Posttranslational modification of *E. coli* histone-like protein H-NS and bovine histones by short-chain poly-(R)-3-hydroxybutyrate (cPHB)

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Abstract Short-chain poly-(R)-3-hydroxybutyrate (cPHB), a highly flexible, amphiphilic molecule with salt-solvating properties, is a ubiquitous constituent of prokaryotic and eukaryotic cells, wherein it is mainly conjugated to proteins. The solvating properties and cellular distribution of cPHB suggest it may be associated with proteins that bind and/or transfer DNA. Here we examine *Escherichia coli* protein H-NS and calf thymus histones, H1, H2A, H2B, H3, and H4, for the presence of cPHB. The proteins are related in that all bind to DNA and are implicated in the compact organization of the chromosome. The presence of cPHB in *E. coli* H-NS was first detected in Western blots of two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels of total cell proteins, probed with anti-cPHB IgG, and then by Western blot analysis of the purified protein. Western blot analysis of the calf thymus histones indicated that each contained cPHB. The presence of cPHB in H-NS and histones was confirmed by chemical assay. The in vivo size of conjugated cPHB could not be established due to the lack of standards and degradation of cPHB during protein purification and storage. The molecular characteristics of cPHB and its presence in histone-like and histone proteins of diverse organisms suggest it may play a role in DNA binding and/or DNA organization. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Histones; H-NS; Polyhydroxybutyrate; Protein modification

1. Introduction

Poly-(R)-3-hydroxybutyrate (PHB), a linear homopolymer of the metabolic intermediate R-3-hydroxybutyrate, is a ubiquitous component of biological cells [1–8]. High molecular weight PHB (60 000 to >1 000 000 residues) is synthesized only by certain eubacteria and archaea [9–13], but all prokaryotes and eukaryotes synthesize short-chain PHB (<200 residues). The universal short-chain form of the polyester has thus far been found complexed to other macromolecules; thus it is referred to as cPHB. cPHB, like PHB, is a water-insoluble, amphiphilic molecule, with the molecular characteristics of salt-solvating polymers; i.e. it has a highly flexible backbone [14–17] bearing coordinating oxygens that are spaced at suitable distances to allow the formation of multiple coordinate bonds with cations [18–21]. Accordingly, cPHB may surround and solvate salts of inorganic polyphosphates or polynucleotides within membranes and hydrophobic pockets of proteins. In this regard, cPHB forms non-specific ion channels in planar lipid bilayers and liposomes [22–24], cPHB complexes with inorganic polyphosphate, located in the plasma membranes of bacteria, form calcium ion channels in planar bilayers [25,26], and cPHB was found complexed to DNA during uptake of exogenous DNA by genetically competent *Escherichia coli* [27].

In *E. coli*, the preponderance of cPHB (>99%) is conjugated to proteins [28]. cPHB-conjugated proteins are located in all cell fractions, but the majority are in the ribosomal fraction (>70%) which includes the nucleoid. In eukaryotes, cPHB-proteins have been found in a wide variety of tissues, organelles, and intracellular fluids [1–8]. The solvating properties of cPHB and its cellular distribution suggest it may be a constituent of proteins involved with binding and/or transfer of polynucleotides. Consequently, we were interested in examining prokaryotic and eukaryotic DNA-binding proteins for the presence of cPHB.

H-NS of *E. coli* is a small (136 amino acids) DNA-binding protein, localized in the nucleoid [29], where it is involved in the structural organization of the chromosome [30–34], and functions as a versatile regulator of transcription [35–39]. The protein consists of an oligomerization domain (residues 15–64) and a DNA-binding domain (residues 90–121) connected by a flexible linker region [40]. This histone-like protein shows little sequence specificity, but in vitro studies show that H-NS binds with higher affinity to strongly curved DNA [41–45].

Histones are the most abundant proteins associated with chromatin in the eukaryotic nucleus [46]. They are responsible for packing DNA into nucleosomes and then compacting nucleosomes into higher order structures. These relatively small proteins have a globular core domain involved in nucleosome formation, and highly charged tail domains that protrude from the nucleosome and are involved in histone–DNA interactions. The nucleosome consists of ~147 bp of DNA wrapped around an octameric core, composed of two molecules each of histones H2A, H2B, H3 and H4 [47]. Histone H1 binds as a monomer to nucleosomes and internucleosomal linker-DNA to facilitate DNA compaction [48].

In this study, we examine *E. coli* H-NS and calf thymus

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histones H1, H2A, H2B, H3, H4 for the presence of cPHB using immunological and chemical assays.

