The Polypeptide Chain of Eukaryotic Initiation Factor 5A Occurs in Two Distinct Conformations in the Absence of the Hypusine Modification

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ABSTRACT: Eukaryotic initiation factor 5A (eIF-5A) requires posttranslational modification of lysine at position 50 to hypusine for its biological activity. We have expressed an unmodified variant of eIF-5A in *Escherichia coli* and show that it has structural properties different from those of the native protein in terms of its near- and far-UV circular dichroism spectra and its equilibrium unfolding transition with guanidinium chloride. In contrast to the hypusine-modified protein, which unfolds in a two-state process, the complex unfolding transition of unmodified eIF-5A suggests that this variant occurs in two differently folded conformations, F_1 and F_2. Both conformations are populated under near-physiological conditions at a ratio of 60 to 40, respectively. Equilibrium unfolding consists of parallel events: unfolding of F_1 to one or several intermediate states (I), and unfolding of F_2 to the unfolded state (U). Although the establishment of each of these individual equilibria is fast, the interconversion is slow at guanidinium chloride concentrations between 0 M and 3 M. Kinetic analysis reveals activation energies of 24.3 kcal mol^{-1} for the reaction of F_1 to F_2 and 24.1 kcal mol^{-1} for the reaction of F_2 to F_1. Both F_1 and F_2 possess well-defined secondary and tertiary structure. However, the tertiary structures of the two conformations differ as indicated by their distinct near-UV circular dichroism spectra. These differences may be restricted to the C-terminal part of the protein as 2-dimensional ^{1}H-NMR spectra of unmodified eIF-5A reveal no doubled set of proton resonances for aromatic amino acid and histidine residues, of which almost all are located in the N-terminal region. Since the F_2 conformation has structural properties very similar to those of the hypusine-modified protein, the importance of this modification is likely to be connected with the hypusine residue itself and not with an induced conformational change *per se*. Our results further suggest that not every polypeptide chain has the inherent capacity to fold to a unique energy minimum. Since the observed energy minima between F_1 and F_2 are separated by a high energy barrier, we argue in favor of a "kinetic hypothesis" of protein folding.

Eukaryotic initiation factor 5A is the only protein that contains the amino acid hypusine [for review, see Park et al. (1993)]. The single hypusine residue is derived by specific posttranslational modification of a lysine side chain at position 50. The modification occurs in two enzyme-catalyzed reactions that involve (i) the transfer of the aminobutyl moiety of spermidine to the ϵ-amino group of lysine at position 50 in eIF-5A[1] and (ii) subsequent hydroxylation of this intermediate:

$$\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-NH}_2 \rightarrow\rightarrow \quad \text{(lysine)}$$

$$\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-NH-CH}_2\text{-CHOH-CH}_2\text{-CH}_2\text{-NH}_2 \quad \text{(hypusine)}$$

The amino acid sequence of eIF-5A (including the hypusine residue) is well conserved throughout all eukaryotes and archaebacteria. However, no protein with a related sequence was found in eubacteria, which also lack the enzymes necessary for hypusine synthesis (Park et al., 1993). Moreover, correct modification of lysine to hypusine is a prerequisite for cell viability and proliferation in eukaryotes and archaebacteria, and thus for eIF-5A to be functional in its cellular environment (Schnier et al., 1991). Although eIF-5A is an essential protein that is abundant in these organisms, its function is not yet fully understood. eIF-5A was originally named after its ability to stimulate the *in vitro* formation of the first peptide bond in protein synthesis (Safer, 1989; Park et al., 1993). Recent findings, however, suggest that the protein may fulfill other functions *in vivo*; e.g., it may participate in mRNA transport [eIF-5A was suggested to be a cellular binding partner of the HIV-1 Rev protein; see Ruhl et al. (1993)] or mediate the synthesis of a specific subset of proteins (Kang & Hershey, 1994).

Human eIF-5A is a monomeric protein that consists of 153 amino acids (Klier et al., 1995). In this work, we have compared the structural properties of a hypusine-modified HeLa cell-derived and an unmodified recombinant version of human eIF-5A to detect alterations in the overall fold of the protein upon modification of K50 to hypusine. Our results suggest that only the native, hypusine-modified protein is able to fold into a unique 3-dimensional structure; unmodified recombinant eIF-5A occurs in two differently folded conformations that are in slow equilibrium. One of these conformations possesses structural properties similar to those of the hypusine-modified form.

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[1] Abbreviations: CD, circular dichroism; DTT, 1,4-dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; eIF-5A, eukaryotic initiation factor 5A; Gdn·HCl, guanidinium chloride.

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MATERIALS AND METHODS

Materials

Sequanal grade Gdn·HCl, which was used for spectroscopic studies, was purchased from Pierce. Factor Xa was obtained from Boehringer Mannheim Corp. GSH-Sepharose was obtained from Pharmacia. All other reagents were of analytical grade.

Protein Purification

Hypusine-modified eIF-5A was purified from HeLa S3 cells as described (Klier et al., 1995). For the production of unmodified eIF-5A protein, the human eIF-5A cDNA (Koettnitz et al., 1994) was amplified by standard PCR techniques (Clackson et al., 1991) and inserted into the *Bam*HI restriction site of plasmid pGEX-3X (Pharmacia). The resulting glutathione *S*-transferase/eIF-5A fusion protein was expressed in *E. coli* BL 21 cells according to the supplier's specifications and purified as follows: 20 g of wet cells were resuspended in 80 mL PBS (10 mM sodium phosphate buffer at pH 7.4, with 150 mM NaCl), containing 1 mM EDTA and 0.05% 2-mercaptoethanol. After sonication at 0 °C, the resulting homogenate was centrifuged at 34 000*g* for 60 min. The supernatant was loaded onto a column of GSH-Sepharose (2.6 cm × 6.0 cm) which was equilibrated and washed with the same buffer (PBS containing 1 mM EDTA and 0.05% 2-mercaptoethanol). Following a second washing step with 150 mL of PBS, containing 1 mM EDTA, 0.05% 2-mercaptoethanol and NaCl at a total concentration of 1 M, the guathione *S*-transferase/eIF-5A fusion protein was eluted from the column with PBS supplemented with 10 mM L-glutathione (reduced form). Average yields were 5 mg of fusion protein per gram of wet cells.

