Covalent Immobilization of Proteins for High-Sensitivity Sequence Analysis: Electroblotting onto Chemically Activated Glass from Sodium Dodecyl Sulfate–Polyacrylamide Gels†

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ABSTRACT: We report a new method for the preparation of proteins in a form suitable for high-sensitivity N-terminal amino acid sequence analysis. Proteins separated by polyacrylamide gel electrophoresis were electrophoretically transferred onto glass fiber filter paper chemically activated by the introduction of phenyl isothiocyanate functional groups. The proteins became covalently coupled to the matrix during the electrotransfer process. Bands containing transferred proteins were detected by fluorescent staining or autoradiography, cut out from the glass fiber filter, and directly loaded into the cartridge of a gas-phase sequenator. The covalent nature of the interactions between protein and glass fiber support permitted the use of more vigorous solid-phase sequencing protocols and of alternative sequencing reagents. This high-efficiency isolation and covalent coupling method provides the essential first step toward enhanced-sensitivity protein sequence analysis. The method has been successfully applied to the isolation of a wide variety of proteins from SDS–polyacrylamide gels, and was shown to be compatible with both the standard Edman reagent phenyl isothiocyanate and alternative sequencing reagents such as 4-(*N*,*N*′-dimethylamino)azobenzene-4′-isothiocyanate (DABITC).

High-sensitivity protein sequence analysis is an indispensable tool in modern biological research. In addition to the relatively abundant traditional targets of protein sequence analysis such as structural proteins, enzymes, and serum proteins, there has been considerable recent interest in proteins available only in much smaller amounts. These proteins include cell membrane receptors, growth factors, peptide hormones, and regulatory proteins as well as proteins involved in intracellular signal transduction. The complete primary structure of these low-abundance proteins is frequently determined by using a combination of protein chemical and recombinant DNA methods, limited protein sequence information serving as a key to the identification and isolation of the corresponding gene from appropriate gene libraries (Ullrich et al., 1985).

Increased sensitivity in protein sequencing has been achieved by an iterative process in which each limiting step was systematically improved. This has resulted in a sensitivity increase of several orders of magnitude in some 3 decades and climaxed in the introduction of the gas-phase sequenator which allowed the sequence analysis of polypeptides at a level of 10–20 pmol of sequenceable protein (Hewick et al., 1981). High-yield purification of such small amounts of protein in a form suitable for sequence analysis is not readily achieved with classical protein separation techniques and has presented a considerable technical challenge. Recently, efficient methods for the direct isolation of microgram or submicrogram amounts of proteins in a form suitable for N-terminal (Kent & Aebersold, 1984; Vandekerckhove et al., 1985; Aebersold et al., 1986; Matsudaira, 1987) or internal sequence analysis (Aebersold et al., 1987) have been developed.

The practical UV detection limit for PTH derivatives, the final product of the Edman degradation, is on the order of 1 pmol and currently limits the sensitivity of protein sequence analysis to an initial signal of 2–5 pmol. Several attempts to enhance detectability of the final cleavage product in automated or manual Edman degradation with radiolabeled, chromophoric, or fluorescent reagents have had only limited success (Silver & Hood, 1975; Ender et al., 1984; Chang, 1977; Salnikow et al., 1982; Muramoto et al., 1984; Jin et al., 1986). Pure radiolabeled sequencing reagents of high specific activity are difficult to produce, and the amount of radioactivity required is prohibitively great for routine use. For this reason, it is desirable to use Edman reagents with inherently greater sensitivity of detection. However, the physicochemical characteristics of chromophoric and fluorescent sequencing reagents are sufficiently different from PITC that a drastically altered degradation chemistry is required. In particular, chromophoric and fluorescent sequencing reagents are generally solid compounds, and therefore these reagents need to be delivered as solutions in inert, high-boiling solvents. Furthermore, highly efficient washing and extraction procedures with strong solvents are necessary to reduce background contamination. The key to the practical implementation of high-sensitivity degradation cycles incorporating these principles is the covalent coupling of polypeptides to a solid support.

Solid-phase sequencing technique was pioneered by Laursen (1971) for nanomole-level sequencing but has not been exploited for microsequencing. Solid-phase sequencing methods have long been limited by problems of handling and immobilizing small amounts of proteins, so that to date the full sensitivity potential of this approach could not be explored. In this paper, we describe a method for the preparation of a chemically activated glass fiber support and its use for the electrophoretic transfer and covalent coupling of picomole amounts of proteins separated by polyacrylamide gel electrophoresis. Proteins isolated this way are suitable for use with solid-phase or other Edman degradation protocols. The general

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utility of the method is demonstrated by sequence data from a variety of proteins isolated from sodium dodecyl sulfate (SDS)[1]–polyacrylamide gels. The method is compatible with drastically altered degradation chemistry and opens the door to the use of alternative sequencing reagents with enhanced detectability, thus potentially overcoming the current sensitivity-limiting step in protein sequence analysis.

