Addition of Sulfhydryl Groups to *Escherichia coli* Ribosomes by Protein Modification with 2-Iminothiolane (Methyl 4-Mercaptobutyrimidate)†

Rodney Jue,‡ John M. Lambert, Leland R. Pierce, and Robert R. Traut*

ABSTRACT: The bifunctional reagent, methyl 4-mercaptobutyrimidate, has been useful in identifying neighboring protein pairs in the *Escherichia coli* 30S ribosomal subunit. The reagent reacts with protein amino groups and upon oxidation forms intermolecular disulfide bonds. The compound has been characterized further employing the techniques of nuclear magnetic resonance spectroscopy, infrared spectroscopy, ultraviolet spectroscopy, mass spectroscopy, elemental analysis, and sulfhydryl group titration. All of the results indicate that the compound formerly considered to be methyl 4-mercaptobutyrimidate is in fact 2-iminothiolane (2-iminotetrahydrothiophene). Unlike most imidates, 2-iminothiolane is highly stable in solution at acidic and neutral pH. Furthermore, we have not observed any protein:protein cross-links produced with the reagent that were not reversibly cleaved by mild reduction indicating the absence of uncleavable protein:protein *N*-alkyl imidate linkages caused by side reactions typical of other imidates. The extent of modification of 30S ribosomal protein by 2-iminothiolane has been investigated. Approximately 43 sulfhydryl groups were added to the proteins of the 30S subunit under conditions employed in previous investigations. The majority of these reacted rapidly with reagents for sulfhydryl groups, suggesting that 2-iminothiolane had reacted preferentially with exposed lysine groups. Unmodified and modified 30S subunits were alkylated with [1-^{14}C]iodoacetamide and ribosomal proteins were separated by two-dimensional polyacrylamide/urea gel electrophoresis. Comparison of radioautographs of the gels demonstrated that all the 30S ribosomal proteins were modified to varying extents by 2-iminothiolane.

Imido esters have proved to be valuable reagents for use in experiments that employ the techniques of chemical modification to study structural and functional properties of proteins. Imido esters react specifically with amino groups of proteins to form positively charged amidine derivatives (Hunter & Ludwig, 1962, 1972; Hand & Jencks, 1962). This retention of surface charge distribution favors the preservation of tertiary and quaternary structure of proteins since it is likely that the local side-chain interactions are maintained. This conclusion was suggested by the work of Wofsy & Singer (1963) and is supported by amidination experiments with tobacco mosaic virus (Perham & Richards, 1968; Perham, 1973), glyceraldehyde-3-phosphate dehydrogenase (Lambert & Perham, 1977), and alcohol dehydrogenase (Plapp, 1970; Dworschack et al., 1975), in which the modified proteins retain biological activity.

Bis(imido esters), such as dimethyl suberimidate, have been extremely useful tools for the examination of the quaternary structure of oligomeric enzymes (Davies & Stark, 1970), and also of the protein topography of the ribosome (Bickle et al., 1972; Lutter et al., 1974; Clegg & Hayes, 1974; Expert-Bezançon et al., 1977). Difficulties in the identification of the ribosomal components of cross-linked dimers thus formed prompted the use of imido esters which permitted ready cleavage of the cross-link (Traut et al., 1973; Lutter et al., 1975; Peretz et al., 1976). The alternative strategy for cross-linking experiments used in this laboratory involves modification of protein with an imido ester that contains a sulfhydryl group, followed by a separate cross-linking step accomplished by oxidation of the sulfhydryl-rich species (Traut et al., 1973). In this two-step cross-linking technique hydrolysis of one end of a bis(imido ester) does not present a problem with respect to cross-link yield insofar as each modified lysine has a free sulfhydryl group potentially capable of forming a cross-link. The hydrolysis of one group of a bis(imido ester), the other group of which reacts with lysine, can be anticipated to lower the yield of cross-linking. The two-step cross-linking procedure using the reagent known as methyl 4-mercaptobutyrimidate has been employed to study the spatial arrangement of ribosomal proteins with substantial success (Sun et al., 1974; Sommer & Traut, 1974–1976; Kenny et al., 1975).

We describe here an investigation of the structure and properties of the reagent originally named methyl 4-mercaptobutyrimidate. All of the results indicate that the compound is in fact 2-iminothiolane (2-iminotetrahydrothiophene), a cyclic thioimidate that has been described by Schramm & Dülffer (1977a,b). The use of 2-iminothiolane in studies involving chemical modification of protein is discussed with regard to its reaction with the 30S ribosomal subunit.

Materials and Methods

Reagents. Methyl 4-mercaptobutyrimidate [2-iminothiolane (2-iminotetrahydrothiophene)][1] was purchased from Pierce Chemical Co. and stored in a vacuum desiccator over Drierite at 4 °C. Anhydrous trifluoroacetic acid (Sequanal grade) was also obtained from Pierce and 3-(trimethylsilyl)-propanesulfonic acid (sodium salt) from Thompson Packard, Inc.; iodoacetamide, 5,5′-dithiobis(2-nitrobenzoic acid),[2] and

† From the Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616. *Received June 6, 1978.* This work was supported by a grant (GM 17924) from the U.S. Public Health Service.

‡ Present address: Department of Chemistry, University of California, San Diego, California 92093.

[1] The bifunctional reagent methyl 4-mercaptobutyrimidate, used previously in this laboratory to cross-link neighboring ribosomal proteins, has been found to have properties indicating its identity with the compound 2-iminothiolane described by Schramm & Dülffer [Schramm, H. J., & Dülffer, T. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 137–139]. The latter name will be used here.

[2] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/78/0417-5399$01.00/0

bistris were from Sigma; urea "Ultrapure" and 2-mercaptoethanol were from Schwarz/Mann. Triethanolamine was purchased from Eastman Organic Chemicals and was redistilled under vacuum before use. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from Eastman and used without further recrystallization. [1-^{14}C]Iodoacetamide (specific radioactivity 57 mCi/mmol) was from Amersham/Searle and diluted 1:100 with nonradioactive iodoacetamide before use.

Structural Studies on 2-Iminothiolane. Elemental analysis and mass spectroscopy were performed at the Department of Chemistry, University of California, Berkeley. Samples of 2-iminothiolane were recrystallized by refrigeration of saturated solutions of the compound in methanol/acetone. The largest crystals (mp 192–193 °C) were dried in a vacuum desiccator over Drierite, crushed to powder and evacuated with a high-vacuum pump for 24 h before sealing under vacuum in glass ampules for storage before analysis.