For specific cleavage of the glutathione *S*-transferase/eIF-5A fusion protein with factor Xa, the protein solution was dialyzed against 50 mM Tris-HCl buffer at pH 8.0, containing 0.1 M NaCl and 1 mM $CaCl_2$, and concentrated by ultrafiltration with Amicon PM 10 filters to a concentration of about 2 mg/mL. Factor Xa (1 mg/mL in the same buffer) was added to a ratio of 1/200 of the fusion protein by weight. After incubation for 16 h at room temperature, the reaction mixture was passed over a column of GSH-Sepharose (2.6 cm × 6.0 cm) and loaded onto a Mono Q column (Pharmacia), which was equilibrated with 50 mM Tris-HCl buffer at pH 8.0, containing 0.5 mM EDTA and 0.05% 2-mercaptoethanol. The unmodified eIF-5A protein was eluted with a linear gradient of 0−0.5 M NaCl and stored at −20 °C after the protein solution was dripped into liquid nitrogen.

Determination of Protein Concentration

The concentrations of hypusine-modified and unmodified eIF-5A were determined by their absorbance at 280 nm in solutions containing 6 M Gdn·HCl. The corresponding molar absorption coefficients were calculated from the number of tryptophan and tyrosine residues, using the tabulated absorption coefficients of tryptophan and tyrosine in model compounds (Gill & von Hippel, 1989). The calculated molar absorption coefficient is 3840 M^{-1} cm^{-1} for both eIF-5A variants.

Circular Dichroism Measurements

CD measurements were performed with a Jasco J710 instrument at 22 °C. Far-UV CD (185−250 nm) spectra were recorded at protein concentrations of 10 to 20 μM in 0.02-cm or 0.1-cm cells; near-UV CD (250−320 nm) spectra, at protein concentrations of 20 to 40 μM in 1-cm or 2-cm cells. The spectra, which were averaged over 10 accumulations, were corrected for the appropriate buffer baseline. Mean residue ellipticity values ($[\Theta]_{mrw}$) were calculated using the expression

$$[\Theta]_{mrw} = 100\Theta_{obs}/lc$$

where Θ_{obs} is the observed ellipticity in degrees, c is the molar residue concentration, and l is the light path in centimeters (Schmid, 1989).

Equilibrium unfolding of hypusine-modified and unmodified eIF-5A was monitored by following the change in circular dichroism at 217 nm and 269 nm. The measurements were conducted on protein solutions of 0.1 mg/mL (at 217 nm) and 0.2 to 0.4 mg/mL (at 269 nm) in cells having a 0.5-cm and 2-cm light path, respectively. Following incubation for 4 days at 4 °C in 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM EDTA, 1 mM DTT, and the indicated concentrations of Gdn·HCl, the ellipticity reached a constant value.

Gel Filtration Chromatography

Gel filtration chromatography was performed on a Superdex 75 column (1 cm × 30 cm) using a Pharmacia FPLC system equipped with a Merck-Hitachi L-4200 variable wavelength detector. The column was equilibrated and eluted with 50 mM potassium phosphate buffer at pH 6.5, containing 1 mM EDTA, 1 mM DTT, and the indicated concentrations of Gdn·HCl at 22 ± 2 °C. Elution profiles were recorded by monitoring the absorption at 230 nm after injection of 100-μL samples. The flow rate was 0.4 mL/min.

Equilibrium unfolding measurements of hypusine-modified and unmodified eIF-5A were carried out on protein solutions of 0.05 mg/mL in the above column buffer. The proteins were incubated with various Gdn·HCl concentrations for 4 days at 4 °C before they were injected onto the column. For equilibrium refolding studies, the proteins (0.3 mg/mL) were unfolded in a solution of 4 M Gdn·HCl for 24 h at 4 °C. Prior to the measurements, the unfolded proteins were diluted into solutions containing the indicated concentrations of Gdn·HCl and incubated for 4 days at 4 °C.

Interconversion of the two conformations of unmodified eIF-5A was measured at different buffer conditions after their separation by gel filtration chromatography. Protein solutions at 2.4 mg/mL in 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM EDTA and 1 mM DTT, were injected onto the column and eluted with the same buffer containing 1.6 M Gdn·HCl. The two peaks (9.8 mL and 10.8 mL elution volumes) were collected separately. Kinetic studies of the interconversion of the species contained in these peaks was done at concentrations of 1.6 and 0.1 M Gdn·HCl in the above buffer either by directly reinjecting aliquots of each of the separated fractions after the indicated time intervals, or by rapidly diluting the samples with 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM EDTA and 1 mM DTT, prior to injection, respectively. The column was eluted with buffer containing 1.6 M Gdn·HCl as described above. In addition, a similar time course was followed after unfolding of the protein in a solution of 6 M Gdn·HCl for 4 days at 4 °C, and refolding by dilution. To

measure the interconversion of the different conformations at 3 M Gdn·HCl, unmodified eIF-5A in 50 mM potassium phosphate buffer, pH 6.5, with 1 mM EDTA and 1 mM DTT, was rapidly mixed with a solution of 6 M Gdn·HCl in the same buffer to give a final Gdn·HCl concentration of 3 M and a final protein concentration of 0.08 mg/mL. Aliquots of this solution were applied to the gel filtration column after various time intervals, and the column was eluted with buffer supplemented with 1.6 M Gdn·HCl.

Equilibrium Unfolding Monitored by Fluorescence

Fluorescence measurements were carried out using a SLM-8000C spectrofluorimeter equipped with JD-490 photomultipliers and a 450 W Xenon Arc lamp. The spectral band width was set to 2 nm for excitation and 4 nm for emission. Measurements were performed at 25 °C in a 750-μL quarz cuvette with a 10-mm optical path length. Protein solutions of 0.1 mg/mL were incubated in 50 mM potassium phosphate buffer at pH 6.5, containing 1 mM EDTA, 1 mM DTT, and the indicated concentrations of Gdn·HCL for 4 days at 4 °C. The change of intrinsic tyrosine fluorescence was monitored at an excitation wavelength of 278 nm and an emission wavelength of 310 nm, with polarizers in the emission and excitation path set at magic angle (Lakowicz, 1983). The measured protein fluorescence values were corrected for the small apparent fluorescence change of the solutions of Gdn·HCl in the buffer.

NMR Methods

Unmodified eIF-5A was dialyzed against buffer (50 mM potassium phosphate, 1 mM DTT, 99.9% D_2O or H_2O/10%D_2O) at pH 6.5 to give a final protein concentration of 1 to 2 mM in 0.5 mL. Sodium 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid was used as an internal reference (0 ppm). Two-dimensional ^{1}H-NMR spectra were recorded on a Bruker AMX500 spectrometer at a probe temperature of 27 °C. For samples made up in H_2O, the jump-and-return sequence for effective water suppression was used in both the homonuclear-Hartmann-Hahn spectroscopy (HOHAHA) and nuclear-Overhauser-spectroscopy (NOESY) experiments (Bax & Davis, 1985), while the correlation spectroscopy (COSY) experiments were performed using SCUBA phase cycling (Brown et al., 1988) to recover resonances under the water peak. For samples made up in D_2O, the transmitter offset was placed on the HOD resonance, which was irradiated during the relaxation time (1.3 s) in order to suppress the HOD signal. All experiments were carried out using a spectral width of 6024 Hz. Typically, 2048 data points were recorded in t_2 for each of 600 to 900 t_1 values with 128 transients/free induction decay. pH values are uncorrected meter readings on a radiometer PHM 84 Research pH meter, using an Ingold combination electrode.

