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Cyclosporin synthetase is a 1.4 MDa multienzyme polypeptide

Re-evaluation of the molecular mass of various peptide synthetases*

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The earlier determined molecular mass of 0.8 MDa for the multifunctional polypeptide, cyclosporin synthetase, was re-evaluated by SDS-PAGE and CsCl density gradient centrifugation. In SDS-PAGE, new molecular mass values as standards were available from sequencing data. In the CsCl density gradient extremly low protein concentrations, such as 10–50 nM could be analysed due to the fluorescence detection system of the analytical ultracentrifuge. Both methods yielded approximately the same value of about 1.4 MDa. Using this molecular mass of cyclosporin synthetase as a reference the molecular masses of various related enzymes could be re-evaluated in SDS-PAGE. The sedimentation coefficient of 26.3 S for cyclosporin synthetase indicates an oblate overall shape of the enzyme.

Cyclosporin synthetase; SDS-PAGE; Analytical ultracentrifugation; Fluorescence; Peptolide SDZ 214–103 synthetase; Sedimentation coefficient

1. INTRODUCTION

The immunosuppressant cyclosporin A (CyA), a cyclic undecapeptide, is synthesized in at least 40 reaction steps by a multienzyme, which is a single polypeptide. The enzyme activates the eleven constituent amino acids of CyA as adenylates and binds them as thioesters. At this stage, 7 amino acids are *N*-methylated. Then peptide bonds and cyclization are carried out [1].

Cyclosporin synthetase is the most complex of a number of peptide synthetases known so far [2]. Its molecular mass was originally determined by SDS-PAGE and glycerol gradient velocity sedimentation to be at least 650–800 kDa [1]. Enniatin synthetase, linear gramicidin synthetase 2 and tyrocidine synthetase 3 were used as standards for SDS-PAGE. When recently the molecular mass of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase, the first enzyme of the penicillin biosynthesis, could be calculated from the nucleotide sequence of the open reading frame [4–6], it became obvious that the earlier data from SDS-PAGE were a drastic underestimation, i.e. about 425 kDa instead of 230 kDa [3]. The basic difficulty in determining very high molecular masses of proteins with SDS-PAGE is the lack of appropriate standards.

In a new effort the M_r values from SDS-PAGE are derived from comparison with known masses in the range of 500,000, and as an alternative approach the molecular mass was determined by CsCl density gradient centrifugation in an analytical ultracentrifuge with fluorescence detection. The latter method yields absolute values for M_r, i.e. not evaluated from comparison with standards, and requires only minute molar concentrations of the enzyme because of the very sensitive mode of fluorescence recording. Since no experiments have been published so far concerning the structure of cyclosporin synthetase, the hydrodyamic shape of the synthetase was studied by sedimentation velocity.

Abbreviations: ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; CyA, cyclosporin A; IAMF, 4′-(((iodoacetyl)amino)methyl)fluorescein.

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2. MATERIALS AND METHODS

2.1. *Enzymes*

Cyclosporin synthetase and peptolide SDZ 214–103 synthetase were prepared as previously described [1,7,8]. Recombinant tyrocidine synthetase 2 was a gift from R. Weckermann (TU Berlin), ACV synthetase was a gift from H. v. Liempt (TU Berlin), gramicidin synthetase 2 was a gift of C. Ullrich (TU Berlin), enriched preparations of bacitracin synthetases 1–3 and linear gramicidin synthetase 2 and tyrocidin synthetase 3 were a gift from H. v. Döhren (TU Berlin).

2.2. *Preparation of fluorescence-labeled cyclosporin synthetase*

For fluorescence labelling of cyclosporin synthease 100 μl of 40 mM 4′-(((iodoacetyl)amino)methyl)fluorescein (Molecular Probes Inc., Eugene, OR, USA) in 50% EtOH were added to 1 ml of enzyme

purified by glycerol gradient ultracentrifugation and the mixture was incubated for 2 h at 4°C in the dark. The enzyme was then separated from fluorescein by passage through a PD-10 column (Pharmacia, Freiburg).

