Isolation and Structural Characterization of Different Isoforms of the Hypusine-Containing Protein eIF-5A from HeLa Cells

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ABSTRACT: Posttranslational modification of a specific lysine residue in eukaryotic initiation factor 5A (eIF-5A) is essential for cell viability and proliferation. The product of this modification is hypusine, an amino acid unique to eIF-5A. We have purified and characterized one major and three minor isoforms of human eIF-5A from HeLa cells. The main form, which accounts for approximately 95% of the total eIF-5A, carries hypusine at position 50 and is amino-terminally acetylated as determined by amino acid composition analysis and electrospray ionization mass spectrometry. Analytical gel filtration indicates that this protein variant possesses a native apparent molecular weight that lies between that expected for a monomeric and dimeric form. Nevertheless, several experiments confirm this protein to be monomeric. It is further shown that eIF-5A has well-defined secondary structure. Both the far-UV circular dichroism spectrum as well as secondary structure predictions using different algorithms suggest this protein to have predominantly β-sheet structure. Two plausible models for the packing of the secondary structure elements are presented. In contrast to the main form, all three minor isoforms of eIF-5A are characterized by acetylation of the ϵ-amino group of lysine at position 47. The minor isoforms are distinguishable by their state of modification of the lysine residue at position 50. Whereas the main form occurs in both the cytoplasmic and the nuclear fraction of HeLa cells, the minor isoforms were not detectable in the preparation of the nuclear fraction. Therefore, acetylation of lysine at position 47 might play a controlling role in the distribution of the minor isoforms to the nucleus.

Only one protein is known to contain the unusual amino acid hypusine: eukaryotic initiation factor 5A (eIF-5A).[1] In this protein, a specific lysine residue is modified via transfer and subsequent hydroxylation of an aminobutyl moiety from spermidine to its ϵ-amino group (Wolff et al., 1990). eIF-5A with its hypusine modification is highly conserved in all eukaryotes and archaebacteria (for review see Park et al., 1993). Since both the protein and its hypusine residue are essential for cell viability (Schnier et al., 1991; Wöhl et al., 1993), eIF-5A is expected to exert a fundamental and largely ubiquitous cellular function. However, despite its essential role and its abundant expression in many organisms, the cellular function of eIF-5A remains unknown. Although eIF-5A was formerly thought to be involved in the formation of the first peptide bond in protein synthesis, recent experiments using unstable eIF-5A mutants rather rule out its general participation in translation initiation (Kang & Hershey, 1994). The protein was also suggested to play a role in mRNA export from the nucleus, since eIF-5A was described as a cellular binding partner of the regulatory HIV-1 Rev protein (Ruhl et al., 1993). Eukaryotic initiation factor 5A, therefore, appears to locate both to the nucleus (Ruhl et al., 1993) and to the cytoplasm (Wolff et al., 1992). Furthermore, different isoforms of the protein were found in various organisms—e.g., in yeast (Klier et al., 1993) and CHO cells (Park, 1988; Park, 1989). The physiological roles of the different modifications are unclear.

In this work, we have identified the modifications of one major and three minor isoforms of human eIF-5A. The major isoform, which carries the hypusine modification and accounts for approximately 95% of the total eIF-5A, is shown to be a monomeric protein with predominantly β-sheet structure. The minor isoforms appear to be inefficiently modified at K50 because of their ϵ-amino acetylation of K47.

MATERIAL AND METHODS

Materials

HeLa S3 cells were purchased from the Computer Cell Culture Center, University of Mons, Belgium. DNase-free RNase and sequencing grade endoproteinase LysC were supplied by Boehringer Mannheim, F.R.G. Chromatography matrices and columns were from Pharmacia, Freiburg, F.R.G., with the exception of the Fractogel columns, which were purchased from Merck, Darmstadt, F.R.G., and the protein C_4 reversed-phase column, which was purchased from Vydac, Hesperia, U.S.A. IPG DryPlates for isoelectric focusing were also from Pharmacia, Freiburg, F.R.G. Immobiline P membrane was purchased from Millipore, Bedford, U.S.A. The horseradish peroxidase−coupled anti-rabbit-IgG F(ab′)$_2$ fragment, as well as the ECL Western blot detection reagents were supplied by Amersham International

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[1] Abbreviations: DTT, 1,4-dithiothreitol; EDTA, (ethylenedinitrilo)-tetraacetic acid; eIF-5A, eukaryotic initiation factor 5A; ESI-MS, electrospray ionization mass spectrometry; Gdn·HCl, guanidinium chloride; HPLC, high performance liquid chromatography; IPG, immobilized pH gradient; pI, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; 2D, two-dimensional.

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plc, Little Chalfont, England. All other chemicals were of the highest purity available.

Purification of eIF-5A from HeLa Cells

For purification of human eIF-5A, 35 g of HeLa cells were resuspended in 280 mL of 10 mM Tris-HCl, pH 7.0, containing 10 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and 1 mM PMSF, and disrupted in a Dounce homogenizer with 20 strokes (pistil B), allowing the suspension to cool on ice for 10 min after the first 10 strokes. After centrifugation (3 500*g* for 10 min plus 40 000*g* for 45 min, 4 °C), the supernatant was stored on ice for further use. The pellet was resuspended in 60 mL of 50 mM Tris-HCl, pH 7.5, containing 3 mM $MgCl_2$, 1 mM DTE, and 1 mM PMSF, and incubated with 30 μg of RNase for 15 min at 22 ± 2 °C. Following centrifugation at 3 500*g* for 10 min at 4 °C and a second round of RNase treatment, both RNase-extracts were pooled, centrifuged at 40 000*g* for 20 min, and the resulting supernatant was added to the initial cytosolic extract (supernatant of the first centrifugation). Proteins were precipitated with ammonium sulfate in a stepwise manner. Addition of ammonium sulfate to a concentration of 40% (w/v) was followed by a second precipitation step with ammonium sulfate at a final concentration of 80% (w/v). After incubation for 30 min at 4 °C and centrifugation at 27 000*g* for 10 min, the pellet of the second precipitation was dissolved in 500 mL of 25 mM potassium phosphate buffer, pH 7.8, 0.5 mM DTT, and 0.05 mM EDTA, and loaded onto a 300-mL Q-Sepharose Fast Flow column with a flow rate of 10 mL/min. Subsequent to a washing step with 0.2 M NaCl in 50 mM potassium phosphate at pH 7.8, 1 mM DTT, and 0.1 mM EDTA, eIF-5A was eluted in the same buffer at 0.3 M NaCl. The eluted fraction was supplemented with 1/10 of its volume of 1 M KH_2PO_4 and concentrated to a final volume of 50−100 mL in an Amicon PM10 ultrafiltration device. After addition of 1 volume of 3 M ammonium sulfate/NaOH at pH 7.0, the sample was loaded onto a Phenyl Sepharose column. Proteins were eluted with a linear gradient of 1.5−0 M ammonium sulfate in 50 mM potassium phosphate buffer, pH 6.5, 1 mM DTT, and 0.1 mM EDTA at a flow rate of 2 mL/min. The eIF-5A-containing fractions were pooled, concentrated in an Amicon ultrafiltration device to 10 mL, and dialyzed against 50 mM potassium phosphate buffer, pH 6.5, 1 mM DTT, and 0.1 mM EDTA. The protein solution was then applied to a Mono S column. Elution of the proteins was performed by using a linear gradient of 0−0.5 M NaCl in 50 mM potassium phosphate buffer, pH 6.5, 1 mM DTT, and 0.1 mM EDTA, and a flow rate of 0.5 mL/min. eIF-5A was recovered both in the break-through of the cation exchange column and in a single peak, which eluted between 150 mM and 200 mM NaCl.