RESULTS

Expression and Purification of the Proteins

Hypusine-modified eIF-5A was purified from HeLa cells to >95% purity as judged by 2-D gel electrophoresis and silver staining (see Klier et al., 1995). In addition to the modification of lysine at position 50 to hypusine, the protein is N-terminally acetylated such that the first methionine residue is replaced by an acetyl group. The molecular weight of the purified protein was determined by mass spectrometry

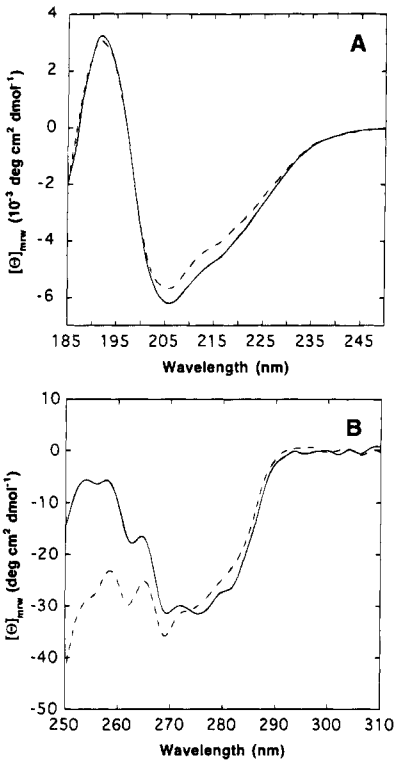

FIGURE 1: CD spectra of hypusine-modified and unmodified eIF-5A. Protein concentrations were 10−40 μM in 50 mM potassium phosphate, pH 6.5, with 0.5 mM EDTA and 0.5 mM DTT. Spectra were recorded at 22 °C. (—) Hypusine-modified; (− − −) unmodified eIF-5A. (A) Far-UV CD spectra measured in a cell with 0.02-cm path length. (B) Near-UV CD spectra recorded in a cell with 1-cm light path.

(M_r 16,830) and agreed well with the deduced M_r of 16 829, the calculation of which is based on the published cDNA sequence (Smit-McBride et al., 1989) and the modifications outlined above (see Klier et al., 1995).

Prokaryotic cells lack the two enzymes that are necessary for hypusine modification. In order to produce large amounts of unmodified eIF-5A, the protein was, therefore, expressed in *E. coli* cells. Insertion of the human cDNA into the *Bam*HI restriction site of plasmid pGEX-3X led to the coding sequence of a glutathione *S*-transferase/eIF-5A fusion protein, which contains a factor Xa cleavage site between the glutathione *S*-transferase and eIF-5A moieties. After expression and purification, the fusion protein was cleaved specifically with factor Xa. This cleavage generated an eIF-5A molecule with three additional amino acids at its N-terminus (GIQ−). The unmodified eIF-5A variant was purified to >95% purity as judged by 2-D gel electrophoresis and silver staining, and verified by N-terminal amino acid sequencing and mass spectrometry. The determined M_r of 17,132 is in good agreement with the calculated molecular weight (M_r 17 131).

Spectroscopic Properties of Hypusine-Modified and Unmodified eIF-5A

Circular dichroism spectra of hypusine-modified and unmodified eIF-5A were measured as a relative indicator of differences in secondary and tertiary structure (Bayley, 1980; Schmid, 1989). Figure 1A shows the far-UV CD spectra of the proteins under native conditions. The spectrum of unmodified eIF-5A is very similar to that of the hypusine-modified variant. Both spectra, which have an ellipticity

maximum at 192 nm and a minimum at 206 nm with a shoulder between 215 and 230 nm, reflect well-defined secondary structure. Deconvolution of the far-UV CD spectrum of hypusine-modified eIF-5A indicates 10% α-helical and 38% β-strand conformation (Klier et al., 1995). Since the CD spectrum of unmodified eIF-5A has only 90% of the amplitude of the hypusine-modified eIF-5A spectrum between 202 and 225 nm, the unmodified protein appears to possess a slightly lower content of secondary structure.

As shown in Figure 1B, the near-UV CD spectra of hypusine-modified and unmodified eIF-5A, which are determined by aromatic amino acids and their environment in the protein, differ qualitatively. The spectrum of both hypusine-modified and unmodified eIF-5A have ellipticity minima at 263, 269, and 276 nm; however, the negative amplitude at 263 nm is more and that at 276 nm is less pronounced for the unmodified protein. Both eIF-5A variants, therefore, appear to have defined tertiary structure. However, at least some of the respective aromatic amino acids seem to be located in different environments in the corresponding proteins.

Equilibrium Folding Studies

Small globular proteins frequently unfold cooperatively, and alterations within the amino acid sequence often cause changes in their unfolding transition (Jaenicke, 1991; Fersht, 1993). We used the Gdn·HCl-induced unfolding transitions of both eIF-5A variants as an additional criterion for assessing the influence of the hypusine modification on the overall structure and stability of the protein. Unfolding at equilibrium was followed by different spectroscopic measurements and by gel filtration chromatography to detect changes in secondary structure (CD at 217 nm; fluorescence emission at 310 nm), in tertiary structure (CD at 269 nm; fluorescence emission at 310 nm), and in hydrodynamic volume (gel filtration chromatography) of the protein. In general, one round of unfolding in concentrated solutions of Gdn·HCl and refolding by dialysis had no effect on the spectroscopic properties of the protein variants.

Hypusine-Modified eIF-5A. The hypusine-containing protein variant unfolds cooperatively and reversibly with Gdn·HCl. The normalized unfolding curves are shown in Figure 2. The unfolding transitions monitored by CD, fluorescence emission, and gel filtration are identical, each having a midpoint at 1.4 M Gdn·HCl. All three spectroscopic signals and the elution volume from the gel filtration column decreased upon unfolding, indicating the loss of secondary and tertiary structure with a simultaneous increase in the protein's hydrodynamic volume. Unfolding of hypusine-modified eIF-5A, therefore, appears to follow a two-state process in which only the native and the unfolded states are stable.

