Carboxylic acid-modified polyethylene: A novel support for the covalent immobilization of polypeptides for C-terminal sequencing

NARMADA R. SHENOY, JEROME M. BAILEY, AND JOHN E. SHIVELY

Beckman Research Institute of the City of Hope, Division of Immunology, 1450 E. Duarte Road, Duarte, California 91010

(RECEIVED May 10, 1991; ACCEPTED August 26, 1991)

Abstract

We have developed a method for the covalent immobilization of peptides, for the purpose of C-terminal sequencing, to a novel solid support, carboxylic acid-modified polyethylene (PE-COOH) film. The peptides are attached by coupling the N-terminal amino group to the activated carboxyl groups of the film. Reagents for carboxyl group activation, including 1,3-dicyclohexylcarbodiimide (DCC), 1,1'-carbonyldiimidazole (CDI), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), and 1,3-diisopropylcarbodiimide (DICD) were compared. The best yields were obtained with DCC for a variety of tested peptides and averaged approximately 50%. The covalent attachment at pH 6.7 of peptides was shown to occur predominantly through the α -amino group for the peptide, SIGSLAK, which after attachment to the PE-COOH support permitted the C-terminal lysine residue to be sequenced in good yield, indicating that the ϵ -amino group of lysine is not covalently attached. This support offers a number of advantages over other solid supports, such as silica and polyvinylidene difluoride, for C-terminal sequencing including (1) stability to base and the high temperatures (65 °C) employed for C-terminal sequencing, (2) wettability with both aqueous and organic solvents, (3) a high capacity (1.6 nmol/mm²) for covalent coupling of polypeptides, and (4) easy divisibility into 1 × 5-mm pieces for use in our continuous flow reactor (CFR), which is also used for automated N-terminal sequencing (Shively, J.E., Miller, P., & Ronk, M., 1987, *Anal. Biochem.* 163, 517–529). Automated C-terminal sequencing on these supports is described in the companion paper (Bailey, J.M., Shenoy, N.R., Ronk, M., & Shively, J.E., 1992, *Protein Sci.* 1, 68–80).

Keywords: carboxylic acid-modified polyethylene; C-terminal sequencing

A goal of our laboratory is the development and automation of C-terminal sequencing chemistry for peptides and proteins with the same speed, sensitivity, and number of repetitive cycles currently possible with Edman (N-terminal) chemistry. As discussed in the following report (Bailey et al., 1992), we have chosen the thiocyanate method, which is based on a procedure originally described by Schlack and Kumpf (1926). This method involves the derivatization of an activated carboxy-terminal amino acid to a thiohydantoin derivative with thiocyanate-based reagents. The derivatized C-terminal amino acid is then specifically cleaved to yield a shortened peptide and a thiohydantoin amino acid. To date, this method has been

the most widely studied of the existing C-terminal sequencing strategies and appears to hold significant promise. We and others (Hawke & Boyd, 1991; Inglis et al., 1991; Wittman-Liebold et al., 1991) have recognized the need to apply this chemistry to samples covalently attached to the solid phase. Covalent immobilization of the sample onto a solid support allows the use of reagents and solvents optimal for sequencing without causing sample washout, permits efficient washing of the sample to remove reaction by-products that might otherwise interfere with identification of the released thiohydantoin amino acids, and prevents mechanical losses associated with most solution phase methods. In general, automated solid phase chemistry is expected to be more efficient and less labor intensive compared to manual solution phase methods.

Since the introduction of the solid phase approach to

Reprint requests to: Jerome M. Bailey, Beckman Research Institute of the City of Hope, Division of Immunology, 1450 E. Duarte Rd., Duarte, California 91010.

N-terminal protein sequencing by Laursen (1966), several different types of functionalized supports have been described for the covalent immobilization of polypeptide samples. These include polystyrene resins, polyacrylamide resins, and glass beads substituted with aminoalkyl or aminophenyl groups (Laursen & Machleidt, 1980). Typically these amino functionalized supports are activated for protein coupling with bifunctional reagents such as phenylene diisothiocyanate (DITC). The DITC group is capable of forming a stable thiourea linkage to the support and the peptide N-terminal amino group or ϵ -amino group of lysine side chains. Recently glass beads derivatized with isothiocyanato, aminophenyl, and aminoethylaminopropyl groups (Song-Ping Liang & Laursen, 1990), glass fiber sheets functionalized with aminophenyl groups (Aebersold et al., 1990), and polyvinylidene difluoride (PVDF) membranes derivatized with aryl amines and DITC (Pappin et al., 1990) have been used for the covalent immobilization of polypeptides for N-terminal sequencing. The polypeptides are either immobilized by coupling between the ϵ -amino groups of the lysine and the isothiocyanate groups on the solid support using the established DITC chemistry or by the coupling of the activated C-terminal carboxyl groups of the polypeptides and the amino groups on the matrix.

Many of the initial studies involving the application of the thiocyanate chemistry for C-terminal sequencing to the solid phase have involved the use of glass beads for the covalent immobilization of peptide samples (Williams & Kassell, 1975; Rangarajan & Darbre, 1976; Meuth et al., 1982; Hawke et al., 1987; Inglis et al., 1989). More recent work has involved the use of carboxylic acid-modified PVDF (Bailey & Shively, 1991), DITC-activated amino PVDF (Miller et al., 1989; Inglis et al., 1991), and a disuccinimidoyl carbonate polyamide resin (Hawke & Boyd, 1991). As described in this report, the use of the glass and PVDF supports were found by our laboratory to be unsatisfactory for C-terminal sequencing. A more suitable support was found to be a carboxylic acid-modified polyethylene (PE-COOH) film. This novel support for C-terminal sequencing offers a number of advantages over the existing supports. These include (1) stability of the support to the conditions employed for C-terminal sequencing, (2) the ability, due to the hydrophilic nature of the surface groups, to use both aqueous and organic solvents for performing chemistry on covalently coupled polypeptide samples, (3) a high capacity (1.6 nmol/mm^2) to covalently couple polypeptides, and (4) easy divisibility into $1 \times 5\text{-mm}$ pieces for use in our continuous flow reactor (CFR), which is also used for automated N-terminal sequencing (Shively et al., 1987).