2.3. *Analytical ultracentrifugation*

CsCl density gradient centrifugation and sedimentation velocity runs were carried out in an analytical ultracentrifuge (Spinco model E, Beckman Instruments, Munich). The optical systems were either the UV absorption optics equipped with a high-intensity illumination system [9] and photoelectric scanner or a newly developed fluorescence detection system [10,11]. An argon ion laser (Spectra Physics, Darmstadt, Serie 2000 25 20.01, 15 W total light output) served as a light source for fluorescence excitation of IAMF-labeled cyclosporin synthetase at 488 nm. All experiments with the UV optics required double sector, charcoal filled epon centerpieces with an optical pathlength of 12 mm, experiments with the fluorescence optics single sector centerpieces of 3 mm optical pathlength. All runs were performed in a four-hole rotor AN F-Ti.

CsCl density gradient centrifugation of cyclosporin synthetase was carried out in 50 mM Tris-HCl, pH 7.8, 2 mM EDTA, 2 mM DTT, 7.5% (w/v) glycerol and 2.325 M CsCl corresponding to a starting density of $\rho_0 = 1.290$ g·cm^{-3}. The runs were carried out at 40,000 rpm and a temperature between 25 and 27°C in different experiments.

Sedimentation velocity runs were performed in 100 mM Tris-HCl, pH 7.8, 4 mM EDTA, 4 mM DTT, 15% (w/v) glycerol at 48,000 rpm and at a temperature between 11 and 14°C in different experiments.

2.4. *SDS-PAGE*

SDS-PAGE (3% w/v) was performed as described [1].

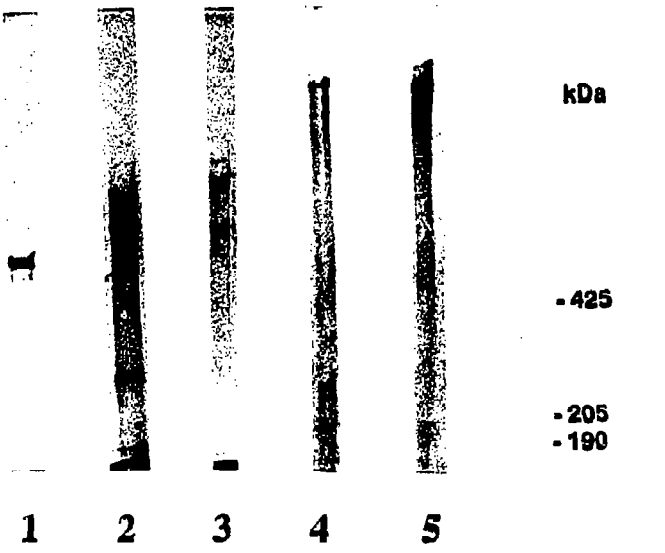

Fig. 1. Molecular mass estimation of peptide synthetases in one 3% SDS-PAGE. The molecular masses of the reference proteins recombinant tyrocidine synthetase 2 (190 kDa), myosin (205 kDa) and ACV synthetase (425 kDa) are indicated. Extrapolation results in molecular masses of 570 kDa for gramicidin synthetase 2 (lane 1), 310, 730 and 870 kDa for bacitracin synthetases 2, 1 and 3 (lane 2,), 640 and 880 kDa for linear gramicidin synthetase and tyrocidine synthetase 3 (lane 3), 1530 kDa for cyclosporin synthetase (lane 4) and 1460 kDa for peptolide SDZ 214–103 synthetase (lane 5).

3. RESULTS

3.1. *Molecular mass*

Using the molecular mass calculated from sequence analyses of the ACV synthetase from *Aspergillus nidulans* [6] as a standard we evaluated the molecular masses of several peptide synthetases in 3% SDS-PAGE (Fig. 1). Linear regression analysis of the mobilities of the enzymes in 3 different SDS-PAGEs, of which one is shown in Fig. 1, results in molecular masses of 580 kDa for gramicidin synthetase 2 (Fig. 1, lane 1) and 1,525 kDa for cyclosporin synthetase (Fig. 1, lane 4). The value for gramicidin synthetase 2 is in good agreement with a value of 556 ± 5 kDa obtained recently by laser desorption mass spectroscopy (M. Vestal and H. von Döhren, personal communication) and a value of 510 kDa from sequencing [12]).

