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Methyl 4-Mercaptobutyrimidate as a Cleavable Cross-Linking Reagent and Its Application to the *Escherichia coli* 30S Ribosome†

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ABSTRACT: The compound, methyl 4-mercaptobutyrimidate, of which the synthesis is described, has been used to produce disulfide-linked dimers and higher oligomers between neighboring proteins on the intact 30S ribosome from *Escherichia coli*. The imidate function of the reagent is first allowed to react with amino groups on ribosomal proteins, following which the particles modified by the addition of extra SH groups are oxidized under mild conditions to form disulfide cross-links. The formation of new products of molecular weight greater than existing protomeric ribosomal proteins is monitored by gel electrophoresis in the presence of sodium dodecyl sulfate. Upon reduction of the oxidized ribosome or the extracted proteins, the normal protein pattern on gels in sodium dodecyl sulfate is completely restored. Similarly the reduction products, or proteins bearing extra SH groups, retain their characteristic behavior during electrophoresis in buffers containing 8 M urea at pH 8.6 and 4.5. Identification of the specific protein components of purified dimers by two-dimensional slab gel electrophoresis is thus feasible. Possible minor alterations in electrophoretic mobility due to partial ionization of SH groups are completely avoided by alkylation of the proteins with iodoacetamide. The compound thus represents a new cleavable cross-linking reagent which should be applicable not only to the investigation of the topography of ribosomal proteins but also protein–protein interactions in a variety of other biological systems.

Of major importance in current research on the mechanism of protein synthesis and ribosome structure is the determination of the spatial arrangement of ribosomal proteins and the identification of ribosomal binding sites for initiation and elongation factors. Recent work has concentrated on the protein topography of the *Escherichia coli* 30S ribosomal subunit. A variety of methods are being used toward these ends (for a complete review, see Wittmann and Stöffler, 1972).

One of the most direct experimental approaches for the determination of the overall topography of ribosomal proteins as well as the identification of those specific proteins comprising the binding sites for protein factors involved in protein synthesis is the use of protein-specific bifunctional reagents to cross-link either neighboring ribosomal proteins or ribosome-bound factors and ribosomal proteins. At this time bisimido esters (Bickle *et al.*, 1972; Clegg and Hayes, 1972; Lutter *et al.*, 1972) and bismaleimides (Chang and Flasks, 1972; Kurland *et al.*, 1972) have been used to identify certain pairs of 30S ribosomal proteins covalently joined by the reagent employed.

Several approaches are available for the identification of

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the components of purified cross-linked products: (1) cleavage of the dimer or oligomer into its constituent protomers; (2) selective labeling of individual proteins in the ribosome by reconstitution techniques and demonstration of the label in specific cross-linked products; (3) analysis of purified cross-linked products with antibodies specific for each pure 30S protein. The first approach would seem to be the simplest provided that cleavable bifunctional reagents which would not alter the electrophoretic and chromatographic properties of the resulting protomers were available. Early experiments of this kind in this laboratory (Bickle *et al.*, 1972) employed bisimido esters (Davies and Stark, 1970), which seemed highly suitable for studies of this kind, first, because ribosomal proteins are rich in potentially reactive lysine amino groups; second, because the covalent bond formed is in principle cleavable by ammonolysis. Although a number of specific cross-linked products were formed, as indicated by analysis by electrophoresis on sodium dodecyl sulfate gels, identification of their protomeric components by ammonolysis was hindered by the low yield of the cleavage reaction. Therefore, we have sought new bisimido esters which could be cleaved more efficiently than the simple bisimido esters used in our earlier studies. The report by Perham and Thomas (1971) of the synthesis of a mercaptopropionimidate[1] which can introduce additional thiol groups to proteins suggested the application of this class of reagents to protein–protein cross-linking. Oxidation of the SH-charged ribosome should produce disulfide bonds between neighboring ribosomal proteins and the dimers or oligomers thus formed should be readily cleaved by mild reduction.

The present report describes improved methods for the synthesis of mercaptopropionimidate and a mercaptobutyrimidate in high yield and preliminary results on the application of these reagents to the cross-linking of 30S *Escherichia coli* ribosomal proteins. The typical procedure consists of three steps: (1) reaction of amino groups on ribosomal proteins in the intact ribosome with the imidate function of the mercaptoimidate; (2) mild oxidation of the SH-charged ribosome to form S–S linked protein dimers (or higher oligomers). The cross-linked products thus formed are readily observed on sodium dodecyl sulfate gels and can be isolated by conventional methods. (3) Reductive cleavage of the cross-linked products results in essentially 100% yield to the original protein monomers. The resulting monomeric proteins, although modified by conversion of the original lysine amino groups to mercaptobutyroamidine groups, retain their normal electrophoretic mobilities in several different gel electrophoresis systems when the SH-charged proteins are first treated with iodoacetamide. The identification of the cross-linked monomers by the conventional methods used for analysis of ribosomal proteins can therefore be readily accomplished. The method should be generally applicable to studies of protein–protein interaction in a variety of other biological structures.