This report describes the optimization of methods for the covalent attachment of peptide samples, via the N-terminal amino group, to this PE-COOH support for the purpose of C-terminal sequencing. Variables examined

include, selection of the optimal activation reagent and conditions for activation of the surface carboxylic acid groups, determination of the optimal time for coupling peptides to these activated surface groups, choice of the best solvent for maintaining the solubility of the peptide being coupled while at the same time maintaining compatibility with the coupling reaction, and an examination of the generality of this procedure for peptides by determining the covalent coupling yields of a variety of peptides with different properties (hydrophobic, hydrophilic, etc.).

Results

Quantitation of covalently coupled peptides

The quantitation of covalently coupled peptides to the carboxylic acid-modified PVDF and PE-COOH supports was performed by direct amino acid analysis of the membrane strip. Application of this procedure to the tripeptide, AFP (2.7 nmol), and the hexapeptide, VGVAPG (550 pmol), noncovalently absorbed to PVDF strips of the size routinely used for sequence analysis ($1 \times 10 \text{ mm}$), yielded only 24% of the expected amounts of Ala, Phe, and Pro from the AFP peptide and 35% of the expected amounts of Val, Gly, and Ala from the peptide, VGVAPG, and none of the expected Pro. However, if after acid hydrolysis the PVDF strip is first extracted with 400 μL of 50% acetonitrile/water solution, the extracts evaporated in a vacuum centrifuge, and then taken up in the usual sodium citrate buffer for amino acid analysis, quantitative recoveries of all the expected amino acids from these two peptides were obtained. This extraction procedure was also tested with the peptide, DRVYIHPFHLVIH (2.8 nmol), in order to test this procedure with a wider variety of amino acid side chains. High recoveries were obtained for all of the amino acids (Table 1). The extraction procedure with acetonitrile/water was found to be unnecessary with the polyethylene support. The peptide, DRVYIHPFHLVIH (1.1 nmol), was noncovalently spotted on four strips of PE-COOH ($2 \times 10 \text{ mm}$). After acid hydrolysis, two of the strips were extracted with 50% acetonitrile/water, dried, and taken up with sodium citrate buffer as described above and analyzed. The other two strips were taken up in sodium citrate buffer immediately after hydrolysis. All four strips showed equivalent quantities of the expected amino acids. In order to test if covalent coupling of the peptides would affect the ability to obtain quantitative data by amino acid analysis, four strips ($1 \times 12.5 \text{ mm}$) of PE-COOH containing 830 pmol of YGGFL covalently coupled were analyzed. Two strips were extracted with 50% acetonitrile in water prior to taking up the sample in sodium citrate buffer, whereas the other two were taken up in sodium citrate buffer immediately after hydrolysis. All four strips showed equivalent amounts of the expected amino acids (Table 2).

Table 1. Amino acid analysis of a test peptide (DRVYIHPFHLVIH) noncovalently immobilized on PVDF^a

Amino acid	Control, pmol (no. residue)	Extracted with 50% acetonitrile/water, pmol (no. residue)
Asx	3,009 (1.05)	2,775 (1.00)
Pro	2,901 (1.01)	2,883 (1.05)
Val	5,655 (1.98)	5,604 (2.04)
Ile	5,055 (1.77)	4,968 (1.81)
Leu	3,060 (1.07)	3,057 (1.11)
Tyr	2,721 (0.95)	2,565 (0.93)
Phe	3,132 (1.09)	3,027 (1.14)
His	8,748 (3.06)	8,067 (2.93)
Arg	2,940 (1.03)	2,808 (1.02)

^a A solution of the peptide, DRVYIHPFHLVIH (3 μ L in 50% acetonitrile, 2.8 nmol) was applied to a 2 $\times$ 12-mm strip of PVDF (Immobilon P, Millipore). An identical amount of peptide was added directly to a hydrolysis tube. After hydrolysis, the PVDF strip was dried by vacuum centrifugation and rinsed once with 400 μ L of 50% acetonitrile/water. This solution was dried by vacuum centrifugation and the amino acids dissolved in sodium citrate buffer for analysis. The data shown are the average of two determinations.

Table 2. Amino acid analysis of leucine-enkephalin covalently coupled to PE-COOH^a

Amino acid	No extraction, pmol (no. residue)	Extracted with 50% acetonitrile/water, pmol (no. residue)
Gly	1,728 (2.14)	1,756 (2.05)
Leu	796 (0.99)	880 (1.03)
Tyr	704 (0.87)	800 (0.93)
Phe	796 (0.99)	852 (0.99)

^a Four strips (1 $\times$ 12.5 mm) of PE-COOH containing 830 pmol of leucine-enkephalin (YGGFL) covalently coupled to PE-COOH as described in Table 3. Two strips were extracted with 50% acetonitrile in water prior to taking up the sample in sodium citrate buffer, and the other two were taken up in sodium citrate buffer immediately after hydrolysis. After hydrolysis, the PVDF strip was dried by vacuum centrifugation and rinsed once with 400 μ L of 50% acetonitrile/water. This solution was dried by vacuum centrifugation and the amino acids dissolved in sodium citrate buffer for analysis. The data shown are the average of two determinations.

