Sticholysin II, a cytolysin from the sea anemone *Stichodactyla helianthus*, is a monomer-tetramer associating protein

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Abstract Sticholysin II (Stn-II) is a pore-forming cytolysin. Stn-II interacts with several supports for size exclusion chromatography, which results in an abnormal retardation precluding molecular mass calculations. Sedimentation equilibrium analysis has revealed that the protein is an associating system at neutral pH. The obtained data fit a monomer-tetramer equilibrium with an association constant K_4^c of 10^9 M^{-3}. The electrophoretic pattern of Stn-II treated with different cross-linking reagents, in a wide range of protein concentrations, corroborates the existence of tetrameric forms in solution. A planar configuration of the four monomers, C4 or D2 symmetry, is proposed from modelling of the cross-linking data.

Key words: Protein cross-linking; Sedimentation equilibrium; Actinoporin

1. Introduction

Sticholysin II (Stn-II) is a cytolytic protein isolated from the sea anemone *Stichodactyla helianthus*. This organism, like other coelenterates, produces many toxic polypeptides, mainly neurotoxins and cytolysins, involved in mechanisms of defence and/or attack. Stn-II was described more than 20 years ago [1] as a potent hemolytic protein, formerly named cytolysin III. More than 20 cytolysins have been isolated from different sea anemones [2], tenebrosin C from *Actinia tenebrosa* [3], equinatoxin II from *Actinia equina* [4], and Stn II [5] being the most studied. These cytolysins belong to a protein family called actinoporins [6–8] and are composed of a single polypeptide chain of about 175 amino acid residues (lacking cysteines), which display a strong sequence similarity. These cytolysins also show a similar action on lipid model membranes promoting the formation of pores [5,9–13]. The kinetic analysis of this leakage of aqueous contents of model vesicles revealed that oligomerization of these proteins, three to four monomers, is apparently involved in the membrane permeabilization [5,10,12,13]. Experiments performed with chemical cross-linkers and ultracentrifugation studies with the native protein have revealed that Stn-II displays a monomer to tetramer associating equilibrium in the absence of membranes. This finding opens the possibility of explaining the cytolytic activity of these proteins in terms of their intrinsic tendency to oligomerization. We herein report the results obtained from such studies.

2. Materials and methods

Stn-II has been isolated from extracts of specimens of *S. helianthus* purchased from Nayeco (Barcelona, Spain) as previously described [5]. The purification procedure includes two chromatographic steps, gel filtration on Sephadex G-50 and ion exchange on CM-cellulose CM-52. This procedure renders sequence-degree purified protein [5].

Ultracentrifugation measurements were performed on a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics, in an An60Ti rotor. The protein was equilibrated in 15 mM MOPS, pH 7.0, containing 0.1 M NaCl. Short column experiments (70 μl of protein sample at a concentration ranging from 8 to 400 μM; 0.15–10 mg/ml) were carried out at two consecutive speeds (15 and 20 krpm) by taking absorbance scans (0.001 cm step size, 10 averages) at the appropriated wavelength (250, 295 or 300 nm) after the sedimentation equilibrium was reached. Double-sector centerpieces of charcoal-filled Epon (3 or 12 mm optical path) were used. The equilibrium temperature was 25°C. High speed sedimentation (40 krpm) was conducted afterwards for baseline correction in all the cases. The buoyant molecular mass of the protein was estimated by fitting a sedimentation equilibrium model for a single sedimenting solute to individual data sets with the EQASSOC program (supplied by Beckman Instruments; see [14]). These values were converted to the corresponding average molecular masses by considering 0.736 ml/g as the partial specific volume of Stn-II, which was calculated according to [15] from the amino acid composition of the protein. Several models of self-association (monomer-dimer, monomer-trimer, and monomer-tetramer; see [16]) were globally fitted to multiple sedimentation equilibrium using the MULTEQ3B program kindly supplied by Dr. Allen Minton (National Institutes of Health, USA).

