Torti, F. M., Dieckmann, B., Beutler, B., Cerami, A., & Ringold, G. M. (1985) *Science 229*, 867–869.
Wang, S. A., & Gudas, L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5880–5884.
Weiner, F. R., Czaja, M. J., Jefferson, D. M., Giambrone, M. A., Tur-Kaspa, R., Reid, L. M., & Zern, M. A. (1987) *J. Biol. Chem. 262*, 6955–6958.
Yamada, K. M. (1982) in *Cell Biology of the Extracellular Matrix* (Hay, E. D., Ed.) pp 95–114, Plenum Press, New York.
Yoshizato, K., Taira, T., & Yamamoto, N. (1985) *Biomed. Res. 6*, 61–72.
Zern, M. A., Leo, M. A., Giambrone, M. A., & Lieber, C. S. (1985) *Gastroenterology 89*, 1123–1131.

Comparative Cross-Linking Study on the 50S Ribosomal Subunit from *Escherichia coli*

Jan Walleczek,[‡] Thomas Martin,[‡,§] Bernhard Redl,[∥] Marina Stöffler-Meilicke,[*,‡,⊥] and Georg Stöffler[∥]

Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, D-1000 Berlin 33 (Dahlem), FRG, and Institut für Mikrobiologie, Medizinische Fakultät der Universität Innsbruck, Fritz-Pregl-Strasse 3, A-6020 Innsbruck, Austria

Received July 22, 1988; Revised Manuscript Received January 13, 1989

ABSTRACT: We have carried out an extensive protein–protein cross-linking study on the 50S ribosomal subunit of *Escherichia coli* using four different cross-linking reagents of varying length and specificity. For the unambiguous identification of the members of the cross-linked protein complexes, immunoblotting techniques using antisera specific for each individual ribosomal protein have been used, and for each cross-link, the cross-linking yield has been determined. With the smallest cross-linking reagent diepoxybutane (4 Å), four cross-links have been identified, namely, L3–L19, L10–L11, L13–L21, and L14–L19. With the sulfhydryl-specific cross-linking reagent *o*-phenylenedimaleimide (5.2 Å) and *p*-phenylenedimaleimide (12 Å), the cross-links L2–L9, L3–L13, L3–L19, L9–L28, L13–L20, L14–L19, L16–L27, L17–L32, and L20–L21 were formed; in addition, the cross-link L23–L29 was exclusively found with the shorter *o*-phenylenedimaleimide. The cross-links obtained with dithiobis(succinimidyl propionate) (12 Å) were L1–L33, L2–L9, L2–L9–L28, L3–L19, L9–L28, L13–L21, L14–L19, L16–L27, L17–L32, L19–L25, L20–L21, and L23–L34. The good agreement of the cross-links obtained with the different cross-linking reagents used in this study demonstrates the reliability of our cross-linking approach. Incorporation of our cross-linking results into the three-dimensional model of the 50S ribosomal subunit derived from immunoelectron microscopy yields the locations for 29 of the 33 proteins within the larger ribosomal subunit.

The catalysis of peptide bonds to form proteins from aminoacyl-tRNAs under direction of mRNAs occurs on ribosomes. In order to understand this mechanism, which is fundamental to all organisms, a detailed knowledge of the structure of the ribosome is essential.

Whereas the overall topography of the 30S subunit has been revealed (Stöffler-Meilicke & Stöffler, 1987; Capel et al., 1988; Brimacombe et al., 1988), the knowledge of the topography of the 50S subunit is by far not as complete (Stöffler & Stöffler-Meilicke, 1986; Nowotny et al., 1986). Therefore, we initiated an extensive protein–protein cross-linking study with the aim of providing the missing topographical data, necessary for constructing a considerably more complete three-dimensional model of the protein topography of the 50S subunit. In this study, we have used four different cross-linking reagents of varying length and specificity, namely, diepoxybutane (DEB),[1] *o*-phenylenedimaleimide (oPDM), *p*-phenylenedimaleimide (pPDM), and dithiobis(succinimidyl propionate) (DSP). A total of 12 cross-links have been identified with DSP, 10 with oPDM, 9 with pPDM, and 4 with DEB as cross-linking reagent. As we have pointed out previously, two-dimensional diagonal gel electrophoresis (Kenny et al., 1979; Traut et al., 1980, 1986) can yield ambiguous results when used for the identification of cross-linked complexes (Walleczek et al., 1989). For an unambiguous identification, we have thus used specific immunoreaction in the analysis of the individual members of the cross-linked protein complexes (Stöffler et al., 1988). In addition, the cross-linking yield of each cross-linked protein complex has been determined in order to minimize the possibility of identifying cross-linked complexes that are derived from a small subpopulation of protein-depleted or functionally inactive ribosomal particles (Walleczek et al., 1989).

Together with the cross-linking reagents used by us in previous cross-linking studies (Walleczek et al., 1989; Redl et al., 1989), a total of six different cross-linking reagents have

* To whom correspondence should be addressed.
‡ Max-Planck-Institut für Molekulare Genetik.
§ Present address: Institut für Genbiologische Forschung Berlin GmbH, Ihnestrusse 63, D-1000 Berlin 33, FRG.
∥ Institut für Mikrobiologie, Medizinische Fakultät der Universität Innsbruck.
⊥ Present address: Institut für Klinische und Experimentelle Virologie, Hindenburgdamm 27, D-1000 Berlin 45, FRG.

[1] Abbreviations: DEB, diepoxybutane; oPDM, *o*-phenylenedimaleimide; pPDM, *p*-phenylenedimaleimide; DSP, dithiobis(succinimidyl propionate); DMS, dimethyl suberimidate; SDS–PAG, sodium dodecyl sulfate–polyacrylamide gel; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IEM, immunoelectron microscopy; TP_{50}, total protein from 50S ribosomal subunits; TP_{50_X}, total protein from cross-linked 50S ribosomal subunits.

