel electrophoresis (PAGE) and blotted to sequencer-stable supports such as modified glass fiber (GF) and poly(vinylidene difluoride) (PVDF) membranes (Vandekerckhove et al., 1985; Aebersold et al., 1986; Bauw et al., 1987; Matsudaira, 1987). If such methods were of a more general applicability, they would enable researchers to circumvent many tedious and costly steps often required to purify proteins to homogeneity in a form suitable for microsequence analysis. The protein losses inherent in multistep purification procedures would also be circumvented.

Here we present N-terminal sequences of fourteen of the acidic Hma proteins separated by high-resolution two-dimensional electrophoresis (2DE), blotted, and then excised for direct N-terminal gas-phase and gas–liquid-phase sequencing. In addition, we report internal amino acid sequences of two large proteins of the 50S subunit of Hma; these sequences were also obtained exclusively with the use of gel electrophoresis and blotting methods. Finally, we report new methods for enhancing the binding capacity of supports used in electroblotting for sequencing applications and discuss optimization of sequencing protocols for obtaining extended Edman degradation on electroblotted samples.

Materials and Methods

Materials. Sodium dodecyl sulfate (SDS), *N,N'*-methylenebis(acrylamide), Coomassie Blue R250, *N,N,N',N'*-tetramethylethylenediamine, and ammonium persulfate were obtained from Bio-Rad; ultrapure urea was obtained from Schwartz-Mann or from BRL. 2-Mercaptoethanol, methanol, ethanol, acetone, glycine, tris(hydroxymethyl)aminomethane (Tris), sodium bicarbonate, glycine, methylamine, amido black, thioglycolic acid, hydrochloric acid, and dithiothreitol were from Merck. Hydrogen fluoride (38–40%) was from Fluka; glutathione (free acid) and standard proteins were from Sigma. Polybrene [poly(1,5-dimethyl-1,5-diazaundecamethylene dibromide)] was obtained from Aldrich, and fluorescamine was from Serva. 5-[[2-[(Iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid (1,5-IAEDANS) and 3,3'-dipentyloxacarbocyanine iodide (DPOCC) were from Molecular Probes; 1,4-butanediol diglycidyl ether, sodium borohydride, and 2-aminothiophenol were from Aldrich. Ribonuclease A was obtained from Boehringer, Mannheim, and Staphylococcal V8 protease was from Miles. Glass fiber sheets (GF/C, 47 cm × 56 cm) were from Whatman, 589/1 Red Ribbon paper (20 cm × 20 cm) was from Schleicher & Schuell, and Polybase-coated glass fibers sheets were a gift from Janssen Ltd. Poly(vinylidene difluoride) membranes (Immobilon) were from Millipore. Capillaries (200 μL) were from Assistent (catalog no. 7087 57). If not otherwise stated, all chemicals were analytical grade or higher.

Preparation of Hma Cells, Ribosomes, and Ribosomal Subunits. The growth of Hma cells and the isolation of ribosomes and ribosomal subunits were according to the methods of Visentin et al. (1972) with modifications described by Shevack et al. (1985). 50S subunits from *Bacillus stearothermophilus* (Bst) were obtained from T. Pohl (this institute).

Extraction of r-Proteins. The proteins were extracted from the 70S ribosome of Hma and from the 50S ribosomal subunits of Hma and Bst as described (Hardy et al., 1969) except that all dialysis buffers contained 10 mM 2-mercaptoethanol. The final dialysis was against 2% acetic acid, which maintained the r-proteins from both organisms in a soluble state before lyophilization.

Nomenclature for Hma r-Proteins. The numbering of the halophilic r-proteins is based solely on the apparent molecular weight of the individual major protein spots after 2DE. No attempt has been made to directly correlate protein spots separated on the gel system described here to those proteins previously separated essentially by the method of Strom and Visentin (1973).

One- and Two-Dimensional Analytical Gel Electrophoresis. The proteins of the 50S subunit and 70S ribosome of Hma were separated by the procedure of O'Farrell (1975) with modifications. The first dimension isoelectric focusing (IEF) gel had the following composition: 4% acrylamide, 9.2 M urea, 2% ampholytes (from a stock solution containing 3 parts of pH range 3.5–5 and 1 part each of pH range 4–6, 6–8, and 3.5–10 ampholytes). The nonionic detergent NP-40 was omitted. Analytical IEF was done in disposable 200-μL glass capillaries. The gel solution was prepared, degassed, and pipetted into 5- or 10-mL measuring cylinders to the desired height. The capillaries were then inserted into the cylinder. After polymerization, the capillaries were cut to a length about 1 cm above the level of the gel to allow for sample application, rinsed gently with IEF sample buffer, and then assembled in a microchamber. The catholyte and anolyte buffers (20 mM sodium hydroxide and 10 mM phosphoric acid, respectively) were added, and the samples containing 10–50 μg of protein were applied under the catholyte. Prefocusing was omitted, and IEF was carried out at 600 V for 4 h, which was adequate to resolve the proteins of Hma as sharp spots. For the second dimension, the tube gels were extruded as described (Brockmöller & Kamp, 1986) and pushed between the glass

[1] Abbreviations: 1,5-IAEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]-amino]naphthalene-1-sulfonic acid; 2DE, two-dimensional gel electrophoresis; AP-GF, aminopropyl GF; Bst, *Bacillus stearothermophilus*; DITC-GF, diisothiocyanate GF; DPOCC, 3,3'-dipentyloxacarbocyanine; GF, glass fiber; Hma, *Halobacterium marismortui*; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; PB-GF, polybrene-coated GF; PTH, phenylthiohydantoin; PVDF, poly(vinylidene difluoride); QA-GF, quaternary ammonium GF; r-proteins, ribosomal proteins.

plates of an SDS–PAGE gel (Laemmli, 1970) with the aid of a spatula. The use of a stacking gel enhanced the sharpness of the spots. The gels after 2DE were stained with Coomassie Blue R250 or according to silver nitrate technique (Oakley et al., 1980).

One-Dimensional and Two-Dimensional Gels for Blotting and Sequencing Applications. IEF gels used as first dimension for electroblotting and sequencing were polymerized in 16 cm long glass tubes (5-mm i.d.). The gel solution was of the same composition as used for capillary IEF. The glass tubes were first immersed in sodium dichromate–sulfuric acid overnight, washed with deionized water, and dried. To prevent slippage of the tube gels during IEF, the glass tubes were additionally treated with potassium hydroxide saturated ethanol for several hours, washed in distilled water, and dried. After the gels were poured, they were overlaid with water. After polymerization, the water was removed and replaced with IEF sample buffer which contained 9.4 M urea, 2% ampholytes, 1–2% dithiothreitol, 20 mM methylamine, pH 7.6, and 15 mM thioglycolic acid. In all cases, gels were polymerized 6–12 h before use and then preelectrophoresed at 300 V for 30 min and then at 600 V for 3 h with fresh IEF sample buffer. Subsequently, the catholyte and sample buffer were removed, new catholyte and overlay buffer (O'Farrell, 1975) containing 15 mM thioglycolic acid was added, and the sample was layered under the overlay buffer. Usually, 500 μg of total 50S or 70S protein was applied, but as much as 1.5 mg could be applied without a serious impairment of resolution. After IEF, the gels were extruded into SDS–equilibration buffer (O'Farrell, 1975) and stored at −80 °C. SDS–PAGE gels for the second dimension were 1 or 1.5 mm thick with a 16-cm resolving gel and a 4 cm long 4% stacking gel. The gels were prepared the day before use and preelectrophoresed with 700 μL of thioglycolic acid per liter of upper chamber running buffer, until the buffer front had migrated 10 cm into the resolving gel. IEF gels were then thawed, equilibrated on a shaker for 15 min, and sealed in place by using 0.5% agarose in SDS–equilibration buffer. Thioglycolic acid was again added to the running buffer. The SDS used in all buffers was recrystallized twice (Hunkapiller et al., 1983).

