

Evidence for a Dimerisation State of the *Bacillus subtilis* Catabolite Repression HPr-Like Protein, Crh

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Abstract

The *Bacillus subtilis* catabolite repression HPr (Crh) exhibits 45% sequence identity when compared to histidine-containing protein (HPr), a phosphocarrier protein of the phosphoenolpyruvate:carbohydrate phosphotransferase system. We report here that Crh preparations contain a mixture of monomers and homodimers, whereas HPr is known to be monomeric in solution. The dissociation rate of dimers is very slow ($t_{1/2}$ of about 10 hours), and the percentage of dimers in Crh preparations increases with rising temperature or protein concentration. However, at temperatures above 25°C and a protein concentration of 10 mg/ml, Crh dimers slowly aggregate. Typically, NMR spectra recorded at 25°C showed the coexistence of both forms of Crh, while in Crh solutions kept at 35°C, almost exclusively Crh monomers could be detected. Circular dichroism analysis revealed that the monomeric and dimeric forms of Crh are well folded and exhibit the same overall structure. The physiological significance of the slow Crh monomer/dimer equilibrium remains enigmatic.

Introduction

The histidine-containing protein (HPr) is a phosphocarrier protein which plays a central role in the uptake of

Abbreviations:

HPr, histidine containing protein
Crh, catabolite repression HPr
CcpA, catabolite control protein A
PEP, phosphoenolpyruvate
PTS, phosphoenolpyruvate carbohydrate phosphotransferase system
3D, three-dimensional
CD, circular dichroism
NMR, nuclear magnetic resonance
ppm, parts per million
HPLC, high-pressure liquid chromatography

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carbohydrates via the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS). It is phosphorylated by the PEP-dependent protein kinase enzyme I at His-15 and transfers the phosphoryl group to several enzymes II, each specific for a particular carbohydrate (for a review, see Postma *et al.*, 1993). HPr of low-GC gram-positive bacteria is also phosphorylated by the ATP-dependent HPr kinase at Ser-46 (Deutscher and Saier, 1983; Galinier *et al.*, 1998; Reizer *et al.*, 1998). P-Ser-HPr functions as corepressor in carbon catabolite repression by interacting with the catabolite control protein A (CcpA) (Deutscher *et al.*, 1995; Jones *et al.*, 1997), a member of the LacI/GalR family of repressors (Henkin *et al.*, 1991; Weickert and Adhya, 1992). The complex between P-Ser-HPr and CcpA binds to the catabolite response element (*cre*) (Fujita *et al.*, 1995; Galinier *et al.*, 1999; Gosseringer *et al.*, 1997; Kim *et al.*, 1998; Martin-Verstraete *et al.*, 1999a; Presecan-Siedel *et al.*, 1999), an operator-like sequence present in most catabolite-repressed genes and operons (Henkin *et al.*, 1991).

During the *B. subtilis* genome sequencing programme (Kunst *et al.*, 1997) a novel gene encoding a HPr like protein, Crh (for catabolite repression HPr), was discovered (Galinier *et al.*, 1997). Crh is composed of 85 aminoacids and exhibits 45% sequence identity with HPr. As HPr, Crh is phosphorylated by the ATP-dependent HPr kinase at Ser-46 (Galinier *et al.*, 1997; Galinier *et al.*, 1998). It was shown that Crh is involved in catabolite activation or repression of several genes and operons: together with P-Ser-HPr, P-Ser-Crh regulates genes encoding inositol dehydrogenase (*iol*) (Galinier *et al.*, 1997), levanase (*lev*) (Galinier *et al.*, 1997; Martin-Verstraete *et al.*, 1999a), β -xylosidase (*xyn*) (Galinier *et al.*, 1999; Galinier *et al.*, 1997), acetyl coenzyme A synthetase (*acsA*) (Zalieckas *et al.*, 1998), histidine utilization enzymes (*hut*) (Zalieckas *et al.*, 1999), acetate kinase (*ackA*) (Turinsky *et al.*, 1998) and phosphotransacetylase (*pta*) (Presecan-Siedel *et al.*, 1999). Using DNase I footprinting experiments, it was shown that similarly to P-Ser-HPr, P-Ser-Crh stimulates binding of CcpA to the *cre* sequences of the *xyn*, *lev* and *pta* genes (Galinier *et al.*, 1999; Martin-Verstraete *et al.*, 1999a; Presecan-Siedel *et al.*, 1999).