Experimental Procedures

Materials

The glass filter paper used was Whatman GF/F (Whatman, Ltd., Maidstone, England). This is available in large rectangular sheets or in large circles. Trifluoroacetic acid (TFA) was purchased from Halocarbon Chemicals (Hackensack, NJ) and was glass redistilled before use. *CAUTION: Because of the extremely corrosive nature of TFA, all manipulations involving this compound should be performed in an efficient fume hood using Neoprene full-length gloves and apron, together with face and eye protection.* (3-Aminopropyl)triethoxysilane was obtained from Pierce Chemical Co., Rockford, IL. DITC (1,4-phenylene diisothiocyanate) was purchased from Kodak, Rochester, NY. Diisopropylethylamine and *N*-ethylmorpholine were obtained from Sigma Chemical Co., St. Louis, MO. Fluorescent stain, 7-(diethylamino)-3-[4-[(iodoacetyl)amino]phenyl]-4-methylcoumarin (DCIA), was from Molecular Probes, Junction City, OR. Acrylamide and bis(acrylamide) were purchased from National Diagnostics, Highland Park, NJ. All other polyacrylamide gel electrophoresis chemicals were of electrophoresis grade. Reagent-grade dithiothreitol and dimethylformamide (DMF) were purchased from Aldrich, Milwaukee, WI. A standard blotting apparatus, the Bio-Rad "Trans-Blot" cell, and a Model 250/2.5 constant-voltage power supply were used. Proteins were ^{125}I labeled by the chloramine-T method (Hunter & Greenwood, 1962). Activities of radiolabeled proteins were determined by γ scintillation counting. All other chemicals were of analytical reagent grade. Auro-dye colloidal gold protein staining solution was obtained from Janssen Life Science Products, Beerse, Belgium.

Methods

SDS–Polyacrylamide Gel Electrophoresis. Minigels (8 cm × 8 cm × 0.5 mm) were run essentially according to Laemmli (1970). Modified procedures and specific precautions to minimize amino-terminal blocking of proteins were applied as described (Hunkapiller et al., 1983), except that no thioglycolate was used.

In addition, acrylamide was batch-tested in the following fashion for the absence of contaminants which blocked amino groups. An aliquot of a ^{125}I-labeled preparation of α-lactalbumin yielding an initial PTH signal of 10–20 pmol was directly applied onto a Polybrene-coated glass fiber disk and inserted into the sequenator cartridge. An identical aliquot was electrophoresed in an SDS–polyacrylamide gel prepared from the acrylamide batch to be tested, electroblotted onto charge-modified GF/F glass fiber sheets, and sequenced after detection of the protein band on the electroblot (Aebersold et al., 1986). The specific initial signal (picomoles per 1000 cpm) was determined in both sequencing runs. Acrylamide batches causing less than 10% amino-terminal blocking were accepted for electroblotting experiments.

Preparation of DITC–GF/F Sheets. (*A*) *Acid Etching.* Whatman GF/F filters (11-cm diameter circles, or rectangular sheets cut to size) were etched in neat TFA for 1 h at room temperature on a rotating platform and dried on Whatman 3 paper in a fume hood as previously described (Aebersold et al., 1986).

(*B*) *Aminopropyl-GF/F.* One or two acid-etched filters were placed in plastic bags containing 50 mL of 2% (v/v) (aminopropyl)triethoxysilane in H_2O and heat sealed. Incubation was carried out at room temperature for 3–5 h on a rocker table. Derivatized filters were washed 10 times (5 min each) in acetone on a shaking platform to remove excess reagent. Curing of silane linkages was achieved by drying the damp-derivatized sheets on Whatman 1 paper in an oven for 45 min at 110 °C. The quantitative ninhydrin reaction (Sarin et al., 1981) was used to assay the amount of free 3-aminopropyl groups on the glass filters as follows: a circular sample disk of 1-cm diameter cut out from the GF/F sheet was weighed and placed at the bottom of a 100 x 7.5 mm glass test tube. Twenty-five microliters of reagent A and 50 μL of reagent B and 25 μL of reagent C were added. [Reagent A was prepared by mixing 40 g of reagent-grade (unstabilized) phenol with 10 mL of absolute ethanol. The mixture was warmed until the phenol dissolved, stirred with 4 g of Amberlite mixed-bed resin MB-3 for 45 min, and filtered. Reagent B was prepared by dissolving 65 mg of KCN in 100 mL of water. Twelve milliliters of the KCN solution was diluted to 100 mL with pyridine (freshly distilled from ninhydrin). The solution was stirred with 4 g of Amberlite mixed-bed resin MB-3 and filtered. Reagent C was prepared by dissolving 2.5 g of ninhydrin in 50 mL of absolute ethanol. Reagent C was stored in a wrapped bottle to exclude light.] To another tube were added only the reagents. Both tubes were mixed well, and care was taken to completely immerse the glass fiber disks. Both tubes were put into a heating block at 100 °C. After 10 min, 3 mL of 60% ethanol/water was added to each tube, and the product, Rhueman's purple, was dissolved by extensive vortexing. The absorbance of the sample supernatant was measured against the reagent blank supernatant at 570 nm and the amount of amine calculated (Sarin et al., 1981). Typical quantities of bound amino groups were between 12 and 24 nmol/mg of glass. Aminopropyl-GF/F sheets prepared in this way could be used for the preparation of proteins for N-terminal sequence analysis by electroblotting after SDS–PAGE (Aebersold et al., 1986), or for further derivatization to DITC–GF/F.

DITC–glass filters were prepared by modifying the procedure of Wachter (Wachter et al., 1973). One sheet of dried aminopropyl-GF/F was incubated at 37 °C overnight on a rocker platform in a sealed plastic bag containing a 10-fold molar excess of DITC over amino groups dissolved in 50 mL of diispropylethylamine/DMF (1:9 v/v). The product DITC–GF/F sheets were washed on a shaking platform, 3 times in MeOH and 7 times in acetone, and finally dried on Whatman 1 paper at 37 °C for 10 min. Each washing cycle was for 5 min. Residual amino groups on the DITC–glass filters were quantitatively assayed by the ninhydrin reaction as described above. Typical values indicated a 40–60% reduction in amino groups compared to the starting aminopropyl-GF/F.

