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GRAMICIDIN S-SYNTHETASE

ELECTROPHORETIC CHARACTERIZATION OF THE MULTIENZYME

HANS KOISCHWITZ and HORST KLEINKAUF

Max-Volmer-Institut für Physikalische Chemie und Molekularbiologie, Abteilung Biochemie, Technische Universität Berlin (Germany)

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Summary

1. A method characterizing the fully active gramicidin S-synthetase (EC. 6.3.2.—) multienzyme in protein mixtures by a combination of sedimentation and polyacrylamide gel electrophoretic mobility data has been described.

2. The molecular weight of 280 000 has been reevaluated by gradient centrifugation, gel filtration, and polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate. The size of the multienzyme is not changed by sodium dodecyl sulfate treatment.

3. In polyacrylamide gel electrophoresis dimerisation occurs in Tris, while two bands, which may represent monomer and dimer, are observed in phosphate.

4. Reliability of molecular weight determinations of sodium dodecyl sulfate-protein complexes of sizes up to 300 000 daltons has been determined, correlating either mobilities or retardation coefficients.

Introduction

The multienzyme of gramicidin S-synthetase (EC 6.3.2.—) catalyses activation of the amino acids proline, valine, ornithine, and leucine as aminoacyl adenylates, which are transferred to thiols. By interaction with phenylalanine racemase (EC 5.1.1.11.) peptide synthesis is initiated with the formation of D-phenylalanyl-proline, which remains covalently linked on the multienzyme. Subsequent peptidyl-transfers to 4′-phosphopantetheine and from the cofactor to the specific amino acid give rise to pentapeptides enzyme-S-Leu-Orn-Val-Pro-D-Phe which combine antiparallel to the symmetric decapeptide gramicidin S.

It has been pointed out that peptide formation is a relatively unstable function of the enzyme, as compared to amino acid activation [7]. The reason for this inactivation may be partial proteolysis or specific cleavage of 4′-phospho-

pantetheine by a peptidyl carrier protein hydrolase. We have observed that inhomogeneous preparations may contain forms of the enzyme with altered substrate specificity, or fragments down to 70 000 daltons, with no peptide formation activity (Ref. 4, and unpublished results). To obtain information on the structure of the fully active enzyme, we analysed the protein composition of inhomogeneous preparations by a combination of the sedimentation profile with polyacrylamide gel electrophoresis or sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Only one component catalysed the synthesis of gramicidin S, while others may carry out partial reactions, or influence partial reactions by protein-protein interactions (unpublished results). The active multienzyme dimerized during polyacrylamide gel electrophoresis, and was stable against sodium dodecyl sulfate-treatment.

Methods and Materials

Enzyme purification

For protein maps, enzymes were prepared according to Kleinkauf et al. [2] with some modifications [4]. DEAE-cellulose purified enzymes were concentrated by precipitation with twice the volume saturated ammonium sulfate solution (pH 7.5, 0.1 M EDTA), dialysed, and centrifuged in a linear glycerol gradient (10—30%, v/v) for 24 h at 0°C in a SW 27-rotor at 27 000 rev./min; usually 1 ml protein solution was applied onto 32 ml of gradient solution. Unless otherwise indicated, pure enzyme was prepared according to the previously described procedure [1].

Polyacrylamide gel electrophoresis

Protein mapping. Gels contained 8% acrylamide and 0.45% bis, and were run in 0.1 M Tris · HCl, pH 9.1, at 4°C; the buffer contained 1 mM dithiothreitol. 100 µl of sample were applied in 20 mM triethanolamine, 10 mM $MgCl_2$, 0.25 mM EDTA, 1 mM dithiothreitol, pH 7.5; the buffer contained 20% glycerol, and 5 µl bromphenol blue (0.02%) were added. Electrophoresis was performed at 1—3.5 mA/tube, staining was done in a colloidal solution of Coomassie brilliant blue R 250 [5], for 30 min at 60°C, after the gels had been cut at the dye-position. Destaining was done in 1% acetic acid. For protein estimation absorbance of stained gels was measured at 590 nM.