Contrary to HPr, Crh does not seem to play a role in the PTS-catalysed carbohydrate transport and phosphorylation. Since the catalytic His-15 of HPr is replaced with a glutamine in Crh, no PEP-dependent, enzyme I-catalysed phosphorylation of this protein could be detected (Galinier *et al.*, 1997). However, it has previously been demonstrated that Q15H mutant Crh could be phosphorylated by PEP and enzyme I, and could carry out some of the catalytic and regulatory functions of P-His-HPr. By contrast, Q15H mutant Crh did not restore growth of a *ptsH* mutant strain on PTS sugars and glycerol (Martin-Verstraete *et al.*, 1999b). In this paper, we report a new difference between HPr and Crh, which was

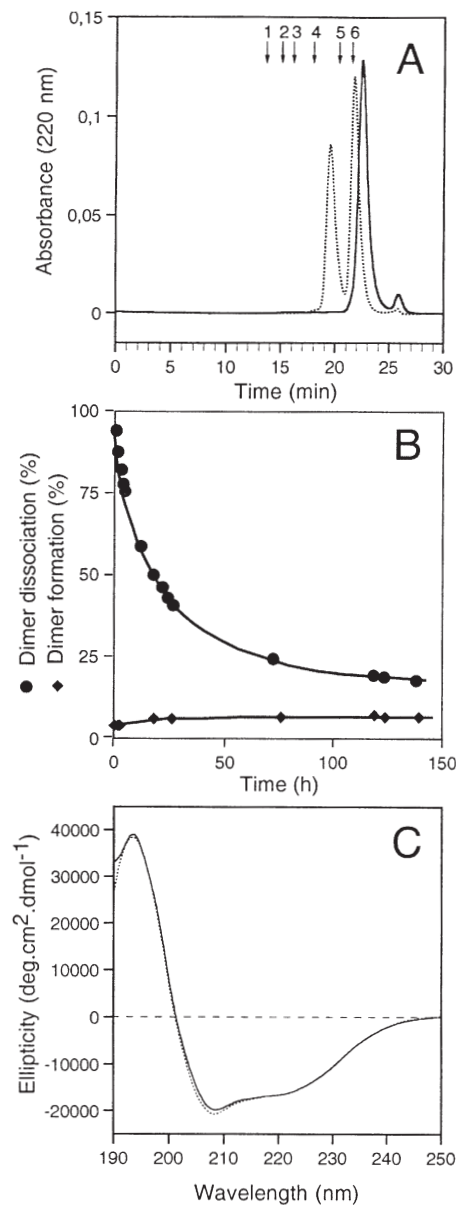


Figure 1. Characterisation of dimers in Crh preparations. (A) Gel filtration chromatography of purified Crh (10 μ g, dotted line) and HPr (7 μ g, solid line) using a Protein PAK Glass 200SW column (Waters, 0.8 x 30 cm) equilibrated with 100 mM sodium phosphate, pH 7.0. The flow rate was 0.5 ml/min. Numbered arrows indicate the elution time of molecular weight markers: 1, Blue Dextran (2,000,000); 2, Alcohol dehydrogenase (150,000); 3, Bovine Serum Albumin (66,000); 4, β -Lactoglobulin (35,000); 5, Myoglobin (17,800); 6, Cytochrome C (12,400). The molecular weight of HPr(His)₆ is 10,100. V_t was 27.7 min. The presence of Crh in the two eluted peaks was proven by SDS-PAGE (data not shown). (B) Kinetics of Crh dimerisation and dimer dissociation. Monomers and dimers of Crh were purified by gel filtration as described in (A). Monomer preparation (0.16 mg/ml) and dimer preparation (0.17 mg/ml) were incubated at 20°C. At the indicated times, aliquots were analysed by gel filtration to estimate the content of monomers and dimers. The percentage of each form was determined by integration of the corresponding peak. (C) Comparison of far UV CD spectra of Crh monomers and dimers. Monomers (solid line) and dimers (dotted line) of Crh were separated by gel filtration as described above and concentrated rapidly by ultrafiltration on ultrafree 5,000 (Waters). Protein concentrations (0.85 mg/ml and 0.74 mg/ml for the monomers and the dimers, respectively) were estimated from UV absorbance of tyrosine using a molar extinction coefficient of 1536 M⁻¹.cm⁻¹ at 280 nm. After CD record, it was checked by gel filtration that the presence of monomer in dimer preparation and dimer in monomer preparation represented less than 5% of protein.

discovered by analysing the oligomerisation state of Crh: in contrast to the monomeric HPr, Crh was found to form dimers in solution.

