Use of a New Membrane-Impermeable Guanidinating Reagent, 2-*S*-[^{14}C]Thiuroniumethanesulfonate, for the Labeling of Intracytoplasmic Membrane Proteins in *Rhodobacter sphaeroides*†

Bhupinder S. Hundle and William R. Richards*

Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

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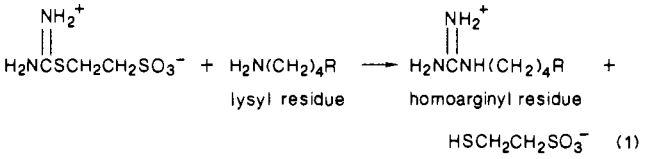
ABSTRACT: This study has demonstrated the effectiveness of 2-*S*-thiuroniumethanesulfonate as a new membrane-protein labeling reagent with several desirable characteristics: (1) it can easily be synthesized in a single-step reaction and can be radiolabeled readily by using [^{14}C]thiourea; (2) it has conveniently mild optimum labeling conditions, giving good yields of labeled membrane fractions by treating them for 1–2 h at 30–37 °C with 0.2 M concentrations of reagent in aqueous buffers at pH 9.0–10.5; (3) it is a low molecular weight zwitterion that has been shown to be impermeable to the intracytoplasmic membranes of *Rhodobacter sphaeroides* and should be useful for studies on the localization of membrane proteins with accessible reactive residues; and (4) it is a specific reagent that reacts with amino- and thiol- (but not with phenol-, imidazole-, or hydroxyl-) containing amino acid side chains of proteins, converting them to guanidinium and thiuronium ions, respectively. Hence, lysyl residues are converted to homoarginyl residues, stable amino acid residues with very similar properties to the parent lysyl residues. The reaction of 2-*S*-thiuroniumethanesulfonate with *N*-acetyl-L-cysteine yielded the thiuronium-containing derivative *N*-acetyl-*S*-amidino-L-cysteine, which may be cyclized by anhydrous trifluoroacetic acid to 2-iminothiazolidine-4-carboxylic acid; hence, the reagent may also be adapted for use as a cysteine-specific protein cleavage reagent in peptide analysis.

Greenstein (1935) first demonstrated that *O*-methylisourea could be used to convert lysine (either as the free amino acid or in peptides) to homoarginine. Over the ensuing 50 years, the guanidination of a large number of proteins has been accomplished with this reagent almost exclusively. Schütte (1943) had also used *S*-methylisothiourea as a guanidinating reagent; however, Hughes et al. (1949) pointed out that it was much less reactive than *O*-methylisourea. The usual procedures for guanidination with *O*-methylisourea have required long incubation times (4–6 days) at 0–4 °C and pH 10–11 (Kimmel, 1967; Means & Feeney, 1971). Even though the reagent is rather unstable in aqueous systems, it is fairly specific and reacts with the ε-amino groups of lysyl residues but not the *N*-terminal amino groups of proteins (Chervenka & Wilcox, 1956). Banks and Shafer (1972) have found, however, that whereas the guanidination of lysyl residues predominated at pH 10.5, the methylation of cysteinyl residues predominated at pH <10. More recently, *O*-methyl[^{13}C]isourea has been prepared and used to ^{13}C-guanidinate various cytochromes *c* (Stellwagen et al., 1977; Kennelly et al., 1981).

Amidination using ethyl acetimidate (Wofsy & Singer, 1963) has also been used to alter the lysyl residues of proteins. The reaction requires much less time than does guanidination with *O*-methylisourea, and it may be carried out at lower pH values (8–9). However, the product (ε-acetimidolysine) is less stable than homoarginine and is partially destroyed during acid hydrolysis. Ethyl acetimidate is able to penetrate membranes readily; however, Whiteley and Berg (1974) have synthesized a zwitterionic derivative, isethionyl acetimidate, which is impermeable to membranes. Both ^{3}H- and ^{14}C-labeled ethyl and isethionyl acetimidates have been synthesized and used to study the localization of membrane proteins in human erythrocyte membranes (Whiteley & Berg, 1974).

A variety of other lysine-specific labeling agents have been employed for the modification of soluble proteins (Lundblad & Noyes, 1984) or for the localization of membrane proteins (Hubbard & Cohn, 1976). In an earlier report from this laboratory, pyridoxal phosphate plus [^{3}H]KBH_4 was used to study the localization of membrane proteins in the ICM[1] of *Rhodobacter sphaeroides* (Francis & Richards, 1980). We now wish to report the use of 2-*S*-[^{14}C]thiuroniumethanesulfonate as an effective lysine guanidinating reagent (eq 1).



This reagent combines the advantages of *O*-methylisourea, by yielding the more stable guanidino group of homoarginine, with those of ethyl acetimidate, by reacting in much shorter times and under milder conditions (1–2 h at pH 9 and 30–37 °C). Also, since it is a zwitterion (as is isethionyl acetimidate), it is impermeable to biomembranes and can be used for membrane protein localization studies. In addition to reacting with lysyl residues, 2-*S*-thiuroniumethanesulfonate also reacts with cysteinyl residues, converting them to thiuronium derivatives

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* Author to whom correspondence should be addressed.

[1] Abbreviations: ICM, intracytoplasmic membrane; *t*-BOC, *tert*-butoxycarbonyl; BOC-ON, *N*-(*tert*-butoxycarbonyl)oxyimino-2-phenylacetonitrile; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; SDV, spheroplast-derived vesicles; CF_α and CF_β, α- and β-subunits of the coupling factor ATPase; RC_H, RC_M, and RC_L, heavy, medium, and light subunits of the reaction center; LH, light harvesting; 2-*S*-TES, 2-*S*-thiuroniumethanesulfonate.

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rather than leading to alkylation as with *O*-methylisourea.