Unmodified eIF-5A. In contrast to eIF-5A carrying a hypusine residue, unfolding of the unmodified protein is more complex. As shown in Figure 3A, gel filtration chromatography yielded two peaks at intermediate concentrations of Gdn·HCl. Both peaks shifted towards smaller elution volumes (i.e., increased hydrodynamic volume of the polypeptide chain) with increasing concentrations of denaturant. Further, the peak with the larger elution volume decreased and that with the smaller elution volume increased in peak area with increasing Gdn·HCl concentrations. The unfolding transition was fully reversible in respect to both elution

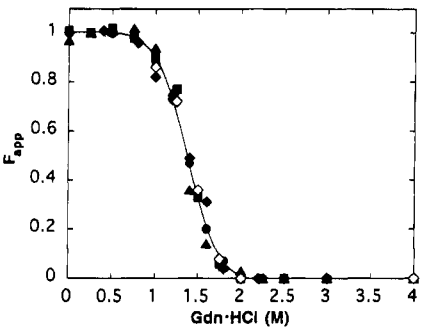

FIGURE 2: Equilibrium unfolding of hypusine-modified eIF-5A. Unfolding was induced with Gdn·HCl and followed either by different spectroscopic signals or gel filtration chromatography as described under Materials and Methods. The unfolding and refolding curves were normalized by extrapolating the linear change of the individual signals above 2.5 M Gdn·HCl to 0 M Gdn·HCl. F_{app} is the change of the individual signals expressed as a fractional deviation from this baseline. Unfolding monitored by (●) CD at 217 nm, (■) CD at 268 nm, (▲) fluorescence emission at 310 nm, (◆) gel filtration chromatography; (◇) refolding monitored by gel filtration chromatography.

volume and peak area, and equilibrium refolding experiments gave identical results (data not shown for clarity of Figure 3A). Thus, unfolding of unmodified eIF-5A at equilibrium occurs via two fast equilibria, which are manifested by the individual shift of both peaks, and one slow equilibrium, evident from the finding that the two peaks do not interconvert to any significant extent during the column run (Uversky & Ptitsyn, 1994).

Unfolding followed by spectroscopic measurements yielded different transitions (Figure 3B). The midpoints of the curves varied between 1.4 M Gdn·HCl for the loss of tertiary structure (CD at 269 nm) to 1.6 M Gdn·HCl for the loss of secondary structure (CD at 217 nm). This discrepancy between the different unfolding curves indicates that parts of the secondary structure of unmodified eIF-5A unfold after the tertiary structure of the protein is lost. Comparison of all four unfolding transitions (Figure 3C) reveals that the loss of tertiary structure coincides with the shift in elution volume of one of the peaks from gel filtration chromatography, namely that with the smaller elution volume, which increases in peak area with increasing Gdn·HCl concentrations.

Comparison of the Unfolding Curves. An overlay of the normalized unfolding curves of hypusine-modified and unmodified eIF-5A followed by CD at 269 nm is shown in Figure 4. Both transitions are very similar and possess identical midpoints. The similarity of the unfolding transitions suggests that the tertiary structures of the two eIF-5A variants have an almost identical unfolding behavior. However, whereas the secondary structure of hypusine-modified eIF-5A unfolds simultaneously with the tertiary structure, unfolding of some of the secondary structure of unmodified eIF-5A takes place at higher concentrations of Gdn·HCl.

The complex unfolding transition as determined by gel filtration chromatography suggests that the unmodified protein occurs in two differently folded conformations, F_1 and F_2 (see discussion). The tertiary structures of both conformations unfold in a similar manner; however, F_1 unfolds to a intermediate state or states, I, with which it is in rapid equilibrium at moderate concentrations of Gdn·HCl, and F_2 unfolds to the unfolded state (cf. Figure 8). Transition of F_1 to I is reflected by the shift of the peak with the larger elution volume, whereas transition of F_2 to

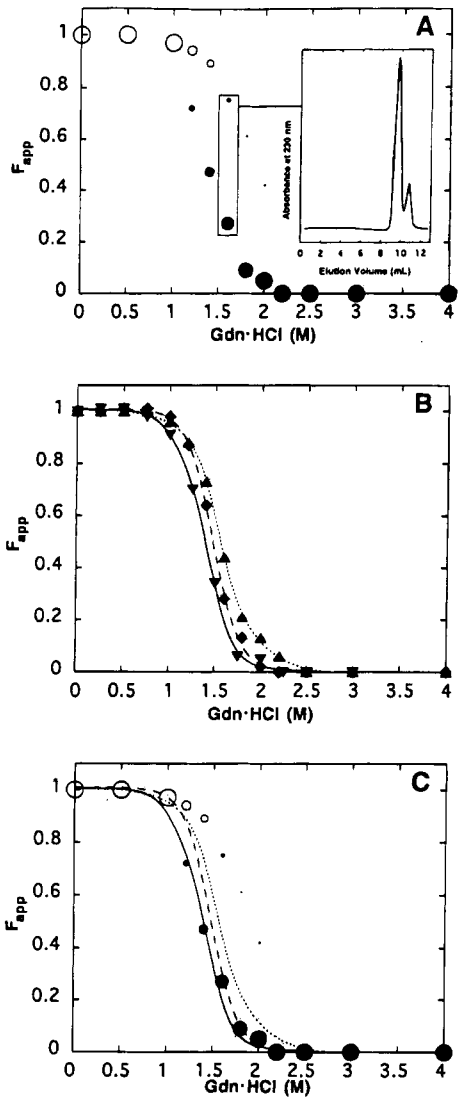

FIGURE 3: Equilibrium unfolding of unmodified eIF-5A. Unfolding was induced with Gdn·HCl and followed either by different spectroscopic signals or by gel filtration chromatography as described under Materials and Methods. F_{app} is defined in Figure 2. (A) Gel filtration chromatography. The protein elutes in two peaks (● and ○) at intermediate Gdn·HCl concentrations. The corresponding peak areas are proportional to the respective marker size. The inset shows a representative elution profile of unmodified eIF-5A at 1.6 M Gdn·HCl. (B) Unfolding monitored by (.....▲.....) CD at 217 nm; (—▼—) CD at 268 nm; and (— — ◆ — —) fluorescence emission at 310 nm. (C) Comparison of the unfolding transitions from (A) and (B). (● and ○) Gel filtration chromatography: (.....) CD at 217 nm; (—) CD at 268 nm; (— — —) fluorescence emission at 310 nm.

the unfolded state is manifested by the shift of the peak with the smaller elution volume on the gel filtration column. In the presence of increasing concentrations of Gdn·HCl, the former transition is converted into the latter one. As shown in Figure 4, unfolding of F_2 to the unfolded state overlays with the loss of tertiary structure of eIF-5A; i.e., F_2 appears to have a similar unfolding transition as the hypusine-modified protein.