The eIF-5A isoforms which appeared in the break-through of the Mono S column were further purified on a 1 mL Mono Q column with a flow rate of 0.5 mL/min using a linear gradient of 0−0.5 M NaCl in 50 mM potassium phosphate, pH 7.8, 1 mM DTT, and 0.1 mM EDTA. Finally, the isoform-containing fractions were pooled and subjected to a reversed-phase HPLC on a Vydac protein C_4 column (250 × 4.6 mm/10 μm). Elution was carried out with a flow rate of 1 mL/min using the following gradient in 0.1% (v/v) trifluoroacetic acid: 15−25% acetonitrile in 5 min, 25−40% acetonitrile in 15 min, 40−50% acetonitrile in 20 min, 50−60% acetonitrile in 5 min, and 60−15% acetonitrile in 5 min.

Throughout the entire purification procedure, the eIF-5A content was assayed by SDS−PAGE with subsequent silver staining or Western blot analysis (see below) on the Phast System (Pharmacia) according to the instructions of the manufacturer.

Purification of eIF-5A from HeLa Cell Nuclei

For preparation of HeLa nuclei, 10 g of HeLa cells were homogenized as described above. After centrifugation (1 600*g*, 10 min, 4 °C), the pellet was resuspended in 50 mL of 60% sucrose (w/v), 50 mM Tris, pH 7.0, 5 mM $MgCl_2$, and 1 mM PMSF, layered onto a cushion of 100 mL of the same buffer, and centrifuged at 130 000*g* for 30 min at 4 °C. The pellet was extracted by two RNase treatments in a volume of 30 mL each, as described above. The supernatants of both RNase extractions were pooled and centrifuged at 24 000*g* for 20 min at 4 °C. The resulting supernatant was titrated to a pH of 7.5 by addition of a 50 mM Tris solution, and the proteins were subsequently precipitated with ammonium sulfate as outlined. The proteins precipitating between 40% (w/v) and 80% (w/v) of ammonium sulfate were dissolved in 50 mM potassium phosphate, pH 7.8, 1 mM DTE, and 0.1 mM EDTA to give a final conductivity of <16 mS. After centrifugation at 4 000*g* for 5 min at 4 °C, the supernatant was loaded onto a 10-mL Fractogel EMD TMAE-650 (S) anion exchange column. Proteins were eluted with a flow rate of 1 mL/min, using a gradient of 0−0.5 M NaCl in the same buffer. The eIF-5A-containing fractions were diluted with 50 mM potassium phosphate containing 1 mM DTE and 0.1 mM EDTA to yield a final pH of 6.2−6.5 and a conductivity of 9 mS, and applied to a 10-mL Fractogel EMD SO_3^--650 (S) cation exchange column with a flow rate of 1 mL/min. The proteins were eluted from the column using a linear gradient of 0−0.4 mM NaCl in 50 mM potassium phosphate, pH 6.5, 1 mM DTE, and 0.1 mM EDTA.

Determination of Protein Concentration

The protein concentration of eIF-5A was determined spectrophotometrically in solutions containing 6 M Gdn·HCl. The corresponding molar absorption coefficient of 3840 M^{-1} cm^{-1} was calculated from the number of aromatic residues according to the method of Gill and von Hippel (1989). The values thus obtained are identical to those determined by the Bradford (1976) assay, using bovine serum albumin as standard.

Two-Dimensional Gel Electrophoresis

For two-dimensional (2D) gel electrophoresis, protein samples in aqueous buffers were diluted with at least one volume of lysis buffer [9 M urea, 1% (v/v) NP-40, 5% β-mercaptoethanol, 0.4% (v/v) Pharmalytes 3.5−10, and 0.4% (v/v) Ampholine carrier ampholytes (Görg et al., 1988; Werner et al., 1990)] to a final volume of 50−100 μL. Protein samples in organic solvents, as obtained from reversed-phase HPLC, were concentrated to a final volume of 10 μL in a Speed Vac Concentrator, washed with 100 μL H_2O, concentrated again to a final volume of 10 μL, and diluted with lysis buffer as described above. Isoelectric focusing on immobilized pH gradients pH 4−7 was carried

out as described (Klier & Lottspeich, 1992) except that the final focusing step was at 3500 V instead of 4000 V.

For SDS−PAGE in the second dimension the equilibrated IPG strips were transferred onto Laemmli gels (Laemmli, 1970). Following the electrophoretic separation (115 V in the stacking gel of 0.1 × 16 × 2 cm and 250 V in the resolving gel of 0.1 × 16 × 12 cm), the gels were stained with either Coomassie Brilliant Blue or silver nitrate (Heukeshoven & Dernick, 1985).

Western Blot Detection of eIF-5A

For Western blot analysis of column fractions, a sample aliquot was separated on 12.5% homogeneous SDS−polyacrylamide gels and electroblotted onto nitrocellulose membranes using the Phast System (Pharmacia) according to the instruction of the manufacturer. The membranes were blocked in a solution of 5% skimmed milk powder in PBS, 0.1% Tween 20, and then incubated with the anti-eIF-5A antibody (Ruhl et al., 1993). Detection was with a horseradish peroxidase−coupled anti-rabbit-IgG $F(ab')_2$ fragment in a 1:5 000 dilution using ECL Western blotting reagents according to the supplier's instructions.

For Western blot detection of eIF-5A after 2D-gel electrophoresis, the proteins were electroblotted onto Immobiline P membrane using a current of 1.5 mA/cm^2 (Eckerskorn et al., 1988). In order to visualize the major protein spots on the membrane (for comparison with the silver stained gels), the membrane was reversibly stained for 5 min in 0.2% (w/v) Ponceau S, 10% (v/v) acetic acid. The appearing spots were marked by small scratches using an injection needle prior to addition of the blocking solution.

Circular Dichroism Spectroscopy

CD measurements were carried out with a Jasco J710 instrument at 20 °C. The protein was dialyzed against 25 mM potassium phosphate at pH 6.5 containing 0.25 mM DTT, and the spectrum was recorded at a protein concentration of 22.5 μM in a 0.02-cm cell. The CD spectrum, which was averaged over five accumulations, was corrected for the buffer baseline. Mean residue ellipticity values ($[\Theta]_{mrw}$) were calculated according to

$$[(\Theta)]_{mrw} = 100\ \Theta_{obs}/lc \text{ (deg cm}^2\text{ dmol}^{-1}\text{)}$$

where Θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeters. Calculation of the percentage of secondary structure present in eIF-5A was performed using the program SELCON (Sreerama & Woody, 1993).

