On the Relationships between Molecular Conformation, Affinity towards Penicillin-Binding Proteins, and Biological Activity of Penicillin G-Sulfoxide

Frank Beise, Harald Labischinski, and Hans Bradaczek

Robert Koch-Institute of the Federal Health Organization, A V.2, Nordufer 20, D-1000 Berlin 65, Bundesrepublik Deutschland
Institute of Crystallography, Free University of Berlin, Takustraße 6, D-1000 Berlin 33, Bundesrepublik Deutschland

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The binding capacity of penicillin G-sulfoxide towards the penicillin-binding proteins (PBP) of *Staphylococcus aureus* H was studied. The sulfoxide and its parent compound, penicillin G, differ only in two aspects, the sulfur-bound oxygen and an altered conformation of the five-membered thiazolidine-ring system. These minor alterations of the penicillin structure resulted in a drastical decrease of binding activity (about two orders of magnitude) of the sulfoxide derivative towards its target enzymes. Furthermore, the sulfoxide did not exhibit the selectivity of subinhibitory doses for PBP 3, as could be observed for penicillin G.

The biological consequences of this behaviour were monitored *via* growth curves, uptake of cell wall label, and analysis of the cell wall. Binding studies revealed that comparable growth inhibition and impairment of cell wall label uptake were achieved by at least a 100-fold higher penicillin G-sulfoxide concentration, compared to its parent compound.

In cell wall analysis, the application of high doses of the antibiotics, *i.e.* nearly saturated PBP, verified the above mentioned observation. Surprisingly, small but significant differences in cell wall composition occurred using subinhibitory doses, probably due to the altered affinity towards PBP 3, supporting the hypothesis of an important role of this PBP in peptidoglycan transpeptidation.

Introduction

It is now well known that an important prerequisite for the antibacterial activity of β-lactam antibiotics is the binding to and the acylation of membrane-associated bacterial proteins, which are involved in final steps of the assembly of the bacterial cell wall (penicillin-binding proteins; for review see [1, 2]).

However, in spite of considerable progress in determining the molecular structure of the active site of some penicillin-sensitive enzymes ([3–6], *cf.* also [7]), relatively few details are known about the steric requirements for β-lactam antibiotics promoting their ability to inactivate their target enzymes. In order to enlighten this situation, the study of the interaction of PBPs with β-lactam antibiotics which differ chemically and/or conformationally only in minor and well defined aspects but exhibit large variations in biological activity seems to be one of several possible strategies.

Penicillin G (Pen G) and its corresponding 1-β-sulfoxide (PSO) represent nearly ideal candidates for such purpose:

i) they differ chemically only with respect to oxidation of the sulfur atom,

ii) the conformational differences between both compounds are well known from a recent comparative X-ray diffraction study. They concern mainly the thiazolidine ring conformation, which was found to occupy the 3-α-COOH-equatorial ring puckering in case of the sulfoxide, but the 3-α-COOH-axial type for its parent compound [8], similar as has been observed for cloxacillin sulfoxide and penicillin V-sulfoxide in respect to their parent compounds [9, 10],

iii) as other β-lactam sulfoxides carrying the penam nucleus (but, interestingly, in contrast to β-lactams equipped with a cephem nucleus [11]) the penicillin G-sulfoxide exhibits a drastically reduced biological activity compared to its parent compound.

Abbreviations: PAGE, polyacrylamide gel-electrophoresis; PBP, penicillin-binding protein; Pen G, penicillin G; PMSF, phenylmethanesulfonylfluoride; PPO, 2,5-diphenyloxazole; PSO, penicillin G-sulfoxide; SDS sodium-dodecylsulfate; TEMED, tetramethyl-ethylenediamine; Tris, tris-hydroxymethylaminomethane.

Reprint requests to F. Beise.

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Based on their X-ray study on the conformation of Pen G and PSO mentioned above, the authors claimed that the low activity of the sulfoxide might to be due to a reduced binding capacity towards its target enzymes and/or missing capability of the drug to persist in the inactivated enzyme-drug complex, not as a consequence of a reduced chemical activity but caused by conformational factors.

We, therefore, compared the effects of various doses of both Pen G and PSO on growth, cell-wall synthesis, and wall composition, as well as PBP-saturation of *Staphylococcus aureus*.