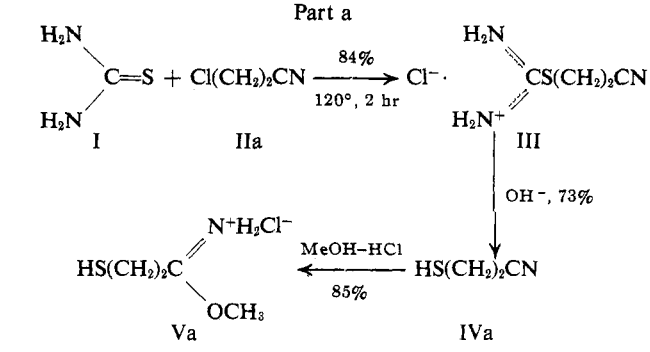
Methods

Organic Syntheses

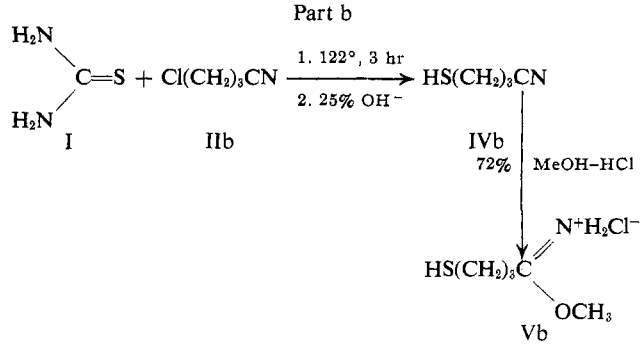
Methyl 3-Mercaptopropionimidate Hydrochloride. An improved procedure based on that of Perham and Thomas (1971) was used for the synthesis of methyl 3-mercaptopropionimidate hydrochloride. Since the overall yield (44%) is substantially greater than that previously reported, a detailed procedure is presented here. The synthesis together with yields obtained at each step is summarized in Scheme Ia. Redistilled 3-chloropropionitrile (I) (16.6 g, 0.185 mol (bp 62–63°) (11 mm)) was combined with 19.0 g (0.25 mol) thiourea (IIa) and 12.6 ml of water and refluxed in a 120° oil bath for 2 hr. After cooling the solution to room temperature, 250 ml of cold acetone was added. The crystalline isothiouronium salt, 3-isothioureidopropionitrile (III), precipitated immediately and was collected by suction filtration after standing for 15 min at 4° and washed with 3 × 50 ml of cold acetone and 2 × 50 ml of diethyl ether, and air-dried overnight (yield 25.8 g, 84%, mp 163–165°).

SCHEME I

Part a



Part b



In the next step, 23.0 g (0.139 mol) of the isothiouronium salt (III) was dissolved in 30.0 ml of degassed H_2O and treated with 14.0 ml of 50% (w/v) NaOH solution (0.175 mol) in a 50° water bath for 30 min with stirring under nitrogen. The mixture was cooled quickly to room temperature in an ice bath, 41 ml of diethyl ether was added through an addition funnel, and the mixture was stirred for 5 min at room temperature. The reaction mixture was transferred to a nitrogen-purged separatory funnel, the ether layer was removed, and a second extraction with 41 ml of ether was performed under nitrogen. The aqueous layer was brought to pH 7 by the addition of approximately 22 ml of 6 N H_2SO_4 and extracted with 3 × 41 ml of diethyl ether under N_2. The combined ether extracts were dried 40 min with 15 g of anhydrous sodium sulfate and the solvent was removed by rotary evaporation at room temperature. The residue, 3-mercaptopropionitrile (IVa, yield 8.9 g, 73%), was obtained as a light yellow oil. This was vacuum distilled (bp 57–59° (6 mm)) and a clear colorless oil was collected.

An ice-cold solution of 3.2 g (0.088 mol) of anhydrous HCl in 4.0 ml (0.1 mol) of absolute methanol was prepared by passing hydrogen chloride through a drying tube filled with Drierite, through concentrated H_2SO_4 in a washing bottle, and finally into the cold methanol in a reaction flask fitted with a Drierite exit tube. This was continued until the ap-

[1] Abbreviations used are: TEA, triethanolamine; mercaptopropionimidate, methyl 3-mercaptopropionimidate hydrochloride; mercaptobutyrimidate, methyl 4-mercaptobutyrimidate hydrochloride.

propriate gain in weight due to added HCl was obtained. The distilled 3-mercaptopropionitrile (IVa; 2.75 g) was introduced quickly into the cold methanolic HCl and the flask was stoppered tightly and left overnight at 0°.

The reaction mixture was transferred to a three-neck flask fitted with a nitrogen inlet tube, an addition funnel for adding washing solvents, and an exit tube covered with a scintered-glass disk leading to a filtrate trap. Diethyl ether (5 ml) was added and slender white needles formed. An additional 20 ml of cold ether was added and the flask was left on ice for 1 hr. The supernatant was decanted through the scintered-glass filtered and the precipitate was washed two times with 5 ml of cold 1:3 absolute methanol–anhydrous ether and then two times with cold ether. The system was kept at 0° and under N_2 throughout these operations. The product, methyl 3-mercaptopropionimidate hydrochloride (Va; 4.2 g; 85% yield, 44% overall yield; mp 78–79° dec, lit. (Perham and Thomas, 1971) mp 73–74°) was obtained. The compound was stored in a vacuum desiccator over Drierite at 4°. Its purity was analyzed periodically on thin-layer chromatographic (tlc) plates (E. Merck Cellulose F) by using 8:2 (95%) ethanol–ethyl acetate; spots were visualized by exposure to iodine vapor. The compound decomposed gradually over a period of several months.