bis-(Sulfosuccinimidyl) suberate (BS^3), dithio*bis*-(sulfosuccinimidyl-propionate) (DTSSP) and glutaraldehyde were used as cross-linking reagents. Purified Stn-II (sequence-degree; see [5]) shows a certain amount of dimeric form by SDS-polyacrylamide gel electrophoresis. This has also been reported for equinatoxin II from *A. equina* [10]. The proportions of the different oligomer fractions resulting from the cross-linking were calculated without any correction for this small, variable fraction of non-covalent dimer. The cross-linking reaction was performed by adding a small aliquot (2–3 μl) of a concentrated freshly prepared cross-linker solution to the protein sample, at the required concentrations. Both protein and cross-linker were prepared in 15 mM MOPS, pH 8.0, containing 0.1 M NaCl. The reaction mixture was incubated at room temperature and it was quenched at the required time intervals by addition of either Tris 1 M (0.1 M final concentration) or $NaBH_4$ dissolved in 0.1 M NaOH (50 mM final concentration) for BS^3 and DTSSP or glutaraldehyde, respectively. The cross-linking produced by DTSSP can be reversed by treatment with 25 mM dithiothreitol at 37°C for 30 min upon reduction of the disulfide bridge of the reagent. After addition of 5 ml of the electrophoresis loading buffer 3× to each aliquot, the cross-linked products

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Abbreviations: BS^3, *bis*-(sulfosuccinimidyl) suberate; DTSSP, dithio*bis*-(sulfosuccinimidyl) propionate; Stn-II, *Stichodactyla helianthus* sticholysin II

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were analyzed by SDS-polyacrylamide gel electrophoresis following standard procedures [17]. Silver staining of the gels, when required, was performed as described [18]. Volumograms from the stained gels were obtained on a photodocumentation system UVI-Tec using the software facility UVIsoft UVIband Windows Application V97.04.

Circular dichroism measurements were performed on a Jasco J-715 spectropolarimeter at 25°C with thermostatted cylindrical cells. Data were expressed in units of degree cm^2 $dmol^{-1}$ of residue (110 mean residue mass for the amino acids of this protein).

Estimation of the apparent molecular mass of Stn-II in neutral solutions (15 mM MOPS, pH 7.0, containing 0.1 M NaCl) by size exclusion chromatography was performed on different columns: (conventional techniques) Sephadex G-50 superfine gel (cross-linked dextran-derived matrix) from Pharmacia, and Biogel P-60 (polyacrylamide-derived gels) from Bio-Rad; (FLPC techniques) Superdex 75 HR 10/30 (dextran covalently bonded to cross-linked agarose beads) from Pharmacia; (HPLC techniques) Ultraspherogel-SEC 3000 (silica bonded with hydrophilic coating) from Beckman Instruments. In all cases, appropriated molecular mass markers were used for calibration.

3. Results and discussion

Sedimentation equilibrium analysis of Stn-II reveals that this protein is an associating system at neutral pH and in the presence of 0.1 M NaCl, conditions at which the cytolysin promotes leakage of aqueous contents of lipid model vesicles [5]. At low protein concentration (7.8–10.0 μM), the average molecular mass of Stn-II resulting from the centrifugation analysis is 19 600, which closely agrees with the theoretical mass value (19.3 kDa) calculated from the amino acid sequence of the protein [21]. That molecular mass value increases with protein concentration, a typical behavior of an associating system [19,20]. The association system that best fits the experimental data corresponds to a monomer-tetramer equilibrium, with an association constant K_4^C of 10^9 M^{-3} (Fig. 1).

It is well known [22–27] that chemical cross-linking with bifunctional reagents is a valuable tool for studying the quaternary structure of proteins. When an oligomeric protein is treated with a cross-linking reagent, many derivatives can be expected: (i) adducts in which one of the reactive groups of the reagent remains intact; (ii) cross-links within each polypeptide chain; (iii) cross-links between polypeptides of the oligomer; (iv) cross-links between oligomers. This latter possibility becomes less possible at lower protein concentration (<10 μM [22]; in this case <0.2 mg/ml), whereas the probability of cross-linking within an oligomer is unaffected. Thus, the number of bands observed after SDS-polyacrylamide gel electrophoresis of a cross-linked protein at low protein concentration should correspond to the number of subunits in the original protein structure. A tetramer protein renders four bands corresponding to the monomer, dimer, trimer and tetramer cross-linked forms, although within each molecular mass category there are a variety of species with different numbers of intra- and intermolecular cross-links. The relative amount of each observed band depends on the probability of intermolecular cross-linking, which arises from the particular three-dimensional structure of the protein. Fig. 2 summarizes the results obtained from cross-linking experiments of Stn-II. Glutaraldehyde, BS^3 and DTSSP were used as cross-linkers and the obtained results were similar in terms of the number of bands observed after electrophoresis, although a larger extent of cross-linking can be obtained with glutaraldehyde (nevertheless, higher concentrations of the dialdehyde are required for cross-linking). SDS-polyacrylamide gel electrophoresis of cross-linked Stn-II reveals the existence of protein bands ranging from monomer to tetramer (Fig. 2A,B). The cross-linked oligomers resulting from treatment with DTSSP render the monomer form upon reduction of the disulfide