2-Iminothiolane (70 mg) was dissolved in 0.4 mL of trifluoroacetic acid containing 3-(trimethylsilyl)propanesulfonic acid (sodium salt) as a zero reference for nuclear magnetic resonance spectroscopy performed with a Variant Anaspect EM 360 NMR spectrometer. Higher resolution was obtained using a Japan Electron Optics Laboratory Co. Ltd. Model JNM-MH-100 NMR spectrometer. The trifluoroacetic acid solvent was found to have a resonance at 11.73 ppm, close to the published value of 11.67 ppm (Sadtler, Standard NMR Spectra, No. 15078 M).

Infrared spectroscopy was performed with a Beckman IR 8 infrared spectrometer at slow dual beam speed. 2-Iminothiolane was prepared for infrared spectroscopy by mixing with KBr (5% 2-iminothiolane, w/w) which was pressed into a translucent pellet. Polystyrene (Beckman) was used to calibrate the spectrum at the wave numbers 2849.9, 1601.0, and 906.5 cm^{-1}.

Ultraviolet spectroscopy was performed with a Cary 16 spectrophotometer, a Cary Recorder Interface Model 1626, and a Varian C-2000 recorder. The absorption spectrum was determined using 2-iminothiolane dissolved in acetonitrile to give an absorbance of about 1.0 unit (about 0.1 mM).

Ribosomes. The 30S ribosomal subunits were prepared from *Escherichia coli* strain MRE600 grown in rich medium using techniques already described (Hershey et al., 1977; Eikenberry et al., 1970). Ribosomal subunits (25–30 mg/mL) were stored at −70 °C in 10 mM Tris-HCl, pH 7.2, containing 10 mM $MgCl_2$, 100 mM NH_4Cl, and 14 mM 2-mercaptoethanol.

Modification of 30S Ribosomal Subunits with 2-Iminothiolane. The 30S subunits were diluted to about 3 mg/mL and dialyzed overnight against 50 mM triethanolamine hydrochloride, pH 8.0, containing 50 mM KCl, 1.0 mM $MgCl_2$, and 1% 2-mercaptoethanol (TEA–SH buffer). A stock solution of 2-iminothiolane (0.5 M; 68.8 mg/mL) was prepared immediately before use by dissolving the solid reagent in a solution consisting of 1.0 M triethanolamine hydrochloride, pH 8.0, and 1.0 M triethanolamine, free base (1:1 v/v); the final pH was about 8.0. Modification was performed at 0 °C after adding reagent to the desired final concentration (12 mM in most experiments). After incubation for 20 min, the reaction mixture was dialyzed for 3 h at 4 °C against 100 vol of 50 mM triethanolamine hydrochloride, pH 8.0, containing 50 mM KCl and 1.0 mM $MgCl_2$ (TEA buffer) with a buffer change each hour as described previously (Traut et al., 1973; Sommer & Traut, 1975). Alternatively, the modification reaction was rapidly stopped by gel filtration of samples as described in the following section.

Determination of Sulfhydryl Groups with Nbs_2. Samples (about 0.5 mL) of 30S ribosomal subunits (about 3.0 mg/mL) in TEA–SH buffer were applied to columns (15.5 × 0.7 cm) of Bio-Gel P-2 (100–200 mesh) equilibrated with 10 mM Tris-HCl, pH 7.4, containing 30 mM NH_4Cl and 10 mM $MgCl_2$. The column buffer was deaerated and then saturated with nitrogen in order to prevent air oxidation of sulfhydryl groups during gel filtration. Columns were developed at 4 °C. The peak fraction (A_{260}) was collected (approximately 1.1 mL), and 1.0 mL was transferred immediately to a cuvette and mixed with 100 μL of 10 mM Nbs_2 dissolved in the column buffer. The increase in absorbance at 412 nm was followed using a Gilford 2400-S recording spectrophotometer. After 10 min at 25 °C, 50 μL of 20% sodium dodecyl sulfate was added to denature the 30S subunit and the increase in absorbance was followed for a further 10 min. The molar extinction coefficient of the thionitrobenzoate ion at 412 nm was assumed to be 1.36×10^4 M^{-1} cm^{-1} (Ellman, 1959).

Duplicate samples (15 μL) of the 30S subunit peak fraction were diluted into 1.0 mL of column buffer to determine 30S ribosomal subunit concentration, assuming $E^{1\%}_{260nm}$ to be 145.0, and the molecular weight of the 30S ribosomal subunit to be 0.9×10^6 (Hill et al., 1969).

The same procedure was used to determine sulfhydryl groups of 30S subunits modified with 2-iminothiolane. Modified 30S ribosomal subunits were passed through columns of Bio-Gel P-2 to remove 2-mercaptoethanol and excess reagent, after the modification procedures already described.

Reaction of 30S Ribosomal Subunits with [1-^{14}C]Iodoacetamide. Samples of unmodified 30S subunits or 30S subunits modified with 2-iminothiolane, at approximately 2.0 mg/mL and free of 2-mercaptoethanol after gel filtration, were added to an equal volume of 8.0 M urea, 6.0 M LiCl, 0.2 M Tris-HCl, pH 8.0, containing 50 mM [1-^{14}C]iodoacetamide (specific radioactivity, 0.57 mCi/mmol). After 1 h at 25 °C, excess reagent was quenched by the addition of 2.5% 2-mercaptoethanol. Duplicate samples (5.5 μg) of the alkylated subunits were precipitated with 2.5 mL of 5% trichloroacetic acid along with 100 μg of bovine serum albumin carrier protein, to determine the extent of radioactive alkylation. After 30 min at 94 °C, the samples were filtered through glass filters (Whatman, 2.4 cm GF/C) and washed well with 5% trichloroacetic acid. The filters were dried and counted in a Beckman LS-200B liquid scintillation counter in 5.0 mL of toluene containing 2,5-diphenyloxazole (4 mg/mL) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.5 mg/mL). The counting efficiency was 80%.

Ribosomal protein from the alkylated samples of ribosomal subunits was extracted with 66% acetic acid and 34 mM $MgCl_2$ (Hardy et al., 1969). After removal of the RNA precipitate by centrifugation at 10 000 rpm for 15 min in a Sorvall RC2-B centrifuge (SS-34 rotor), the supernatant fraction was exhaustively dialyzed against 6% acetic acid and the protein recovered by lyophilization.

