Eur. J. Biochem. *138*, 83–87 (1984)

Study of the Zn-containing DD-carboxypeptidase of *Streptomyces albus* G by small-angle X-ray scattering in solution

Harald LABISCHINSKI, Peter GIESBRECHT, Erwin FISCHER, Gerhard BARNICKEL, Hans BRADACZEK, Jean-Marie FRÈRE, Claude HOUSSIER, Paulette CHARLIER, Otto DIDEBERG, and Jean-Marie GHUYSEN

Robert-Koch-Institut des Bundesgesundheitsamtes, Berlin; Institut für Kristallographie der Freien Universität, Berlin; Service de Microbiologie, Institut de Chimie, Université de Liège au Sart Tilman; Service de Chimie Physique, Institut de Chimie, Université de Liège au Sart Tilman; and Laboratoire de Cristallographie, Institut de Physique, Université de Liège au Sart Tilman

(Received July 4/October 11, 1983) – EJB 83 0705

Study of the Zn^{2+}-containing D-alanyl-D-alanine-cleaving carboxypeptidase of *Streptomyces albus* G by small-angle X-ray scattering in solution yielded the following molecular parameters: radius of gyration $R = 1.82 \pm 0.05$ nm; largest diameter $D = 5.9 \pm 0.2$ nm; relative molecular mass $M_r = 17000 \pm 2000$; volume $V \approx 35 \pm 2$ nm^3; degree of hydration: 0.25 ± 0.02 g water/g protein. By reference to theoretical scattering curves of rigid triaxial homogeneous bodies, a model which fits all experimental data is an elliptical cylinder. Such a model is compatible with that observed in the crystal structure. At those high concentrations necessary to form inactive enzyme-ligand associations the non-competitive β-lactam inhibitors, cephalothin and cephalosporin C, drastically altered the scattering behaviour of the protein.

The G, R61 and R39 D-alanyl-D-alanine-cleaving peptidases (in short DD-peptidases), isolated from *Streptomyces albus* G, *Streptomyces* R61 and *Actinomadura* R39 respectively, have been used extensively as model enzymes for the study of the reactions involved in the last stages of bacterial wall peptidoglycan synthesis and the mode of action of the β-lactam antibiotics [1]. The R61 and R39 enzymes perform catalysis via an active serine residue and are respectively very and exceedingly sensitive to penicillin. In contrast, the G enzyme operates via a Zn^{2+} cofactor and is highly resistant to penicillin. Knowledge of the exact atomic level structures and functioning of these enzymes is also relevant to the proposed peptidoglycan network models [2, 3]. The G and R61 DD-peptidases have been crystallized. The solution of the R61 enzyme structure has proceeded to a resolution of 0.28 nm, showing, via difference Fourier maps, the binding site of cephalosporin C and 6,6-dichloro-4-deaza-2,2-didemethylpenicillanic acid [4, 5]. The amino acid sequence (212 residues) [6] and the three-dimensional structure at 0.45 nm and 0.25 nm resolution of the G Zn^{2+} DD-peptidase have been established [7, 8] and a plausible picture of how this enzyme performs catalysis has been proposed [9]. The overall shape of the enzyme in the crystal structure is that of an elliptical cylinder with a height of 4.8 nm and an axial ratio of 1.4:1:0.8 (height:long axis:short axis). The enzyme conformation in aqueous solution has now been studied by small-angle X-ray scattering. The results obtained are presented here.

MATERIALS AND METHODS

Enzyme

The enzyme was purified to protein homogeneity [10]. The enzyme solutions were prepared by extensive dialysis at 4 °C against 50 mM Tris/HCl buffer pH 8.3 (buffer I) or 10 mM Hepes/NaOH buffer pH 7.75 containing 5 mM $MgCl_2$ (buffer II). Enzyme concentrations ranging between about 0.2 mM and 0.8 mM were determined spectrophotometrically at 280 nm, using $A^{1\%}_{1\,cm} = 10$.

