BIOORGANIC CHEMISTRY **24,** 50–58 (1996)
ARTICLE NO. 0006

Syntheses and Transglutaminase-Catalyzed Incorporation of Novel Spin-Labeled Primary Amines into Proteins[1]

CHAKRAVARTHY NARASIMHAN,*,[2] CHING-SAN LAI,† AND JOY JOSEPH†

*Biochemistry Department and †National Biomedical ESR Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226

Received August 2, 1995

A series of spin-labeled primary amine derivatives, namely, 2,2,6,6-tetramethyl-piperidinyl-1-oxyl-4-amidoalkylamines with varying alkyl chain lengths, have been synthesized. The spin-labeled primary amine with a tetramethylene or a pentamethylene chain covalently modifies human plasma fibronectin with a stoichiometry of 0.97-to-1.0 (probe-to-subunit) in the presence of coagulation factor XIIIa. The labels with two or one methylene chain also similarly modify fibronectin, but with a stoichiometry of only about 0.3–0.4 per subunit. The spin-labeled primary amine with a trimethylene chain does not label fibronectin. The labeling site appears to be the glutamine-3 residue at the amino-terminal region of fibronectin. Electron spin resonance studies show that the bound labels are partially immobilized with an effective rotational correlation time of 0.4–0.6 ns. The spin-labeled primary amine with tetramethylene chain also is shown to covalently incorporate into bee venom melittin in the presence of guinea-pig liver transglutaminase. The syntheses of the various spin-labeled primary amines and their applications in the study of structure and dynamics of different proteins and peptides are discussed. The observations from this study suggest that these spin-labeled primary amines have potentially wide application as structural probes.

INTRODUCTION

The enzymes that belong to the transglutaminase family catalyze the calcium-dependent cross-linking of glutaminyl residues of proteins and glycoproteins to the epsilon-amino group of lysine residues to create inter- or intramolecular isopeptide bonds (*1, 2*). This class of enzymes can also covalently link a variety of primary amines to peptide-bound glutamine residues. Cellular transglutaminases occur widely in differentiating cells and tissues in mammals. Coagulation factor XIII, the proenzyme of plasma transglutaminase, is present in blood plasma at a concentration of 10–20 μg/liter. Upon activation by thrombin, factor XIII is converted into factor XIIIa which catalyzes an acyl transfer reaction that involves glutaminyl residues in proteins as acyl donors and a variety of primary amines as acyl acceptors, resulting in the formation of monosubstituted γ-amide bonds (*3, 4*). Coagulation factor XIII is the best studied among the transglutaminases known to date.

The site-specific, transglutaminase-directed labeling of proteins with primary amines such as the fluorescent probe, dansylcadaverine (*1*), has opened up a general

[1] This work was supported by NIH Grants GM35719, RR01008, and GM22923.
[2] To whom correspondence should be addressed. Fax: (414) 266-8497.

0045-2068/96 $18.00

strategy for exploring cross-linking domains of proteins by fluorescence spectroscopy (*5–8*). Of equal importance is the possibility of studying posttranslational modification of proteins by small molecular weight primary amines catalyzed by transglutaminases. Further, primary amines such as isoniazid and hydrazaline that are used in the treatment of tuberculosis and hypertension, respectively, require transglutaminase-catalyzed incorporation into their respective targets in order to impart their drug effect (*9*). As the physiological relevance of transglutaminase sites in a variety of proteins and peptides are identified (*1*), the need to develop novel probes that will help to study their structure and function becomes important.

Plasma fibronectin (Fn),[3] a glycoprotein, circulates in vertebrate blood at a concentration of about 0.3 mg/ml and participates in many important processes including blood coagulation and wound healing (*10*). The protein is a dimer consisting of 240–250 kD subunits linked near the carboxyl termini by two disulfide bonds. Fn contains one plasma transglutaminase-reactive site per subunit which has been identified as the glutamine-3 residue near the amino-terminus (*11*). The presence of transglutaminase sites on Fn has made it possible to selectively label the protein with a variety of fluorescent primary amine derivatives including dansylcadaverine and fluorescenylcadaverine for structural analyses using fluorescence techniques (*5–7*).

Melittin is a basic peptide of 26 amino acid residues which constitutes about 50% of the dry weight of bee venom (*12*). The peptide has Gln residues in positions 25 and 26. Perez-Paya *et al.* showed that in the presence of guinea-pig liver transglutaminase, the fluorescent probe, dansylcadaverine, could be introduced into Gln25 (*12*).