Methyl 4-Mercaptobutyrimidate Hydrochloride. Methyl 4-mercaptobutyrimidate hydrochloride was synthesized by a procedure similar to that for methyl 3-mercaptopropionimidate (Scheme I, part b) but with minor modifications. The principal difference was that the intermediate isothiouronium salt (III) was not isolated. Redistilled 4-chlorobutyronitrile (38.3 g, 0.37 mol, bp 192–194°) was combined with 38 g of thiourea (0.5 mol) and 25.2 ml of water and the mixture was refluxed with stirring in a 122° oil bath for 3 hr. Analysis of the reaction mixture on tlc plates (Baker-Flex silica gel IBF) developed in methanol and visualized with uv or iodine vapor indicated quantitative conversion of 4-chlorobutyronitrile to a new product after 3 hr. Three-quarters of the cooled solution was then used directly for the next step in which the product was treated with 25.8 ml of 50% (w/v) NaOH (0.49 mol) under N_2 for 30 min at 40°. The oil layer which resulted was removed and the aqueous layer was extracted with 2 × 75 ml of diethyl ether, adjusted with 6 N H_2SO_4 to pH 7, and extracted with 3 × 75 ml of ether. The combined ether layers were extracted with 2 × 75 ml of H_2O and the combined water washings were reextracted with 2 × 35 ml of ether. The ether layers were combined and dried with anhydrous Na_2SO_4. The ether was removed by rotory evaporation, with the formation of 4-mercaptobutyronitrile (IV, 7.59 g, 26%) as a light yellow oil. This was purified by vacuum distillation (bp 61–64° (5 mm)). The purity of the distillate was confirmed by thin-layer chromatography (Baker-Flex silica gel IBF) using 1:1 chloroform–acetone. The imidate (V) was prepared by the addition of 1.4 g of this distillate ($HS(CH_2)_3CN$), 0.014 mol) to a solution of 1.9 g (0.052 mol) of dry HCl in 2.2 ml (0.069 mol) of absolute methanol and the mixture was held overnight at 0°. The product (1.7 g, 72%; 19% overall yield) was isolated in the manner previously described for methyl 3-mercaptopropionimidate hydrochloride. This solid product was recrystallized by refrigeration of saturated solutions of the compound in methanol–acetone mixture with the formation of large blunt white needles (mp 192–193° dec). The recrystallized product produced a single spot (R_F 0.58) on thin-layer chromatography (Baker-Flex silica gel IBF) using 8:2 (95%) ethanol–ethyl acetate plus 0.1% 2-mercaptoethanol.

Procedure for Cross-Linking Ribosomal Proteins by Disulfide Formation

Step I. Reaction of Ribosomes with Imidate Function. Buffers containing primary or secondary amino groups are unsuitable for reactions involving imido ester, since the rate of reaction with buffer would greatly exceed that with protein amino groups. Accordingly, the buffer triethanolamine (TEA) at pH 8 was chosen as the standard condition for the reaction of mercaptoimidates with the ribosome. As shown previously (Bickle *et al.*, 1972), under appropriate ionic conditions in TEA the 30S ribosome retains its native physical properties, does not dimerize and retains substantial activity in poly(U)-directed poly(phenylalanine) synthesis. The standard buffer for the reaction was therefore TEA-HCl (50 mM, pH 8.0)–$Mg(OAc)_2$ (1 mM)–KCl (50 mM)–3% β-mercaptoethanol (TEA·SH buffer). Some experiments were carried out at 5 mM $Mg(OAc)_2$ and 100 mM KCl; the results were the same as those reported here.

The presence of a reducing agent such as β-mercaptoethanol was found advantageous to insure that the ribosomal SH groups were in the reduced state during reaction with mercaptoimidate, and that no disulfide-bond formation took place prior to the controlled second oxidation step.

The ribosomes were allowed to react with the mercaptoimidate (concentration given in text or figure legends) at 3 mg/ml in TEA·SH buffer for 20 min at 0° followed by dialysis at 5° against TEA·HCl (50 mM, pH 8.0)–$Mg(OAc)_2$ (1 mM)–KCl (50 mM) (TEA buffer) in order to remove β-mercaptoethanol and free mercaptoimidate or its hydrolysis products.

Step II. Oxidation of Ribosomes Modified by Addition of Mercaptoimidate Residues. Following dialysis, ribosomes were incubated for 30 min at room temperature in TEA buffer with the addition of H_2O_2 to give a final concentration of 40 mM.

The reaction was either stopped by adding sodium dodecyl sulfate sample buffer (without β-mercaptoethanol) and analyzed on gels immediately, or alternatively by addition of catalase to remove excess H_2O_2. In other experiments, the ribosomes were concentrated by centrifugation at 4° of the oxidizing reaction mixture and the pellets were resuspended in TEA buffer. The pattern and yield of cross-linked products was the same regardless of the procedure used. The formation of disulfide-linked products seemed to depend more on the number of SH groups introduced than the conditions used for oxidation.

Step III. Cleavage of Disulfide-Linked Proteins. The solution of oxidized ribosomes in TEA buffer was adjusted to 3% β-mercaptoethanol (TEA·SH) and incubated for 30 min at 37°. Alternatively, the standard procedure for electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate in which the sample is heated in the presence of reducing agent was used to reduce the disulfide linkages.

30S Ribosomes. Subunits were prepared from *E. coli* strain MRE600 grown in rich medium as described previously (Eikenberry *et al.* (1970), zonal centrifugation; Fakunding and Hershey (1973), growth conditions).