Automated sequencing of peptides covalently coupled to PVDF and glass supports

Automated C-terminal sequencing was performed on leucine-enkephalin covalently coupled to aminopropyl glass, carboxylic acid-modified PVDF, and DITC-activated amino PVDF from Milligen/Biosearch as described in the adjacent report (Bailey et al., 1992). Only one cycle of degradation was obtained when the glass supports were used. Amino acid analysis of the glass support after three cycles of automated sequencing showed very lit-

tle peptide remaining on the support. Although the derivatized PVDF supports gave better sequencing yields than glass, these supports rapidly turned progressively blacker and more brittle with each cycle of sequencing. The PE-COOH support was found to be stable under these conditions.

Activation of the surface carboxylic acid groups

A number of reagents were tested for their ability to activate the surface carboxylic acid groups of PE-COOH. As shown in Table 3 the highest yields of covalently attached peptide were obtained when 1,3-dicyclohexylcarbodiimide (DCC) was used as an activating reagent. Of the two solvents tested for the coupling reaction, 50% dimethylformamide (DMF)/water gave higher yields of covalent coupling than did 50% acetonitrile/water. During activation of the surface carboxylic acid groups with DCC, an anhydride, which is capable of covalent reaction with peptide amino terminal groups or protein amino terminal and lysine ϵ -amino groups, is initially formed. Depending on the conditions of the reaction, this activated anhydride can rearrange to an *N*-acylurea derivative (Khorana, 1953), which can prevent the carboxylic groups from further reaction. The conditions in Table 4 examine both the optimal time for the activation of the surface carboxyl groups by DCC and for the sub-

Table 3. Effect of different coupling reagents and solvents on the yield of leucine-enkephalin coupled to PE-COOH^a

Activating reagent ^b	nmol covalently coupled to PE-COOH (% yield)	
	50% acetonitrile	50% DMF
DCC	0.2 (4.4)	1.8 (39.6)
CDI	0.07 (1.5)	0.23 (5.1)
EDC	0.32 (6.9)	0.12 (2.6)
BOP	0.58 (12.7)	0.29 (6.4)
DICD	0.68 (15.0)	0.57 (12.6)

^a A strip of PE-COOH (1 $\times$ 0.1 cm) was placed in a 1.5-cm CFR (Shively et al., 1987) sealed at one end. The strips were reacted with 30 μ L (30 nmol) of the activating reagent for 30 min at room temperature. The solvent used in all cases was 100% DMF, except when EDC was the activating agent, in which case water was the solvent. After the activation reaction, excess reagent was removed with the solvent used for the activation reaction (3 mL). Each sample was then washed with acetonitrile (1 mL) and dried in a vacuum centrifuge. The activated supports were then reacted with a 30- μ L solution (4.54 nmol) of the CFR reactor (sealed at one end) for 16 h. The strips were washed with either 50% acetonitrile or 50% DMF, followed by acetonitrile, and then dried in a vacuum centrifuge. The amount of coupled peptide was determined by amino acid compositional analysis of the derivatized support.

^b DCC—1,3-dicyclohexylcarbodiimide; CDI—1,1'-carbonyldiimidazole; EDC—1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; BOP—benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; DICD—1,3-diisopropylcarbodiimide.

Table 4. Effect of activation time and coupling time on the yield of covalent attachment of leucine-enkephalin^a

Activation time (h)	Coupling time (h)	Amount coupled (nmol)	Yield
0.5	1	0.54	4.0
0.5	2	2.11	15.6
0.5	4	3.33	24.7
0.5	20	5.19	38.4
1.0	1	1.01	7.5
1.0	2	1.59	11.8
1.0	4	2.73	20.2
1.0	20	5.57	41.3
2.0	1	2.44	18.1
2.0	2	2.45	18.1
2.0	4	5.25	38.9
2.0	20	7.2	53.3
4.0	1	0.52	3.9
4.0	2	0.52	3.9
4.0	4	3.4	25.2
4.0	20	4.1	30.4

^a The strip of PE-COOH (1 × 12.5 mm) were activated by an excess of DCC in anhydrous DMF (1 g/1 mL) for the amount of time indicated. At the end of the activation reaction, the excess reagent was removed with anhydrous DMF, and the activated strip dried in a vacuum centrifuge. Each activated PE-COOH strip was inserted into a CFR (Shively et al., 1987) containing a 100-μL solution of leucine-enkephalin (13.5 nmol) in 50% aqueous DMF for the indicated time at 22 °C. The microbore tubing on one end of the CFR was sealed. After the coupling reaction, the support was rinsed with coupling solvent and acetonitrile, and then dried in a vacuum centrifuge. The amount of covalently coupled peptide was determined by amino acid analysis as described in the Materials and methods.

sequent covalent attachment of the peptide, YGGFL. The data show that the optimal activation time is between 1 and 2 h with an overnight coupling reaction with the peptide. Maximum yields for covalent attachment of the peptide range from 41 to 53%. The coupling yields are lower when the supports are activated for more than 2 h presumably because the active *O*-acyl form of the carboxyl group can rearrange to an *N*-acylurea, which is not capable of covalently coupling to the peptide (Khorana, 1953).