Analytical Gel Filtration

Gel filtration chromatography was performed on a Pharmacia FPLC system equipped with a Merck-Hitachi L-4200 variable-wavelength detector using a column of Superdex 75 (1 cm × 30 cm). Experiments were conducted at 22 ± 2 °C in 50 mM potassium phosphate buffer, pH 6.5, containing 150 mM KCl, 0.5 mM EDTA, and 0.5 mM DTT. The flow rate was 0.4 mL/min. After 200-μL samples of protein were injected, the elution profiles were monitored by absorption at 230 nm. The column was calibrated with proteins of known M_r: aprotinin (6 500), horse heart cytochrome c (12 300), horse myoglobin (17 800), bovine erythrocyte carbonic anhydrase (29 000), chicken ovalbumin (44 000), bovine serum albumin (66 300); and gave a linear relationship between the logarithm of M_r and the elution volume.

Folding Studies

Equilibrium unfolding of eIF-5A was monitored by analytical gel filtration. The measurements were performed on protein solutions of 0.002−0.15 mg/mL (0.2−10 μM) in 50 mM potassium phosphate, pH 6.5, 150 mM KCl, 0.5 mM EDTA, and 0.5 mM DTT, and the indicated concentrations of Gdn·HCl. Following incubation of the protein samples for 24 h at 4 °C, the corresponding elution profiles were recorded in the same buffers. No further change in the elution profiles occurred after longer incubation times.

Secondary Structure Prediction

Four different algorithms were used for secondary structure prediction. These include the PHD program as developed at the EMBL-Heidelberg (Rost & Sander, 1993, 1994; Rost et al., 1994), the ETH program (Benner & Gerloff, 1991; Benner et al., 1994), the method of Chou−Fasman (Chou & Fasman, 1978; Nishikawa, 1983), and that of Garnier−Osguthorpe−Ribson (Garnier, 1978). The latter two algorithms were used within the GCG software package (Wisconsin) Package Version 8, Genetics Computer Group, U.S.A. 1994). The PHD predictions are performed by a system of neural networks making use of multiple sequence alignment. In contrast, both the Chou−Fasman and Garnier−Osguthorpe−Robson prediction methods use only the given protein sequence, without making use of knowledge from homologous sequences, in predicting secondary structure. In the case of eIF-5A, 11 sequences were used in the alignment; the degree of sequence similarity varied from 99% down to 33% (relative to the human eIF-5A sequence), i.e., closely resembling the ideal situation for optimal results being obtained (Rost & Sander, 1993; Rost & Sander, 1994; Sanders & Schneider, 1991). In the secondary structure predictions obtained from the ETH program, sequence alignment was made using 11 evolutionary diverse sequences. The sequences used in the alignment by the PHD and ETH programs are: eif-5a_human, eif-5a_rabit, eif-5a_chick, eif-5a_dicdi, eif-51_yeast, eif-52_yeast, eif-5a_caeel, eif-51_nicpl, eif-52_nicpl, eif-5a_medsa and hypu_sulac. These sequences, however, are not representative of the full evolutionary tree.

Tryptic Peptide Mapping

After separation of the eIF-5A isoforms by 2D-gel electrophoresis and subsequent Coomassie Brilliant Blue staining, the respective spots were excised from the gel and excessively washed with deionized water for more than 15 h. The gel matrix was then passed through two wire meshes with a pore size of 100 μm and 32 μm, respectively and dried under vacuum to a residual humidity of about 10%. The gel pellet was soaked with 50 μL of 25 mM Tris-HCl, pH 8.5, and 1 mM EDTA containing 0.15 μg endoproteinase LysC per μg of eIF-5A (estimated from Coomassie Brilliant Blue staining). The cleavage reaction was carried out at 37 °C for 16 h and stopped by addition of 1 mL of 60% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. The elution of the peptides was carried out repeatedly at 37 °C. The eluate

was finally passed through an Anotop filter with a pore size of 0.02 μm (Merck), and dried under vacuum; the peptides were then dissolved in 20% acetic acid. The peptide mixture was separated on a reversed-phase HPLC column (Supersper RP60 Select B; 125 × 2 mm/4 μm; Merck) using a linear gradient of 0−60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 mL/min.

Protein Chemical Analysis

Electrospray ionization mass spectrometry was carried out as described in (Klier et al., 1993) on a quadrupole instrument API III (Sciex, Thronhill, Ontario, Canada). Ortho-phthaldialdehyde amino acid composition analysis was performed according to Wöhl et al. (1993). Amino acid sequence analyses were performed on an Applied Biosystems 477A gas phase sequencer equipped with a 120A PTH amino acid analyzer according to the instructions of the manufacturer. For confirmation of acetyl-lysine in the sequence, synthetic PTH-acetyl-lysine was used.

RESULTS

Purification of Human eIF-5A

Human eIF-5A was purified from HeLa cells in order to investigate its primary and secondary structure and to characterize possible isoforms. Since eIF-5A occurs both in the cytosolic and in the insoluble fraction (i.e., associated with the nucleus (Ruhl et al., 1993) after cell lysis, it was necessary to solubilize the protein from the latter fraction. Repeated RNase treatment was found to be most efficient in extracting eIF-5A from the insoluble pellet after Dounce homogenization. According to Western blot analysis, using a polyclonal anti−eIF-5A antibody (Ruhl et al., 1993), the initial nuclear fraction contained approximately 5−10% of the total eIF-5A protein. The extracts from the RNase treatment were combined with the cytosolic fraction, and eIF-5A and its isoforms were purified from this pool by ammonium sulfate precipitation and several subsequent chromatographic steps. Following an initial anion exchange chromatography and a hydrophobic interaction chromatography on a Phenyl Sepharose column, different forms of eIF-5A were separated by cation exchange chromatography. Whereas approximately 5% of the total eIF-5A protein interacted only weakly with the column material (designated "minor isoforms"), the remaining ~95% of eIF-5A eluted in a single, symmetrical peak between 0.15 and 0.2 M NaCl ("main form"). The immunopositive proteins in both fractions showed identical apparent molecular weights as judged by SDS−PAGE. The main form, which bound to the cation exchange material, was pure. It gave a single spot after 2D-gel electrophoresis (Figure 1). However, the eIF-5A minor isoform(s) in the break-through of the cation exchange column had to be further purified. Following an additional anion exchange chromatographic step on a Mono Q column, where all the minor isoform(s) eluted in a single peak, reversed-phase HPLC yielded pure eIF-5A. 2D-gel electrophoresis revealed two immunopositive spots (see Figure 2).

In summary, the total purification procedure yielded 1.8 mg of eIF-5A from 35 g of HeLa cells with 1260 mg of soluble protein. This corresponds to a 700-fold purification of eIF-5A.