Small-angle X-ray scattering

Weighed amounts of cephalosporin C and cephalothin (from Ely Lilly and Sigma respectively) were added to the enzyme solutions followed by rapid agitation. The molar ratios enzyme: antibiotic were 1:20 for cephalosporin C in buffer I and 1:17 for cephalothin in buffer II. Prior to the measurements, the solutions were kept at room temperature for at least 1 h. The complex between cephalosporin C and the enzyme was studied using a highly stabilized X-ray generator (Seiffert, Debyeflex 1500) with a copper target tube (50 kV, 50 mA) and a Kratky camera. Samples, placed in Mark capillaries (1 mm diameter), were kept at either 4 °C or 37 °C during irradiation by means of a temperature cuvette (Anton Paar KG; Graz). The scattered intensities were recorded by a scintillation counter with pulse-height discrimination set to receive the Cu lines Kα and Kβ. Scattering angles ranging from 3×10^{-3} to 1.2×10^{-1} radians were set by an electronically programmed step-scanning device (EFG GmbH, Berlin). In order to reduce the statistical errors to 0.3%, a minimum of 10^5 pulses was counted at each point. The absolute intensities were determined via a Lupolen sample [11] that had been calibrated at the Institut für Physikalische Chemie in Graz. The cephalothin-enzyme complex was studied with the Kratky compact camera, equipped with a position-sensitive counter (Braun, Munich, and Siemens AG, Munich). Samples were irradiated at 4 °C in quartz capillaries supplied with the camera. In all cases, antibiotic-free enzyme solutions were used as controls.

Evaluation of the scattering data was done using several computer programs. Examination of the statistical reliability of the data and subtraction of the background scattering was carrried out by a program written by Zipper [12]. Corrections for collimation effects caused by the line-shaped primary beam and for the presence of Cu Kβ radiation were made using either

one of the usual methods [12] or the so-called indirect Fourier transform developed by Glatter [13, 14]. The experimental data were compared with the theoretical scattering curves generated by various models of uniform electron density using tabulated values for simple triaxial bodies and a computer program which handled models built up from a series of surfaces of second degree using a Fast Fourier transform algorithm [15]. Comparative density measurements between solutions of G enzyme and antibiotic-treated enzyme samples were carried out with the digital densitometer DMA 002 (Anton Paar KG, Graz) as described in [16].

Fluorescence polarization

Fluorescence polarization measurements were performed at 20 °C, using a Baird Atomic SF 100 EE spectrofluorimeter with a Glan-Thomson prism in the excitation beam (295 nm) and a polarizing coat in the emission beam (350 nm). The fluorescence anisotropy $\mu = (I_{\text{parallel}} - I_{\text{perpendicular}})/(I_{\text{parallel}} + 2\,I_{\text{perpendicular}})$ was corrected for the transmittivity factor of the emission monochromator [17]. Solutions with variable sucrose contents from 0 to 40 % were used to vary the T/η factor of the Perrin equation [18]

$$\mu^{-1} = \mu_0^{-1}\,[1 + (RT/V_h\eta)\tau]$$

where V_h is the sphere hydrated volume, τ the fluorescence lifetime, T the absolute temperature and η the viscosity. The enzyme (45 μM in 10 mM Hepes/NaOH buffer pH 8.0, containing 3 mM $MgCl_2$) was incubated at 37 °C for 6 h in the absence and in the presence of 10 mM cephalosporin C or cephalothin. At the end of the incubation no activity could be detected in the samples containing the cephalosporins. The samples were then divided in two fractions. One set of fractions were dialysed against the Hepes/HCl buffer, and the second set against the same buffer containing 40 % w/v sucrose. The dialysis was performed at 4 °C and no recovery of activity was observed in the treated samples. Samples containing various final sucrose concentrations were obtained by mixing in appropriate proportions the samples dialysed against the buffer and those dialysed against the sucrose-containing buffer.

RESULTS

Measurements carried out on the native enzyme at 4 °C and 37 °C essentially yielded the same results. Consequently, the data given below should be regarded as temperature-independent at least in the 4–37 °C range. All the data were recorded and evaluated twice from measurements carried out with the usual Kratky camera equipped with a normal counter accessory, and with the compact camera and a position-sensitive detector. Essentially the same results were obtained in spite of the fact that the measuring time with the compact camera was about 50-fold smaller.