Electroblotting onto DITC–GF/F Sheets. SDS–polyacrylamide gels were removed from glass plates and immersed into transfer buffer, 25 mM *N*-ethylmorpholine hydrochloride,

[1] Abbreviations: TFA, trifluoroacetic acid; DITC, 1,4-phenylene diisothiocyanate; DCIA, 7-(diethylamino)-3-[4-[(iodoacetyl)amino]-phenyl]-4-methylcoumarin; DMF, dimethylformamide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

pH 8.3. The blotting sandwich was assembled as previously described (Aebersold et al., 1986). Electroblotting was carried out at 50 V (200–400 mA) at 37 °C for 2 h, for 12.5% total acrylamide, 0.5 mm thick gels. Thicker gels or higher percent total acrylamide required somewhat longer transfer times.

Covalent Coupling of Proteins to DITC–GF/F from Solution. Forty microliters of the protein-containing solution was spotted onto DITC–GF/F disks of 1-cm diameter. The disk was air-dried, and if necessary, additional portions of the protein solution were spotted and dried. For coupling, the disk was placed at the bottom of a 17 × 100 mm plastic tube with approximately 1 mL of pH 8.3/25 mM *N*-ethylmorpholine buffer and covered. Alternatively, 50 μL of coupling buffer was added to the disk and coupling was performed in a water-saturated atmosphere. Coupling was continued for 2 h at 37 °C. It was important to maintain a pH of 8.3–9.0 during the coupling process. To determine the degree of covalent coupling, ^{125}I-radiolabeled proteins were coupled as described above, and after completion of the coupling reaction, the disks were extensively washed with TFA and DMF in a small sintered glass funnel connected to a water aspirator. Covalent coupling yield was calculated from the number of trichloroacetic acid precipitable counts applied to the disk and the number of counts remaining on the disk after the washing process.

Protein Detection. Staining of proteins electroblotted onto DITC–GF/F was accomplished through the use of the sulfhydryl-specific fluorescent reagent DCIA. Either cysteines were maintained in a reduced state after electrophoresis under reducing conditions by addition of 0.5 mM DTT to the transfer buffer or after electrophoresis and electroblotting under nonreducing conditions proteins were reduced on the DITC–GF/F sheet by incubating the sheet in 100 mL of transfer buffer containing 10 mM DTT at 37 °C for 10 min. Before being stained, filters were rinsed 3 times with distilled H_2O. One or two filters were stained at one time using 0.01% DCIA (w/v) in 200 mM Na_2HPO_4/propanol, 60:40 v/v, pH 7.0. DCIA was first dissolved with 100–200 μL of DMF and then added to the staining buffer on a shaking platform for 20 min or until bands were visible under longwave UV light. Filters were destained by rinsing 4 times in fresh MeOH.

Storage. DITC–glass filters were kept at room temperature, in plastic bags in a vacuum desiccator under nitrogen, for up to several months before use. Electroblotted proteins were excised and loaded into a sequenator, or the protein-containing bands were stored in plastic Eppendorf tubes at −20 °C for up to several months before sequencing. The whole stained blots could also be stored at −20 °C for weeks before proteins were cut out and sequenced.

Sequencing of Covalently Immobilized Proteins. Protein bands, detected after fluorescent staining or by autoradiography, were cut out of the glass fiber sheet and placed in the cartridge of a gas-phase sequenator (Hewick et al., 1981) without any further treatment. Protein sequence determination was performed as described (Aebersold et al., 1986), except that the sequencing program was started with an extensive S2 (ethyl acetate) washing step directly followed by the first coupling with Edman reagent. Typically, 20–30% of N-terminal amino groups were coupled to the DITC–GF/F, so that exposure to TFA prior to the coupling step would lead to cyclization and cleavage of the derivatized N-terminal amino acid, generating a "ragged" sequence corresponding to the amount of N-terminal modified by reaction with the DITC–GF/F. HCl/methanol-converted PTH derivatives were analyzed by HPLC on IBM cyano columns as described (Hunkapiller & Hood, 1983). PTH-amino acids were quantitated by peak integration in a data acquisition/data processing system developed in our laboratory (Kent et al., 1987).

Results and Discussion

Activation and Characterization of the Support. The principles of electroblotting of proteins for direct sequence analysis have been described previously. After the introduction of this concept (Kent & Aebersold, 1984), a variety of supports have been used for electroblotting of proteins for sequence analysis. These include covalently modified glass fiber sheets (Aebersold et al., 1986), glass fiber sheets coated with polybrene or polybases (Vandekerckhove et al., 1985), and sheets of the synthetic polymer poly(vinylidine difluoride) (PVDF) (Matsudaira, 1987). On all of these matrices, proteins are adsorbed noncovalently, presumably by hydrophobic and/or ionic interactions. We decided to develop a new matrix, on which the proteins were covalently bound during the electroblotting process, for the following reasons: (i) Proteins covalently coupled to a chemically inert support are compatible with solid-phase degradation chemistry (Laursen, 1971). This type of sequence analysis offers greater flexibility in the choice of conditions for the Edman degradation. More stringent washes with strong solvents to remove contaminants, excess reagents, and reaction byproducts are possible. (ii) Post-translationally modified amino acids such as phosphorylated residues can be readily extracted from the cartridge for analysis. (iii) The use of alternative sequencing reagents can be explored.