2. Materials and methods

2.1. Materials and strains

E. coli W3110 was used for the two-dimensional gels. *E. coli* MRE 600 was used for purification of H-NS. Calf thymus histones were obtained from Boehringer Mannheim.

2.2. Acrylamide gel electrophoresis and Western blotting

Two-dimensional PAGE of *E. coli* W3110 proteins was performed with the Investigator System (Millipore Corp.). Ampholines at pH 4 to 9 were used for the first dimension and 11.5% Duracryl (Millipore Corp.) and Trizma pre-set (pH 8.8; Sigma Chem. Co.) were used for the second dimension. Each gel was loaded with 106 cpm of a [^{35}S]methionine-labeled sample.

For one-dimensional SDS–PAGE, the sample in loading buffer (Bio-Rad) was heated in a boiling water bath for 3 min and resolved on a 16.5% polyacrylamide gel (bis) using SDS–tricine–glycine buffer, pH 8.3. Half the gel was stained with Coomassie brilliant blue R250, and the other half was transferred to supported nitrocellulose membrane using a Mini Trans-Blot electrophoretic cell (Bio-Rad). For H-NS, transfer was performed using Tris–glycine buffer, pH 8.3; for histones transfer was in CAPS buffer, pH 11.3.

2.3. Antibody to cPHB

The cPHB antibody was produced in rabbits to synthetic 8 mer of HB conjugated to electrophoresis-pure gelatin (Bio-Rad) by Metabolix Corp., and purified by protein A chromatography.

2.4. Probing blots with anti-cPHB IgG

The membranes were blocked with 2% gelatin (electrophoresis grade; Bio-Rad) in Tris-buffered saline, pH 7.5, 0.1% Tween-20. Primary incubation was with anti-cPHB-IgG in blocking buffer. Second antibody was goat anti-rabbit IgG–alkaline phosphatase conjugate in the same buffer. Color development was performed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

2.5. Purification of H-NS

E. coli MRE600 cells were grown to mid-log phase. (No overexpression system). Ca. 50 g frozen cells was disrupted with 80 g aluminum oxide (Alcoa) at 4°C (20 min). The disintegrated cells were homogenized with 150 ml extraction buffer (20 mM Tris–Cl, pH 7.5, 10 mM MgOAc, 10 mM 2-mercaptoethanol, 100 mM NH_4Cl) and incubated with 13.5 U DNaseI/ml for 30 min at 0°C. Cell debris and Alcoa were removed by centrifugation (5000 rpm, 4°C, 20 min), and the supernatant was centrifuged at 7000 rpm, 4°C, 30 min to obtain a clear lysate. A third centrifugation 40 000 rpm, 19 h, 4°C was performed to remove ribosomes. The ribosome-free supernatant was fractionated with NH_4SO_4 at 0°C. The precipitate from the 35% to 60% fraction was dissolved in 10 ml PG buffer (50 mM $KHPO_4$, pH 7.2, 50 mM KCl, 10 mM 2-mercaptoethanol, 10 mM EGTA 10% (v/v) glycerol, 23 μg/ml PMSF), and dialyzed against PG buffer. The solution was added to phosphocellulose P11 and eluted with a gradient of KCl in PG buffer (50 mM to 1.5 M KCl). Active fractions were dialyzed against PG buffer, and fractionated on Heparin Sepharose 6B using a KCl gradient in PG buffer (50 mM to 1.5 M).

2.6. Chemical assay for cPHB

The procedure was essentially as reported by Huang and Reusch [28]. Protein (100 μg) was precipitated by addition of 5 ml ice-cold 95% ethanol. After standing at 4°C for 2 h, the precipitate was collected by centrifugation and dried by lyophilization. Concentrated sulfuric acid was added (0.6 ml) and the sample was heated at 120°C for 40 min. The solution was cooled on ice, diluted with 1.2 ml of saturated ammonium chloride, and extracted three times with a two-fold volume of methylene chloride. The methylene chloride solutions were combined in a glass vial containing 100 μl of 5 N NaOH to convert crotonic acid to the sodium salt. Methylene chloride was then evaporated with a stream of N_2. The samples were made acidic by addition of 5 N H_2SO_4 and the sample was chromatographed on an Aminex HPX-87H ion exclusion organic acid analysis column (Bio-Rad; 4.1×250 mm) using 0.014 N H_2SO_4 as eluent. The crotonic acid peak was identified by its elution time, UV absorption curve, and mass spectrum, and quantitated by comparison of peak area with that of crotonic acid standards. PHB standards from *Alcaligenes* spp. (Aldrich), subjected to the same procedure, produced ~50% of the theoretical yield of crotonic acid.