Gel Electrophoresis. The following polyacrylamide gel electrophoresis systems were employed: (1) a modification of the stacking sodium dodecyl sulfate gel system first described by Laemmli (1970); modified according to R. F. Gesteland (personal communication; see legend to Figure 1 for details); (2) sodium dodecyl sulfate gel electrophoresis in gels containing 10% acrylamide in phosphate buffer as previously described (Weber and Osborn, 1969; Bickle and Traut, 1971);

FIGURE 1: Comparison of mercaptopropionimidate and mercaptobutyrimidate. Ribosomal subunits (30 S) were treated with imidate at 10 mM, oxidized and analyzed on discontinuous polyacrylamide gels in sodium dodecyl sulfate, without mercaptan in the sample buffer. Gel system is that referred to in Methods with the following final composition: stacking gel: 5% acrylamide–2.6% bisacrylamide –0.1% sodium dodecyl sulfate–0.125 M Tris·HCl (pH 7.8); running gel: 15% acrylamide–0.08% bisacrylamide–0.1% sodium dodecyl sulfate 0.372 M Tris·HCl (pH 8.7); reservoir buffer: 0.005 M in Tris, 0.384 M in glycine, 0.1% in sodium dodecyl sulfate (pH 8.5). The sample buffer is the same as that described by Laemmli (1970): (a) control ribosomes; (b) ribosomes treated with mercaptopropionimidate; (c) ribosomes treated with mercaptobutyrimidate.

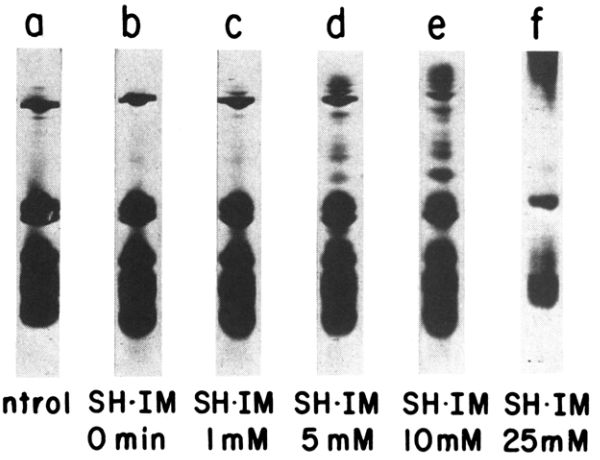


FIGURE 2: Effect of mercaptobutyrimidate concentration on the formation of products of higher molecular weight. Ribosomes were treated with mercaptobutyrimidate at the concentrations indicated and oxidized as described in Methods, and then analyzed on 10% sodium dodecyl sulfate gels in which mercaptan was omitted from the sample buffer. (d) Zero-time control at 10 mM imidate. The sodium dodecyl sulfate sample buffer was added at 0° immediately after the addition of imidate.

(3) gel electrophoresis at pH 4.5 in 8 M urea (Traut, 1966); (4) two-dimensional electrophoresis (Kaltschmidt and Wittmann, 1970; Howard and Traut, 1973). For the analysis of oxidized products reducing agents were omitted from the sodium dodecyl sulfate sample buffers; for analysis of the reduced products, reducing agents were present according to standard procedures.

Alkylation. Ribosomal proteins were extracted from control and SH-charged 30S ribosomes in TEA buffer by treatment with ribonuclease T-1 and ribonuclease A in the presence of 10 mM EDTA (Mizushima and Nomura, 1970). The precipitated proteins were solubilized by addition of solid urea to 8 M and the solution was adjusted to 17 mM iodoacetamide and allowed to react at 30° for 30 min (Battell *et al.*, 1968).

Materials

β-Mercaptoethanol was obtained from Schwarz-Mann; hydrogen peroxide AR 30% solution from Mallinckrodt Chemical Works; sodium dodecyl sulfate "Salzfrei" from SERVA, Heidelberg; 3-chloropropionitrile and 4-chlorobutyronitrile were obtained from Aldrich Chemical Co.; thiourea from Matheson, Coleman & Bell; iodoacetamide from Calbiochem. Acrylamide and bisacrylamide were used without recrystallization as obtained from Eastman Organic Chemicals. Triethanolamine was also obtained from Eastman and was redistilled before use.

Results

Comparison of Mercaptopropionimidate and Mercaptobutyrimidate. Preliminary experiments showed that both mercaptopropionimidate and mercaptobutyrimidate when allowed to react with intact *E. coli* 30S ribosomes modified the appearance of the band pattern when the proteins from the oxidized ribosomes were analyzed by sodium dodecyl sulfate gel electrophoresis. Figure 1 shows the analysis on sodium dodecyl sulfate gels of the reaction products formed with both reagents. As shown in Figure 1b, treatment of the 30S ribosome with mercaptopropionimidate resulted in the disappearance of several bands including proteins S1, S2, and S3. However, no *discrete* new cross-linked products were apparent. We suspect that the shorter compound produces mainly intramolecular cross-links, and large aggregates involving S1, S2, and S3. As shown in Figure 1c, with mercaptobutyrimidate, discrete bands of cross-linked products with high molecular weight are clearly visible. The contrast between these results comparing the two reagents led us to concentrate our efforts on the use of mercaptobutyrimidate for further studies, although it is highly likely that under appropriate conditions the shorter reagent would also prove useful for determining protein–protein interactions.

Effect of Mercaptobutyrimidate Concentration on Formation of Higher Molecular Weight Products. A variety of concentrations of mercaptobutyrimidate were tested. The results are shown in Figure 2. The standard condition 10 mM mercaptobutyrimidate was selected for further experiments in order to maximize the formation of the discrete new bands without the appearance of a preponderance of aggregates of extremely high molecular weight. The latter probably represent oligomers higher than dimers and trimers for which subsequent isolation and identification of their protomeric components would be technically complex and the results ambiguous.