Separation of Bst 50S Subunit r-Proteins. The r-proteins of Bst were separated as described (Brockmöller & Kamp, 1986). If required, the tube gels following the first dimension electrophoresis could be stored frozen in the second dimension running buffer containing 10% glycerol. When the second dimension separation of Bst proteins was done by using SDS–PAGE, they were first washed three times for 10 min each time in SDS running buffer and then equilibrated for 10 min with SDS–equilibration buffer before application to the second dimension gel.

Peptide Mapping in Gels. After 2DE, selected gels were immersed in 4 M sodium acetate for detection of separated proteins. Individual spots were excised, rinsed thoroughly with distilled water, then equilibrated briefly in protease buffer (Cleveland, 1983) containing 0.1% or 0.2% SDS, and used immediately for digestion in gels or frozen at −20 °C. Gel pieces were placed in wells of a second SDS–PAGE gel containing a 5% stacking gel and a 18% resolving gel. Protease buffer containing 15% glycerol was added followed by addition of running buffer. The gel pieces were then overlaid with 5 μg of staphylococcal V8 in protease buffer containing 0.2% SDS. Electrophoresis was commenced at 20 V to allow proteolysis to occur until the dye front reached the interface of the stacking and resolving gel and was then continued at 200 V. As for the regular SDS–PAGE gels, prerunning with thioglycolic acid was done, but the latter was omitted for the SDS–PAGE of the samples for in situ proteolysis. When it was necessary to separate peptides from more extensive digests of proteins, longer (18–20 cm) gels with a gradient of 15–22.5% acrylamide were used, and the concentration of Tris in the resolving gel and of Tris and glycine in the running buffer was twice normal (Fling & Gregerson, 1986); such gels resolve peptides to at least 3 kDa.

Activation and Derivatization of Glass Fiber (GF) and Paper. GF/C sheets were activated by immersion in 100% trifluoroacetic acid (TFA) for 4 h. Additional activation methods used were immersion in potassium hydroxide saturated ethanol for 12 h, 3 N hydrochloric acid for 12 h, or 0.1% hydrogen fluoride in water for 15 min. Polybrene-treated GF (PB-GF) was prepared as described (Vandekerckhove et al., 1985) except the GF was submerged in a glass dish containing a suspension of Polybrene in water until uniformly wetted and was then dried hanging in a hood. The same solution may be used for treatment of at least 15 sheets of GF. In most cases, the GF treated with Polybrene had first been activated with TFA. An estimate of the amount of Polybrene attached to the GF was obtained by weighing sheets of GF before immersion in the Polybrene solution, after thorough drying and washing in water and drying once more. All GF supports were stored at room temperature except for DITC-GF and aminophenyl thioether paper, which were stored dry at −20 °C. Aminopropyl (AP) and quaternary ammonium (QA) GF were prepared by reacting acid-treated GF with (3-aminopropyl)-triethoxysilane and *N*-[(trimethoxysilyl)propyl]-*N*,*N*,*N*-trimethylammonium chloride, respectively, and diisothiocyanate GF (DITC-GF) was prepared by reacting AP-GF with 1,4-phenylenediisothiocyanate as described (Aebersold et al., 1986). Diazotization of paper and GF/C was done by the procedure of Seed (1982).

Determination of Protein Binding Capacity. The protein binding capacity of the various supports when used in electroblotting applications was determined by separating standard proteins on SDS–PAGE in amounts sufficient to saturate the binding capacity of each support. The blotted proteins were excised and hydrolyzed with 200 μL of 5.7 N HCl at 110 °C for 24 h. The supernatant was recovered, measured, and then dried in a desiccator. Amino acid analysis was carried out by using precolumn derivatization with *o*-phthaldialdehyde. Selected Hma proteins separated by 2DE were blotted, excised, and also hydrolyzed for protein quantitation as done for standard proteins. The capacity of DITC-GF to bind protein covalently was determined by application of serial dilutions of bovine serum albumin, as 25-μL aliquots, to 1-cm diameter disks of DITC-GF. Covalent coupling was achieved by placing of the DITC-GF disk on a glass frit inside a test tube containing 25% trimethylamine and heating the sealed test tube at 55 °C for 1 h. The disks were removed, dried in a desiccator, and placed in the wells of an SDS–PAGE gel and electrophoresed. The gel was then stained, and the appearance of protein in the appropriate lane of the gel indicated that the capacity of the support to bind protein covalently was exceeded. Myoglobin and albumin were pipetted onto diazotized paper or diazotized GF in 50 mM Tris-HCl buffer, pH 8. The supports were dried, and the covalently attached protein was quantitated by the gel electrophoresis assay as described for DITC-GF.

Electroblotting. Blotting was conducted in an apparatus of standard design (Towbin et al., 1979) using a chamber with an interelectrode distance of 10.5 cm. After 2DE, gels were first equilibrated for 30–60 min in transfer buffer which was

25 mM Tris-HCl, pH 8.4, with 0.5 mM dithiothreitol (Aebersold et al., 1986). The transfer buffer was prechilled, and blotting was conducted at 4 °C. The standard procedure for Hma proteins was 150–200 mA for 1 h and then 650 mA for 4 h. Bst proteins separated on acetic acid–urea gels (Brockmöller & Kamp, 1986) were preequilibrated for 30–60 min in 1% acetic acid with 0.5 mM dithiothreitol, and blotting was conducted toward the cathode for 60 min, also at 650 mA. PVDF membranes were first wetted in methanol for 20 s and then rinsed in distilled water before placement on the gel.