3. Identification of cPHB in H-NS

The presence of cPHB in *E. coli* H-NS was first detected on Western blots of two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels of total *E. coli* W3110 proteins. When the blots were probed with anti-cPHB IgG, one of the proteins that showed a positive response to the antibody was tentatively identified by its position on the gel as H-NS. H-NS was then purified from *E. coli* MRE600 and subjected to SDS–PAGE and Western blot analysis. The purified protein displayed a strong reaction to the antibody (Fig. 1).

The presence of cPHB in purified H-NS was confirmed by a chemical assay in which the cPHB-protein is converted by β-elimination to its unique degradation product, crotonic acid, by heating in concentrated sulfuric acid [28]. The crotonic acid is extracted, purified by HPLC chromatography and quantified by peak area (Fig. 2, arrow).

There are at present no proper protein-cPHB standards; consequently, the peak area was compared with that of crotonic acid produced by standards of high molecular weight PHB from *Alcaligenes* spp. treated by the same protocol. This uncomplexed high molecular weight PHB is converted to crotonic acid at a much faster rate (20 min at 90°C) than protein-complexed cPHB (40 min at 120°C). Since the product, crotonic acid, is also labile, and degrades at a substantial rate at the higher temperature used in the cPHB assay, the amounts obtained represent relative and not absolute values. Using this protocol, a 200 μg H-NS sample produced 24.2 μg crotonic acid, indicating an average of 21 cPHB units per molecule H-NS (Table 1).

cPHB is highly insoluble in water but soluble in chloroform. To determine whether some or all of the cPHB was only weakly associated with the protein, H-NS was extracted with warm chloroform. The chloroform extract was filtered and tested for cPHB content by dot-blot immunoassay with anti-cPHB IgG and by chemical assay. The dot-blots did not show a positive reaction to anti-cPHB IgG and chemical assay of the dried extracts did not produce detectable crotonic acid. These results indicate that the association of cPHB with H-NS is strong and likely covalent.

Table 1
cPHB content of *E. coli* H-NS and calf thymus histones

Protein	cPHB (μg/mg protein)	HB U/mol protein
E. coli H-NS	121	21
Calf thymus H1	11.2	2.8
Calf thymus H2A	18.3	2.7
Calf thymus H2B	16.9	2.8
Calf thymus H3	16.8	3.0
Calf thymus H4	20.5	2.7

Deviations are ±15%. Values given are relative to granule PHB from *Alcaligenes* spp.

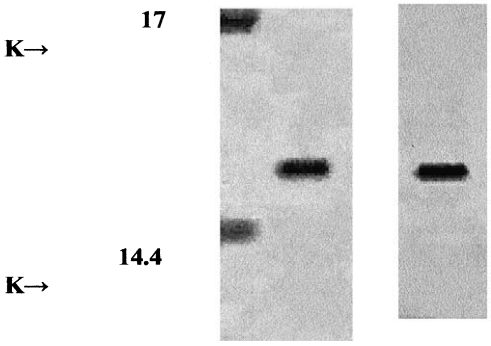

Fig. 1. Left: SDS–PAGE gel of purified *E. coli* MRE60 H-NS Coomassie brilliant blue R250; right: Western blot of an identical gel probed with anti-cPHB-IgG.

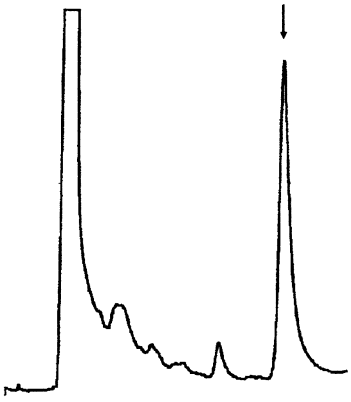

Fig. 2. HPLC elution profile showing the presence of crotonic acid (arrow) in concentrated sulfuric acid digests of H-NS.

4. Identification of cPHB in calf thymus histones

Pure samples of the calf thymus histones H1, H2A, H2B, H3 and H4, obtained commercially, were separated by SDS–PAGE (Fig. 3, left). The proteins were transferred to PVDF membranes and probed with anti-cPHB IgG. Each protein reacted positively to the antibody (Fig. 3, right). The identities of the major bands, at molecular weights corresponding to each histone protein, were confirmed by N-terminal Edman degradation.

Each histone was then examined by the chemical assay for cPHB as above. All histone proteins produced a small but significant amount of crotonic acid, confirming the identity of cPHB. The cPHB concentrations were calculated from peak areas, relative to standards of crotonic acid produced by *Alcaligenes* spp. PHB (Table 1). It is obvious from the Western blots that some samples were contaminated with other cPHB-proteins or with other histones, consequently, some of the values are slightly high.