Due to the transparent nature of the PE-COOH film, the activation of the surface carboxylic groups by various reagents could be monitored by infrared (IR) spectroscopy. Pieces of PE-COOH film (12 × 14 mm) were activated with the various reagents listed in Table 1 for the desired time at 22 °C. The activated supports were then rinsed with anhydrous DMF and the IR spectra recorded. Infrared spectra collected at various times during the course of activation of the surface carboxylic groups by DCC are shown in Figure 1. The carbonyl stretching band at 1,707 cm⁻¹ and the broad OH stretch at 2,500–3,600 cm⁻¹ disappear after the 1-h activation time indi-

Fig. 1. Infrared spectra of the PE-COOH support at various times during activation by DCC. Strips of PE-COOH film (12 × 14 mm) were activated with DCC in anhydrous DMF (1 g/mL) for the desired time at 22 °C. The activated supports were washed with anhydrous DMF (4 mL), dried in a vacuum centrifuge, and the IR spectra recorded.

cating complete reaction with DCC. The new absorption bands are in agreement with the expected activated anhydride. The doublet at 1,760 and 1,795 cm^{-1} is expected for the asymmetrical and symmetrical carbonyl stretching modes of the anhydride, and the stretching band at 1,648 cm^{-1} is due to the C=N bond. As the reaction is allowed to proceed, the gradual appearance of amide absorption bands at 1,689 and 1,507 cm^{-1} and the carbonyl band at 1,707 cm^{-1} indicate that the activated anhydride both rearranges to an inactive *N*-acylurea and hydrolyzes back to the free carboxylic acid. The data show that the optimal reaction time for activation is 1–2 h, which is in good agreement with the peptide coupling data in Table 4. Examination of the IR spectra with time of reaction for the other coupling reagents examined in Table 3 supports the choice of DCC in DMF as the optimal coupling reagent. The reaction of 1,3-diisopropylcarbodiimide (DICD) with the surface carboxylic groups was as efficient as DCC, but the anhydride formed rearranged to the acylurea more rapidly than that formed with DCC, resulting in lower coupling yields of the peptide with the solid support. Reaction of water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) with the surface carboxylic groups was rapid and occurred within 1–2 min; however, the activated carboxylic acid either rapidly rearranged to the acylurea or hydro-

lyzed back to the free carboxylic acid resulting in low yields of peptide coupling. Alternative activating reagents, such as 1,1'-carbonyldiimidazole (CDI) and benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), showed incomplete reaction with the surface carboxylic groups, thus explaining the low yields observed during the peptide coupling experiments with these two reagents (Table 3). The peptide-coupling data (Tables 3, 4) and the IR data both support the conclusion that the optimal method for the covalent attachment of a peptide to PE-COOH is a 1–2 h activation with DCC in DMF, followed by an overnight coupling of the peptide in a 50% DMF/water solution.

Generality of the peptide-coupling protocol to PE-COOH

Experiments to address the generality of this peptide-coupling procedure are shown in Table 5. Sixteen different peptides at two concentration levels (1 and 10 nmol) were applied to strips of PE-COOH (1 × 12.5 mm) activated with an excess of DCC in anhydrous DMF and coupled overnight (20 h) to the peptides in a CFR as described in Tables 3 and 4. A variety of peptides with differing chemical and physical properties were studied and most of these coupled efficiently to the support surface

Table 5. Effect of peptide nature on the coupling yields on carboxyl-activated PE-COOH

Peptides ^a	10 nmol applied		1 nmol applied	
	Amount coupled (nmol)	% Yield	Amount coupled (nmol)	% Yield
YGGFMRGL	1.79	18	0.45	45
KVILF	8.37	84	0.74	74
YGGFMRF	4.72	47	0.76	76
YGGFM	4.97	50	0.48	48
SIGSLAG	4.50	45	0.62	62
ADSGEGDFLAEGGVR	0.75	8	0.06	6
SYSMEHFRWG	6.09	61	0.66	66
AFP	1.50	15	0.14	14
TVL	3.69	37	0.29	29
FAN	1.90	19	0.23	23
RGYALG	5.10	51	0.48	48
YGGFLRRIRP	4.95	50	0.57	57
YGGFMRRVGRPE	5.77	58	0.62	62
YKW	2.23	22	0.56	56
PFAL	3.68	37	0.49	49
YGGFL	2.38	24	0.42	42

^a Peptides were dissolved in water containing 1% hexafluoroacetone. Hexafluoroacetone was included to improve peptide solubility. Aliquots of these solutions (2–5 μL) corresponding to 10 and 1 nmol were transferred to the CFR. Ten microliters of a 1:1 solution of 25 mM HEPES buffer (pH 6.7) and DMF was added to each reaction mixture, and the reaction volume was made up to 15 μL with the addition of the appropriate volume of 1% hexafluoroacetone in water. The activated PE-COOH films (prepared as described in Table 2) were immersed into each of these peptide solutions and the coupling reaction was carried out overnight at 22 °C. At the end of the reaction, each strip was washed with 2 mL 25 mM HEPES buffer:DMF (1:1), water (2 mL), and methanol (2 mL) and dried in a vacuum centrifuge. The amount of covalently coupled peptide was determined by amino acid analysis as described above.

(average yield is approximately 50%). A peptide with proline at the N-terminus (PFAL), which has a secondary amino group, was also found to couple to the support surface in relatively good yield.

Capacity of the PE-COOH to covalently couple to leucine-enkephalin

Experiments to determine the capacity of a 1×12.5 -mm strip of PE-COOH (the size typically used for sequencing) for the covalent coupling of leucine-enkephalin, YGGFL, are shown in Figure 2. Each strip was activated with DCC in dry DMF for 2 h and incubated with amounts of YGGFL ranging from 1 to 100 nmol in 50% aqueous DMF for 20 h at 22 °C as described above. As indicated by this experiment, the capacity of these small strips for short peptides is approximately 40 nmol (1.6 nmol/mm^2).