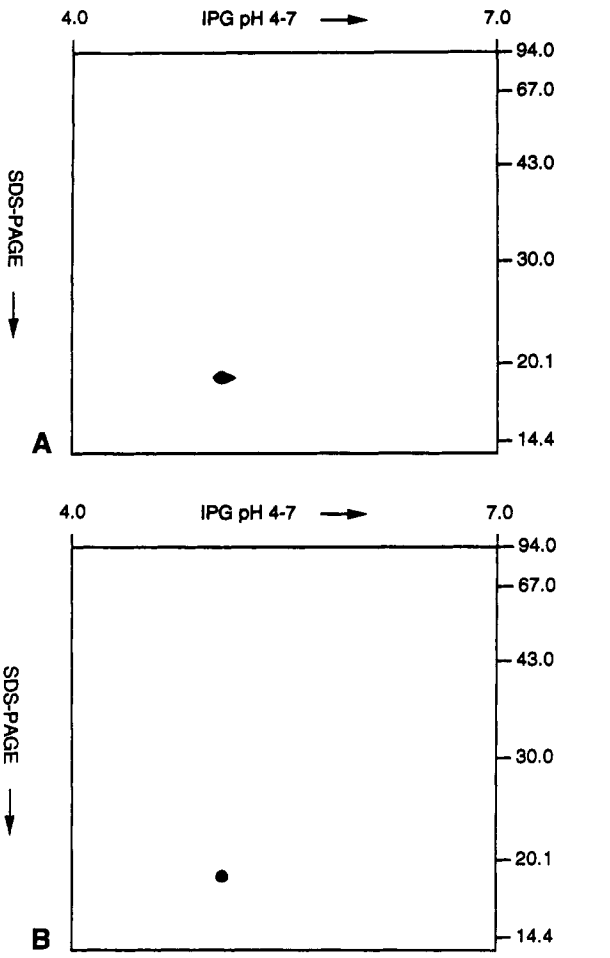

FIGURE 1: 2D gel analysis of the main form of eIF-5A. Silver staining (A) and Western blot (B). Protein separation was performed by anodic isoelectric focusing in the first dimension on an immobilized linear pH gradient from pH 4.0 to 7.0 (as indicated on top), which was followed by SDS−PAGE separation (12% T; 2.6% C) in the second dimension. The molecular masses of the marker proteins are given in kiloDaltons on the right margin of the gels. The main form of eIF-5A appears as a single spot with an apparent molecular mass of 18 kDa and an isoelectric point of 5.2.

Properties of eIF-5A Main Form

The eIF-5A Main Form Contains a Hypusine Residue. The purified main form of eIF-5A appears as one protein spot on 2D gel electrophoresis corresponding to an apparent molecular mass of 18 kDa and an isoelectric point (pI) of 5.2 (Figure 1A). It is immuno-positive with the anti-eIF-5A antibody (Figure 1B). Amino acid composition analysis revealed that the protein contains hypusine, an amino acid unique to eIF-5A (Park et al., 1981). Furthermore, electrospray ionization mass spectrometry of this protein form yielded an average molecular mass of 16 830.7 ± 1.5 Da. This value is in agreement with the calculated average mass of 16 829.3 Da, which is based on the cDNA sequence (Smit-McBride et al., 1989) assuming that (i) the lysine residue at position 50 is modified to hypusine and (ii) the α-amino group of the first alanine residue is acetylated, i.e., the first methionine residue is replaced by an acetyl group (Tsunasawa & Sakiyama, 1992). Note that the numbering of the amino acid positions in this publication refers to the amino acid sequence, which is deduced from the cDNA sequence, containing the methionine residue in position 1.

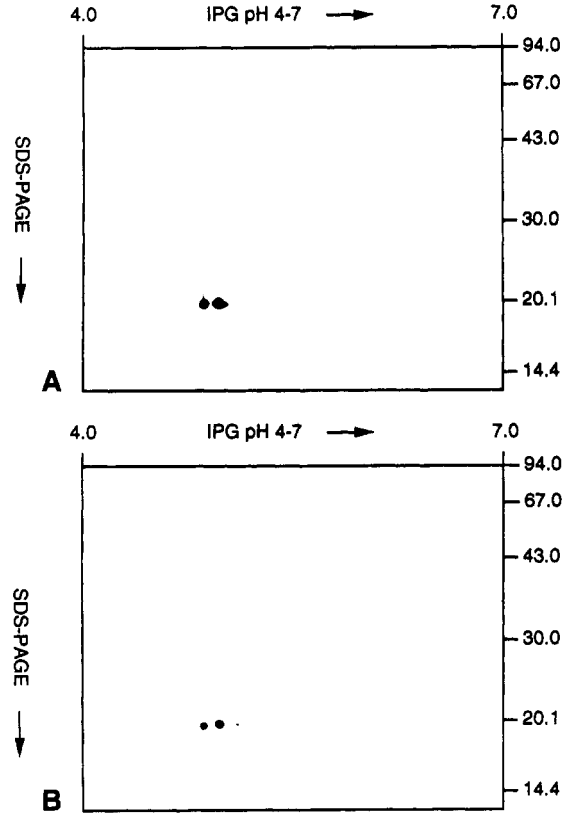

FIGURE 2: 2D gel analysis of the minor isoforms of eIF-5A. Silver staining (A) and Western blot (B), performed as described in Figure 1. The minor isoforms appear as two spots with isoelectric points of 5.0 and 5.1, respectively. Traces of the main form (pI of 5.2) are detectable after Western blot development (B) of the 2D gels.

The Secondary Structure of eIF-5A. Secondary structure predictions were made using four different prediction algorithms: Chou−Fasman (CF), Garnier−Osguthorpe−Robson (GOR), the PHD, and the ETH program. A comparison of the secondary structure predictions is given in Figure 3. Whereas the different algorithms predict rather different secondary structure elements for the N- and C-terminal end regions (amino acid residues 1−25 and 140−154) and residues 110 to 114, the predictions for the central residues (ca. 80 residues surrounding K50, the site of the hypusine modification) are highly consistent. This region is predicted to have predominantly β-sheet conformation. The site of hypusine modification is flanked on the N-terminal side by a β-strand, and on the C-terminal side by either a β-strand (GOR, PHD, ETH-B) or an internal α-helix (ETH-A). [On the basis of the low reliability of prediction results obtained using the Chou-Fasman method (Gerloff et al., 1993; Gomis-Rüth et al., 1993), the results obtained from secondary structure predictions on eIF-5A using this method are included in Table 1 and Figure 3 for completeness, but are not discussed in detail.] A long loop appears to follow this stretch (residues 65−88). The main difference in secondary structure prediction for the core region of the protein surrounding the site of the hypusine modification is that the Chou-Fasman method predicts an α-helix on the N-terminal side of the modification rather than a β-sheet (see Figure 3). Furthermore, an analysis of patterns of conservation and variation in the aligned 11 homologues sequences led to the identification of a solid "parse" (break in secondary structure) at residue positions 95−97. This is consistent with

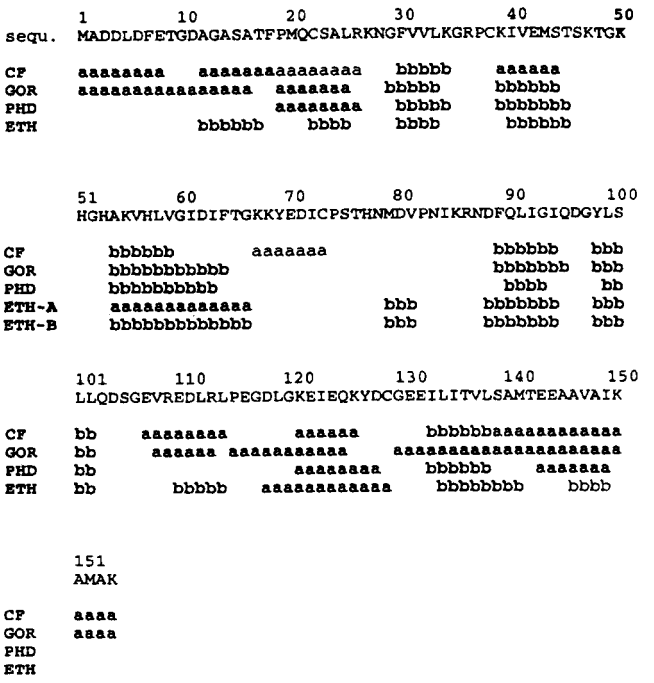

FIGURE 3: Secondary structure prediction of human eIF-5A. Given below the sequence numbers are the letters "a" and "b" corresponding to whether the residue in the sequence is predicted to be in an α-helix or β-sheet, respectively, with the method of prediction given at the edge of the figure. CF, GOR, PHD, and ETH represent the predictions according to Chou−Fasman, Garnier−Osguthorpe−Robson, EMBL−Heidelberg, and the ETH, respectively (see text for references). The predictions not given in bold type are weak. The ETH program predicts either a β-strand (ETH-B) or, preferentially, an internal α-helix for residues 54−66 (ETH-A).

