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Matrix-assisted *in vitro* refolding of *Pseudomonas aeruginosa* class II polyhydroxyalkanoate synthase from inclusion bodies produced in recombinant *Escherichia coli*

Bernd H. A. REHM*[1], Qingsheng QI*, Br. Bernd BEERMANN†, Hans-Jürgen HINZ† and Alexander STEINBÜCHEL*

*Institut für Mikrobiologie der Westfälischen Wilhelms-Universität Münster, Corrensstrasse 3, 48149 Münster, Germany, and †Institut für Physikalische Chemie der Westfälischen Wilhelms-Universität Münster, Schlossplatz 3, 48149 Münster, Germany

In order to facilitate the large-scale preparation of active class II polyhydroxyalkanoate (PHA) synthase, we constructed a vector pT7-7 derivative that contains a modified *phaC1* gene encoding a PHA synthase from *Pseudomonas aeruginosa* possessing six N-terminally fused histidine residues. Overexpression of this *phaC1* gene under control of the strong Ø10 promoter was achieved in *Escherichia coli* BL21(DE3). The fusion protein was deposited as inactive inclusion bodies in recombinant *E. coli*, and contributed approx. 30 % of total protein. The inclusion bodies were purified by selective solubilization, resulting in approx. 70–80 % pure PHA synthase, then dissolved and denatured by 6 M guanidine hydrochloride. The denatured PHA synthase was reversibly immobilized on a Ni^{2+}-nitrilotriacetate–agarose matrix. The matrix-bound fusion protein was refolded by gradual removal of the chaotropic reagent. This procedure avoided the aggregation of folding intermediates which often decreases the efficiency of refolding experiments. Finally, the refolded fusion protein was eluted with imidazole. The purified and refolded PHA synthase protein showed a specific enzyme activity of 10.8 m-units/mg employing (*R*/*S*)-3-hydroxydecanoyl-CoA as substrate, which corresponds to 27 % of the maximum specific activity of the native enzyme. The refolding of the enzyme was confirmed by CD spectroscopy. Deconvolution of the spectrum resulted in the following secondary structure prediction: 10 % α-helix, 50 % β-sheet and 40 % random coil. Gel filtration chromatography indicated an apparent molecular mass of 69 kDa for the refolded PHA synthase. However, light-scattering analysis of a 10-fold concentrated sample indicated a molecular mass of 128 kDa. These data suggest that the class II PHA synthase is present in an equilibrium of monomer and dimer.

Key words: PhaC1, PHA biosynthesis, PHA synthase, polyhydroxyalkanoic acid.

INTRODUCTION

Polyhydroxyalkanoate (PHA) synthases are the key enzymes of PHA biosynthesis, and catalyse the conversion of 3-hydroxyacyl-CoA substrates into PHAs with the concomitant release of CoA. More than 40 different PHA synthases have been cloned and assigned [1]. The multiple alignment of the primary structures of these PHA synthases shows an overall identity of 21–88 %, with only eight strictly conserved amino acid residues. PHA synthases have been assigned to three classes based on their substrate specificity and subunit composition. The class I PHA synthases, with *Ralstonia eutropha* synthase as prototype, are composed of a single type of polypeptide chain and use mainly (*R*)-3-hydroxybutyryl-CoA, (*R*)-3-hydoxyvaleryl-CoA and other short-carbon-chain-length hydroxyalkanoic acid CoA thioesters as substrates. The class III PHA synthases, as represented by the *Allochromatium vinosum* enzyme, are composed of two different subunits, each of approx. 40 kDa [2]. The substrate specificity is similar to that of class I synthases, although some medium-chain-length 3-hydroxyfatty acids are incorporated [3]. Both types of PHA synthase have been purified, and *in vitro* activity has been demonstrated [4–6]. The catalytic reaction mechanism of these enzymes has been studied intensively. The post-translational modification of a conserved serine residue with 4-phosphopantetheine described for class I PHA synthase from *R. eutropha*, which was supposed to provide a catalytically active SH group, was not supported by studies of the enzyme in the natural host [7]. Mutagenesis studies with the *A. vinosum* PHA synthase revealed that His-331 is the general base catalyst that activates the nucleophile Cys-149 for covalent catalysis. Asp-302 is assumed to function as a general base catalyst by deprotonating the 3-hydroxy group of 3-hydroxybutyryl-CoA or that of the bound 3-hydroxybutyrate to render possible the nucleophilic attack on the covalently linked thiol ester intermediate [8].