Characterization of Two Differently Folded Forms of Unmodified eIF-5A

Kinetic Studies. The two conformations of unmodified eIF-5A, which were detected in the equilibrium unfolding experiment, can best be resolved by gel filtration chromatography in the presence of 1.6 M Gdn·HCl. Because of

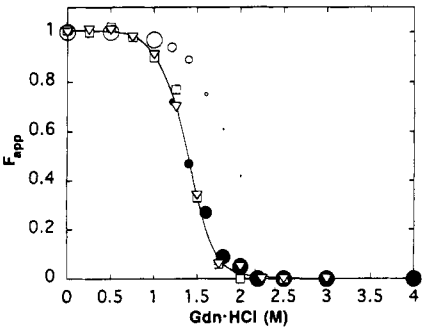

FIGURE 4: Comparison of the equilibrium unfolding transmission of hypusine-modified and unmodified eIF-5A. The unfolding transitions were taken from Figures 2 and 3. Unfolding of (□) hypusine-modified and (▽) unmodified eIF-5A followed by CD at 268 nm; (● and ○) unfolding of unmodified eIF-5A followed by gel filtration chromatography. The solid line represents the unfolding transition of the hypusine-modified protein.

their different unfolding behaviors they possess different elution volumes at intermediate concentrations of denaturant: F_1, which appears to be in rapid equilibrium with a partially folded form (I), has an elution volume of 10.8 mL; and F_2, which appears to be in rapid equilibrium with the unfolded state, has an elution volume of 9.8 mL (inset to Figure 3A). Since the two peaks are both present at 1.6 M Gdn·HCl in the equilibrium unfolding experiment, the two equilibria appear to be established in parallel, but interconvert only slowly.

To distinguish between the two equilibria, the eIF-5A forms were eluted from the gel filtration column in the presence of 1.6 M Gdn·HCl in all of the following experiments. Direct injection of unmodified eIF-5A, which was dialyzed extensively against a buffer of near-physiological salt concentration and pH (50 mM potassium phosphate, pH 6.5, containing 150 mM KCl, 1 mM EDTA, and 1 mM DTT), resulted in an elution profile similar to that obtained in equilibrium unfolding, where the protein was incubated in the presence of 1.6 M Gdn·HCl prior to the column run. The protein incubated under near-physiological conditions eluted in two peaks with 9.8 and 10.8 mL elution volume, respectively. However, a different ratio in the peak areas was observed as compared to the equilibrium unfolding experiment. The first peak (9.8 mL elution volume) accounted for 40%, and the second (10.8 mL elution volume) for 60% of the protein molecules. Since the elution volumes are equal to those obtained in the equilibrium unfolding experiment, interconversion between F_1 and I as well as between F_2 and the unfolded state is fast compared to the time required for elution. Further, assuming that the interconversion of both equilibria, i.e., the differently folded forms contained in both peaks, is sufficiently slow, the above results suggest that the F_1 and F_2 conformations occur under native conditions at a ratio of about 60 to 40, respectively.

Neither F_1 nor I appears to be in rapid exchange with the unfolded state. Therefore, to measure the kinetics for the interconversion of F_2 to F_1 under native conditions, the protein was unfolded in a solution of 6 M Gdn·HCl and refolded by dilution (the final concentration of Gdn·HCl was 0.1 M). Aliquots of this solution were injected onto the column after different time intervals. The column was eluted in the presence of 1.6 M Gdn·HCl and the peak areas of the resulting elution profiles were integrated (Figure 5A). Direct injection of the refolded protein yielded a single peak with 9.8 mL elution volume, indicating exclusive formation of

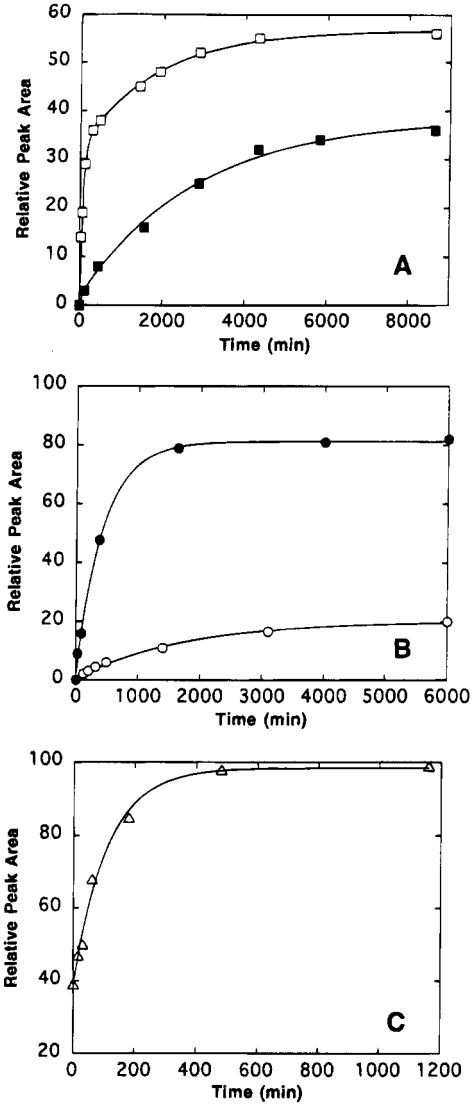

FIGURE 5: The differently folded conformations of unmodified eIF-5A interconvert slowly. Interconversion was followed by gel filtration chromatography in the presence of 1.6 M Gdn·HCl. Aliquots of the protein solutions were injected onto the column after various time intervals, and the corresponding peak areas of the resulting elution profiles were integrated. The solid lines represent the data fitted to eq (1) or (2); see text. (A) Interconversion of (■)$F_1 \rightarrow F_2$ and (□) $F_2 \rightarrow F_1$ in 50 mM potassium phosphate, pH 6.5, with 1 mM EDTA, 1 mM DTT, and 0.1 M Gdn·HCl. The initial F_1 and F_2 conformations were individually generated either by refolding unmodified protein after separation of the respective molecule species by gel filtration chromatography in the presence of 1.6 M Gdn·HCl (F_1), or by refolding the protein after incubation in 6 M Gdn·HCl (F_2). (B) Interconversion of the two fast equilibria at 1.6 M Gdn·HCl. The corresponding peaks (cf. Figure 3A) were individually collected after gel filtration chromatography in the presence of 1.6 M Gdn·HCl and reinjected onto the column. (●) Time-dependent formation of the peak with 9.8 mL elution volume, corresponding to the fast equilibrium between F_2 and U; (○) time-dependent formation of the peak with 10.8 mL elution volume, corresponding to the fast equilibrium between F_1 and I. (C) Interconversion of I to U. Prior to the measurement, unmodified eIF-5A was rapidly unfolded by addition of Gdn·HCl to a final concentration of 3 M, thus yielding a mixture of I and U.

the F_2 conformation. However, with longer incubation time a second peak with 10.8 mL elution volume became apparent. With the assumption that the reaction from U to F_2 is fast compared to the interconversion of F_2 to F_1, the presence of this second peak reflects the formation of F_1 from F_2. The increase in peak area at 10.8 mL with time, which was analyzed by a nonlinear regression procedure, failed to fit to a single exponential, but fitted well to a double exponential process with an offset [eq. (1)]:

$$A(t) = A_{0,1} \exp(-k_{obs,1}t) + A_{0,2} \exp(-k_{obs,2}t) + C \quad (1)$$

where $A(t)$ is the peak area at time t, $A_{0,1}$ and $A_{0,2}$ are the relative amplitudes, $k_{obs,1}$ and $k_{obs,2}$ are the rate constants of the two phases, and C is the offset. Interconversion of F_2 to F_1 proceeds with two phases, the first having a rate constant of $(1.5 \times 0.1) \times 10^{-2}$ min^{-1} and an amplitude of (32 ± 1)%, and the second with a rate constant of $(5.5 \pm 0.7) \times 10^{-4}$ min^{-1} and an amplitude of (25 ± 1)%.