Materials and Methods

Chemicals

Bacto-peptone and yeast extract were from Difco (Detroit, Mich.), Sodium-dodecylsulfate, Tris-base, DMSO, and PMSF were purchased from Fluka (Buchs, Switzerland). Penicillin G, acrylamide, N,N′-methylenebisacrylamide, TEMED, Coomassie brilliant blue R250, DNase, trypsin, and Triton X-100 were obtained from Serva (Heidelberg, F.R.G.); PPO and lysostaphin were from Sigma (St. Louis, U.S.A.), Dimilume was from Packard (Illinois, U.S.A.), [^{3}H]benzylpenicillin (999 GBq·mmol^{-1}) and [^{14}C]N-acetylglucosamine (1.924 GBq·mmol^{-1}) were purchased from Amersham-Buchler (Braunschweig, F.R.G.).

Penicillin G-sulfoxide was a kind gift of W. Gruszecki, Berlin, the *Chalaropsis* B enzyme was a kind gift of J. Gmeiner, Darmstadt.

Bacterial strain and growth conditions

The organism used was the penicillin-sensitive and β-lactamase-free *Staphylococcus aureus* H. It was grown either in PYK-broth (0.5% bacto-peptone, 0.5% yeast extract, 0.3% K_2HPO_4, supplemented with 0.2% glucose) or 2.5% bacto-peptone (pH 7.2) containing 0.5% NaCl.

To the pre-warmed medium a bacterial suspension of an overnight culture (5×10^9 cfu/ml) was added, to give a concentration of about 1.5×10^8 cfu/ml. The bacteria were allowed to grow at 37 °C under vigorous shaking with aeration, until the selected optical density (A_{578}) was reached.

Preparation of membrane proteins

Bacteria were grown until late log-phase ($A_{578} = 1.0$), cooled down in an ice-bath and harvested by centrifugation (10 min, 4 °C, 12,000 × *g*). The cells were resuspended in 50 mM Tris/HCl, pH 7.4, containing 1 mM PMSF, followed by a lysostaphin treatment with 100 mg·l^{-1} lysostaphin and 20 mg·l^{-1} DNase for 30 min at 37 °C to degrade the cell walls. Membranes were pelleted by centrifugation in a Beckman L2-65B ultracentrifuge (Beckman, Stanford, U.S.A.) with a type 65 rotor (3 h, 4 °C, 220,000 × *g*), washed once by resuspension with a Braun-Sonic 300 S sonicator (Braun, Melsungen, F.R.G.) in Tris-buffer, followed by ultracentrifugation for 90 min (140,000 × *g*, 4 °C). The pellet was resuspended in a buffer consisting of 20 mM Tris/HCl, pH 7.4, 20 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM PMSF, 0.2% Triton X-100, and 10% glycerol (solubilization buffer) and brought to a concentration of 10 mg·ml^{-1} total protein (according to Lowry *et al.* [12]). The membranes were stored frozen at −25 °C.

The competition test was performed by the incubation of a suspension with 130 µg total protein with the appropriate antibiotic amount at 37 °C for 15 min and subsequent labelling as described below.

Preparation of membrane proteins from antibiotic-treated staphylococci

Bacteria ($A_{578} = 0.5$) were pipetted into centrifugation tubes containing the antibiotic solution; the amount needed for one PBP-assay was 2.5 ml per concentration. After 30 min of incubation at 37 °C a mixture of lysostaphin and DNase (final concentration 20 mg·l^{-1} and 5 mg·l^{-1}, respectively) was added and after 3–5 min of further incubation the now weakly opaque solutions were cooled down in an ice-bath and subjected to ultracentrifugation (90 min, 4 °C, 140,000 × *g*). The pellets were resuspended in solubilization buffer (see above; 25 µl per assay) and either labelled and prepared for electrophoresis at once or after storage at −25 °C.

Labelling and detection of PBPs

To the membrane preparation 1 µCi [^{3}H]benzylpenicillin was added. After 15 min of incubation at 37 °C the reaction was terminated by the addition of 50 µl of 0.1 M Pen G. The proteins were precipitated by a 4-fold excess of ice-cold acetone, and centrifuged in a Kontron-Hermle ZK 400 centrifuge (Kontron, Munich, F.R.G.) with an A8.24 fixed angle rotor (with adaptors for small tubes) for 30 min

at 15,000 rpm and 4 °C. The pellets were then prepared for SDS-PAGE. The apparatus used was a LKB-multiphor with a LKB 2103 power supply (LKB, Bromma, Sweden). We used the discontinuous gel system of Laemmli and Favre [13], employing a 3.5% stacking gel and a 5–15% linear gradient resolving gel of about 19 cm in length. Electrophoresis was performed at 4 °C with a constant current of 10 mA for 16–18 h.