Materials and Methods

Materials. *N*-Acetyl-*S*-(2-sulfoethyl)-L-cysteine was synthesized from *N*-acetyl-L-cysteine and 2-bromoethanesulfonate by the method of Niketic et al. (1974). *N,N'*-Diacetyl-L-cystine was synthesized from *N*-acetyl-L-cysteine by treating with cystamine. N^{α}-*t*-BOC-L-homoarginine was synthesized from L-homoarginine and BOC-ON by the method of Itoh et al. (1975). The following materials were purchased from the sources indicated: [^{14}C]thiourea (2.15 GBq/mmol) from Amersham Canada Ltd., Oakville, Ontario; Aquasol and En^{3}Hance from NEN Products, DuPont Canada, Inc., Lachine, Quebec; BOC-ON, poly(L-lysine), and protein molecular weight standards from Sigma Chemical Co., St. Louis, MO; MN-Polygram Sil G-22 (silica gel G) TLC sheets from Brinkmann Instruments (Canada) Ltd., Rexdale, Ontario; Merck silica gel 60F_{254} TLC sheets from BDH Chemicals Canada Ltd., Vancouver, BC; J. T. Baker Si 250 PA (19C) TLC plates from American Hospital Supplies, Richmond, BC; and Kodak XRP film from Kodak Canada Inc., Toronto, Ontario. All other chemicals were of reagent grade.

Synthesis of the Guanidinating Reagent. 2-*S*-Thiuroniumethanesulfonate was synthesized from 25 mmol each of sodium 2-bromoethanesulfonate and thiourea in 10 mL of water by the method of Schramm et al. (1955). After recrystallization in water, the yield was 78%. The product decomposed upon heating to 265 °C. Anal. Calcd for $C_3H_8N_2O_3S_2$: C, 19.55; H, 4.37; N, 15.21. Found: C, 19.30; H, 4.52; N, 14.99. The ^{13}C NMR spectrum (recorded on a Bruker Model WM 400 in dimethyl sulfoxide) showed three resonances assigned as follows: ethylene C1 (26.711 ppm); ethylene C2 (50.066 ppm); thiuronium C (170.598 ppm). 2-*S*-[^{14}C]Thiuoniumethanesulfonate was synthesized in a similar manner from 1 mmol each of sodium 2-bromoethanesulfonate and [^{14}C]thiourea (37.7 GBq/mol). The product was used without recrystallization or diluted with unlabeled 2-*S*-thiuroniumethanesulfonate as required (cf. below).

Reaction of the Guanidinating Reagent with Derivatives of Amino Acids. Poly(L-lysine) (10 mg; equivalent to 78 μmol of lysine residues) was reacted with a 50-fold excess of unlabeled 2-*S*-thiuroniumethanesulfonate in 10 mL of 0.1 M potasium phosphate buffer, pH 10.5, for 1 h at 37 °C. After dialysis against distilled water, the solution was concentrated to 1 mL in vacuo and an equal volume of 12 M HCl added. The sample was hydrolyzed for 26 h at 110 °C in a sealed tube under vacuum. The hydrolysate was analyzed by TLC in system A [silica gel G sheets in chloroform/methanol/9 M ammonia (2:2:1 v/v)] and by a Beckman Model 116 amino acid analyzer with a PA28 ion-exchange column.

The N^{α}-*t*-BOC derivatives of L-lysine, L-tyrosine, L-histidine, and L-serine plus *N*-acetyl-L-cysteine (20 μmol each) were reacted with a 20-fold molar excess of unlabeled 2-*S*-thiuroniumethanesulfonate in 2 mL of 0.1 M potassium phosphate buffer, pH 9.0, for 2 h at 37 °C. The samples were freeze-dried, and each of the four *t*-BOC derivatives was treated with 2 mL of trifluoroacetic acid for 30 min at 20 °C and the solvent removed in vacuo. The samples were then analyzed by TLC in system B [silica gel 60F_{254} sheets [or Si 250 PA (19C) plates when used with the alkaline ferricyanide/nitroprusside spray] in 2-propanol/acetic acid/water (4:1:1 v/v)].

N-Acetyl-L-cysteine (30.6 mmol) was reacted with 68.5 mmol of unlabeled guanidinating reagent in 340 mL of the above buffer for 3 h at 37 °C. Following complete removal of water in vacuo, the residue was extracted with three 75-mL aliquots of acetone. The acetone extract was filtered and evaporated, and the residue was reextracted with three 25-mL aliquots of 2-propanol. The 2-propanol extract was filtered, its volume was reduced to 4 mL, and 2 mL of this solution was added to a column (2.8 × 36 cm) of Kieselgel 60 silica (230–400 mesh) and developed with 2-propanol. The fraction eluted nearest the solvent front exhibited only a single component at R_f 0.60 during TLC in system B (cf. Results). The solvent was completely removed in vacuo for 48 h. The ^{13}C NMR spectrum (in D_2O) showed six resonances assigned as follows: acetylmethyl (24.610 ppm); cysteine C3 (36.568 ppm, shifted from 27.952 ppm in *N*-acetyl-L-cysteine); cysteine C2 (56.850 ppm); thiuronium C(174.122 ppm); acetylcarboxyl and cysteine C1 (176.955 and 178.022 ppm, found at 176.664 and 177.301 ppm in *N*-acetyl-L-cysteine). The ^{1}H NMR spectrum (in D_2O) showed three resonances assigned as follows: acetylmethyl (singlet at 2.040 ppm); cysteine C3 (octet centered at 3.513 ppm, shifted from doublet centered at 2.974 ppm in *N*-acetyl-L-cysteine); cysteine C2 (triplet centered at 4.532 ppm).

Both N^{α}-*t*-BOC-L-lysine and *N*-acetyl-L-cysteine were also treated with a 20-fold excess of 2-*S*-[^{14}C]thiuroniumethanesulfonate (diluted 25-fold) and analyzed by TLC in system B both before and after treatment with trifluoroacetic acid. Spots were scraped from the TLC plates, eluted with developing solvent, and counted in 10 mL of Aquasol on an LKB-Wallac Model 1217 RackBeta liquid scintillation counter.