0006-2960/89/0428-4099$01.50/0

Table I: Reagents Used for Cross-Linking Proteins within the 50S Ribosomal Subunit of *E. coli*

cross-linking reagent	length (Å)	specificity	water-soluble	ref
2-iminothiolane	14.5	NH_2	+	Traut et al. (1980)
p-phenylenedimaleimide	12.0	SH	−	Chang & Flaks (1972)
dithiobis(succinimidylpropionate)	12.0	NH_2	−	as reported by supplier (Pierce)
dimethyl suberimidate	11.0	NH_2	+	as reported by supplier (Pierce)
o-phenylenedimaleimide	5.2	SH	−	Chang & Flaks (1972)
diepoxybutane	4.0	NH_2, SH	+	Brockmüller & Kamp (1986)

been used, ranging from 4 to 14.5 Å in length. About half of the cross-links have repeatedly been found with different cross-linking reagents, irrespective of their length. This good agreement between the different sets of cross-linking data demonstrates the reliability of our cross-linking approach. Our cross-linking results will be discussed in terms of their contribution to the knowledge of the protein topography of the 50S ribosomal subunit.

Materials and Methods

Materials. DSP, pPDM, oPDM, and DEB were from Sigma. Nitrocellulose was from Schleicher & Schuell. All other reagents were from Merck and of analytical grade.

Preparation and Cross-Linking of 50S Ribosomes. 50S subunits were isolated as described (Hindenach et al., 1971; Noll et al., 1973). The ribosomes were biologically active as assayed by poly(U)-dependent polyphenylalanine synthesis (Traub & Nomura, 1968). For each cross-linking experiment, 500 A_{260} 50S ribosomal subunits (3 mg/mL) were dialyzed against 50 mM triethanolamine hydrochloride, pH 8.0, 50 mM potassium chloride, and 6 mM magnesium chloride. The cross-linking reagents used in this study and their length, amino acid specificity, and solubility are summarized in Table I.

Cross-linking of ribosomes with DSP (1 mM) was in principle done as described (Lomant & Fairbanks, 1976). DSP (5 mg/150 μL of dimethyl sulfoxide) was added to the ribosomes until the concentration of DSP was 1 mM. After incubation for 1 h at 0 °C, excess reagent was neutralized by adding glycinamide (pH 8.0) to a final concentration of 50 mM. The ribosomes were then incubated for 45 min at 37 °C.

Cross-linking of ribosomes with pPDM and oPDM was in principle done as described by Chang and Flaks (1972). oPDM or pPDM (135 mg/mL dimethylformamide) was added to the ribosome solution until the concentration of oPDM or pPDM, respectively, was 7.5 mM. After incubation at 37 °C for 1 h, the reaction was stopped by addition of 0.1 M 2-mercaptoethanol for 5 min.

Cross-linking of ribosomes with DEB was performed according to Brockmüller and Kamp (1986); 1% (v/v) DEB was added to the ribosomes, and they were incubated for 1 h at 37 °C. Excess reagent was removed by incubation with 50 mM glycinamide (pH 8.0) for 45 min at 37 °C.

After the cross-linking procedure, 50S ribosomal subunits were separated from excess reagent and 50S–50S dimers by centrifugation in a linear sucrose gradient (10–30% sucrose in 100 mM ammonium chloride, 50 mM Tris-HCl, pH 7.4, and 20 mM magnesium chloride) using a Beckman SW27 rotor (23 000 rpm for 16 h at 4 °C). The monomeric ribosomes were pooled and pelleted by centrifugation in a 50Ti rotor (40 000 rpm for 22 h at 4 °C). Ribosomal proteins were extracted according to Leboy et al. (1964).

Identification of Cross-Links. The cross-linked proteins were identified by immunoblotting of one-dimensional SDS–PAGs as described in detail (Stöffler et al., 1988). In order to construct a calibration curve for the determination of the molecular weight of the cross-links, total protein from 50S ribosomal subunits (TP_{50}) was separated in one lane of each SDS–PAG. After transfer of the proteins to nitrocellulose by electroblotting, the blot was incubated with a mixture of antisera against three selected proteins (L2, L5, and L7/12) used as references and immunostained. Measurements for constructing the calibration curve were made directly on the immunoblots.

The yield of cross-linking was determined as described by Walleczek et al. (1989). Decreasing amounts of TP_{50_X} were separated by one-dimensional SDS–PAGE, transferred to nitrocellulose, and incubated with the respective antiserum. The yield of cross-linking (percent) was calculated according to

$$\text{yield (\%)} = \frac{X}{X + Y} \times 100$$

where X represents the lowest amount of TP_{50_X} that still gives a visible band for the un-cross-linked protein band and Y represents the lowest amount of TP_{50_X} that gives a visible band of approximately the same intensity for the cross-linked protein band.

Results

Characterization of the Modified 50S Ribosomal Subunits. When 50S subunits treated with pPDM, oPDM, DSP, and DEB were analyzed by sucrose gradient centrifugation, between 5 and 15% of the subunits sedimented as cross-linked 50S–50S dimers (data not shown). Therefore, the monomeric subunits, sedimenting at 50 S, were isolated as described under Materials and Methods and used for all further analyses.

Identification of the Protein Pairs Cross-Linked with DEB. Identification and quantification of the individual members of the cross-linked pairs were done by immunoblotting from one-dimensional SDS–PAGs as described (Stöffler et al., 1988; Walleczek et al., 1989). For illustration of the strategy used to identify the proteins in a given cross-linked complex, the analysis of the cross-links obtained with DEB shall be described in detail. In order to screen for cross-linked complexes, TP_{50} from un-cross-linked ribosomal subunits and TP_{50} extracted from cross-linked 50S subunits (TP_{50_X}) were separated by one-dimensional SDS–PAGE and transferred to nitrocellulose sheets. The nitrocellulose sheets were incubated with antisera specific for each of the individual ribosomal proteins of the large subunit (sera against proteins L31, L35, and L36 were not available). Cross-linked proteins given rise to a new band on the immunoblot with a molecular weight higher than that of the un-cross-linked component. In the first set of experiments, it was found that only proteins L3, L10, L11, L13, L14, L18, L19, L21, and L25 had been cross-linked by DEB. Only cross-links occurring in a yield of 5% or more have been analyzed in the course of this study. Proteins L18 and L25 were cross-linked in yields well below 5% and thus were not further analyzed.