After staining the gels with Coomassie brilliant blue R-250 and destaining, they were impregnated with a scintillator (PPO) and the PBPs detected by fluorography with a pre-sensitized Kodak-X-Omat XAR-5 X-ray film (Kodak, Rochester, U.S.A.) in a Siemens exposure-cassette (Siemens, Berlin, F.R.G.) according to the method of Bonner and Laskey [14]. The exposure-time was 7–10 days at −75 °C. For quantification the fluorograms were scanned with a Joyce-Loebl microdensitometer MK III CS (Joyce, Loebl Co., Gateshead, G.B.).

Uptake of cell wall marker

Growth was initiated with a (3% vol./vol.) inoculum from an overnight culture (5×10^9 cfu/ml). Bacteria were grown to late log-phase ($A_{578} = 1.0$) and then added to pre-warmed broth to yield a concentration of 3% (vol./vol.; 3×10^7 cfu/ml). At an optical density of approximately $A_{578} = 0.5$ the culture was divided into several 25 ml portions. To each one, the selected amount of the antibiotic was added together with the cell wall marker [^{14}C]N-acetylglucosamine (3500–4000 dpm/ml), which was shown to be incorporated nearly quantitatively into the staphylococcal cell wall [15, 16]. The optical density was measured as well as the uptake of the label. The uptake was expressed as the difference between the total label of the cell suspension and the one of the cell-free supernatant, divided by the total label. After addition of 3 ml Dimilume scintillation cocktail the β-radiation of 500 µl of the supernatant was measured using a LKB model 2604 Rac β-counter (LKB, Bromma, Sweden).

Preparation of labelled cell walls

Bacteria with an A_{578} of approx. 0.5 (as described above) were treated with the chosen amount of Pen G or its corresponding sulfoxide. Furthermore the cell wall marker [^{14}C]N-acetylglucosamine was added to label the new-built cell wall material. Due to the fact that increasing antibiotic amounts decreased the uptake of cell wall marker the concentration ranged from 0.2 µCi up to 0.8 µCi per assay.

After 1 h of further growth, when maximum incorporation occurred, cells (cooled down rapidly) were harvested by centrifugation (10 min in an A6.14 rotor, 4 °C, $12{,}000 \times g$). Resuspended cells were broken with glass beads of 0.1–0.11 mm in diameter using a Dyno-Mill KDL laboratory mill (Bachofen, Bruck, Switzerland) for 3–5 min at 4 °C. The mixture was poured into a 4% SDS solution, the glass beads were removed by suction filtration and the filtrate washed several times with 1% SDS. To remove the cytoplasmic membrane and to denature degrading enzymes the filtrate was heated for 30 min at 60 °C. After several washings of the labelled wall material, wall-linked proteins (*e.g.* protein A) were removed by a trypsin treatment (incubation of the suspended cell wall material in 150 mM Tris/HCl, pH 7.4, containing 0.2 mg per ml trypsin at 37 °C for 24 h). Cell wall material was washed (150 mM Tris/HCl, pH 7.4, 150 mM Tris/HCl, pH 7.4 + 2 M NaCl, 150 mM Tris/HCl, pH 7.4, 3× water) and the purified wall material was lyophilized.

Fragmentation of the cell walls and chromatography

About 5 mg cell wall material were suspended in 1 ml 0.1 M ammonium acetate buffer, pH 5.4, and incubated with 1 µg *Chalaropsis* B muramidase (which cuts the sugar backbone of the peptidoglycan, but leaves peptide cross-links intact), overnight at 37 °C. The resulting fragments were separated by gel filtration on a LKB AcA 202 column (95 cm in length, 16 mm in diameter; LKB, Bromma, Sweden). They were eluted with 50 mM ammoniumacetate, pH 7, at a flow rate of 9.75 ml per hour. Fractions were collected in 15 min intervals, and aliquots of each were counted.

For better comparison of the results, the fraction numbers were changed into K_D-values (see [17]).

The degree of cross-linkage Q was calculated using the following equation:

$Q(\%) = 0.93\ N_{Oligomer} + 0.75\ N_{Tetramer} + 0.67\ N_{Trimer} + 0.5\ N_{Dimer}$ were N_x is the percentage of the activity of the actual peak in relation to the total amount of radioactivity [18].