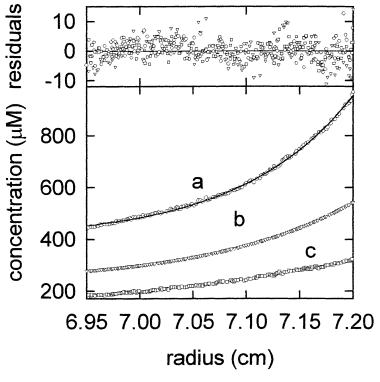

Fig. 1. Sedimentation equilibrium analysis of Stn-II at different protein concentrations: (a) 10.05 mg/ml; (b) 7.57 mg/ml; (c) 5.03 mg/ml. The residuals (difference between the experimental data and the fitted data for each point) are shown in the top panel.

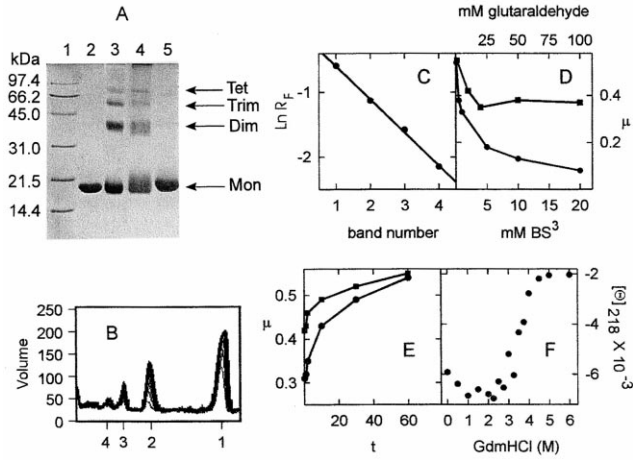

Fig. 2. A: Representative SDS-polyacrylamide gel electrophoresis pattern of chemically cross-linked Stn-II: (1) molecular mass (kDa) markers; (2) uncross-linked Stn-II; (3) cross-linked Stn-II after treatment with 100 mM glutaraldehyde for 10 s at 1 mg/ml protein concentration; (4) cross-linked Stn-II after treatment with 1 mM DTSSP for 1 min at 0.5 mg/ml protein concentration; (5) sample cross-linked as in lane (4) after reduction with 25 mM dithiothreitol at 37°C for 30 min. The arrows point to the bands corresponding to: (Mon) monomer; (Dim) dimer; (Tri) trimer; and (Tet) tetramer. Gels were stained either with Coomassie blue or with silver depending on the cross-linked protein concentration. B: Volumogram of lane 3 in the gel shown in A. The optical density volume is expressed in arbitrary units. (1) Monomer; (2) dimer; (3) trimer; and (4) tetramer forms. C: Natural logarithm of the relative electrophoretic mobilities (ln R_F) versus scale of successive integers for the bands obtained after SDS-PAGE of cross-linked Stn-II. Values are the average for 10 different determinations. D: Plot of μ, the overall degree of cross-linking, calculated as $\mu=\Sigma_n\ (n-1)\times[\text{fraction}_{(n-\text{mer})}]$ [30] from the image analysis of the corresponding SDS-PAGE plates, versus (●) BS^3 and (■) glutaraldehyde concentration, for a 2.5 μM Stn-II concentration. E: Kinetic analysis of the μ value determined from the electrophoretic analysis of the cross-linking obtained with (■) 0.1 mM BS^3 and (●) 1 mM glutaraldehyde at 2.5 μM Stn-II concentration. F: Ellipticity at 218 nm of Stn-II, expressed in units of degree cm^2 $dmol^{-1}$ of residue, versus guanidinium hydrochloride concentration.