Radius of gyration R *and largest diameter* D

The radius of gyration R of a particle is defined as the root mean square of the distance of all the electrons from the center of the electronic mass. The R value of the G enzyme was calculated from the slopes of the innermost portions of the scattering curves (Fig. 1) using Guinier's approximation [19]. The Guinier plots did not show any protein concentration effect and yielded an R value of 1.82 ± 0.05 nm. Alternatively, an R value of 1.81 ± 0.05 nm was obtained on the basis of the distance distribution function $p(r)$ (calculated using the indirect Fourier transform algorithm developed by Glatter [13, 14]) (Fig. 2).

$$R^2 = \frac{\int_0^{D_{max}} p(r)\, r^2\, dr}{2\int_0^{D_{max}} p(r)\, dr}$$

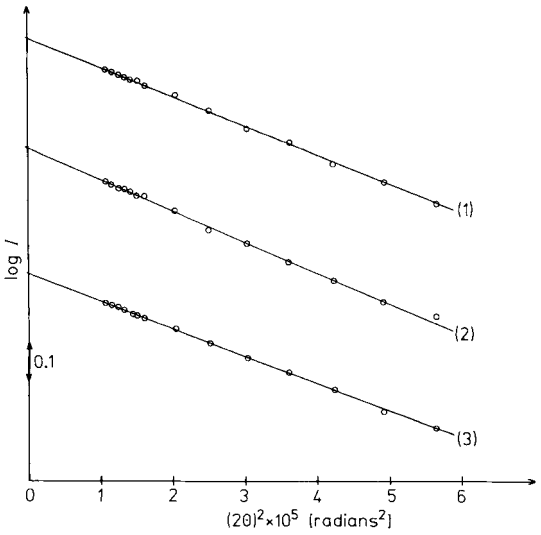

Fig. 1. *Guinier plots [log* I(h) *versus* h^2, $h = 4\pi\sin\theta/\lambda$; λ = *wavelength; 2θ = scattering angle] for concentrations of the G* DD*-peptidase of 19 mg/ml (1), 9.5 mg/ml (2) and 6.3 mg/ml (3).* The corresponding radii of gyration were 1.81 nm (1), 1.85 nm (2) and 1.79 nm (3)

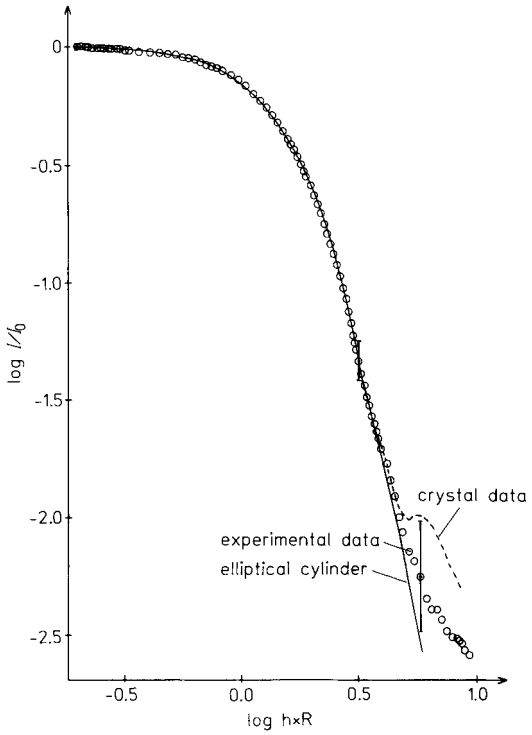

Fig. 2. *Comparison between the experimental and calculated scattering curves for the G* DD*-peptidase.* (○) Experimental data; (——) elliptical cylinder; (– – –) crystal data

where the upper integration limit, D_{max}, was varied from 6 nm to 9 nm. Since $p(r) = 0$ when $r = D$, the largest diameter of the particle, plotting $p(r)$ versus r, yielded a D value of 5.9 $\pm$ 0.2 nm.