Manual C-terminal sequencing of leucine-enkephalin immobilized on PE-COOH

The first cycle of C-terminal sequencing on leucine-enkephalin, YGGFL, covalently attached to PE-COOH is shown in Figure 3. The sequencing chemistry used is described under Materials and methods and is essentially

Fig. 2. Capacity of PE-COOH to covalently couple the model peptide, leucine-enkephalin (YGGFL). A 1×12.5 -mm strip of PE-COOH film was activated with DCC in anhydrous DMF (1 g/mL) for 2 h and incubated with 1–100 nmol of YGGFL in 50% aqueous DMF for 20 h at 22 °C. The quantity of the peptide coupled on the support was determined by amino acid composition of the derivatized film after thoroughly washing with 50% DMF/water and acetonitrile and drying it. Control experiments were performed with noncovalently attached peptide to ensure that the wash solvents used completely removed all noncovalently absorbed peptide sample.

as previously described for the solution phase (Bailey & Shively, 1990).

Discussion

Any successful strategy for the development of an automated sequencer capable of extended sequential degradation from the C-terminus of proteins and peptides involves the combination of several research areas. Not only must the reagents and solvents used for the sequential degradation not interact with the solid support, they must also be able to derivatize specifically and hydrolyze

Fig. 3. Manual sequencing of the C-terminal amino acid of leucine-enkephalin (YGGFL) covalently coupled to PE-COOH. One cycle of C-terminal sequencing of leucine-enkephalin (YGGFL) covalently attached to PE-COOH was manually carried out in a 1.6-mL Eppendorf tube. A strip (1×12.5 mm) PE-COOH containing covalently coupled YGGFL (3.9 nmol) was incubated with 100 μ L of a 5% aqueous triethylamine solution for 2 min, the strip was dried in a vacuum centrifuge, activated with 100 μ L of acetic anhydride (50 °C, 15 min), dried in a vacuum centrifuge, and reacted with 30% TMS-ITC in acetonitrile (50 °C, 30 min). The support was washed with 1 mL each of 50% methanol in water, 0.1% TFA in acetonitrile, and water. It was cleaved with 100 μ L of 5% triethylamine in water (50 °C, 20 min). The solution containing the released thiohydantoin derivative was dried in a vacuum centrifuge, dissolved in 100 μ L of 0.1% aqueous TFA, and analyzed by HPLC.

only the C-terminal amino acid, which can possess any 1 of 20 naturally occurring side chains, many of them having potentially reactive side chains, and leave the shortened peptide or protein capable of continued degradation. Finally, the released amino acid must be identified and quantitated. Each one of these areas of research has its own considerations that must be optimized. In order to achieve the desired goal of C-terminal sequencing each area must be considered with all the others in mind. The advantages of solid phase sequencing outlined can only be realized if efficient methods for the covalent immobilization of polypeptide samples to a solid support can be achieved. A good support would be one that is inert and stable to all the reagents and solvents that are optimal for the sequencing chemistry and has a large capacity to couple peptide samples covalently.

The solid support

As discussed above, the nature of the solid support has been shown to affect the results of C-terminal sequencing. Although derivatized PVDF supports for the covalent immobilization of polypeptide samples have shown promise for solid phase N-terminal sequencing techniques (Coull et al., 1989; Pappin et al., 1990), when applied to the conditions used in our laboratory for C-terminal sequencing, these derivatized PVDF membrane supports were found to be unstable. When subjected to the basic conditions used for cleavage of the peptidylthiohydantoin, these supports rapidly turned brown, and then black and brittle. This observation is consistent with the results of Shinohara (1979) who showed that derivatized PVDF was much more susceptible to degradation (dehydrofluorination) by a basic hydroxide solution than was underivatized PVDF. In general, it seems that the introduction of any surface groups onto a PVDF support will make that support unstable to our current C-terminal sequencing conditions and thus may be a limiting factor to an automated extended sequential degradation from the C-terminus. Additionally, during the course of the base-catalyzed dehydrofluorination reaction, UV-absorbing products are formed (Faingold-Murshak & Karvaly, 1985). Because this reaction occurs during the course of the cleavage reaction, these UV-absorbing compounds cannot be washed away and must be injected into the high performance liquid chromatograph (HPLC) along with the thiohydantoin amino acid. These derivatives can subsequently interfere with identification of released thiohydantoin amino acid. Other laboratories have also utilized chemically activated glass beads for covalent coupling of peptides and proteins for C-terminal sequence analysis (Williams & Kassell, 1975; Rangarajan & Darbre, 1976; Meuth et al., 1982; Inglis et al., 1989). Our preliminary results with the automated sequencing of the pentapeptide, YGGFL, on DITC-activated aminopropyl

glass permitted only one cycle of sequencing with high background absorbance. Amino acid analysis after three cycles of sequencing indicated that the covalently attached peptide was removed during the sequencing chemistry. As a result, sequencing on this support was not further pursued. An expected drawback of the aminopropyl glass is the base liability of the siloxane bond. This type of bond is susceptible to nucleophilic cleavage under basic conditions (Taylor, 1991), making it unstable to the basic conditions used for C-terminal sequencing. The novel PE-COOH support described in this report has been shown in this study and in the following report (Bailey et al., 1992) to be optimal for our automated C-terminal sequencing procedure. This support is stable to the sequencing conditions and provides for the lowest level of UV absorption background of any of the supports tested. The importance of the nature of the solid support used for C-terminal sequencing is clear from these experiments.