Ferguson-plots. Gels contained the same acrylamide/bis ratio as above. Acrylamide concentrations of 6 to 9% were used for estimation of retardation coefficients. The buffer was Tris · HCl, pH 8.5, containing 0.1% 2-mercaptoethanol.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Gels were either polymerized with 0.1% sodium dodecyl sulfate or equilibrated at 2 mA/tube for at least 2 h. Acrylamide/bis ratio was as above, ammonium persulfate was used at 100 mg/100 ml final concentration, so that polymerisation took about 10 min. Buffers used were 0.1 M Tris · HCl, pH 8.4, 0.1 or 0.05 M sodium phosphate, pH 7.2, both containing 0.2% 2-mercaptoethanol and 0.1% sodium dodecyl sulfate. Samples were treated usually for 10 min at 50°C with 0.2% sodium dodecyl sulfate/mercaptoethanol; the electro-

phoresis pattern could not be changed by more extensive treatment (see below). Samples were applied directly in triethanolamine buffer (mapping), in Tris-buffer (sodium dodecyl sulfate/gradient centrifugation analysis, Ferguson plots) or in 20 mM phosphate (Ferguson plots). Electrophoresis was carried out at 1—8 mA/tube. Staining was done according to Weber and Osborn [6], destaining by diffusion in methanol/acetic acid/water (1 : 1 : 8, v/v). After the run gels were cut at the bromphenol blue position, and mobilities were related to the length of the gel after destaining. Protein was estimated by absorbance at 590 nm.

Proteins used were (mol. wt. $\times 10^{-3}$): trypsin inhibitor (21.5), aldolase (40), dimer (80), bovine serum albumin (68), dimer, trimer, tetramer (136, 204,272), rabbit serum albumin dimer (139), phosphorylase *a*(100), dimer and trimer (200 and 300), *Eschericia coli* RNA polymerase α(39), δ(90), $\beta\beta'$(160), pyrophosphatase (126), catalase (60), dimer and tetramer (120 and 240), hemocyanin (290).

Sodium dodecyl sulfate gradient centrifugation

Enzyme (1 ml) purified by glycerol gradient procedure and enriched in the active high molecular weight component was dialysed against 0.1 M Tris · HCl, pH 8.5, containing 0.1% sodium dodecyl sulfate and 2-mercaptoethanol. Centrifugation was carried out in 32 ml of a linear 10—30% glycerol gradient (v/v) of the same buffer for 24 h at 20°C in a SW 27 rotor at 27 000 rev./min. Fractions of approximately 1 ml were collected, and 0.1 ml analysed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

Gel filtration on Sepharose 6B

Elution volume (V_e, reduced by exclusion volume V_o) was confirmed to be a linear function of logarithm of molecular weight. Marker proteins were ferrithin, catalase, racemase of gramicidin S-synthetase, and cytochrome *c*. Chromatography has been carried out on a 5 × 65 cm column, using 20 mM sodium phosphate, 2 mM $MgCl_2$, 2 mM dithiothreitol, and 0.25 mM EDTA, pH 7.2, at 4°C and a flow rate of 120 ml/h. The initial concentration of marker proteins was 0.5 mg/ml, except for racemase (0.02 mg/ml) and multienzyme (0.05 mg/ml).

Materials

Sodium dodecyl sulfate was obtained from Merck/Schuchardt and was used without further purification. Proteins were obtained from Boehringer, except for rabbit serum albumin and hemocyanine which were from Calbiochem. Ribulose diphosphate carboxylase was a gift from Dr. J. Vater.

Results and discussion

1. Correlation of protein and activity

We tried to purify the heavy enzyme of gramicidin S-synthetase by a combination of Sephadex G-200 filtration and DEAE-cellulose chromatography as has been suggested by the work of Kleinkauf et al. [2] and Gilhuus-Moe et al. [3] with an additional gradient centrifugation step [4]. As is shown in Fig. 1