Molecular weight M_r

Using a partial specific volume $\bar{V}_2$ of 0.713 $\pm$ 0.003 $cm^3\ g^{-1}$ (calculated according to [20] from the amino acid composition of the protein), and the procedure of Kratky et al. [21], a M_r value of 17000 $\pm$ 2000 was obtained. Taking into account the strong dependence of M_r on $\bar{V}_2$, this value compared satisfactorily with that of 22000 derived from the amino acid sequence [6].

Volume V *and degree of hydration* H

The V value was calculated according to [22] using the relation

$$V = 0.000291\ I_0/Q\ (nm^3)$$

where Q, the invariant, is defined by $Q = \int_0^\infty I(2\theta)^2\ d(2\theta)$, and I_0 corresponds to the intensity at zero angle. The integration was performed numerically in the 0 – 0.14-h range. The tail end of the scattering curve oscillated around K/h^4 and in the 0.14 h – ∞ range, the intogration was performed analytically after determination of the k constant value. This procedure led to a V value of 35 $\pm$ 2 nm^3. By comparing this value with the volume calculated for the unhydrated protein (on the basis of a molecular weight of 22000 and a partial specific volume of 0.713 $cm^3\ g^{-1}$), a degree of hydration of 0.25 $\pm$ 0.2 g water/g protein was estimated.

Shape

In small-angle scattering studies of proteins in solution the protein particle is usually assumed to be a rigid body inside which the electron density distribution is almost constant. By comparing the experimental scattering curves with those calculated for a series of simple triaxial bodies with homogeneous electron density distribution, a good fit was obtained with an elliptical cylinder exhibiting the following parameters: height = 4.53 nm; baseplate semiaxes = 1.13 nm and 2.26 nm. As shown in Fig. 2 this model fitted well the experimental scattering data, except for small deviations in the outermost portion of the curve, where the experimental errors were the most important ones and where a homogenous electron density was no longer a good approximation. In addition, the model agreed well with the R, D and V values determined as described above,

Comparison with single crystal data

The scattering curves were compared with the electron density data obtained on the crystal enzyme at 0.45 nm resolution [7]. One protein molecule was isolated and its electron density digitized using a three-dimensional grid. The spacing was 0.3 × 0.3 × 0.3 nm^3 and the function was set equal to zero (except when the electron density exceeded one-fourth of the maximum value found in the map). After transformation using a Fast Fourier algorithm [15], the data gave rise to the theoretical scattering curve shown in Fig. 2, and from this, to an R value of 1.78 nm. The two curves superimposed each other at least up to h values of about 0.25, under which conditions the intensity dropped to 1% of the initial value. An excellent

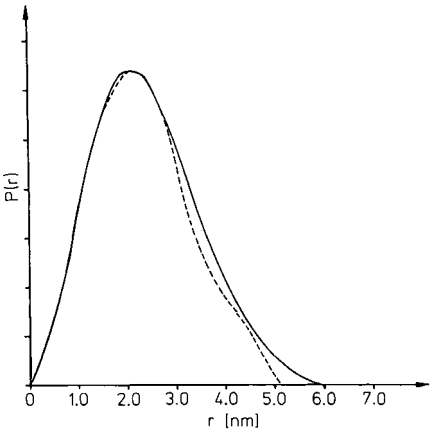

Fig. 3. *Comparison between distance distribution functions [*p(r)*] for the G* DD*-peptidase in solution (——) and in its crystalline state (– – –)*

agreement was also observed using larger scattering angles and the side maximum was apparent in both the 'solution curve' and the 'crystal data curve'. Finally, good fit was obtained between the distance distribution function of the enzyme in solution and that in the crystalline state (Fig. 3).