Interconversion of F_1 to F_2 was measured after both forms were separated by gel filtration chromatography in the presence of 1.6 M Gdn·HCl. The second peak (10.8 mL elution volume), which contained protein molecules involved in the fast equilibrium between F_1 and I, was collected, and the equilibrium shifted toward F_1 by dilution (the final concentration of Gdn·HCl was 0.1 M). Time-dependent formation of F_2 from F_1 (Figure 5A) was measured by injecting aliquots of this solution onto the gel filtration column as described above. Direct injection of the refolded protein molecules resulted in a single peak with 10.8 mL elution volume. With increasing incubation time, a second peak with 9.8 mL elution volume occurred, thus indicating formation of F_2 from F_1. The increase in peak area at 9.8 mL with time fitted to an equation describing a single exponential process [eq (2)]:

$$A(t) = A_0 \exp(-k_{obs}t) + C \quad (2)$$

where A_0 is the amplitude and k_{obs} the rate constant. The final peak area was acquired in a slow single-exponential process with a rate constant of $(3.4 \pm 0.3) \times 10^{-4}$ min^{-1} and an amplitude of (40 ± 1)%. Therefore, in contrast to the formation of F_1 from F_2, which appears to involve two distinguishable forms that fold to F_1, the formation of F_2 from F_1 proceeds via a single reaction.

Interconversion of the two peaks at 1.6 M Gdn·HCl is slow (Figure 5B). Following their separation, aliquots from each peak were reinjected after different time intervals, and the formation of the corresponding second peak was monitored by integrating the respective peak areas. The increase in peak area of the second peak was fitted to a single exponential process described by equation (2). Formation of the peak with 9.8 mL elution volume proceeded with a rate constant of $(2.3 \pm 0.2) \times 10^{-3}$ min^{-1} and an amplitude of about 81%, and that of the peak with 10.8 mL elution volume proceeded with a rate constant of $(5.9 \pm 0.6) \times 10^{-4}$ min^{-1} and an amplitude of 19%. Thus, the two equilibria—i.e., that between F_1 and I and that between F_2 and the unfolded state—interconvert slowly in the presence of 1.6 M Gdn·HCl. These results also underline the finding that neither F_1 nor I is in rapid exchange with F_2 and/or the unfolded state.

Since both the unfolding of F_1 to I and F_2 to U are fast, addition of 3 M Gdn·HCl results in a mixture of I and U only. Aliquots of this mixture were injected onto the gel filtration column after different time intervals to detect the subsequent interconversion of I to the unfolded state. The column was eluted in the presence of 1.6 M Gdn·HCl, and the fast reestablishment of the individual equilibria (between F_1 and I and between F_2 and U) enabled the evaluation of

Table 1: Influence of Ionic Strength and pH on the Relative Stabilities of F_1 and F_2[a]

buffer conditions[b]	F_1 (%)[c]	F_2 (%)[c]
pH 6.5	62	38
pH 6.5 + 0.75 M $(NH_4)_2SO_4$	65	35
pH 6.5 + 1 M KCl	64	36
pH 4.5	72	28
pH 4.5 + 0.25 M KCl	82	18
pH 4.5 + 1 M KCl	98	2
pH 8.5	1	99
pH 8.5 + 1 M KCl	40	60

[a] Unmodified eIF-5A was dialyzed against the respective buffers for 5 days. The protein solutions were analyzed by gel filtration chromatography in the presence of 1.6 M Gdn·HCl as described under Materials and Methods. [b] All buffers contained 1 mM EDTA and 1 mM DTT: pH 6.5: 50 mM potassium phosphate at pH 6.5; pH 4.5: 50 mM sodium acetate at pH 4.5; pH 8.5: 50 mM Tris-HCl at pH 8.5. [c] The ratio of F_1 to F_2 was measured by integrating the peak areas at 10.8 mL and 9.8 mL elution volume, respectively.

the ratios of I and U at 3 M Gdn·HCl (Figure 5C). Unfolding of I proceeded in a slow single-exponential process with a rate constant of $(9.3 \pm 0.9) \times 10^{-3}$ min^{-1}, suggesting that it is separated by a high energy barrier from the unfolded state.

Influence of Ionic Strength and pH. The relative solubilities of the differently folded conformations of unmodified eIF-5A appear to be dependent on the buffer conditions (Table 1). After extensive dialysis of the protein against buffers of different ionic strength and pH, the protein solution was subjected to gel filtration chromatography. Gdn·HCl was added to the elution buffer to a concentration of 1.6 M in order to distinguish between the two conformations. Whereas at near-physiological pH (pH 6.5) the ratio of F_1 to F_2 does not significantly change with the salt concentration, there is a strong influence of the ionic strength on the relative stabilities of F_1 and F_2 at acidic and alkaline pH values. At pH 4.5, increasing salt concentrations lead to almost exclusive formation of F_1. In contrast, low concentrations of salt favor the formation of the F_2 conformation at pH 8.5.