Results

Gel filtration chromatography (Figure 1A) clearly revealed that HPr was monomeric (solid line), whereas Crh preparation gave 2 major peaks (dotted line). Calibration of the gel filtration column allowed us to conclude that the faster (apparent molecular weight 24 kDa) and slower (apparent molecular weight 12 kDa) migrating peaks correspond to Crh dimers and monomers, respectively. It should be mentioned that monomer and dimer of Crh could be also separated by anion exchange chromatography (data not shown). To verify that the 6xHis tag was not involved in the dimerisation process, we overexpressed and purified a Crh after cleavage from a GST-Crh fusion protein (Martin-Verstraete *et al.*, 1999a) or Crh without any tag. By carrying out gel filtration experiments, solutions of Crh and GST-Crh were also found to contain dimers and monomers, indicating that the 6xHis tag was not involved in dimerisation process (data not shown). It should be mentioned that Crh(His)₆ was used for all the experiments since it was easily to obtain highly purified at very high quantity (50 mg / culture litre) required for RMN experiments while Crh without tag was difficult to purify.

In addition, monomers and dimers were also observed in Q15H mutant Crh preparations (Martin-Verstraete *et al.*, 1999b), suggesting that Gln-15 is not involved in dimerisation. The presence of both monomers and dimers was also observed in Crh phosphorylated on serine 46 by the HPr kinase (data not shown). This result suggests that Ser-46 is not located at the dimer interface and that this ATP-dependent phosphorylation is not inhibited by Crh dimerisation.

When studying the characteristics of Crh, we noticed that the proportion of monomers and dimers in Crh preparations was rather variable and depended on the protein concentration, pH, temperature and storage conditions, suggesting that the monomer-dimer exchange rate was fairly slow. To measure of the kinetics of dimer formation and dissociation reported in Figure 1B, monomers and dimers of Crh were first purified by gel filtration. Then, monomer and dimer preparations were incubated separately and analysed by gel filtration to evidence the apparition of dimer in monomer preparation and monomer in dimer preparation. The Figure 1B shows the very slow dissociation of dimers (circles; $t_{1/2}$ of about 10 hours), and the equilibrium was not reached even after 6 days. By contrast, the dimerisation process appeared to be faster (diamond) although the percentage of dimers remained very low when low concentration of monomers was used (0.16 mg/ml in this experiment). In fact, the amount of dimers increased when the Crh concentration was elevated and a 1:1 monomer-dimer ratio can be reached at about 10 mg/ml Crh, but total dimerisation of Crh was never reached (data not shown). At this concentration, Crh solutions were found to be rather stable at temperatures below 25°C, whereas Crh dimers tend to slowly aggregate when the temperature was increased above 25°C (see below). As a consequence of the slow