The class II enzymes which, like class I PHA synthases, are composed of only one type of subunit, are found mainly in pseudomonads such as, for example, *Pseudomonas aeruginosa*. One major difference between class II and both class I and class III PHA synthases is the substrate specificity. Class II PHA synthases incorporate preferentially 3-hydroxyfatty acids of medium chain length (C_6–C_{14}) into PHA, and the resulting product is a latex-like polymer [9–12]. *In vivo* these substrates are derived mainly from intermediates of fatty acid β-oxidation [10,12,13] or from fatty acid *de novo* biosynthesis [14,15], provided that fatty acids or simple non-related carbon sources (e.g. carbohydrates) respectively are available. Only recently have class II PHA synthases been purified by immobilized metal chelate affinity chromatography and *in vitro* activity been achieved [16]. Ren et al. [17] were able to produce the PHA synthase from *P. oleovorans* simultaneously as active soluble

Abbreviations used: PHA, polyhydroxyalkanoate; Ni^{2+}-NTA, Ni^{2+}-nitrilotriacetate; DLS, dynamic light scattering.

[1] To whom correspondence should be addressed (e-mail rehm@uni-muenster.de).

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enzyme and as inactive aggregates in recombinant *P. putida*. However, they were not able to refold the PHA synthase from these aggregates solublized with 8 M urea, whereas activation and solubilization of the aggregated enzyme was achieved by the addition of S-Sepharose. However, the efficiency of these purification protocols was rather low, due to the low expression level and weak binding of the native His_6-tagged fusion protein to Ni^{2+}-nitrilotriacetate (Ni^{2+}-NTA)–agarose and to the low recovery (10 %) of the aggregated enzyme [16,17]. In order to achieve a more detailed understanding of the biochemical and enzymological mechanism of class II PHA synthases, and to establish a new enzyme system for the *in vitro* synthesis of novel polyesters, we have overproduced His_6-tagged PhaC1 (class II PHA synthase from *P. aeruginosa*) by applying the strong T7 RNA polymerase promoter Ø10, purified and refolded the protein from inclusion bodies by matrix-assisted procedures, and assessed its activity and its secondary and quaternary structure.

EXPERIMENTAL

Bacterial strains, plasmids and growth of bacteria

Pseudomonas aeruginosa A.T.C.C. 15692 (wild type, *alg*$^-$) was used to prepare genomic DNA, and *Escherichia coli* JM109 [*e14*$^-$ (*mcrA*) *recA1 endA1 gyrA96 thi-1 hsdR17* (r_k^-, m_k^+) *supE44 relA1* Δ(*lac-proAB*) (*Fí traD36 proAB lacI*^{q} *Z*Δ*M15*)] was used for cloning and plasmid isolation [18]. Strain *E. coli* BL21(DE3), containing the T7 RNA polymerase under *lacUV5* promoter control integrated in the chromosome, was used for overexpression and production of inclusion bodes [19]. *E. coli* LS1298 (*fadB*) and *E. coli* RS3097 (*fadR*) were used to determine the *in vivo* activity of class II PHA synthase. *E. coli* was grown at 37 °C in Luria–Bertani medium. *P. aeruginosa* was grown at 30 °C either in nutrient broth complex medium (0.8 %, w/v) or in a mineral salts medium with 0.05 % (w/v) ammonium chloride. Vector pT7-7 served as expression vector [19].

Isolation, analysis and manipulation of DNA

All genetic procedures and manipulations of DNA were conducted as described by Sambrook et al. [18]. DNA sequencing was carried out by the dideoxy chain-termination method with single-stranded or double-stranded alkali-denatured plasmid DNA, but with 7-deazaguanosine 5′-triphosphate instead of dGTP. The coding region of the PHA synthase gene *phaC1* from *P. aeruginosa* was amplified by tailored PCR, introducing restriction sites for *Nde*I and *Bam*HI at the 5′ and 3′ ends respectively. The following oligonucleotides were used: 5′-GG-GGAAGACATATGCATCACCATCACCATCACAGTCAG-AAG AACAATAACAGC-3′ (encoding the N-terminus plus the His_6 tag and an *Nde*I site) and 5′-AAACGCGGATCCTT-TTCATCGTTCATGCA-3′ (encoding the C-terminus including a *Bam*HI site). The resulting PCR product was inserted into the corresponding restriction sites of vector pT7-7, resulting in plasmid pQQ7, under the control of T7 RNA polymerase promoter Ø10. *E. coli* BL21(DE3) was used to express the gene encoding the His_6-tagged fusion protein.

