The N-terminal domain of the enzyme I is a monomeric well-folded protein with a low conformational stability and residual structure in the unfolded state

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The bacterial phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) involves a cascade of phosphorly-transfer steps from PEP to the sugar-specific enzyme II permeases via phosphointermediates of the general cytosolic non-sugar-specific phosphotransferase enzyme I, EI (EC 2.7.3.9), and the histidine phosphocarrier, HPr (Postma et al., 1993; Deutscher et al., 2006). EI is auto-phosphorylated on a histidine residue by PEP and then, the phosphoryl group is transferred to HPr. EI is one of the best conserved proteins in the prokaryotic kingdom, and it has no similarity to animal proteins. The EI and HPr proteins could be considered as targets to control the function of the PTS, since any compound able to bind to EI at the natural HPr-binding site should interrupt the flow of phosphoryl groups along the cascade for any sugar.

The 64 kDa EI protein is a homodimer, whose association is stronger in the phosphorylated state than in the unphosphorylated one (Waygood et al., 1979; Kukuruzinska et al., 1984; Seok et al., 1998). Phosphorylation requires the presence of Mg2+, and it targets a histidine in the N-terminal region of the protein. Proteolytic cleavage of EI from Escherichia coli, EIa, yields two domains (LiCalsi et al., 1991; Lee et al., 1994). The first one (the EI N-terminal domain, EIna) comprises, roughly, the first 230 residues of the protein; it contains the HPr-binding domain and the active-site histidine (Weigel et al., 1982). And the second one (the EICa, C-terminal domain) mediates dimerization and binds PEP in the presence of Mg2+ (Zhu et al., 1999; Broxk et al., 2000). The structure of EIna has been determined by X-ray and NMR (Liao et al., 1996; Garrett et al., 1997), and its complex with HPr has been characterized by state-of-the-art NMR techniques (Garrett et al., 1999; Suh et al., 2008). The isolated EIna, which consists of an α-helical domain (where HPr is bound) and an α/β domain, contains the phosphorylation centre, and it is structurally similar to the phospho-histidine domain of the pyruvate-phosphate dikinase (PPDK) (Teplyakov et al., 2006). The structure of the C-terminal domain of EI is that of an (α/β)8
barrel protein, and it is structurally similar to the PEP-binding domain of PPDK (Teplyakov et al., 2006).

Streptomyces is a soil-dwelling actinomycete that grows on a variety of carbon sources. The complete genome of Streptomyces coelicolor has been sequenced, and it shows the largest number of genes found in any bacteria (Bentley et al., 2002). The presence of the different components of the PTS in S.coelicolor has been reported, and the corresponding proteins have been cloned and expressed (Parche et al., 1999; Nothaft et al., 2003a,b). We have undertaken an extensive description of the structures and conformational stabilities of the HPrsc and EIsc proteins (Fernández-Ballester et al., 2003; Neira and Gómez, 2004; Hurtado-Gómez et al., 2005, 2006) as a first step to understand the binding between both proteins. For instance, we have characterized and described the affinity of EIsc for HPrsc, and found that the reaction is enthalphy driven, whereas in other species is entropy driven (Hurtado-Gómez et al., 2008); furthermore, the value of the dissociation constant for the EIsc-HPrsc complex is larger (~100 μM) than those measured in E.coli (9 μM) or Mycoplasma capricolum (7 μM) (Chauvin et al., 1996; Zhu et al., 1999). Therefore, it is necessary to know whether the binding mechanism between HPrsc and EIsc is different from that observed in other bacterial species, and to understand, if any, the structural determinants responsible for those differences, specifically in the HPr binding region of EIsc (that is, in EIsc). In this work, we characterize the structure and the conformational stability of EINsc in a wide pH range (from pH 2.0 to 13.0); it is important to indicate at this stage that the conformational stability of Salmonella typhimurium EIN has been only addressed by differential scanning calorimetry (DSC) in a narrow pH range (6.5–8.5) (LiCalisi et al., 1991), but at the best of our knowledge, this work is the first extensive characterization of an EIN domain at different pHs by using chemical and thermal denaturations (DSC or circular dichroism (CD)). We found that EINsc is a well-folded protein, with a low conformational stability and a substantial amount of residual structure in the denatured state. Since the affinity of EINsc for HPrsc is in the range of 10 μM (Olga Abian and Adrián Velázquez-Campoy, unpublished results, personal communication), and thus higher than that of the whole EIsc for HPrsc (Hurtado-Gómez et al., 2008), the difference in both values must rely on the presence of the C-terminal domain in the intact EIsc or, alternatively, in slight structural changes in the isolated EINsc occurring upon addition of HPrsc, when compared with other EIN domains.

Materials and methods

Materials

Ultrapure urea and GdmCl were purchased from ICN Biochemicals (USA). Urea was used for chemical- and thermal denaturations followed by circular dichroism (CD); GdmCl was used for DSC measurements. Exact concentrations of both denaturants were calculated from the refractive index of the solution (Pace, 1986). Standard suppliers were used for all other chemicals. Water was deionized and purified on a Millipore system.

Cloning of EINsc

Plasmid pFT35, containing the cloned pstl gene, from S.coelicolor M145 (EI) (a kind gift from Dr. F. Tietge, Germany), was used as a source of DNA (Nothaft et al., 2003a). The primers used to clone the 738 bp EI N-terminal fragment were designed based on the GenBank sequence accession number AL939108 (gene SC1A8A.11) (Bentley et al., 2002). These were EIN5 (5′-AAACATGCTgaga caacgtcgcagg-3′) and EIN3 (5′- gagcteccgegcttgccgg-3′), including NcoI and SacI restriction sites, respectively. The PCR fragment obtained was purified from agarose gel using QIAquick (Qiagen, Barcelona) and subcloned using a StrataClone™ PCR Cloning Kit (Stratagene, Barcelona). The isolated sub-cloning plasmid was purified using QIAprep Spin miniprep kit (Qiagen), and then digested by using NcoI and SacI. The digested fragment was purified from agarose gel using QIAquick, then ligated into pET52b(+) plasmid (Novagen, Barcelona), which was cut with the same enzymes, to create plasmid pSER70. Once the fragment was cloned, it was sequenced twice by using standard T3 and T7 primers, using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied-Biosystems, Barcelona). The resulting construction allows the production of the recombinant EIN-terminal fragment with a polyhistidine tag (His10-tag) at its C terminus and a thrombin cleavage site.

Expression and purification of EINsc

EINsc was expressed in BL21(DE3) strain (Novagen) using LB medium supplemented with ampicillin. Induction was carried out by addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside when the culture had an OD600 = 0.6–0.8. Growth was continued overnight at 37°C. Cells were centrifuged at 6 400 g in a Beckman Coulter J2-HS (Germany) centrifuge during 15 min and frozen at −80°C until they were used. The cell pellet from 8 l of LB media was treated with 50 ml of lysis buffer (500 mM NaCl, 5 mM imidazole, 20 mM Tris (pH 8.0) Tr, and a thrombin cleavage site.

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more than 95%, as judged from SDS gels. Approximately 10–15 mg of protein per litre of culture were obtained; protein concentration was determined from the extinction coefficient of tyrosine residues (Gill and von Hippel, 1989). The protein was dialyzed against water and frozen at −80 °C until its use.

Fluorescence

Fluorescence spectra were collected on a Cary Eclipse spectrofluorometer (Varian, USA) interfaced with a Peltier temperature-controlling system. Sample concentration was in the range of 1–2 μM, and the final concentration of the buffer was, in all cases, 10 mM. All fluorescence spectra in the pH range from 2.0 to 13.0 were collected at 25 °C (unless it is stated otherwise) by using a 1-cm-pathlength quartz cell (Hellma).