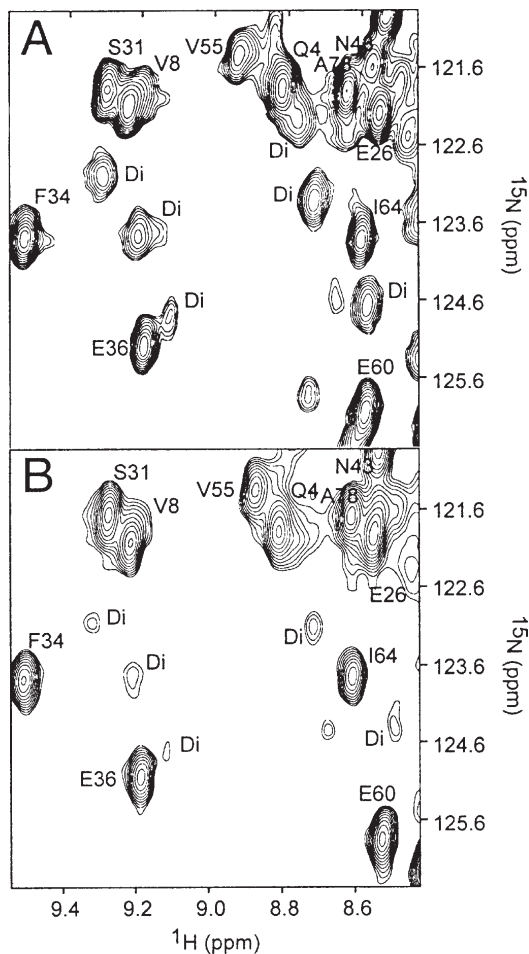


Figure 2. [^1H - ^{15}N]HSQC spectra of ^{15}N -labeled Crh. Selected amide region of 2D ^1H - ^{15}N HSQC NMR spectra of Crh recorded at 25°C (A) and 35°C (B). The assigned cross-peaks correspond to the monomeric form of Crh and crosspeaks labelled "Di" correspond to the dimeric form of the protein.

kinetic of dimer dissociation and of the aggregation process of Crh it was not possible to properly measure association and dissociation constants.

The CD spectra of Crh monomers and dimers recorded immediately after their separation by gel filtration were found to be almost identical and typical for well-folded proteins (Figure 1C). This result indicates that dimerisation has no significant effect on the folding of Crh. The appearance of a maximum at 193 nm, a minimum at 208 nm, and a large shoulder in the 220-225 region of the CD spectra denote the presence of both α -helices and β -sheets. Deconvolution of the spectra for secondary structure estimation did not reveal significant differences between monomer and dimer and yielded an α -helix and β -sheet content of 39% and 24%, respectively.

Crh was analysed by NMR in order to resolve its 3D structure. A selected region of the 2D ^1H - ^{15}N HSQC spectra of Crh obtained at 25°C and at 35°C are presented in Figure 2. At 25°C (Figure 2A), the intensity of the dimer crosspeaks were 20 to 40% weaker than the crosspeaks of the monomeric form. The HSQC NMR spectra confirmed that both populations of Crh were well folded and that the

monomer/dimer transition was slow. When increasing the temperature to 35°C, a slow precipitation of Crh was observed. This precipitate was not soluble by dilution, except when moderate amounts of chaotropic agents were added (*i.e.* 1.5 M guanidinium hydrochloride or 2 M urea). Interestingly, the removal of these agents by dialysis yielded soluble Crh exhibiting the same properties than the initial preparation (*i.e.* mixture of monomers and dimers and surimposable NMR spectra). These features indicate that Crh aggregates when increasing the temperature. In addition, Figure 2B shows that the intensity of the dimer signals in NMR spectra recorded at 35°C represented less than 10% of the monomer signals. This result indicated that rising the temperature induced the selective aggregation of Crh dimers. This property allowed us to prepare NMR samples largely enriched in Crh monomers, which are currently used for its 3D structure determination.

Discussion-Conclusion

The main difference between HPr and Crh (the catabolite repression HPr) is that Crh plays apparently no role in PTS-catalysed carbohydrate transport since it is not phosphorylated by enzyme I due to the replacement of the catalytic His-15 with a glutamine (Galinier *et al.*, 1997). We describe here another difference between these two proteins: Crh exists as a mixture of monomers and dimers, which are in very slow exchange (several hours), whereas HPr was found to form only monomers in solution. However, although previous NMR data and biochemical characterisation of HPr did not mention any dimerisation process, it could not be excluded that, under particular conditions, dimerisation of HPr might take place.

The monomer/dimer equilibrium was also observed for CrhQ15H and P-Ser-Crh preparations, suggesting that neither Gln-15 nor Ser-46 are involved in the dimerisation process. The results obtained by CD spectroscopy showed identical global folding for Crh monomers and dimers. In addition, the secondary structure composition of Crh (39% α -helix and 24% β -sheet) was found to be quite similar to that of HPr, which was reported to contain between 35-38% α -helices and 23.5-28% β -sheets, depending on the structure determination studies reported (see (Waygood, 1998)). In all studies, HPr was described as a monomer. By contrast, the presence of Crh dimers could be observed in the ^{15}N ^1H HSQC NMR spectrum recorded at 25°C, whereas at 35°C, almost exclusively monomers of Crh could be detected. The good quality of these NMR spectra should allow to resolve the 3D structure of Crh and to identify the residues located at the dimer interface.