Table 1: Predicted Secondary Structure Elements[a]

prediction method[b]	α-helix	β-sheet	others
CF	42.9	18.2	38.9
GOR	42.2	22.1	35.7
PHD	14.9	24.0	61.0
ETH-A	7.8	42.2	50.0
ETH-B	16.2	33.8	50.0
CD analysis	10	38	52

[a] Results are given in %. [b] As defined in the legend to Figure 3.

the folding of eIF-5A into two domains, the first from residues 1−95 and the second from 96−154.

The scores of the individual structure predictions are summarized in Table 1. In addition, the far-UV circular dichroism spectrum of the main form of eIF-5A was measured to determine experimentally the relative amounts of secondary structure (Figure 4). The CD spectrum, which has an ellipticity minimum at 206 and a maximum at 192 nm, reflects well-defined secondary structure (Johnson, 1990). Analysis of the CD spectrum using the program SELCON (Sreerama & Woody, 1993) indicated that the secondary structure of the protein is dominated by β-sheet conformation with 38% β-sheet, 10% α-helix, and 52% other structure. Based on a comparison of the results of the CD analysis with the secondary structure predictions, it is evident that both the PHD and ETH-A and -B predictions correlate best with the experimentally determined secondary structure.

In addition to the secondary structure, the solvent accessibility of individual residues was also predicted. The Kyte−Doolittle (GCG package), PHD, and ETH algorithms gave

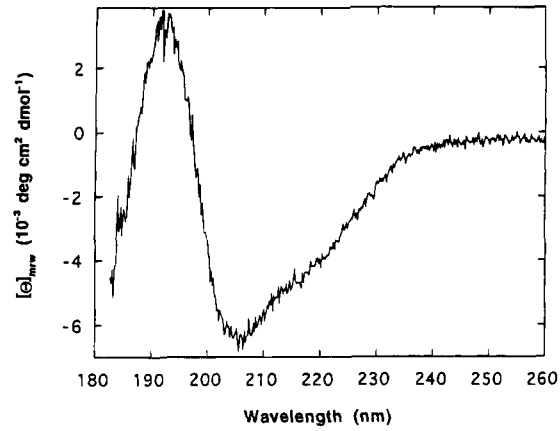

FIGURE 4: Far-UV CD spectrum of the main form of eIF-5A. The protein concentration was 22.5 μM in 25 mM potassium phosphate buffer at pH 6.5 containing 0.25 mM DTT. The spectrum was recorded at 20 °C in a cell with 0.02-cm path length.

consistent results: (i) The predicted β-strands ranging from residues 30 to 33, 40 to 44, 54 to 64, 89 to 94, 98 to 102, and 134 to 138 contain almost exclusively buried residues and should therefore be internal β-strands (note that the β-strand from residue 54 to 64 might also be an internal α-helix; see Figure 3). (ii) The predicted α-helix from residue 121 to 126 contains predominantly exposed residues, i.e., appears to be a helix on the surface of the protein. (iii) All other predicted secondary structure elements contain a mixture of both exposed and buried residues, suggesting that they are close to the protein surface. Structural models / folding patterns have been suggested on the basis of secondary structure and solvent accessibility predictions as obtained from the ETH and PHD programs (see Discussion section, Figure 7).

eIF-5A Is a Monomer. Analysis of eIF-5A by gel electrophoresis in the presence of SDS showed good agreement with the determined M_r of 16 830.7 (Figure 1). However, the protein elutes from a calibrated gel filtration column at a volume corresponding to an apparent M_r of 26 300 (Figure 5A). This value lies midway between that expected for a monomeric (16 829.3) and dimeric (33 658.6) protein, and may be interpreted in terms of a loosely folded monomeric, or a compactly folded dimeric polypeptide chain, respectively, in the absence of any further information. In the following we provide experimental evidence that eIF-5A is a monomeric protein with a slightly expanded Stokes radius.

First, a 100-fold dilution of a 10 μM solution of native eIF-5A in 50 mM potassium phosphate buffer at pH 6.5, containing 150 mM KCl, 1 mM EDTA, and 1 mM DTT, did not affect its elution volume in analytical gel filtration or its migration in 7.5% native polyacrylamide gels (data not shown). Second, the folding of eIF-5A is independent of the protein concentration (Figure 5B), as expected for a monomeric protein (folding is a first-order reaction). The folding of native eIF-5A is fully reversible and follows a two-state process at equilibrium (João et al., 1995). The equilibrium unfolding transition with Gdn·HCl is not affected by the protein concentration between 0.2 and 10 μM, and exhibits a midpoint at 1.41 M Gdn·HCl. Third, incubation of eIF-5A with glutaraldehyde (up to 2%; v/v) or with dimethyl 3,3′-dithiobispropionimidate (DTBP; up to 3 mM) in 0.1 M triethanolamine-HCl buffer at pH 8.5, containing

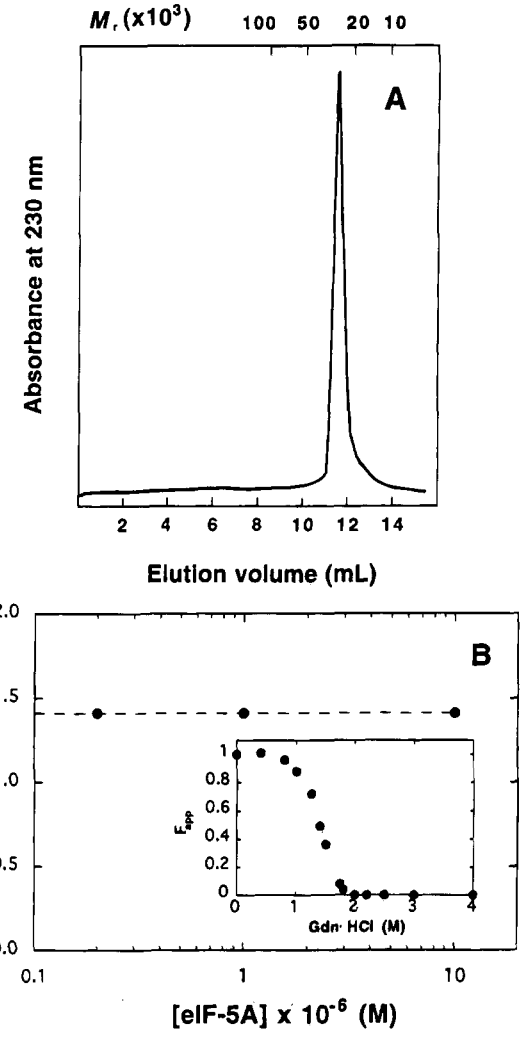

FIGURE 5: eIF-5A is a monomeric protein. (A) Gel filtration chromatography on a Superdex 75 column. The elution buffer was 50 mM potassium phosphate, pH 6.5, containing 150 mM KCl, 0.5 mM EDTA, and 0.5 mM DTT. The elution profile was monitored by absorption at 230 nm at a flow rate of 0.4 mL/min. M_r values on upper ordinate from calibration with marker proteins. (B) Equilibrium unfolding by guanidinium chloride. Unfolding of eIF-5A was followed by gel filtration chromatography. The midpoint of the unfolding curve ($[Gdn\cdot HCl]_{50\%}$) is independent of the concentration of eIF-5A. A typical unfolding curve with $C_{eIF\text{-}5A}$ = 1 μM is shown in the insert. The unfolding curves were normalized by extrapolating the linear change of the elution volume above 2.5 M Gdn·HCl to 0 M Gdn·HCl. F_{app} is the change of the profile expressed as a fractional deviation from this baseline.