Molecular masses in the MDa range are too high for a determination with sedimentation diffusion equilibrium centrifugation. The appropriate rotor speed of 3,500–5,000 rpm leads easily to hydrodynamic instabilities, and the concentration for UV measurements has to be quite high in order to obtain an accurate concentration profile. Therefore we decided to carry out CsCl density gradient centrifugation with fluorescence recording. The high molecular mass leads to a narrow distribution which can be recorded with high accuracy and the fluorescence detection system developed earlier in our laboratory allowed us to use very low protein concentrations. Prerequisites of this method were the solubility of low concentrations of the protein in 2.3 M CsCl and a method for fluorescence labeling of the enzyme.

In vitro synthesis of CyA is inhibited by several thiol blocking agents as could be shown for iodoacetamide, *N*-ethylmaleimide and 2,2'- and 4,4'-dithiodipyridine (J. Dittmann and A. Lawen, unpublished). Also the fluorescence label, IAMF, inhibits CyA synthesis, for example by 60% if incubated with a concentration of 1 mM IAMF at 4°C for 10 min. For the preparation of fluorescence-labeled cyclosporin synthetase a concentration of 3.6 mM IAMF was used for 1 h. Binding to the enzyme shifts the fluorescence emission maximum from 512 to 520 nm. After separation from unbound IAMF the enzyme preparation could be used for the analytical ultracentrifuge experiments.

Fig. 2 shows the concentration profiles in the density gradient for three different starting protein concentrations. The molecular mass was calculated from the formula of Meselson et al. [13],

$$M_r = \frac{RT\rho}{\sigma^2(d\rho/dr)_{r_c}\omega^2 r}$$

with the gas constant R, the absolute temperature T, the buoyant density of the protein ρ, the half width 2σ of the band at 0.607 of the height δ, the slope of the density gradient $d\rho/dr$, the angular velocity ω and the radial position of the protein r. Taking into consideration the finding of Schmid and Hearst [14] that lower M_r values were determined at higher macromolecular concentra-

tions caused by thermodynamic non-ideality, the dependence of M_r upon the protein concentration was studied in our experiments. When starting concentrations of 150 and 30 nM cyclosporin synthetase were recorded with the UV optics (profiles not shown), an easily measurable peak could be detected with 150 nM protein at a density of 1.286 g·cm^{-3} whereas no protein peak was detected with 30 nM. However, 150 nM cyclosporin synthetase showed very strong aggregation and yielded bands with nearly the same width, 2σ, as tobacco mosaic virus particles of a molecular mass of 40 MDa and a buoyant density of 1.325 g·cm^{-3} [15] which were added to the solution as internal standards (data not shown). For recording lower concentrations, fluorescence-labeled cyclosporin synthetase was analyzed at 12 different protein concentrations in 16 experiments of which three are depicted in Fig. 2. The good agreement with the Gaussian curve (dots in Fig. 2) demonstrates the one-component character of the cyclosporin synthetase sample. From the concentration profile of Fig. 2b and the best fit according to the Gaussian curve, respectively, a molecular mass of 1.46 MDa was calculated. A clear indication of protein aggregation is seen from the profile in Fig. 2c, because firstly a M_r of 4×10^6 was obtained and secondly the profile is characteristic for high M_r in the center and lower M_r at the peripheries of the band. The signal-to-noise ratio decreased drastically at concentrations lower than 10 nM, but still lead to a fair agreement with the Gaussian distribution (Fig. 2a). The deviation at the high density side might be due to protein degradation.

All M_r values and maximum errors are shown in the plot of Fig. 3. Starting protein concentrations higher than 50 nM lead to aggregation, e.g. 19×10^6 for 150 nM as measured with the UV optics. Below 50 nM the molecular mass was found to be independent of concentration. The findings of Schmid and Hearst [14] who reported lower apparent M_r values with higher macromolecular concentrations, cannot be related in more detail to ours because nucleic acids were studied in their case. From 9 measurements between 10 and 44 nM an average molecular mass of 1.4 MDa with a maximum error of ±160 kDa was derived. In this average the values from concentrations below 10 nm were not included because of their lower accuracy. The M_r value of 1.4×10^6 is in good agreement with the value of 1.5×10^6 from SDS-PAGE (Fig. 1). Corrections of M_r due to the compressibility of the protein and CsCl solution and due to salt binding and hydration of the protein were carried out according to the studies of Ifft and Vinograd [16] on bovine mercaptalbumin and were found to compensate for a small value compared to the experimental error mentioned above (B. Schmidt, thesis, Heinrich-Heine-Universität Düsseldorf, Germany, in preparation).