In the past, a variety of procedures for the covalent coupling of proteins from a solution onto solid supports have been proposed (Laursen, 1971, 1977; Machleidt & Wachter, 1977; Horn & Laursen, 1973). Of these procedures, coupling of proteins by their α-amino groups and the ε-amino groups of lysine side chains to controlled-pore glass beads modified with DITC has been the most generally applicable method. This procedure is also relatively insensitive to the chemical composition of the coupling solution as long as a basic pH value is maintained and there is no excess primary amine. However, it was very difficult to obtain pure protein in a form suitable for coupling without excessive losses. This was particularly true for very small amounts of proteins. We felt we could adapt the electroblotting procedure to the efficient covalent attachment of microgram or submicrogram amounts of proteins without excessive losses. Therefore, we adapted the chemistry for the generation of DITC–CPG to the production of DITC–glass fiber sheets. The chemistry of activation is schematically shown in Figure 1.

To reproducibly obtain high-performance electroblotting supports, good quality control on the preparation of chemically activated glass fiber paper was essential. We used the quantitative ninhydrin determination of free amine (Sarin et al., 1981) to monitor the quality of DITC–GF/F and the intermediates involved in its production. From each sheet, three aliquot disks of 1-cm diameter were cut out at the following stages of preparation of the support: (i) after TFA etching; (ii) after derivatization with (aminopropyl)triethoxysilane; and (iii) after derivatization of the aminopropyl-GF/F with DITC. At stage 1, no amino groups should be detectable. At stage 2, best results were obtained with approximately 20 nmol of primary amino groups/mg of glass fiber matrix. At stage 3, typically about 50% of the amino groups present after the second stage were derivatized with DITC. In addition, 2-mL aliquots of the last two acetone washing solutions after the aminopropyl derivatization were dried down and analyzed by the ninhydrin reaction. They

Table I: Yields of Electrophoretic Transfer and Covalent Attachment

protein[a]	$M_r \times 10^{-3}$	pI	protein on blot (%)	protein in gel (%)	covalently coupled (% of protein transferred)	covalently coupled (% of protein loaded onto gel)
horse heart cytochrome *c*	12	9.6	31	38	98	30
equine myoglobin	17	8.3	83	18	96	80
soybean trypsin inhibitor	21	4.5	61	40	96	59
bovine carbonic anhydrase	29	6.0	98	<1	91	89
bovine serum albumin	67	4.9	105	<1	96	100

[a] Ten micrograms of protein loaded onto the gel.

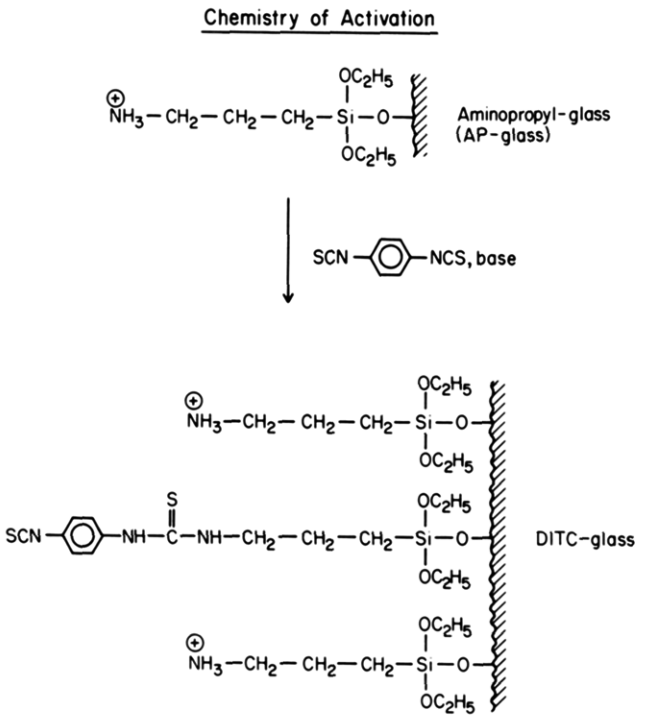

FIGURE 1: Chemistry of activation. 3-(Aminopropyl)triethoxysilane was used to produce glass fiber sheets containing aminopropyl groups. Aminopropyl-glass could be further derivatized with *p*-phenylene diisothiocyanate (DITC) to yield glass fiber sheets containing reactive aryl isothiocyanate functional groups. Aminopropyl-glass can serve as an electroblotting substrate capable of noncovalent interactions with proteins and is compatible with gas-phase sequencing chemistry. Proteins may be covalently linked to DITC–glass fiber supports. DITC–glass is compatible with gas-phase and solid-phase sequencing chemistries.

should not contain any detectable quantities of amino groups.

The extent of aminopropyl functionalization of the TFA-etched filters depended on the reaction time, the amount of water present, and the temperature at which the reaction was carried out. The degree of derivatization of aminopropyl groups with DITC was a function of the DITC concentration, reaction time, reaction temperature, and pH. Conditions which in our hands reproducibly yielded good DITC–GF/F are described under Methods. For good electroblotting results, it was essential that a substantial amount of aminopropyl groups remained underivatized in the final DITC–GF/F blotting substrate. The positively charged aminopropyl groups initially trapped the SDS-coated protein by ionic interactions until the slower formation of the covalent linkage occurred (see below). Residual amino groups apparently did not react with the isothiocyanate moieties even if a high molar excess of DITC over the amino groups was used. It is not clear whether this is due to steric reasons or to some form of chemical protection (Schiff base formation or protonation). For the production of DITC–glass fiber sheets, we routinely used a 10-fold molar excess of DITC over the amino groups present on the filter.

