Enhanced Hydrolytic Stability of Short-Chain Poly[(*R*)-3-hydroxybutyrate] Conjugated to Native *E. coli* Cytoplasmic Proteins

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Dedicated to Professor *Dieter Seebach* on the occasion of his 65th birthday

Many prokaryotic and eukaryotic proteins are modified by post-translational conjugation to short-chain poly[(*R*)-3-hydroxybutyrate] (cPHB). The relative lability of ester bonds raises the concern that the cPHB may be substantially degraded by chemical hydrolysis during protein purification, thus increasing the difficulty of its detection and measurement. Here, we compare rates of acid- and base-catalyzed hydrolysis of cPHB conjugated to native and denatured proteins at room temperature. *E. coli* cytoplasmic proteins, native or denatured by addition of guanidium hydrochloride, were treated with aqueous solutions of H_2SO_4 or NaOH at concentrations ranging from 0.1 – 2.0N. The loss of cPHB was measured as a function of time by a chemical assay. We find that cPHB conjugated to native proteins is surprisingly resistant to both acid- and base-catalyzed hydrolysis, whereas cPHB conjugated to denatured proteins is proficiently degraded at rates proportional to acid or base concentration. The results suggest that cPHB occupies a highly protective environment within native proteins.

1. Introduction. – Poly[(*R*)-3-hydroxybutyrate] (PHB) is a linear polymer of (*R*)-3-hydroxybutyrate, a common metabolic intermediate derived from acetate [1]. High molecular weight PHB (>60,000 residues) is synthesized and stored in cytoplasmic storage granules only by certain prokaryotes, but short-chain PHB (<200 residues) is a ubiquitous constituent of both prokaryotic and eukaryotic cells [2]. In all cases studied to date, the short-chain polyester is complexed to other macromolecules, chiefly proteins, and, thus, it is referred to as complexed PHB or cPHB.

cPHB is a linear, amphiphilic, highly flexible polyester [3] with salt-solvating properties [4]. These molecular characteristics suggest that conjugation to cPHB may significantly influence protein structure and function. In a corresponding fashion, the protein may modify the properties of cPHB. In *Escherichia coli*, over 1% of the proteins contain cPHB [5]; the cPHB-conjugated proteins are located in all cell fractions, but more than 90% are in the cytoplasm, and these are predominantly in the ribosomal fraction (>70%).

Modification of proteins by conjugation to cPHB has escaped notice until recently. This may be partly attributed to its simple molecular structure, which lacks unusual atoms or functional groups and has no characteristic UV absorption. Moreover, cPHB tends to disintegrate during mass spectrometry, giving rise to a multiplicity of very low intensity peaks (unpublished observation), and is 'invisible' in X-ray crystallographic structures due to its extreme flexibility and molecular resemblance to crystallographic solvents such as polyethylene glycol.

The study of cPHB-conjugated proteins requires that the polyester remain as intact as possible during protein isolation. The greater lability of ester bonds compared to

amide bonds raises the concern that cPHB may be significantly degraded during purification procedures. In many protocols, the cPHB-proteins remain suspended in dilute solutions, sometimes in denaturing solvents, for long periods at room temperature. Since many cPHB-proteins have very low or very high pI values (unpublished observations), they may be maintained for substantial periods at an elevated or reduced pH.

In this study, we examined the chemical stability of cPHB conjugated to native, and, alternatively, to denatured proteins, in acidic or basic solutions at room temperature.

2. Results. – *Acid- and Base-Catalyzed Hydrolysis of cPHB Conjugated to Native Proteins.* The rate of hydrolysis of cPHB conjugated to cytoplasmic proteins was examined as a function of acid or base concentration at room temperature. *E. coli* JM109 stationary-phase cells were collected by centrifugation, suspended in 10 mM *Hepes* buffer, pH 7.4, and disrupted by ultrasonication. Unbroken cells and cell walls were removed by low-speed centrifugation, and plasma membranes were pelleted by ultracentrifugation. The supernatant, which contains the cytoplasmic fraction, was decanted and used for these studies.

H_2SO_4 or, alternatively, NaOH, at final concentrations ranging from 0.1 to 2N, was added to aqueous solutions of the native *E. coli* cytoplasmic proteins (2 mg) in a final volume of 0.5 ml, and the samples were incubated with stirring at room temperature. At selected times, the proteins were precipitated with cold 95% EtOH, dried by lyophilization, and the cPHB content was measured by a chemical assay in which cPHB is converted to its unique degradation product, crotonic acid, by heating in concentrated H_2SO_4 [5]. The crotonic acid was then separated by HPLC, detected by UV absorption, and quantified by peak area.