Here we report the syntheses and applications of a series of new, spin-labeled primary amine derivatives, namely, 2,2,6,6-tetramethyl-piperidinyl-1-oxyl-4-amidoalkylamines (TEMPO-C_n; Fig. 1).Transglutaminase-catalyzed incorporation of these new spin labels into the Fn molecule and into melittin are examined, and their usefulness in deriving structural and dynamic information by electron spin resonance (ESR) spectroscopy is discussed.

MATERIAL AND METHODS

Tris(hydroxymethyl)aminomethane (Tris), phenylmethylsulfonyl fluoride, guinea-pig liver transglutaminase, and melittin were obtained from Sigma (St. Louis, MO). Coagulation factor XIII and thrombin were obtained from Calbiochem (San Diego, CA). Plasma Fn was isolated from fresh frozen human plasma using gelatin-Sepharose affinity chromatography (*13*). The purity of the protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Other chemicals and solvents were obtained from Aldrich chemical Co., (Milwaukee, WI). The TLC plates were from Whatman (Kent, England). Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

[3] Abbreviations used: ESR, electron spin resonance; Fn, fibronectin; TEMPO, (2,2,6,6-tetramethyl-1-piperidinyl-1-oxyl-); FMOC, fluorenylmethyloxycarbonyl; THF, tetrahydrofuran.

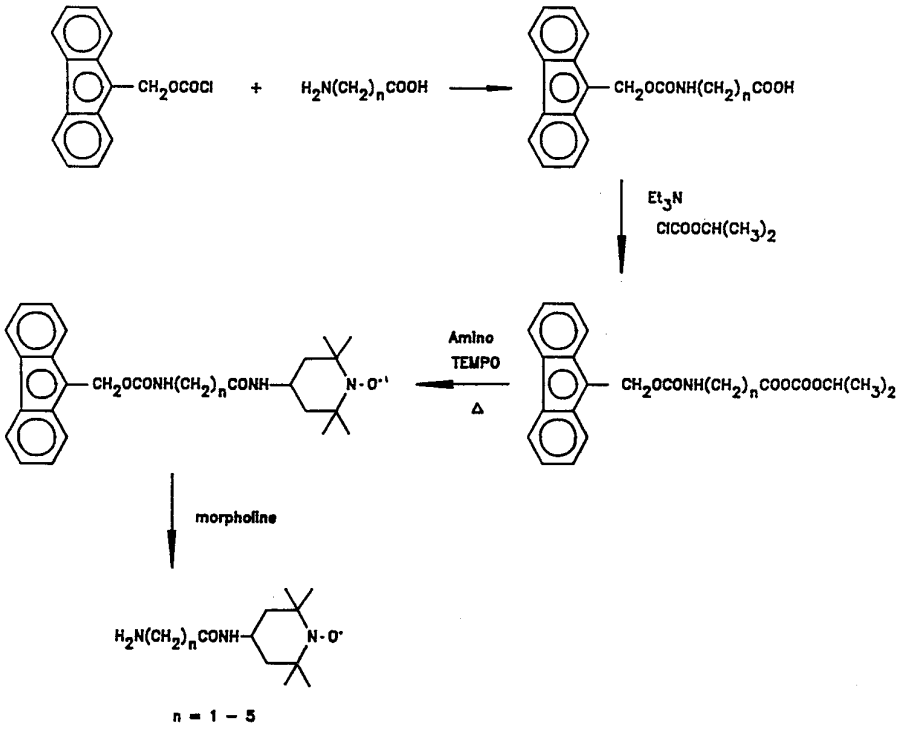

FIG. 1. Schematic diagram for the synthetic routes of spin-labeled primary amines, $n = 1$, TEMPO-C 1; $n = 2$, TEMPO-C2; $n = 3$, TEMPO-C3; $n = 4$, TEMPO-C4; $n = 5$, TEMPO-C5. The chemical names of compounds involved in the synthesis of TEMPO-C4 are given under Material and Methods.