Detection of Blotted Proteins. Subsequent to blotting, GF sheets and PVDF membranes were rinsed twice in distilled water for 5 min each time. Proteins blotted to activated GF sheets were detected as described (Aebersold et al., 1986). Proteins blotted onto PB-GF and derivatized GF were detected with DPOCC except that 3 mg of DPOCC per 100 mL of staining solution was used. Destaining was most efficiently done by compressing the stained sheets between Kimwipes several times. The sheets were then dried by compression between paper towels, and all clearly detectable spots were cut out as disks or fragments of GF and stored in plastic microcentrifuge tubes at −20 °C under nitrogen. In some cases, excised disks of GF detected with DPOCC were destained with ethyl acetate and dried under nitrogen before storage. Proteins separated by 2DE were also detected by using 0.5–1 mg of fluorescamine/L of acetone for 1–2 s. Proteins blotted to PVDF were detected by using DPOCC or fluorescamine as for GF or by using 0.1% amido black or Coomassie Blue R250 in 50% methanol and destaining briefly with 30% methanol or with water only. In most cases, however, proteins on PVDF were detected without staining as follows. First, as the PVDF dries, spots become detectable as areas much grayer than the surrounding membrane; second, with further drying, areas containing protein are easily seen as zones more intensely white than the surrounding PVDF when transilluminated with white light. In some experiments, proteins were prelabeled by carboxymethylation with 1,5-IAEDANS as described (Gorman, 1987) or after separation by analytical IEF and incubating the tube gel in the same buffer. Blots stained with fluorescent compounds were photographed on an ultraviolet transilluminator using a Polaroid 445 camera and Polaroid type 665 P/N film with exposure and development times of 40–60 s.

Sequence Analysis of Blotted Proteins. Blotted proteins were sequenced in a gas-phase sequencer constructed in the institute workshop (Reimann & Wittmann-Liebold, 1986) and also in a pulse–liquid-phase sequencer, Model 477A (Applied Biosystems), equipped with the Model 120 PTH-aa analyzer. Prior to commencement of the normal sequencing programs, GFs and membranes were washed in the reactor with *n*-heptane, ethyl acetate, and chlorobutane. Usually, a total of 1 or 1.5 mg of Polybrene was added to the pieces of GF or PVDF containing the protein before insertion into the reactor. When several fragments of support were used, they were always covered with a disk of GF or PVDF. Polybrene applied to PVDF disks was first diluted in 60% methanol.

Results

Two-Dimensional Electrophoresis of Hma Proteins. Figure 1 shows 2DE gels of proteins from the 50S subunit and 70S ribosome of Hma. Experiments using nonequilibrium pH gradient gel electrophoresis (O'Farrell et al., 1977) establish that all proteins extracted from the Hma ribosome are resolvable by the pH gradient employed by use of the 4 cm × 4 cm gels. The marked variation in stain intensity for many proteins reflects in part the differential staining of proteins

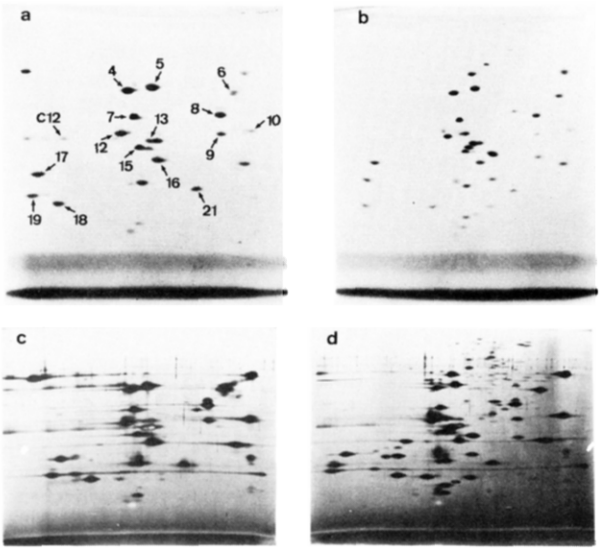

FIGURE 1: Hma r-proteins were extracted, dialyzed, and lyophilized; the proteins were then suspended in sample buffer and separated in the first dimension in capillaries; the second dimension was SDS–PAGE. Proteins were detected by staining with Coomassie Blue R250 (a and b) or by the silver nitrate technique (c and d). The numbered proteins (a) indicate the proteins that were subjected to N-terminal sequencing. Panels a and c are 50S subunit proteins, and panels b and d are from the 70S ribosome.

Table I: Protein Binding Capacity of Various Activated and Derivatized Supports

support	capacity[a]
activated glass fibers	
trifluoroacetic acid treated	5–8
hydrogen fluoride treated	5–8
hydrochloric acid treated	5–8
potassium hydroxide treated	5–8
derivatized supports (glass fibers)	
Polybrene	25–35
aminopropyl	4–6
quaternary ammonium	4–6
diazotized diisothiocyanate	4–6
Polybase-coated GF (Janssen Ltd.)	20–30
poly(vinylidene difluoride) (Millipore)	30–40

[a] Values are given in μg of protein/1-cm disk of support. The values for activated supports are for proteins blotted from acetic acid–urea gels; the values for the other supports are for proteins blotted from SDS–PAGE gels.

and the incomplete extraction of some proteins when the method of Hardy et al. (1969) for extraction of r-proteins from eubacterial ribosomes is employed. Silver-stained 2DE gels of 50S and 70S proteins (Figure 1c,d) show at least 30 spots for the 50S subunit and about 60 spots for the 70S ribosome. Some of the fainter spots in the high molecular weight part of the gels may be incompletely extracted r-proteins or proteins that copurify with ribosomes such as protein biosynthesis factors.

Choice of Supports for Electroblotting for Sequencing. The binding capacity for standard proteins, Bst ribosomal proteins (mostly very basic), and Hma r-proteins (generally acidic) was evaluated for eleven supports (Table I). Since DITC-GF may bind proteins during blotting by interactions other than covalent coupling under basic blotting conditions, the gel assay (see Materials and Methods) proved very useful in ascertaining the maximum covalent protein binding capacity of the support. The gel assay indicated that both diazotized paper and DITC-GF bind a maximum of 6 μg of bovine serum albumin per 1-cm diameter disk. This value would be expected to show some variability depending on the protein or peptide coupled.

FIGURE 2: Hma proteins were separated by 2DE and blotted to PB-GF. Protein L5 (arrow) was excised from both the first and second layer of PB-GF and placed in the wells of an SDS–PAGE gel. The first (lane a) and second layers (lane b) of PB-GF had been treated with 4 and 8 mg/mL Polybrene, respectively. The ladder pattern of bands below protein L5 derives from the Polybrene, which forms micelles with the SDS and stains intensely with Coomassie Blue R250.

The diazotization procedure used for paper was also carried out on GF, but no evidence for covalent attachment of protein to the GF by the gel assay was found. Presumably, a procedure used to diazotize glass beads (Weetal, 1976) would be effective but would be difficult to perform on a laboratory scale with GF sheets.