bridge of the bifunctional reagent (Fig. 2A), thus discarding the possibility of non-covalent SDS-resistant species originated upon cross-linker treatment [28]. The relative electrophoretic mobility of each observed band is a regular function of its number of polypeptide chains (Fig. 2C), which is one of the reassurances that should be provided in this type of experiments. This result was obtained in a wide range of protein concentrations, 0.05–7.50 mg/ml (2.5–375.0 μM). It is also important to mention that this pattern is observed even at a very short reaction time (1 min) and remains constant during the studied period. The kinetics of the cross-linking can be followed only when very low protein and reagent concentrations are used (Fig. 2E). In some cases, particularly at low protein concentration and high reagent concentration, the observed bands are split into several sub-bands of equal or higher electrophoretic mobility (equal or lower apparent molecular mass). This has also been observed for other cross-linked proteins and it was interpreted in terms of the presence of polypeptide chains not completely unfolded by SDS because of the stabilizing effect of the cross-linker [29]. Increase of the SDS concentration in the electrophoresis did not alter the pattern, thus suggesting that a large number of intrachain cross-links is formed under such conditions avoiding saturation of the polypeptides by the detergent. Higher molecular weight bands are observed when the protein concentration and/or the reaction time is increased. At protein concentrations higher than 1.5 mg/ml and reaction times longer than 5 min, the cross-linking reaction results in the production of some insoluble material. The characteristic cross-linking pattern of Stn-II is observed even in the presence of 3 M guanidinium hydrochloride. An increase of the denaturant concentration abolishes the cross-linking. This fact is related to protein unfolding, which occurs at concentrations higher than 4 M guanidinium hydrochloride as revealed by circular dichroism measurements (Fig. 2F).

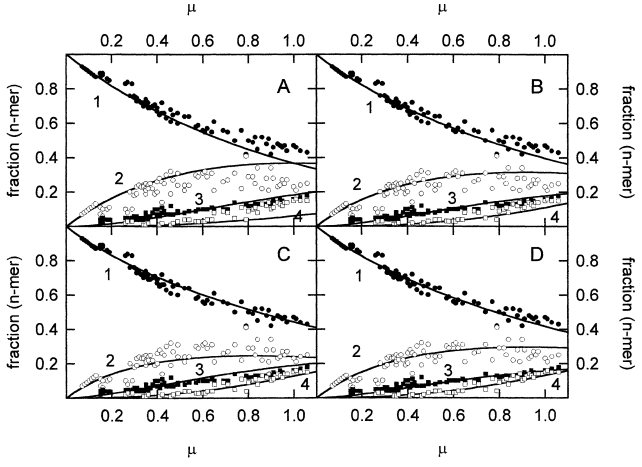

Fig. 3. Statistical composition of the mixture of (1) monomer, (2) dimer, (3) trimer and (4) tetramer after theoretical cross-linking of a protein composed of four polypeptide chains, assuming: (B) a linear arrangement; (C) a tetrahedral arrangement; (D) a planar arrangement with C_4 symmetry (a planar arrangement with D_2 symmetry, p and 0.8 p being the probabilities of cross-linking at the two different interfaces [29], would render similar results). The results obtained for (A) a random collisional cross-linking [30] are also included. The plots (continuous lines) represent the relative amount of the n-mer compound (fraction n-mer) versus μ, the overall degree of cross-linking, calculated as $\mu = \Sigma_n\ (n-1) \times [\text{fraction}_{(n-\text{mer})}]$ [30]. The experimental results obtained from the cross-linking experiments with Stn-II for (●) monomer, (○) dimer, (■) trimer, and (□) tetramer are included. The relative amount of each n-mer compound was calculated from the volumetric analysis of the image of either the Coomassie blue- or silver-stained SDS-polyacrylamide gels of the cross-linked samples at different protein and reagent concentrations and reaction times. The best fit between theoretical and experimental results is observed in panel D.

The cross-linking results were analyzed using model calculations based on different potential arrangements of four monomers and the probabilities of forming cross-links within each oligomeric structure [29,30]. Fig. 3 summarizes this analysis. Linear, tetrahedral and cyclic planar arrangements were compared with the theoretical distribution resulting from a random collisional cross-linking. Random collisional cross-linking between monomeric proteins may be of significance at high protein concentrations. For instance, it must be considered for membrane-bound proteins since they can exist at sufficiently high local densities. However, this phenomenon should not be of significance for proteins in aqueous solution at low concentrations. Nevertheless, we also considered this possibility in the model calculations for comparison. The experimental results show the best fit to a planar arrangement of the four protein monomers, C_4 symmetry, although a D_2 symmetry, in which the molecule behaves as two pairs of subunits, with a small difference between the probabilities of cross-linking at the two different interfaces cannot be discarded (Fig. 3).