Reaction of the Guanidinating Reagent with Whole Cells of R. sphaeroides. *R. sphaeroides* NCIB 8253 was grown as described by Francis and Richards (1980). Washed whole cells were resuspended in 0.1 M potassium phosphate buffer, pH 9.0, at a concentration of 0.5 g/mL, and incubated with various concentrations of 2-*S*-[^{14}C]thiuroniumethanesulfonate (diluted 8-fold) for 2 h at 35 °C. The cells were then thoroughly washed 4 times with 0.05 M potassium phosphate buffer, pH 7.4, and then incubated 3 times for 20 min in 15% (w/v) sucrose and phosphate buffer alternately, followed by four more washings. This procedure should have osmotically shocked the guanidinating agent out of the periplasm of the bacteria. It was continued until no more radioactivity appeared in the supernatants. The bacteria were then sonicated 5 times for 1-min pulses at a setting of 50 on a Bronwill Biosonik III ultrasonicator, and the chromatophore fractions were isolated and purified by the method of Francis and Richards (1980) by centrifugation in discontinuous sucrose density gradients in a Beckman Model L5-75 ultracentrifuge using an SW40 rotor for 10 h at 21 000 rpm. The chromatophores were finally resuspended in 0.5 mL of 1% aqueous SDS; 0.05 mL was used to assay for protein content by the method of Lowry et al. (1951), using bovine serum albumin as a standard. To the remaining 0.45 mL was added 0.225 mL of 10% aqueous SDS, and the samples were boiled for 1 min and added to 10 mL of Aquasol for scintillation counting.

Reaction of the Guanidinating Reagent with Purified Membrane Fractions of R. sphaeroides. Chromatophores were prepared by French press disruption of *R. sphaeroides* and purified by discontinuous sucrose density gradient centrifugation by the method of Francis and Richards (1980) using an SW27 rotor for 16 h at 20 000 rpm. The chromatophores were finally resuspended in potassium phosphate buffer at a concentration of 4 mg of protein/mL and 0.2-mL aliquots were added to enough solid 2-*S*-[^{14}C]thiuroniumethanesulfonate (diluted 36-fold) to give the concentrations desired. In order to find the optimum labeling conditions, the time, concentration of guanidinating reagent, pH of the buffer, and tem-

perature were varied as indicated in Figure 1. When the effect of one variable was studied, the others were maintained at the standard conditions of 0.2 M 2-*S*-[^{14}C]thiuroniumethanesulfonate incubated for 2 h at 30 °C at pH 9.0. When the concentration of the guanidinating reagent was varied, however, the radioactive reagent was diluted only 10-fold. Following incubation, the chromatophore suspensions were centrifuged at 100000*g* and resuspended in 0.1 M potassium phosphate buffer, pH 7.4, several times until the supernatant showed no more radioactivity. The chromatophores were then analyzed for protein content and radioactivity as described above.

SDV were also prepared from *R. sphaeroides* grown in very high light intensities (Francis & Richards, 1980), disrupted by the lysozyme/osmotic shock procedure of Michels and Konings (1978), and purified by centrifugation in continuous sucrose density gradients by the method of Takemoto and Bachmann (1979), using an SW40 rotor for 10 h at 21 000 rpm. A chromatophore fraction was also purified as described above except that centrifugation was carried out in continuous sucrose density gradients (between 5% and 45% sucrose w/w) using an SW27 rotor for 16 h at 20000 rpm. It and the SDV were then labeled with 0.1 M 2-*S*-[^{14}C]thiuroniumethanesulfonate (undiluted) in 0.1 M potassium phosphate buffer, pH 9.0, for 2 h at 35 °C. The membrane fractions were washed several times as described above until the supernatants showed no radioactivity. The labeled membrane fractions were then analyzed by SDS–polyacrylamide gel electrophoresis by the method of Laemmli (1960). Fluorography was accomplished by treating the dried gels with En^{3}Hance and leaving them in contact with Kodak XRP film for 8 weeks at −70 °C.

Results

Two spots were visible with ninhydrin during TLC analysis (in solvent system A) of the hydrolysate of poly(L-lysine) after it had been treated with the guanidinating reagent, corresponding to homoarginine (R_f 0.23) and lysine (R_f 0.47), respectively. Integration of the peak areas of eluates from an analysis of the hydrolysate by an amino acid analyzer [and comparing the products to authentic samples of lysine (retention time 93 min) and homoarginine (retention time 368 min)] revealed that the conversion of lysine to homoarginine was 87%.

Studies were then carried out with other amino acid derivatives (blocked in the α-amino groups) to see if the guanidinating reagent reacted with functional groups other than the ϵ-amino group of lysine. The compounds present after treating the N^{α}-*t*-BOC derivatives of L-lysine, L-tyrosine, L-histidine, and L-serine with the guanidinating reagent (followed by decomposition of the *t*-BOC derivatives) were analyzed by TLC in system B. As expected, two spots were visible with ninhydrin for the treated lysine sample; these corresponded to authentic lysine (R_f 0.08) and homoarginine (R_f 0.13), respectively. Only single spots, identical with the parent amino acids, were visible for the other amino acid derivatives, indicating that the guanidinating reagent had not reacted at pH 9.0 with the imidazole of histidine (R_f 0.06), the hydroxy group of serine (R_f 0.22), or the phenol of tyrosine (R_f 0.44).

N^{α}-*t*-BOC-L-lysine was also reacted with 2-*S*-[^{14}C]thiuroniumethanesulfonate, and the products were analyzed by TLC in system B after treatment with trifluoroacetic acid to decompose the *t*-BOC derivatives; the results are shown in Table I. The latter treatment became necessary because the *t*-BOC derivatives of both L-lysine and L-homoarginine were found to migrate with the same R_f (0.75) in the solvent system used. When the TLC plates were analyzed for radioactivity

Table I: Thin-Layer Chromatography of the Products of the Guanidination of N^{α}-*t*-BOC-L-lysine with 2-*S*-Thiuroniumethanesulfonate

sample	R_f values[a]				
N^{α}-*t*-BOC-L-lysine					0.75
N^{α}-*t*-BOC-L-homoarginine					0.75
L-lysine	0.08				
L-homoarginine		0.13			
2-*S*-thiuroniumethanesulfonate				0.35	
N^{α}-*t*-BOC-L-lysine + 2-*S*-[^{14}C]thiuroniumethanesulfonate + CF_3COOH	0.08 [64]	0.13 [629]	[64]	0.35 [10994]	[51]

[a] On silica gel 60F_{254} sheets in 2-propanol/acetic acid/water (4:1:1 v/v); total dpm recovered from spots in square brackets.