Quantitation of covalently coupled peptides

Because literature data suggested difficulties with the extraction of the hydrolyzed amino acids from peptide samples hydrolyzed on PVDF (Tous et al., 1989), we checked to see if similar problems would occur with peptide samples on PE-COOH. We first developed an efficient extraction procedure for peptides hydrolyzed on PVDF and then tested whether or not such an extraction would be necessary with peptides hydrolyzed on polyethylene. Unlike the PVDF supports, amino acid analysis could be reliably performed without special extraction conditions of the hydrolyzed amino acids from the PE-COOH support prior to analysis.

The covalent immobilization of peptides to PE-COOH

The covalent immobilization of a peptide onto a solid support requires a solvent that is capable of solubilizing a wide variety of peptides, can wet the support, and is unreactive with the activated surface carboxylic groups. Due to the hydrophilic nature of the PE-COOH support, aqueous solvents can be used. The buffered aqueous solvent system used in this report has been found to be satisfactory for dissolving all of the peptides so far tested and is currently being used routinely in our laboratory. In this solvent system, the covalent attachment of peptides is postulated to occur predominantly through the α -amino group due to the pH of the buffer used in the coupling reaction (pH 6.7). The covalent coupling of the peptide, SIGSLAK, to the PE-COOH support and subsequent application of our automated C-terminal sequencing procedure permitted the C-terminal lysine res-

idue to be sequenced in good yield indicating that the ϵ -amino group of lysine is not covalently attached. The coupling protocol described above has been found to be unsatisfactory for most peptides greater than 15 residues and for protein samples. The two peptides with the lowest coupling yields, ADSGEGDFLAEGGVR and AFP, both have an N-terminal alanine residue. This may suggest that peptides with a particular N-terminal residue, in this case alanine, may be more difficult to covalently couple to PE-COOH. Experiments are currently in progress in our laboratory to address this issue. Because we have observed that many of the longer samples are not as soluble as the shorter peptides in the 50% DMF/water solvent used in this study, different activating reagents are being evaluated in order to permit the coupling chemistry to be carried out in 100% aqueous buffered solutions in which the majority of protein samples are more soluble.

Manual sequencing of peptides immobilized on PE-COOH

The current state of the chemistry allows the stepwise degradation of peptides containing most of the naturally occurring amino acids (with the exception of proline) by manual solution phase methods (Bailey & Shively, 1990). However, these methods are not appropriate for extended C-terminal degradation as the shortened peptide must be isolated at each step. In addition to being time consuming, the sample losses inherent in each separation step will limit the number of cycles obtainable for a given amount of starting material. The initial activation of the C-terminal carboxylate with acetic anhydride causes both racemization of the C-terminal amino acid and partial acetylation of internal amino acid side chains (particularly Thr, Ser, and Tyr) resulting in the formation of multiple peptidylthiohydantoin further complicating manual solution phase C-terminal analysis. Elimination of the purification step of the peptidylthiohydantoin is not practical as there are UV-absorbing side products formed during the derivatization of the C-terminal amino acid that will subsequently interfere with identification of the C-terminal thiohydantoin amino acid after cleavage of the peptidylthiohydantoin. Application of this chemistry to the solid phase has the potential to solve these problems. In addition to being useful for automated C-terminal sequencing, the covalent attachment of a polypeptide sample to PE-COOH should more easily permit manual C-terminal sequencing. The hydrophilic nature of the PE-COOH support allows the chemistry described by our laboratory (Bailey & Shively, 1990), involving derivatization with trimethylsilylisothiocyanate (TMS-ITC) and cleavage by dilute aqueous triethylamine, to be directly applied to covalently attached samples. Sequencing of peptides covalently attached to PE-COOH can be

carried out more efficiently and conveniently than in solution phase with minimal sample loss from cycle to cycle. The background is low as the support can be thoroughly washed with solvents and a single cycle of degradation can be carried out within 2–3 h.

In summary, we have described a novel solid support for C-terminal sequencing that eliminates the drawbacks associated with previously described supports. We also have described methodology for the covalent attachment of peptide samples to this support suitable for C-terminal sequencing. Continuing work in our laboratory involves the development of methods for the routine covalent attachment of protein samples. It is also anticipated that this PE-COOH support can be exploited as a solid support for other techniques such as solid phase N-terminal sequencing and solid phase peptide synthesis. Further work is necessary to tap the full potential of this solid support.

Materials and methods

Materials

Acetic anhydride was purchased from Fisher Chemical Co.; TMS-ITC, anhydrous DMF, BOP, 1,3-diisopropylcarbodiimide (DIIC), and CDI were from Aldrich. Water was purified on a Millipore Milli Q system. All of the peptides used in this study were either obtained from Bachem or Sigma; and DCC, EDC, and triethylamine (sequanal grade) were obtained from Pierce. The PE-COOH film was provided by the Pall Corporation (Long Island, New York). The amino acid thiohydantoins used in this study were synthesized as described previously (Bailey & Shively, 1990).

Covalent coupling of peptides to carboxylic acid-modified PVDF

A strip of PVDF-COOH (1 × 0.1 cm) was placed in a CFR (Shively et al., 1987), and 2 μ L of DCC (2 mmol/mL) in DMF was added through a Hamilton syringe. The microbore tubing on one end of the CFR was sealed by first heating and then pinching closed with pliers. The CFR was then placed in a sealed Eppendorf tube. After a 2-h activation (25 °C), the activated membrane was rinsed with 2 mL of DMF and then dried under a stream of argon. Leucine-enkephalin (2 μ L of a 14-nmol/ μ L solution in DMF) was carefully layered on the dry membrane and allowed to react overnight. The membrane was washed with methanol (5 mL) and dried under a stream of argon. The yield of coupled peptide was determined, after acid hydrolysis of the derivatized membrane, by amino acid composition analysis. Typical yields were approximately 50%.