Two-Dimensional Polyacrylamide/Urea Gel Electrophoresis and Identification of Radioactive Alkylated Ribosomal Proteins. Lyophilized samples of radioactively alkylated ribosomal protein (300 μg) were dissolved in 8.0 M urea (50 μL) and analyzed by two-dimensional polyacrylamide/urea gel electrophoresis according to Knopf et al. (1975). The sample was applied to a 4% (w/v) polyacrylamide gel (10 × 0.3 cm) containing 0.066% (w/v) *N,N'*-methylenebisacrylamide, 38 mM bistris, 6.0 M urea, and adjusted to pH 5.5 with acetic acid after mixing the components. A stacking gel (0.5 × 0.3 cm) was of the same composition adjusted to pH 4.7 with acetic acid. Electrophoresis was performed for 5 h at 1 mA/gel toward the cathode using pyronine G as marker dye. The upper

TABLE I: Elemental Analysis of 2-Iminothiolane.

elements	calcd $C_5H_{12}ONSCl$[a]	calcd C_4H_8NSCl[b]	obsd
C	35.40	34.91	35.04
H	7.13	5.86	5.85
N	8.26	10.18	10.17
S	18.90	23.29	23.38
Cl	20.90	25.76	25.62
O	9.43	0.00	0.00
total	100.02%	100.00%	100.60%

[a] Methyl 4-mercaptobutyrimidate hydrochloride. [b] 2-Iminothiolane hydrochloride.

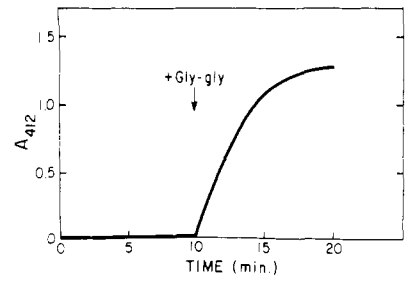


FIGURE 1: Reaction of 2-iminothiolane with Nbs_2. 2-Iminothiolane (13 μg in 10 μL of a stock solution in acetonitrile) was added to 1.0 mL of TEA buffer, pH 8.0, containing 100 μL of 10 mM Nbs_2 at 7 °C. The absorbance change at 412 nm was followed. After 10 min, glycylglycine (pH 8.0) was added to a final concentration of 17.5 mM.

buffer chamber contained 0.02 M bistris-acetate, pH 3.7, and the lower chamber 0.02 M bistris-acetate, pH 7.0. The gel tube was embedded at the origin of an 18.0% (w/v) polyacrylamide gel slab (13.0 × 11.0 × 0.15 cm) containing 0.24% (w/v) *N,N'*-methylenebisacrylamide, 6.0 M urea, 48 mM KOH, and 5.2% (v/v) acetic acid, pH 4.6. Electrophoresis was at 80 V toward the cathode for 12–16 h until the pyronine G tracking dye was 1.0 cm from the base of the gel, using a well buffer consisting of glycine (14 g/L) and acetic acid (1.5 mL/L). Gels were stained with 0.55% (w/v) Amido black in methanol: water:acetic acid (5:5:1 by volume) and destained in the same solution without dye. Gels were prepared for radioautography by drying onto filter paper (Howard & Traut, 1973), and exposed to Kodak No-Screen Medical X-ray film for 40 days.

Results

Reaction of 2-Iminothiolane with Nbs_2. Nbs_2 titration was employed to test for sulfhydryl groups in freshly prepared solutions of 2-iminothiolane. When 2-iminothiolane was added to TEA buffer containing Nbs_2 at 7 °C, there was no initial increase in absorbance at 412 nm as shown in Figure 1, indicating the absence of free sulfhydryl groups. Addition of glycylglycine to the reaction (17.5 mM; about 180-fold excess over 2-iminothiolane) led to the rapid appearance of sulfhydryl groups. Similar titrations at 25 °C showed that sulfhydryl groups were very slowly exposed in the absence of any primary amine. The initial rate of formation of sulfhydryl groups from 2-iminothiolane (at concentrations of 0.1 mM and 1.0 mM) in TEA buffer at 25 °C was 0.9% per min of the total sulfhydryl groups that were generated by addition of glycylglycine. This appearance of sulfhydryl groups in the absence of amines was almost undetectable at 7 °C (Figure 1).

The weight of 2-iminothiolane equivalent to 1 mol of free sulfhydryl group released upon treatment with glycylglycine was determined. The Nbs_2 titration procedure shown in Figure 1 was used, and the maximum absorbance change at 412 nm promoted by glycylglycine was measured. Five determinations of the sulfhydryl group concentration derived from 2-iminothiolane (5.527 μg/mL) gave values of 38.67, 39.55, 40.29, 40.29, and 40.66 μM. From these values, it was calculated that 138 g (±2 g) of 2-iminothiolane released 1 mol of free sulfhydryl group upon treatment with a primary amine.

Chemical Analysis of 2-Iminothiolane. The results of elemental analysis shown in Table I were consistent with the formula C_4H_8NSCl which is the chemical formula of 2-iminothiolane hydrochloride, and not with the chemical formula of $C_5H_{12}ONSCl$ for methyl 4-mercaptobutyrimidate hydrochloride. The mass spectrum gave an intense peak at *m/e* 101 corresponding to loss of HCl from 2-iminothiolane hydrochloride, and the fragmentation pattern was quite consistent with the structure 2-iminothiolane while the spectrum contained no evidence to support a structure corresponding to methyl 4-mercaptobutyrimidate (results not shown).

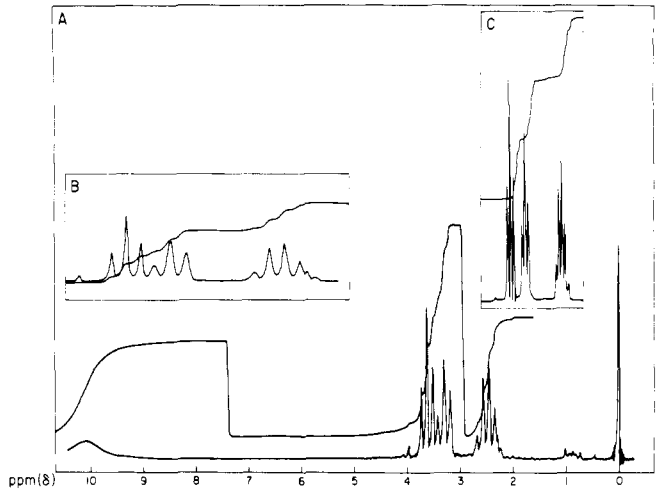


FIGURE 2: Nuclear magnetic resonance spectrum of 2-iminothiolane. The spectrum A was obtained at 600 Hz with sweep time 300 s. The spectrum B was the 2–4-ppm region of spectrum A run at 120 Hz with sweep time 120 s. The spectrum C was the 2–4-ppm region of spectrum A run at 1080 Hz with sweep time 250 s. For other details, see text.

Nuclear Magnetic Resonance Spectroscopy of 2-Iminothiolane. Four major peaks were seen in the NMR spectrum A shown in Figure 2. There was a broad peak with a chemical shift of 10.07 ppm, two triplet peaks at 3.63 ppm and 3.30 ppm, and a quintet peak at 2.48 ppm. The inset C of Figure 2 shows the 2–4-ppm region determined with the JNM-MH-100 NMR instrument, which clearly resolves the triplets as two peaks and not one complex peak. The inset B of Figure 2 shows an enlargement of the 2–4-ppm region of the spectrum A, from which it was possible to show that the triplet peaks at 3.63 and 3.30 ppm were each coupled to the quintet peak at 2.48 ppm with a coupling constant *J*, determined as 6.4 and 7.0 cps, respectively.