Steady-state fluorescence measurements. Since EINsc only contains four tyrosine residues and no tryptophans, samples were excited at 275 nm and the fluorescence spectra were recorded between 290 and 320 nm to be sure that the maximum emission intensity of the tyrosine amino acids was collected (Schmid, 1997) using 5 nm slits both for excitation and emission lights. The signal was averaged for 1 s and the wavelength increment was 1 nm. Blank corrections were made in all spectra. The salts and acids used in buffer preparation were: pH 2.0–3.0, phosphoric acid; pH 3.0–4.0, formic acid; pH 4.0–5.5, acetic acid; pH 6.0–7.0, NaH2PO4; pH 7.5–9.0, Tris acid; pH 9.5–11.0, Na2CO3; pH 11.5–13.0, Na3PO4. The pH was measured with an ultrathin electrode and interfaced with a Peltier unit. The instrument was periodically calibrated with an Aldrich electrode in a Radiometer (Copenhagen) pH-meter.

ANS binding. Excitation wavelength was at 380 nm and emission fluorescence was measured from 400 to 600 nm. Stock solutions of ANS were prepared in water and diluted into the samples to yield a final 100 μM dye concentration. Dye concentrations were determined using an extinction coefficient of 8000 M−1 cm−1 at 372 nm. In all cases, blank solutions were subtracted from the corresponding spectra.

Circular dichroism

Circular dichroism spectra of EINsc were collected on a Jasco J810 spectropolarimeter (Japan) fitted with a thermostated cell holder and interfaced with a Peltier unit. The instrument was periodically calibrated with (+) 10-camphorsulphonic acid. Unless it is stated otherwise, all the experiments were carried out at 25 °C.

Steady-state measurements. Isothermal wavelength spectra at different pHs were acquired at a scan speed of 50 nm/min with a response time of 2 s and averaged over four scans at 25 °C. Far-UV measurements were performed using 10 μM of EINsc in 0.1-cm-pathlength quartz cells (Hellma). Near-UV spectra were acquired using 30–40 μM protein in a 0.5-cm pathlength cell. All spectra were corrected by subtracting the proper baseline. The molar ellipticity, [θ], and the helical content were calculated as described (Muro-Pastor et al., 2003). In addition to changes in secondary structure and aromatic side chains, far-UV CD is also sensitive to distortions of helices such as bends or tilting of the amide planes relative to the helix axis (Woody, 1995; Kelly and Price, 2000; Seeramana and Woody, 2004). To take into account these factors, we have also used modern deconvolution methods of CD analysis, such as CDNN (Bohm et al., 1992) or DICROWEB (Whitmore and Wallace, 2004, 2008).

Every chemical denaturation was repeated at least three times with new samples. The chemical denaturations were fully reversible (data not shown). In the pH-induced unfolding experiments, the pH was measured after completion of the experiments, and essentially no differences were observed with those pHs calculated from the buffer stock solutions. The pH range explored was 2.0–13.0. Buffer concentration was 10 mM in all cases, and the buffers were the same used in fluorescence.

Thermal denaturation. Thermal denaturations of EINsc in the absence of urea were performed at a constant heating rate of 60 °C h−1 and a response time of 8 s. Thermal scans were collected in the far-UV region by following the ellipticity at 222 nm from 25 °C to 85 °C in 0.1-cm pathlength cells with a total protein concentration of 10 μM. The reversibility of thermal transitions was tested by recording a new steady-state spectrum after cooling down to 25 °C the thermally denatured samples, and comparing it with the spectrum obtained before heating; in all cases, both spectra were identical (data not shown). The possibility of drifting of the CD spectropolarimeter was tested by running two samples containing only buffer, before and after the thermal experiments; no difference was observed between the scans. Every thermal denaturation experiment was repeated at least twice with new samples.

To determine the ΔCp of the EINsc unfolding reaction, thermal denaturations in the presence of urea (at concentrations of 0.5, 0.75 and 1.0 M) were carried out with the same experimental set described above.

NMR spectroscopy

The NMR experiments were acquired on a Bruker Avance DRX-500 spectrometer (Bruker GmbH, Karlsruhe, Germany) equipped with a triple resonance probe and z-pulse field gradients. Protein concentration was 1 mM, unless it is stated. All measurements were carried out at 25 °C.

1H-1D-NMR spectra. NMR samples were prepared by dissolving the lyophilized protein in a 9:1 H2O:D2O solution, in phosphate buffer (50 mM, pH 7.5). The solution was centrifuged briefly to remove insoluble protein and then transferred to a 5-mm NMR tube. Homonuclear 1D-NMR spectra were acquired using 16 K data points, averaged over 512 scans and using 6501.69 Hz spectral width (13 ppm), with the residual water signal removed by the WATERGATE sequence (Piotto et al., 1993). Data were zero-filled, resolution-enhanced and baseline corrected with the XWINNMR software (Bruker GmbH, Karlsruhe, Germany) working on a PC computer. Qualitative exchange experiments were carried out by using Amicon centrifugal devices (Millipore), with a 10-kDa molecular weight cut-off, after 4 h of exchange at pH 7.0 and 5 °C. Spectra were referenced to external TSP.

Translational diffusion measurements (DOSY experiments). The following relationship exists between the translational self-diffusion parameter, D, and the delays used
(Czypionka et al., 2007):

\[
\frac{I}{I_0} = -\exp \left( D \gamma^2 \delta^2 G^2 \left( \Delta - \frac{\delta}{3} - \frac{\tau}{2} \right) \right),
\]

where \( I \) is the measured peak intensity of a particular (or alternatively a group of) resonance(s); \( I_0 \) is the maximum peak intensity of the same resonance(s) at the smaller gradient strength; \( D \) is the translational self-diffusion constant (in \( \text{cm}^2 \text{s}^{-1} \)); \( \delta \) is the duration (in s) of the gradient; \( G \) is the gradient strength (in T cm\(^{-1} \)); \( \Delta \) is the time (in s) between the gradients; \( \gamma_1 \) is the gyromagnetic constant of the proton; and, \( \tau \) is the recovery delay between the bipolar gradients (100 \( \mu \)s). Data were plotted as the \( -\ln(I/I_0) \) versus \( G^2 \) and the slope of the resulting line is \( D \gamma^2 \delta^2 (\Delta - \delta/3 - \tau/2) \), from where \( D \) can be obtained. The duration of the gradient varied between 2.2 and 3 ms and the time between both gradients was changed from 100 to 150 ms. The most up-field shifted methyl groups (those between 0.5 and 1.0 ppm) were used to measure the changes in intensity of the EIN\( ^{13} \)C spectra.

To determine the \( D \) of the domain, we used the approach previously described (Czypionka et al., 2007). Briefly, the value of \( D \) is protein-concentration dependent; therefore, we obtained the \( D \) at infinite dilution concentration by measuring it at different protein concentrations. The gradient strength was calibrated by measuring the \( D \) for the residual proton water line in a sample containing 100% D\(_2\)O in a 5-mm tube (Czypionka et al., 2007).

Measurements of the \( T_2 \)-relaxation time. Measurements of the \( T_2 \) (transverse relaxation time) provide a convenient method to determine the molecular mass of a macromolecule, since the correlation time, \( \tau_c \), is approximately equal to \( 1/(5T_2) \) (Anglister et al., 1993). We measured the \( T_2 \) for EIN\( ^{13} \)C with the 1–1 echo sequence (Sklenar and Bax, 1987); the calculation of the \( \tau_c \) was carried out as described (Anglister et al., 1993). It is important to keep in mind that the uncertainty inherent to this technique was 10% (Anglister et al., 1993).