Table II: Thin-Layer Chromatography of the Products of the Guanidination of *N*-Acetyl-L-cysteine with 2-*S*-Thiuroniumethanesulfonate

sample	R_f values[a]				
2-*S*-thiuroniumethanesulfonate		0.35			
2-*S*-thiuroniumethanesulfonate + CF_3COOH		0.42			(0.59)
urea				0.52	
thiourea					0.59
thiourea + CF_3COOH					0.59
N-acetyl-L-cysteine				0.51	
N,*N*′-diacetyl-L-cystine		0.38			
N-acetyl-*S*-(2-sulfoethyl)-L-cysteine		0.35			
N-acetyl-L-cysteine + 2-*S*-thiuroniumethanesulfonate	(0.21)	0.36		0.50	0.60
N-acetyl-L-cysteine + 2-*S*-[^{14}C]thiuroniumethanesulfonate + CF_3COOH	(0.21) [64]	0.38 [3792]	0.45 [371]	(0.50) [60]	

[a] On silica gel 60F_{254} sheets in 2-propanol/acetic acid/water (4:1:1 v/v); minor bands in parentheses; total dpm recovered from spots in square brackets.

(Table I), the level in the homoarginine spot was ca. 10-fold above that in the lysine spot (which was the same as background), confirming the presence of the ^{14}C-guanidinating group in the former. The level of radioactivity in the unreacted guanidinating reagent spot (detected as a dark spot at R_f 0.38 by viewing the plates under UV light) was some 19-fold higher than in the homoarginine spot due to the use of a 20-fold molar excess of guanidinating reagent to amino acid derivative in the labeling reaction.

The products of the reaction of 2-*S*-thiuroniumethanesulfonate with *N*-acetyl-L-cysteine were not visible with ninhydrin but could be visualized by two methods: (1) by spraying with a KI/starch solution after treatment of the plate with chlorine gas (Rydon & Smith, 1952), a method that detects peptides, amides, ureides, and thioureides; and (2) by spraying with an alkaline ferricyanide/nitroprusside solution (Walker, 1955), a method that detects guanidinium and thiuronium ions by a specific color reaction. The results (Table II) of TLC analysis in system B demonstrated that the thiol of *N*-acetyl-L-cysteine had reacted with 2-*S*-thiuroniumethanesulfonate at pH 9.0. The product of this reaction exhibited a major unidentified (red)[2] spot at R_f 0.60, together with unreacted *N*-acetyl-L-cysteine (a blue spot at R_f 0.50), the guanidinating reagent (a red-brown spot at R_f 0.36), and another minor unidentified spot at R_f 0.21. The major unidentified product at R_f 0.60 had an R_f similar to that of thiourea (a purple spot at R_f 0.59) but was different from that

[2] The colors are those generateed by the nitroprusside reagent.

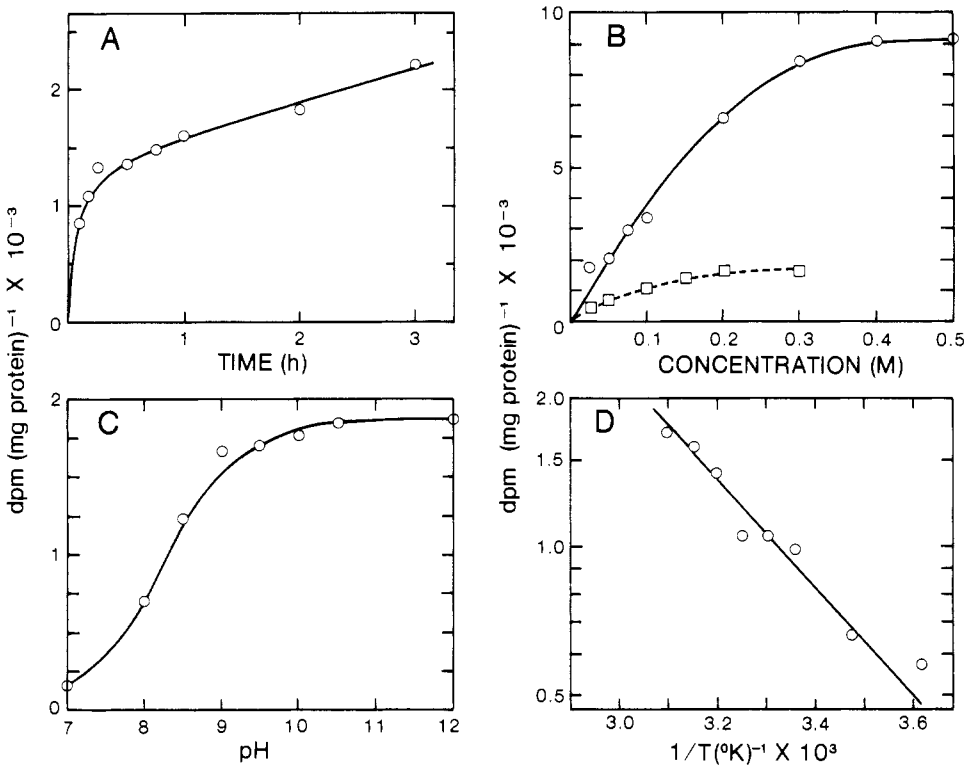


FIGURE 1: Specific activities (per milligram of protein) of washed chromatophores after labeling purified chromatophores (○) or whole cells (□) of *R. sphaeroides* with 2-*S*-[^{14}C]thiuroniumethanesulfonate as a function of (A) time of incubation, (B) concentration of guanidinating reagent, (C) pH of the incubation buffer, and (D) temperature of the incubation.

of *N*,*N*′-diacetyl-L-cystine (R_f 0.38), eliminating the possibility of air oxidation of *N*-acetyl-L-cysteine to the disulfide during TLC. It was also found not to be *N*-acetyl-*S*-(2-sulfoethyl)-L-cysteine (R_f 0.35), which is a possible reaction product of *N*-acetyl-L-cysteine with 2-S-thiuroniumethanesulfonate (cf. eq 2, Discussion). We could not detect, however, whether *some* of this product had in fact formed, as it exhibited the same R_f value as the guanidinating reagent in the solvent system used.