Effect of cephalothin and cephalosporin C

The penicillins and cephalosporins react with the G DD-peptidase according to: $E + I \overset{K}{\rightleftharpoons} E.I. \xrightarrow{k_2} E-I^* \xrightarrow{k_3} E + P$ where E = enzyme; I = antiobiotic; E – I* = inactive complex; P = degradation product; K = dissociation constant; k_2 and k_3 = first-order rate constants [1]. Although the k_3 values are generally small, the penicillins are very weak inhibitors of the G enzyme because of very large K and very small k_2 values (150 mM and $8 \times 10^{-4}\ s^{-1}$, respectively, for methoxyphenylpenicillin, which is the best penicillin inactivator so far tested). The cephalosporins are somewhat better inactivators mainly because of more favourable K values (1 – 10 mM) [10]. Kinetically, cephaloglycine and β-iodobenzyl-7-aminocephalosporanate behaved as competitive inhibitors. The crystal enzyme derivative formed with this latter compound showed satisfactory isomorphism [7, 8] and provided direct evidence that binding occurred at the active site. In contrast, cephalothin and cephalosporin C acted non-competitively [9] and, at 1.66 mM and 10 mM concentrations respectively, destroyed the crystal lattice. This unexpected behavior was re-examined by X-ray scattering.

After reaction with cephalothin as described in Materials and Methods, the measurements carried out at 4 °C yielded an R value of 2.58 nm and the intensity I_0 at zero angle was about 25% higher than that observed with the native enzyme at the same temperature. The invariant Q was not modified and comparative density measurements did not reveal any change in the partial specific volume. But, the distance distribution function (Fig. 4) showed marked increased values as the r values increased, yielding a D value of 8.6 nm. Cephalosporin C had even more drastic effects. At 4 °C, a stable state could not be reached and a faint precipitate occurred. At 37 °C, the scattering was stable (did not vary with time) and gave rise to an R value of 3 nm. In parallel to this, the intensity I_0 at zero angle was about 30% higher than that observed with the native enzyme at that temperature. At high r values, the $p(r)$ function had a particular pattern (Fig. 4) reminiscent of that found for the theoretical $p(r)$ curves calculated for dimeric particles [23].

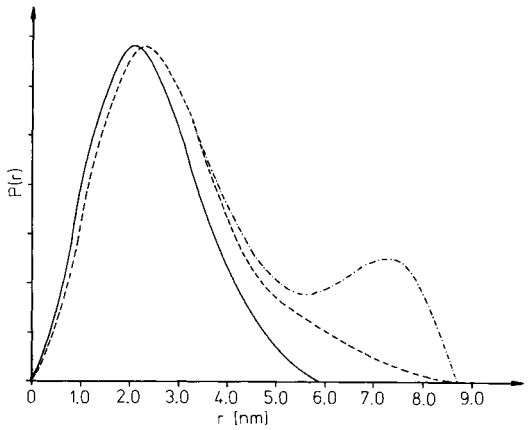

Fig. 4. *Distance distribution functions [p(r)] for the native (——), cephalothin-treated (– – –) and cephalosporin-C-treated (–·–·–) G DD-peptidase*

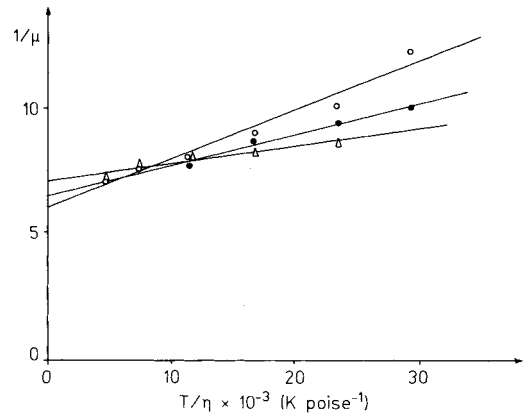

Fig. 5. *Perrin plots. Reciprocal of emission anisotropy (1/μ) versus* T/η *(η = viscosity; T = absolute temperature) for control (○), cephalothin-treated (●) and cephalosporin-C-treated (△) samples.* For experimental conditions, see Materials and Methods

The effects of the two cephalosporins under consideration were also investigated by fluorescence polarization. As shown in Fig. 5, the slopes of the Perrin plots for the cephalothin-treated and cephalosporin-C-treated enzyme samples (for conditions, see Materials and Methods) were 63% and 36%, respectively, of that of the control sample. Assuming that the fluorescence lifetime of tryptophan did not change during the interaction, these observations indicated that the hydrated volumes of the enzyme molecules treated with cephalothin and cephalosporin C were about 1.6 and 2.8-fold larger than that of the native protein. One should also note that a small precipitate occurred during dialysis of the cephalosporin-C-treated enzyme.