Spectroscopic Studies. It is possible to generate unmodified eIF-5A exclusively in its F_1 or F_2 conformation by using the appropriate buffer conditions (cf. Table 1). In order to detect any differences in secondary and tertiary structure between these conformations, the far- and near-UV CD spectra of the protein were measured in 50 mM sodium acetate at pH 4.5, containing 1 M KCl (exclusive formation of F_1) and in 50 mM Tris-HCl (exclusive formation of F_2). The spectra, which are shown in Figure 6, are almost identical to those obtained when both forms were separated on a gel filtration column in the presence of 1.6 M Gdn·HCl and rapidly refolded by dilution into 50 mM potassium phosphate buffer at pH 6.5. The far-UV CD spectrum of unmodified eIF-5A in its F_1 conformation has an ellipticity minimum which is shifted toward shorter wavelength as compared with the F_2 conformation (Figure 6A). In addition, the shoulder between 210 and 220 nm is of smaller amplitude in the F_1 spectrum. These spectra suggest that F_2 has a slightly higher content of secondary structure than has F_1. Comparison of the near-UV CD spectra (Figure 6B) of both conformations reveals a qualitative difference in the chiral environment of one or more aromatic amino acid residues. Although both spectra possess similar ellipticity minima, the amplitudes of these minima are considerably different. Further, the spectrum of the F_2 conformation equals that of

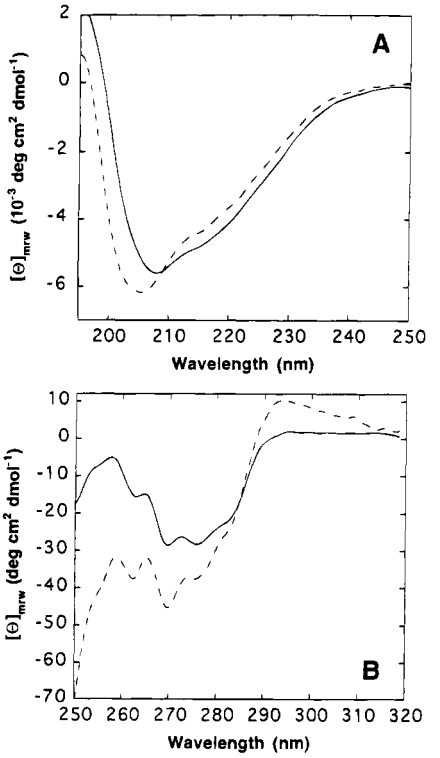

FIGURE 6: CD spectra of the F_1 and F_2 conformations of unmodified eIF-5A. The individual conformations were generated by incubating the protein under different buffer conditions. (– – –) F_1, 50 mM sodium acetate, pH 4.5, 1 M KCl, 0.5 mM EDTA, 0.5 mM DTT; (—) F_2, 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM DTT. Measurements were made at 22 °C. (A) Far-UV CD spectra measured in a cell with 0.1-cm light path. (B) Near-UV CD spectra measured in a cell with 2-cm light path.

the hypusine-modified eIF-5A qualitatively and quantitatively (cf. Figure 1B).

The 2-dimensional NMR spectra (COSY, TOCSY, and NOESY) of unmodified eIF-5A were measured to analyze the differences in tertiary structure between the two conformations in more detail. Despite the differences in the near-UV CD spectra of F_1 and F_2, the NMR spectra (Figure 7) do not show an obvious doubled set of proton resonances for the side chains of the aromatic (3 tyrosines and 5phenylalanines) and the 4 histidine amino acids at conditions where both conformations are significantly populated (50 mM potassium phosphate, pH 6.5). In fact, most of these residues appear to have only one set of ^{1}H-resonance signals in this region of the spectrum, indicating that they interact with the same groups of the polypeptide chain in each of the conformations. Therefore, the structural differences between F_1 and F_2 are restricted to a relatively small region of the protein involving only a minimum number of aromatic amino acids or histidine residues.

DISCUSSION

The unique modification of a lysine to a hypusine residue is necessary for the eukaryotic initiation factor 5A to fulfill its cellular function (Schnier et al., 1991). To study the structural implications imposed on the protein upon this modification, two variants of eIF-5A were compared in their overall structural properties and their stability toward unfolding by guanidinium chloride. A hypusine-modified form of the protein was purified from HeLa cells and an unmodified form was obtained by expression of the corresponding eIF-5A gene in bacterial cells, which naturally lack the two

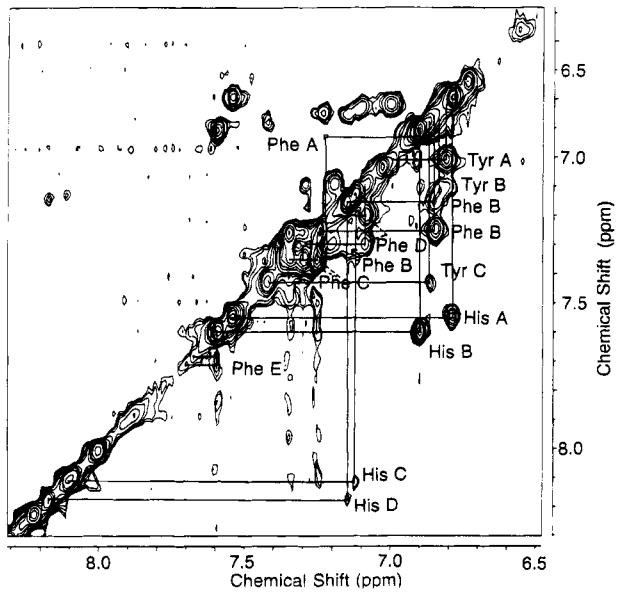

FIGURE 7: Aromatic region of the 500 MHz TOCSY spectrum of unmodified eIF-5A. The spectrum was recorded at 27 °C in 50 mM potassium phosphate, pH 6.5. Intra-residue NOE cross-peaks for 4 histidine, 5 phenylalanine, and 3 tyrosine residues are labeled.

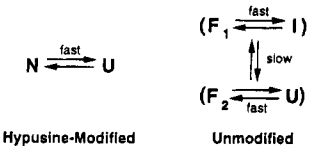

FIGURE 8: Proposed folding mechanism of hypusine-modified and unmodified eIF-5A. N, U, and I are the native, unfolded, and intermediate states, respectively. F_1 and F_2 are two distinctly folded conformations of the unmodified protein.

enzymes that are responsible for this modification. Since both variants differ, apart from modification of lysine at position 50, only in their N-terminus (hypusine-modified eIF-5A lacking the first methionine and being N-terminally acetylated, and unmodified eIF-5A carrying three additional amino acids), we assume that the proteins are representative of eIF-5A in its hypusine-modified and unmodified state, respectively. Both protein forms possess well-defined secondary and tertiary structure. However, the overall structure of unmodified eIF-5A appears to be different from that of the hypusine-modified protein as indicated by their different circular dichroism spectra and unfolding transitions.

Unfolding of hypusine-modified eIF-5A follows a two-state process in which only the native (N) and unfolded (U) states are stable (see Figure 8). Similar unfolding transitions are frequently observed for small globular proteins and imply that no folding intermediates are significantly populated on the folding pathway at equilibrium (Kim & Baldwin, 1990). By contrast, unfolding of the unmodified protein is more complex. The tertiary structure of this eIF-5A variant unfolds prior to the loss of all secondary structure. Further, the equilibrium unfolding transition followed by gel filtration chromatography suggests the existence of two equilibria with fast rate constants. The molecule conformations involved in each of these equilibria are in slow exchange with those of the corresponding other equilibrium. According to the results obtained from equilibrium and kinetic refolding experiments, we propose a folding mechanism that consists of parallel events (Figure 8). One folding process involves the fast equilibrium between a folded conformation (F_1) and one or more intermediate or partially folded states (I). In the presence of increasing concentrations of Gdn·HCl, this equilibrium converts to a second folding process involving the fast equilibrium between another, differently folded conformation (F_2) and the unfolded state. Both fast equilibria are in slow exchange since the corresponding conformations do not interconvert during their separation by gel filtration chromatography (ca. 25 min). Therefore, neither F_1 nor I is in rapid exchange with F_2 or the unfolded state.