Results

In order to test the interaction of Pen G and PSO with the PBPs of *S. aureus*, proliferating bacteria

were treated with various drug doses for 30 min. Determination of the amount of antibiotic bound at the different PBPs was accomplished by labelling the free binding sites with radioactive penicillin G, which was subsequently detected by fluorography of the electrophoresis gel as described in detail in Material and Methods. Fig. 1 shows the densitograms of the fluorograms obtained from bacteria pre-treated with penicillin G (Fig. 1a) and with its corresponding sulfoxide (Fig. 1b). This kind of representation allows an easy quantitative comparison of the effects of the various doses on the binding of the drugs, since the peak areas for the PBPs directly represent the residual binding capacity of each PBP after pre-treatment with either Pen G or PSO.

Fig. 1a shows the effects measured for Pen G treatment. Even at the lowest concentration investigated (10^{-8} M) a severe decrease in the amount of free PBP 3 was found with no significant changes in the other ones. However, already at 10^{-7} M Pen G only minor residual activities for both PBP 1 and 2 could be detected, *i.e.* nearly all of the PBPs were saturated under this condition. On the other hand only concentrations higher than 10^{-4} M caused complete saturation.

In Fig. 1b the corresponding result for PSO is shown. Obviously, much higher concentrations of PSO were required to saturate the PBPs. To achieve binding, PSO concentrations of at least 10^{-6} M were needed (*i.e.* concentrations two orders of magnitude higher than in case of Pen G). Increasing concentrations of PSO caused a steady increase in PSO-binding for all of the PBPs.

To insure that the observed effects were not due to the use of living cells (*e.g.* drug permeation problems to reach the target enzymes etc.), corresponding *in vitro* experiments were also performed. However, we found no significant differences in the case of PSO while Pen G needed much higher concentrations to achieve the same *in vitro* effects as the *in vivo* experiments. This latter result was possibly due to the low amount of antibiotic present, *i.e.* the number of penicillin molecules per PBP was too small, even if the (overall) concentration might have been sufficient (*cf.* [19], p. 359).

Growth curves and uptake of cell wall label

The inhibitory effect of the two β-lactams Pen G and PSO was monitored by the measurement of the

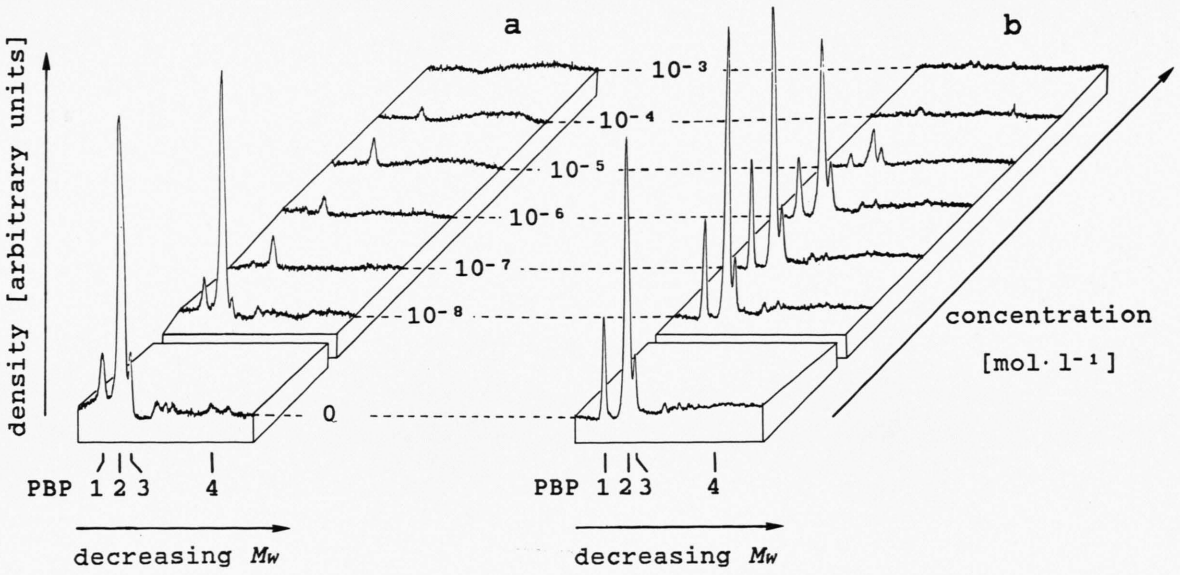


Fig. 1. Densitograms of PBP-patterns of *S. aureus* H pretreated with various concentrations of a) non-radioactive penicillin G and b) non-radioactive penicillin G-sulfoxide. Free binding sites of the PBPs were subsequently labelled with radioactive penicillin G. The figure shows the optical density (in arbitrary units) of the fluorograms *versus* the direction of migration of the PBPs on the SDS-gel. Note that a decrease in peak area for each PBP correspondends to an increase in binding of the non-radioactive drug applied.