Covalent coupling of peptides to PE-COOH

Samples of PE-COOH film were treated with a 1 N solution of HCl for 1–2 min at room temperature, in order to convert the surface carboxylic acid groups to the free acid form, prior to the covalent attachment of peptide samples. Peptide samples were covalently coupled to the surface carboxylic acid groups as described in Tables 3 and 4.

Covalent coupling of peptides to DITC-activated glass

The DITC-activated aminopropyl glass was prepared as described by Bridgen (1975). Peptides were coupled as described by Laursen (1977). The peptide, leucine-enkephalin (YGGFL), was dissolved in DMF/*N*-methyl-morpholine/water, 4:1:1, v/v/v, pH 9.0, and this solution was added to the DITC-glass. The mixture was kept at 45 °C for 30 min. After addition of excess ethanolamine, the mixture was allowed to stand an additional 30 min at 45 °C. The yellow glass particles were washed several times with methanol by centrifugation and dried under vacuum.

IR spectroscopy

The IR spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrometer using barium fluoride windows. Pieces of PE-COOH film (12 × 14 mm) were activated with the various reagents listed in Table 3 for the desired time at 22 °C. The activated supports were washed with anhydrous DMF (4 mL) or with 50% aqueous methanol (in the case of EDC), dried in a vacuum centrifuge, and the IR spectra recorded.

Quantitation of covalently coupled peptides

The quantitation of covalently coupled peptides to the polyethylene supports was performed by direct amino acid analysis of the support strips. Our typical analysis procedure involved gas phase hydrolysis with 6 N HCl, containing 0.3% β-mercaptoethanol, for 24 h. The sample was then dried in a vacuum centrifuge and washed with aqueous sodium citrate buffer (200 μL/μg sample) and analyzed with a Beckman 6300 amino acid analyzer.

Manual sequencing of leucine-enkephalin covalently attached to the PE-COOH

Leucine-enkephalin (YGGFL) covalently attached to PE-COOH was manually sequenced in an Eppendorf tube (1.6 mL). YGGFL (3.92 nmol), covalently attached to a strip of film (1 × 12.5 mm), was incubated with 100 μL of a 5% (v/v) aqueous triethylamine solution for 2 min.

The strip was then dried in a vacuum centrifuge, activated with 100 μL of acetic anhydride (50 °C, 15 min), dried in a vacuum centrifuge, and reacted with 30% TMS-ITC in acetonitrile (50 °C, 30 min). The support was washed with 1 mL each of 50% methanol in water, 0.1% trifluoroacetic acid (TFA) in acetonitrile and water. The derivatized C-terminal amino acid was cleaved with 100 μL of 5% aqueous triethylamine (50 °C, 20 min). The cleaved solution was dried in a vacuum centrifuge, dissolved in 100 μL of 0.1% aqueous TFA, and analyzed by HPLC.

High performance liquid chromatographic analysis of leucine-thiohydantoin

Reverse-phase HPLC was performed on a Vydac C18 column (2.1 × 25 mm) on a Beckman 126 pump module with a Shimadzu (SPD-6A) detector. UV absorbance was monitored at 265 nm. A linear gradient from 98% solvent A (water/TFA, 99.9/0.1, v/v) to 60% solvent B (water/TFA/acetonitrile, 9.9/0.1/90, v/v/v) over 30 min at a flow rate of 0.25 mL/min was employed at room temperature.

Acknowledgments

We thank Dr. Harvey Brandwein of the Pall Corp. (Long Island, New York) for providing us with the carboxylic acid-modified PVDF and polyethylene supports, Jim Sligar for performing the amino acid compositions, and Dr. Gabor Dibo for covalently coupling peptide samples to the PVDF and glass supports. This work was supported in part by NSF grant BBS-8714266 and by the Tosoh Corporation.

References

- Aebersold, R.H., Pipes, D.G., Wettenhall, R.E.H., Nika, H., & Hood, L.E. (1990). Covalent attachment of peptides for high sensitivity solid-phase sequence analysis. *Anal. Biochem.* 187, 56–65.
- Bailey, J.M., Shenoy, N.R., Ronk, M., & Shively, J.E. (1992). Automated carboxy-terminal sequence analysis of peptides. *Protein Sci.* 1, 68–80.
- Bailey, J.M. & Shively, J.E. (1990). Carboxy-terminal sequencing: Formation and hydrolysis of C-terminal peptidylthiohydantoin. *Biochemistry* 29, 3145–3156.
- Bailey, J.M. & Shively, J.E. (1991). Carboxy terminal sequencing: Automation and application to the solid phase. In *Techniques in Protein Chemistry: II* (Villafranca, J.J., Ed.), pp. 115–129. Academic Press, Inc., New York.
- Bridgen, J. (1975). Solid phase sequencing: A new support for the high sensitivity degradation of peptides and proteins. *FEBS Lett.* 50, 159–162.
- Coull, J.M., Dixon, J.D., Laursen, R.A., Koester, H., & Pappin, D. (1989). Development of membrane supports for the solid phase sequence analysis of proteins and peptides. In *Methods in Protein Sequence Analysis* (Wittmann-Liebold, B., Ed.), pp. 69–78. Springer-Verlag, Berlin.
- Dias, A.J. & McCarthy, T.J. (1984). Synthesis of a two dimensional array of organic functional groups: Surface-selective modification of poly(vinylidene fluoride). *Macromolecules* 17, 2529–2531.
- Faingold-Murshak, V. & Karvaly, B. (1985). Halogenated poly(acety-