What is the structure of the F_1 compared with the F_2 conformation? Separation of the two fast equilibria by gel filtration chromatography in the presence of 1.6 M Gdn·HCl and reequilibration of the resulting protein solutions in the absence of denaturant suggests that both forms are stable under conditions that favor the native state. The F_1 and F_2 conformations are in slow equilibrium and are present at a ratio of 60 to 40, respectively. Further, it is possible to shift the equilibrium between F_1 and F_2 to either side by incubating the protein under the appropriate buffer conditions; low pH and high ionic strength favors F_1, high pH and low ionic strength favors F_2 formation. The CD spectra of the individual conformations suggest well-defined secondary and tertiary structure of both forms. The almost equal stability of F_1 and F_2 under native conditions, therefore, implies that the tertiary structures of the two conformations must unfold at very similar denaturant concentrations. Accordingly, we find that unfolding of the tertiary structure of unmodified eIF-5A, i.e., a mixture of F_1 and F_2, coincides with the transition of F_2 to the unfolded state (see Figure 3C). Since (i) both of these transitions overlay with the unfolding process of the hypusine-modified eIF-5A variant (see Figure 4) and (ii) the near-UV CD spectrum of the F_2 conformation is virtually identical to that of the hypusine-modified protein, the F_2 conformation appears to possess most of the structural properties of the native, hypusine-modified eIF-5A.

The F_1 differs from the F_2 conformation in several important aspects. First, it has a slightly lower content of secondary structure. Second, it has a different unfolding transition: F_1 unfolds to one or several intermediate states, which, as judged by the different unfolding transitions (see Figure 3B), have no tertiary structure, are less compact, but still retain some of the secondary structure. Third, one or more aromatic amino acids appear to experience a different chiral environment as compared with the F_1 conformation. The primary sequence of eIF-5A (Smit-McBride et al., 1989) is biased in the distribution of aromatic amino acids and histidine. eIF-5A contains 5 phenylalanine (F7, F18, F30, F64, F89), 3 tyrosine (Y69, Y98, Y127) and 4 histidine (H51, H53, H57, H77) residues (no tryptophan residues), and only one of these (Y127) is located in the C-terminal third of the amino acid sequence. Based on the analysis of the 2-dimensional NMR spectra of the unmodified protein, the conformational differences between F_1 and F_2 may be restricted to a small region that resides in the C-terminal part of eIF-5A.

Refolding of unmodified eIF-5A, which was incubated in a solution of 6 M Gdn·HCl for several days, appears to yield exclusively the F_2 conformation in a first folding process. Since unfolded polypeptide chains isomerize into a mixture of *cis* and *trans* peptide bonds at proline residues (Kiefhaber et al., 1992), proline isomerization *per se* is unlikely to be the major determinant for the different folds of F_1 and F_2. However, after refolding of U to F_2, the latter converts slowly to F_1 in a double exponential process. The F_2 conformation, therefore, either represents not a completely homogeneous

population itself, or folding of U to F_2 includes two parallel reactions with different rate constants, most probably due to proline isomerization. We favor the latter possibility since the observed ratio of F_1 to F_2 of 60 to 40, respectively, conflicts with the corresponding rate constants for the interconversion of the conformations. F_1 isomerizes to F_2 with a first-order rate constant of 3.4×10^{-4} min^{-1}. The faster phase of the reaction of F_2 to F_1 proceeds with a rate constant of 1.5×10^{-2} min^{-1}, and the slower phase with a rate constant of 5.5×10^{-4} min^{-1}. Assuming that the faster phase results from a molecule species that is on the folding pathway of U to F_2, but isomerizes slower to the final F_2 than to the F_1 conformation, the relevant rate constant for $F_2 \rightarrow F_1$ interconversion is 5.5×10^{-4} min^{-1}. The expected ratio of F_1 to F_2 according to these rate constants (3.4×10^{-4} min^{-1} for $F_1 \rightarrow F_2$ and 5.5×10^{-4} min^{-1} for $F_2 \rightarrow F_1$) is 62 to 38, respectively, and in good agreement with the experimental results. The corresponding activation energies for interconversion are high: 24.3 kcal mol^{-1} for $F_1 \rightarrow F_2$ and 24.1 kcal mol^{-1} for $F_2 \rightarrow F_1$.

CONCLUSION

It is probably not the primary role of the hypusine-modification to induce a conformational change in the overall protein structure since 40% of the molecules of unmodified eIF-5A are in a conformation that is already very similar to that of the hypusine-modified protein. Therefore, the importance of the modification for the function of the protein is likely to be connected with the hypusine residue itself, possibly by its direct interaction with other cellular components (e.g., proteins, RNA, DNA). However, our most important finding is that the hypusine-modification is necessary to define a unique energy minimum on the folding free energy surface of eIF-5A, and that the unmodified polypeptide chain is unable to distinguish between two alternate energy minima in a thermodynamically controlled reaction. Similar observations have been made for insulin-like growth factor I which folds *in vitro* to yield two distinct disulfide-bonded forms. Both forms differ in two of three disulfide bonds, are in equilibrium with each other, and possess similar thermodynamic stabilities (Owers Narhi et al., 1993; Hober et al., 1994). It is evident from our findings that not every polypeptide chain has *a priori* the capacity to fold to a unique energy minimum and that several energy minima of similar magnitudes can be present on the energy surface. These minima can be separated by substantial energy barriers. Since native proteins usually adopt unique structures, they may have to overcome this multiple-minima problem either by stabilizing one energy minimum relative to all others (thermodynamic control) or by increasing the energy barriers between these minima to trap the polypeptide chain in a local energy minimum (kinetic control). Although it has not yet been possible to obtain unambiguous experimental evidence for a thermodynamically controlled folding reaction (Baker & Agard, 1994), there is a growing number of proteins being found for which the folding reactions are kinetically controlled (Schultes & Jaenicke, 1991; Baker et al., 1993; Eder et al., 1993; Bennett et al., 1994; Baker & Agard, 1994). *Ab initio* calculations of a protein structure from a given amino acid sequence may, therefore, have to take into account not only the free energy of the folded state but also its accessibility for the folding polypeptide chain.

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