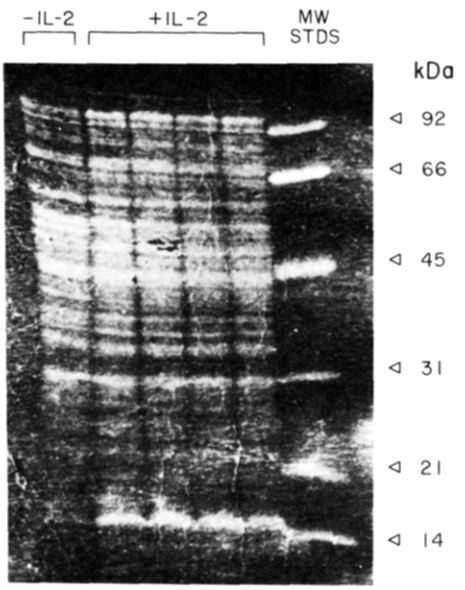

FIGURE 2: Electroblotting of *E. coli* whole cell lysate onto DITC–GF/F. Crude whole cell lysates of *E. coli* containing the IL-2 coding plasmid (+IL-2) or a control strain without the plasmid (–IL-2) were analyzed by SDS–PAGE (12.5% total acrylamide). The separated proteins were electroblotted onto DITC–GF/F and stained with the fluorescent stain DCIA. After being destained, the blot was photographed after destaining on a long-wavelength UV transilluminator. The protein band of ~15 kDa represents IL-2. The right lane contains molecular weight standards.

Using a higher molar excess of DITC over the amino groups did not change the amount of underivatized amino groups and had no effect on the coupling capacity of the paper.

Electroblotting. We have used electroblotting onto DITC–GF/F to prepare a wide variety of proteins for sequence analysis. Figure 2 shows the stained blot obtained from an experiment in which proteins in a total *Escherichia coli* cell lysate were separated by SDS–PAGE. The intense staining of protein bands on the electroblot over the entire separation range of the gel and the absence of residual protein in the gel after electroblotting (as determined by Coomassie blue staining) showed that most proteins were transferred efficiently.

Transfer yields were quantitatively determined by measuring the transfer of ^{125}I-radiolabeled proteins ranging in size from 12 000 to 68 000 daltons (Table I). Transfer yields ranged from 31% to 100% and did not show a significant correlation with the molecular weight of the protein. Very basic proteins such as horse heart cytochrome *c* were difficult to efficiently transfer under these conditions. Conditions to improve transfer yields of these proteins are discussed below. These quantitative transfer data were in general agreement with the qualitative results shown in Figure 2.

A critical aspect of electroblotting onto DITC–GF/F is the degree of covalent coupling. We investigated this using ^{125}I-radiolabeled proteins. Test samples were separated by

SDS–PAGE and electroblotted onto DITC–GF/F, and protein bands were detected by fluorescent staining with DCIA. Protein-containing regions were cut out of the blots, and the bands were counted by γ-radiation counting. The glass fiber disks were then extensively washed with TFA and DMF, and the degree of protein retention was determined by γ-radiation counting after the washing procedure. Results are shown in Table I. The term covalent coupling is used here as a strictly operational term, describing the fact that proteins were retained on DITC–GF/F under washing conditions which efficiently removed the same proteins from aminopropyl-GF/F. The degree of covalent attachment varied from 91% to 98% of the total transferred protein. Even the smaller proteins near the lower end of the SDS–PAGE separation range coupled efficiently. These proteins on the average contain a lower number of amino groups available for the coupling to DITC–GF/F than larger proteins. Furthermore, there was no obvious correlation between the coupling yield and the isoelectric point (pI) of the proteins (Table I).

The success of the blotting experiment was strongly dependent on the transfer conditions. For high-efficiency covalent coupling, it was essential that the protein was initially immobilized on the DITC–GF/F filter by noncovalent interactions, to allow the slower formation of the covalent linkage to take place. Conditions which interfered with this initial trapping, such as SDS in excess of 0.01% in the transfer buffer, resulted in low protein recoveries. This was demonstrated in the following electroblotting experiment. ^{125}I-radiolabeled equine myoglobin and bovine carbonic anhydrase were separated on an SDS–polyacrylamide gel and electroblotted onto DITC–GF/F in the presence of 0.5% SDS. Transferred protein bands were detected by fluorescent staining. After extensive washing of the protein-containing bands with liquid TFA and DMF, 44% and 46% of the protein originally present in the gel band, respectively, were recovered in the blotted bands. This represents an approximately 2-fold reduction of the protein recovered after electroblotting without excess SDS in the transfer buffer (cf. Table I). A pH value of the transfer buffer significantly above the pK_a of the aminopropyl groups (pK_a $\sim$10) also resulted in low recoveries (P. Vo, personal communication).

Electroblotting onto DITC–GF/F was performed at 37 °C without noticeable effects on the sequenceability of transferred proteins. At 37 °C, the rate of formation of the covalent linkage was enhanced as compared to room temperature, and thus the time of electroblotting experiments could be reduced.

The low transfer efficiency observed with very basic or very insoluble proteins could be attributed to two factors: a low negative charge density of the protein and the (partial) stripping of the SDS coat from the protein. Basic proteins, with isoelectric points higher than the pH of the transfer buffer, will contain a net negative charge during the electroblotting experiment only if the SDS coat provided additional negative charges. Proteins insoluble in aqueous environments (e.g., membrane proteins) tended to precipitate in the gel as soon as the SDS concentration was significantly reduced. Even after prolonged electroblotting, such precipitated proteins could not be efficiently transferred. To obtain enhanced transfer rates of these basic or insoluble proteins, stripping of the SDS coat should therefore be prevented. This was best achieved by omitting methanol, which is known to remove SDS from proteins, from the transfer buffer and by adding up to 0.01% SDS to the transfer buffer during the electroblotting. The addition of such small amounts of SDS to the transfer buffer did not show any noticeable effect on the initial trapping of the molecules on the DITC–GF/F, but did result in substantially improved electroblotting yields for basic and hydrophobic proteins.