The physiological significance of the dimerisation of Crh is actually unknown and the intracellular oligomerisation state of Crh remains to be determined. It is possible that the slow Crh monomer-dimer transition is involved in a fine regulation mechanism of catabolite repression. Indeed, similarly to P-Ser-HPr, P-Ser-Crh was found to act as corepressor for the dimeric repressor CcpA during carbon catabolite repression and catabolite activation (Galinier *et al.*, 1999; Martin-Verstraete *et al.*, 1999a; Presecan-Siedel *et al.*, 1999). Moreover, DNase I footprinting experiments had suggested that P-Ser-Crh was only slightly less efficient than P-Ser-HPr in stimulating

binding of CcpA to the *xyn cre* (Galinier *et al.*, 1999). In addition, titration experiments indicated that CcpA interacts with P-Ser-HPr and these proteins form a specific ternary complex composed of two molecules of P-Ser-HPr, a CcpA dimer, and the *cre* DNA sequence (Jones *et al.*, 1997). Taken together, these data suggest that two molecules of P-Ser-Crh might bind to the dimeric repressor CcpA and it is tempting to speculate that dimerisation of Crh occurs when P-Ser-Crh interacts with CcpA. The resolution of the 3D structure of Crh, phosphorylated or not, the identification of the residues involved in dimerisation, and the comparison with 3D structures of HPr and P-Ser-HPr (free and complexed with CcpA) should help us to evaluate this hypothesis. In addition, the influence of the dimerisation of the corepressor P-Ser-Crh on the interaction of the dimeric repressor CcpA with its DNA targets remains to be determined.

Experimental Procedures

Crh and HPr Purification

Crh(His)₆ and HPr(His)₆ were overproduced and purified on Ni-NTA-agarose columns as described previously (Galinier *et al.*, 1997). The purification of Crh was completed by anion exchange chromatography on a resource Q column (Pharmacia) equilibrated with 20 mM Tris-HCl buffer, pH 8.5, and run with a 0 to 1 M NaCl gradient in this buffer. Crh-containing fractions were pooled, concentrated by ultrafiltration, and dialysed against 20 mM sodium phosphate pH 7.5, 50 mM NaCl. Purified protein concentrations were estimated by Bio-Rad Protein assay (Bio-Rad) except when they were used for CD analysis (see legend of figure 1). For NMR analysis, ¹⁵N isotopic labelling of Crh was achieved by growing bacteria in Celtone-N medium (Martek). NMR samples were supplemented with D₂O (8%) and sodium azide (0.05%); the final concentration of Crh was 5.7 mg/ml. The overproduced proteins exhibited the expected molecular mass as measured by electrospray mass spectroscopy (10392 and 9327 MH⁺ average mass for Crh(His)₆ and Crh, respectively).

Circular Dichroism Measurements

CD spectra were recorded on a Jobin-Yvon CD6 spectrometer in the 190-250 nm wavelength range with 0.2 nm increments and 2 s integration time at 20°C in 0.02 cm path length quartz cuvettes. The baseline-corrected spectra were smoothed by using a third-order least squared polynomial fit. The secondary structure percentages of Crh were estimated from the measured ellipticities using the Varselec method (Johnson, 1990). The VARSLC1 program was kindly provided by W.C. Johnson Jr.

Nuclear Magnetic Resonance (NMR)

Two dimensional ¹H-¹⁵N heteronuclear single-quantum correlation (HSQC) spectra (Bodenhausen and Ruben, 1980) were recorded on a Varian INOVA 600 spectrometer. Data sets were acquired with 1024 (¹H) X 256 (¹⁵N) complex points and 4 scans per *t*₁ increment resulting in an experimental time of 40 minutes. The spectral widths in the ¹H and ¹⁵N dimensions were set to 9000 and 2500 Hz, respectively. After signal apodization using a squared cosine function and zero filling, the time domain data were Fourier transformed to final matrices of 2048 (¹H) X 1024 (¹⁵N) spectral points.

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