Isolation and purification of inclusion bodies

Cells were dissolved in 50 mM phosphate buffer, pH 8.0, containing lysozyme and DNase I, and incubated on ice for 30 min. Then EDTA was added to a final concentration of 10 mM and the cells were incubated on ice for another 20 min. After French press treatment, the crude extract was centrifuged at 10000 ***g*** for 20 min to obtain the insoluble fraction. The resulting sediment was suspended in 50 mM phosphate buffer, pH 8.0, containing 0.15 M NaCl and 1 mM EDTA. After incubation on ice for 20 min, the inclusion bodies were sedimented as described above, and were then washed in phosphate buffer containing 2 M urea and 0.5 % Triton X-100. Finally, the purified inclusion bodies were dissolved in phosphate buffer containing 6 M guanidine hydrochloride and 3 mM 2-mercaptoethanol. The guanidine hydrochloride-insoluble materials were removed by centrifugation at 10000 ***g*** for 10 min.

Matrix-assisted refolding and purification of the class II PHA synthase

The dissolved protein was subjected to metal chelate affinity chromatography using a Ni^{2+}-NTA resin (Qiagen, Hilden, Germany) with a bed volume of 5 ml. It was equilibrated on an FPLC apparatus (Pharmacia) with 50 mM phosphate buffer, pH 8.0, containing 300 mM NaCl, 5 mM imidazole, 8 M urea, 1 mM 2-mercaptoethanol and 10 % glycerol. Refolding was achieved by applying a gradient from 8 to 0 M urea at a flow rate of 0.1 ml/min in a total volume of 30–40 column volumes. The refolded enzyme was then eluted by increasing the imidazole concentration from 30 to 500 mM.

Analytical gel filtration chromatography

The native molecular mass of the refolded PHA synthase was estimated by gel filtration chromatography on a Superdex 200 HR 10/30 column (10 mm × 300 mm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl, at a flow rate of 1 ml/min. The analysed protein sample was taken directly from the refolding experiment; a sample of 4 ml was applied with a protein concentration of approx. 30 μg/ml. Protein in the eluate was monitored at A_{280}; fractions were collected and analysed by SDS/PAGE. For calibration, standard proteins (Amersham Pharmacia Biotech) were chromatographed under the same conditions.

Dynamic light scattering (DLS)

All light-scattering experiments were performed with a DynaPro molecular sizing instrument (Protein Solutions) at a fixed angle of 90°. The Dynamics 5.25.44 software package was used for data analysis. The translational diffusion coefficient (D_t) of the sample particles was determined by measuring the fluctuations in the intensity of scattered light with an autocorrelation function. The hydrodynamic radius (R_h) of the particles was calculated using the Stokes–Einstein equation ($D_t = k_b T / 6\pi\eta R_h$, where k_b is the Boltzmann constant, T is the absolute temperature and η is the solvent viscosity). Assuming that the particles are spherical and of standard density, the molecular mass of the particles was estimated from R_h. As the autocorrelation function was analysed initially with a bimodal exponential cumulant analysis (one particle size), the polydispersity and error gave an indication of the homogeneity of particle sizes in the solution.

All protein solutions for DLS experiments were filtered through a 0.02 μm filter (Anodisc 13; Whatman). DLS of refolded PHA synthase was measured at 15 °C using a concentration of 0.3 mg·ml^{-1} in 50 mM potassium phosphate buffer (pH 8.0) containing 250 mM imidazole, 300 mM NaCl and 10 % (v/v) glycerol; 50 independent measurements were acquired for each sample, and the reported values are the calculated arithmetic means.

SDS/PAGE and Western immunoblotting

SDS/PAGE was performed according to Sambrook et al. [18]. Proteins were separated in 12.5 % (w/v) polyacrylamide gels in

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the presence of SDS and stained with Coomassie Brilliant Blue R-250. Western blotting was performed using the Semidry Fastblott (Biometra, Göttingen, Germany). On Western blots [20] using nitrocellulose membranes, PhaC1 from *P. aeruginosa* was detected by applying a monospecific polyclonal anti-PhaC1 antiserum and an alkaline-phosphatase–antibody conjugate as second antibody. Bound antibodies were detected using Nitro Blue Tetrazolium chloride and the toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate.