Fourier transform infrared spectroscopy

Attenuated total reflection infrared (ATR-FTIR) spectra were obtained on a Bruker IFS55 FTIR spectrophotometer (Ettlingen, Germany) equipped with an MCT detector (with a broad band of 12 000–12 420 cm\(^{-1} \), cooled with liquid N\(_2\), and 24 h hold time). The resolution was 2 cm\(^{-1} \) with an aperture of 3.5 mm, and the spectra were acquired in the double-sided, forward–backward mode. Two levels of zero filling of the interferogram prior to Fourier transform allowed encoding the data every 1 cm\(^{-1} \). The spectrometer was continuously purged with dry air (Whatman 75-62, Haverhill, MA). The experimental set has been previously described (Aguado-Llera et al., 2010); the experiments were acquired at 25°C, and at pH 2.5 and 7.0. All spectra were recorded by attenuated total reflection (ATR) FTIR technique. A diamond internal reflection element was used on a Golden Gate Micro-ATR from Specac (Orpington, UK). The angle of incidence was 45. The small volume of 0.5 \( \mu \)l of the protein solution (20 mg/ml) was quickly evaporated under N\(_2\) stream to obtain a homogenous film and the cover of the flow cell from the Golden Gate Micro-ATR was closed.

Protein secondary structure was obtained as described (Goormaghtigh et al., 2006). For hydrogen/deuterium exchange experiments, N\(_2\) gas was saturated with D\(_2\)O by bubbling through a series of four vials containing D\(_2\)O; a flow rate of 50 ml min\(^{-1} \) was controlled by a flow tube (Fisher Bioblock Scientific, Illkirch, France). Sample deuteration started by connecting the measurement chamber to the D\(_2\)O-saturated N\(_2\) flow from the output of the first sample chamber.

The area of the Amide I and II peaks were obtained by automatic integration. For each spectrum, the area of Amide II was divided by the area of Amide I to take into account the swelling of the sample layer due to the presence of D\(_2\)O (Raussens et al., 2004). All kinetic curves were analysed as three-exponential decays using a nonlinear-least-square procedure. Spectra were collected every 30 s (32 scan each) during the first 4 min, and then every 2 min.

Gel filtration

The standards used in column calibration, and their corresponding Stokes radii were: ovalbumin (30.5 Å); bovine serum albumin (35.5 Å); aldolase (48.1 Å); ferritin (61 Å) and thyroglobulin (85 Å) (Hinkle et al., 1999; Carvalho et al., 2006). The bed and the void volumes were determined by using riboflavin and blue-dextran, yielding 23.6 and 7.0 ml, respectively. The samples were loaded in a Superdex G200 HR 10/30 (GE Healthcare, Spain) column, equilibrated in 25 mM Tris (pH 7.3) with 150 mM NaCl at 1 ml min\(^{-1} \), running on an AKTA-FPLC system (GE Healthcare) at 25°C, and monitored with an on-line detector at 280 nm. Gel filtration chromatography was used to determine the protein Stokes radius, \( R_s \), as it has been described (Darlin et al., 2000; Muro-Pastor et al., 2003). Briefly, such approach uses the weight average partition coefficient (\( \sigma \)) of a protein and takes the inverse of the error function complement of \( \sigma \) (erf\(^{-1} \) (\( \sigma \))), which has a linear relationship with the \( R_s \) (Darlin et al., 2000): \( R_s = a + b \text{erf}^{-1} (\sigma) \), where \( a \) and \( b \) are the particular calibration constants for the column. Measurements were repeated three times with 7 \( \mu \)M of EIN\( ^{13} \)C at 25°C.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed at 25°C in an Optima XL-A (Beckman-Coulter Inc.) analytical ultracentrifuge equipped with UV-visible optics, using an An50Ti rotor, with a 12 mm double-sector charcoal-filled Epon centrepiece. Samples of freshly dialyzed protein at 150 \( \mu \)M in 50 mM Tris (pH 8.0) were loaded into the cell, and the dialysate was transferred to the reference sector. The velocities used were 2 600 g, 5 900 g, 10 500 g, 16 400 g, 23 600 g and 32 100 g. Short column (90 \( \mu \)l), for sedimentation equilibrium, was performed and the system was assumed to be at equilibrium when the successive scans overlaid. The equilibrium scans were obtained at 280 nm. The baseline signal was measured after high-speed centrifugation (5 h at 42 000 rpm). The whole-cell apparent molecular weight of the protein was obtained using the program EQASSOC (Minton, 1994); the goodness of the fit was judged by the values of residuals (in all cases less than \( \pm 0.02 \)).
The sedimentation velocity experiments were carried out in an XL-A analytical ultracentrifuge (Beckman-Coulter Inc.) at 128,400 g and 25°C, using an An50Ti rotor and a 12 mm charcoal-filled Epon double-sector centrepiece. Absorbance was measured at 280 nm. Protein concentration was determined by spectrophotometry at 280 nm in 50 mM Tris (pH 8.0). Data were modelled as a superposition of Lamm equation solutions with the SEDFIT software (available at www.analyticalultracentrifugation.com/default.htm) (Schuck, 2000). The sedimentation coefficient distribution, \( c(s) \), was calculated at a confidence level of \( P = 0.68 \). The experimental sedimentation values were determined by integration of the main peak of \( c(s) \) and corrected to standard conditions to get the corresponding \( s_{20,w} \) values with the SEDNTERP program (Laue et al., 1992). Calculation of frictional coefficient ratio was performed with the SEDFIT program to obtain the \( c(M) \) distribution (Schuck, 2000).

**Differential scanning calorimetry**

DSC experiments were carried out with a VP-DSC calorimeter (MicroCal, Northampton, MA). Protein solutions were prepared by exhaustive dialysis against the working buffer (10 mM MES, pH 6.5, 200 mM NaCl) at 5°C. To minimize the amount of gas dissolved in the solutions, all the samples were degassed under vacuum for 10 min at room temperature with gentle stirring before being loaded into the calorimetric cells. Samples were heated at a constant scan rate of 1.0°C min\(^{-1} \) (60°C h\(^{-1} \)) and held under an extrapressure of 2 bars (28 psi) to avoid bubble formation and evaporation at high temperatures. Several buffer–buffer scans were performed to ensure proper instrument equilibration. The heat capacity change upon protein unfolding, \( \Delta C_p \), was determined from the temperature dependence of the calorimetric enthalpy change upon unfolding, \( \Delta H(T_m) \), obtained from experiments carried out in the presence of low concentrations of denaturant (0 < [GdmCl] < 1 M); solutions were prepared volumetrically using the previously dialyzed stock protein solution and a 2 M solution of GdmCl prepared in the dialysis buffer. In those experiments, the reference cell (and the sample cell for the buffer–buffer scans) was loaded with a matching solution of denaturant in the same buffer. To test whether the heat-induced denaturation of the protein was reversible, protein solutions were cooled in situ to 20°C for 30 min immediately after the first scan was completed (usually ranging from 20 to 80°C), and rescanned under the same experimental conditions. To correct for small mismatches between the two cells, an instrumental baseline (i.e., buffer–buffer baseline) was subtracted from the protein endotherm before data analysis. All traces were dynamically corrected to account for the time-delayed response of the detector to the heat event that evolved within the calorimetric cell. After normalization to concentration, a chemical baseline calculated from the progress of the unfolding transition was subtracted. Fitting was carried out by using the Origin 7.0 package supplied with the instrument.

**Analysis of the pH-, thermal- and chemical denaturation curves**

The pH-denaturation experiments were analysed by assuming that both species, protonated and deprotonated, contribute to the ellipticity of the CD spectrum at 222 nm, and/or to the fluorescence spectral intensity, according to the acid–base equation (Aguado-Llera et al., 2010):

\[
X = \frac{(X_a + X_b10^{(\text{pH}-\text{pK}_a)})}{(1 + 10^{\text{pH}-\text{pK}_a})^2},
\]

where \( X \) is the physical property being measured (ellipticity and/or fluorescence intensity at any particular wavelength), \( X_a \) is that at low pHs (acidic form), \( X_b \) is that at high pHs (basic form) and \( \text{pK}_a \) is the apparent \( pK \) of the titrating group. The apparent \( \text{pK}_a \) reported was obtained from three measurements, carried out with new samples.