Identification of the Cross-Links L13–L21, L10–L11, L14–L19, and L3–L19. Upon incubation of a nitrocellulose blot with anti-L13, a single cross-link band with an approximate molecular weight of 28 000 and a cross-linking yield of

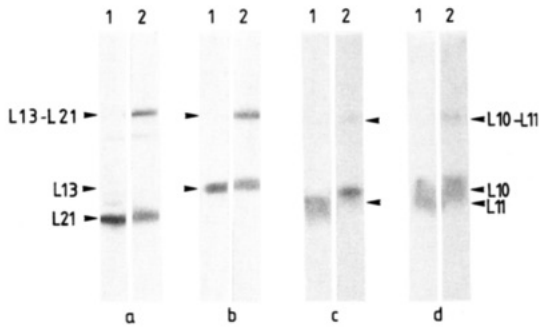

FIGURE 1: Identification of the cross-links L13–L21 and L10–L11 formed with DEB by immunoblotting from one-dimensional SDS–PAGs. 15 μg of TP_{50} (lane 1) and 15 μg of TP_{50_X} (lane 2) were separated by one-dimensional SDS–PAGE and electroblotted to nitrocellulose sheets, which were incubated with (a) anti-L21, (b) anti-L13, (c) anti-L11, and (d) anti-L10. For details, see Results.

Table II: Summary of the Cross-Links Obtained with DEB[a]

cross-link	M_r	cross-linking yield (%)
L3–L19 (25 300–15 000)	39 000	5
L10–L11 (16 500–15 700)	33 500	10
L13–L21 (18 500–10 200)	28 000	50
L14–L19 (15 700–15 000)	30 000	5

[a] The molecular weights of the monomeric proteins given in parentheses are taken from Dijk and Littlechild (1979), and the apparent molecular weights of the cross-linked protein complexes have been calculated from the calibration curve (see Materials and Methods). The cross-linking yields have been determined by immunoblotting (see Materials and Methods; Walleczek et al., 1989) and are rounded to the nearest 5%.

50% appeared on the immunoblot (Figure 1a). A cross-link band with approximately the same molecular weight and the same yield was observed on the immunoblot obtained with anti-L21. Since addition of the molecular weight of L13 (18 500) and L21 (10 200) yields a molecular weight of 28 700, a value in agreement with the molecular weight determined for the cross-link band, it was concluded that proteins L13 and L21 had been cross-linked by DEB. Incubation of an immunoblot with the two antisera simultaneously confirmed this result (data not shown).

Similarly, comparison of the immunoblots obtained after incubation with anti-L10 and anti-L11 demonstrates that protein L10 (M_r 16 500) had been cross-linked to protein L11 (M_r 15 700) since a cross-link band with equal apparent molecular weight (33 500) can be observed on both immunoblots (compare Figure 1c and Figure 1d). Again, incubation of a nitrocellulose sheet with anti-L10 and anti-L11 simultaneously confirmed this result (data not shown). The cross-linking yield for this protein pair was 10% (Table II).

Incubation of a blot with anti-L19 revealed two different cross-link bands, one with a molecular weight of approximately 30 000 and the other with a molecular weight of approximately 39 000 (Figure 2b). The immunoblot obtained with anti-L14 (M_r 15 700) also revealed a cross-link band with a molecular weight of approximately 30 000, and the one obtained with anti-L3 revealed a band with a molecular weight of about 39 000, indicating that these two proteins had been cross-linked to L19 (M_r 15 000) (compare lanes 2 in Figure 2a,b and Figure b,c). The yield of both cross-links was 5% (Table II). All cross-links obtained with DEB are summarized in Table II.

Identification of the Protein Pairs Cross-Linked with pPDM and oPDM. In the first set of experiments, it was found that proteins L1, L4, L6, L15, L22, L24, L26, L30, L33, and L34 had not been cross-linked at all, neither by pPDM nor by the shorter reagent oPDM. As with DEB, proteins L18 and L25

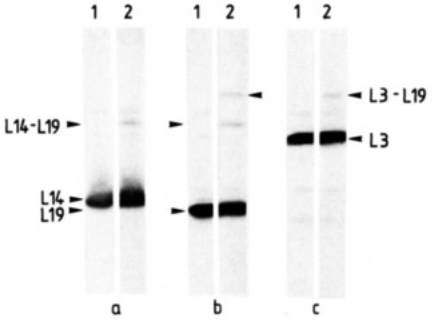

FIGURE 2: Identification of the cross-links L3–L19 and L14–L19 formed with DEB by immunoblotting from one-dimensional SDS–PAGs. Lane 1, 15 μg of TP_{50}; lane 2, 15 μg of TP_{50_X}. Blots were incubated with (a) anti-L14, (b) anti-L19, and (c) anti-L3. For details, see legend to Figure 1 and Results.

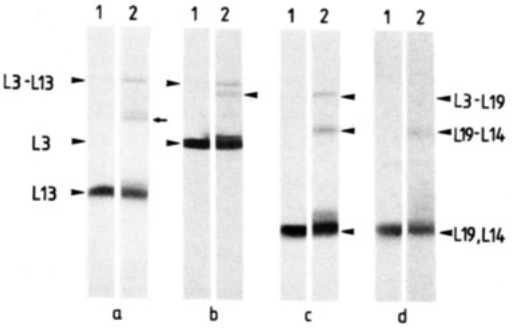

FIGURE 3: Identification of the cross-links L3–L13, L3–L19, and L19–L14 formed with oPDM. Lane 1, 15 μg of TP_{50}; lane 2, 15 μg of TP_{50_X}. Blots were incubated with (a) anti-L13, (b) anti-L3, (c) anti-L19, and (d) anti-L14. The identification of the cross-link with the low molecular weight in (a) (see arrow) is described in Figure 4. For details, see legend to Figure 1 and Results.