The proton integration ratios for the four major peaks were measured to be 1.01 (10.07 ppm):0.94 (3.63 ppm):1.08 (3.30 ppm):0.95 (2.48 ppm), showing that each peak represented an equal number of protons. The evidence was consistent with the three peaks in the 2–4-ppm region being derived from a $-CH_2CH_2CH_2-$ system in the molecule, each peak corresponding to two protons. The broad peak with the largest chemical shift at 10.07 ppm, and which also corresponds to two protons determined from the integration ratios, was probably derived from the imine group. Thus all four proton peaks can be accounted for. There was no peak to correspond with protons from a methoxy group (CH_3-O-), which appears as a

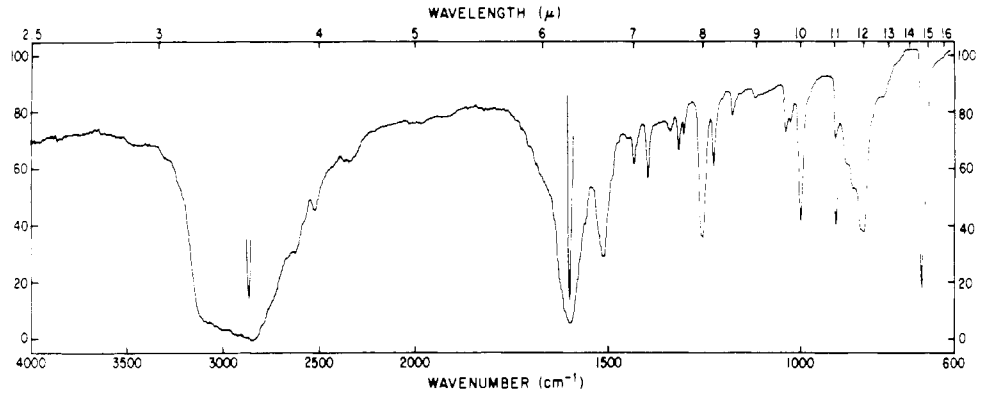


FIGURE 3: Infrared spectrum of 2-iminothiolane. For other details, see text.

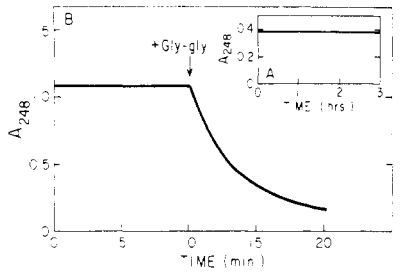


FIGURE 4: The effect of glycylglycine on the absorbance of 2-iminothiolane at 248 nm. Inset A shows the absorbance at 248 nm of 2-iminothiolane (0.05 mM) dissolved in TEA buffer, pH 8.0, followed for 3 h at 7 °C. Graph B shows the absorbance at 248 nm of 2-iminothiolane (0.11 mM) dissolved in TEA buffer, pH 8.0, at 7 °C. After 10-min incubation, glycylglycine (pH 8.0) was added to 17.5 mM, final concentration.

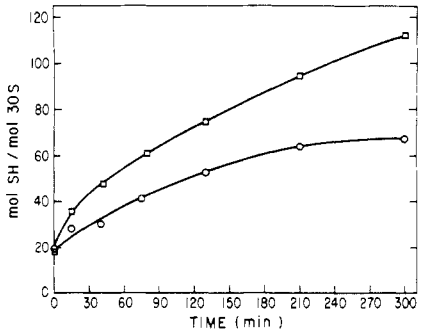


FIGURE 5: Modification of 30S ribosomal subunits with 2-iminothiolane. 30S subunits (5 mg/mL) in TEA-SH buffer at 0 °C were treated with 10 mM (— O —) or 20 mM (— □ —) 2-iminothiolane. The number of sulfhydryl groups per 30S ribosomal subunit was determined at various times by Nbs_2 using procedures described in the text.

singlet at 4.30 ppm in the NMR spectrum of methyl acetimidate dissolved in trifluoroacetic acid (Bates et al., 1975). There was also no evidence for a proton resonance from a sulfhydryl group, which would probably appear as a triplet in the 1-2-ppm region (Dyer, 1965) if the imido ester existed as the open chain methyl 4-mercaptobutyrimidate.

Infrared Spectroscopy of 2-Iminothiolane. The infrared spectrum of the compound is shown in Figure 3. The spectrum was interpreted with the aid of chemical references (Dyer, 1965) and by comparison with the spectrum of 4-mercaptobutyric acid, γ-(thiolactone) (Sadtler, Standard Infrared Spectra, no. 31708). The presence of a nonsubstituted imine was suggested by the peak at 1600 cm^{-1} (C=N stretch) and the broad peak at 2400–3300 cm^{-1} (N-H with C-H stretch). The peak at 690 cm^{-1} may be accounted for by CH_2-S-C rocking, and other features of the spectrum were entirely consistent with the proposed structure, 2-iminothiolane.

Ultraviolet Spectroscopy of 2-Iminothiolane. 2-Iminothiolane (about 0.1 mM) dissolved in acetonitrile at 25 °C gave a spectrum with a peak at λ_{max} 248 nm. Exactly the same spectrum was obtained if the reagent (about 0.1 mM) was added to 0.1 M HCl at 25 °C from an acetonitrile stock solution (5–10 mM). The spectrum was quite stable in acidic aqueous solution, though the absorbance maximum at 248 nm was abolished by exposure to base (pH 10). The extinction coefficient at 248 nm of 2-iminothiolane in 0.1 M HCl was determined to have a value of ϵ_{248} = 8840 M^{-1} cm^{-1} (±5%).

2-Iminothiolane, from a stock solution in acetonitrile (about 5 mM), was added to TEA buffer, pH 8.0, to a final concentration of about 0.05 mM. Figure 4A shows that the absorbance at 248 nm was very stable at 7 °C, with less than 3% decrease in 3 h. When glycylglycine (17.5 mM) was added to the imido ester in TEA buffer at 7 °C, the absorbance at 248 nm rapidly decreased as shown in Figure 4B at a rate similar to the appearance of free sulfhydryl groups in a similar experiment monitored with Nbs_2 (Figure 1). Thus the absorbance of 2-iminothiolane at 248 nm, abolished by base which is likely to catalyze hydrolysis, provides a method for directly observing the stability of the reagent to hydrolysis and its reaction with amines, with concomitant ring opening to reveal a free sulfhydryl group.