1 mM EDTA, did not yield specific cross-linking of eIF-5A molecules as judged by gel electrophoresis in the presence of SDS. Fourth, coexpression of GAL4-DNA-binding-domain:eIF-5A and eIF-5A:GAL4-activation-domain fusion proteins using the yeast two-hybrid system (Fields & Song, 1989) did not lead to significant β-galactosidase production, indicating that the individual eIF-5A moieties are unable to interact to produce an active GAL4-transactivator complex (O. Schatz, personal communication). Fifth, simultaneous refolding of eIF-5A together with a glutathione-*S*-transferase:eIF-5A fusion protein at a stoichiometric ratio did not result in mixed dimer/multimer formation as judged by analytical gel filtration. Instead, the proteins, which were refolded from concentrated Gdn·HCl solutions by dialysis against the gel filtration column buffer, eluted in separate peaks having elution volumes of 11.7 and 8.4 mL, respectively. It should

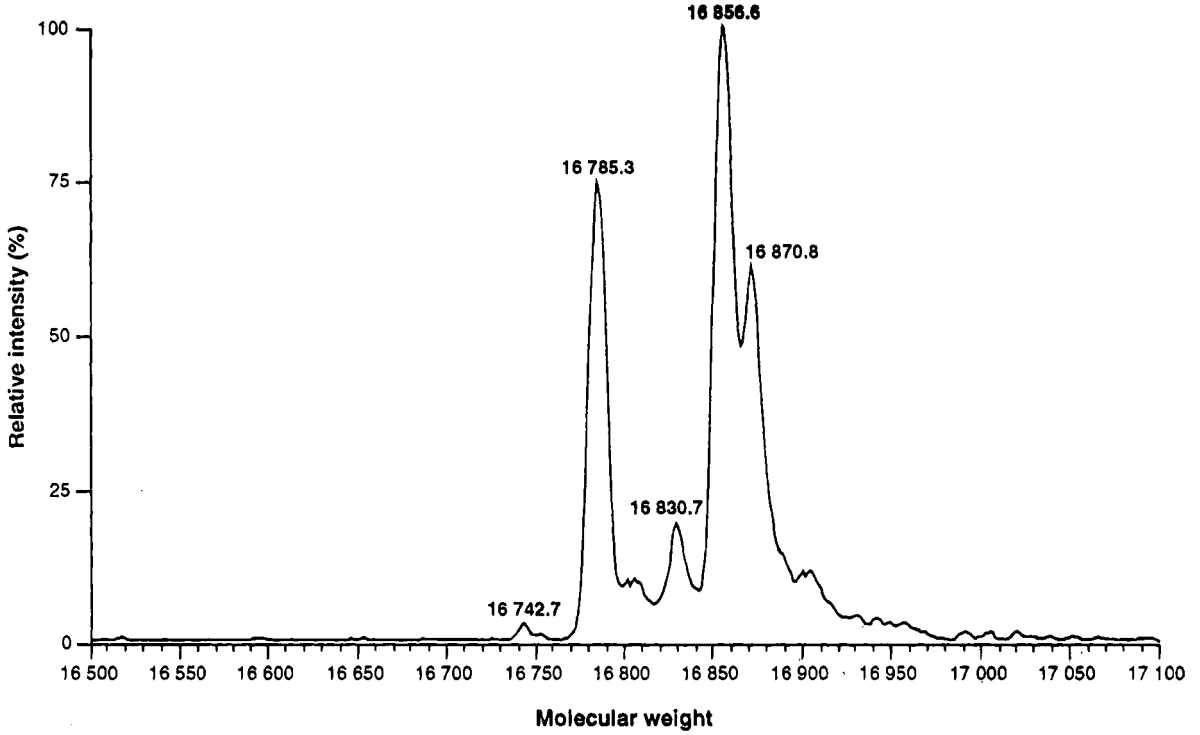

FIGURE 6: Reconstruction of the ESI-MS spectra of the purified minor isoform fraction. The precise peak masses (Daltons) are given in the figure, showing that this preparation contains three major isoform species (16 785.3 Da, 16 856.6 Da, and 16 870.8 Da, respectively). Small amounts of main form eIF-5A (16 830.7 Da) were also found in this preparation, as was also seen by 2D gel analysis (see Figure 2B). The mass of 16 742.7 Da corresponds to that of the α-N-acetylated precursor form of eIF-5A, which is not modified on K50.

be noted that the proteins used in the latter experiment were produced in *Escherichia coli* and were found to be unmodified at K50 (João et al., 1995). However, the hypusine modification does not affect the elution profile in gel filtration chromatography, and the unmodified protein possesses the same apparent M_r as the native, modified variant.

Protein Chemical Characterization of the eIF-5A Isoforms

In contrast to the main form of eIF-5A, the fraction containing the minor isoforms (break-through of the cation exchange chromatography) led to two major protein spots after 2D gel electrophoresis (Figure 2A). Both spots were recognized by the anti−eIF-5A antibody (Figure 2B), thus confirming that they are eIF-5A isoforms. The apparent molecular weight of both minor isoforms is 18 000 as determined by this method, which is identical to that of the main form. However, the minor isoforms are more negatively charged than the eIF-5A main form (pI = 5.2) possessing isoelectric points of 5.1 and 5.0, respectively. The reduced isoelectric points of the two minor isoforms are in accordance with their weaker interaction with the cation exchange column, as compared with the main form of eIF-5A.

In contrast to the two spots on the 2D-gel (indicative of two isoforms) mass spectrometry of the minor isoforms revealed three prominent masses of 16 785.3 Da, 16 856.6 Da, and 16 870.8 Da for these proteins (Figure 6). These three masses cannot be explained by any of the known modifications of eIF-5A (Klier et al., 1993). We, therefore, postulate additional modification(s) of the human eIF-5A protein for these isoforms. For further analysis of these modifications the main form as well as the minor isoforms of eIF-5A were excised from Coomassie brilliant blue−stained 2D-gels and subjected to a tryptic digest. After elution from the gel matrix the tryptic peptides of the three different protein spots were separated on reversed-phase HPLC. Comparison of the respective elution profiles suggests an apparent difference in the retention time of only one peptide peak. Following their separation, the corresponding peptides were subjected to amino acid sequence analysis and mass spectrometry (summarized in Table 2). The peptide obtained from the eIF-5A main form contained the amino acid sequence I-V-E-M-S-T-S-K corresponding to the eIF-5A sequence from I40 to K47. Its experimentally determined molecular mass is 893.5 ± 0.3 Da and is identical to the calculated average mass of 893.5 Da.