Table I. Turnover induced effects on the binding capacity of PSO-pretreated PBP[a].

	Incubation time				
	5	7.5	10	15	30 min
PBP 1	43%	28%	40%	42%	43%
PBP 2	30%	31%	36%	40%	45%
PBP 3	8%	14%	24%	22%	43%

[a] Membrane preparations of bacteria grown in the presence of 5.5×10^{-5} M PSO were allowed to stay at 37 °C before the usual labelling procedure. For each PBP the residual binding sites available to radioactive Pen G are given in percent of the untreated control. An increase in % binding with time would indicate PSO-turnover.

optical density (A_{578}). The results are shown in Fig. 2a and b. In the case of Pen G, the first inhibitory effect could be detected at 10^{-7} M. Higher concentrations resulted in increasing growth inhibition.

Comparable effects could be achieved with PSO only at concentrations two orders of magnitude higher than with Pen G, in good accordance with the MIC values (0.01 $\mu g \cdot ml^{-1}$, *i.e.* 3×10^{-8} M, and 3 $\mu g \cdot ml^{-1}$, *i.e.* 10^{-5} for Pen G and PSO, respectively).

The uptake of [^{14}C]N-acetylglucosamine was used for quantitation of cell wall synthesis, for it is predominately linked to the staphylococcal cell wall [2, 15]. Concentrations in the range of the MIC's of either β-lactam antibiotic already reduced the uptake of the wall label, and higher doses (10 to 100 × MIC) drastically inhibited incorporation of radioactive wall precursors (data not shown).

Thus, both, growth as well as the label uptake, showed a parallel behaviour under the influence of both compounds PSO and Pen G, but needing an about 100-fold higher concentration for PSO.

Fragmentation of the cell wall

The PBP-labelling experiments indicated that the specificity of Pen G and PSO towards the individual staphylococcal PBPs differed slightly from each other. Although the detailed functions of the staphylococcal PBPs are only partly understood [17, 20], it can be assumed that differential inhibition of the PBPs might be reflected in different chemical qualities of the cell wall material, especially in respect to the degree of peptide cross-linking. This was studied by muramidase fragmentation of the isolated and