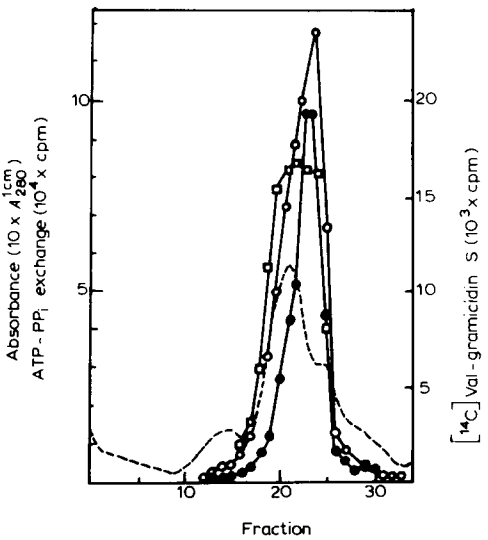


Fig. 1. Glycerol gradient centrifugation of gramicidin S-synthetase heavy enzyme, which has been purified by $(NH_4)_2SO_4$-fractionation, Sephadex G-200 filtration, and DEAE-cellulose chromatography according to Kleinkauf et al. [2]. Experimental details are described in Methods. Dotted line shows absorption at 280 nm, full circles: gramicidin S formation by [^{14}C] valine incorporation with complementary racemase; open circles: L-ornithine-dependent ATP-PP_i exchange reaction; open squares: L-valine-dependent exchange reaction. Activities have been measured according to Gevers et al. [21]. The obtained protein fraction is not homogeneous, since partial activation activities, gramicidin S-synthesis, and protein concentration (which has also been measured by polyacrylamide gel electrophoresis) cannot be correlated.

activation of amino acids as measured by ATP-PP_i exchange and gramicidin S-formation with complementary racemase could not be correlated with each other or with protein concentration. We have investigated density gradient fractions by polyacrylamide gel electrophoresis and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Fig. 2), thus preparing protein maps of the enzyme preparation.

This permitted correlation of concentration profiles of individual proteins with enzymatic activities. As shown in Fig. 3, only component A3 or B1 were apparently identical with the fully active enzyme. The identity of the two proteins A3 and B1 has been demonstrated by two dimersional polyacrylamide gel electrophoresis [results not shown] and by extensive purification studies [1].

Other enzymatic activities have not been assigned, since until now proteolytic modification of the active form has not been studied in detail, and the purified proteins A1 and A5 with the subunit composition 6 × B15 and 8 × B19 show no partial activity, while other proteins could not be isolated so far in homogeneous form.

2. Molecular weight of native protein

Sucrose gradient centrifugation. Kleinkauf et al. [2] and Kurahashi et al. [8] have evaluated sucrose gradients of the native enzyme according to Martin and Ames [9]. Standards were catalase (244 000), alcohol dehydrogenase (150 000), and β-glucuronidase (280 000). Their estimate of 280 000 daltons was confirmed by the more extensive studies of Kambe et al. [10].

Glycerol gradient centrifugation. We have been particularly interested in an

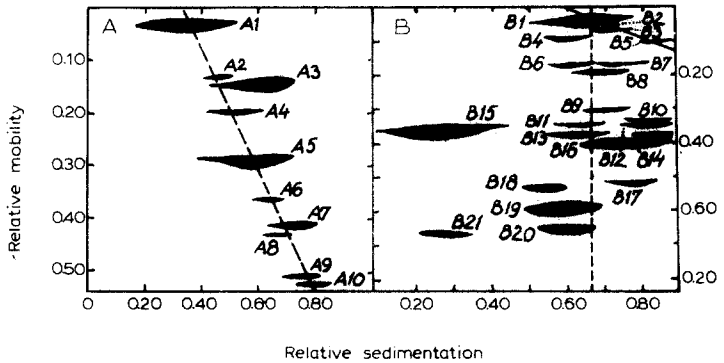


Fig. 2. Protein maps correlating sedimentation velocity and mobility in polyacrylamide gel electrophoresis of enzyme preparation as described in Fig. 1. (2A), and sedimentation velocity and mobility of sodium dodecyl sulfate-protein complexes in polyacrylamide gel electrophoresis (2B). Aliquots of density gradient fractions have been analysed by polyacrylamide gel electrophoresis. Protein bands have been numbered according to their electrophoretic mobility. The area indicates amount of protein in individual fractions, which has been estimated from stained gels, taking the size of the protein zones. Minor components are exaggerated by the method of documentation. The dashed line in Fig. 2A indicates a linear relationship between sedimentation velocity and mobility, which had been measured at pH 9.1. Protein A3 shows a significant deviation from this function together with an asymmetric sedimentation profile. The dashed line in Fig. 2B indicates position of maximum of protein B1 in the gradient. No other sodium dodecyl sulfate-peptide can be correlated exactly with this position. A size-mobility relationship apparently holds for proteins B1, B2, B3, and B5 (solid line), while all other proteins can be split into subunits. Relative sedimentation is the ratio of density gradient fraction/total number of fractions, while mobility is related to bromophenolblue.