DISCUSSION

In spite of the inherent errors that occur in the outermost parts of the scattering curves, the possible errors due to the smoothing procedure that precedes the desmearing one, the limitations of the enzyme crystal data and, finally, the influence (which was neglected) of the solvent on the scattering behavior, the present work led to two main conclusions. First, the conformations of the native G enzyme in solution (Table 1) and in the crystal are, at least, very similar. (Note that the G enzyme crystals are enzymatically active [24].) Second, in parallel to the non-competitive type of inhibition that they exert on the enzyme activity and the disrupting effects that they exert on the protein crystal, both cephalothin and, to a still greater extent, cephalosporin C profoundly modify the physical state of the protein molecules in solution. At present, however, one can only make suggestions about the possible underlying mechanism(s).

Table 1. *Small-angle X-ray parameters for the native* Streptomyces albus *G DD-peptidase*

Parameter	Value
Radius of gyration (R)	1.82 ± 0.05 nm[a]
	1.81 ± 0.05 nm[b]
Molecular weight (M_r)	17.000 ± 2.000[c]
Volume (V)	35 ± 2 nm^3[d]
Hydration	0.25 ± 0.02 g water/g protein[d]
Maximum diameter (D)	5.9 ± 0.2 nm

[a] Calculated from Guinier plot.
[b] Calculated from distance distribution function.
[c] Using partial specific volume of 0.713 ± 0.003 cm^3 g^{-1}.
[d] Using molecular mass of 22000 from amino acid sequence.

It is hardly possible to visualize how a simple change in the protein conformation could cause a shift of the radius of gyration from 1.82 nm to 2.58 nm and even 3 nm. An ellipsoid of revolution having the volume of the native enzyme molecule but a radius of gyration of 3 nm would have semiaxes of about 1.1 nm and 6.5 nm, and thus a diameter of 13 nm, i.e. twice that of the native enzyme (5.9 nm; see above). Alternatively a view of the enzyme-cephalosporin complexes as open random coils is not compatible with the observed scattering behaviour. Indeed, the scattering function of a random coil shows an $I \approx h^{-2}$ dependence for values near the Guinier region and a transition to $I \approx h^{-4}$ [25] at increasing scattering angles. Such scattering behaviour was not observed.

Considering the high absolute concentrations (≈ 10 mM) of antibiotic used in the present study, unspecific binding of β-lactam molecules to the enzyme might explain, at least partly, the apparent increased volume and molecular weight. Binding of about 10 to 12 cephalothin molecules could explain the observed phenomenon and is not at variance with the Guinier-plots and the $p(r)$ function. In the case of cephalosporin C, however, the data also suggest very large conformational changes in the protein like partial unfolding and/or, more likely, aggregation of the enzyme molecules.

The work in Berlin was supported by the *Deutsche Forschungsgemeinschaft* and in Liège by the *Fonds de la Recherche Scientifique Médicale*, Brussels (grant 3.4501.79) and the Belgian Government (*Action concertée; convention no.* 79/84-I1).

REFERENCES

1. Ghuysen, J. M., Frère, J.-M., Leyh-Bouille, M., Dideberg, O., Lamotte-Brasseur, J., Perkins, H. R. & De Coen, J. L. (1981) in *Topics in Molecular Pharmacology* (Burgen, A. S. V. & Roberts, G. C. K., eds) pp. 63–97, Elsevier/North-Holland Biomedical Press.
2. Labischinski, H., Barnickel, G., Bradaczek, H. & Giesbrecht, P. (1979) *Eur. J. Biochem. 95*, 147–155.
3. Barnickel, G., Labischinski, H., Bradaczek, H. & Giesbrecht, P. (1979) *Eur. J. Biochem. 95*, 157–165.
4. Kelly, J. A., Moews, P. C., Knox, J. R., Frère, J.-M. & Ghuysen, J. M. (1982) *Science (Wash. DC) 218*, 479–480.