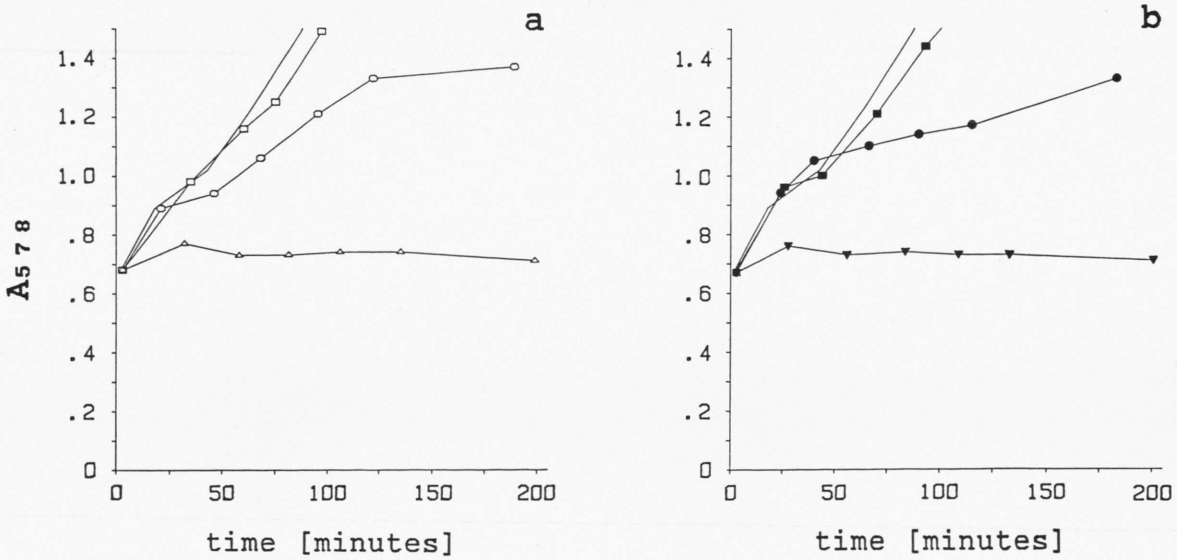
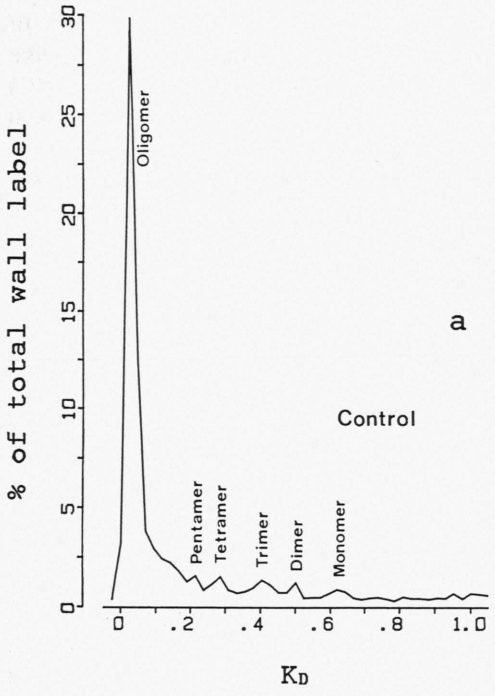
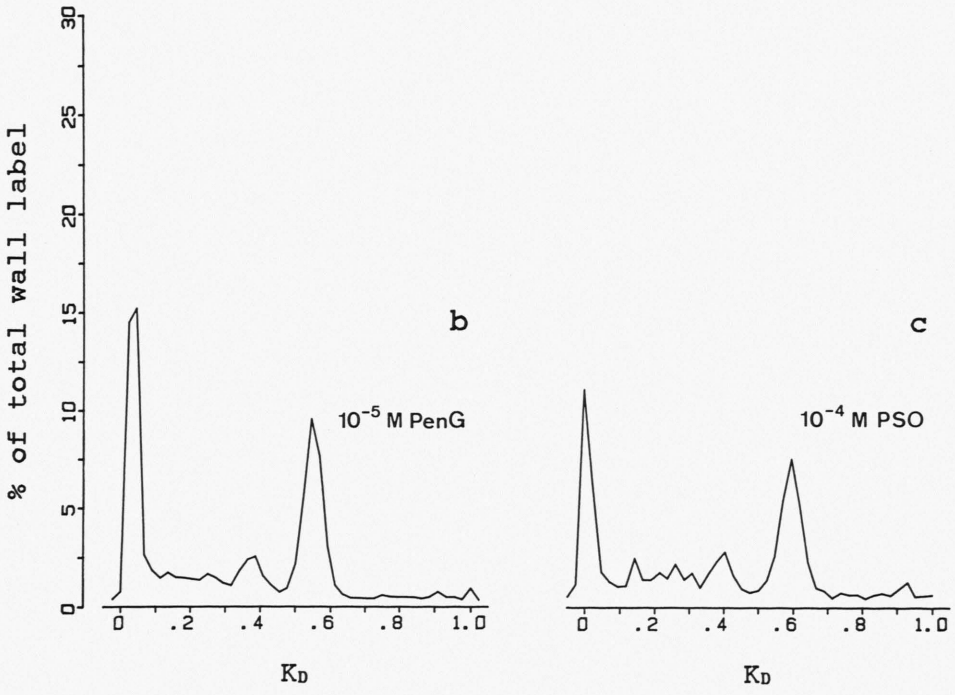


Fig. 2. Growth curves of *S. aureus* H in peptone broth (t = 0: time of drug application; A_{578} = optical density at 578 nm). a) Pen G treatment: −:Control, □——□: 10^{-8} M, ○——○: 10^{-7} M, △——△: 10^{-5} M. 10^{-8} M Pen G had only a minor effect. At 10^{-7} M a clear inhibitory effect was observed. Higher concentrations caused even stronger effects (10^{-6} M Pen G has been omitted for reason of better readability). b) PSO treatment: −: Control, ■——■: 10^{-6} M, ●——●: 10^{-5} M, ▼——▼: 10^{-4} M. The same course of events took place under the influence of PSO, but at concentrations two orders of magnitude higher.



labelled wall material. The resulting soluble products (*i.e.* monomers, dimers, trimers etc. of the disaccharide-peptide subunits of peptidoglycan) were separated by gel chromatography as described in Materials and Methods. A typical fragmentation pattern of cell walls of untreated *S. aureus* is shown in Fig. 3a. According to its high degree of cross-linking (>75%), a large and dominating oligomer peak could be observed, accompanied by some smaller peaks of the smaller fragments (< four disaccharide-oligopeptide units). The amount of monomer was nearly negligible.