Table III: Summary of the Cross-Links Obtained with oPDM and pPDM[a]

cross-link	M_r	cross-linking yield[b] (%)
L2–L9 (33 000–15 700)	46 000	5
L3–L13 (25 300–18 500)	43 000	5
L3–L19 (25 300–15 000)	39 500	5
L9–L28 (15 700–12 200)	27 500	10
L13–L20 (18 500–16 200)	33 000	5
L14–L19 (15 700–15 000)	30 000	5
L16–L27 (17 300–14 000)	29 000	20
L17–L32 (16 500–8 400)	23 000	ND[c]
L20–L21 (16 200–10 200)	26 000	10
L23–L29[d] (14 000–7 600)	20 000	15

[a] For explanations, see legend to Table II. [b] The cross-linking yield is given for the cross-links with oPDM. [c] ND means not determined (for details, see text). [d] This cross-link was found with oPDM only.

were found cross-linked in yields below 5% and were not further analyzed. With the exception of the cross-link L23–L29 which was only found with oPDM, all cross-links formed with pPDM were also found with oPDM. Thus, we shall only illustrate the cross-links obtained with oPDM. All cross-links obtained with these two reagents are summarized in Table III.

Immunoblots from one-dimensional SDS–PAGs obtained by incubation with anti-L3 revealed two cross-link bands, one with a molecular weight of 43 000 and the other with a molecular weight of 39 500 (Figure 3b). The band with the molecular weight of 43 000 was shown to contain the cross-link L3–L13, since the immunoblot obtained with anti-L13 revealed a cross-link band with the same molecular weight (compare Figure 3a and Figure 3b), and the cross-link band with the molecular weight of 39 500 was shown to contain the cross-link L3–L19 (compare Figure 3b and Figure 3c). The cross-linking

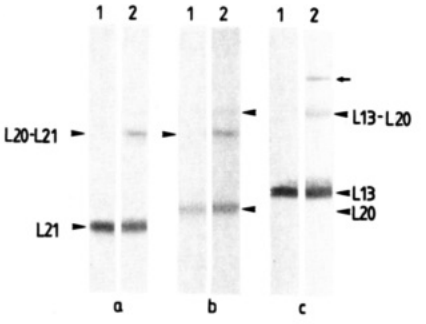

FIGURE 4: Identification of the cross-links L13–L20, and L20–L21 formed with oPDM. Lane 1, 15 μg of TP_{50}; lane 2, 15 μg of TP_{50_X}. Blots were incubated with (a) anti-L21, (b) anti-L20, and (c) anti-L13. The identification of the cross-link with the high molecular weight in (c) (see arrow) is shown in Figure 3. For details, see legend to Figure 1 and Results.

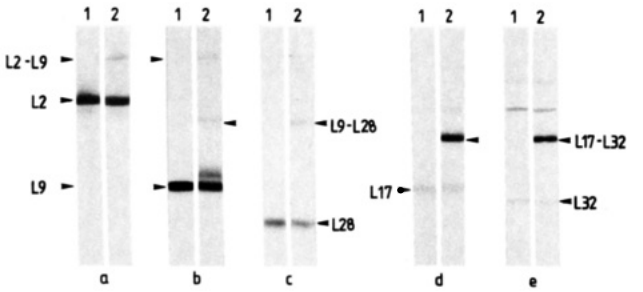

FIGURE 5: Identification of the cross-links L2–L9, L9–L28, and L17–L32 formed with oPDM. Lane 1, 15 μg of TP_{50}; lane 2, 15 μg of TP_{50_X}. Blots were incubated with (a) anti-L2, (b) anti-L9, (c) anti-L28, (d) anti-L17, and (e) anti-L32. The additional immunostained band in (e) is due to some unspecific reaction of the antiserum with a ribosomal proteins of high molecular weight. For details, see legend to Figure 1 and Results.

yield for both cross-links was determined to be 5% (Table III). On the immunoblot obtained with anti-L19, a second cross-link band with a molecular weight of 30 000 was observed, which was shown to contain the cross-link L14–L19 (compare Figure 3c and Figure 3d).

The immunoblot obtained after incubation with anti-L20 revealed two cross-link bands. The cross-link band with the high apparent molecular weight (33 000) was shown to contain the cross-link L13–L20 (cross-linking yield 5%; compare Figure 4b and Figure 4c). The cross-link band with the low apparent molecular weight (26 000) was shown to contain the cross-link L20–L21 (cross-linking yield 10%; compared Figure 4a and Figure 4b).

The immunoblot with anti-L2 showed one cross-link band with an apparent molecular weight of approximately 46 000. The only other immunoblot revealing a cross-link band with the same molecular weight was the one obtained after incubation with anti-L9. Thus, we concluded that proteins L2 and L9 are the members of one cross-linked complex (yield 5%; compare Figure 5a and Figure 5b). The immunoblot incubated with anti-L28 revealed one cross-link band with an apparent molecular weight of 27 500. Comparison of this immunoblot with the blot obtained with anti-L9 showed that protein L28 had been cross-linked to protein L9 (yield 10%; compare Figure 5b and Figure 5c).