Detection of Proteins. Proteins electroblotted onto DITC–GF/F were detected by staining with the fluorescent dye DCIA which specifically reacts with sulfhydryl groups and had a detection limit on the order of 100–200 ng of protein per band, under long wavelength UV light. Detection sensitivity was dependent on the number of sulfhydryl residues per protein chain. Proteins electroblotted from nonreducing gels needed to be reduced before the staining process, and a slightly reducing milieu was maintained during the electroblotting process even after electrophoresis under reducing conditions (0.5 mM DTT). DCIA, as other sulfhydryl-reactive dyes, was not absolutely sulfhydryl-specific so that even proteins without cysteine residues could be detected, although at a lower sensitivity ($\sim$1 μg/band).

The use of alternative protein stains for the detection of transferred proteins was investigated. Anionic dyes such as Coomassie blue were less effective because of strong interaction with the DITC–aminopropyl sheets and consequent high backgrounds. Hydrophobic interaction dyes showed a strong background staining with DITC–GF/F, presumably due to the relatively hydrophobic DITC groups. Colloidal gold staining is a potentially extremely sensitive method to detect proteins on DITC blots (detection limit less than 10 ng of protein per band). However, in our hands, the method was not reproducible and was plagued by erratic background problems.

We investigated the influence of protein detection by DCIA and colloidal gold on the sequenceability of the stained proteins. Sperm whale myoglobin was coupled to an 11-cm-diameter DITC–GF/F disk at a density of approximately 40 pmol of sequenceable protein per cm^2. Aliquot disks of 1-cm diameter were cut out and subjected to the various staining procedures, and then sequenced. Initial coupling yields, repetitive stepwise yields, and the occurrence of additional, dye-induced contaminants in the final PTH analysis by HPLC were investigated. Proteins detected by DCIA (following the procedure described under Methods section), or by colloidal gold staining following the manufacturer's protocol, could be sequenced without interference from the dyes with no reduction in initial yields. No additional contaminants were observed in the analysis of the resulting PTH derivatives. Potentially, DCIA-derivatized cysteine residues should be efficiently detectable by their fluorescence in the reverse-phase HPLC analysis of PTH-amino acids.

Covalent Attachment of Proteins from Solution. Proteins prepared by means other than SDS–PAGE were applied directly onto DITC–GF/F disks of 1-cm diameter for covalent attachment. The coupling reaction was insensitive to chemicals present during the coupling process, as long as the required basic pH in the range of 8.3–9.0 was maintained and no excess free amino groups were present. Yields under various conditions were determined as follows. Thirty micrograms of ^{125}I-labeled bovine serum albumin in 30 μL of water was spotted onto DITC–GF/F disks, and disks were γ-radiation counted and air-dried. Forty microliters of coupling buffer, 100 mM *N*-ethylmorpholine hydrochloride, pH 8.3, containing up to 2% SDS or up to 4 M guanidine hydrochloride with the pH readjusted to 8.3, was added to the dried disks, and coupling was carried at 37 °C for at least 2 h. The disks were then extensively washed with TFA and DMF to remove noncovalently bound protein, and the amount of protein retained was determined by γ-radiation counting. Coupling

Table II: Sequencing Yields of Proteins Electroblotted onto DITC–GF/F

(A) Repetitive Yield and Sample Washout

protein	sample application	initial yield (pmol)	repetitive stepwise yield	sample loss/ cycle (%)
bovine α-lactalbumin	spotted onto AP-GF/F	69	92	1.4
bovine α-lactalbumin	electroblotted onto DITC–GF/F	40	94	0.3
equine myoglobin	spotted onto AP-GF/F	27	91	1.3
equine myoglobin	electroblotted onto DITC–GF/F	20	92	0.3

(B) N-Terminal Blocking during Isolation Procedure

protein	application	PTH-Leu_3 (pmol/1000 cpm)	ratio	N-terminal blocking (%)
bovine α-lactalbumin	spotted onto AP-GF/F	9.5	0.96	4
bovine α-lactalbumin	electroblotted onto DITC–GF/F and DCIA stained	9.2		

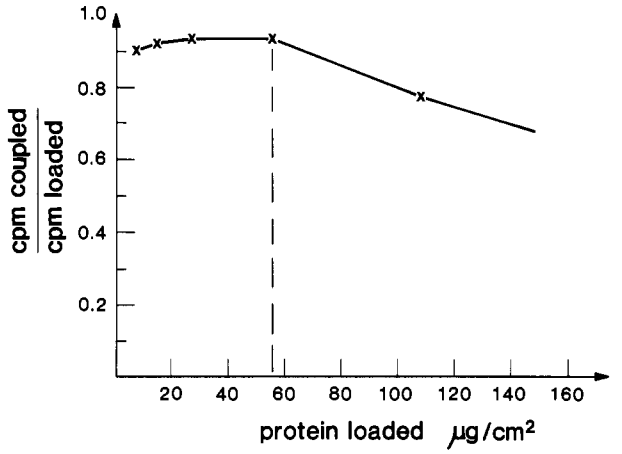

FIGURE 3: Capacity of DITC–GF/F for protein coupling from solution. ^{125}I-Radiolabeled bovine α-lactalbumin was applied to 1-cm-diameter disks of DITC–GF/F, and the protein was coupled for 2 h at 37 °C in coupling buffer as described in the text. Noncovalently coupled protein was removed by extensively washing the disks with TFA and DMF. Specific radioactivity was determined by amino acid composition analysis of radiolabeled, trichloroacetic acid precipitated protein.

buffer containing up to 2% SDS and up to 4 M guanidine hydrochloride did *not* affect the coupling yield.