Class II PHA synthase activity *in vivo*

PHA synthase activity was confirmed by expression of the respective PHA synthase gene in various metabolic backgrounds favouring PHA_{MCL} synthesis, e.g. *E. coli* RS3097 (only in the presence of the β-oxidation inhibitor acrylic acid) and *E. coli* LS1298 [16]. Recombinant bacteria harbouring the respective plasmid were cultivated in the presence of 0.25% (w/v) decanoate. PHA accumulation was determined by GC–MS analysis of lyophilized cells, and indicated PHA synthase activity *in vivo*.

GC–MS of polyester in cells

PHA was analysed qualitatively and quantitatively by GC–MS. Liquid cultures were centrifuged at 10000 ***g*** for 15 min, and then the cells were washed twice in saline and lyophilized overnight. An aliquot of 8–10 mg of lyophilized cell material was subjected to methanolysis in the presence of 15% (v/v) sulphuric acid. The resulting methyl esters of the constituent 3-hydroxyalkanoic acids were assayed by GC–MS, as described previously [9]. GC analysis was performed by injecting 3 μl of sample into a Hewlett-Packard 6890 gas chromatograph/mass spectrometer (Hewlett-Packard, Palo Alto, CA, U.S.A.). The same column as for the GC analysis was applied using a temperature profile as described previously [9].

Analysis of (*R*,*S*)-3-hydroxydecanoyl-CoA

The substrate (*R*,*S*)-3-hydroxydecanoyl-CoA was synthesized as described previously [14]. (*R*,*S*)-3-Hydroxydecanoyl-CoA was purified using Sep-Pak cartridges (reverse-phase C18 column; Waters) and by eluting the CoA thioester with 0.01 M NaOH in 20% (v/v) methanol. The purified (*R*,*S*)-3-hydroxydecanoyl-CoA was analysed by HPLC, and its concentration was determined by hydroxylamine treatment, which causes the release of bound CoA. The concentrations of free CoA before and after hydroxylamine treatment [21] were analysed by the Ellman method [22].

Class II PHA synthase assay *in vitro*

The activity of class II PHA synthase *in vitro* using (*R*,*S*)-3-hydroxydecanoyl-CoA as substrate was determined as described previously [16].

CD spectroscopy

UV absorption

The protein concentration was determined after dialysis and final centrifugation by absorption measurements at 280 nm using a diode array spectrophotometer X-Dap (1024) from IKS. The molar absorption coefficient ($\epsilon = 0.6061\ \mathrm{ml \cdot mg^{-1} \cdot cm^{-1}}$) for the PhaC1 fusion protein was calculated according to Pace et al. [23].

CD

CD spectra were recorded on a Jobin-Yvon (Paris, France) model CD6 spectropolarimeter equipped with a custom-made peltier element cuvette holder for temperature control.

The fusion protein was dialysed against a solution of 50 mM KCl, of pH 7.2 if not indicated otherwise. The spectra were recorded at 20 °C. The protein concentrations used ranged from 49 to 256 μg/ml. For concentrations below 150 μg/ml, a 0.5 mm quartz cuvette was used, and for higher concentrations the cuvette path length was 0.1 mm.

The results were expressed as mean residue ellipticity, $[\Theta]_{MRE} = (0.1 \cdot \mathrm{MRE} \cdot \theta_{obs}/c \cdot d)$, where θ_{obs} is the observed ellipticity (m-degrees), MRW is the mean residue weight of PhaC1 fusion protein (10672 Da), d is the cuvette path length (cm) and c is the protein concentration (mg/ml). Deconvolution of CD spectra was carried out using the program 'CDNN' described by Bohm et al. [24].

RESULTS

Overexpression of the *P. aeruginosa* type II PHA synthase gene

The expression of the PHA synthase gene *phaC1* under the control of the *lac* promoter was weak, and resulted in PHA synthase production that represented only 5% of total protein [16]. Therefore we employed the expression vector pT7-7 to obtain overexpression under control of the Ø10 promoter. The plasmid pQQ7 was constructed, which contained the coding region encoding the His_6-tagged fusion protein co-linear to the Ø10 promoter in restriction sites *Nde*I and *Bam*HI, as described in detail in the Experimental section. The plasmid was transferred into *E. coli* BL21(DE3) and, upon induction of the T7 RNA polymerase gene with 1 mM isopropyl β-D-thiogalactoside, overproduction of the His_6-tagged fusion protein was observed, which amounted to approx. 30% of total cell protein (Figure 1).