As shown in Table I, PB-GF has the highest protein binding capacity of the modified GF supports. In preliminary experiments to ascertain the optimal concentration of Polybrene to obtain maximum protein binding, sheets of GF were immersed in water containing 3, 4, 8, and 15 mg of Polybrene/mL. Immersion of sheets in increasingly concentrated solutioins of Polybrene led to proportionately greater amounts of Polybrene bound to the GF. About 20 μg of Polybrene/mg of GF remained on GF treated with 3 mg/mL Polybrene, and 75 μg/mg of GF remained when immersed in 15 mg of Polybrene/mL. The Polybrene remained adherent even after washing in water for 10 min before blotting, prolonged immersion in buffer during blotting, and subsequent staining and destaining (see Figure 2). However, immersion of GF sheets in Polybrene at a concentration greater than 4 mg/mL resulted in considerable transfer of Polybrene from the GF onto the SDS–PAGE gel. Furthermore, the detection of the blotted proteins with DPOCC was made more difficult (see next section). For this reason, a concentration of 4 mg/mL Polybrene for GF treatment was chosen to assure high protein binding capacity and reasonable ease of detection on the first-layer blot, while the second layer was 4 or 8 mg/mL PB-treated sheets of GF/C. Prior acid or base treatment increased the capacity of the PB-GF to bind protein as judged by the quantity and number of protein spots on the second layer of the blotting support.

Another effect from use of excess Polybrene is shown in Figure 3. Here, six proteins were separated by SDS–PAGE and blotted to two layers of PB-GF (both treated with 8 mg/mL Polybrene). The gel was stained after electroblotting and showed virtually complete transfer of the three largest proteins, but the three smaller proteins which are basic in charge were only partially eluted from the gel and retained on the PB-GF (Figure 3).

The basic ribosomal proteins of Bst were also separated in the first dimension by the standard procedure (Brockmöller & Kamp, 1986) and then in the second dimension by SDS–

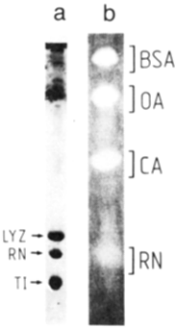

FIGURE 3: Six proteins were separated by SDS–PAGE and blotted to two layers of PB-GF; both were pretreated with 8 mg/mL Polybrene. The gel was stained after transfer (lane a) and shows incomplete elution of the three lower molecular weight proteins. Of the three low molecular weight proteins only ribonuclease can be seen clearly on the blot (lane b) after DPOCC staining. Abbreviations: BSA, bovine serum albumin; OA, ovalbumin; CA, carbonic anhydrase; LYZ, lysozyme; RN, ribonuclease; TI, trypsin inhibitor.

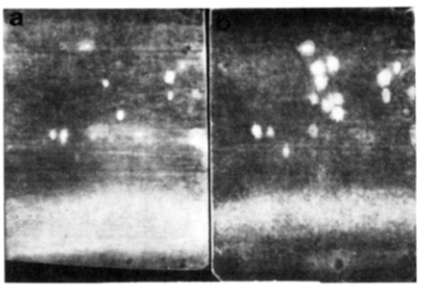

FIGURE 4: Hma 50S subunit proteins separated by 2DE were blotted to PVDF or PB-GF. In both cases, the second layer was PB-GF. Less protein penetrates to the second layer when PVDF is used (a) compared with PB-GF (b). Proteins were detected with DPOCC.

PAGE. Only when the concentration of Polybrene was kept at or below 4 mg/mL for both sheets of GF were Bst proteins efficiently eluted and bound to the PB-GF. The binding was markedly reduced and protein elution from the gel was markedly hindered when sheets of GF with higher concentrations of Polybrene were used (data not illustrated). Thus, the concentration of Polybrene used to pretreat GF for blotting basic proteins should not exceed 4 mg/mL.

Lastly, PVDF was evaluated for utility in blotting and sequence analysis applications. PVDF was found to be stable in all reagents and solvents used in the Edman degradation. Like PB-GF it has been found to have a high binding capacity for protein (see Table I and Figure 4) and requires no derivatization or activation before use.

Blotting from Acetic Acid–Urea Gels. Acetic acid–urea gels are frequently used for the separation of basic proteins such as histones and r-proteins. Blotting with acidic transfer buffers is commonly used for such gels (Towbin et al., 1979) as well as for IEF gels. PB-GFs are not useful for these applications because of charge repulsion between Polybrene and the basic proteins. Instead, TFA-activated GF is used directly for blotting. Three additional methods, namely, hydrofluoric and hydrochloric acid treatment and potassium hydroxide treatment, were used to activate GFs in this study (see Materials and Methods). All four procedures for activation gave similar results in terms of protein binding as illustrated for hydrofluoric acid treated and TFA-treated GF in Figure 5. On the other hand, GFs treated with the alternative activating methods are more fragile than TFA-treated GF. PVDF binds protein as well as activated supports under acidic blotting conditions, but the overall protein binding capacity is considerably less than when PVDF is used in blotting from SDS–PAGE gels. During acid blotting to PVDF much of the

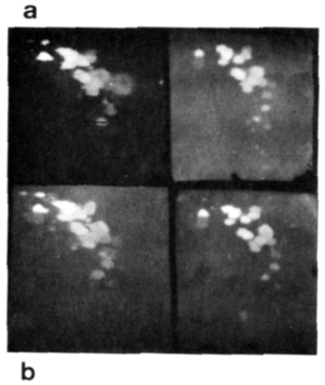

FIGURE 5: The r-proteins of Bst were electroblotted under acidic conditions to GF activated by trifluoroacetic acid, hydrogen fluoride, potassium hydroxide, or hydrochloric acid or to PVDF membrane. Only the results for TFA (a) and hydrogen fluoride (b) activated GF are shown in the figure; the second layer of GF (activated by hydrofluoric acid) is shown on the right side of the figures in (a) and (b).

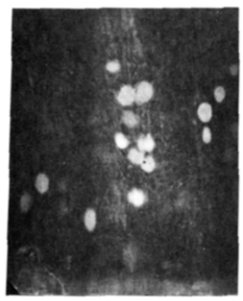

FIGURE 6: Hma r-proteins were separated by 2DE and blotted to PB-GF; the GF was first activated by immersion in potassium hydroxide saturated ethanol. The proteins on the first layer of the blot were detected with DPOCC. Many additional spots were seen on irradiation with ultraviolet light but are not seen in the photo.

total protein penetrates onto the second layer even though only a few micrograms of protein are contained in each spot.

Detection of Proteins on Blots. Unmodified and activated GFs can be stained with the common dyes such as amido black and Coomassie Blue R250 and with fluorescent compounds such as DPOCC and fluorescamine (Figure 6). PB-GF cannot be stained with amido black or with Coomassie Blue R250 because of interaction of the stains with the Polybrene. DPOCC detects proteins at the low picomole level, it does not cause N-terminal modification, and it is promptly removed from GF by a brief ethyl acetate wash after staining or in the reactor before the start of the Edman degradation (Aebersold et al., 1986). However, when it is used with PB-GF, substantial background may cause difficulties in the identification of protein spots. To circumvent this, a lower concentration of DPOCC (3 mg/100 mL of stain solution) was used, and the blots were stained only until spots became visible under ultraviolet light. Destaining for repeated compression between Kimwipes and paper towels was found to be the most effective method for destaining (Figure 6). The background is not entirely attributable to interactions between the DPOCC and the Polybrene since the second PB-GF layer of the sandwich pretreated with 8 mg/mL Polybrene showed negligible background (Figure 4). Most likely, contaminants from the gel are trapped on the first PB-GF layer and cause the background problems.