The protein binding capacity of DITC–GF/F was approximately the same for proteins coupled from solution as for proteins coupled by electroblotting as shown in the following experiment. Increasing amounts of ^{125}I-labeled bovine α-lactalbumin were spotted onto 1-cm-diameter disks of DITC–GF/F and covalently coupled as described under Methods. Nonbound protein was removed by TFA and DMF washes after the coupling, and the fraction of coupled protein was determined by γ-radiation counting before and after the washing process. The data in Figure 3 show that 50 μg of bovine α-lactalbumin could be efficiently coupled to a 1-cm-diameter (~0.75 cm^2) disk. In electoblotting experiments, the upper limit of the binding capacity was in the range of 15–20 μg of myoglobin per (3–4) × 10 mm band. This capacity for blotted proteins is adequate, since the resolving power of SDS–polyacrylamide gels decays rapidly if more than 10–15 μg of protein per band is loaded onto the gels.

Sequence Determination. The stained protein-containing regions of the blots were cut out and transferred without further treatment to the cartridge of a gas-phase sequenator for automated Edman degradation as described (Aebersold et al., 1986). For proteins electroblotted onto DITC–GF/F, repetitive stepwise yields of 92–94% at the 20–70 pmol level were obtained (Table IIA). These values were similar to or slightly higher than the values for proteins prepared by conventional noncovalent electroblotting techniques. Repetitive yields were measured on the basis of the amount of PTH derivatives present in successive cycles of the Edman degradation. Radiolabeled proteins were used to determine whether the 6–8% reduction of sequenceable protein per cycle was due to release of protein from the support ("washout"). Proteins were electroblotted onto DITC–GF/F, and the amount of protein present was determined by γ-radiation counting before sequencing and after 17–20 degradation cycles. Table IIA shows that for conventional gas–liquid-phase degradation chemistry the loss of radioactivity per sequencing cycle was negligible for proteins electroblotted onto DITC–GF/F and was in the range of only 1–1.5% on aminopropyl-GF/F paper even for the relatively small proteins used in the experiments. Using the much more vigorous washing and extraction conditions of solid-phase degradation chemistry, we obtained similar results for proteins bound to DITC–GF/F (<1% loss of radioactivity per cycle) (Nika et al., unpublished results).

We then investigated the extent of amino-terminal blocking during the SDS–PAGE/electroblotting. ^{125}I-Radiolabeled bovine α-lactalbumin was spotted onto aminopropyl-GF/F and N-terminally sequenced. The specific yield of the initial signal was calculated, based on trichloroacetic acid precipitable counts and the magnitude of the PTH-Leu signal in step 3 of the sequencing process. An aliquot of the same sample was then electrophoresed in a SDS–polyacrylamide gel, electroblotted onto DITC–GF/F, and stained on the blot with DCIA. The protein band was N-terminally sequenced after γ-radiation counting, and the specific initial signal was calculated as for the spotted protein. As shown in Table IIB, the degree of NH_2-terminal blocking in this procedure was minimal (<5%), provided that gel chemicals selected for minimal blocking were used and precautions were taken to minimize N-blocking reactions (see Methods).

After electroblotting onto DITC–GF/F, typically 20–30% of each amino group (N-terminal α-amino group and ε-amino group of lysine side chains) was attached to the solid support, as measured from the reduced yields of N-terminal amino acids and lysine residues compared with neighboring residues. This degree of derivatization was in strong contrast to the covalent coupling of proteins to DITC controlled-pore glass from solution, where typically each amino group was almost quantitatively coupled (Wachter et al., 1973). The lower degree of covalent coupling of individual amino groups may reflect the lower density of DITC groups present on DITC–GF/F as compared to DITC–CPG (maximum of 20 μmol/g versus 30–50 μmol/g) (Machleidt & Wachter, 1977). Most polypeptides separated by SDS–PAGE contain multiple lysine residues, so that the overall coupling yield is not compromised by the low coupling yield of individual amino groups (Table I). The low degree of attachment per amino group offered the advantage that both the lysine residues and the N-terminal amino acid residues could readily be detected on DITC–GF/F-immobilized proteins.

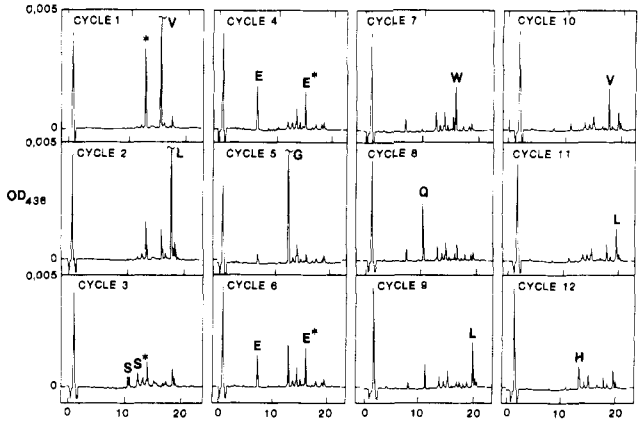

FIGURE 4: Solid-phase sequence analysis using 4-(dimethylamino)-azobenzene-4′-isothiocyanate (DABITC). Sperm whale myoglobin was spotted and coupled onto DITC–GF/F and sequenced in a modified Caltech sequenator using a DABITC–phenyl isothiocyanate double-coupling protocol (Nika et al., unpublished results). DABTH degradation products (66% injected from 60 pmol of sequenceable protein) were separated by reverse-phase HPLC and detected at 436 nm according to Chang (1981). DABTH derivatives are denoted in the single-letter code of the corresponding amino acid. Asterisks indicate unidentified reaction byproducts; S*, unidentified byproduct of DABTH–Ser; E*, byproducts of DABTH–Glu, possibly DABTH–Glu methyl ester. x axis, retention time (minutes).