From the experiments below 50 nM the buoyant density of 1.274 g·cm^{-3} for cyclosporin synthetase was determined. This is the value for the non-aggregated protein and is 0.012 g·cm^{-3} lower than the value of the aggregated protein, i.e. at 150 nm, but in agreement with the results of Ifft [17] who reported a difference of 0.006–0.009 g·cm^{-3} between the aggregated and soluble state of some proteins in CsCl density gradient centrifugation.

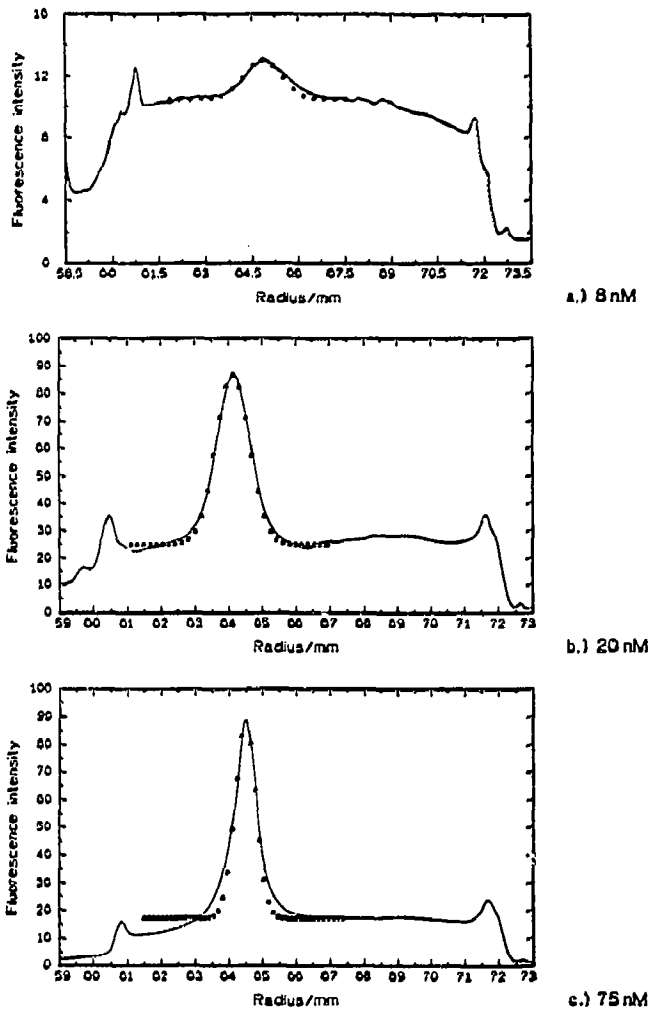

Fig. 2. CsCl density gradient centrifugation profiles of cyclosporin synthetase. Concentrations were: a): 8,3 nM, b): 20 nM, c): 75 nM. Rotor speed was 40,000 rpm, ρ_0 was 1.290 g·cm^{-3}, profile (a) was monitored after 9 h, profiles (b) and (c) after 17 h. Open circles designate the best fit to the Gaussian distribution equation.

3.2. *Molecular shape*

In order to obtain information on the overall shape of cyclosporin synthetase, sedimentation velocity runs were performed. Because of the higher stability of the enzyme at lower temperature, sedimentation runs were carried out at temperatures between 11 and 14°C and in a buffer in which in vitro enzyme activity is high [7]. Sixteen runs were performed at 8 different protein concentrations between 580 and 28 nm either with the UV

