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Structure and conformational stability of the enzyme I of *Streptomyces coelicolor* explored by FTIR and circular dichroism

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Abstract

The bacterial phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS), formed by a cascade of several proteins, couples the translocation and phosphorylation of specific sugars across cell membranes. The structure and thermal stability of the first protein (enzyme I, EI) of the PTS in *Streptomyces coelicolor* is studied by using far-UV circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR) at pH 7.0. The deconvolution of FTIR spectra indicates that the protein is mainly composed by a 35% of α-helical structure and 30% of β-sheet. The thermal denaturation curves, as followed by both techniques, show only a midpoint at 330 K. This thermal denaturation behaviour is different to that observed in other members of the EI family.

Keywords: Circular dichroism; Enzyme I of the bacterial phosphoenol pyruvate:sugar phosphotransferase system; Fourier transform infrared spectroscopy; Thermal unfolding

1. Introduction

The bacterial phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) [1,2] is a complex system restricted to the bacterial world, whose main function is phosphorylation and concomitant transport of carbohydrates and their derivatives across the membrane. During the transport process, a phosphoryl group is transferred from phosphoenolpyruvate (PEP) to the carbohydrate. The PTS operates through a cascade of phosphoryl transfers from PEP via phosphointermediates of the general non-sugar-specific phosphotransferases enzyme I (EI) and the histidine-phosphocarrier protein (HPr) to substrate-specific enzyme II permeases. Enzyme I (an approximately 60 kDa protein) is phosphorylated on a histidine residue by PEP in the presence of Mg^{2+} ([3,4] and references therein); the phosphoryl is then transferred to the low molecular weight HPr (approximately 9 kDa) at other histidine position. Both proteins can be considered the gateway to the system, and can be used as targets to design molecules, which could hamper sugar phosphorylation.

Streptomyces are soil-dwelling actinomycetes which grow on a variety of carbon sources. They are the origin of approximately two thirds of all natural antibiotics currently produced by the pharmaceutical industry. The complete genome of *Streptomyces coelicolor* has been sequenced, showing the largest number of genes found in any bacteria [5]. The presence of the different components of the PTS in *S. coelicolor* has been reported, and the corresponding proteins cloned and expressed [6–8]. As a part of an approach to design new antibiotics, we are studying the structure and conformational stability of the PTS proteins. There is a growing interest in determining to which extent related proteins of the same family share the same conformational stability features [9]. For instance, we have shown that HPr of *S. coelicolor* shows different stability and folding properties than those HPrs of *B. subtilis*

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and *E. coli* [10,11], although the structures are similar. Stability studies of EI from *E. coli* [4,12] and *M. capricolum* [3,13] have been carried out using several spectroscopic techniques, namely, fluorescence and CD, and calorimetry (DSC). Such studies show the presence of two thermal transitions, which have been attributed to unfolding of the N- and C-terminal domains of EI. Here, we use FTIR and CD to probe the structure and stability of EI of the *S. coelicolor* (scEI). Our results show that scEI has a large content of α-helix, but it does unfold via one thermal transition.

2. Experimental

2.1. Materials

Imidazole, Trizma acid, its base, and NaCl were from Sigma. β-mercaptoethanol was from BioRad, and the Ni^{2+}-resin was from Invitrogen. Standard suppliers were used for all other chemicals. Water was deionized and purified on a Millipore system.

2.2. Protein expression and purification

The scEI clone comprises residues 1–576, with the extra methionine and the His_6-tag at the N terminus. Wild type scEI protein was expressed in *E. coli* BL21(DE3) strain, and purified using Ni^{2+}-NTA chromatography. To eliminate any protein or DNA bound to the resin, co-eluting with the protein, an additional gel filtration chromatography step was carried out by using a Superdex 200 16/60 gel filtration column (Amersham Biosciences) running on an AKTA-FPLC. Protein was more than 95% pure as judged by SDS–PAGE. The samples were dialysed extensively against 12 mM Tris (pH 7.0) and 100 mM NaCl, frozen in liquid nitrogen, and stored at −80 °C. Protein concentration was calculated from the absorbance of stock solutions measured at 280 nm, using the extinction coefficients of model compounds [14].

2.3. Circular dichroism measurements

Circular dichroism spectra were collected on a Jasco J810 spectropolarimeter fitted with a thermostated cell holder and interfaced with a Neslab RTE-111 water bath. The instrument was periodically calibrated with (+) 10-camphorsulphonic acid.

2.3.1. Steady-state measurements

Spectra were acquired at a scan speed of 50 nm/min with a response time of 4 s and averaged over four scans at the desired temperature. Protein concentration was 4 μM in 12 mM Tris (pH 7.0), 100 mM NaCl using 0.1-cm-pathlength cells. All spectra were corrected by subtracting the proper baseline. The mean residue ellipticity, $[\Theta]$, was obtained from the raw ellipticity data, Θ, as reported elsewhere [10]. The helical content of scEI was calculated from its mean residue ellipticity at 222 nm [15]: $f_h = [\Theta]_{222}/\left[[\Theta]^{\infty}_{222}\left(1-\frac{k}{n}\right)\right]$, where f_h is the helical fraction of the protein, $[\Theta]_{222}$ is the observed mean residue ellipticity, $[\Theta]^{\infty}_{222}$ is the mean ellipticity for an infinite α-helix at 222 nm (−34,500 deg cm^2 $dmol^{-1}$), k is a wavelength-dependent constant (2.57 at 222 nm), and n is the number of peptide bonds (576 for the His-tagged scEI).