Effect of Reduction Analyzed by Sodium Dodecyl Sulfate Gel Electrophoresis. As shown in Figure 2, the reaction of the ribosome with 10 mM mercaptobutyrimidate followed by oxidation resulted in the formation in discrete bands of molecular weight higher than the normal 30S gel pattern. Exposure of this cross-linked ribosome to 3% β-mercaptoethanol (see Methods) completely restores the normal sodium dodecyl sulfate gel pattern (Figure 3d). From this result, it is concluded that the formation of higher molecular weight products is caused by the formation of disulfide bridges which can be readily and completely cleaved by mild reduction with mercaptans (see Discussion).

Effect of Reduction Analyzed by One- and Two-Dimensional Urea Gel Electrophoresis. Since methods other than electro-

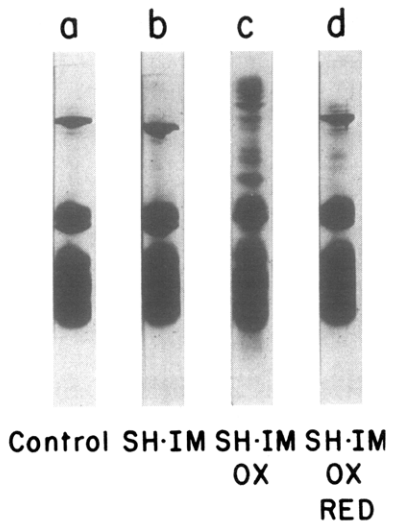


FIGURE 3: Analysis on 10% polyacrylamide gels in sodium dodecyl sulfate of ribosomes before (a) and after (b) reaction with mercaptobutyrimidate, oxidation (c), and reduction (d). The proteins were analyzed by polyacrylamide gel analysis in sodium dodecyl sulfate as referred to in Methods (Bickle and Traut, 1971). Only the sample buffer for samples b and d contain reducing agent.

phoresis in sodium dodecyl sulfate will ultimately be useful for identification of cross-linked proteins, the SH-charged ribosomal proteins extracted from ribosomes after step I were also analyzed by gel electrophoresis in buffers containing urea. After modifications of the ribosomal proteins by addition of mercaptobutyrimidate residues (40 mM imidate), changes in the mobilities of the proteins on pH 4.5 urea gels were observed both as a slight broadening or fuzziness in each band, and a systematic lowering of the mobility of all the proteins in the gel pattern (Figure 4c). The abnormal behavior could be eliminated by prior treatment of the extracted reduced protein with iodoacetamide (Figure 4d) and this was adopted as a standard procedure for analysis of the proteins in urea gel systems.

Figure 5 compares the two-dimensional gel patterns of untreated total 30S proteins with those derived from modified, oxidized, and reduced ribosomes following exhaustive treatment of the reduced proteins with iodoacetamide. Even without treatment with iodoacetamide, the patterns are similar except that the spots are less compact (results not shown). Thus, two-dimensional gel electrophoresis can be used for the identification of the components of purified cross-linked products.

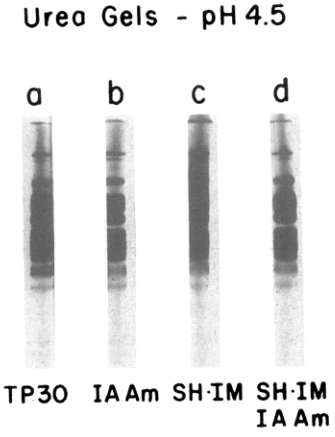


FIGURE 4: Analysis on polyacrylamide gels containing urea at pH 4.5 of 30S ribosomal proteins treated with mercaptobutyrimidate: (a) total 30S protein, untreated; (b) total protein reacted with iodoacetamide; (c) total 30S protein extracted from ribosomes following treatment with 10 mM mercaptobutyrimidate; (d) proteins from (c) after reaction with iodoacetamide.

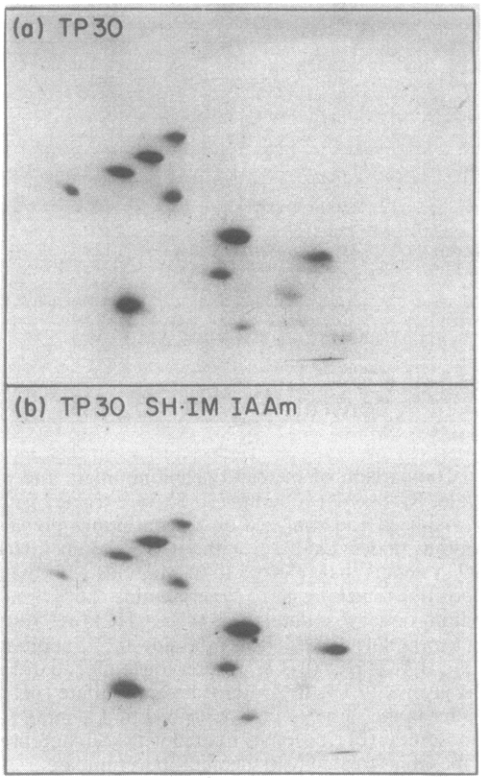


FIGURE 5: Analysis by two-dimensional polyacrylamide electrophoresis of 30S ribosomal proteins: (a) control total 30S protein; (b) protein extracted from 30S ribosomes following reaction with 10 mM mercaptobutyrimidate and carboxymethylation with iodoacetamide. Only those proteins migrating toward the cathode in the first dimension are shown. The mobility of proteins S1, S2, S6, and S10 (not shown) are also unchanged.