The relevant peak obtained from the peptide map of the eIF-5A isoform having a pI of 5.1 contained a peptide with the sequence: I-V-E-M-S-T-S-K^{Ac}-T-G-_-H-G-H-A-K (whereby "K^{Ac}" refers to an unusual PTH-amino acid with a retention time between those of PTH-alanine and PTH-histidine). The "_" in the position of K50 represents an amino acid residue that is not detectable under routine conditions, such as hypusine. This peptide resembles the sequence between the amino acid residues I40 and K55 and eIF-5A. Since (i) neither of the lysine residues at position 47 and 50 (Smit-McBride et al., 1989) were recognized by trypsin and (ii) the amino acid residues at position 47 and 50 were not detected as PTH-lysine by amino acid sequence analysis, both of these lysine residues must be modified. One possible modification of lysine side chains is the acetylation of their ε-amino groups (Krishna & Wold, 1993). To test this possibility for modification of K47, a synthetic peptide containing an ε-acetylated lysine residue was also sequenced. The retention time of this PTH−ε-acetyl-lysine was identical to that of the PTH-amino acid found in position 47 of the isoform peptide and thus supported our hypothesis.

Table 2: Differences in the Primary Structure of eIF-5A Isoforms from HeLa Cells[a]

Isoform	sequence of differently modified tryptic peptides[b]	average peptide mass [Da] (calculated value)	average protein mass [Da] (calculated value)	relative charge
	40 55			
main form	I-V-E-M-S-T-S-K T-G-**Z**-H-G-H-A-K	[c]	16 830.7 ± 1.5 (16 829.3)	0
intermediate form I	I-V-E-M-S-T-S-K-T-G-**Z**-H-G-H-A-K (Ac)	1 838.8 ± 0.4 (1 839.1)	16 870.8 ± 0.8 (16 871.3)	-1
intermediate form II	I-V-E-M-S-T-S-K-T-G-**X**-H-G-H-A-K (Ac)	1 822.6 ± 0.6 (1 823.1)	16 856.6 ± 1.4 (16 855.3)	-1
acidic form	I-V-E-M-S-T-S-K-T-G-**K** (Ac)	1 221.7 ± 1.6 (1 222.4)	16 785.3 ± 1.3 (16 784.2)	-2

[a] Results as obtained from direct sequencing and electrospray mass spectrometric analysis of the respective tryptic peptide. [b] As depicted in the respective peptide sequences from I40 to K55, all isoforms differ by the state of modification of K47, which can be acetylated, and their modification level at position 50 (highlighted in bold letters), which can be hypusine (Z), deoxyhypusine (X), or free lysine (K). [c] No average peptide mass is given for the main form peptide, because the sequence of I40 to K55 is covered by two tryptic peptides (free K47), as indicated by the gap between K47 and T48.

Analysis of this peptide peak by mass spectrometry revealed two different species of mass 1 838.8 ± 0.2 Da and 1 822.6 ± 0.2 Da, respectively. A possible explanation for the observed mass difference of 16 Da for the above-defined amino acid sequence is, that while the lysine residue at position 47 is ϵ-amino terminally acetylated, the lysine at position 50 is either modified to hypusine or deoxyhypusine; the latter being the proposed intermediate for the hypusine biosynthesis, and lacking the hydroxyl group (Wolff et al., 1990). The hydroxyl group would thus account for the mass difference of 16 Da for the two peptides species. Since neither hypusine nor deoxyhypusine is detectable by Edman degradation, these two peptides cannot be interdistinguished by this sequencing technique.

The respective peptide of the eIF-5A isoform with a pI of 5.0 gave rise to the sequence I-V-E-M-S-T-S-K^{Ac}-T-G-K, corresponding to position I40 to K50. As in the above case, the PTH−amino acid in position 47 was identified as acetyllysine. In agreement with (i) the experimentally determined molecular mass of this peptide (1 221.7 ± 1.6 Da, Table 2), (ii) the peptide pattern of the tryptic digest, and (iii) the determined amino acid sequence, the eIF-5A isoform is acetylated at K47 and possesses a free lysine side chain in the place of hypusine at position 50.

The posttranslational modifications, which were identified by detailed peptide analysis of the respective isoforms, are sufficient to explain the differences in the prominent protein masses determined for the intact minor isoforms in comparison to the mass of the main form of eIF-5A. Therefore, all eIF-5A isoforms are N-terminally acetylated, i.e., the first methionine residue is replaced by an acetyl group. We find that the main form, and also one of the three minor isoforms of eIF-5A are modified such that they contain hypusine in place of lysine at position 50. All three minor isoforms, but not the main form, have an additional modification at position 47; K47 is ϵ-acetylated. These results are also in good agreement with the relative charges of the different eIF-5A variants deduced from their pI values (see Table 2). The main form of eIF-5A possesses two positively charged amino groups at the hypusine residue as well as one positive charge at K47 and is therefore the most basic isoform. Two minor isoforms both contain two positive charges at position 50 (namely at deoxyhypusine or hypusine) but lack the positive charge of K47 due to its acetylation. The third minor isoform of eIF-5A is the most acidic isoform as it contains only one positive charge at position 50 (having a free lysine residue) and no charge at the acetylated K47. It should be noted, however, that minor traces of posttranslationally unmodified precursor of eIF-5A (having free lysine residues in position 47 and 50) are also detectable by mass spectrometry (16 742.7 Da; see Figure 6) for the isoform preparations from HeLa cells (without prior interference with the polyamine metabolism of the cells).

eIF-5A Isoforms from the HeLa Nuclear Fraction

In order to investigate the cellular distribution of the different eIF-5A isoforms, we also purified the protein from a nuclear fraction of HeLa cells. In contrast to the purification of eIF-5A from complete HeLa cells, all the eIF-5A prepared from the nuclear fraction eluted from the final cation exchange column in a single peak. No eIF-5A was detected in the break-through of the column, and none of the minor isoforms could be detected by 2D gel electrophoresis or mass spectrometry. Therefore, all the K47 acetylated eIF-5A minor isoforms appear to be excluded from the nuclear fraction, and are localized in the cytoplasmic fraction after cell lysis.