Figure, *a* and *b*, show the relative cPHB concentrations in native cytoplasmic proteins after incubation at room temperature in 0.1 to 1.0N H_2SO_4 and NaOH, respectively. The dashed lines at 100% in both *a* and *b* indicate that the cPHB concentration in native proteins is virtually unaffected over an 8-h period by even the strongest acid or base treatment studied. In fact, when the incubation time was

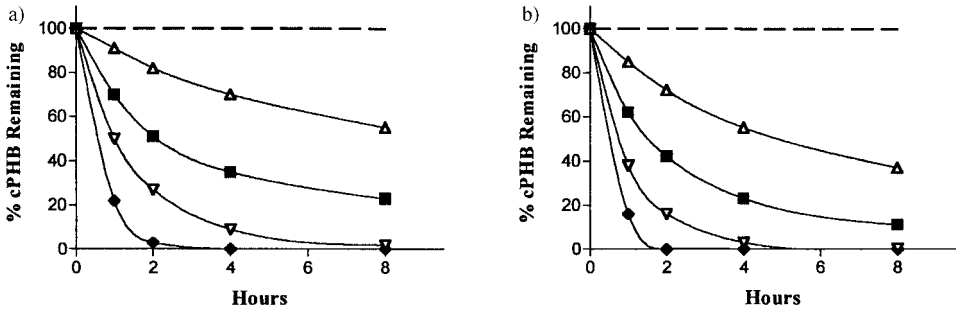

Figure. a) *Rates of hydrolysis of cPHB conjugated to cytoplasmic* E. coli *proteins at room temperature in H_2SO_4.* Native proteins in 0.1N, 0.5N, 1.0N, and 2.0N H_2SO_4 represented by the dashed line; denatured proteins: △: 0.1N; ■: 0.5N; ▽: 1.0N; ◆: 2.0N. b) *Rates of hydrolysis of cPHB conjugated to cytoplasmic* E. coli *proteins at room temperature in NaOH.* Native proteins in 0.1N, 0.5N, 1.0N and 2.0N NaOH represented by the dashed line; denatured proteins: △: 0.1N; ■: 0.5N; ▽: 1.0N; ◆: 2.0N.

extended, cPHB remained essentially unchanged for 24 h, either at room temperature or at 37°. This contrasts dramatically with the sensitivity to hydrolysis of cPHB in denatured protein, discussed below.

Acid- and Base-Catalyzed Hydrolysis of cPHB Conjugated cPHB to Denatured Proteins. E. coli cytoplasmic proteins were prepared in the same manner as described above and denatured by addition of guanidium hydrochloride to a final concentration of 6M. H_2SO_4, or alternatively NaOH, was added to solutions containing 2 mg of denatured protein at final concentrations ranging from 0.1 to 2.0N, and the samples were incubated with stirring at room temperature. As described above, samples were taken at selected times, the proteins were collected, dried, and the cPHB content was measured by chemical assay.

The sensitivity of cPHB conjugated to denatured cytoplasmic proteins to both acid- and base-catalyzed hydrolysis at room temperature is clearly shown in *Figure*, *a* and *b*, respectively. As may be expected, the rates are dependent on concentration, with hydrolysis significantly more efficient in basic solutions. cPHB conjugated to denatured proteins was completely hydrolyzed at room temperature after 3 h in 2N H_2SO_4 and 2 h in 2N NaOH. After 4 h at 0.1N concentrations, *ca.* 30% of cPHB is lost from denatured proteins in H_2SO_4 and *ca.* 45% in NaOH.

3. Discussion. – This study reveals a striking difference in the hydrolytic stability at room temperature of cPHB conjugated to native *vs.* denatured proteins. We find that cPHB in native proteins is highly resistant to hydrolysis during extended exposure to strong acids or bases, whereas cPHB in denatured proteins is proficiently hydrolyzed at rates proportional to acid or base concentration.

The observed hydrolytic stability of cPHB conjugated to native proteins in aqueous acid or base is consistent with the surprisingly slow rate at which cPHB conjugated to proteins undergoes β-elimination when heated in concentrated H_2SO_4 [5]. At 90°, uncomplexed PHB is converted to crotonic acid in 20 min, but 12 h are required for protein-conjugated cPHB under the same conditions (or 40 min at 120° as used in this study).