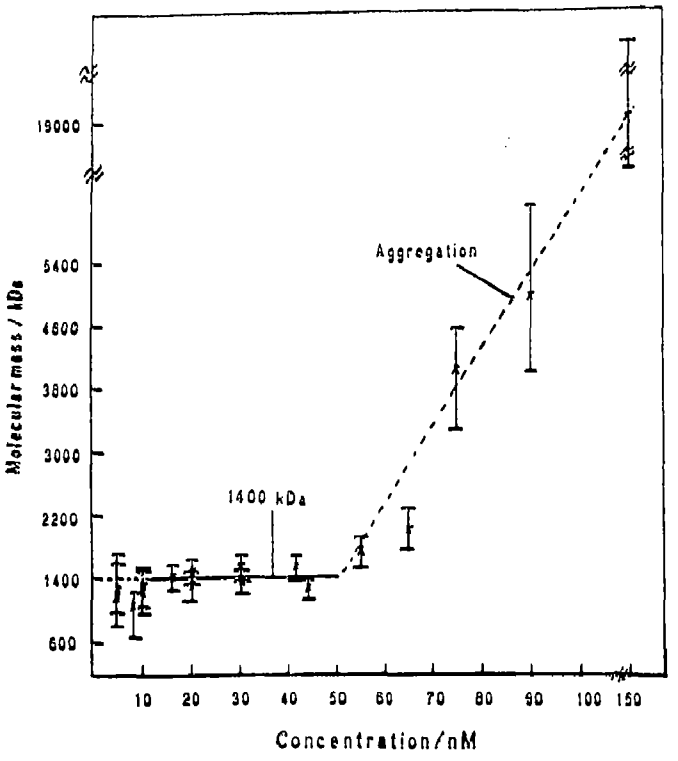

Fig. 3. Molecular mass of IAMF-labeled cyclosporin synthetase determined by CsCl density gradient centrifugation is dependent upon protein concentration. The molecular mass is marked with an x, the maximum error is given by the margins. Mensurements at 5, 10, 20 and 30 nM were carried out twice. The average molecular mass from concentrations between 10 and 44 nM is indicated. The phenomenon of aggregation is reflected by the dashed line.

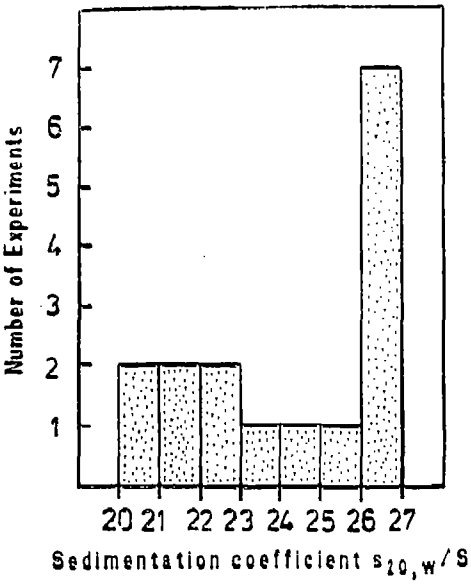

Fig. 4. Numbers of experiments yielding the measured sedimentation coefficients of cyclosporin synthetase. The lower values are probably due to protein degradation and unfolding. The highest s values reflect the value for the intact protein.

optics or with the fluorescence optics. The sedimentation profiles had the same quality as those which were depicted for other proteins in earlier publications [11,18]. A dependence of the sedimentation constant from the protein concentration was not found, neither at the higher protein concentrations recorded with the UV optics nor at lower concentrations with the fluorescence optics. Sedimentation coefficients between 20.6 and 26.6 were obtained in different experiments. The s values obtained from all experiments are presented in form of a histogram (Fig. 4) in order to demonstrate that it is not the distribution of error statistics but most probably a systematic deviation due to the enzyme behaviour. The average $s_{20,w}$ value of 7 experiments is 26.3 S, which was taken as the value of the undegraded cyclosporin synthetase in its native conformation, whereas the lower values, particular those around 20–22 S indicate degradation and/or unfolding.

For estimating the shape the model of the ellipsoid of revolution was applied [19,20]. By using M_r of 1.4×10^6 in the Svedberg equation [21] the translational friction coefficient was determined to be 2.2×10^{-7} g·s^{-1}. A specific volume $\bar{v}$ was assumed to be 0.75 cm^3·g^{-1} [22]. The translational frictional ratio is the ratio of the friction coefficient of the molecule under study and the friction coefficient of a hydrated sphere of the same mass and volume [23]. This ratio is characteristic for the overall shape of the molecule. A sphere of 1.4 MDa and a specific partial volume of 0.75 cm^3·g^{-1} would have a hydrated volume of 2.66×10^6 Å^3 and a radius of 86 Å if 0.4 g bound water/g protein was assumed [19]. From this a friction coefficient of 1.62×10^{-7} g·s^{-1} and an s value of 36 S is obtained for the sphere. From the $s_{20,w}$ value of 26.3 of cyclosporin synthetase a frictional ratio of 1.36 was determined. If this ratio is interpreted in terms of an oblate ellipsoid an axial ratio a:b of 7.2 is obtained (cf. [19,20]). The oblate ellipsoid would look like a discus of 330 Å in diameter and a thickness of 46 Å. Considering the function of the protein, i.e. the multi- step synthesis of a cyclic peptide, and taking into