Rate of Reaction of 30S Ribosomal Subunits with 2-Iminothiolane. The 30S subunits (5 mg/mL) in TEA-SH buffer at 0 °C were treated with 10 mM or 20 mM 2-iminothiolane. Samples were withdrawn at various times and the number of sulfhydryl groups per 30S subunit was determined using Nbs_2, following gel filtration of the samples to remove 2-mercaptoethanol and unreacted imidate. Figure 5 shows the rate of amidination of the 30S subunit when treated with 10 mM or 20 mM 2-iminothiolane. Approximately 60 mol of sulfhydryl group/mol of 30S subunit was reached after 180 min using 10 mM reagent, or in about half the time (80 min) using 20 mM 2-iminothiolane.

In previous procedures used in this laboratory 30S ribosomal subunits (3 mg/mL) in TEA-SH buffer were incubated with 12 mM 2-iminothiolane for 20 min at 0 °C and then dialyzed against TEA buffer as described in the Materials and Methods section. The extent of modification using this procedure was determined with Nbs_2, following gel filtration to remove the remaining reagent and 2-mercaptoethanol: estimation by Nbs_2 showed the presence of between 0.5 and 1.0 mM sulfhydryl groups after the 3-h dialysis. The Nbs_2 titration of a typical sample of 30S ribosomal subunits so modified showed that

TABLE II: Reaction of 2-Iminothiolane with Amines at 22 °C.[a]

amine	time for 50% reaction of 2-iminothiolane[b] (min)
no addition (hydrolysis)	59.2
glycylglycine	1.6
glycylglycine/$MgCl_2$ (1:1 mol/mol)	1.5
glycinamide	1.6
glycine	3.5
Tris	45.3
NH_4Cl	36.3

[a] Amines (20 mM) from stock solutions (1.0 M), adjusted to pH 8.0, were added to 2-iminothiolane (0.1 mM) in 50 mM-triethanolamine hydrochloride, pH 8.0, containing 1 mM Nbs_2. [b] Uncorrected for hydrolysis.

TABLE III: Inhibition of Amidination of 50S Ribosomal Subunits by Amines.[a]

amine[b]	concn (M)	extent of modification with 2-iminothiolane (mol of SH groups added/mol of 50S subunit)
no addition		43.0
glycylglycine/$MgCl_2$ (1:1 mol/mol)[c]	0.05	4.9
	0.10	0.0
	0.50	0.7
lysine	0.10	10.3
ammonium chloride	0.50	25.0
Tris	0.10	47.5

[a] Refer to text for experimental details. [b] Amines were added to the reaction mixture from stock solutions adjusted to pH 8.0 with HCl. [c] An equal molar amount of $MgCl_2$ was added with glycylglycine to prevent unfolding of the 50S subunit since glycylglycine can chelate magnesium ions.

there were 61 sulfhydryl groups per 30S subunit (Lambert et al., 1978). The number of sulfhydryl groups per unmodified 30S ribosomal subunit, determined as 18 by Nbs_2 titration, was close to the values of cysteine content determined by Moore (1975; Bakardjieva & Crichton, 1974; Acharya & Moore, 1973). Thus, in a typical modification experiment performed by methods used previously (Sommer & Traut, 1976), about 43 sulfhydryl groups were added per 30S subunit during modification with 2-iminothiolane. The titrations with Nbs_2 showed that the sulfhydryl groups added to the ribosomal subunit during modification reacted rapidly and were therefore presumed to be at the surface of the 30S particle, accessible to solvent and reagents.

Reaction of 2-Iminothiolane with Amines. Conditions for controlling more precisely the extent of modification of ribosomes may be achieved by addition of excess amines to terminate the protein modification reaction by removal of 2-iminothiolane. The rate of reaction of 2-iminothiolane (0.1 mM) at pH 8.0 with various amines (20 mM) at 22 °C is shown in Table II. The reaction was monitored by measuring the rate of appearance of free sulfhydryl groups with Nbs_2 (Figure 1). The evidence in Table II shows that glycylglycine and glycinamide reacted rapidly with 2-iminothiolane, while Tris and ammonium only reacted slowly at rates barely greater than the rate of hydrolysis of the thioimidate. Tris was particularly unreactive even though the amine was at 200-fold molar excess over 2-iminothiolane. This result suggests that it is not necessary to carry out the modification reaction in triethanolamine buffers in the absence of Tris.

Inhibition of Ribosome Modification by Amines. Experiments were performed with 50S ribosomal subunits (3 mg/mL) in TEA-SH buffer, pH 8.0, at 0 °C. Modification was initiated by addition of 10 mM 2-iminothiolane. After 10 min, a sample of 50S subunits was freed of reagent and 2-mercaptoethanol by gel filtration and the sulfhydryl groups per 50S subunit (mol/mol) determined by assay with Nbs_2. To other samples various amines, from stock solutions adjusted to pH 8.0 with HCl, were added to final concentrations shown in Table III. After a further 60-min incubation, samples of 50S subunits were assayed for sulfhydryl groups with Nbs_2 following gel filtration. The number of sulfhydryl groups added per 50S subunit after the 10-min incubation with 2-iminothiolane is shown in Table III. It was found that a tenfold excess of glycylglycine over 2-iminothiolane completely inhibited further amidination of the ribosomal subunits, while ammonium and Tris were relatively ineffective in preventing protein amidination. These results support those shown in Table II, and make it possible to define conditions for limiting the extent of modification of ribosomes by reacting excess modifying reagent with glycylglycine or glycinamide.

Alkylation of 30S Ribosomal Subunits with [1-^{14}C]Iodoacetamide. Unmodified and modified 30S ribosomal subunits in TEA-SH buffer were eluted from Bio-Gel P-2 columns. Samples (1.0 mg) were taken for Nbs_2 assay, while the rest of each sample (0.8 mL; 1.8 mg) was alkylated by incubation for 60 min at 25 °C with 1 vol of 8.0 M urea, 6.0 M LiCl, 0.2 M Tris-HCl, pH 8.0, containing 50 mM [1-^{14}C]-iodoacetamide (specific radioactivity 0.57 mCi/mmol). These conditions (4.0 M urea, 3.0 M LiCl), which destroy the ribosome structure (Leboy et al., 1964), have been shown to lead to the exposure of all the sulfhydryl groups of cysteine residues of the 30S particle to modification both with Nbs_2 and iodoacetamide (Lambert et al., 1978). Samples of ribosomal subunits were assayed for protein-bound radioactivity following trichloroacetic acid precipitation. Values of 17.1 mol/mol unmodified 30S subunit and 67.4 mol/mol modified 30S subunit for the number of radioactively alkylated groups per 30S ribosomal subunit were obtained. These values were in good agreement with the numbers of sulfhydryl groups per 30S particle determined by Nbs_2 assay to be 16.4 mol/mol unmodified 30S subunit and 62.8 mol/mol modified 30S subunit in this experiment.