The chemical-denaturation curves were analysed using a two-state model for the native/unfolded equilibrium, according to the linear extrapolation model:

\[
\Delta G = m(U)_{50\%} - [U],
\]

(Pace and Scholtz, 1997), where \( \Delta G \) is the free energy of denaturation, \([U]\) is the denaturant concentration and \([U]_{50\%}\) is the denaturant concentration at the midpoint of the transition. The denaturation data from far-UV CD and fluorescence were fitted to the two-state equation (Clarke and Fersht, 1993):

\[
X = \frac{(X_N + X_De^{(-\Delta G/RT)})}{(1 + e^{-\Delta G/RT})},
\]

where \( X_N \) and \( X_D \) are the corresponding ellipticities at 222 nm of the folded (N) and denatured states (D), respectively, for which a linear relationship with the denaturant (U) (i.e., \( X_N = \alpha N + \beta N \) and \( X_D = \alpha_D + \beta_D(U) \)) is assumed; \( R \) is the gas constant and \( T \) is the temperature in K.

In thermal-denaturations, the \( \Delta G \) was given by Privalov (1992):

\[
\Delta G(T) = \Delta H(T) - T\Delta S(T)
\]

\[
= \Delta H_m + \int_{T_m}^{T} \Delta C_p dT - T\Delta S_m - \int_{T_m}^{T} \frac{\Delta C_p}{T} dT,
\]

where \( \Delta S_m \) and \( \Delta H_m \) are the entropy and enthalpy of unfolding at the thermal denaturation midpoint, \( T_m \), and \( \Delta C_p \) is the heat capacity of the unfolding reaction. The shape of the two-state equation does not impose restrictions on the value of \( \Delta C_p \); that is, the exact determination of the \( T_m \) does not rely on a pre-fixed value of the heat capacity (Itzhaki et al., 1997). In fact, we did not find any difference in the final fitted value of \( T_m \) when \( \Delta C_p \) was either fixed (to 1200 cal mol\(^{-1} \) K\(^{-1} \), a usual standard value) or left to float in the fitting procedure.

In the thermal denaturations in the presence of different concentrations of denaturant monitored either by CD or DSC, the \( \Delta H_m \) was obtained from the fitting, and used to determine the \( \Delta C_p \), since \( \Delta C_p = \partial \Delta H_m / \partial T_m \) (Privalov, 1992). Fitting by non-linear least-squares analysis to the above equations was carried out by using Kaleidagraph (Abelbeck software) on a PC computer.

**Results**

**EIN\(^{\text{ac}} \) is a monomeric protein**

To determine the oligomerization state of EIN\(^{\text{ac}} \), we used a three-part approach. First, we calculated the Stokes radius, \( R_s \) and the molecular weight of the EIN\(^{\text{ac}} \) by using two different experimental NMR procedures. Second, we calculated the \( R_s \) by gel filtration; and finally, we determined the oligomerization state and the molecular weight of EIN\(^{\text{ac}} \) by AU.

The straight line of the translational diffusion coefficient, \( D \), measured at different concentrations yields a y-axis intercept of (8.59 ± 0.06) \( \times 10^{-7} \) cm\(^2\) s\(^{-1} \) (Fig. 1A); from the
Stokes-Einstein equation (Atkins and De Paula, 2006) the \( R_s \) is 20.3 Å. Dobson and co-workers (Wilkins et al., 1999) have suggested that the \( R_s \) of a folded protein is given by:
\[
R_s = (4.75 + 1.11) N_0^{0.29},
\]
where \( N_0 \) is the number of residues; this equation leads to a value of 24.0 ± 4 Å for EIN\(^{ec}\), which is similar to that determined by DOSY-NMR, and suggests that the protein is a monomer. The difference between both values could be due to an elongated molecular shape, or to the fact that we measure the average translational diffusion value of all species present in solution.

We also measured the \( T_2 \) of the most down-field shifted signals of the \( ^1\)H-\( ^1\)D-NMR spectrum of EIN\(^{ec}\) (at 10.30 ppm, Fig. 2B, see below). The value is 16.26 ms, which yields a correlation time of 12.19 ns, leading to a molecular weight of 24.59 kDa, similar to that calculated from the sequence (27.9 kDa).

EIN\(^{ec}\) eluted at 13.98 ml. Since the equation for the column is
\[
R_s = 12.974 + 41.958 \text{erfc} \left( \frac{s}{1} \right),
\]
this yields an experimental \( R_s \) for EIN\(^{ec}\) of 27.60 Å. The theoretical value of the Stokes radius, \( r_0 \), for a spherical molecule is (Atkins and De Paula, 2006):
\[
r_0 = \sqrt{\frac{3M\bar{V}}{4N\pi}},
\]
where \( M \) is the molecular mass of the protein; \( \bar{V} \) is the partial specific volume of the protein (0.720 cm\(^3\) g\(^{-1}\)) and \( N \) is the Avogadro’s number. This yields a value of 19.9 Å, which is smaller than that measured experimentally by gel filtration measurements, further supporting that EIN\(^{ec}\) has an elongated shape.

Since gel filtration and translational diffusion measurements cannot distinguish contributions of mass and shape to molecular diffusion, altogether the above results suggest the presence of a monomeric elongated species in solution. Conversely, analytical ultracentrifugation (AU) can be used to determine directly the molecular weight of proteins (Schuck, 2000). The monomeric nature of the protein was supported by sedimentation equilibrium measurements, since the distribution of mass along the cell could be fitted to a solute with an apparent molecular mass of 26.9 kDa (at 21 000 rpm) (Fig. 1B, top panel). In the sedimentation velocity experiments, a dominant species was observed with an estimated molecular weight of 28.2 kDa (Fig. 1B, bottom panel), slightly larger than that obtained by sedimentation equilibrium. Interestingly enough, there was a small broad peak at approximately 60 000 Da, which could be due to the presence of small population of dimeric species present (less than 10% as judged by the value of \( c(M) \), Fig. 1B, bottom panel). This dimeric species could not be detected in gel filtration experiments due to the low protein concentration used (7 versus 150 \( \mu \)M in AU), and the inherent dilution effect when loading into the column, nor in the NMR spectra due to the large molecular weight of the associated species, which would result in peak broadening.

To conclude, the EIN\(^{ec}\) construction described in this work is mainly a monomeric protein with an elongated shape. It is interesting to note that the shape of the monomeric EIN\(^{ec}\) in solution is elongated (Garrett et al., 1997, 1999); furthermore, the modelled structure of the intact EIN\(^{ec}\) also shows an elongated shape (Hurtado-Gómez et al., 2006).

**What is the structure of EIN\(^{ec}\)?**

We used several spectroscopic probes to address the structure of the protein, and to compare it with that of EIN\(^{ec}\)
and with that of the whole intact EIsc (Hurtado-Gómez et al., 2006).

The fluorescence spectrum of EIsc showed a maximum at 307 nm, which is characteristic of the emission of a tyrosine residue (data not shown) (Schmid, 1997).

The far-UV CD spectrum of EIsc shows the typical shape of an α + β helical protein, with minima at 208 and 222 nm (Fig. 2A), and it is similar to that of EIsc (Nosworthy et al., 1998). Deconvolution of the far-UV spectrum by CDNN and the DICHROWEB website yields the results of Table 1. The different procedures fairly agree in the percentages of the different types of secondary structure, and show that the predominant contribution to the far-UV spectrum of EIsc is that of random-coil conformations.

The near-UV of EIsc was very weak and it did not show any particular feature, probably due to the relatively low content of aromatic residues (four tyrosine and three phenylalanine residues) (data not shown).