Analysis by Sucrose Gradient Centrifugation of Modified, Oxidized, and Reduced Ribosomes. Figure 6 shows sucrose gradient analyses of 30S ribosomes at each step of the procedure adopted. The results show the following points: (1) no dimer formation between 30S particles occurs at any stage; (2) the particles sediment at 30S throughout the procedure; (3) the reaction with mercaptobutyrimidate leads to a slight broadening of the 30S peak; (4) this broadening is reversed by the oxidation step. It is concluded that the conditions used do not cause any major conformational change in the 30S ribosome and that therefore any protein–protein interactions detected by the formation of specific protein products of higher molecular weight reflect the proximity of the constituent proteins in the native 30S ribosomal particle.

Dependence of the Formation of Specific Cross-Linked Products on the Native 30S Ribosome Conformation. When total protein was first extracted from the 30S ribosome and then treated under conditions similar to those employed with the native particle (equal total protein concentration) no cross-linked material was formed (Figure 7). Therefore, it is concluded that the formation of the cross-linked products depends upon the relative orientation of the proteins involved and requires the native conformation of the 30S particle. Furthermore, this result makes it seem highly likely that random disulfide bond formation between free proteins during their extraction from the oxidized ribosome and further analysis does not occur.

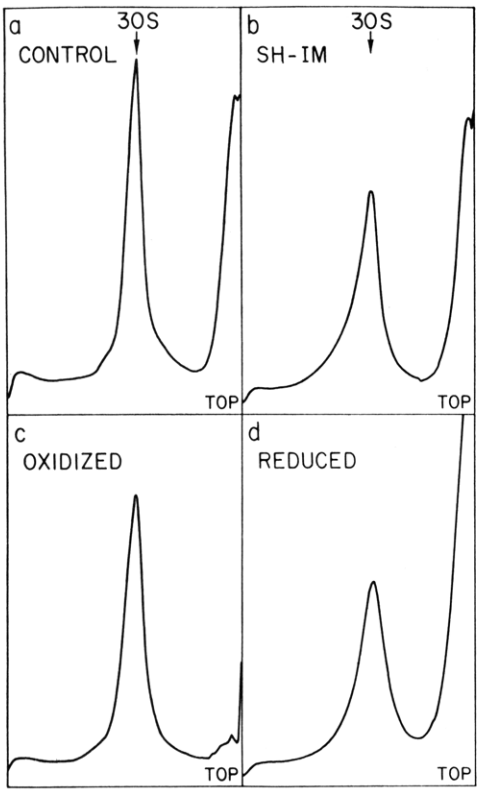


FIGURE 6: Analysis by sucrose gradient centrifugation of 30S particles at various stages of cross-linking and cleavage procedure: (a) control particles: (b) 30S ribosomes after reaction with 10 mM mercaptobutyrimidate; (c) particles from b following oxidation; (d) particles from c after reduction by incubation with 3% β-mercaptoethanol at 37° for 20 min. The ribosomes were centrifuged for 110 min at 55,000 rpm in the Beckman SW 56 rotor in linear sucrose gradients (7–25%) in TEA buffer.

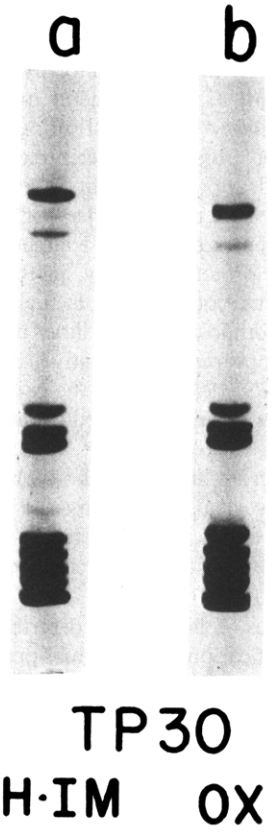


FIGURE 7: Effect of oxidation of free total 30S protein previously treated with 10 mM mercaptobutyrimidate. Total 30S protein was extracted from the ribosomal subunit as described in the text and analyzed on the discontinuous gel system described in Figure 1 in the absence of reducing agent: (a) protein from ribosomes treated with mercaptoimidate; (b) protein from (a) after the standard oxidation procedure.

Comparison of the Cross-Linking Caused by Bismethylsuberimidate and Mercaptobutyrimidate. Previous studies have shown that treatment of the 30S ribosome with bismethylsuberimidate resulted in the formation of discrete products of higher molecular weight. Figure 8 compares the spectrophotometric tracings of sodium dodecyl sulfate gels stained with Coomassie Blue of cross-linked 30S proteins prepared with bismethylsuberimidate and methyl mercaptobutyrimidate. The patterns are quite similar in the positions of the new products of elevated molecular weight; the identity of the constituents of these new products remains to be established. The experiment illustrates the greater efficiency of the mercaptobutyrimidate oxidation procedure. The concentration of this reagent was 10 mM; the concentration of bismethylsuberimidate which gave approximately the same yield of discrete cross-linked products was 25 mM, or 50 mM in total imidate. The greater efficiency of the mercaptoimidate reagent is very likely due to the fact that hydrolysis of the free imidate-terminal group of the monoreacted bisimidate competes with the cross-linking reaction to another protein. The free mercapto group, on the other hand, is stable and remains available for oxidation to the disulfide.