DISCUSSION

The purification of eIF-5A from HeLa cells led to the isolation and characterization of several different isoforms. The main form of eIF-5A, which represents approximately 95% of the total eIF-5A, was shown to be modified only by replacement of the first methionine with an acetyl group and by carrying hypusine at position 50. An identically modified

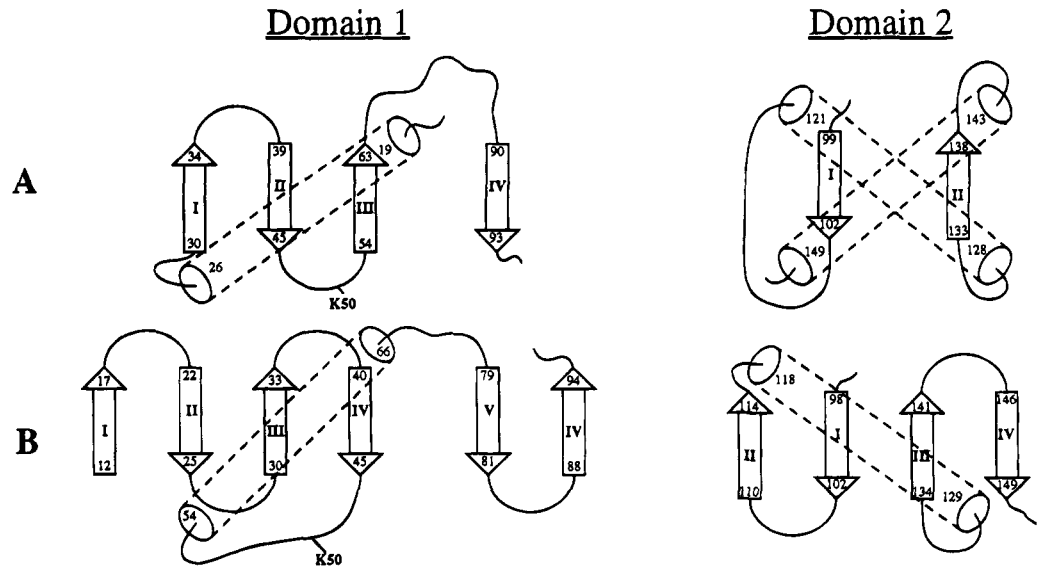

FIGURE 7: Structural models built from the (A) PHD and (B) ETH (preferred prediction B) secondary structure and solvent accessibility predictions.

form of eIF-5A seems to occurs in chicken and mammals (Wolff et al., 1992) and was also recently described for the two eIF-5A homologues Hyp1p and Hyp2p, in the yeast *Saccharomyces cerevisiae* (Magdolen et al., 1994; Wöhl et al., 1993). However, while phosphorylation at the first serine residue in Hyp2p gives rise to an additional isoform (Klier et al., 1993), no phosphorylation was found in any of the purified human eIF-5A isoforms. Since hypusine is central to eIF-5A function (Magdolen et al., 1994; Schnier et al., 1991) and efficient cell proliferation requires the protein to be present in large amounts (Kang & Hershey, 1994; Park et al., 1994; Wöhl et al., 1993), we believe that the N-terminally acetylated, hypusine-containing main form represents a (the) physiologically active eIF-5A component. Further structural characterization has, therefore, been done predominantly on this protein isoform.

In agreement with previously published work describing gel filtration and analytical ultracentrifugation experiments (Chung et al., 1991) we find that the eIF-5A main form possesses a hydrodynamic volume that is larger than that expected for a compactly folded, monomeric protein. However, the native protein does not exist in a dimeric form. Several independent experiments as described in this manuscript suggest eIF-5A to be a monomer, and therefore, its native structure is slightly more expanded than that expected for an ideal globular protein. As indicated by its far-UV CD spectrum, native eIF-5A appears to have predominantly β-sheet structure. In addition, four separate secondary structure predictions using different algorithms gave consistent results for a region of about 80 amino acid residues surrounding the site of hypusine modification (K50; see Figure 3). Five distinct secondary structure elements were predicted in this sequence. Those that directly flank K50 might be the most important determinants for the structure recognized by the hypusine-modifying enzymes: β-strands covering residues 30−34, 39−45, and 54−64 (the latter β-strand might also be an internal α-helix according to the ETH-A prediction). These theoretical considerations may explain the recent experimental findings by Joe and Park (1994). In this work, N- and C-terminal truncation mutants of recombinant human eIF-5A precursor led the authors to define the corresponding minimal eIF-5A sequence required for modification by the deoxyhypusine synthase to be located between F30 and E70.

The reliability of prediction results have previously been judged in a contest in which the secondary structure of metallohemorrhagic protease was predicted by the above four methods before the crystallographic structure was published (Gerloff et al., 1993; Gomis-Rüth et al., 1993). The results of this competition showed that the most reliable predictions were obtained using the PHD and ETH methods, while the predicted structure by the Chou−Fasman method deviated most from the crystal structure. The different secondary structure predictions obtained for human eIF-5A, when compared to the secondary structure as determined by CD, would agree with this conclusion. With the information obtained from the PHD and ETH programs on the secondary structure and solvent accessibility, we suggest structural models/folding patterns as outlined in Figure 7. According to the PHD and ETH predictions, we favour a structure for eIF-5A that consists of two domains. Based on the PHD predictions, the first domain (residues 1−95) has an α-helix (residues 19−26) packing against an anti-parallel β-sheet. An alternative model based on the ETH-A prediction data, is an internal α-helix (residues 54−66) packing against two internal anti-parallel β-strands (residues 30−34 and 40−45). Packing against this β-sheet are two sets of anti-parallel β-strands. The second domain (residues 96−154) is modeled to be two α-helices (residues 121−128 and 143−149) packing against a buried anti-parallel β-sheet (PHD) or a surface α-helix packing against a β-sheet consisting of four anti-parallel β-strands (ETH-A and -B).

In addition to the main form of eIF-5A, three minor isoforms were purified. They account for approximately 5% of the total eIF-5A. Detailed analysis of their primary structure by mass spectrometry and peptide mapping revealed that all minor isoforms carry an additional acetylation at K47. A similar modification is likely to occur also in the eIF-5A isoform in CHO cells, since acetylation of K47 in these isolated isoforms would explain the observed charge shift relative to the main form and their resistance to tryptic digest at K47 (Park, 1988, 1989). The ϵ-acetylation of K47 of

human eIF-5A seems to conflict with efficient formation of hypusine at position 50. As compared to the minute amounts of eIF-5A precursor, which are neither acetylated at K47 nor modified at K50, significant amounts of K47 acetylated isoforms with an incompletely modified K50, carrying a free lysine or deoxyhypusine, were found. Since the modification of K50 to hypusine is an irreversible process (Park et al., 1993), acetylation of K47 appears to reduce the rate of hypusine synthesis. Therefore, introduction of amino acid replacements at position 47 might lead to eIF-5A forms that are less efficiently hypusine modified at K50. Studying the physiological consequences of such mutations by complementation analysis in yeast might give further insight into the *in vivo* function of this protein.

Acetylations of the ϵ-amino groups of lysine residues can be reversible posttranslational events (Han & Mertinage, 1992) and may exert regulatory functions. For example, the state of lysine acetylation in histones is discussed in terms of influencing the functions involved in DNA replication and gene activation [for review see Csordas, (1990)]. In the case of the eIF-5A isoforms, however, the physiological implications of the K47 acetylation remain unclear. Nevertheless, this modification might influence the cellular distribution of this isoform. The main form, but not the K47 acetylated isoforms, was found in the preparation of eIF-5A from the nuclear fraction. As the amino acid sequence around the hypusine residue (position 50) is the only region in the eIF-5A sequence where positively charged amino acid residues are clustered, this cluster possibly acts as a nuclear localization signal (Agutter & Prochnow, 1994). Reducing the positive charge in this part of eIF-5A by acetylating K47 might be sufficient to inactivate this putative nuclear localization signal [as shown for nuclear localization signals of other proteins, (Agutter & Prochnow, 1994)] and the K47 acetylated isoforms are kept cytoplasmic. Mutational studies that define the putative nuclear localization signal more precisely are ongoing.

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