The 1H-1D-NMR spectrum shows up-field shifted methyl groups (Fig. 2B, panel A) close to 0.0 ppm, and dispersion in the amide region (Fig. 2B, panel B). However, the spectrum also shows a mixture of broad and sharp lines suggesting that several regions of the protein have different mobility (Cavanagh et al., 1996), as also indicated by the 15N-1H HSQC spectrum, where different signals show different broadening (data not shown). Interestingly enough, there are two proton resonances at 10.30 ppm (as shown by the 15N-1H HSQC spectrum, data not shown), and since the protein does not contain any tryptophan residues (as concluded from the sequence, and the fluorescence spectrum), the resonances must be attributed to two down-field shifted amide protons. The presence of down-field shifted Hα protons at 5.5 ppm (Fig. 2B, panel B) also indicates the presence of β-sheet structure. Exchange experiments, after 4 h at 5°C by using the Amicon devices, suggest that at pH 7.0 a large proportion of the amide protons remain in the solution (roughly a 70%, as judged by integration of the whole amide region).

Since CD predicts a high percentage of disordered structure and NMR shows the presence of broad and sharp peaks, together with the dispersion in the amide and methyl regions, we decided to use a complementary spectroscopic technique to determine the percentages of secondary structure. The FTIR is a powerful method for investigation of protein secondary structure. The main advantage in comparison with CD and fluorescence is that FTIR is much more sensitive to the presence of β-structure or random-coil conformations. In 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 (Surewicz and Mantsch, 1988). The Amides I and II of the FTIR spectra of EI either at pH = 7.0 or 2.5 show maxima at 1654 and 1545 cm⁻¹, respectively (data not shown), which are characteristic of an α-helix (Goormaghtigh et al., 2006). The blind application of the methods described in the Materials and

### Table 1. Secondary structural analysis of EIsc as determined by CD and FTIR

<table>
<thead>
<tr>
<th>Structural assignment</th>
<th>CD</th>
<th>FTIR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDNN</td>
<td>k2D</td>
</tr>
<tr>
<td>α-Helix</td>
<td>17.8</td>
<td>20</td>
</tr>
<tr>
<td>β-Sheet</td>
<td>32.4</td>
<td>29</td>
</tr>
<tr>
<td>β-Turns</td>
<td>16.4</td>
<td>0</td>
</tr>
<tr>
<td>Random coil</td>
<td>33.4</td>
<td>51</td>
</tr>
</tbody>
</table>

The far-UV spectrum of EIsc in aqueous solution, phosphate buffer (continuous line, open circles) and at 6 M urea, pH 7.0 (dotted line, open squares). (B) The (a) methyl and (b) amide and aromatic regions of the 1H-1D-NMR spectrum of EIsc. Conditions were 25°C and pH 7.0, in 50 mM phosphate buffer. (C) Hydrogen exchange curves at two pHs (circles at pH 2.5, and crosses at pH 7.0), as determined by FTIR. The line through the points is the fitting to the three-exponential decay curve (see section Materials and methods for details); the y-axis is the normalized intensity.
Methods section yields the percentages of secondary structure of Table I. As it can be observed, the percentages of secondary structure are different at both pHs, with a larger percentage of α-helix at pH 2.5, whereas at pH 7.0 the percentages of β-sheet and random coil are larger than those at pH 2.5.

Exchange experiments at both pHs indicate that at pH 7.0 a 65% of the amide protons (the slowest ones) exchanged with a time constant of 10 000 min, whereas at pH 2.5, only a 50% of the amide protons exchanged with a time constant of 347 min. The exchange kinetics curves at both pHs, as monitored by FTIR, are shown in Fig. 2C.

To conclude, the EINsc cloned and purified in this work is a folded protein with a large content of disordered regions at pH 7.0, but a well-packed core of hydrogen-bonded amide protons (see section Discussion).

Is the structure of EINsc pH dependent?

We have shown that EINsc has a well-folded structure, but is this structure stable in a wide pH-range?, if not, in which pH range is stable?, which types of residues are responsible for possible pH-dependent conformational changes? Trying to address these questions, we carried out a spectroscopic characterization of EINsc at different pHs.

The fluorescence emission intensity at 307 nm showed two transitions (Fig. 3A, filled squares). The first one at acidic pHs, with a $pK_a$ of 3.9 ± 0.4, which could be due to an aspartic and/or glutamic residue (Thurlkill et al., 2006). The $pK_a$ of the second transition at basic pHs could not be determined since there is not a long basic baseline; this transition could be due to tyrosine residues.

The pH changes followed by the ellipticity also showed a sigmoidal transition at acidic pHs (Fig. 3A, blank circles) with a $pK_a$ of 3.4 ± 0.2. This value agrees, within the error, with that obtained by intrinsic fluorescence. It is important to note that the ellipticity at 222 nm at the lowest pHs was $-2800$ deg cm$^2$ dmol$^{-1}$, that is, more than two-thirds of the final value at pH 7.0 ($-3970$ deg cm$^2$ dmol$^{-1}$); this finding suggests that at acidic pHs there was an important amount of residual secondary structure, and EINsc was not completely unfolded, as it has been previously suggested by FTIR (see above).

ANS is used as a fluorescence probe, which binds to spatially close solvent-exposed hydrophobic patches (Semisotnov et al., 1991), shifting the fluorescence maxima from 520 (isolated ANS) to 480 nm (ANS-bound to a polypeptide hydrophobic patch). At low pHs, the ANS fluorescence intensity was large in the presence of EINsc, indicating solvent-exposure of hydrophobic patches; as the pH was increased, the intensity at 480 nm decreased, and thus, burial of solvent-exposed hydrophobic patches occurs. The $pK_a$ of the transition was 3.8 ± 0.1, which agrees with that obtained by the above techniques (Fig. 3B).

To sum up, all the spectroscopic techniques show a sigmoidal transition in EINsc, when folded from acidic pHs with an average $pK_a$ of $3.7 ± 0.3$.

The conformational stability of EINsc

From the above studies, we can conclude that EINsc acquires a native conformation at pHs >5.0, and that this structure remains unchanged until pH 8.5 (as shown by fluorescence, Fig. 3A, filled squares). However, how are the EINsc thermal and chemical stabilities in that pH interval?; although the structure does not change significantly, does the stability do?; furthermore, are both thermal and chemical denaturations reversible? Trying to address these questions, we carried out the description of the thermal and chemical stabilities of EINsc followed by CD and DSC.

Thermal denaturations. We explored the thermal stability of EINsc at pHs 5.4, 7.0, 8.0, 9.0 and 9.5; except for pH 5.4, which is on the verge of the acidic titration (Fig. 3), the rest of the denaturations was reversible. Furthermore, the stability, as determined from the $T_m$ value, was similar at all pHs, ranging from $56.6 ± 0.4^\circ$C (at pH 5.4) to $58 ± 0.3^\circ$C (at pH 9.5) (Fig. 4A). At the highest temperature reached during the denaturations (85°C), there is still evidence of structure (around 10%), as concluded from the remaining ellipticity.

Since the thermal denaturations were reversible, we decided to determine the $\Delta C_p$ of the EINsc unfolding reaction by CD. However, since the $T_{mS}$ were pH independent, we carried out thermal-denaturation measurements at low concentrations of urea (Fig. 4B). The fitting of the traces to a two-state equation allows the determination of the $T_m$ and the van’t Hoff enthalpy change upon unfolding, $\Delta H_{vH}(T_m)$. The variation of $\Delta H_{vH}(T_m)$ with $T_m$ is linear, yielding a $\Delta C_p = 836 ± 816$ cal mol$^{-1}$ K$^{-1}$ (data not shown); we obtained a similar value, within the error, when GdmCl was used as denaturant ($2192 ± 1317$ cal mol$^{-1}$ K$^{-1}$).
To determine more precisely the DCp, we monitored the heat-induced unfolding process by high-sensitivity DSC in the presence of different amounts of GdmCl. At pH 7.0, the protein seems to unfold following a two-state mechanism with a Tm of $59.3 \pm 0.1$ °C (Fig. 5A). Under these conditions, the reversibility of the process is high (around 80%, as judged by the enthalpy change upon unfolding of the refolded sample) and the Tm for the unfolding of the previously refolded protein amounts to $59.7 \pm 0.1$ °C, very close to the value obtained in the first scan. However, important differences were observed between the calorimetric and van't Hoff enthalpies ($41 \pm 4$ and $134 \pm 5$ kcal mol$^{-1}$, respectively), which might suggest the presence of large amounts of disordered structure in the folded state (see section Discussion) or aggregated species. Moreover, the low enthalpy change upon unfolding points towards an incomplete unfolding of the polypeptide chain leading to a partially folded conformation, which may retain a large percentage of secondary and/or tertiary structure. The presence of increasing [GdmCl] (from 0.125 to 1 M) decreased the thermal stability of the protein as evidenced by a large and linear decline in Tm, with the slope of the linear regression, $\partial Tm/\partial$[GdmCl], being $-18.4$ K M$^{-1}$ (Fig. 5B). Figure 5C shows the variation of the calorimetric enthalpy change, for the scans performed at different [GdmCl]. The slope of the linear regression yields a value for the DCp of 1.47 kcal K$^{-1}$ mol$^{-1}$.