Completion of the cross-linking process so that only the highest order oligomer is detected (in this case the tetramer) is not always obtained even for well established tetramers, because at a high cross-linker concentration the probability of finding two free reacting target residues within the protein would be very low. It is important to note that an intermolecular cross-link requires two free ε-amino groups, each of them in a different monomer. The case of cross-linking of a monomer to tetramer associating protein such as Stn-II is more complicated. The intermolecular cross-linking within the tetramer would shift the mass equilibrium towards the associated form since the cross-linked monomers cannot dissociate; however, intramolecularly cross-linked monomers could not participate in intermolecular reactions as mentioned above. Regarding this, it is known that chemical modification of amino acid side chains can lead to dissociation of multisubunit proteins [31]. Thus, as the cross-linker concentration increases, the extent of the Stn-II cross-linking should decrease for a fixed protein concentration, which is precisely the observed behavior (Fig. 2D). The parallel trend obtained for the two different reagents somehow eliminates the possibility of particular details of the chemical reactions being responsible for this behavior, pointing to an intrinsic property of Stn-II.

Chemical cross-linking (with dimethyl suberimidate in PBS buffer at pH 9.0) experiments performed with equinatoxin II from *A. equina* have revealed the existence of protein bands corresponding to up to seven monomers in the presence of small unilamellar vesicles composed of sphingomyelin/phosphatidylcholine (4:1 molar ratio) [10]. Analysis of the relative probability of occurrence of each oligomeric state for the membrane-bound protein revealed the best fit for a model of a preexisting tetramer [10]. No protein aggregate of size larger than two was observed in the absence of membranes [10]. This discrepancy with the cross-linking observed for Stn-

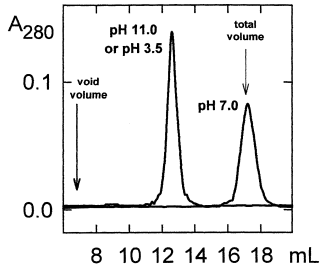

Fig. 4. Elution profile of Stn-II on a Superdex 75 column at pH 7.0 and 11.0. The flow rate was 1 ml/min. The absorbance at 280 nm (A_{280}) was recorded by a continuous flow spectrophotometer (Pharmacia FPLC chromatographic system). The arrows indicate the position of both void and total volume of the column employed, which was calibrated with selected marker proteins of known molecular mass.

II may be related to the pH employed for equinatoxin II (pH 9.0), in opposition to the pH used in this case (pH 8.0), since it has been reported that equinatoxin II may exist in a slightly altered conformation at around pH 9.0 [32]. However, it should also be emphasized that at pH 9.0 the chemical reactivity of the ε-amino group is higher than at pH 8.0 because the reactive form is the de-protonated species and, in addition, such an experiment was performed at 5.5 mM reagent concentration, conditions under which the extent of the intermolecular cross-linking would be very low, as discussed above, if equinatoxin II also behaves as an associating protein. In addition, dimethylsuberimidate usually renders lower yields of cross-linked products due to the competition between hydrolysis of the free imidoester group and cross-linking at each reagent molecule bound to a lysine amino group [33].

Stn-II has been considered to be a single polypeptide chain protein based on the results obtained from SDS-polyacrylamide gel electrophoresis. Gel filtration experiments with the native protein failed to give valuable results due to a non-specific interaction with the chromatographic matrix, resulting in an abnormal retardation of the protein. Fig. 4 shows the elution profile of native Stn-II from chromatography on Superdex-75. Protein elutes at the total volume of the column. Similar results have been obtained with other molecular exclusion chromatographic beds (Sephadex, Sepharose, Ultrogel, BioGel, HPLC-Ultraspherogel; data not shown). Such an abnormal behavior is abolished at pH 11.0 or 3.5. In fact, the protein elutes at a position corresponding to a molecular mass of 19 kDa at these extreme pH values (Fig. 4).

The obtained results demonstrate that Stn-II behaves as a monomer-tetramer associating system. The interaction with lipid membranes would promote an increased protein concentration at the bilayer surface favoring the tetrameric form of the protein, this form being responsible for the cytolytic properties of the protein as all the kinetic data of the membrane permeabilization suggested. The properties of the pore may be due to the particular structure of the tetramer. The different susceptibility of lipid vesicles with different composition to be permeabilized may be explained in terms of the lateral diffusion of the protein in the plane of the membrane.