Table I

Re-evaluation of molecular masses of peptide synthetases

Synthetases	Molecular masses (kDa)		
	Described	Ref.	Re-evaluated
Linear gramicidin synthetase 2	350	[26]	630
Bacitracin synthetase 1	380	[27]	650
Bacitracin synthetase 2	230	[27]	290
Bacitracin synthetase 3	420	[27]	780
Tyrocidine synthetase 3	450	[28]	820
Cyclosporin synthetase	650–800	[1]	1,400
Peptolide SDZ 214-103 synthetase	650–1,500	[8]	1,380

Three 3% PAGEs were performed. The table lists the average values of the three regression analyses.

account the electron micrographs of gramicidin synthetase 2 [24], the oblate ellipsoid is more appropriate than the prolate form.

4. DISCUSSION

The availability of high M_r standards for SDS-PAGE and the use of analytical ultracentrifugation with fluorescence detection allowed us to determine a M_r value as high as 1.4×10^6 for the single peptide enzyme cyclosporin synthetase. Although CsCl density gradient centrifugation is an unusual method for M_r determination of proteins, the very high molecular mass lead to narrow banding and therefore good accuracy in M_r determination. Furthermore, fluorescence detection allowed us to measure the M_r values in concentrations as low as 10 nM and to exclude aggregation effects. A heterogeneity of the protein sample which might limit the accuracy of M_r in other techniques could be excluded in these studies due to the good agreement of the theoretical and experimental curves. As such, the homogeneity of the protein sample could be measured as an independent parameter, which exhibits a specific advantage of the method. Since the samples for gel electrophoresis were boiled in 4 M urea, 0.5% SDS and 72 mM β-mercaptoethanol, the value of 1.5 MDa is the value of the molecular mass of a single peptide chain. It is noteworthy that in 2 M urea the CyA synthesis activity is totally inhibited (J. Dittmann and A. Lawen, unpublished), probably due to the unfolding of the polypeptide chain. Nearly the same value, i.e. 1.4 MDa, was found under non-aggregating conditions in 2.34 M CsCl. Thus, in both experiments the single subunit protein was analyzed. At low ionic strength, as applied in the velocity sedimentation experiments, we cannot exclude dimerisation or higher aggregation. However, this is highly improbable because of the s value. A reasonable interpretation in structural terms could be achieved only for a monomer. In summary, this enzyme appears to be the largest enzymatically active polypeptide chain described to date.

The most convincing interpretation of the hydrodynamic data in terms of the model of an ellipsoid of revolution, especially considering the function of the protein, is the oblate ellipsoid. The discus- like structure of the enzyme would have a diameter of about 330 Å and a thickness of 46 Å. This result should be discussed taking into consideration the electron micrographs of gramicidin synthetase 2, which show flat circular structures of about 120 Å diameter and 60 Å height with a central opening of 60 Å [24]. Cyclosporin synthetase could have a central opening of 50–60 Å, too. The growing chain of cyclosporin A might need a space up to 10–15 Å, according to X-ray data [25] of cyclosporin A. An additional 20 Å could be needed for moving putative phosphopanthetein arms. A central opening of 50–60 Å, however, would contribute to only 2.4–4.3%, respectively, of the total volume and therefore would have no drastic effect on the friction coefficient and density of the enzyme.

Using in addition to the molecular mass standards from Fig. 1 the average value for gramicidin synthetase 2 from laser desorption mass spectroscopy and sequencing data (533 kDa) and that of cyclosporin synthetase obtained from ultracentrifugation studies (1.4 MDa), molecular masses of other peptide synthetases can be easily re-evaluated from SDS-PAGE. Thus, for bacitracin synthetases 1, 2 and 3 apparent molecular masses of 650, 290 and 780 kDa have been estimated; linear gramicidin synthetase 2 has a molecular mass of about 630 kDa and tyrocidine synthetase 3 has a molecular mass of about 820 kDa. Peptolide SDZ 214–103 synthetase [8] appears to have a molecular mass only slightly lower than that of cyclosporin synthetase. Table I compares the published and the newly estimated molecular masses of some peptide synthetase polypeptide chains. It demonstrates that due to the re-evaluation of one synthetase our present knowledge of a whole series of synthetases has improved markedly.