Chemical denaturations. We carried out chemical denaturations followed by CD in the same pH interval as the thermal

![Fig. 4. Thermal denaturations of EINsc followed by CD. (A) Thermal denaturations followed by the changes in the ellipticity at 222 nm (far-UV CD) at selected pHs (filled squares, pH 7.0; open circles, pH 8.0; open diamonds, pH 9.5). The y-axis for each trace has been scaled for reader's sake. (B) Thermal denaturations at different urea concentrations (filled squares, 0 M; open circles, 1 M) followed by the changes in the ellipticity at 222 nm. The y-axis for each trace has been scaled for reader's sake.

Fig. 5. DSC of EINsc. (A) Superposition of the excess heat capacity functions for the heat-induced unfolding of EINsc at different GdmCl concentrations; the endotherm in all cases is consistent with a two-state unfolding mechanism. The concentrations of GdmCl used in each experiment were (curves from higher to lower, $Tm$): 0, 0.125, 0.25, 0.50, 0.75 and 1.0 M, respectively. (B) Linear dependence of the decrease in the thermal stability of the protein ($Tm$, °C) with the concentration of denaturant ([GdmCl], in M). The slope of the linear regression amounts to $-18.4$ K M$^{-1}$. Fitting errors in the $Tm$ were smaller more than 0.1 °C. (C) Linear dependence of the calorimetrically (DSC) determined calorimetric temperature molar enthalpy change upon unfolding, $\Delta H_m$ ($Tm$), with the melting temperature, $Tm$. The slope of the linear regression yields the molar heat capacity change upon unfolding, DCp, which amounts to 1.47 kcal K$^{-1}$ mol$^{-1}$. Errors in the $\Delta H_m$ ($Tm$) were fitting errors to the two-state unfolding mechanism; fitting errors in the $Tm$ were smaller than 0.1 °C.

ones (that is, from pH 6.0 to 8.5, where the spectroscopic properties of the protein did not change). This allowed us to obtain the apparent free energy profile of EINsc as a function
The structure of EIN<sup>sc</sup>

The modelled structure of EIN<sup>sc</sup> (Hurtado-Gómez et al., 2006), which is based on the X-ray structure of EIN<sup>ec</sup> (Liao et al., 1996), consists of a four-helix bundle, comprising α-helices 1–4; and an α/β subdomain, comprising a β-sandwich, formed by a four-stranded parallel β-sheet (β-strands 1–4) and a three-stranded antiparallel β-sheet (β-strands 1, 5 and 6), and three short α-helices (helices 5-7) that contain the phosphorylation site (His186). The percentages of secondary structure as shown by the modelled structure, are: α-helices: 51% (133 residues), β-sheet: 16% (43 residues) and random coil: 32% (82 residues). These percentages agree well with those obtained by FTIR deconvolution at pH 7.0 (Table I); however, the CD deconvolution programs predict a higher content of random-coil conformations than the FTIR data and the modelled structure (Table I). We do not know the exact reasons of the discrepancy, but they could be due to: (i) the number of tyrosine and phenylalanine residues (four tyrosine and three phenylalanine residues) present in the protein, and which absorb at 222 nm (Vuilleumier et al., 1993; Woody, 1995); (ii) large distortions of α-helices or β-sheets, which could not be taken into account by the deconvolution algorithms (Sreerama and Woody, 2004); (iii) the fact that the FTIR measurements were acquired with the ATR technique, where a film of the protein is prepared (see Materials and Methods section); and, (iv) the fact that deconvolution algorithms are still not good enough for predicting accurately the structure of some proteins. Thus, the atomic-detailed structure of EIN<sup>sc</sup> must wait until high-resolution NMR methods are applied, if the apparent different segmental mobility of EIN<sup>sc</sup> (as suggested by the 1D-NMR (Fig. 2) and <sup>15</sup>N-H HSQC spectrum) does not hamper assignment (JLN, unpublished results). However, (i) the identical shape of the far-UV CD spectra of EIN<sup>ec</sup> and EIN<sup>sc</sup> (Nosworthy et al., 1998) and (ii) the agreement among the predicted secondary structure populations by FTIR and the modelled structure of EIN<sup>sc</sup> suggest that the EIN<sup>sc</sup> structure must be similar to that of other members of the EIN family.

The conformational stability of EIN<sup>sc</sup>

Figure 6 shows the pH-dependence of the apparent Gibbs free-energy of EIN<sup>sc</sup> at 25°C. Its maximum conformational stability averages 4.1 ± 0.4 kcal mol⁻¹, and occurs as a broad maximum between pH 6.0 and 8.5, where the spectroscopic probes indicate that the protein maintains a native-like structure (Fig. 3). At low pH, where native EIN<sup>sc</sup> is positively charged, the pKₐ's of the carboxyl groups in the folded protein are expected to be lower than in the unfolded species (Tandford, 1968). Then, unfolded EIN<sup>sc</sup> binds protons more tightly than folded EIN<sup>sc</sup>, and this seems to be the main reason in the stability decrease as the pH is lowered. Similar pH behaviour has been observed in RNase A and RNase T1 (Pace et al., 1990; Yao and Bolen, 1995), barnase (Pace et al., 1992), in SAMP73 (Barrera et al., 2002) and staphylococcal nuclease (Ionescu and Eftink, 1997; Whitten and García-Moreno, 2000; Whitten et al., 2001).

The m-value of the denaturations of EIN<sup>sc</sup> was constant in the pH interval from 6.0 to 8.5, and it decreased at the extreme of pHs (Fig. 6B). Whether or not the m-value of pH. The chemical denaturations are completely reversible at the different pHs, as shown by the superposition of the curves started from the fully folded and unfolded protein (data not shown); however, it is important to indicate that the ellipticity at 6 M urea was not null, but it had around 15% of the ellipticity at 0 M, suggesting the presence of residual structure in the unfolded state. The results suggest that EIN<sup>sc</sup> is moderately stable at acid and neutral pHs; its maximum apparent conformational stability at 25°C is ΔG = 4.1 ± 0.4 kcal mol⁻¹ (the average from the free-energy values measured between pH 6.0 and 8.5) (Fig. 6A), occurring in a broad maximum between pH 6.0 and 8.5. The m-values of the chemical denaturation are fairly constant, except at low and high pHs (see Discussion) (Fig. 6B), with an average value of 1.3 ± 0.2 kcal mol⁻¹ M⁻¹.