The His_6-tagged fusion protein occurred as inactive inclusion bodies. This was concluded from microscopic observations of light-scattering inclusions in cells, as well as from the presence of isolated insoluble fusion protein. No activity of the over-

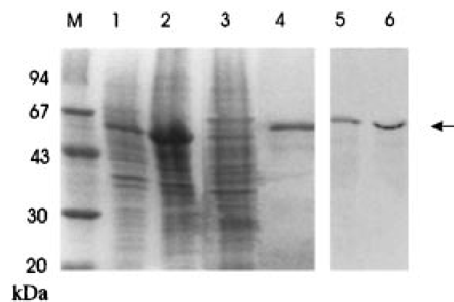

Figure 1 SDS/PAGE analysis and Western immunoblotting of the overproduced, refolded and purified PHA synthase fusion protein

Lane M, molecular mass standards; lane 1, crude extract of *E. coli* BL21(DE3) harbouring pQQ7; lane 2, purified inclusion bodies from *E. coli* BL21(DE3) harbouring pQQ7; lane 3, crude extract of *E. coli* BL21(DE3) habouring pT7-7 (negative control); lane 4, refolded and purified PHA synthase fusion protein; lane 5, immunoblot of crude extract of *E. coli* BL21(DE3) harbouring pQQ7; lane 6, immunoblot of refolded and purified PHA synthase fusion protein. Monospecific anti-PhaC1 antibodies were applied. The arrowhead indicates the position of the PHA synthase fusion protein.

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Table 1 Refolding and purification of class II PHA synthase

Abbreviation: nd, not detectable.

Fraction	Protein concentration (mg/ml)	Total protein (mg)	Estimated PHA synthase content (%)	PHA synthase activity (m-units/mg)
Crude extract	8.4	42	~30	nd
Inclusion bodies	2.2	5.5	70–80	nd
Purified PHA synthase	0.13	1.2	>95	10.8

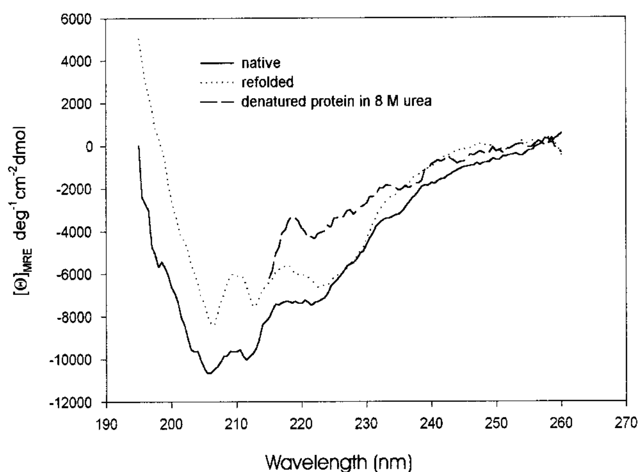

Figure 2 CD spectroscopic analysis of native, refolded and denatured PHA synthase fusion proteins

Spectra were recorded from 195 to 260 nm with the native and refolded proteins, and from 212 to 260 nm in the case of the denatured protein. Measurements were carried out at 20 °C in 50 mM KCl solution, pH 7.2. For denaturation, 8 M urea was added to the KCl solution. Concentrations were 49 μg/ml for the native, 157 μg/ml for the denatured and 256 μg/ml for the refolded PhaC1 fusion proteins. Protein concentrations above 150 μg/ml were measured in a 0.1 mm cuvette, whereas for lower concentrations a path length of 0.5 mm was used. All spectra were recorded five times, averaged and smoothed, taking the mean of five data points. The integration time was 1 s per data point.

produced fusion protein could be obtained *in vivo*, as concluded from the absence of PHA accumulation, as described previously [12]. For this test, fatty acids were provided as the carbon source, and the fatty acid β-oxidation pathway was inhibited with acrylic acid or a *fadB* mutant impaired in fatty acid β-oxidation was used, in order to provide substrates for class II PHA synthases. Enzymic activity is correlated with the accumulation of PHA, which can be determined by gas chromatographic analysis. Cells overproducing the fusion protein did not show any accumulation of PHA. Moreover, no enzymic activity was detected *in vitro* (Table 1)