Proteins were also detected by immersion of PB-GF or PVDF in fluorescence solution (0.5–1 mg/L of acetone) for 1 s. N-Terminal blockage is a potential complication especially when only low picomoles of material are present in protein spots. In addition, the procedure is relatively insensitive (Vandekerckhove et al., 1985). Proteins on PVDF membranes were detected with Coomassie Blue R250, amido black, fluorescamine, and DPOCC. Amido black gives very satisfactory results. Staining requires only 20–30 s, and destaining with water or 30% methanol to remove excess dye is completed in several minutes. This method will allow visualization of low picomole amounts of protein, and in practice, if the protein could not be seen by this method, there was not enough material for a sequence determination. Although DPOCC and fluorescamine can be used to visualize proteins on PVDF membranes, both methods when used on PVDF give rise to unacceptable contaminant peaks during sequence analysis. Therefore, neither of these reagents should be used if samples are intended for sequencing. In addition, proteins can often be detected without prior staining by visual inspection during drying as described earlier (Materials and Methods); between 25 and 50 pmol of β-lactoglobulin was detected easily by this method. Finally, we investigated prelabeling the total Hma r-protein extract with 1,5-IAEDANS (Gorman, 1987) both prior to the 2DE separation and by incubating the first dimension IEF tube gel with this reagent. Prelabeling gave rise to substantial heterogeneity in mobility of individual protein spots indicating nonuniform labeling, and labeling of the IEF gel required excess reagent which could not be extracted from the tube gel and gave rise to high background fluorescence in the second dimension gel.

Sequence Analysis of Electroblotted Proteins. Table II lists the N-terminal sequences obtained on the blotted proteins. The sequence analysis was usually performed by combining several pieces of support containing the protein inside the reactor and then overlaying the pieces with an intact disk of PVDF or activated GF. Between 40 and 500 pmol of individual proteins was present on these combined pieces of support. However, the quantity of protein in the cartridge was not the important determinant of the number of N-terminal residues identified; rather, it was dependent on the other factors enumerated in the next section. The 2DE location of the proteins used for sequence analysis is indicated in Figure 1a. For most economic use of the sequencers, the sequencing of several proteins was terminated before PTH-amino acid signals were exhausted.

In the initial phase of our studies, the effects of adding more Polybrene to the PB-GF before sequencing was evaluated for four Hma proteins. Usually, an additional 1–1.5 mg of Polybrene was added. This measure doubled the initial yield and increased repetitive yield by 4–6% in the case of PB-GF. Therefore, all subsequent sequencing of the Hma proteins was done was added Polybrene. For PVDF, although satisfactory repetitive yields can be obtained without addition of Polybrene (Table III), sequencing results using standard proteins suggest that the addition of PB to the bottom layer of membrane does increase the repetitive yields (J. McDougall, unpublished results). A systematic determination of repetitive yields for all the proteins sequenced here is not possible because of (1) a marked increase in overlap and general increase in the chromatographic background after 8–12 cycles for most of the proteins sequenced and (2) numerous seryl and threonyl residues which are usually recovered in lower yield from Edman degradation and numerous glutamic and aspartic acid residues which are recovered in low yield from the PVDF support [see also Bauw et al. (1987)]. Low yields (~40–50%) of these residues are also obtained when more Polybrene is added to PB-GF directly before sequencing. Nevertheless, the range of values shown in Table III is comparable to the results we

Table II: N-Terminal Sequences of Blotted Proteins and Peptides[a]

		1				5					10					15					20					25					30				
L4	(41K)	M	Q	A	T	I	Y	D	L	D	G	N	T	D	G	E	V	D	L	P	D	V	F	E	T	P	V	R						
L5	(42K)	A	D	N	E	E	D	V	E	A	E	E	E	Y	T	E	L	T	D	I	S	G	V	G	P	R	K	A	E	S	R			
L7	(33K)	D	S	K	K	K	R	Q	R	G	S	R	T	H	G	G	G	S	H	K	N	R	R	G	A	G	H	R	G	G	N	R		
L8	(34K)	A	D	Q	E	I	E	N	A	V	S	R	A	L	E	D	A	P	E	R	N	F	R	E	T	V	D	L	A	V	N	L	R	D
L9	(29K)	P	S/R	V	E	L	E	I	P	E	D	V	D	A	E	Q	D	H	L	D	I	T	V	S/E	G	D	N	G	S	V	T			
L10	(30K)	A	G	T	I	E	V	L	V	P	G	G	E	A	N	P	G	P	P	L	G	P	E	L	G	P	T	P						
L12	(27K)	A	T	G	P	R	Y	K	V	P	M	R	R	R	R	E	A	A	T	D	Y	H	Q	R	L									
LC12	(27K)	A	R	S	A	Y	S	Y	I	R	D	A	T	K	?	P	G	D	G	?	L													
L13	(26K)	S	S	E	S	E	S	G	G	D	F	H	E	M	R	E	P	?	I	E	K	V	V											
L15	(25K)	S	K	Q	P	D	K	Q	R	K	S	Q	R	R	A	P	L	H																
L16	(21K)	M	H	A	L	V	Q	L	R	G	E	V	N	M	H	T	D	I	Q	D	T	L	E	M	L	N	I	H	H	V	N			
L17	(20K)	G	I	S	Y	S	V	E	A	D	?	D	T	T	A	K	A	M	L	R	E	R	Q	M	S	F	K							
L19	(14K)	S	K	T	N	P	R	L	S	S	L	I	A	D	L	K	S	A	A	R	S	S	G	G										
L21	(18K)	S	W	D	V	I	K	H	P	H	V	T	E	K	A	M	N	D	M	D	F	Q	N											
L4	(peptide)	V	K	S	L	L	E	A	L	D	V	H	A	D	I	D	R	A	D	E	T	K	I	K	A	G	Q	G	S	A				
L5	(peptide)	L	Q	A	R	G	L	T	E	K	T	P	D	S	L	D	E																	

[a] Apparent molecular weight (M_r) is indicated in parentheses.

Table III: Repetitive Yields (RY) for Selected Proteins

	residue	support	Polybrene (mg)	RY (%)
L4	Leu 8, 18	PVDF	0.7	92.5
	Leu 8, 18	GF	1.2	93.7
L5	Ala 1, 9, 27	PVDF	1.0	93.1
	Val 7, 22	PVDF	1.0	91.4
	Ala 1, 9	GF	0.6	93.5
L7	Ser 2, 10, 17	GF	1.3	93.7
L8	Ala 8, 12, 16	PVDF	0.7	94.0
L9	Val 3, 11, 22, 29	PVDF	1.0	90.9
	Pro 1, 8	GF	1.3	95.0
L10	Leu 7, 19, 23	PVDF	1.0	91.3
	Pro 9, 15, 21, 25	PVDF	1.0	92.5
	Tyr 6, 20	GF	1.5	95.3
C12	Ala 1, 4, 11	PVDF/GF	1.0	91.9
L16	Met 1, 13, 23	PVDF	1.0	93.7
L17	Ala 8, 14	GF	1.6	94.0
L4 (peptide 3)	Val 1, 10	PVDF	1.0	90.7
	Lys 2, 21	PVDF	1.0	89.5