2.3.2. Thermal denaturation measurements

Experiments were performed at constant heating rates of 15 K/h, 30 K/h, and 60 K/h, and a response time of 8 s. Measurements were acquired every 0.2 K. Thermal scans were collected in the far-UV region at 222 nm from 298 to 353 K in 0.1-cm-pathlength cells with a total protein concentration of 4 μM. The reversibility of thermal transitions was tested by recording a new scan after cooling down to 298 K the thermally denatured sample, and comparing the thermal denaturation curve with that obtained in the first scan. The experiments were not reversible at any heating rate; all the heating rates yielded essentially the same thermal midpoint. Fitting of thermal denaturations was carried out by using Kaleidagraph running on a PC, with the equation corresponding to a two-state model as described in other EI members [3,4,12].

2.4. Fourier transform infrared spectroscopy

The protein was speed-vacuum dried and dissolved in deuterated buffer Tris 12 mM and 100 mM NaCl (pH 7.0). No pH corrections were done for the isotope effects. Samples of scEI at a final concentration of 10 mg/ml were placed between a pair of CaF_2 windows separated by a 50 μm thick spacer in a Harrick demountable cell. Spectra were acquired on a Bruker FTIR instrument equipped with a deuterated triglycine sulphate detector and thermostated with a Braun water bath. The cell holder was continuously filled with dry air.

2.4.1. Steady-state measurements

Three-hundred scans per sample were taken, averaged, apodized with a Happ–Genzel function, and Fourier transformed to give a final resolution of 2 cm^{-1}. The signal to noise ratio of the spectra was better than 1000:1. Buffer contributions were subtracted, and the resulting spectra were used for analysis. To quantify the secondary structure components, the amide I band was decomposed into its constituents by curve-fitting (based on a combination of Gaussian and Lorentzian functions), using the number and position of bands obtained from the deconvolved (with a Lorentzian bandwidth of 18 cm^{-1} and a resolution enhancement factor of 2) and the Fourier derivative (using a power of 3 and a breakpoint of 0.3) of the spectra [16–19].

2.4.2. Thermal denaturation measurements

Thermal denaturation experiments were carried out with a scanning rate of 50 K/h, and acquired every 3 K. Fifty scans per temperature were averaged. The heating experiments were not reversible under these conditions. Fitting was carried out by using Kaleidagraph running on a PC, to a two-state model, as described in other EI members [3,4,12].

3. Results and discussion

3.1. Circular dichroism measurements

We used far-UV CD in the analysis of scEI as a spectroscopic probe that is sensitive to protein secondary structure [20,21]. The CD spectrum of scEI at 298 K showed two intense minima at 208 and 222 nm, characteristic of proteins with an α-helical structure [20,21] (Fig. 1A). Similar findings have been observed in other EI members [3,4,12]. The estimated population of α-helix from the equation above is 16%. The same shape was also observed at low temperature (278 K).

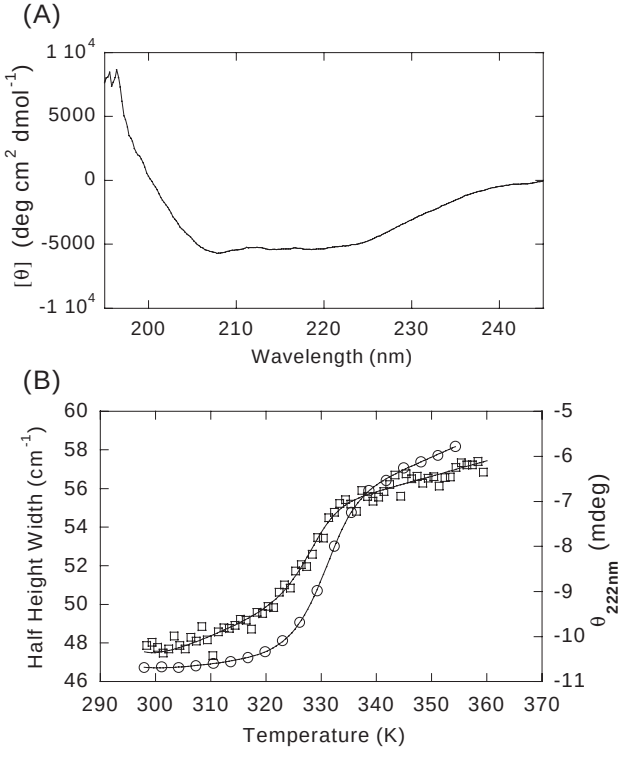

Fig. 1. (A) Steady state CD spectrum of scEI at pH 7.0. (B) Thermal denaturation profile of scEI at pH 7.0 followed by the changes in the ellipticity (blank squares, right axis) at 222 nm and the half height width band of the FTIR spectra (blank circles, left axis). The conditions for the CD were: 4 μM of protein, buffer concentration (Tris) was 12 mM in all cases (pH 7.0) and 100 mM NaCl. Spectra were acquired in 0.1-cm-pathlength cells. The conditions for the FTIR spectra were deuterated buffer Tris 12 mM and 100 mM NaCl (pH 7.0), with a final scEI concentration of 10 mg/ml. The lines are the fit to the equation corresponding to a two-state model.