Synthesis of Spin-Labeled Primary Amines

A schematic diagram of the general synthetic route is outlined in Fig. 1 in which the major steps in the preparation of the spin-labeled primary amines are shown. A typical procedure for the preparation of 4-(5-amino-pentaneamido)-2,2,6,6-tetramethyl-piperidinyl-1-oxyl (TEMPO-C4; Fig. 1) is described below. Fluorenylmethyloxycarbonyl (FMOC) derivative of 5-amino pentanoic acid was synthesized following the published protocol (*14*). In a typical procedure, 5-amino pentanoic acid (4 mmol) and sodium carbonate (8 mmol) were taken in 8 ml of water and the mixture was stirred with 7 ml of dioxane at 0°C. To this mixture, fluorenylmethyloxycarbonylchloride (4 mmol) in 7 ml dioxane was added dropwise and stirred for 1 h at 0°C and for 18 h at room temperature. The contents were poured into 100 ml of cold water and extracted with 3 × 50 ml ether. The ether extract was discarded and the aqueous phase was adjusted to pH 2–3 and extracted with 3 × 50 ml ethylacetate. The organic layer was dried by rotary evaporation. The compound was recrystallized from ethylacetate by adding hexane to get white solid (1.1 g, 81%). The progress of the synthesis was followed by IR (KBr) spectroscopy. The synthesized compound showed the following characteristic IR spectral features: 3310(m) 2920(s) 2850(s) 1700(s) cm^{-1}.

TABLE 1
Analytical Data for TEMPO Alkyamines

	Elemental analysis						
	Calculated (%)			Found (%)			
Sample	C	H	N	C	H	N	R_f[a]
TEMPO-C1	53.66	9.76	17.07	53.60	9.92	17.12	0.62
TEMPO-C2	55.38	10.00	16.15	55.33	9.98	16.21	0.50
TEMPO-C3	56.93	10.21	15.33	56.86	10.09	15.43	0.40
TEMPO-C4	58.33	10.42	14.58	58.35	10.51	14.62	0.33
TEMPO-C5	59.60	10.59	13.91	59.65	10.56	14.02	0.33

[a] Values measured on silica gel-60 plates using the solvent system: NH_4OH (Conc.): CH_3OH: $CHCl_3$ (1:10:26).

The spin label was introduced by reacting the FMOC aminopentanoic acid with 4-amino-TEMPO by the mixed anhydride method (*15*). FMOC aminopentanoic acid (1 mmol) in 5 ml of tetrahydrofuran (THF) was kept cooled in an ice bath. To this 1.1 mmol of triethylamine was added followed by 1 mmol of isopropylchloroformate in 5 ml THF and stirred at 0°C for 30 min. After stirring, 5 ml of 4-amino TEMPO (1 mmol) in THF was added to the mixture, stirred for 1 h, and then boiled under reflux for 20 min, rotary evaporated, and extracted with chloroform. The chloroform layer was washed with 0.1 N HCl until washings were acidic and later washed with $NaHCO_3$ solution to remove acid. The resulting solution was dried over $MgSO_4$ and then rotary evaporated. The residue was then chromatographed on silica gel 60 and eluted with ethyl acetate. The brown band that eluted was collected and rotary evaporated to get a brown solid (0.405 g, 82%). $R_f = 0.40$ (ethylacetate). IR (KBr) 3320(m) 3050(w), 2925(s), 2850(s), 1690(s), 1640(s), 1600(w)cm^{-1}

The FMOC protecting group was removed by stirring the solid in 2 ml each of CH_2Cl_2 and morpholine for 6 h. The solution was rotary evaporated and the residue was dissolved in $CHCl_3$ and applied to a silica gel 60 column. The column was first washed with chloroform and the bound product was eluted with $CHCl_3$: CH_3OH (90:10). Homogeneous fractions were combined and rotary evaporated to get a brown–red semisolid (125 mg, 56%). R_f = 0.33 ($CHCl_3$: $CH_3$0H: NH_4OH 75:25:2.5). IR(KBr) 3400(m) 3280(m) 2920(s) 2850(s) 1650(s) 1530(m) cm^{-1}. The spin-labeled amines were isolated as monohydrates. *Anal. Calcd* for $C_{14}H_{30}N_3O_3$: C, 58.33; H, 10.42; N, 14.58. Found: C, 58.35; H, 10.51; N, 14.62. In addition, the spin quantitation using ESR double integration technique revealed a spin-mass ratio of 1-to-1 stoichiometry. Other spin-labeled primary amines were synthesized and characterized similarly and their analytical data are summarized in Table 1.

Labeling of Plasma Fibronectin

Labeling of Fn with the spin-labeled primary amines was essentially the same as the method of Wolff and Lai (*6*) modified from that of Mosher *et al.* (*16*) for introducing fluorescenylcadaverine probes into Fn using thrombin-activated factor