apparent monomer-dimer equilibrium of the multienzyme in glycerol containing gradients, since an asymmetric profile was obtained (Fig. 3). We have not been able, however, to change this profile by varying the protein concentration from 0.1 to 1.0 mg/ml. Using aldolase (147 000) and catalase (232 000) as standards, a molecular weight of 275 000 ± 15,000 was calculated.

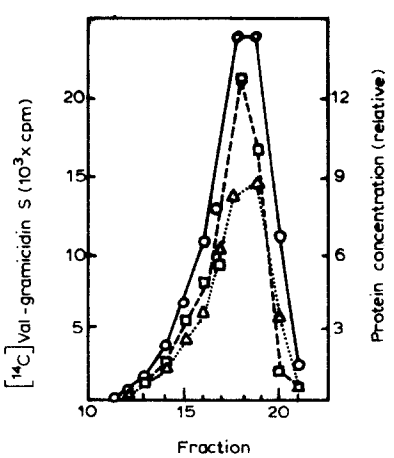


Fig. 3. Correlation of gramicidin-S synthesizing activity (circles) with complementary racemase, protein A3 of Fig. 2A (triangles), and protein B1 of Fig. B2 (squares). The identity of the two proteins had been confirmed by two dimensional polyacrylamide gel electrophoresis (unpublished results). Within experimental error, the activity is related to this protein, which has been substantiated by purification studies [1]. Protein was estimated by absorbance of stained polyacrylamide gels at 590 nm, and is plotted in relative units. Activity was determined by incorporation of [^{14}C] L-valine into gramicidin S [4].

Gel filtration. A linear relation between the elution volume and the logarithm of the molecular weight has been established for a Sepharose 6B column using ferritin, catalase, gramicidin S-synthetase light enzyme (racemase), and cytochrome *c* as standards. The position of the heavy enzyme corresponded to a molecular weight of 280 000 ± 30 000.

Polyacrylamide gel electrophoresis. As demonstrated by Hedrick and Smith [11], the molecular weight of globular proteins can be correlated to retardation coefficients obtained from Ferguson plots [12]. We obtained a linear plot using pepsin, catalase, protein 5 of Fig. 2, ribulose diphosphate carboxylase, and ferritin monomer, dimer, and trimer. The molecular weight of the heavy enzyme has been estimated to be 550 000 ± 60 000, indicating dimerisation of the protein in Tris · HCl. In sodium phosphate, however, two protein bands with an intensity ratio of about 2 to 1 can be observed, although sodium dodecyl sulfate/polyacrylamide gel electrophoresis in sodium phosphate displays a single band (Fig. 4). From the observed mobilities we conclude the presence of monomer and dimer under these conditions.

3. Molecular weight of protein/sodium dodecyl sulfate/complex

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis. It was of great importance to establish the molecular weight of the sodium dodecyl sulfate/peptide as 280 000 (molecular weight refers to denaturated peptide chain