The calf thymus histone proteins were each extracted with warm chloroform as above to determine whether their association with cPHB was partially or completely non-covalent. The filtered extracts were examined by dot-blot and chemical assays. None of the extracts showed a positive reaction to anti-cPHB IgG, and none produced detectable amounts of crotonic acid in the chemical assay.

5. Conclusions

These studies show that prokaryotic histone-like protein, *E. coli* H-NS, and eukaryotic calf thymus histone proteins, H1, H2A, H2B, H3 and H4, are posttranslationally modified by conjugation with cPHB. The presence of cPHB in proteins of similar function in such diverse organisms suggests that cPHB plays a significant role in shaping the structure and/or in facilitating the function of these important proteins.

The molecular characteristics of cPHB suggest that it may act as a 'solvent' for DNA, in much the same manner as has been proposed for its solvation of inorganic polyphosphates to form ion channels [25,26]. The polyester may be regarded as a slender, amphiphilic, flexible chain on which hydrophobic methyl groups alternate with hydrophilic ester carbonyl oxygens. As such, it may insert itself between protein and DNA; the methyl groups may associate with and thereby mask hydrophobic amino acids of the protein while the ester oxygens may form coordinate bonds to cations ionically bonded to the phosphate groups of DNA.

Despite their similarities in function, H-NS and histones have significantly different amino acid compositions and structures. Most strikingly, H-NS is a slightly acidic protein (pI = 5.4) whereas the histones are very basic (pI = 10–11.5). However, it should be noted in this regard that the pI of the DNA-binding region of H-NS, C-terminal residues 90–121, is 9.7. Posttranslational modification of H-NS is suggested by the existence of isoforms with pI close to 7.5 [49], although no modifying agent has been identified. Though not all cPHB-proteins are basic, it may be mentioned that the cPHB-conjugated *Streptomyces lividans* potassium channel, KcsA, has a pI of 10.3. Histones are well-known to undergo extensive and varied posttranslational modifications including acetylation, methylation, amidation, ubiquitination, ADP-ribosylation and phosphorylation [50–54].

The amount of cPHB in H-NS (av. 21 residues per molecule) is substantial; nevertheless, it is important to realize that the chemical assay provides only minimal values. The assay

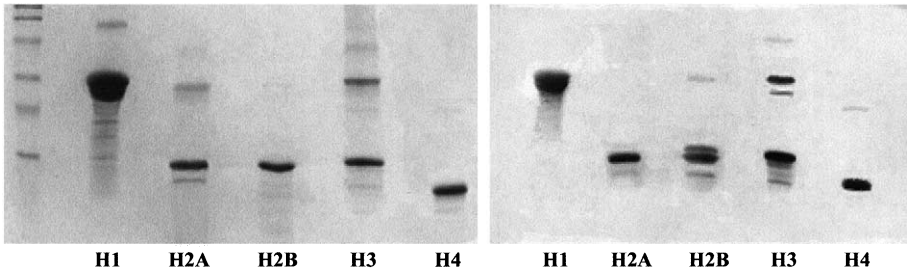

Fig. 3. Left: SDS–PAGE gel of calf thymus histones Coomassie brilliant blue R250; right: Western blot of an identical gel probed with anti-cPHB IgG.

confirms the identity of cPHB, and affords values that are reproducible within ± 15%, but due to lack of standards these values are relative and not absolute. Yet another problem in accurate determination of cPHB is its lability during protein isolation and storage due to enzymatic and/or chemical hydrolysis. The lability of cPHB is likely responsible for the conspicuously small amounts of cPHB found in the histones as compared to H-NS. The protocol used to prepare H-NS used in this study did not subject the protein to extremes of pH. On the other hand, purification procedures for the commercial samples of histone proteins include steps such as strong acid extraction that cause substantial degradation of cPHB. Perhaps of greater importance, the H-NS was freshly prepared and the histones had been stored for considerable periods. In our experience, the cPHB content of proteins diminishes at a constant rate during storage (unpublished observation). The responsible degradative agent has not been identified.

We consider it unlikely that histones in vivo are modified by dimers or very small oligomers of HB as the data suggest (Table 1). Not only do the data represent minimum values, but HB oligomers of less than four residues are not recognized by anti-cPHB IgG and they do not contribute significantly to production of crotonic acid in the chemical assay. It is more likely that a small fraction of histone molecules are conjugated to significantly longer oligomers while the majority have little or none. This difference in degree of modification may be caused by chemical or enzymatic degradation during isolation, but it is also possible that cPHB modification is a dynamic process similar to that of acetylation and only a small fraction of histone molecules are conjugated with cPHB at any given time.

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