After alkylation was completed (60 min), the ribosomal protein was extracted from RNA by the acetic acid method of Hardy et al. (1969). Samples of alkylated protein from both unmodified and modified 30S ribosomal subunits were analyzed on polyacrylamide/urea gels. A typical example is shown in Figure 6A. The amount of protein applied to each gel, as judged by the intensity of protein stain, was about the same for each sample. Radioautographs of the dried gels are shown in Figure 6 (panels B and C). The pattern of radioactively labeled protein from unmodified 30S ribosomal subunits (Figure 6B) was as expected from previous results in other laboratories (Bakardjieva & Crichton, 1974; Kahan et al., 1974; Moore, 1975), and from amino acid sequence evidence (Stöffler & Wittmann, 1977). Radioactivity was found to coincide with S2, S4, S8, S9/11, S12, S13, S14, S17, S18, and S21: no distinction was made between S9 and S11, although other evidence points to there being two cysteine residues in S11 and none in S9 (Moore, 1975; Chen & Wittmann-Liebold, 1975).

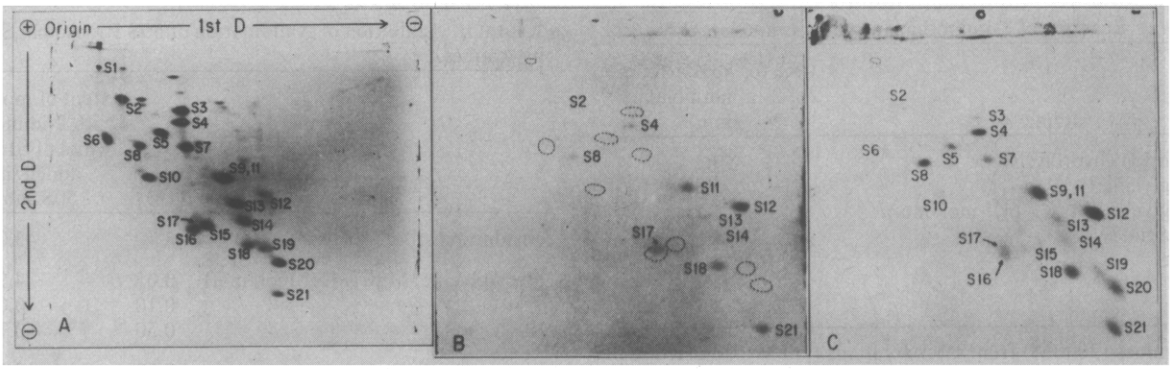
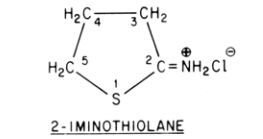
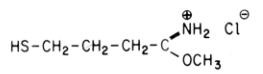


FIGURE 6: Analysis of 30S ribosomal proteins by two-dimensional polyacrylamide/urea gel electrophoresis, following alkylation with [1-^{14}C]iodoacetamide. (Panel A) Protein (300 μg) stained with Amido black. (Panel B) Radioautograph of proteins from unmodified 30S ribosomal subunits. (Panel C) Radioautograph of proteins from 30S ribosomal subunits modified with 2-iminothiolane.



2-IMINOTHIOLANE



METHYL 4-MERCAPTOBUTYRIMIDATE

FIGURE 7: The chemical structures of 2-iminothiolane and methyl 4-mercaptobutyrimidate.

Protein S1 was found only in low yield. It was probable that a large proportion of this fractional protein did not migrate into the gel.

Figure 6C shows the radioautograph of a polyacrylamide/urea gel of alkylated protein from modified 30S subunits. All the proteins were found to be radioactively labeled, though to differing extents. Protein S1 was again found in very low yield. The proteins S4, S5, S7, S8, S9/11, and S20 became prominent while proteins S15 and S16, which have not been found as constituents of a cross-linked protein pair (Sommer & Traut, 1976), were only weakly radiolabeled. A more complete determination of protein stoichiometry of the alkylation was impractical owing to possible variable protein losses during analysis. The evidence in Figure 6 shows that all the 30S proteins do become more alkylated following modification of the 30S particle with 2-iminothiolane and therefore that all were modified by the thioimidate, with the introduction of sulfhydryl groups.

Discussion

The properties of the bifunctional reagent, methyl 4-mercaptobutyrimidate, were investigated by a variety of techniques. The evidence of chemical analysis, sulfhydryl titration, NMR, infrared, and mass spectroscopy demonstrated that the compound has a structure identical with that of the cyclic thioimidate, 2-iminothiolane. The two chemical structures are shown in Figure 7. The finding that freshly prepared aqueous solutions of the compound did not react with Nbs_2 was consistent with the other evidence that showed the structure of the compound to be identical with 2-iminothiolane. Reaction of 2-iminothiolane with primary amines to form amidine derivatives resulted in the appearance of titratable sulfhydryl groups as expected for the newly defined correct structure. The calculated molecular weight for 2-iminothiolane hydrochloride (137.6) was close to the experimental value for the weight of reagent equivalent to 1 mol of titrable sulfhydryl group; this was found to be 138 g ($\pm$2 g).

Recently, 2-iminothiolane has been described by Schramm & Dülffer (1977a,b) as an alternative to mercaptoimidates for the modification of proteins. Our evidence shows that the compound previously known as methyl 4-mercaptobutyrimidate is in fact identical with 2-iminothiolane (Figure 7). Cyclization to form the stable five-membered ring structure very likely occurred during chemical synthesis of the imido ester (Traut et al., 1973). The ring structure of this compound contrasts with the structure of methyl 3-mercaptopropionimidate, which was first synthesized by Perham & Thomas (1971). This mercaptoimidate, which has been used in cross-linking studies on histones (Thomas & Kornberg, 1975), was found to have a titratable sulfhydryl group in freshly prepared solutions and so is likely to have an open chain structure (Perham & Thomas, 1971).

2-Iminothiolane was found to have an absorption maximum at 248 nm which disappeared following reaction of the imidate with a primary amine. The absorption at 248 nm was also lost by exposure to pH 10, probably owing to rapid base-catalyzed hydrolytic ring opening of the cyclic thioimidate. The absorption of 248 nm characteristic of the ring structure provides a direct spectroscopic method for measuring the reaction of 2-iminothiolane with amines and water. Alternatively, the ring-opening of 2-iminothiolane upon reaction with amines and water may be followed by titration of newly exposed sulfhydryl groups with Nbs_2.