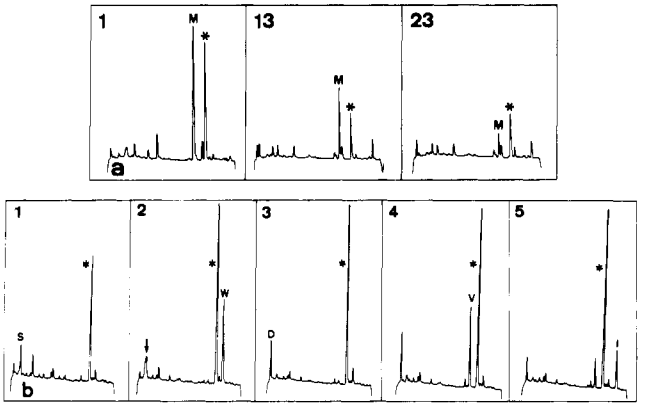

FIGURE 7: HPLC chromatograms of degradation cycles obtained from Hma proteins L16 and L21. Three methionine residues at positions 1, 13, and 23 of Hma L16 (a) and the first five cycles of Hma L21 (b) are shown. (M, S, W, D, V, and I indicate PTH-methionine, -serine, -tryptophan, -aspartic acid, -valine, and -isoleucine, respectively.) The arrow indicates a peak often seen with PTH-tryptophan. The asterisk indicates the position of DPTU. Absorbance was measured at 269 nm and the absorbance on the chromatograms set at 0.025 A_{269} unit full scale.

obtain routinely with larger proteins applied directly to precycled Polybrene-coated GF, and most of the values reported were obtained before the Edman degradation sequence program for blotted proteins was optimized (see next section). Initial yields for proteins L4, L5, and L7 were determined by submitting identical PVDF disks containing these proteins either to sequencing or to amino acid analysis for quantitation, and values of 120%, 78%, and 73%, respectively, were obtained. These values indicate that most, if not all, of the blotted protein was available for Edman degradation.

The use of additional Polybrene must be accompanied by preliminary washes of the GF or PVDF disk in the reactor, otherwise contaminant peaks in the PTH chromatograms of the first cycles obscure the identification of authentic PTH-amino acids. Washes in the reactor were carried out by using sequential deliveries of *n*-heptane, ethyl acetate, and chlorobutane. When this was done, the chromatograms of the PTH-aa from the first cycle of Edman degradation were usually comparable to the patterns obtained when HPLC-purified proteins were applied to precycled Polybrene-containing GF (see Figure 7). As well, the use of amido black for detection of proteins on PVDF gave clean first-cycle chromatograms comparable to that obtained if the protein was detected by methods other than staining as described under Materials and Methods. As reported previously (Aebersold et al., 1986), a brief ethyl acetate wash efficiently removes the DPOCC used for protein detection from GF supports, but we found it ineffective in the case of PVDF.

In addition to high initial yields obtained for blotted proteins, there are two other notable findings. First, the incidence of N-terminal blockage was low. One protein, Hma L18, did not yield an N-terminal sequence although there was 150 pmol of protein on the blot; Hma L6 did not yield a sequence possibly because the amount was not sufficient. Second, methionine was recovered at expected levels, indicating there was no significant destruction of this labile amino acid, and averaged repetitive yield for methionine at positions 1, 13, and 23 of Hma L16 was 93.7%. Tryptophan, which is reported to be especially liable to destruction during electrophoresis, was encountered once and was present at levels consistent with

little or no procedure-related destruction (Figure 7b). In contrast, Matsudaira (1987) has reported substantial destruction of tryptophan blotted from ultrathin SDS–PAGE gels. Presumably, the measures used here are effective in protecting tryptophan even when blotting is done from 2DE gels.

One of the proteins whose N-terminal sequence was determined here by sequence analysis of blots has been partially sequenced elsewhere. Subsequent to our sequence determinations, Dijk et al. (1987) recently reported an N-terminal sequence of a protein named L6 from Hma, which corresponds to our L9 protein.

Sequence Analysis of Blotted Peptides. Proteins of the 50S subunit and the 70S ribosome of Hma were separated by 2DE. The separated proteins were detected without staining by using sodium acetate or potassium chloride. Proteins were excised and after equilibration were placed in wells of a second SDS–PAGE gel of higher concentration. Limited staphylococcal V8 protease digestion of the proteins was performed (Cleveland, 1983), and the resulting peptides were then electroblotted from the gel onto multiple layers of PVDF. The binding capacity of peptides of M_r ~3000, or higher, is comparable to that of proteins. For smaller peptides, the binding capacity of the support was difficult to quantitate since the peptide band on the gel was more diffuse and therefore covers a larger area of the blotting membrane when transferred. Four sequences were obtained, two of which corresponded to the N-terminus of L4 and L5. Two additional sequences of L4 and L5 peptides are shwon in Table III.

Performance of the ABS Sequencer. Four observations were made in regard to the performance of the ABS sequencer 477A in sequence analysis of moderately large Hma proteins obtained from blots: (1) The 300-s cleavage time after the liquid delivery of TFA routinely leads to 30% overlap if a proline residue is present and 50% overlap if a proline–proline sequence or a proline–hydrophobic amino acid sequence is encountered. When the cleavage time was increased to 600 s, overlap attributable to incomplete cleavage was essentially eliminated. Protein L10, which contains 7 prolines in the first 27 residues sequenced, including the proline–proline sequence, was degraded using a 600-s cleavage throughout. (2) For the very acidic proteins of Hma, prolonging the cleavage time to 600 s caused a considerable increase of background of other amino acids, which sometimes made interpretation of PTH-aa chromatograms of long sequences difficult even with computerized methods for chromatogram analysis. The increase in background and overlap may reflect two processes, namely, acid cleavage of the protein and N–O acyl shift at seryl and threonyl residues (Thomsen et al., 1976). The tendency for acid cleavage may be exacerbated by the large number of acidic residues, often consecutive, which are found in Hma r-proteins; for example, 10 of the first 18 residues of L5 are acidic. Halobacterial proteins are also rich in threonine and serine (Rao & Argos, 1981), and the rapid rise in background seen for Hma protein L13, which has 4 four seryl residues in the first 6 amino acids, may reflect this mechanism. Apparently, the 20-s pretreatment with trimethylamine is not sufficient to reverse the shift caused by anhydrous acid in the case of proteins very rich in serine and threonine. (3) It was found that an additional delivery of phenyl isothiocyanate and trimethylamine also contributed to reduction of the overlap that occurred with larger acidic proteins. Therefore, trimethylamine was delivered four times for 350 s rather than three times for 400 s. (4) When several fragments of GF or PVDF were used, even though they were covered with an intact disk of GF or PVDF, the tendency for overlap was aggravated presumably because of nonuniform delivery of reagents and solvents. Also, the fixed volume of TFA used (~20 μL) for cleavage in the 477A sequencer may not be sufficient to wet the additional volume of support. In the situation where many pieces of support are used, vapor-phase delivery may give a more homogeneous exposure to TFA. On the other hand, the volumes of phenyl isothiocyanate and TFA used for GF may be excessive for PVDF disks, and shorter drying times after phenyl isothiocyanate and TFA treatment may further improve the performance of PVDF.