Discussion

A variety of methods are available and are being used in many laboratories for the formation and identification of products resulting from the treatment of ribosomal subunits with bifunctional reagents. In those cases in which the cross-linked products are not cleaved into their constituent protomeric components, rather elaborate and technically complex procedures have been employed. These studies rely either upon the availability of antibodies against each pure ribosomal protein and the testing of pure dimers one by one with each specific antibody; or on the reconstruction of ribosomes with specific proteins assumed to be cross-linked radioactively labeled. The first approach, while completely general, requires the prior preparation of purified proteins and their

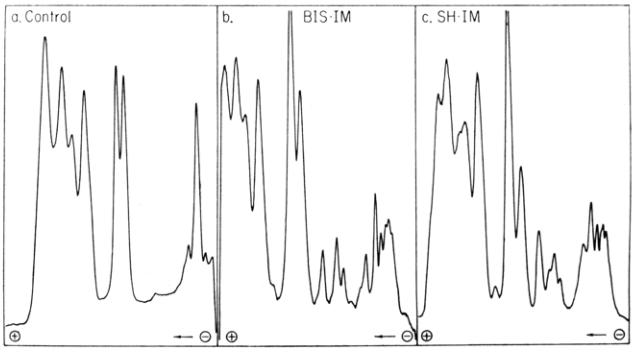


FIGURE 8: Comparison of bissuberimidate and mercaptobutyrimidate as cross-linking reagents. The 30S ribosomes were treated either with 25 mM bissuberimidate (Bickle *et al.*, 1972) or with 10 mM mercaptobutyrimidate and electrophoresed on 10% polyacrylamide gels with sodium dodecyl sulfate. The gels, following staining with Coomassie Blue, were scanned in a Gilford gel scanner (Bickle and Traut, 1971).

cognate antibodies. The second approach is particularly applicable to confirm the composition of protein dimers when the protomeric constituents are already suggested from other results, but is not a simple general method because of the technical complexity of reconstituting ribosomes with all permutations of possible dimerizable proteins selectively labeled. A third approach might utilize an analysis of the tryptic peptides of isolated dimers or oligomers and comparison of the peptides with those already characterized from studies on pure proteins. Finally, in the exceptional case in which dimer formation proceeds in high yield, and with high specificity, the composition of a dimer may be inferred by the coordinate disappearance of putative cross-linked protomers (Chang and Flaks, 1972). Given the extremely powerful analytical techniques for identifying ribosomal proteins (*e.g.*, two-dimensional gel electrophoresis), and the need for a completely general cross-linking method which is capable of detecting even low yields of all possible cross-linked proteins without making any prior assumptions, we have concentrated our efforts on the development a readily cleavable bifunctional reagent.

The advantages of the method described here are clear. (1) Large-scale purification of ribosomal proteins is not required either to perform peptide analysis, nor to reconstruct selectively labeled ribosomes, nor to prepare antibodies. (2) The cleavage of the cross-linked products occurs in 100% yield. (3) The cleaved protomeric proteins retain their characteristic behavior in the most powerful analytical method available for ribosomal proteins, two-dimensional gel electrophoresis (Kaltschmidt and Wittmann, 1970). A modification of this technique developed in this laboratory (Howard and Traut, 1973) permits the analysis of amounts of each ribosomal protein in the range of 1–5 μg. Thus, large amounts of cross-linked materials need not be isolated; indeed, since the modified method permits radioautography, the entire procedure can be carried out on a radioactive scale. (4) All ribosomal proteins are modified by the addition of SH groups (results in progress, not shown) and thus are potentially able to form cross-links to neighboring proteins. (5) Since the mercaptoimidate is a heterogeneous bifunctional reagent, the reaction of each function can be carried out in a separate step. Thus, the method is suitable for experiments in which one protein, either a ribosomal protein or an initiation or elongation factor, is modified with the imidate function prior to its incorporation into or binding to the ribosome, after which in the controlled oxidation step, the cross-links are formed.

While it seems abundantly evident that the cross-links formed are due to disulfide-bond formation, we have compared the results of treating the SH-charged ribosomes by oxidation and by reaction with a sulfhydryl group specific bifunctional, bisphenylmaleimides (R. L. Heimark and R. R. Traut, unpublished results). The pattern of cross-linked products observed on sodium dodecyl sulfate gels was identical in two cases. This result confirms that the cross-linking is between SH groups; moreover, it indicates that the added length of possible cross-links introduced with the bismaleimide does not lead to the appearance of new products. In addition, the oxidized form of the compound, the disulfide dimer of the mercaptoimidate, has been employed directly as a bifunctional reagent. In general, the pattern of cross-linked products resembled that obtained by the procedure described here. However, yields were lower, probably due to hydrolysis of the monoreacted imido ester. Nevertheless, the disulfide diimidate should find useful application in other systems.

Preliminary experiments on the utilization of mercaptobutyrimidate reagent to identify specific *E. coli* 30S protein neighbors have been carried out. A number of well-separated cross-linked bands were isolated from sodium dodecyl sulfate gels with radioactive proteins. Reduction of these products resulted in their quantitative conversion to new protein bands of lower molecular weight. The molecular weights of the cross-linked bands and their reduction products as determined by sodium dodecyl sulfate gel analysis are as follows. A band with an apparent molecular weight of 35,000 gave rise on reduction to two products with molecular weights of 20,000 and 14,000. This result is entirely consistent with the dimer formation between the two monomeric proteins to form the observed dimer. Another product with an apparent molecular weight of 32,000 gave products with molecular weights of 20,000, 14,000, and 13,000, and hence is most likely a mixture of two dimers. Similarly, a third product of mol wt 26,000 yielded products of mol wt 14,000, 13,000, and 11,000 and again is likely to contain two dimers. Further identification of these products is in progress.