From comparison of the immunoblots incubated with anti-L17 and anti-L32, it was concluded that protein L17 is cross-linked to L32, since both immunoblots showed one cross-link band with an apparent molecular weight of 23 000 (compare Figure 5d and Figure 5e). In this latter case, a remarkable observation was made: The cross-link band was significantly more stained than both un-cross-linked proteins

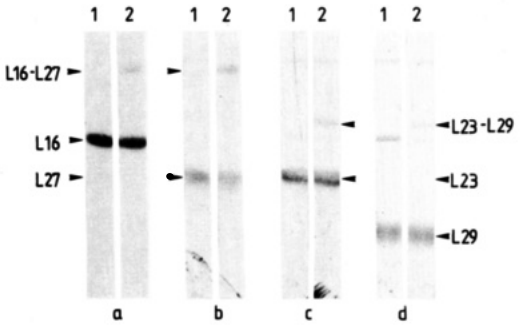

FIGURE 6: Identification of the cross-links L16–L27 and L23–L29 formed with oPDM. Lane 1, 15 μg of TP_{50}; lane 2, 15 μg of TP_{50_X}. Blots were incubated with (a) anti-L16, (b) anti-L27, (c) anti-L23, and (d) anti-L29. The additional band in (d), lane 1, is due to some unspecific reaction of the antiserum and cannot be observed with TP_{50_X} (lane 2). For details, see legend to Figure 1 and Results.

Table IV: Summary of the Cross-Links within the 50S Ribosomal Subunit Obtained with Six Different Cross-Linking Reagents[a]

L1–L33[b,c,d]	L7/12–L11[d]	L17–L32[b,c,d,e,f]
L2–L9[b,c,e,f]	L9–L28[b,c,d,e,f]	L18–L22[d]
L2–L9–L28[b,c]	L10–L11[d,g]	L19–L25[b,c,d]
L3–L13[e,f]	L13–L20[d,e,f]	L20–L21[b,c,e,f]
L3–L19[b,c,e,f,g]	L13–L21[b,c,g]	L22–L32[b]
L5–L7/12[d]	L14–L19[b,c,e,f,g]	L23–L29[f]
L6–L19[d]	L16–L27[b,c,d,e,f]	L23–L34[b,c]
L7/12–L10[d]	L17–L30[b]	L27–L33[d]

[a] Proteins which have been localized by IEM on the surface of the 50S ribosomal subunit (Stöffler & Stöffler-Meilicke, 1986; Hackl et al., 1988) are underlined. The cross-linked protein complexes have been formed by the following: [b] 2-Iminothiolane (Walleczek et al., 1989). [c] DSP (this paper). [d] DMS (Redl et al., 1989). [e] pPDM (this paper). [f] oPDM (this paper). [g] DEB (this paper).

L17 and L32 in the lane with the control TP_{50} (compare lanes 1 and 2 in Figure 5d,e), the reason for which is not clear. The cross-linking yield for this protein pair could thus not be determined by the quantification method applied by us.

After comparison of the immunoblots obtained upon incubation with anti-L16 and anti-L27, a single cross-link band with equal apparent molecular weight (29 000) appeared on both immunoblots, indicating that protein L16 (M_r 17 300) had been cross-linked to protein L27 (M_r 14 000; compare Figure 6a and Figure 6b). The cross-linking yield was calculated to be 20%. Also, the immunoblots with anti-L23 and anti-L29 revealed a cross-link band with the same apparent molecular weight (20 000)8 thus showing that protein L33 (M_r 14 000) had been cross-linked to protein L29 (M_r 7600; compare Figure 6c and Figure 6d).

Protein Complexes Cross-Linked with DSP. Upon cross-linking of 50S ribosomal subunits with DSP, a very similar cross-linking pattern was observed on the immunoblots from one-dimensional SDS–PAGs as upon cross-linking with 2-iminothiolane (Walleczek et al., 1989). With the exception of the cross-links L17–L30 and L22–L32, all cross-links found with 2-iminithiolane have also been observed with DSP, namely, L1–L33, L2–L9, L2–L9–L28, L3–L19, L9–L28, L13–L21, L14–L19, L16–L27, L17–L32, L19–L25, L20–L21, and L23–L34. The analysis of these cross-links will not be illustrated here; they are summarized in Table IV.

Cross-Links with Proteins L5, L7/12, L10, and L11. Immunoblots of 50S ribosomal subunits cross-linked either with oPDM and pPDM or with DSP incubated with anti-L7/12 showed multiple smeared cross-link bands (data not shown). This was also observed for the immunoblots obtained with anti-L5, anti-L10, and anti-L11. These proteins had apparently been cross-linked to each other in a highly complex

manner. Since the neighborhood relationships of these proteins has already been shown by IEM (Stöffler & Stöffler-Meilicke, 1986), no further effort was made to identify the cross-links.

Discussion

This report completes a comparative protein–protein cross-linking study within the 50S ribosomal subunit of *Escherichia coli* using four different cross-linking reagents, namely, DEB, oPDM, pPDM, and DSP. The results of two further studies in which we used 2-iminothiolane and DMS as cross-linking reagents are reported elsewhere (Walleczek et al., 1989; Redl et al., 1989). All the cross-links obtained with the six different cross-linking reagents are summarized in Table IV.

Altogether, 14 cross-links with 2-iminothiolane, 13 with DMS, 12 with DSP, 10 with oPDM, 9 with pPDM, and 4 with DEB have been identified. Of this total of 62 cross-linked protein pairs, 24 were different from each other. Of these cross-links, seven, namely, L2–L9, L3–L19, L9–L28, L14–L19, L16–L27, L17–L32, and L20–L21, were formed independently of the length of the cross-linker or the respective amino acid specificity, since they were found both with amino-specific and sulfhydryl group specific reagents, as well as with short (approximately 5 Å) and long (approximately 12 Å) cross-linking reagents (see Table IV). Our results indicate that, furthermore, cross-links with L7/12 as a member, that have so far only been identified for DMS (Table IV), are also formed with other cross-linking reagents with the exception of DEB.

Nine cross-links, namely, L1–L33, L5–L7/12, L6–L19, L17–L30, L18–L22, L19–L25, L22–L32, L23–L34, and L27–L33, were only found with cross-linking reagents of a length of at least 11 Å and a specificity for free amino groups (see Table IV). In contrast, the cross-linked protein pairs L3–L13 and L23–L29 were formed only with the sulfhydryl group specific reagent oPDM or pPDM. The finding of these latter cross-links is surprising since it is known that none of the proteins involved in the two cross-links contains any sulfhydryl groups (Wittmann-Liebold, 1986). Apparently, oPDM and pPDM were able to react with amino acid residues other than SH groups in order to cross-link these proteins.