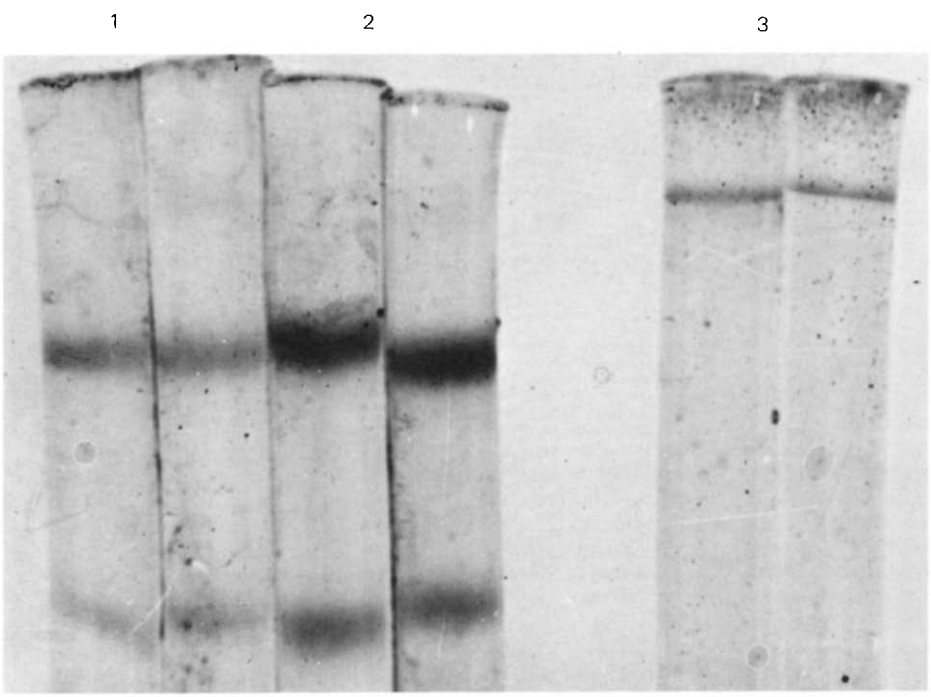


Fig. 4. Behaviour of multienzyme on polyacrylamide gel electrophoresis in phosphate buffer. Although apparently homogeneous on sodium dodecyl sulfate-phosphate gels (gel 3), two bands were observed in simple phosphate buffer. The intensity ratio of the two bands seems not to be affected by protein concentration (compare gels 1 with 2). Mobilities correspond approximately to monomer and dimer of multienzyme. No evaluation using retardation coefficients has been carried out.

without bound sodium dodecyl sulfate), since this meant evidence for either an unusually tight subunit structure resistent to sodium dodecyl sulfate, or an unusually large polyfunctional single chain protein.

The mobility of a protein in sodium dodecyl sulfate/polyacrylamide gel electrophoresis depends on its size, as well as sodium dodecyl sulfate binding, determining its charge, conformation, and new molecular weight. Generally, no other parameter than size is used, and molecular weight estimations are carried out in the range up to 200 000 daltons. Payne [13] has shown that mobility varied linearly with log molecular weight for minimal cross-linked protein polymers up to more than 900 000 daltons. Care has to be taken in work with unreduced proteins, or proteins containing extensive cross-links [14], as well as with discontinuous buffer systems [15]. There appears to be no nonlinear function of mobility and logarithm of molecular weight as has been discussed by Neville [16] and Hayashi et al. [17]. Instead, in the high molecular weight region a different slope is observed (compare data of Refs. 6, 13 and 16). In our evaluation we have used "cross-linked" proteins, that have formed apparently by self-condensation either before or during electrophoresis. So these proteins contained minimal cross-links, and using a continuous buffer system

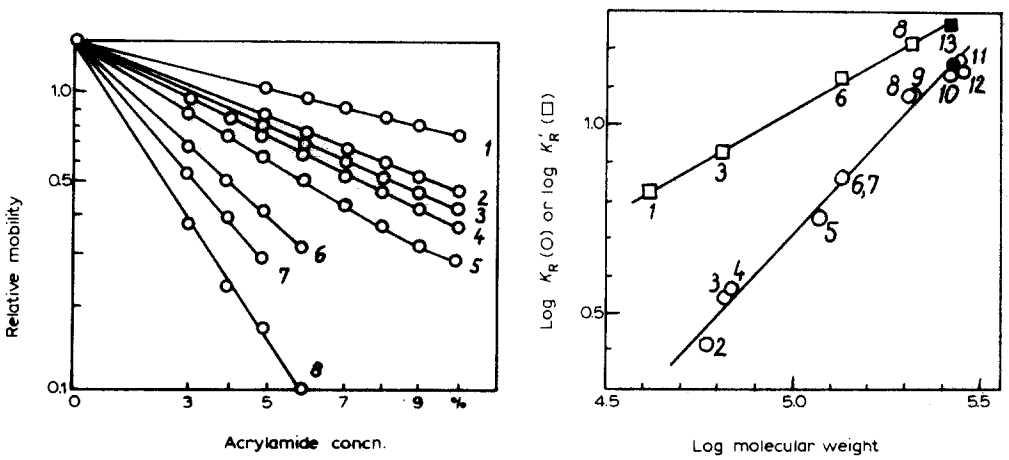
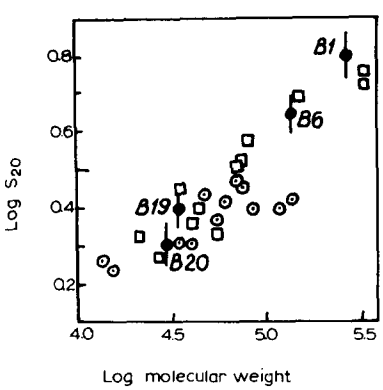