Hydrolysis was found to be negligible at 7 °C, relative to the rate of reaction with the primary amino group of glycylglycine or glycinamide. The stability of 2-iminothiolane in aqueous solution contrasts with the more rapid hydrolysis of other imido esters (Hunter & Ludwig, 1962; Browne & Kent, 1975). Previous studies with thioimido esters showed that they had a greater stability to hydrolysis than that of the corresponding oxygen derivatives (Hartigan & Cloke, 1945). The slow hydrolysis rate of 2-iminothiolane makes it suitable for modification of proteins at low concentrations of reagent (Schramm & Dülffer, 1977a).

The work of Hand & Jencks (1962) on the mechanism of the reaction of imido esters with amino groups has been revised by Browne & Kent (1975). The latter study, with ethyl acetimidate, offered an explanation for the cross-linking sometimes seen when proteins were modified with monofunctional imido esters (Hitchcock, 1975). *N*-Alkyl imidates can result from the reaction of amino groups with imido esters at pH 8.0; these can then react further with amino groups to give disubstituted amidines (Browne & Kent, 1975). This explanation may ac-

count for the fact that some of the cross-linked proteins, observed when erythrocyte membrane proteins were cross-linked with dimethyl 3,3′-dithiobis(propionimidate) (Ruoho et al., 1975), were not cleaved by reduction (Browne & Kent, 1975). We have not observed any cross-linked proteins that were not cleaved by reduction of disulfide bonds (Traut et al., 1973) when the reagent methyl 4-mercaptobutyrimidate, now known to be 2-iminothiolane, was used. It is possible that 2-iminothiolane preferentially forms amidine derivatives rather than *N*-alkyl imidates, even at pH values around 8.0.

The use of 2-iminothiolane as a cross-linking reagent has already provided valuable evidence about protein:protein neighborhoods of the ribosomal subunits of *E. coli* (Traut et al., 1973; Sun et al., 1974; Sommer & Traut, 1974–1976; Kenny et al., 1975; Traut & Kenny, 1977). Titration of the 30S subunit with Nbs_2 following modification showed that about 43 sulfhydryl groups were added to the subunit by modification with 2-iminothiolane under conditions identical with those employed in previous experiments. This corresponded to an average of 2 sulfhydryl groups per 30S protein using an average value of 21 proteins of equal lysine content per 30S subunit. Alkylation of the modified 30S proteins with [1-^{14}C]iodoacetamide suggested that all the proteins become modified. The low degree of modification was consistent with the earlier finding that oxidation of the modified particle to form disulfide cross-links resulted predominantly in protein:protein dimers rather than higher oligomers (Traut & Kenny, 1977). These may be readily analyzed by diagonal polyacrylamide gel techniques (Sommer & Traut, 1974), whereas a preponderance of higher oligomers would be difficult to analyze.

2-Iminothiolane is a valuable tool in studies of cellular organelles and oligomeric enzymes, using the techniques of protein:protein cross-linking. It may also be used to introduce reactive sulfhydryl groups into proteins for subsequent reaction with alkylating agents or heavy metal compounds (Perham & Thomas, 1971). The reagent itself possesses no free sulfhydryl group, and so is more stable to air oxidation than reagents such as methyl 3-mercaptopropionimidate which contain free sulfhydryl groups. More importantly, the reagent is more stable to hydrolysis than most of the common imido esters that are in current use in studies involving chemical modification of proteins (Peters & Richards, 1977). The reagent may be readily synthesized in radiolabelled form from [^{35}S]thiourea according to procedures described earlier (Traut et al., 1973). It is likely that 2-iminothiolane will find many more useful applications in the study of biological ultrastructures.

Acknowledgments

We thank Drs. William Benisek and Stephen Smith for valuable advice in the execution and interpretation of the experiments concerning the physical characterization of the compound. Dr. Paul Smith of Pierce Chemical Co. has informed us that new data obtained by them are consistent with the conclusions documented here.

References

Acharya, A. S., & Moore, P. B. (1973) *J. Mol. Biol. 76,* 207–221.

Bakardjieva, A., & Crichton, R. R. (1974) *Biochem. J. 143,* 599–606.

Bates, D. L., Perham, R. N., & Coggins, J. R. (1975) *Anal. Biochem. 68,* 175–184.

Bickle, T. A., Hershey, J. W. B., & Traut, R. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 1327–1331.

Browne, D. T., & Kent, S. B. H. (1975) *Biochem. Biophys. Res. Commun. 67,* 126–132.

Chen, R., & Wittmann-Liebold, B. (1975) *FEBS Lett. 52,* 139–140.

Clegg, C., & Hayes, D. H. (1974) *Eur. J. Biochem. 42,* 21–28.

Davies, G. E., & Stark, G. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66,* 651–656.

Dworschack, R., Tarr, G., & Plapp, B. V. (1975) *Biochemistry 14,* 200–203.

Dyer, J. R. (1965) in *Applications of Absorption Spectroscopy of Organic Compounds,* Prentice Hall, Inc., Englewood Cliffs, N.J.

Eikenberry, E. F., Bickle, T. A., Traut, R. R., & Price, C. A. (1970) *Eur. J. Biochem. 12,* 113–116.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82,* 70–77.

Expert-Bezançon, A., Barritault, D., Milet, M., Guérin, M. F., & Hayes, D. H. (1977) *J. Mol. Biol. 112,* 603–629.

Hand, E. S., & Jencks, W. P. (1962) *J. Am. Chem. Soc. 84,* 3505–3514.

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

Hartigan, R. H., & Cloke, J. B. (1945) *J. Am. Chem. Soc. 67,* 709–715.

Hershey, J. W. B., Yanov, J., Johnston, K., & Fakunding, J. L. (1977) *Arch. Biochem. Biophys. 182,* 626–638.

Hill, W. E., Rossetti, G. P., & Van Holde, K. E. (1969) *J. Mol. Biol. 44,* 263–277.

Hitchcock, S. E. (1975) *Biochemistry 14,* 5162–5167.

Howard, G. A., & Traut, R. R. (1973) *FEBS Lett. 29,* 177–181.

Hunter, M. J., & Ludwig, M. L. (1962) *J. Am. Chem. Soc. 84,* 3491–3504.

Hunter, M. J., & Ludwig, M. L. (1972) *Methods Enzymol. 25,* 585–596.

Kahan, L., Held, W. A., & Nomura, M. (1974) *J. Mol. Biol. 88,* 797–808.

Kenny, J. W., Sommer, A., & Traut, R. R. (1975) *J. Biol. Chem. 250,* 9434–9436.

Knopf, U. C., Sommer, A., Kenny, J., & Traut, R. R. (1975) *Mol. Biol. Rep. 2,* 35–40.

Lambert, J. M., & Perham, R. N. (1977) *Biochem. J. 161,* 49–62.

Lambert, J. M., Jue, R., & Traut, R. R. (1978) *Biochemistry 17* (following paper in this issue).