It is assumed as a general rule that the probability of cross-linking proteins is greatly enhanced with increasing length of the cross-linking reagents used. The validity of this rule is demonstrated by the fact that we find the largest number of cross-links (14) with the longest cross-linking reagent used in this work, namely, 2-iminothiolane (14.5 Å), whereas we find only 4 cross-links upon cross-linking with the shortest reagent, DEB (4 Å). However, the cross-links found with short cross-linking reagents are not necessarily found with long cross-linking reagents. An example is the cross-link L23–L29, which has exclusively been found cross-linked by the short cross-linking reagent oPDM, a reagent of 5.2 Å in length (Table I).

Our method of quantification allowed us to detect cross-links in a yield of 0.1–3.0%, depending on the titer of the antiserum and the amount of TP_{50x} used for the analysis. For practical reasons, only cross-links occurring in a yield of 5% or more have been analyzed. We found that the longer cross-linking reagents generally gave a higher cross-linking yield than the shorter reagents. However, one striking exception is the cross-link L13–L21: This cross-link was found in a yield of 5% with 2-iminothiolane (length 14.5 Å), whereas in a yield of 50% when DEB (length 4.0 Å) was used as cross-linker. Again, this is an example of the specificity of the cross-linking approach.

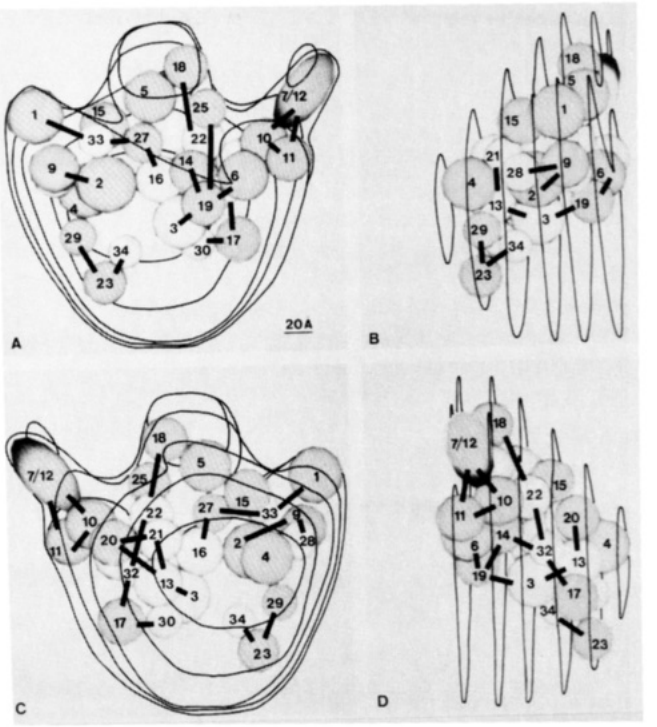

FIGURE 7: Computer graphics model of the protein topography of the 50S ribosomal subunit after Walleczek et al. (1988). The individual members of cross-linked protein complexes (Table IV) with the exception of L5–L7/12 (see Discussion) are connected by bars. The proteins whose position has been taken from the IEM model (Stöffler & Stöffler-Meilicke, 1986; Hackl et al., 1988) are shaded. The orientations of the four views are (A) 360°, (B) 90°, (C) 180°, and (D) 270°. The views in (A) and (C) correspond to the crown views from EM; the views in (B) and (D) are slightly different from the kidney views. The cross-link L14-L32 is from Th. Pohl and B. Wittmann-Liebold (unpublished results).

Since half of the reagents were soluble in organic solvents, whereas the other half was water soluble (Table I), it was possible to control the effect of organic solvents (final concentration was 1–2%) on ribosome structure and the formation of cross-links. We found that of the 14 different cross-links that were formed with the water-soluble reagents (2-iminothiolane, DMS, and DEB), 12 were also formed with the water-insoluble reagents (pPDM, oPDM, and DSP). Thus, we concluded that no significant changes in the relative arrangement of the proteins within the 50S subunit have occurred which might have been induced by the presence of organic solvents during the cross-linking procedure.

Other groups have also reported protein–protein cross-links within the 50S ribosomal subunit using different methods of identification (Clegg & Hayes, 1974; Expert-Bezancon et al., 1975; Traut et al., 1986; Brockmüller & Kamp, 1986; Schönfeld & Foulaki, 1987). The cross-link L13–L21, using DEB as a cross-linking reagent, has already been described by Schönfeld and Foulaki in 1987 (compare Table IV). In *Bacillus stearothermophilus*, a cross-link L23–L29 has been identified with this same cross-linking reagent (Brockmüller & Kamp, 1986). Although in *E. coli* we did not find this latter cross-link with DEB, the cross-link L23–L29 was found with the almost equally short cross-linking reagent oPDM (see Table I). This cross-link confirms the immunoelectron microscopic finding that protein L23 is located at the back of the 50S subunit, in vicinity to protein L29 (Hackl & Stöffler-Meilicke, 1988). Cross-links within the 50S subunit of *E. coli* with DSP, oPDM, and pPDM have so far not been described.

The comparison of our cross-linking data obtained with

DMS (Redl et al., 1989) with those of Expert-Bezançon et al. (1975) gave a good agreement between the two sets of data, whereas the agreement with the results obtained by Clegg and Hayes (1974) was less good. There was also considerable disagreement between our data (Walleczek et al., 1989) and Traut's (Traut et al., 1986) cross-linking data obtained with 2-iminothiolane. A detailed discussion comparing the two sets of data and pointing out difficulties and possible errors when cross-links are exclusively analyzed by two-dimensional gel electrophoresis is given by Walleczek et al. (1989). Clearly, the advantage of our method is that the identification of the individual members of a cross-linked complex is solely based on the immunoreaction of the individual ribosomal proteins with a specific antibody and thus is independent of alterations in the electrophoretic mobility of a protein due to modifications by the cross-linking procedure.