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REFERENCES

[1] Lawen, A. and Zocher, R. (1990) J. Biol. Chem. 265, 11355–11360.
[2] Kleinkauf, H. and von Döhren, H. (1990) Eur. J. Biochem. 192, 1–15.
[3] van Liempt, H., von Döhren, H. and Kleinkauf, H. (1989) J. Biol. Chem. 264, 3680–3684.
[4] Smith, D.J., Earl, A.J. and Turner, G. (1990) EMBO J. 9, 2743–2750.
[5] Diez, B., Gutiérrez, S., Barredo, J.L., van Solingen, P., van der Voort, L.H.M. and Martín, J.F. (1990) J. Biol. Chem. 265, 16358–16365.
[6] MacCabe, A.P., van Liempt, H., Palissa, H., Unkles, S.E., Riach, M.B.R., Pfeifer, E., von Döhren, H. and Kinghorn, J.R. (1991) J. Biol. Chem. 266, 12646–12654.
[7] Lawen, A., Traber, R., Geyl, D., Zocher, R. and Kleinkauf, H. (1989) J. Antibiot. 42, 1283–1289.
[8] Lawen, A., Traber, R. and Geyl, D. (1991) J. Biol. Chem. 266, 15567–15570.
[9] Floßdorf, J. (1980) Macromol. Chem. 181, 715–724.
[10] Rappold, W. (1986) PhD thesis, Universität Düsseldorf.
[11] Schmidt, B., Rappold, W., Rosenbaum, V., Fischer, R. and Riesner, D. (1990) Colloid Polym. Sci. 268, 45–54.
[12] Turgay, K., Krause, M. and Marahiel, M.A. (1992) Mol. Microbiol. 6, 529–546.
[13] Meselson, M., Stahl, F.W. and Vinograd, J (1957) Proc. Natl. Acad. Sci. USA 43, 581–588.
[14] Schmid, C.W. and Hearst, J.E. (1969) J. Mol. Biol. 44, 143–160.
[15] Francki, R.J.B., Fauquet, C.M., Knudson, D.L. and Brown, F. (1991) Classification and Nomenclature of Viruses, Fifth Report of International Committee on Taxonomy of Viruses, Archives of Virology, Suppl. 2, Springer, Wien.
[16] Ifft, J.B. and Vinograd, J. (1966) J. Phys. Chem. 70, 2814–2822.
[17] Ifft, B. (1976) Biophys. Chem. 5, 137–157.
[18] Schmidt, B. and Riesner, D. (1992) in: Analytical Ultracentrifu-

gation in Biochemistry and Polymer Science (S.E. Harding, and A.J. Rowe, eds.) Royal Society of Chemistry, in press.
[19] Cantor, C.R. and Schimmel, P.R. (1980) Biophysical Chemistry, Part II, W.H. Freeman and Company, San Francisco.
[20] Tanford, C. (1961) Physical Chemistry of Macromolecules, J. Wiley, New York.
[21] Svedberg, T. and Nicols, J.B. (1923) J. Am. Chem. Soc. 45, 2910–2917.
[22] Durchschlag, H. (1986) in: Thermodynamic Data for Biochemistry and Biotechnology (H.-J. Hinz, ed.) Springer, Berlin.
[23] Perrin, E. (1934) J. Phys. Radium 5, 497–511.
[24] Vater, J. (1990) in: Biochemistry of Peptide Antibiotics, Recent Advances in the Biotechnology of β-Lactams and Microbial Bioactive Peptides (H. Kleinkauf and H. von Döhren eds.) Walter de Gruyter, Berlin.
[25] Petcher, T.J., Weber, H.-P. and Rüegger, A. (1976) Helv. Chim. Acta 59, 1480–1489.
[26] Akers, H.A., Lee, S.G. and Lipmann, F. (1977) Biochemistry 16, 5722–5729.
[27] Kleinkauf, H. and von Döhren, H. (1988) CRC Crit. Rev. Biotechnol. 8, 1–32.
[28] Lee, S.G., Roskoski Jr., R., Bauer, K. and Lipmann, F. (1973) Biochemistry 12, 398–405.