Leboy, P. S., Cox, E. C., & Flaks, J. G. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52,* 1367–1374.

Lutter, L. C., Bode, U., Kurland, C. G., & Stöffler, G. (1974) *Mol. Gen. Genet. 129,* 167–176.

Lutter, L. C., Kurland, C. G., & Stöffler, G. (1975) *FEBS Lett. 54,* 144–150.

Moore, P. B. (1975) *J. Mol. Biol. 98,* 439–444.

Peretz, H., Towbin, H., & Elson, D. (1976) *Eur. J. Biochem. 63,* 83–92.

Perham, R. N. (1973) *Biochem. J. 131,* 119–126.

Perham, R. N. & Richards, F. M. (1968) *J. Mol. Biol. 33,* 795–807.

Perham, R. N., & Thomas, J. O. (1971) *J. Mol. Biol. 62,* 415–418.

Peters, K., & Richards, F. M. (1977) *Annu. Rev. Biochem. 46,* 523–551.

Plapp, B. V. (1970) *J. Biol. Chem. 245,* 1727–1735.

Ruoho, A., Bartlett, P. A., Dutton, A., & Singer, S. J. (1975) *Biochem. Biophys. Res. Commun. 63,* 417–423.

Schramm, H. J., & Dülffer, T. (1977a) *Hoppe-Seyler's Z. Physiol. Chem. 358,* 137–139.

Schramm, H. J., & Dülffer, T. (1977b) in *Protein Crosslinking*

(Freidman, M., Ed.) Part A, pp 197-205, Plenum Publishing Corp., New York, N.Y.
Sommer, A., & Traut, R. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 3946-3950.
Sommer, A., & Traut, R. R. (1975) *J. Mol. Biol. 97,* 471-481.
Sommer, A., & Traut, R. R. (1976) *J. Mol. Biol. 196,* 995-1015.
Stöffler, G., & Wittmann, H. G. (1977) in *Molecular Mechanisms of Protein Biosynthesis* (Weissbach, H., & Pestka, S., Eds.) pp 117-202, Academic Press, New York, N.Y.
Sun, T. T., Bollen, A., Kahan, L., & Traut, R. R. (1974) *Biochemistry 13,* 2334-2340.
Thomas, J. O., & Kornberg, R. D. (1975) *FEBS Lett. 58,* 353-358.
Traut, R. R., & Kenny, J. W. (1977) in *Protein Crosslinking* (Friedman, M., Ed.) Part A, pp 215-233, Plenum Publishing Corp., New York, N.Y.
Traut, R. R., Bollen, A., Sun, T. T., Hershey, J. W. B., Sundberg, J., & Pierce, L. R. (1973) *Biochemistry 12,* 3266-3275.
Wofsy, L., & Singer, S. J. (1963) *Biochemistry 2,* 104-116.

Disulfide Cross-Linking of *Escherichia coli* Ribosomal Proteins with 2-Iminothiolane (Methyl 4-Mercaptobutyrimidate): Evidence That the Cross-Linked Protein Pairs Are Formed in the Intact Ribosomal Subunit†

John M. Lambert, Rodney Jue,‡ and Robert R. Traut*

ABSTRACT: The 30S ribosomal subunits of *Escherichia coli* were modified with 2-iminothiolane with the formation of amidine-linked sulfhydryl groups attached to ribosomal protein. The modified particle contained 61 sulfhydryl groups, 17-18 due to endogenous cysteine residues and the remainder from modification. The modified ribosomal subunits were oxidized to promote disulfide bond formation (cross-linking). About 15 free sulfhydryl groups per 30S particle remained after oxidation even when performed in the presence of 2-mercaptoethanol. Treatment of modified, oxidized particles with 4.0 M urea, 3.0 M LiCl exposed these sulfhydryl groups which reacted with iodoacetamide only after disruption of the native structure. The presence of these sulfhydryl groups prompted an investigation of possible sulfhydryl/disulfide interchange and random oxidation during extraction of cross-linked ribosomal proteins and/or the preparation of protein for diagonal polyacrylamide/dodecyl sulfate gel electrophoresis. Experiments were carried out to obtain direct evidence concerning the quantitative contribution of disulfide interchange and/or random oxidation during protein extraction to the pattern of cross-linked dimers previously reported. A radiolabeled cross-linked protein fraction of about 35 500 molecular weight was purified from cross-linked ^{35}S-labeled 30S subunits. The radiolabeled protein was added to nonradioactive cross-linked 30S ribosomal subunits immediately before extracting the protein under several different conditions including that with acetic acid used in earlier studies from this laboratory. The radioactivity was subsequently shown to migrate only at 35 500 molecular weight following analysis of the cross-linked protein by polyacrylamide/dodecyl sulfate gels. There was no evidence that disulfide interchange produced new cross-linked radioactive protein bands of different molecular weight. Similar results were obtained using the ^{35}S-labeled 30S ribosomal protein S4, which contains a single cysteine group. Radioactive S4 was found only as the monomeric protein. The results confirmed that earlier results on the cross-linking of the 30S ribosomal subunit reflect interprotein disulfide linked dimers formed in the intact particle. Random diagonal gel patterns have been deliberately formed by promoting oxidation of ribosomal proteins in solution. These patterns had no similarity with those previously published nor to those in which free sulfhydryl groups were blocked by alkylation. Additional evidence supports the conclusion that disulfide linked proteins reflect specific arrangements in the intact ribosome: cross-linked 30S subunits retained 60% of their activity in reassociation with 50S subunits and 30% in polyphenylalanine synthesis.

Chemical cross-linking of biological structures containing several polypeptide chains can provide useful information on the topography of the protein constituents. Such evidence is particularly valuable in the study of molecular structures for which X-ray crystallographic evidence is lacking or inadequate (Peters & Richards, 1977). A fundamental requirement is that the cross-linking reaction not perturb the native conformation of the biological structure. Cross-linking reagents based on bis(imido esters), such as dimethyl suberimidate (Davies & Stark, 1970), have been particularly useful since they react under mild conditions specifically with amino groups of proteins to yield amidine derivatives that retain positive charge (Hand & Jencks, 1962; Browne & Kent, 1975).

The topography of proteins of *Escherichia coli* ribosomal subunits has been extensively studied by a variety of experimental techniques in recent years (Brimacombe et al., 1976; Kurland, 1977a). Cross-linking studies using bis(imido esters) have been prominent in providing evidence on the spatial arrangement of ribosomal proteins (Bickle et al., 1972; Lutter

† From the Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616. *Received June 6, 1978.* This work was supported by a grant (GM 17924) from the U.S. Public Health Service.

‡ Present address: Department of Chemistry, University of California, San Diego, California 92023.

0006-2960/78/0417-5406$01.00/0