Comparison of the IEM model (Stöffler & Stöffler-Meilicke, 1986; Hackl et al., 1988) with the cross-linked complexes in which both members have been located on the ribosomal surface (Table IV) shows that the agreement between the two independent sets of data is very good, with the exception of the cross-link L5–L7/12. Protein L5 has been mapped at the base of the central protuberance, whereas proteins L7/12, which occur in four copies per 50S subunit, have been localized along the L7/12 stalk (Stöffler & Stöffler-Meilicke, 1986); thus, the cross-linking reagent would have to span a distance of approximately 70 Å. There is, however, evidence that one dimer of L7/12 may not be located in the stalk (Boublik et al., 1975; McKuskie-Olson et al., 1985; Traut et al., 1986) and that the stalk may be flexible (Cowgill et al., 1984), two findings which could easily explain the cross-link L5–L7/12. The fact that multiple cross-links with L7/12 are found with most cross-linking reagents may be an indication for the flexibility of the stalk. Clearly, further investigations using methods other than IEM and cross-linking are necessary to define the exact arrangement of proteins L7/12 within the 50S subunit.

The consistency of the cross-links obtained with the different cross-linking reagents (Table IV) proves that cross-linking of ribosomal proteins in situ is a powerful technique for obtaining valuable structural information provided that the members of the cross-links are analyzed by immunological or equally reliable means. Combination of our cross-linking results with the IEM data allowed us to generate a three-dimensional model of the protein topography for 29 of the 33 proteins within the 50S ribosomal subunit using interactive computer graphics (Walleczek et al., 1988). Input in the modeling process served the immunoelectron microscopically determined network of epitope–epitope distances on the surface of the large ribosomal subunit together with the in situ cross-linking data summarized in Table IV. Four views of the model are shown in Figure 7, with the proteins that have been localized on the surface of the 50S subunit by IEM shaded and the individual members of cross-linked protein complexes connected by bars, with the exception of the cross link L5–L7/12 which was not included in the modeling process (Walleczek et al., 1988). Clearly, additional topographical information is necessary for a more precise placement of proteins not mapped by IEM and with a single cross-link only. However, our model contributes considerably to the current knowledge of the protein topography within the 50S ribosomal subunit and establishes a strong basis for future elucidation of ribosomal structure and function.

Acknowledgments

We thank Dr. H. G. Wittmann for continued support. We thank Renate Hasenbank and Karl-Heinz Rak for characterization of the antisera and Renate Albrecht for preparation of the figures.

References

Boublik, M., Hellman, W., & Roth, H. E. (1976) *J. Mol. Biol. 126*, 123–140.

Brockmüller, J., & Kamp, R. (1986) *Biol. Chem. Hoppe-Seyler 367*, 925–935.

Capel, M. S., Engelmann, D. M., Freeborn, B. R., Kjeldgaard, M., Langer, J. A., Ramakrishnan, V., Schindler, D. G., Schneider, D. K., Schoenborn, B. P., Sillers, I.-Y., Yabuki, S., & Moore, P. B. (1987) *Science 238*, 1403–1406.

Chang, F. N., & Flaks, J. G. (1972) *J. Mol. Biol. 68*, 177–180.

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

Cowgill, C. A., Nichols, B. G., Kenny, J. W., Butler, P., Bradbury, E. M., & Traut, R. R. (1984) *J. Biol. Chem. 259*, 15257–15263.

Dijk, J., & Littlechild, J. (1979) *Methods Enzymol. 59*, 481–502.

Expert-Bezancon, A., Barritault, D., Clegg, J. C. S., Milet, M., Khouvine, J., & Hayes, D. H. (1975) *FEBS Lett. 59*, 64–69.

Hackl, W., & Stöffler-Meilicke, M. (1988) *Eur. J. Biochem. 174*, 431–435.

Hackl, W., Stöffler-Meilicke, M., & Stöffler, G. (1988) *FEBS Lett. 223*, 119–123.

Hindennach, I., Stöffler, G., & Wittmann, H. G. (1971) *Eur. J. Biochem. 23*, 7–11.

Kenny, J. W., Lambert, J. M., & Traut, R. R. (1979) *Methods Enzymol. 59*, 534–550.

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

Lomant, A. J., & Fairbanks, G. (1976) *J. Mol. Biol. 104*, 243–261.

McKuskie-Olson, H., Sommer, A., Tewari, D. S., Traut, R. R., & Glitz, D. G. (1985) *J. Biol. Chem. 261*, 6924–6932.

Noll, M., Hapke, B., Schreier, H. M., & Noll, H. (1973) *J. Mol. Biol. 75*, 281–294.

Nowotny, V., May, R. P., & Nierhaus, K. H. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 101–111, Springer-Verlag, New York.

Redl, B., Walleczek, J., Stöffler-Meilicke, M., & Stöffler, G. (1989) *Eur. J. Biochem.* (in press).

Schönfeld, H. J., & Foulaki, K. (1987) *Anal. Biochem. 164*, 23–30.

Schüler, D., & Brimacombe, R. (1988) *EMBO J. 7*, 1509–1513.

Stöffler, G., & Stöffler-Meilicke, M. (1986) in *Structure Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 28–46, Springer-Verlag, New York.

Stöffler, G., Redl, B., Walleczek, J., & Stöffler-Meilicke, M. (1988) *Methods Enzymol. 164*, 64–76.

Stöffler-Meilicke, M., & Stöffler, G. (1987) *Biochimie 69*, 1049–1064.

Traub, P., & Nomura, P. (1968) *J. Mol. Biol. 40*, 391–413.

Traut, R. R., Lambert, J. M., Boileau, G., & Kenny, J. W. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 89–110, University Park Press, Baltimore.