Fig. 5. Ferguson plots of sodium dodecyl sulfate protein complexes, showing logarithm of mobility as a function of acrylamide concentration. Proteins used were 1, RNAase; 2 and 3, proteins 19 and 20 of Fig. 2B; 4, ovalbumin; 5—7, bovine serum albumin monomer, dimer, trimer, 8, gramicidin S-synthetase multienzyme. Apparent free mobility of bovine serum albumin has been estimated in electrophoresis buffer containing 10% glycerol to 1.35 ± 0.07 relative to bromphenol blue (experiment not shown). Plots of all proteins can be extrapolated to approximately this value, indicating a constant charge to mass ratio. Experimental conditions were similar to those of Hayashi et al. [17], who obtained the same result for proteins up to the size of albumin.

Fig. 6. Retardation coefficients obtained from Ferguson plots as a function of molecular weight. Proteins were: 1, ovalbumin; 2, catalase, 3, bovine serum albumin (BSA), 4, rabbit serum albumin (RSA); 5, catalase dimer; 6, BSA-dimer; 7, RSA-dimer; 8, BSA-trimer; 9, RSA-trimer; 10, catalase tetramer; 11, BSA-tetramer, 12, hemocyanin; 13, gramicidin S-synthetase multienzyme. Evaluation of upper function (squares) has been done using Fig. 8 (Tris/buffer), plotting K'_R derived with equal free mobility. In the other function K_R-values were obtained from 50 mM phosphate with no assumptions on the apparent free mobility. The results show that a linear function is obtained, and the method appears useful for size determinations of peptide chains up to at least 300 000 daltons.



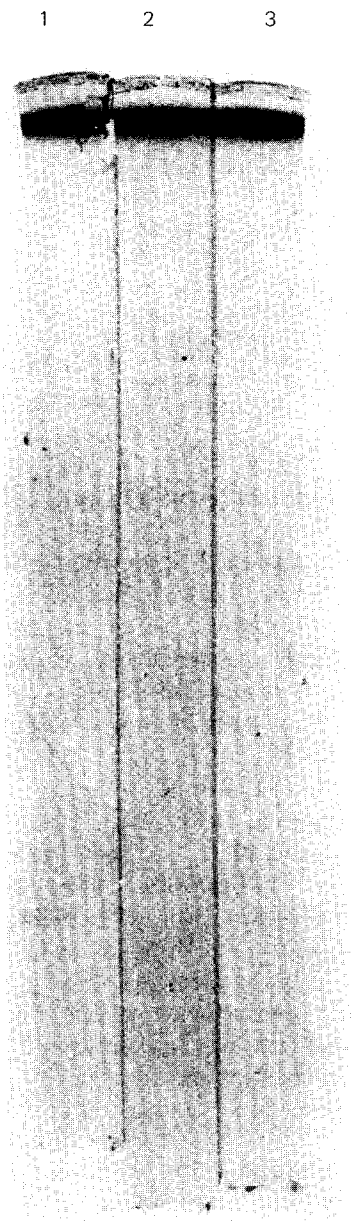


Fig. 7. Plot of sedimentation coefficients and initial molecular weight of sodium dodecyl sulfate-protein complexes. Data have been taken from Bais et al. [19] and Nelson [20], full circles have been calculated relative to the bovine serum albumin/sodium dodecyl sulfate complex [19] by the method of Martin and Ames [9]. Experimental details are given in Methods, numbers refer to proteins in Fig. 2B. Apparently the initial molecular weight is not sufficient information to correlate sedimentation values.