The results indicate that the protein creates an exceptionally protective environment for cPHB. As a H_2O-insoluble, amphiphilic homopolymer, cPHB likely resides within hydrophobic pockets. The extreme conformational flexibility of cPHB has been amply demonstrated by *Seebach et al.* by NMR spectroscopy, fluorescence-resonance-energy-transfer measurements, and molecular dynamics [3]. The Me groups of this amphiphilic polyester can lie against hydrophobic peptide segments, while the ester carbonyl O-atoms form H and/or coordinate bonds to amino acid side chains. The individual bonds may be weak, but collectively they establish a strong connection between the polyester and the protein. These noncovalent interactions with amino acid residues may diminish the reactivity of the ester groups. cPHB may also be protected from pH changes in its microenvironment by the buffering action of nearby amino acid residues. Although strong acids and bases may induce some denaturation, they do not appear to disrupt the close association between cPHB and protein. Strong denaturing agents, such as 6M guanidium chloride, succeed in breaking many of the noncovalent bonds between cPHB and protein, thus exposing the ester bonds to hydrolytic agents.

The results provide guidance in developing protocols for purification of cPHB-conjugated proteins. A number of cPHB-proteins have very low or high p*I* values (unpublished observation), and some of the current protocols for purification of such proteins call for extraction with strong acids or bases. The cPHB proteins may also be subjected to elevated or reduced pH for prolonged periods during HPLC or enzymatic digestions. From this study, one may conclude that cPHB-proteins should be maintained in the native state whenever possible, and that exposure to high or low pH should be strictly limited when denaturation cannot be avoided.

Possible roles of cPHB-conjugation may be inferred from the signal ability of cPHB to solvate salts [4]. This capacity derives from the spacing of the ester carbonyl O-atoms along the flexible backbone at suitable intervals to form multiple coordinate bonds to cations [6]). This polymeric solvent may solvate polyanionic salts such as inorganic polyphosphates [7] and polynucleotides [8] that reside in or migrate through hydrophobic pockets of proteins. It is notable in this regard that cPHB-proteins are predominantly in the ribosomal fraction, which includes the nucleoid. Taken together, these factors suggest that cPHB may assist proteins in their interactions with nucleic acids by providing specific hydrophobic regions with a hydrophilic coating formed by repetitive ester carbonyl O-atoms.

Experimental Part

Preparation of E. coli *Cytoplasmic Proteins. E. coli* JM101 were grown in *Luria Broth* at 37° in a water shaker bath to late stationary phase. Cells were collected by centrifugation and resuspended in 10 mM *KHepes*, pH 7.4. RNase-free DNase 1 was added (20 μg/ml) and the cells were disrupted by ultrasonication. Unbroken cells were removed by centrifugation at 10,000 rpm for 20 min at 4°. The supernatant was centrifuged at 35,000 rpm for 45 min at 4° in a *Ti 50* rotor, to pellet membranes and cell walls. The supernatant, containing the cytoplasmic fraction, was stored at −20°.

Hydrolysis of cPHB Conjugated to Protein. Cytoplasmic proteins (*ca.* 2 mg) were added to 15 ml of *Corex* tubes. The samples were diluted to 0.5 ml with dist. H_2O, or with H_2SO_4 or NaOH solns. to give the designated concentrations. The samples were incubated with stirring at r.t. At designated times, the proteins were precipitated by addition of 10 ml ice-cold EtOH. The samples were maintained at −20° for 1 h, and the precipitate pelleted by centrifugation at 7000 rpm for 30 min. The supernatant was discarded, and the residue was dried by lyophilization. cPHB Content was then measured as described below.

Chemical Assay for Protein-Conjugated cPHB. The method used was a variation of the method of *Karr et al.* [9] as described in [5]. Conc. H_2SO_4 (0.5 ml) was added to the dried sample, the tube was covered with a glass marble and the samples were heated in a dry heating block (*Pierce Chem. Co.*) at 120° for 40 min. The samples were cooled on ice, diluted by addition of 2 volumes of sat. Na_2SO_4, and extracted 3 times with 2 volumes of CH_2Cl_2. NaOH (100 μl, 5N) was added to the extracts to convert crotonic acid to its Na salt. CH_2Cl_2 was removed by evaporation with a stream of dry N_2, and the samples were stored at −20°. Before chromatography, the samples were acidified to pH 2 with 5N H_2SO_4. An aliquot of the sample was chromatographed on an *Aminex HPX-87 H* column (*Bio-Rad*) with 0.014N H_2SO_4 as eluent. The crotonic acid peak was identified by comparison of elution time with that of commercial samples of crotonic acid (*Aldrich*), by its UV spectrum and by mass spectrometry, and quantified by comparison of peak area with that of crotonic acid standards and with crotonic acid produced by commercial samples of PHB (*Aldrich*) treated according to the same protocol.

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