The major product (at R_f 0.60) was shown to be another possible reaction product, *N*-acetyl-*S*-amidino-L-cysteine (cf. eq 3, Discussion), by the following results: (1) it gave a red color with the alkaline ferricyanide/nitroprusside reagent characteristic of an amidino group (e.g., homoarginine and 2-*S*-thiuroniumethanesulfonate both give red colors with this reagent, whereas *N*-acetyl-L-cysteine gives a blue color and thiourea a purple-blue color); (2) the structure was consistent with the assignment of peaks in both the ^{13}C and ^{1}H NMR spectra of the major product purified from a large-scale reaction of the guanidinating reagent with *N*-acetyl-L-cysteine (cf. Materials and Methods); and (3) the product was shown to contain radioactivity when 2-*S*-[^{14}C]thiuroniumethanesulfonate was used as the guanidinating reagent.

Table II also shows the results of TLC analysis of the reaction products of such a reaction. Analysis of the radioactivity contained in the product spot at R_f 0.60 was not possible because it cochromatographed with traces of [^{14}C]thiourea (present as an impurity in the guanidinating reagent). Treatment with trifluoroacetic acid, however, was found to convert the R_f 0.60 spot to another spot at R_f 0.45, which was now well separated from both the guanidinating reagent and thiourea. Treatment with 2.4 M HCl also yielded the same spot at R_f 0.45 (results not shown). Neither the guanidinating reagent nor thiourea was altered significantly by treatment with trifluoroacetic acid, although the former compound yielded some thiourea (cf. Table II) due, perhaps, to an elimination reaction. When the TLC plates were analyzed for radioactivity (cf. Table II), the level in the R_f 0.45 product was ca. 6-fold above background (but some 12-fold less than that found in the guanidinating reagent spot), confirming the fact that it still contained the ^{14}C atom derived from the thiuronium group of the guanidinating reagent. Hence, it is very likely, although it has not been shown definitively, that the labeled compound at R_f 0.45 is 2-iminothiazolidine-4-carboxylic acid (cf. eq 4, Discussion).

Figure 1 shows the results of varying the incubation conditions during the labeling of purified chromatophores with 2-*S*-[^{14}C]thiuroniumethanesulfonate. Only in two cases [varying the concentration of the reagent (Figure 1B) and varying the pH (Figure 1C)] was there an apparent saturation of the extent of membrane labeling under standard conditions, reaching ca. 90% of the saturation level at 0.3 M concentration (which represented the approximate saturation point for the reagent in water) and pH 9.5, respectively. In the other two cases [varying the time (Figure 1A) and varying the temperature (Figure 1D)] the extent of labeling had not reached saturation at the highest values measured (3 h and 50 °C, respectively).

In comparing the extent of the labeling of purified chromatophores to that obtained when whole cells were labeled (followed by isolation and purification of the chromatophore fraction), it can be seen (Figure 1B) that, in the latter case, the isolated chromatophores were labeled to only about 20% of the level of purified chromatophores at equivalent concentrations of guanidinating reagent. An experiment labeling whole cells with undiluted 2-*S*-[^{14}C]thiuroniumethanesulfonate (at an estimated concentration of 0.1 M) yielded a purified chromatophore fraction with a specific activity of 5100 dpm/mg of protein. This was considered to be too low a value to study the labeling of membrane proteins from the periplasmic side of the membrane; hence, SDV were prepared from *R. sphaeroides* in a manner designed to ensure that the ma-

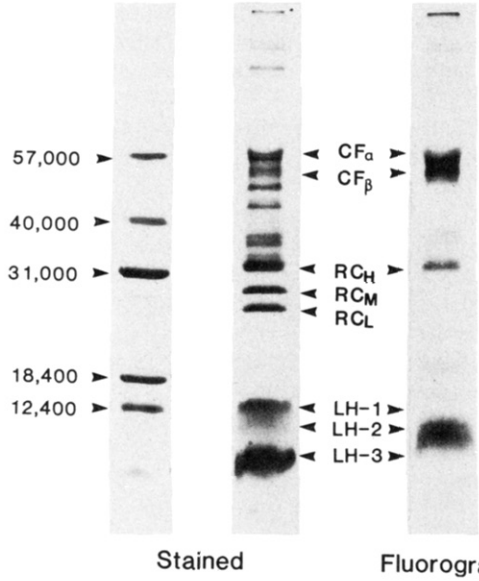


FIGURE 2: SDS–polyacrylamide gel electrophoretic separation and fluorographic detection of polypeptides labeled by addition of 2-*S*-[^{14}C]thiuroniumethanesulfonate to purified chromatophores. Identified bands are described in the text. Protein M_r standards employed were pyruvate kinase (57 000), ovalbumin (40 000), carbonic anhydrase (31 000), β-lactoglobulin (18 400), and cytochrome *c* (12 400).