Table 1
Secondary structural analysis of scEI as determined by FTIR[a]

Wavenumber (cm^{-1})	Structural assignment[b]	% of total secondary structure
1693	β-hairpins	0.2
1679	Turns/loops/(0,π) β-sheet	2.7
1668	β-turns	12.4
1657	Loops/disordered structure/α-helix	9.3
1649	α-helix	35.2
1633	Anti-parallel β-sheet	29.4
1620	β-hairpins	10.8

[a] Errors in the wavenumber are estimated to be ±2 cm^{-1}.

[b] There is one more band which has not been indicated in the table, centered at 1606 cm^{-1}. This band is assigned to side chains [23] and it accounts for 2% of the whole area of the amide I band. The percentages of secondary structure on the third column of the table do not take into account this band. The 1620 and 1693 cm^{-1} components correspond to β-hairpin structure [24]; the 1679 and 1633 cm^{-1} bands are assigned to turns/loops/(0,π) β-sheet structure and β-sheet, respectively [23]; the 1649 and 1657 cm^{-1} bands are assigned to α-helix or disordered structure, and α-helix, respectively [18,23,25], and the 1668 cm^{-1} is assigned to β-turns [26].

Conversely to the results observed in *E. coli* EI (ecEI), where the thermal denaturation followed by CD yielded two midpoints (at 314 and 327 K) [3,22], scEI only showed a single transition at 330 K (Fig. 1B). It has been argued that the second thermal transition in ecEI is due to the melting of the N-terminal domain, and, as it can be observed there is a large similarity between the values of the thermal midpoints of scEI and the second one of ecEI. The absence of a second thermal midpoint in the thermal unfolding experiments of scEI could be due to either: (i) the unfolding of the N terminus is only being observed in scEI, because the C-terminal region of scEI is rather disordered; or (ii) there is a strong coupling between the unfolding of the C and N-terminal regions of scEI, as it has been observed under some conditions in wild-type ecEI and in some of their mutants ([12] and references therein). The C-terminal domain of ecEI is flexible and less structured than the N-terminal domain ([3,4,12] and references therein), and its thermal denaturation, followed by far-UV CD appears as a small shoulder of the main unfolding transition (that of the N terminus) in the wild-type ecEI. It might be that the C-terminal domain of scEI was more disordered than that of ecEI, and its N-terminal was more ordered.

3.2. Fourier transform Infrared spectroscopy (FTIR)

FTIR is a powerful method for investigation of secondary protein structure. The main advantage in comparison with CD is that FTIR is much more sensitive to the presence of β-structure and random-coil. In the case of proteins, structural information can be obtained by analysing the amide I region of the spectrum (1700–1600 cm^{-1}). The absorbance of this band is mainly due to the

stretching vibration of the carbonyl peptide bond, whose frequency is highly sensitive to hydrogen bonding and thus to protein secondary structure [23]. The deconvolution of the band showed maxima centered at 1693, 1679, 1668, 1657, 1649, 1633, 1620, and 1606 cm^{-1}. The percentages of secondary structure calculated from the area of those fitted bands are in Table 1. It can be observed that the predicted percentage of helical structure is larger in FTIR (at least 35%) than that obtained by CD (16%). The differences between the values reported by both techniques could be due to: (i) the deconvolution procedures (in the case of FTIR); (ii) the presence of aromatic or cysteine residues [21,22] (CD), which also absorb at 222 nm (scEI has one tryptophan, twelve histidines, eight tyrosines, five cysteines, and thirteen phenilalanines); or (iii) to the empirical character of the equation used in predicting the helical structure (CD) (see the Experimental section). The determination of the percentage of secondary structure by FTIR has been used for a long time, and it has proved to be a robust method by comparing the calculated percentage of structure obtained by FTIR with that obtained either by X-ray in several model proteins [27]. In all cases the results from FTIR and X-ray are in good general agreement. Based on these findings in other proteins, we favor the percentage of α-helix predicted by FTIR rather than that obtained by CD.

On the other hand, the FTIR thermal denaturation results agree with those observed by CD (Fig. 1B): only a single transition was observed with a thermal midpoint at 330 K. Since by following the changes in the bandwidth upon heating in FTIR we are mapping the melting of the whole secondary structure in scEI, the agreement between the results from different spectroscopic techniques suggests that there is one single unfolding event in the thermal unfolding of the protein, and this might be the unfolding of the N-terminal region of scEI. Moreover, since both techniques use different protein concentrations (see the Experimental section), this thermal unfolding event seems to be not concentration-dependent (even at the low concentrations used in the CD experiments the protein is already dimeric, as concluded from CD titration experiments, unpublished results).

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