When the bacteria were treated with high concentrations of the antibiotics (10^{-5} M Pen G or 10^{-4} M PSO; Fig. 3b and c) prior to isolation of the walls, the amount of the smaller fragments especially of the monomer portion, increased drastically, while the oligomer portion showed a strong decline. Thus, at concentrations well above their MIC-values, both antibiotics led to similar effects. Interestingly, there was a different behaviour when subinhibitory doses of Pen G or PSO were administered. The treatment of the staphylococci with 10^{-6} M PSO led to no detectable differences in the wall fragmentation pattern as compared to control cells (Fig. 3e). The degree of



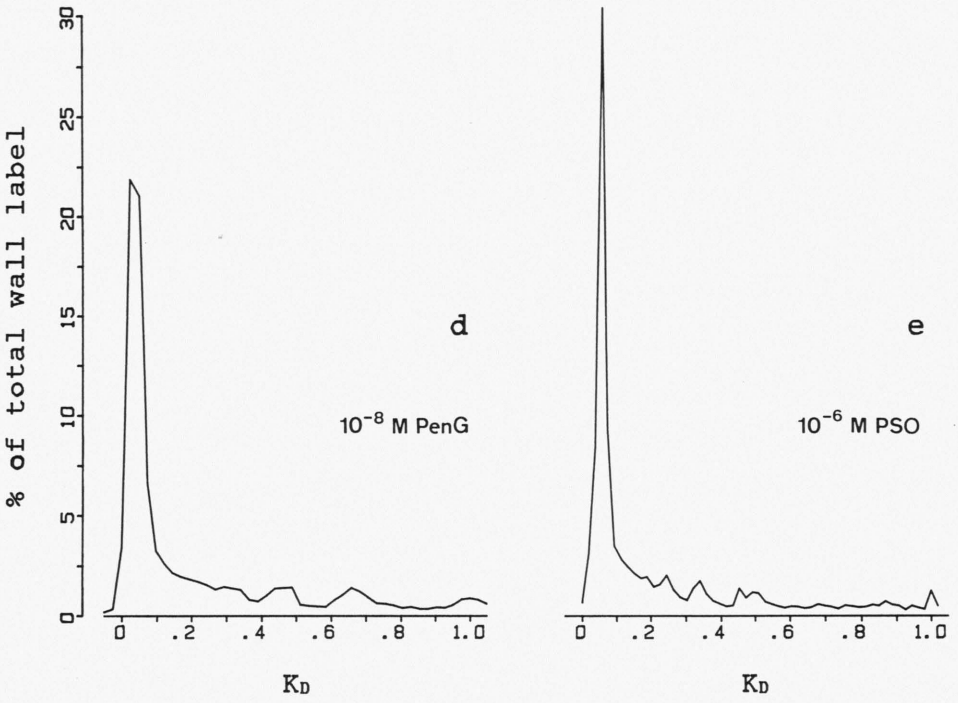


Fig. 3. Cell wall fragmentation pattern of staphylococci. a) Log-phase cells. Most of the cell wall label ([^{14}C]N-acetyl-glucosamine), was concentrated in the oligomer peak. Smaller fragments occurred only in minor quantities. b) Treated with 10^{-5} M Pen G. A large portion of the activity was found in the monomer fraction, reflecting a dramatic decrease in cross-linking as compared to the control values (*cf.* Fig. 3a). c) Treated with 10^{-4} M PSO. The result was essentially equivalent to that observed for high Pen G concentrations (Fig. 3b). d) Treated with 10^{-8} M Pen G. The oligomer peak decreased in intensity and showed a distinct broadening towards smaller fragment sizes. The low molecular weight fragments (especially the monomer) were significantly increased. In contrast to the situation in Fig. 3e, significant changes as compared to the control (Fig. 3a) were observed. e) Treated with 10^{-6} M PSO. No significant differences to control cell walls could be detected.

cross-linkage was as high as in untreated cells. On the other hand the corresponding subinhibitory concentration of 10^{-8} M Pen G led to a significant alteration in the cell wall composition (Fig. 3d): the degree of cross-linkage decreased below 70% and the fragmentation pattern revealed a shift towards smaller fragments. Additionally, the broadening of the oligomer peak indicated an increase of oligomer fragments of slightly reduced molecular weight.