Fig. 6. Stability of EIN<sup>sc</sup> measured by CD urea-denaturation experiments. (A) Curve of ΔG with the pH of EIN<sup>sc</sup>. (B) Curve of the m-values. (C) The midpoints of the denaturation curves [urea]¹/₂. The conditions were 10 μM of protein, at 25°C; buffer concentration was 10 mM in all cases. Spectra were acquired in 0.1-cm pathlength cell.
depends on pH differs from protein to protein. For instance, in RNase A, a linear relationship has been found (Pace et al., 1990); the m-value remains constant in SAMp73 (Barrera et al., 2002), but a more complex behaviour has been observed in RNase T1 (Pace et al., 1990), barnase (Pace et al., 1992), and staphylococcal nuclease (Whitten and García-Moreno, 2000; Whitten et al., 2001). It has been suggested that a variation in the m-value when the pH decreases, is related to the pKₐ values of a putative conformational intermediate (Whitten et al., 2001). Then, according to this model, the decreasing of the m-values at acidic pHs in EIN⁰ would mean that a possible intermediate should have unfolded-like pKₐ's. Since there was: (i) evidence of secondary structure at low pHs as shown by FTIR (Table I); (ii) a large amount of residual ellipticity (Fig. 3A; blank circles) at low pHs; and, (iii) ANS-binding occurring at acidic pHs (Fig. 3B), then we can speculate that the decrease in the m-values of EIN⁰ at low pHs could be monitoring the presence of an intermediate, which could be a molten-globule species (see below).

We can compare the experimental m-values with the value calculated theoretically; to that end we need to estimate the solvent accessible surface area (ASA) of both the native and the completely unfolded protein. Both ASA values were obtained by using the program GETAREA (Fraczkiewicz and Braun, 1998) (http://curie.utmb.edu/getarea.html). An m-value can be estimated from the relationship between m and ΔASA_total (Myers et al., 1995): m (cal mol⁻¹ M⁻¹) = 368 + 0.11 ΔASA_total. Since the ΔASA_total (the difference in the total accessible surface area between the folded and completely unfolded state) from the modelled EIN⁰ is 21 782 Å², this yields a value of 2.76 kcal mol⁻¹ M⁻¹, significantly higher than that observed experimentally. Furthermore, the average experimental m-value of EIN⁰ is smaller (1.3 ± 0.3 kcal mol⁻¹ M⁻¹) than that obtained by multiplying the number of helical residues in EIN⁰ (133 residues, according to the modelled structure (Hurtado-Gómez et al., 2006)) and the per residue m-value for helix unfolding (0.023 kcal mol⁻¹ M⁻¹) (Scholtz et al., 1995): 3.06 kcal mol⁻¹ M⁻¹. We hypothesize that these differences are due to the fact that the EIN⁰ does not completely unfold in the presence of urea (as it is further supported by the finding that the [θ] at 222 nm at 6 M urea was not null, data not shown).

We also used the three-dimensional modelled structure of EIN⁰ to estimate theoretically the heat capacity change upon complete unfolding of the protein, ΔC_p. It has been shown (Gómez et al., 1995) that the molar heat capacity, C_p, of a given conformational state of a protein can be considered as composed by the sum of: (i) an intrinsic term (arising from the contribution to C_p of the covalent bonds present in the molecule and the noncovalent internal interactions within the protein); and, (ii) a term due to the interactions between the surface-exposed residues of the protein and the water molecules solvating it (hydration). Therefore, the ΔC_p for the transition between two conformational states can be calculated as the difference between the molar heat capacity, C_p, of the final state and that of the initial state. Since the contribution arising from the covalent bonds to C_p is identical for all the accessible conformational states of the protein, ΔC_p is comprised only by the other two main contributions: the one due to internal interactions (among solvent buried groups of the protein) and that due to hydration (protein–water interaction). These terms can be rationalized by the changes in the ASAs (polar and apolar) induced by the transition between the two conformational states of the protein (Freire, 1995; Gómez and Freire, 1995; Gómez et al., 1995; Hilser et al., 1996): ΔC_p = 0.45 ΔASA_polar - 0.26 ΔASA_polar - 0.0087 ΔASA_total, where ΔASA_polar and ΔASA_polar are the differences in apolar and polar ASAs between the two conformational states. Estimations of the changes in ASA between the fully unfolded (taken as the final denatured state of the protein) and the native state of the protein by taking into account the modelled structure are: ΔASA_polar = 13 116 Å², ΔASA_polar = 8666 Å² and ΔASA_total = 21 782 Å², which yields an estimated value of 3845 cal K⁻¹ mol⁻¹ for ΔC_p for the complete unfolding of the protein. Since this value is significantly larger than the calorimetrically determined (1470 cal K⁻¹ mol⁻¹; Fig. 5C), it can be concluded that EIN⁰ does not unfold completely at high temperatures. Furthermore, the calculated enthalpy change upon complete unfolding of the protein estimated from the changes in ASA (ΔH_m = -8.44 ΔASA_polar + 31.4 ΔASA_polar, Freire, 1995) amounts to 161 kcal mol⁻¹, 4-fold larger than the DSC calorimetrically determined one (41 kcal mol⁻¹). Therefore, the heat-induced denaturation of EIN⁰ yields a partially folded species with a large fraction of its structure intact, although we think that this residual structure is not a molten-globule-like species (since it does not bind ANS, data not shown). These findings are in agreement with the presence of residual helicity at temperatures higher than the T_m.

Finally, a question remains, why are the calorimetric, ΔH_cal, (41 ± 4 kcal mol⁻¹) and van’t Hoff enthalpies, ΔH_vh (134 ± 5 kcal mol⁻¹) of the EIN⁰ thermal denaturation so different if a two-state folding mechanism is proposed? The experimentally determined endotherms (Fig. 5A) fitted nicely to a two-state mechanism yielding low statistical residuals. Nevertheless, the calorimetric to van’t Hoff enthalpies ratio, ΔH_cal/ΔH_vh, significantly deviates from 1, the value that should be expected for a truly two-state unfolding mechanism (Privalov, 1992; Ibarra-Moleró and Sánchez-Ruiz, 2006). We suggest several reasons to explain the low calorimetric to van’t Hoff enthalpies ratio and the good fittings of the endotherms to a two-state model. First, we have observed that at high concentrations (as those used in AU and in DSC measurements), a small population of dimeric species appeared (Fig. 1B, bottom); the presence of this small population of dimeric species would increase the molar enthalpy change of the cooperative unit (the van’t Hoff enthalpy), although at a low extent due to its small fraction. And second, and most importantly in our view, we have observed that the percentage of random-coil structure is high (~30%), as shown by the FTIR deconvolution results (Table I) and the EIN⁰ modelled structure. Freire and co-workers (Xie et al., 1991; Straume and Freire, 1992) have shown that when the transition temperature of the protein under study is relatively low, and the percentages of random-coil conformations are high (or in other words, the native state is not fully populated) the ΔH_cal tends to be underestimated while ΔH_vh is overestimated, leading to a ratio between them lower than 1. This could be the case for EIN: the large content of random-coil conformations of EIN could...
lead to an overestimation of $\Delta H_{\text{un}}$. However, we do not think that EIN$^{\text{nc}}$ is partially or badly folded, or alternatively in a molten-globule-like conformation (as in the example described by Freire and co-workers, Xie et al., 1991) as a consequence of the deletion of the C-terminal domain. We have several pieces of evidence to support the correctly fold of EIN$^{\text{nc}}$. First, we think that EIN does not populate a molten-globule species at pH 7 and 25°C, since: (i) a sigmoidal thermal transition was observed by spectroscopic techniques (Fig. 4A); and, (ii) no ANS binding was observed at physiological pHs (ANS-binding is one of the signatures of molten-globules, Pitsyn, 1995) at room temperature (Fig. 3B) nor at higher temperatures (as suggested by fluorescence thermal denaturations in the presence of ANS, data not shown). Second, EIN$^{\text{nc}}$ is able to bind HPr$^{\text{sc}}$ with an affinity one order of magnitude larger than the intact EI$^{\text{sc}}$. Furthermore, it is able to recognize peptides containing the His active site also with higher affinity than the intact EI$^{\text{sc}}$ (Olga Abian and Adrián Velázquez-Campoy, unpublished results, personal communication). This situation seems to be incompatible with a partially folded native conformation of EIN$^{\text{nc}}$ if one takes into account the energetic penalty that should be paid by the protein in order to adopt its final conformation.