Fig. 8. Resistance of multienzyme structure to extensive sodium dodecyl sulfate treatment. The enzyme has been purified by sodium dodecyl sulfate gradient centrifugation (1), and is treated further with 0.2 M dithiothreitol and 2% sodium dodecyl sulfate at 95°C for 10 min (2) and for 60 min (3). Samples were run in 8% gels in Tris/buffer.

TABLE I

SUMMARY OF MOLECULAR WEIGHT DETERMINATIONS OF GRAMICIDIN S-SYNTHETASE MULTIENZYME

Method and parameter used		Result ($\times 10^{-5}$)	Remarks
Sucrose gradient centrifugation	s	2.8	[2], [11]
Glycerol gradient centrifugation	s	2.75 ± 0.15	asymmetric peak
Gel filtration (Sepharose 6B)	V_e	2.8 ± 0.3	
Sodium dodecyl sulfate/polyacrylamide gel electrophoresis Tris—HCl, pH 8.5	K_R	5.5 ± 0.6	dimerisation
Sodium dodecyl sulfate/polyacrylamide gel electrophoresis phosphate, pH 7.2	m	2.9 ± 0.2	
Sodium dodecyl sulfate/polyacrylamide gel electrophoresis phosphate, pH 7.2	K_R	2.8 ± 0.3	
Sodium dodecyl sulfate/polyacrylamide gel electrophoresis Tris—HCl, pH 8.5	K'_R	2.8 ± 0.3	

we obtained a molecular weight estimate of 290 000 ± 20 000 from 6% gels for the multienzyme.

One cannot rely on a single estimation, since it has been shown for sodium dodecyl sulfate-low binding proteins like α-amylase [18] that non-linear Ferguson plots are obtained, which means the molecular weight estimate depends on the acrylamide concentration in the gel. If a linear Ferguson-plot can be established, anomalous behaviour toward sodium dodecyl sulfate can be detected by extrapolation of mobilities to the apparent free mobility, which has been shown to be equal for several "normal" proteins [17], but differ significantly for some histones.

So we determined from linear Ferguson-plots (Fig. 5) free mobility, retardation coefficients, and from these again the molecular weight, this procedure giving 280 000 ± 30 000. This is the first time that a linear relation of molecular weight and retardation coefficients up to 290 000 daltons has been established in a continuous buffer system (Fig. 6).

Sodium dodecyl sulfate/glycerol gradient centrifugation. Sodium dodecyl sulfate-gradient centrifugation has been recommended as a supplementary method for confirming calculations from sodium dodecyl sulfate/polyacrylamide gel electrophoresis-data, although evaluation of sedimentation coefficients as a function of molecular weight appears to be strikingly difficult [19,20].

We have calcualted sedimentation data relative to the bovine serum albumin/sodium dodecyl sulfate/complex of the gramicidin S-multienzyme, and of proteins 6, 19, and 20 of Fig. 2B, and compared these to data of Bais et al. [19], see Fig. 7. The molecular weight estimation involves a very large error, but the fit of our data to the available data is reasonable.

Note: extensive sodium dodecyl sulfate treatment

The enzyme as obtained from sodium dodecyl sulfate/glycerol gradient centrifugation has been subjected to more extensive treatment in 0.2 M dithiothreitol, 2% sodium dodecyl sulfate for 60 min at 95°C. As can be seen in Fig. 8 the protein retained its size.

Conclusion

Evidence has been presented that gramicidin S-synthetase heavy enzyme or multienzyme with a molecular weight of 280 000, as determined by gradient centrifugation and gel filtration, readily dimerizes under conditions of polyacrylamide gel electrophoresis. The protein although it displays a normal behaviour in sodium dodecyl sulfate binding, is not dissociable by the agent.

Apparently the structure of this multienzyme is resistant to sodium dodecyl sulfate, and we propose that the subunits are covalently linked, since no protein-protein interaction completely resistant to sodium dodecyl sulfate has been described yet.

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