Traut, R. R., Tewari, D. S., Sommer, A., Gavino, G. R., Olson, H. M., & Glitz, D. G. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.)

pp 286–308, Springer-Verlag, New York.
Walleczek, J., Schüler, D., Stöffler-Meilicke, M., Brimacombe, R., & Stöffler, G. (1988) *EMBO J. 7*, 3571–3576.
Walleczek, J., Redl, B., Stöffler-Meilicke, M., & Stöffler, G. (1989) *J. Biol. Chem.* (in press).
Wittmann-Liebold, B. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 326–361, Springer-Verlag, New York.

^{15}N-Labeled 5S RNA. Identification of Uridine Base Pairs in *Escherichia coli* 5S RNA by ^{1}H–^{15}N Multiple Quantum NMR†

Darrell R. Davis,‡,§ Ziro Yamaizumi,‖ Susumu Nishimura,‖ and C. Dale Poulter*,‡

Department of Chemistry, University of Utah, Salt Lake City, Utah 84112, and National Cancer Center Research Institute, Chou-ku, Tokyo, Japan

Received September 29, 1988; Revised Manuscript Received January 4, 1989

ABSTRACT: *Escherichia coli* 5S RNA labeled with ^{15}N at N3 of the uridines was isolated from the Sϕ-187 uracil auxotroph grown on a minimal medium supplemented with [3-^{15}N]uracil. ^{1}H–^{15}N multiple quantum filtered and 2D chemical shift correlated spectra gave resonances for the uridine imino ^{1}H–^{15}N units whose protons were exchanging slowly with solvent. Peaks with ^{1}H/^{15}N shifts at 11.6/154.8, 11.7/155.0, 11.8/155.5, 12.1/155.0, and 12.2/155.0 ppm were assigned to GU interactions. Two labile high-field AU resonances at 12.6/156.8 and 12.8/157.3 ppm typical of AU pairs in a shielded environment at the end of a helix were seen. Intense AU signals were also found at 13.4/158.5 and 13.6/159.2 ppm where ^{1}H–^{15}N units in normal Watson–Crick pairs resonate. ^{1}H resonances at 10.6 and 13.8 ppm were too weak, presumably because of exchange with water, to give peaks in chemical shift correlated spectra. ^{1}H chemical shifts suggest that the resonance at 13.8 ppm represents a labile AU pair, while the resonance at 10.6 ppm is typical of a tertiary interaction between U and a tightly bound water or a phosphate residue. The NMR data are consistent with proposed secondary structures for 5S RNA.

The small ribosomal ribonucleic acid 5S RNA is a necessary constituent of the large ribosomal subunit (Gould et al., 1970). Although little is known in detail about the specific functions and interactions of 5S RNA with other molecules in the ribosome, there are indications that 5S RNA is located near the ribosomal binding site of tRNA (Roberts, 1972; Soffer, 1974). A considerable effort has been devoted toward elucidation of the secondary and tertiary structure of 5S RNA and its interactions with other constituents in order to evaluate its role in the ribosome during translation. ^{1}H NMR spectroscopy has been widely used to study the structure of 5S RNA. The majority of these investigations focused on the uridine and guanosine imino resonances, with an emphasis on NOE studies to assign chemical shifts of nearest neighbors (Chan & Marshall, 1986; Chang & Marshall, 1986; Lee & Marshall, 1987). Other factors that influence the specta, such as ionic strength and temperature, were also used to assign resonances (Leontis & Moore, 1986b; Leontis et al., 1986).

^{1}H NMR spectra of 5S RNAs have been hampered by the complexity of the imino region between 9 and 15 ppm and by line broadening. As a result, some recent work utilized fragments obtained from limited nuclease digests (Li & Marshall, 1986; Leontis & Moore, 1986a) to simplify the spectra. Spectral editing techniques based on ^{1}H–^{15}N heteronuclear correlations (Griffey et al., 1985; Griffey & Redfield, 1987) with isotopically enriched molecules have also been employed. Some GU base pairs were identified in a uniformly ^{15}N-labeled fragment (Kime, 1984) by difference decoupling. Recently, ^{1}H–^{15}N spectra were also reported for a partially labeled 5S fragment (Gewirth et al., 1987). Unfortunately, resolution in difference decoupling experiments is inherently poor because of limitations imposed by the magnitude of the one-bond ^{1}H–^{15}N coupling constant, and it is not possible to resolve closely spaced resonances. We now report a 2D ^{1}H/^{15}N chemical shift correlation study with *Escherichia coli* 5S RNA labeled specifically with ^{15}N at N3 of uridine which allowed selective detection of hydrogen-bonded uridine imino protons in slow exchange with solvent without complications from ^{1}H/^{15}N couplings. Of particular interest is the identification of five resonances attributed to G–U interactions.

Materials and Methods

The *E. coli* Sϕ-187 uracil-requiring auxotroph (Griffey et al., 1982) was grown to late log phase in a minimal medium supplemented with 4 μg/mL casamino acids, 40 μg/mL thymidine, 40 μg/mL cytosine, and 18 μg/mL [3-^{15}N]uracil. A soluble RNA fraction was obtained by phenol extraction and 2-propanol precipitation as previously described (Griffey et al., 1985). Crude RNA was fractionated by chromatography on DEAE-Sephadex A-50, pH 7.5, and fractions containing activity for $tRNA_2^{Tyr}$ were purified by chromatography on BD-cellulose (Nishimura, 1971). The first sharp peak to elute from BD-celluose was homogeneous 5S RNA, as determined by polyacrylamide gel electrophoresis (Barrell, 1971). NMR samples were prepared by dissolving 8 mg of 5S [3-^{15}N]RNA in 350 μL of 10 mM phosphate buffer, pH 6.0, containing 5% deuterium oxide, 10 mM magnesium chloride, 100 mM po-

† This work was supported by NIH Grant GM 32490 (to C.D.P.).
‡ University of Utah.
§ Present address: Department of Chemistry, University of Washington, Seattle, WA 98195.
‖ National Cancer Center Research Institute.

0006-2960/89/0428-4105$01.50/0