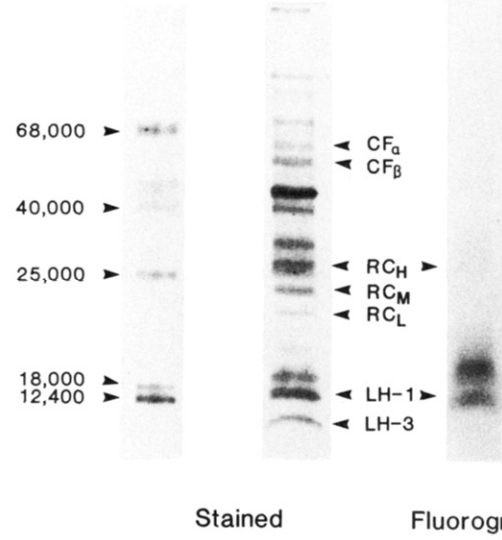


FIGURE 3: SDS–polyacrylamide gel electrophoretic separation and fluorographic detection of polypeptides labeled by addition of 2-*S*-[^{14}C]thiuroniumethanesulfonate to purified spheroplast-derived vesicles. Identified bands are described in the text. Protein M_r standards employed were bovine serum albumin (68 000), ovalbumin (40 000), chymotrypsinogen A (25 000), myoglobin (18 000), and cytochrome *c* (12 400).

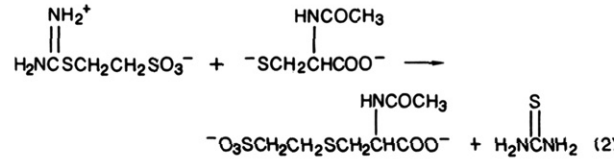
jority were of the "periplasmic side out" orientation (cf. Discussion). Both the purified SDV and chromatophores were labeled with undiluted 2-*S*-[^{14}C]thiuroniumethanesulfonate (at an estimated concentration of 0.1 M) and found to contain specific activities of 52 950 and 79 680 dpm/mg of protein, respectively.

The results of the separation of membrane proteins by SDS–polyacrylamide gel electrophoresis and analysis of the gels for radioactive proteins by fluorography are shown in Figures 2 and 3 for the purified chromatophore and SDV fractions, respectively. The major proteins labeled in the chromatophore fraction (Figure 2) included the α- and β-subunits of the coupling factor (M_r 55 000 and 51 000), together with several other polypeptides in the RC_H (M_r 31 000) and LH-2 (M_r 10 000) regions of the gel. All of these polypeptides are known to be accessible from the cytoplasmic side of the ICM (Francis & Richards, 1980). In the case of the SDV (Figure 3), more polypeptides were visible in the gel due to the presence of cytoplasmic and apparent outer membrane components. Although at least two low M_r polypeptides were observed to be heavily labeled (Figure 3), no activity was detected in the α- and β-subunits of the coupling factor after 8 weeks of fluorography.

Discussion

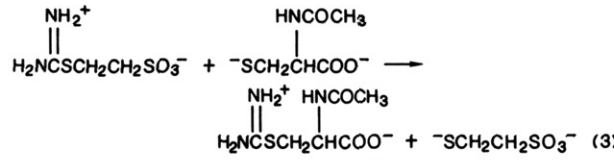
While the functional groups of tyrosine, histidine, and serine do not react with 2-*S*-thiuroniumethanesulfonate at pH 9.0, the ε-amino group of lysine and the thiol group of cysteine do react. Since proteins containing reduced cysteinyl residues are rare, the most common consequence of the use of the guanidinating reagent would be to convert lysyl residues to homoarginyl residues (eq 1) in about 80–90% yield. Since both lysine and homoarginine are basic amino acids (pK_a = 10.5 vs. 12.5, respectively), the properties of polypeptides with altered residues would not be expected to be significantly different from the parent polypeptide; hence, solubilization, isolation, and analysis procedures (including chromatography, gel filtration, detergent gel electrophoresis, etc.) could be carried out and retain altered and unaltered polypeptides in the same fraction.

In order to determine the product of the reaction of the guanidinating reagent with thiol groups, *N*-acetyl-L-cysteine was used as a model compound. At the pH of the guanidinating reaction (>9.0), the attacking species of *N*-acetyl-L-cysteine is very likely the thiolate anion; two reactions are possible. In the first possibility (eq 2), attack of the thiolate

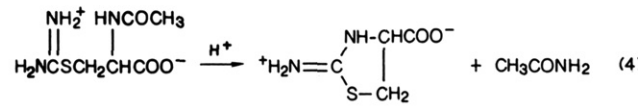


on a saturated carbon (C2 of ethylene) leads to alkylation of the thiol and elimination of thiourea. Banks and Shafer (1972) observed an analogous reaction during the methylation of the cysteinyl residues of papain with *O*-methylisourea.

In the second possibility (eq 3), attack of the thiolate on



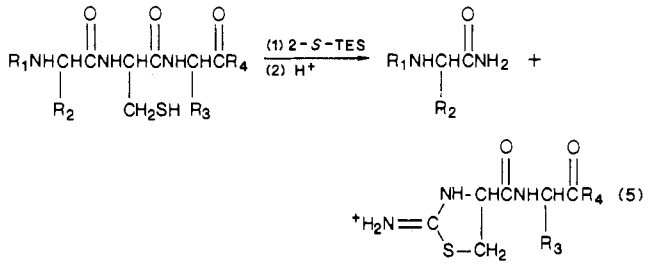
the unsaturated thiuronium carbon leads to elimination of 2-mercaptoethanesulfonate (which would be much more facile than in the case of an O-containing leaving group) and converts *N*-acetyl-L-cysteine to a thiuronium derivative (*N*-acetyl-*S*-amidino-L-cysteine). The major product at R_f 0.60 (Table II) was shown to have this structure (cf. Results). Rambacher (1968) also prepared *N*-acetyl-*S*-amidino-L-cysteine by another procedure and found that it was converted by 2.4 M HCl to 2-iminothiazolidine-4-carboxylic acid (eq 4). Hence, it is very



likely that the labeled compound at R_f 0.45 (Table II) derived from *N*-acetyl-L-cysteine by treatment with trifluoroacetic acid is the above cyclization product.