Comparison with the thermodynamic parameters of the thermal unfolding reaction of other EIN domains

We can also compare the thermal stability of EIN$^{\text{nc}}$ with the thermal stability of other N-terminal domains. One of the advantages of studying proteins within the same family is trying to elucidate whether a similar three-dimensional structure determines the same unfolding thermodynamics, and if not, which are the structural and thermodynamic reasons behind the differences. The N-terminal domain of the EI protein from Salmonella typhimurium, EIN$^{\text{sc}}$, shows pH-independent conformational transitions as explored only by DSC (LiCalsi et al., 1991) with a $T_m$ close to 55°C; that from E.coli, EIN$^{\text{nc}}$, shows also $T_m$ close to 57°C (Dimitrova et al., 2002; Nosworthy et al., 1998). Furthermore, although the $\Delta H_{\text{cal}}$/$\Delta H_{\text{un}}$ rate has not been reported, DSC studies on EIN$^{\text{nc}}$ suggest that the thermal unfolding process follows a two-state unfolding model and that the van’t Hoff enthalpy is $149 \pm 5$ kcal mol$^{-1}$ (Nosworthy et al., 1998; Gingsburg et al., 2000), similar to the value reported in this work for EIN$^{\text{nc}}$ ($134 \pm 5$ kcal mol$^{-1}$); interestingly enough, the van’t Hoff enthalpy estimated from CD measurements in EIN$^{\text{nc}}$ was 127 kcal mol$^{-1}$ (in EIN$^{\text{sc}}$, it accounts for $85 \pm 10$ kcal mol$^{-1}$, data not shown); that is in both proteins the spectroscopic data report a lower estimate of the DSC van’t Hoff enthalpy. However, the $\Delta C_p$ of the EIN$^{\text{nc}}$ unfolding reaction, which was obtained from the DSC profiles at the same pH, was estimated to be $2.7 \pm 0.3$ kcal K$^{-1}$ mol$^{-1}$ (Nosworthy et al., 1998; Gingsburg et al., 2000); this value is similar to the theoretical one which could be expected if the final thermal denatured state was fully unfolded (see above), in contrast to what is observed in EIN$^{\text{sc}}$. Then, from comparison to the values obtained in this work with EIN$^{\text{nc}}$, it seems that the thermal stability (as measured by $T_m$) among the members of the EIN family is similar, but the $\Delta C_p$ and $\Delta H_{\text{un}}$ are different, suggesting that there are key differences in the primary structure among EIN members, which could be responsible for the presence of residual structure in the unfolded state, and possibly to alter the dynamics of the folded state. Although we are tempted to suggest such hypothesis, we do not know at this stage whether the changes in the thermodynamic parameters of the unfolding reaction of the isolated EIN domains could be affecting the thermodynamic parameters of the binding reaction to the corresponding HPr.

Finally, it is interesting to note that the $T_m$ of EIN$^{\text{nc}}$ (this work) is similar to that determined in the intact EI$^{\text{sc}}$ by CD and DSC (Hurtado-Gómez et al., 2006), suggesting that the denaturation of EIC$^{\text{sc}}$ occurs at low temperatures. In fluorescence measurements of the intact EI$^{\text{sc}}$ protein, we have observed another transition at 42°C, which has been tentatively assigned to the denaturation of the EIC$^{\text{sc}}$ in the intact protein. The results of this work show that the fluorescence low-transition temperature corresponds unambiguously to the EIC$^{\text{nc}}$, and that the broad transition observed by CD and DSC (and occurring at 55°C) corresponds to the EIN$^{\text{sc}}$.

pH-induced unfolding of EIN$^{\text{nc}}$

The tertiary and secondary structures of EIN$^{\text{nc}}$ do not change significantly between pH 6.0 and 8.5 as concluded from the fluorescence, CD, and ANS-binding measurements (Fig. 3); this interval is the same where the apparent conformational stability remains unchanged (Fig. 6). However, there are changes observed by the three spectroscopic techniques at low pH, occurring with the same $pK_a$ ($3.7 \pm 0.3$), which must be associated with the titration of an aspartic and/or glutamic acid residues (Thurlkill et al., 2006). Since there are 14 Asp residues and 24 Glu residues in EIN$^{\text{nc}}$, it is not possible to assign such titration to a particular amino acid or a group of them. Furthermore, the fact that all the biophysical probes show the same titration midpoint suggests that the burial of solvent-accessible hydrophobic patches (ANS binding), and the acquisition of tertiary (fluorescence) and secondary (CD) structures occurs concomitantly. However, there is evidence of persistent secondary structure at low pH,
as shown by: (i) the residual ellipticity at low pH (Fig. 3A); (ii) the FTIR Amide I deconvolution methods (Table I); and, (iii) the exchange FTIR results. Since: (i) this low-pH species binds ANS (Fig. 3B); (ii) it lacks most of the tertiary structure (Fig. 3A); (iii) has residual secondary structure (as shown by CD and FTIR, Table I), which is protected from solvent-exchange (as shown by FTIR) (a fraction of 50% amide protons exchange with a rate constant of 346 min, whereas a population of 25% exchange with a rate constant of 14.3 min, close to the expected value for disordered structures (see above)); and, (iv) this low-pH species does not show a thermal sigmoidal transition (as shown by CD thermal denaturations carried out at pH 3.5, data not shown), then, the low-pH species is a molten globule (Pitsyn, 1995).

A question can be raised, how does this pH behaviour of EIN⁰⁸ compare with that of the intact EI⁰⁸? The structure of the intact EI⁰⁸ also remains unaltered between pH 6.0 and 8.0, although a titration in the far-UV CD close to 6.0 was observed. This titration is not observed in EIN⁰⁸ (Fig. 3), suggesting that it must involve residues at the EIC⁰⁸ (tentatively, the sole His residue in the C-terminal domain of EI⁰⁸). The whole EI⁰⁸ also has a molten-globule-like conformation at low pH (Hurtado-Gómez et al., 2006); furthermore, the intact protein acquires concomitantly the secondary and tertiary structures, and the burial of hydrophobic patches. Finally, the folding of EI⁰⁸ at acidic pHs occurs with a pKₐ = 4.0 ± 0.3, identical to the value observed in EIN⁰⁸ (3.7 ± 0.3). Then, we hypothesize that the folding of the intact EI⁰⁸ from acidic pHs might be governed at a large extent by that of isolated EIN⁰⁸.

Biological implications

In the long term, we are interested in describing at atomic detail the structural reasons behind the binding between HPr⁰⁸ and EI⁰⁸. We have previously shown that the affinity of both proteins is ~100 μM (Hurtado-Gómez et al., 2008). This value is larger than that observed in the association of EI-ΔH (see above); and, this low-pH species does not show a thermal sigmoidal transition (as shown by CD thermal denaturations carried out at pH 3.5, data not shown), then, the low-pH species is a molten globule (Pitsyn, 1995).

We have cloned and successfully expressed the N-terminal domain of the enzyme I of the PTS system in S. coelicolor. The protein is well folded, mainly monomeric and behaves as an autonomous folding-unit within the whole intact EI protein. For the first time, we have fully characterized the conformational stability of an EIN family member. Its apparent stability is very low (in the order of 4 kcal mol⁻¹), and it remains unaltered in a wide pH range. Despite its long size (258-residues long) the protein unfolds (chemical and thermally) by an apparent two-state mechanism, but the final denatured state, contains evidence of residual structure as shown by DSC and CD.

Conclusions

We have cloned and successfully expressed the N-terminal domain of the enzyme I of the PTS system in S. coelicolor. The protein is well folded, mainly monomeric and behaves as an autonomous folding-unit within the whole intact EI protein. For the first time, we have fully characterized the conformational stability of an EIN family member. Its apparent stability is very low (in the order of 4 kcal mol⁻¹), and it remains unaltered in a wide pH range. Despite its long size (258-residues long) the protein unfolds (chemical and thermally) by an apparent two-state mechanism, but the final denatured state, contains evidence of residual structure as shown by DSC and CD.

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