Picomole Level Solid-Phase Edman Degradation. The primary impetus for developing a method for the isolation of proteins with efficient covalent attachment to a solid support was to allow the use of fluorescent and chromophoric sequencing reagents. The chromophoric sequencing reagent 4-(dimethylamino)azobenzene-4′-isothiocyanate (DABITC) (Chang et al., 1976) was used as a model compound to explore the compatibility of proteins electroblotted onto DITC–GF/F with solid-phase Edman degradation. A drastically altered degradation chemistry, which could serve as a model for the use of much more sensitive fluorescent sequencing reagents, was used (Nika et al., unpublished results). Figure 4 shows the HPLC analysis of DABITC derivatives from the solid-phase Edman degradation with DABITC of equine myoglobin covalently coupled onto DITC–GF/F. This initial experiment with unoptimized chemistry gave good results at a level of 20–50 pmol of sequenceable protein. While this result does not represent an improvement in sensitivity compared to high-sensitivity gas-phase protein sequence analysis, it is a substantial improvement over the nanomole amounts of protein previously required for automated sequencing using DABITC (Salnikow et al., 1982), and it is clear that this approach holds considerable promise for improving the sequencing sensitivity by the use of alternative degradation reagents. The low level of contaminants and reaction byproducts clearly showed the power and potential of electroblotting with immediate covalent attachment of the protein for solid-phase sequencing.

CONCLUSIONS

We have described an approach to the efficient preparation of proteins for high-sensitivity, solid-phase sequence analysis. Proteins separated by SDS–PAGE were electrophoretically transferred to glass fiber paper containing reactive isothiocyanate moieties, where they were immobilized by covalent attachment through amino groups and detected by fluorescent staining. Protein-containing regions of the blot were cut out and sequenced in a gas-phase sequenator without further manipulations. This procedure provided several advantages over existing electroblotting methods for the isolation of proteins for sequence analysis. (i) Proteins were covalently bound to the matrix, so that there was greater flexibility in the choice of conditions for the Edman reaction. In particular, the method was compatible with degradation chemistry using solid sequencing reagents. (ii) Quantitative ninhydrin determination of amine allowed the control of the derivatization level with amino groups as well as with DITC, providing reliable and easy quality control procedures for the reproducible production of DITC–GF/F sheets, and (iii) the inherently simpler solid-phase degradation will allow the construction of simpler, cheaper, and more efficient protein sequenators (Kent et al., 1987).

Table III: Proteins Sequenced after Electroblotting onto DITC–GF/F

protein	$M_r \times 10^{-3}$	sequenceable amount (pmol)	no. of residues determined
sperm whale myoglobin	17	15	15[a]
equine myoglobin	17	60	20[a]
bovine α-lactalbumin	14	25	15[a]
bovine serum albumin	67	6	12
shark myelin protein P_0	35	25	20
shark myelin protein P_0[a]	33	50	13[a]
hepatitis B virus S protein P 24	24	8	10
hepatitis B virus glycosylated S protein GP 27	27	5	10
interleukin-2 (murine)	15	15	12
antibody κ l chain	27	14	18

[a] The sequenceable amount of protein was quantitated by integrating the peak area of the first and second PTH signals and comparing this area to the peak area of the respective PTH-amino acids in a 10-pmol standard mixture. PTH-amino acid residues either were determined manually on a light box or were determined with a computer-assisted analysis program developed in our laboratory (Kent et al., 1987) until the signal size dropped below the detection limit or until sufficient information was obtained. At this point, the runs were aborted.

We have used this procedure for the isolation for N-terminal sequence analysis of a variety of proteins, some examples of which are shown in Table III. Clean, extended sequencing runs at good repetitive stepwise yields were routinely obtained. The purpose of the method was to develop an efficient protein isolation method, which is compatible with high-sensitivity sequence analysis using fluorescent sequencing reagents. Using radiolabeled proteins, we have found that nanogram and subnanogram amounts of proteins can be efficiently transferred and covalently coupled by using the methods described (data not shown), so that the preparation of subpicomole amounts of proteins for solid-phase Edman degradation level is already a reality.

The method has some limitations. With the exception of autoradiography of radiolabeled proteins, currently no method for the detection of proteins on DITC blots with low nanogram sensitivity is available. The coarse surface texture of the glass fiber disks is not ideal for electroblotting minute amounts of proteins, but the use of GF/F represents a useful compromise until a smoother membrane based on the same principles is available. The method is not suitable for the routine high-efficiency coupling of small (e.g., tryptic) peptides. For the efficient covalent coupling of peptides to solid supports for Edman degradation, alternative methods have been developed (Aebersold et al., unpublished results).

This method has overcome the problem of isolation and efficient covalent attachment to a solid support of microgram and submicrogram amounts of proteins for solid-phase sequence analysis. As described elsewhere (Nika et al., unpublished results), this is a necessary requirement for the use of alternative sequencing reagents with enhanced detectability and is, therefore, a key step toward the removal of the current

sensitivity-limiting step in protein sequence analysis.

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

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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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