If the guanidinating reagent were used on purified proteins in which any cystine disulfides present had been reduced to

thiols, reaction with 2-*S*-thiuroniumethanesulfonate followed by treatment with anhydrous trifluoroacetic acid would result in cleavage of the peptide on the amino side of the cysteinyl residues, yielding the 2-iminothiazolidine-4-carboxamide derivatives of the residue on the carboxyl side, plus the primary amide of the residue on the amino side of each cysteinyl residue (eq 5). A similar peptide cleavage procedure has been de-



veloped by Jacobson et al. (1973) whereby the same 2-iminothiazolidine-4-carboxamide derivative (plus the free carboxylate of the residue on the amino side of the cysteinyl residue) is generated from *S*-cyanocysteinyl residues (generated from residues of cysteine or cystine by reaction with 2-nitro-5-thiocyanobenzoic acid). The 2-iminothiazolidine-4-carboxamide residues may be catalytically reduced with $NiCl_2$ plus $NaBH_4$ to alanyl residues (Schaffer & Stark, 1976); hence, 2-*S*-thiuroniumethanesulfonate represents a new cysteine-specific protein cleavage reagent for peptide analysis that can be employed under relatively mild conditions.

The results of varying the temperature of the incubation of the guanidinating reagent with chromatophores (Figure 1D) have demonstrated that there was a linear relationship between the log of the extent of labeling and $1/T$ in this temperature range (4–50 °C), with no sign of reaching a level of saturation. We did not test whether the highest temperature had led to the unmasking of labeling sites due to partial denaturation of membrane proteins, but this seemed unlikely because of the continuous nature of the temperature variation plot.

The results of varying the time of incubation of the guanidinating reagent with chromatophores (Figure 1A) have indicated that membrane labeling at pH 9.0 is biphasic: an initial rapid labeling rate (which reached apparent saturation in about 15 min) was followed by a much slower labeling rate which remained constant with no sign of reaching saturation after 3 h of incubation. Varying the pH of the incubation buffer from 7 to 12 (Figure 1C) showed that only about 50% of the groups reacting in 2 h at pH >10.5 had reacted at pH 8.3. This would indicate that perhaps the initial rapid labeling rate in Figure 1A (which was carried out at pH 9.0) was due to the labeling of groups with pK_a values <9. Although not specifically tested for, it is likely that the N-terminal amino groups of proteins would react with the guanidinating reagent. The related *S*-ethylisothiourea has been shown (Brand & Brand, 1955) to convert glycine to guanidinoacetic acid. The N-terminal amino groups, together with the thiols of cysteinyl residues, may, therefore, have been labeled in the rapid labeling phase. The results of Figure 2 have demonstrated that lipids as well as proteins are labeled by the guanidinating reagent. Hence, the amino groups of phosphatidylethanolamine and lysyl residues may have been labeled in the slower labeling phase, as they both have pK_a values >10 and would be largely protonated at pH 9.

In order to determine whether 2-*S*-thiuroniumethanesulfonate is impermeable to biomembranes, the guanidinating reagent was added to whole cells of *R. sphaeroides* (Figure 1B). Francis and Richards (1980) found that between 75% and 80% of the photosynthetic membranes of *R. sphaeroides* were labeled by pyridoxal phosphate plus $[^3H]KBH_4$ when these reagents were added to whole cells during their adaptation to phototrophic growth conditions, indicating that the majority of the ICM was accessible to reagents in the periplasm and thus continuous with the cytoplasmic membrane. However, the specific activity of chromatophores isolated from such cells was found to be too low to study by fluorography (Francis & Richards, 1980; cf. also Figure 1B); hence, the labeling of a subcellular membrane system that can be isolated in two distinctly different orientations was studied.

The chromatophore vesicles of purple photosynthetic bacteria (derived from the ICM during cellular disruption by ultrasonication or pressure release in the French pressure cell) are oriented predominantly "inside out" (or cytoplasmic side out), while SDV are oriented predominantly "right side out" (or periplasmic side out; Matsuura & Nishimura, 1977; Michels & Konings, 1978; Lommen & Takemoto, 1978; Takemoto & Bachmann, 1979). The yield of SDV with the right side out orientation can be increased (in the case of *R. sphaeroides*) by growth in very high light intensity during which time the cells produce very little ICM. Numerous studies, using one or both of these membrane vesicle preparations, have been carried out recently to identify the components that have accessible reactive residues on either side of the ICM, including both lipids (Al-Bayatti & Takemoto, 1981) and proteins (Zuber, 1985).

The α- and β-subunits of the coupling factor are extrinsic proteins accessible only on the cytoplasmic side of the ICM (Elferink et al., 1979; Takemoto & Bachmann, 1979). The heavy label detected in these two polypeptides in chromatophores but not in the SDV fraction (after 8 weeks of fluorography), and the different pattern of labeling of the small M_r polypeptides in the two types of membrane preparations (compare Figures 2 and 3), makes it clear that the guanidinating reagent did not penetrate the membrane to any significant extent. An analysis of the labeling of LH proteins purified from the two types of membrane preparations is currently in progress in our laboratory.

References

Al-Bayatti, K. K., & Takemoto, J. Y. (1981) *Biochemistry 20*, 5489–5495.

Banks, T. E., & Shafer, J. A. (1972) *Biochemistry 11*, 110–114.

Brand, E., & Brand, F. C. (1955) *Organic Syntheses*, Collect. Vol. III, pp 440–442, Wiley, New York.

Chervenka, C. H., & Wilcox, P. E. (1956) *J. Biol. Chem. 222*, 635–647.

Elferink, M. G. L., Hellingwerf, K. J., Michels, P. A. M., Seyen, H. G., & Konings, W. N. (1979) *FEBS Lett. 107*, 300–307.

Francis, G. A., & Richards, W. R. (1980) *Biochemistry 19*, 5104–5111.

Greenstein, J. P. (1935) *J. Biol. Chem. 109*, 541–544.

Hubbard, A. L., & Cohn, Z. A. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H., Ed.) pp 427–501, Wiley, New York.