Purification of PHA synthase inclusion bodies

Inclusion bodies from *E. coli* BL21(DE3) harbouring pQQ7 from a 100 ml culture were isolated and purified as described in the Experimental section. After the final washing of the inclusion bodies, a PHA synthase fusion protein with a purity of approx. 80 % was obtained (Figure 1). *E. coli* crude extracts containing the inclusion bodies and purified inclusion bodies were each subjected separately to *in vitro* enzymic activity analysis using (*R*,*S*)-3-hydroxydecanoyl-CoA as substrate, and no enzymic activity was detected in either case. The purified inclusion bodies were dissolved in 6 M guanidine hydrochloride and 3 mM 2-mercaptoethanol. The denatured PHA synthase fusion protein was then applied to metal chelate affinity purification. The matrix-bound fusion protein was subjected to a refolding process using a gradient from 8 to 0 M urea. A flat gradient was used to avoid protein aggregation. The fusion protein was finally eluted employing imidazole. The peak fraction at an imidazole concentration of approx. 200 mM was collected and subjected to SDS/PAGE analysis. Immunoblotting and SDS/PAGE analysis showed that the PHA synthase fusion protein was purified to homogeneity (Figure 1, Table 1). Spectroscopic analysis, however, revealed protein aggregation. Immediate separation of protein aggregates by centrifugation lowered the aggregation tendency of the remaining soluble fusion protein. The purified and partially refolded His_6-tagged PHA synthase was analysed for *in vitro* enzyme activity. A specific enzyme activity of 10.8 m-units/mg of protein was determined (Table 1).

CD spectroscopy of class II PHA synthase

In order to provide further evidence for correct refolding of the PHA synthase fusion protein, CD spectroscopy was employed. The fusion protein was purified both under native conditions, as described previously [16], and also following the protocol established in the present study using denatured protein for matrix-assisted refolding. To allow a comparison of the secondary structure, CD spectra of the purified native protein, the refolded protein and the denatured protein were recorded. The CD spectra of the native and refolded proteins were similar (Figure 2). These spectra show characteristic minima at 208 and 222 nm, indicative of α-helical structure, although the absolute mean residue ellipticity values did not indicate a significant amount of α-helicity. Rather, it appears that other conformational motifs, such as β-sheet and coiled elements, determine the structure. This is supported by the deconvolution of the spectra, which resulted in the following secondary structure composition: 10 % α-helix, 50 % β-sheet and 40 % random coil. The spectrum of the denatured protein in 8 M urea shows the typical random coil ellipticity. Due to the high amounts of urea, no significant data could be recorded at wavelengths below 212 nm. The lower absolute molar ellipticity of the refolded protein is indicative of a degree of refolding of less than 100 %. This is not unexpected for the refolding of high-molecular-mass proteins.

Analysis of the molecular mass of refolded class II PHA synthase

In order to analyse the molecular mass of the active and refolded class II PHA synthase PhaC1 from *P. aeruginosa*, we performed gel filtration chromatography and DLS analysis. PHA synthase obtained after matrix-assisted refolding at a protein concentration of approx. 0.1 mg/ml was subjected directly to gel filtration chromatography on Superdex 200. A single peak was obtained, which indicated an apparent molecular mass of 69 662 Da (results not shown), while the expected molecular mass of His_6-tagged PHA synthase is 63 346 Da. The refolded PHA

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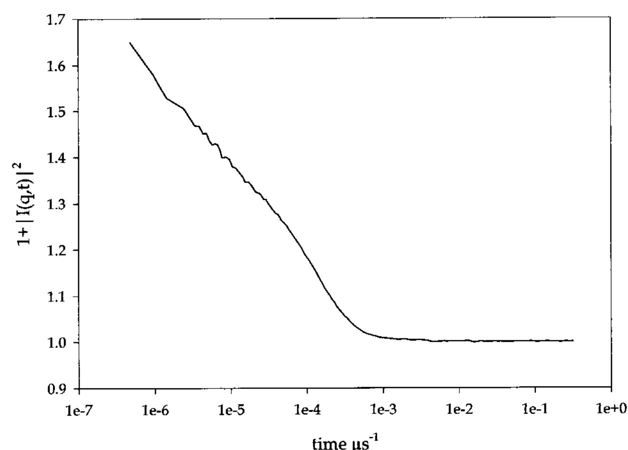

Figure 3 Autocorrelation function as measured by DLS on PHA synthase

Measurements were carried out at 15 °C in 10% glycerol, 300 mM NaCl, 250 mM imidazole and 50 mM potassium phosphate buffer (pH 8.0). The protein concentration (*c*) was 0.3 mg·ml^{-1}. The curve represents the mean values of 50 measurements.

synthase was concentrated 10-fold by applying ultrafiltration, resulting in a protein concentration of approx. 0.3 mg/ml. This sample was subjected to DLS analysis, which indicated a molecular mass of approx. 128 kDa (Figure 3).