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References

[1] Bernheimer, A.E. and Avigad, L.S. (1976) Proc. Natl. Acad. Sci. USA 73, 467–471.
[2] Harvey, H.L. (1990) in: Handbook of Toxicology (Shier, W.T. and Mebs, D., Eds.), pp. 1–66, Marcel Dekker, New York.
[3] Simpson, R.J., Reid, G.E., Moritz, R.L., Morton, C. and Norton, R.S. (1990) Eur. J. Biochem. 190, 319–328.
[4] Belmonte, G., Menestrina, G., Pederzolli, C., Krizaj, L., Gubensek, F., Turk, T. and Macek, P. (1994) Biochim. Biophys. Acta 1192, 197–204.
[5] De los Rios, V., Mancheño, J.M., Lanio, M.E., Oñaderra, M. and Gavilanes, J.G. (1998) Eur. J. Biochem. 252, 284–289.
[6] Turk, T. (1991) J. Toxicol. Toxin Rev. 10, 223–262.
[7] Kem, W.R. (1988) in: The Biology of Nematocysts (Hessinger, D.A. and Lenhoff, H.M., Eds.), pp. 375–405, Academic Press, New York.
[8] Bernheimer, A.W. (1990) in Marine Toxins: Origin, Structure and Molecular Pharmacology (Hall, S. and Stritcharz, G., Eds.), pp. 304–311, A.T.S. Symposium Series 418, American Chemical Society, Washington, DC.
[9] Zorec, R., Tester, M., Macek, P. and Mason, W.T. (1990) J. Membr. Biol. 118, 243–249.
[10] Belmonte, G., Pederzolli, C., Macek, P. and Menestrina, G. (1993) J. Membr. Biol. 131, 11–22.
[11] Macez, P., Belmonte, G., Pederzolli, C. and Menestrina, G. (1994) Toxicology 87, 205–227.
[12] Tejuca, M., Dalla Serra, M., Ferreras, M., Lanio, M.E. and Menestrina, G. (1996) Biochemistry 35, 14947–14957.
[13] Varanda, A. and Finkelstein, A. (1980) J. Membr. Biol. 55, 203–211.
[14] Minton, A.P. (1994) in: Modern Analytical Ultracentrifugation (Schuster, T.M. and Sauer, T.M., Eds.), pp. 81–93, Birkhäuser, Boston MA.
[15] Laue, T.M., Shah, B.D., Ridgeway, T.M. and Pelletier, S.L. (1992) in: Analytical Ultracentrifugation in Biochemistry and Polymer Science (Harding, S.E., Rowe, A.J. and Horton, J.C., Eds.), pp. 90–125, Royal Society of Chemistry, Cambridge.
[16] Muramatsu, N. and Minton, A.P. (1989) J. Mol. Recognit. 4, 166–171.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Chelliah, J. and Jones, D. (1990) J. Gen. Virol. 71, 2353–2359.
[19] Ralston, G. (1993) in: Introduction to Analytical Ultracentrifugation, Beckman Instruments Inc., Palo Alto, CA.
[20] Abril, A.M., Salas, M., Andreu, J.M., Hermoso, J.M. and Rivas, G. (1997) Biochemistry 36, 11901–11908.
[21] Morera, V., Gómez, J., Besada, V., Estrada, R., Pons, T., Álvarez, C., Tejuca, M., Padrón, G., Lanio, M.E. and Pazos, F. (1994) Adv. Mod. Biotechnol. 2, 135.
[22] Davies, G.E. and Stark, G.R. (1970) Proc. Natl. Acad. Sci. USA 66, 651–656.
[23] Hermann, R., Rudolph, R. and Jaenicke, R. (1979) Nature 277, 243–245.
[24] Hermann, R., Jaenicke, R. and Rudolph, R. (1981) Biochemistry 20, 5195–5201.
[25] Burns, D.L. and Schuchman, H.K. (1982) J. Biol. Chem. 257, 8638–8647.
[26] Hermann, R., Rudolph, R., Jaenicke, R., Price, N.C. and Scobbie, A. (1983) J. Biol. Chem. 258, 11014–11019.
[27] Jaenicke, R. and Rudolph, R. (1986) Methods Enzymol. 131, 218–250.
[28] Salmany, J.M., Sloan, R.L. and Cordes, K.A. (1990) J. Biol. Chem. 265, 17688–17693.
[29] Hucho, F., Müllner, H. and Sund, H. (1975) Eur. J. Biochem. 59, 79–87.
[30] Downer, N.W. (1985) Biophys. J. 47, 285–293.
[31] Price, N.C. (1993) in: Mechanisms of Protein Folding (Pain, R.H., Ed.), pp. 160–193, Oxford University Press, Oxford.
[32] Malavasic, M., Poklar, N., Macek, P. and Vesnaver, G. (1996) Biochim. Biophys. Acta 1286, 65–72.
[33] Staros, J.V. (1982) Biochemistry 21, 3950–3955.