Hughes, W. L., Jr., Saroff, H. A., & Carney, A. L. (1949) *J. Am. Chem. Soc. 71*, 2476–2480.

Itoh, M., Hagiwara, D., & Kamiya, T. (1975) *Tetrahedron Lett.*, 4393–4394.

Jacobson, G. R., Schaffer, M. H., Stark, G. R., & Vanaman, T. C. (1973) *J. Biol. Chem. 248*, 6583–6591.

Kennelly, P. J., Timkovich, R., & Cusanovich, M. A. (1981) *J. Mol. Biol. 145*, 583–602.

Kimmel, J. R. (1967) *Methods Enzymol. 11*, 584–589.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lommen, M. A. J., & Takemoto, J. (1978) *J. Bacteriol. 136*, 730–741.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lundblad, R. L., & Noyes, C. M. (1984) *Chemical Reagents for Protein Modification*, Vol. I, pp 127–170, CRC Press, Boca Raton, FL.
Matsuura, K., & Nishimura, M. (1977) *Biochim. Biophys. Acta 459*, 483–491.
Means, G. E. & Feeney, R. E. (1971) *Chemical Modification of Proteins*, pp 93–97, Holden-Day, San Francisco.
Michels, P. A. M., & Konings, W. N. (1978) *Biochim. Biophys. Acta 507*, 353–368.
Niketic, V., Thomsen, J., & Kristiansen, K. (1974) *Eur. J. Biochem. 46*, 547–551.
Rambacher, P. (1968) *Chem. Ber. 101*, 3433–3437.
Rydon, H. N., & Smith, P. W. G. (1952) *Nature (London) 169*, 922–923.
Schaffer, M. H., & Stark, G. R. (1976) *Biochem. Biophys. Res. Commun. 71*, 1040–1047.
Schramm, C. H., Lemaire, H., & Karlson, R. H. (1955) *J. Am. Chem. Soc. 77*, 6231–6233.
Schütte, E. (1943) *Hoppe-Seyler's Z. Physiol. Chem. 279*, 52–59.
Stellwagen, E., Smith, F., Cass, R., Ledger, R., & Wilgus, H. (1977) *Biochemistry 16*, 3672–3679.
Takemoto, J., & Bachmann, R. C. (1979) *Arch. Biochem. Biophys. 195*, 526–534.
Walker, J. B. (1955) *Arch. Biochem. Biophys. 59*, 233–245.
Whiteley, N. M., & Berg, H. C. (1974) *J. Mol. Biol. 87*, 541–561.
Wofsey, L., & Singer, S. J. (1963) *Biochemistry 2*, 104–116.
Zuber, H. (1985) *Photochem. Photobiol. 42*, 821–844.

Identification of Two Segments, Separated by ~45 Kilodaltons, of the Myosin Subfragment 1 Heavy Chain That Can Be Cross-Linked to the SH-1 Thiol†

Kazuo Sutoh‡ and Rennê Chen Lu*,§

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan, and Department of Muscle Research, Boston Biomedical Research Institute, and Department of Neurology, Harvard Medical School, Boston, Massachusetts 02114

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ABSTRACT: The thiol-specific photoactivatable reagent 4-(2-iodoacetamido)benzophenone (BPIA) can be selectively incorporated into the SH-1 of myosin subfragment 1 (S1), and upon photolysis an intramolecular cross-link is formed between SH-1 and the N-terminal 25-kDa region of S1. If a Mg^{2+}–nucleotide is present during photolysis, cross-links can be formed either with the 25-kDa or with the central 50-kDa region [Lu, R. C., Moo, L., & Wong, A. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6392–6396]. Heavy chains with these two types of intramolecular cross-links and un-cross-linked heavy chain have different mobility on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels and therefore can be purified electrophoretically. Each type of heavy chain was cleaved with *Staphylococcus aureus* protease, chymotrypsin, or lysyl endopeptidase. The cleavage points were determined on the basis of the molecular weights of peptides containing the N-terminus, which was identified with the use of an antibody. Locations of the cross-links were deduced by comparing the peptide maps of cross-linked and un-cross-linked heavy chains. The results indicate that the segment located about 12–16 kDa from the N-terminus of the heavy chain can be cross-linked to SH-1 via BPIA independently of the presence of a nucleotide, whereas the segment located 57–60 kDa from the N-terminus can be cross-linked to SH-1 only in the presence of a Mg^{2+}–nucleotide. With use of the avidin–biotin system, it has been shown that SH-1 is located 13 nm from the head/rod junction [Sutoh, K., Yamamoto, K., & Wakabayashi, T. (1984) *J. Mol. Biol. 178*, 323–339]. Since BPIA spans less than 1 nm, our results show that two regions, separated by ~400 amino acid residues and located in the 25- and 50-kDa domains of S1, respectively, are also part of the head structure about 12–14 nm from the head/rod junction.

The head portion of myosin, subfragment 1 (S1),[1] is a key component of the machinery of muscle cells that converts the chemical energy of ATP to mechanical energy. The heavy chains of S1 can be nicked with proteolytic enzymes into three distinct fragments with apparent molecular masses of 50, 25, and 20 kDa on SDS–PAGE (Balint et al., 1975). The nicked S1 retains the K^+- and Ca^{2+}-ATPase activities and the ability to bind actin. The most reactive thiol, SH-1, is located in the C-terminal 20-kDa region (Lu et al., 1978; Balint et al., 1978; Gallagher & Elzinga, 1980); the modification of SH-1 affects the ATPase activities of S1 (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966), but a direct involvement of SH-1 in nucleotide binding has been excluded (Burke & Reisler, 1977;

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* Address correspondence to this author at the Department of Muscle Research, Boston Biomedical Research Institute.
‡ University of Tokyo.
§ Boston Biomedical Research Institute and Harvard Medical School.

[1] Abbreviations: S1, subfragment 1; BPIA, 4-(2-iodoacetamido)-benzophenone; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; kDa, kilodalton(s); $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LEP, lysyl endopeptidase; Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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