Discussion

In 1975, O'Farrell described a 2DE method which features high reproducibility and the capability to resolve most of the proteins of bacterial and mammalian cells. This 2DE procedure is now widely used in basic and clinical research. Likewise, notable advances in protein sequencing methods have occurred chiefly due to radical improvements in the design of sequencer parts, and the sensitivity has been dramatically increased as a result of the use of HPLC for detection of PTH-aa. The procedure of blotting complex mixtures of proteins from 2DE gels followed by sequencing exploits and links these two powerful methodologies. Since microsequencing requires salt-free protein samples, this is usually accomplished at present by reverse-phase HPLC. This technique gives optimal results with small proteins or protein digests but gives variable results when used with larger proteins and highly acidic and hydrophobic proteins. The gel electrophoresis methods now available, in contrast, are completely free from such limitations. When dealing with moderately sized and large proteins, it is also conventional to first cleave the protein chemically or enzymatically and to separate the peptides by HPLC. In this instance, digestion may also be very inefficient without the presence of detergent such as SDS, and many peptide peaks may require sequencing before a pure sequence is obtained. However, SDS causes problems in HPLC separations. In such cases, cleavage in SDS by the method of Cleveland (1983) followed by electrophoretic separation of the fragments and blotting to obtain N-terminal sequences also offers distinct advantages.

Choice of Support for Blotting. Eleven supports listed in Table I were evaluated here by using proteins that had not been sequenced before and standard proteins with known sequence. The supports AP-GF, QA-GF, and DITC-GF function well in blotting applications but have low protein binding capacity. However, this is not disadvantageous when only low picomole amounts of individual proteins are being electroblotted as from 2DE gels of total cellular proteins. These supports have the further advantage that several picomoles of proteins can be detected with DPOCC. This is a better sensitivity than achievable with fluorescamine on PB-GF and appears to carry no risk of N-terminal blockage. However, initial yields of only 15–25% and repetitive yields of less than 90% have been reported with AP-GF and QA-GF although better performance with the addition of Polybrene to the supports before sequencing has been observed (Yuen et al., 1986). If fluorescent Edman reagent homologues (Jin et al., 1986) prove useful in automated gas-phase sequence analysis, then supports to which proteins are covalently bound will be essential since it is necessary to extract the fluorescent phenylthiocarbamyl amino acids from the glass filters under more stringent conditions than used in routine gas-phase sequence analysis, which often solubilizes the protein or peptide if it is not covalently attached (M. J. Walsh, unpublished results). In this context, diazotized GF may also be quite useful pro-

viding a stable, multipoint attachment for the sample but may entail some risk of N-terminal blockage.

When higher binding capacity is required, PB-GF or PVDF are now the supports of choice. One disadvantage of PB-GF is the relative difficulty of clearly visualizing some protein spots with DPOCC; in this case, it may be better to use fluroescamine. The use of additional Polybrene for PB-GF directly before inserting the sample in the reactor is undoubtedly beneficial in obtaining higher repetitive yields in the proteins we have studied.

In examining PVDF for its utility in blotting–sequencing applications, a high protein binding capacity is routinely obtained for all categories of proteins. The support is commercially available and easy to handle, and detection of proteins at a sensitive level is possible with either DPOCC or amido black stain without significant N-terminal blockage. However, DPOCC does cause some interference with the first few PTH-amino acid chromatograms. Direct visualization of proteins or peptides by the drying method outlined under Materials and Methods is still preferred, after which the blots are stained with amido black. Modification of the sequencer programs for instrumental Edman degradation as described here and addition of Polybrene (Matsudaira, 1987) may give better results. Like PB-GF, with PVDF it is generally possible to determine at least the first 10–30 residues blotted from SDS gels, and it is also stable during protein hydrolysis for amino acid determinations. Although the PVDF membrane's ease of use and relatively high protein binding capacity will, no doubt, make it a popular sequencer-stable support, the development of new generations of supports that will give improved repetitive yields and an increased binding capacity are still required. As well, because of the membrane's high hydrophobicity, quantitative elution of proteins from the membrane for further analysis, without the use of detergents such as SDS, has not been possible. If protein elution from the blotting membrane is required, non-sequencer-stable supports such as nitrocellulose and the nylon-based supports may be a better choice.

For blotting under acidic conditions such as from IEF gels and acetic acid–urea gels, PVDF and four activated supports were tested and gave similar results (Table I). In summary, suitable methods now exist for blotting all types of proteins from all gel systems in current use.

Prevention of N-Terminal Blockage. A considerable number of precautions were routinely taken in the preparation of the samples sequenced here. 2-Mercaptoethanol was present in all buffers used for the preparation of ribosomes and subunits and during the dialysis of the protein extracts. IEF gels were prerun, and the sample buffers for the prerun and IEF with sample contained methylamine and thioglycolic acid. SDS was recrystallized, and all SDS gels were polymerized on the day before use and were also preelectrophoresed. All gel solutions were degassed in order to reduce the amount of catalyst needed for polymerization. Several groups have reported taking none or few precautions yet have obtained impressive sequencing results whereas other authors reported that omission of precautions leads to total destruction of tryptophan and methionine and a high incidence of N-terminal blockage (Hunkapiller et al., 1983; Aebersold et al., 1986). Our results show that the measures used here gave excellent recovery of authentic methionine and tryptophan and no evidence for procedure-related N-terminal blockage. We also conclude from these results that there now exists a generally useful procedure for blotting proteins from 2DE gels with minimal risk of modification or significant N-terminal blockage. The implications of these results are also clear in regard to selection of an appropriate strategy for protein purification. Since the resolution of gel electrophoresis exceeds all current methods for separation of complex mixtures of proteins by orders of magnitude and is completely nonrestrictive in regard to protein size, charge, or hydrophobicity, than it is clear that when precautions such as we have utilized here are used, it should often be possible to bypass less reliable and lower resolution methods for protein isolation such as HPLC in order to obtain N-terminal sequences of proteins.

Sequence Analysis of Hma r-Proteins. The results reported here indicate the suitability of blotting–sequencing methodology for the analysis of Hma r-proteins. A prerequisite for such an approach to obtaining N-terminal sequences is the availability of a high-resolution 2DE method for the separation of Hma proteins. The O'Farrell procedure is such a method and gives excellent resolution of all proteins of the Hma 70S ribosome and ribosome subunits. The O'Farrell procedure is also advantageous when used in conjunction with sequencing applications since extensive prerunning with charged reducing agents results in no significant loss of resolution. Thus, gels can be cleared of free radicals, other oxidants, and charged moieties which may cause N-terminal modification or destruction of sensitive amino acids. The sequences obtained for the 14 proteins reported here are sufficiently long to design oligonucleotide probes to isolate the genes coding for these proteins and thus derive complete sequences by molecular cloning. These partial sequences in conjunction with the high-resolution 2DE method used here for the separation of Hma proteins will also be useful for the identification of heavy atom labeled proteins in X-ray analysis of Hma ribosome crystals as well as for identification of specific protein components of cross-links of Hma ribosomes. Already, partial sequences obtained from protein and peptide blots have proved of value in both the characterization of RNA–protein complexes of the extreme halophiles and the synthesis of oligonucleotide probes for multiple regions along the length of halophilic r-protein genes (J. McDougall, unpublished results).