Discussion

The present study has been undertaken in order to check, whether or not the well known low biological activity of β-lactam-1β-sulfoxides as compared to their parent compounds might be due to a drastically reduced conformational adaptability to the binding sites of the penicillin-binding proteins (PBPs) as recently suggested by a X-ray crystallographic investigation [8]. In that investigation the authors proposed that the binding of the sulfoxide to the PBPs should be diminished or the turnover of the sulfoxide-PBP complex could be rather high, due to the steric position of the sulfur-bound oxygen or to the 3-α-COOH-equatorial thiazolidine-ring conformation. In our test system using *Staphylococcus aureus* H, the reduced biological activity of penicillin G-sulfoxide (PSO) was reflected by a more than 100-fold higher MIC-value (3 μg/ml) as compared to its parent compound penicillin G (Pen G) (0.01 μg/ml).

First of all, our data clearly show that the reduced activity of PSO was not due to factors preventing the sulfoxide to reach its target, namely the membrane-bound PBPs (*e.g.* for permeation reasons or because of β-lactamase activity), since the same concentration of PSO was needed to provoke the same level of

PBP binding in our *in vivo* tests as in the *in vitro* system. Secondly our results demonstrate that the very reason for the low activity of PSO was its low affinity to the PBPs of *S. aureus* H. This is shown by the fact that in all experiments an approximately 100-fold increase in concentration of PSO was needed to evoke qualitatively and quantitatively similar effects as observed for Pen G, in nice correlation to the corresponding MIC-values. This applies not only to the drug concentration needed to saturate the PBPs, but also to those required to impair i) growth, measured by optical density, ii) cell wall precursor uptake by the cells, and iii) chemical quality of the wall material as measured *via* cell wall fragmentation analysis and determination of the degree of cross-linking of the peptidoglycan. Obviously, these data strongly support the concept of diminished adaptability of the sulfoxide to interact properly with the PBPs, at least in the case of *S. aureus* H under the conditions applied. Furthermore, our data allow the tentative interpretation that the diminished interaction between PSO and PBPs should be due rather to a reduced binding capacity than to an enhanced turnover of the drug by the PBPs enzymatic activity. This can be deduced from the following observations: i) there was no detectable difference in the binding of PSO under *in vivo* and *in vitro* conditions, ii) if the isolated membranes from bacteria pretreated *in vivo* with high doses of PSO were allowed to stay for up to 30 min at 37 °C before postlabelling with radioactive penicillin G, the corresponding fluorograms were almost identical to those obtained without this time lag, only PBP 3 exerted some release of the bound drug (*cf.* Table I).

Although in general all PBPs needed about a 100-fold increase in PSO concentration relative to Pen G for an equivalent saturation level, there was, interestingly, some difference in the relative affinity of both drugs *versus* the individual PBPs. While PSO was bound by all PBPs with more or less similar affinity, Pen G showed a preference for PBP 3. This led to the interesting effect that under subinhibitory doses (*i.e.* only partial binding to the PBPs) in the case of PSO (effecting the PBPs almost equally) no influence on the degree of cross-linking could be detected at all (*cf.* Fig. 3e), while in the case of Pen G (binding especially to PBP 3), a small but significant decrease in cross-linking was observed (*cf.* Fig. 3d). This could be interpreted in the way that PBP 3 possesses an important transpeptidase activity [21], although it seems not to be an essential one, because even though the binding capacity of this protein at 10^{-8} M Pen G was almost diminished, the bacteria were still able to grow. This is in accordance with the well known suggestion that another hmw PBP may fill the gap of an inactivated one (see *e.g.* [2, 22, 23]). Indeed, recent findings with selectively acting β-lactam antibiotics indicated that only the inhibition of two hmw PBPs was able to induce bacteriolysis [24]. In contrast to the non-essential role of PBP 1 stated so far [22], it was found that the binding to PBP 1 was rather a prerequisite for the induction of lysis [24, 25]. This is reflected by the experiments discussed here: Either Pen G or PSO only induced bacteriolysis when PBP 1 plus at least one further hmw PBP was saturated.

In conclusion our data strongly suggest that the diminished biological activity of penicillin sulfoxides as compared to their parent compounds could be due to a drastically reduced binding affinity towards penicillin-binding proteins for conformational reasons. Of course, our results do not allow to differentiate between the relative contribution of the sulfoxide-oxygen atom and the thiazolidine ring puckering to this phenomenon. This goal could, however, possibly be achieved by similar tests as described in this paper using β-lactam antibiotics with a 3-α-COOH-equatorial thiazolidine ring conformation but without a sulfoxide group. Such tests would be highly desirable since quite different hypotheses had been put forward from theoretical calculations and model-building studies, according to which the 3-α-COOH-equatorial ring puckering is either assumed to be a prerequisite for biological activity [24] or, directly opposed to that, a factor which abolishes this activity nearly completely [25].

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