DISCUSSION

In the present study, we describe for the first time the successful refolding of denatured solublized PHA synthase from inactive inclusion bodies. Purification of the enzyme under native conditions was impaired by the low level of expression of the PHA synthase gene [16], rendering the production of milligram amounts of active enzyme rather difficult. Therefore a new plasmid, pQQ7, a pT7-7 derivative, was constructed. This construct led to overexpression of PHA synthase fusion protein. However, the protein occurred in inactive insoluble inclusion bodies (Figure 1, Table 1). To obtain active enzyme, matrix-assisted refolding procedures were employed using a gradient of decreasing urea concentration. Matrix-assisted refolding was applied, because spatial separation of the refolding intermediates is known to prevent protein aggregation [25]. Moreover, matrix-assisted refolding has also been successful for membrane proteins, which might resemble PHA synthases in their association with phospholipids [26]. It was observed that, directly after elution from the column, the refolded protein showed a tendency to aggregate. When these aggregates were removed by ultracentrifugation, the specific enzyme activity increased from 10 to 11 m-units/mg, and the enzyme remained soluble. Correct refolding was verified by measuring enzyme activity *in vitro* and CD spectra (Figure 2). Approx. 27% of the sample was refolded to active enzyme, as compared with the specific activity of the native enzyme [16]. In view of the molecular mass of approx. 65 kDa characteristic of the class II PHA synthases, this yield is reasonably high. There is a good chance that the present refolding and purification procedure could also be applied to other PHA synthases.

Ren et al. [17] succeeded in overproducing the class II PHA synthase from *P. oleovorans*, which represented 10–25% of total cell protein. Interestingly, the PHA synthase was distributed in active granule-bound enzymes and inactive insoluble aggregates. However, efforts to refold these aggregates after denaturation in 8 M urea using dialysis procedures failed. Moreover, 10% of the aggregated protein was recovered as active enzyme when S-Sepharose was added to the aggregates. The mechanism of this enzyme activation has not been analysed.

The deconvolution of the CD data resulted in a prediction of secondary structure of approx. 50% β-sheet. This value is consistent with a recently developed topological model for class II and class III PHA synthases, in which structural similarities to lipases were reported [8,27]. Determination of the molecular mass of the refolded PHA synthase indicated that at low PHA synthase concentrations the monomer is present, whereas at higher PHA synthase concentrations, as required for light scattering analysis, evidence for a dimeric organization was found (Figure 3). The autocorrelation function shown in Figure 3 can only be fitted using a bimodal function. The bimodal fitting procedure identified two species of significantly different hydrodynamic radii. One is obviously water ($R_H = 0.11$ nm). The second species was found to be the protein. It has a diffusion coefficient of 3.4×10^{-7} cm·s^{-2}, which corresponds to a hydrodynamic radius of 4.75 nm and a molecular mass of 129 kDa, assuming validity of the Stokes–Einstein equation. This molecular mass is slightly higher than that expected for a dimer of the PHA synthase (126 kDa). The hydrodynamic radius appears to be larger than the expected value, due to a strong tendency for aggregation of the PHA synthase under these conditions of protein concentration and buffer composition. This could be seen by the high polydisperity of ± 2.03 nm. Assuming an equilibrium constant of dimerization in the range 6×10^4 to 4×10^5 l·mol^{-1} the monomer would not be detectable in our instrument, as its concentration is below the minimum concentration of 0.215 mg·ml^{-1} required for a reliable signal. The dimer, on the other hand, would not be detectable in gel filtration, as its concentration would be below 5 μg·ml^{-1} at the applied total protein concentration of 30 μg·ml^{-1}. This explanation provides a rationale for the different molecular masses observed by gel filtration and DLS. These data are consistent with the finding that the class I PHA synthase from *R. eutropha* is presumably active as a dimer [7,28]. However, it is still not clear why the refolded PHA synthase achieved only 27% of the maximum specific activity (obtained with the native enzyme) at low protein concentrations.

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