Other cleavable bifunctional reagents have been utilized in a variety of investigations both of the tertiary structure of single polypeptide chains and oligomeric proteins (for review, see Wold, 1972). Of these reagents two types in particular might be applicable to studies on the topography of ribosomal proteins: azo-linked bifunctional reagents cleavable in high yield by treatment with ammonia and dithionite; phenyldisulfonyl chlorides, cleavable with HBr in acetic acid. Neither class of reagent has the advantages of the reagent described here. Cleavage of the azo linkage results in a protomer in which one positive charge per amine group reacted is lost; hence, its electrophoretic mobility will be altered. The disulfonyl chlorides hydrolyze rapidly and are insoluble in water. In addition, the length of the cross-link is 5.6 Å as opposed to the 14.6-Å distance of the cross-link (N $\leftrightarrow$ N) formed by the mercaptobutyrimidate. Previous studies with a homologous series of bis imidates differing in the number of methylene groups (T.-T. Sun, J. W. B. Hershey, R. L. Heimark, T. A. Bickle, and R. R. Traut, unpublished results) have shown that reagents shorter than bisadipimidate (8.7 Å; N $\leftrightarrow$ N) are distinctly less effective in cross-linking ribosomal proteins.

The class of cleavable protein–protein cross-linking reagents described here should provide a valuable tool in determining the ribosomal binding sites for initiation and elongation factors and for the overall protein topography of the ribosome. They should also have a variety of applications in studies on other oligomeric enzyme complexes, more complex cellular organelles, and bacteriophage and virus structures.

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Rate-Determining Step in the Reconstitution of *Escherichia coli* 30S Ribosomal Subunits†

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ABSTRACT: Previous studies on the mechanism of reconstitution of 30S subunits of *Escherichia coli* using 16S RNA and unfractionated 30S proteins indicated that there is a rate-limiting, unimolecular reaction which has a high activation energy. In the present study, the 30S proteins required for this temperature-dependent, rate-limiting step have been examined. Reconstitution was done using 16S RNA and a mixture of 21 purified proteins. The proteins were divided into two parts in various ways, and reconstitution was performed in two steps. In the first step, 16S RNA was incubated with a set of proteins at a high temperature (40°) for 30 min. In the second step the remaining proteins were added and the mixture incubated at a low temperature (30°) for 10 min. It was found that the 30S ribosomal proteins S4(P4a), S8(P4b), S7(P5), S16(P9a), and S19(P13) are required in the first heat step to form active 30S particles. S15(P10b) and S17(P9b) are somewhat less essential in this respect, and S11(P7), S18(P12), S9(P8), S5(P4), and S12(P10) are still less essential but must be present during the first heat step for full functional activity. Some 30S proteins which are found in isolated ribosomal intermediate (RI) particles [S20(P14), S13(P10a), and S6(P3b,c)] do not appear to be required. Some proteins which are required for the temperature-dependent step [S5(P4), S12(P10), and possibly S19(P13)] appear to bind very weakly to RI particles at low temperatures; the isolated RI particles are deficient in these proteins. The temperature-dependent step probably involves a substantial conformational change of the RI particles, as indicated by an increase in the sedimentation coefficient of the particles after heating.

Ribosomal subunits (30S) from *Escherichia coli* can be reconstituted from their dissociated molecular components (Traub and Nomura, 1968). *In vitro* reconstitution, although differing in some details from *in vivo* assembly, may provide information relevant to the latter.

Previous studies on the mechanism of the reconstitution of 30S subunits were done using 16S RNA and a mixture of unfractionated total 30S ribosomal proteins (TP30)[1] (Traub and Nomura, 1969a; Traub and Nomura, 1969b). Kinetic studies on the assembly of 30S subunits showed that the rate-limiting unimolecular reaction has a high activation energy (Traub and Nomura, 1969a). When the components of 30S subunits (16S RNA and TP30) are mixed together under the standard reconstitution conditions and incubated at 0° instead of at the higher temperature (usually 40°) required for formation of active subunits, particles are produced which sediment at about 21–22 S in low Mg^{2+} buffer, are deficient in several proteins, and have no functional activity. These particles (called "21S particles" in this paper) are activated when heated at 40° to form particles which are capable of binding the missing proteins at 0° to form functionally active 30S particles (Traub and Nomura, 1969a). The temperature dependence of this activation step suggests that the isolated 21S particles are similar to or identical with the intermediate which undergoes the rate-limiting unimolecular reaction. The following reaction scheme has been proposed.

$$\text{16S RNA} \xrightarrow{+\text{ RI proteins}} \text{RI particles} \xrightarrow{\text{heating}} \text{RI* particles} \xrightarrow{+\text{ S proteins}} \text{30S ribosomes} \quad (1)$$

† From the Institute for Enzyme Research and Departments of Biochemistry and Genetics, University of Wisconsin, Madison, Wisconsin 53706. *Received March 5, 1973*. This is paper No. 1634 from the Laboratory of Genetics and paper XX in the series Structure and Function of Bacterial Ribosomes. Paper XIX in this series is Higo *et al.* (1973). This work was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin, and by grants from the National Institute of General Medical Sciences (GM-15422) and the National Science Foundation (GB-31086X1).

[1] Abbreviations used are: TP30, unfractionated total 30S ribosomal proteins; RI, reconstitution intermediate; RI*, activated reconstitution intermediate; S proteins, 30S ribosomal proteins which do not bind to RI, but bind to RI* during assembly of 30S ribosomal subunits.