5. Kelly, J. A. (1983) in *The Murein Sacculus of Bacterial Cell Walls. Architecture and Growth. International FEMS Symposium. March 13–18, 1983* (Hakenbeck, R., Höltje, J. V. & Labischinski, H., eds) pp. 387–392, Walter de Gruyter and Co., Berlin, New York.
6. Joris, B., Van Beeumen, J., Casagrande, F., Gerday, Ch., Frère, J.-M. & Ghuysen, J. M. (1983) *Eur. J. Biochem. 130*, 53–69.
7. Dideberg, O., Charlier, P., Dupont, L., Vermeire, M., Frère, J.-M. & Ghuysen, J. M. (1980) *FEBS Lett. 117*, 212–214.
8. Dideberg, O., Charlier, P., Dive, G., Joris, B., Frère, J.-M. & Ghuysen, J. M. (1982) *Nature (Lond.) 299*, 469–470.
9. Charlier, P., Dideberg, O., Dive, G., Dusart, J., Frère, J.-M., Ghuysen, J. M., Joris, B., Lamotte-Brasseur, J., Leyh-Bouille, M. & Nguyen-Distèche, M. (1983) in *The Murein Sacculus of Bacterial Cell Walls. Architecture and Growth. International FEMS Symposium. March 13–18, 1983* (Hakenbeck, R., Höltje, J. V. & Labischinski, H., eds) pp. 369–386, Walter de Gruyter and Co., Berlin, New York.
10. Duez, C., Frère, J.-M., Klein, D., Noël, M., Ghuysen, J. M., Delcambe, L. & Dierickx, L. (1981) *Biochem. J. 193*, 75–82.
11. Kratky, O., Pilz, I. R. & Schmitz, P. J. (1966) *J. Colloid. Sci. 21*, 24–34.
12. Zipper, P. (1972) *Acta Phys. Austriaca, 36*, 27–28.
13. Glatter, O. (1974) *J. Appl. Crystallogr. 7*, 147–153.
14. Glatter, O. (1977) *J. Appl. Crystallogr. 10*, 415–421.
15. Labischinski, H. & Bradaczek, H. (1977) *J. Appl. Crystallogr. 10*, 363–364.
16. Kratky, O., Leopold, H. & Stabinger, H. (1969) *Angew. Phys. 27*, 273–277.
17. Fredericq, E. & Houssier, C. (1972) *Biopolymers, 11*, 2281–2308.
18. Wahl, P. & Weber, G. (1967) *J. Mol. Biol. 30*, 371–384.
19. Guinier, C. R. (1937) *C. R. Hebd. Séances Acad. Sci. 204*, 1115.
20. Schackman, K. H. (1957) *Methods Enzymol. 4*, 32–103.
21. Kratky, O., Porod, G. & Kahovec, I. (1959) *Elektrochemie, 55*, 53–59.
22. Porod, G. (1952) *Kolloid Z. 125*, 51–122.
23. Glatter, O. (1979) *J. Appl. Crystallogr. 12*, 166–175.
24. Dideberg, O., Frère, J.-M. & Ghuysen, J. M. (1979) *J. Mol. Biol. 129*, 677–679.
25. Kratky, O. (1962) *Kolloid Z. Z. Polym. 182*, 7–28.

H. Labischinski and P. Giesbrecht, Robert-Koch-Institut des Bundesgesundheitsamtes, Nordufer 20, D-1000 Berlin (West) 65

E. Fischer, G. Barnickel, and H. Bradaczek, Institut für Kristallographie der Freien Universität Berlin, Takustraße 6, D-1000 Berlin (West) 33

J.-M. Frère, P. Charlier, and J.-M. Ghuysen, Laboratoire de Microbiologie, Faculté de Médicine de l'Université de Liège, Institut de Chimie Organique et de Biochimie, Université de Liège au Sart-Tilman, 4000 Liège, Belgium

C. Houssier, Laboratoire de Chimie Physique, Institut de Chimie, Université de Liège au Sart-Tilman, B-4000 Liège, Belgium

O. Dideberg, Laboratoire de Cristallographie, Institut de Physique, Université de Liège au Sart-Tilman, B-4000 Liège, Belgium