- lene) derivatized by catalyzed dehydrohalogenation of carbochain halopolymers in non-aqueous media. *Polymer Comm.* 26, 358-359.
- Hawke, D. & Boyd, V.L. (1991). Chemical C-terminal sequencing. In *Methods in Protein Sequence Analysis* (Jornvall, H., Hoog, J.O., & Gustavsson, A.M., Eds.), pp. 35-45. Birkhauser-Verlag, Basel.
- Hawke, D.H., Lahm, H.-W., Shively, J.E., & Todd, C.W. (1987). Microsequence analysis of peptides and proteins: Trimethylsilylthiocyanate as a reagent for COOH-terminal analysis. *Anal. Biochem.* 166, 298-307.
- Inglis, A.S., Moritz, R.L., Begg, G.S., Reid, G.E., Simpson, R.J., Graffunder, H., Matschull, L., & Wittman-Liebold, B. (1991). C-terminal sequence analysis. In *Methods in Protein Sequence Analysis* (Jornvall, H., Hoog, J.O., & Gustavsson, A.M., Eds.), pp. 23-34. Birkhauser-Verlag, Basel.
- Inglis, A.S., Wilshire, J.F.K., Casagrande, F., & Laslett, R.L. (1989). C-terminal sequencing: A new look at the Schlack-Kumpf thiocyanate degradation. In *Methods in Protein Sequence Analysis* (Wittmann-Liebold, B., Ed.), pp. 137-144. Springer-Verlag, Berlin.
- Khorana, H.G. (1953). The chemistry of carbodiimides. *Chem. Rev.* 53, 145-166.
- Laursen, R.A. (1966). A solid state Edman degradation. *J. Am. Chem. Soc.* 88, 5344-5346.
- Laursen, R.A. (1977). Coupling techniques in solid phase techniques. In *Methods in Enzymology*, Vol. 47 (Hirs, C.H.W. & Timasheff, S., Eds.), pp. 277-288. Academic Press, New York.
- Laursen, R.A. & Machleidt, W. (1980). Solid phase methods in protein sequence analysis. *Methods Biochem. Anal.* 26, 201-284.
- Meuth, J.L., Harris, D.E., Dwulet, F.E., Crowl-Powers, M.L., & Gurd, F.R.N. (1982). Stepwise sequence determination from the carboxyl terminus of the peptide. *Biochemistry* 21, 3750-3757.
- Miller, C.G., Kong, C.-T., & Shively, J.E. (1989). Carboxy terminal sequence analysis of proteins and peptides by chemical methods. In *Techniques in Protein Chemistry* (Hugli, T.E., Ed.), pp. 67-78. Academic Press, Inc., New York.
- Pappin, D.J.C., Coull, J.M., & Koester, H. (1990). Solid phase sequence analysis of proteins electroblotted or spotted onto polyvinylidene fluoride membranes. In *Current Research in Protein Chemistry* (Villafranca, J.J., Ed.), pp. 191-202. Academic Press, Inc., New York.
- Rangarajan, M. & Darbre, A. (1976). Studies on sequencing of peptides from the carboxyl terminus by using the thiocyanate method. *Biochem. J.* 157, 307-316.
- Schlack, P. & Kumpf, W. (1926). Über eine neue methode zur Ermittlung der Konstitution von Peptiden. *Z. Physiol. Chem.* 154, 125-170.
- Shinohara, H. (1979). Fluorination of polyhydrofluoroethylenes. II. Formation of perfluoroalkyl carboxylic acids on the surface region of poly(vinylidene fluoride) film by oxyfluorination, fluorination and hydrolysis. *J. Polymer Science: Polymer Chem. Ed.* 17, 1543-1556.
- Shively, J.E., Miller, P., & Ronk, M. (1987). Microsequence analysis of peptides and proteins VI. A continuous flow reactor for sample concentration and sequence analysis. *Anal. Biochem.* 163, 517-529.
- Song-Ping Liang & Laursen, R.A. (1990). Covalent immobilization of proteins and peptides for solid-phase sequencing using prepacked capillary columns. *Anal. Biochem.* 188, 366-373.
- Taylor, R.F. (1991). Commercially available supports for protein immobilization. In *Protein Immobilization: Fundamentals and Applications* (Taylor, R.F., Ed.), pp. 139-160. Marcel Dekker, Inc., New York.
- Tous, G.I., Fausnaugh, J.L., Akinyosoye, O., Lackland, H., Winter-Cash, P., Vitorica, F.J., & Stein, S. (1989). Amino acid analysis on polyvinylidene difluoride membranes. *Anal. Biochem.* 179, 50-55.
- Williams, M.J. & Kassell, B. (1975). A solid phase method for peptide sequencing from the carboxyl terminal. *FEBS Lett.* 54, 353-357.
- Wittman-Liebold, B., Matschull, L., Pilling, U., Bradaczek, H.-A., & Graffunder, H. (1991). Modular Berlin microsequencer for the sequential degradation of proteins and peptides from the amino and carboxyl terminal end. In *Methods in Protein Sequence Analysis* (Jornvall, H., Hoog, J.O., & Gustavsson, A.M., Eds.), pp. 9-21. Birkhauser-Verlag, Basel.