Applications of Blotting–Sequencing Methodology. Much discussion has been devoted recently to the use of 2DE of total cellular extracts in conjunction with blotting and sequencing to identify molecular correlates of biologic states and disease processes (Aebersold et al., 1986). Since optimized 2DE separations of total cellular extracts can resolve several thousand proteins, each characterized by unique p*I* and apparent molecular weight, the eventual objective is the ability to sequence any unique protein spot seen on a 2DE gel that may be a correlate of a biological state. Often, however, 2DE analysis of total protein extracts shows changes that are a response to alterations in cell metabolism but are not necessarily clues to etiology or pathogenesis. Changes in levels of growth factors which may be pivotal in physiological regulation or in disease will not be identifiable by 2DE systems that resolve a few thousand proteins. For this reason, we feel the methods used here will be more generally useful when used in conjunction with some preliminary enrichment for proteins of interest such as can be obtained by gradient centrifugation (as done here for Hma r-proteins) or by methods such as lectin and immunoaffinity chromatography. There are now available panels of monoclonal and polyclonal antibodies to hundreds of unique but structurally uncharacterized proteins. In this context, the methods described here for blotting followed by sequencing may be especially useful since even a single passage of a total cell extract through an antibody affinity column will often give sufficient enrichment to permit separation of the

protein of interest from contaminants to allow sequencing after 1D or 2DE separation of the bound proteins. Likewise, subcellular organelles and membranes such as myelin membrane or synaptic vesicles can be enriched for by one of a few centrifugation steps. When followed by 2DE separation, it may often be possible to obtain N-terminal sequences of all the major proteins of such structures from one or a few 2DE gels. In this way, organelle-specific libraries of N-terminal sequences could be constructed analogous to cDNA libraries for whole tissues or organisms. Such libraries would constitute nonoverlapping data bases to define the mRNA and protein complexity of living organisms. As a general guideline, it is now clear that proteins that are present in amounts that can be visualized by Coomassie Blue staining are present in sequencable amounts or may yield peptides by the methods used here to obtain useful sequence information. The availability of such reliable methods, which may attain even higher sensitivity with fluorescent Edman reagents (Jin et al., 1985), should be very useful in molecular biology and in characterizing the molecular basis of disease.

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Registry No. PVDE, 24937-79-9; TFA, 76-05-1; potassium hydroxide, 1310-58-3; hydrochloric acid, 7647-01-0; hydrogen fluoride, 7664-39-3; polybrene, 28728-55-4.

REFERENCES

Aebersold, R. H., Teplow, D. B., Hood, L. E., & Kent, S. B. H. (1986) *J. Biol. Chem. 261*, 4229–4238.

Bauw, G., De Loose, M., Van Montagu, M., & Vandekerckhove, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4806–4810.

Brockmöller, H. J., & Kamp, R. M. (1986) in *Advanced Methods in Protein Microsequence Analysis* (Wittmann-Liebold, Springer-Verlag, Heidelberg and New York.

Cleveland, D. W. (1983) *Methods Enzymol. 96*, 222–229.

Dijk, J., Van Den Broek, R., Nasiulas, G., Beck, A., Reinhardt, R., & Wittmann-Liebold, B. (1987) *Biol. Chem. Hoppe-Seyler 368*, 921–925.

Fling, S. P., & Gregerson, D. S. (1986) *Anal. Biochem. 155*, 83–88.

Gorman, J. J. (1987) *Anal. Biochem. 160*, 376–387.

Hardy, S. J. S., Kurland, C. G., Voynow, P., & Mora, G. (1969) *Biochemistry 8*, 2897–2905.

Hunkapiller, M. W., Lujan, E., Ostrander, F., & Hood, L. E. (1983) *Methods Enzymol. 91*, 227–237.

Jin, S. W., Chen, G. X., Palacz, Z., & Wittmann-Liebold, B. (1986) *FEBS Lett. 198*, 150–154.

Kamp, R. M., & Wittmann-Liebold, B. (1988) *Methods Enzymol.* (in press).

Kimura, J., & Kimura, M. (1987) *J. Biol. Chem. 262*, 12150–12157.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Makowski, I., Frolow, F., Saper, M. A., Shoham, M., Wittmann, H. G., & Yonath, H. (1987) *J. Mol. Biol. 193*, 819–822.

Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.

Oakley, B. R., Kirsch, D. R., & Morris, N. R. (1980) *Anal. Biochem. 105*, 361–363.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

O'Farrell, P. Z., Goodman, H. M., & O'Farrell, P. H. (1977) *Cell (Cambridge, Mass.) 2*, 1133–1142.

Rao, J. K. M., & Argos, P. (1981) *Biochemistry 20*, 6536–6543.

Reimann, F., & Wittmann-Liebold, B. (1986) in *Advanced Methods in Protein Microsequence Analysis* (Wittmann-Liebold, B., Salnikow, J., & Erdmann, V., (Eds.) pp 118–125, Springer-Verlag, Heidelberg and New York.

Seed, B. (1982) *Nucleic Acids Res. 10*, 1799–1801.

Shevack, A., Gewitz, H. S., Hennemann, B., Yonath, A., & Wittmann, H. G. (1985) *FEBS Lett. 184*, 68–71.

Strom, A. R., & Visentin, L. P. (1973) *FEBS Lett. 37*, 274–280.

Thomsen, J., Bucher, D., Brunfeldt, K., Nexo, E., & Oleson, H. (1976) *Eur. J. Biochem. 69*, 87–96.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Vandekerckhove, J., Bauw, G., Puype, M., Van Damme, J., & Van Montagu, M. (1985) *Eur. J. Biochem. 152*, 9–19.

Visentin, L. P., Chow, C., Matheson, A. T., Yaguchi, M., & Rollin, F. (1972) *Biochem. J. 130*, 103–110.

Weetal, H. H. (1976) *Methods Enzymol. 44*, 134–148.

Wittmann, H. G. (1986) in *Structure, Function, and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 1–27, Springer-Verlag, Heidelberg and New York.

Wittmann-Liebold, B., Ashman, K., & Dzionara, M. (1984) *Mol. Gen. Genet. 196*, 439–448.

Yonath, A., Saper, M. A., & Wittmann, H. G. (1986) in *Structure, Function, and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 112–127, Springer-Verlag, Heidelberg and New York.

Yuen, S., Hunkapiller, M. W., Wilson, K. J., & Yuan, P. M. (1986) *Applied Biosystems User Bulletin*, Issue No. 25.