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Structural organization of the human eukaryotic initiation factor 5A precursor and its site-directed variant Lys50 → Arg

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Summary. The molecular properties of the human eukaryotic initiation factor 5A precursor and its site directed Lys50 → Arg variant have been investigated and compared. Structure perturbation methods were used to gain information about the protein architecture in solution. Intrinsic and extrinsic spectroscopic probes strategically located in the protein matrix detected the independent unfolding of two molecular regions. Three cysteines out of four were titrated in the native protein and the peculiar presence of a tyrosinate band at neutral pH was detected. At alkaline pH only two tyrosines out of three were titratable in the native protein, with an apparent pK of about 9.9. Native protein and its Lys50 → Arg variant reacted in a similar fashion to guanidine and to pH variation, but differently to thermal stress. The complex thermal unfolding of both proteins indicated the presence of intermediates. Spectroscopic data showed that these intermediates are differently structured. Consequently, the two proteins seem to have different unfolding pathways.

Keywords: Amino acids – eIF-5A – Hypusine – Protein folding – Post-translational modification

Abbreviations: AEDANS, acetyl-N′-(8-sulpho-1-naphthyl) ethylene-diamine; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ε, molar extinction coefficient; $\Delta\varepsilon$, molar extinction difference; eIF-5A, eukaryotic initiation factor 5A, namely the hypusine-containing protein; eIF-5A precursor [or ec-eIF-5A(Lys)], eukaryotic initiation factor 5A precursor, i.e., the unmodified precursor form of eIF-5A produced in *Escherichia coli* by expression of human eIF-5AcDNA containing Lys in position 50; GdnHCl, guanidinium chloride; I-AEDANS, N-iodo-AEDANS; N-AcCys-AEDANS, N-acetylcysteine-AEDANS, Mr, relative molecular mass;

ODU, optical density unit; RMS, root mean square; TrisHCl, Tris (hydroxymethyl)amino-methane hydrochloride.

Introduction

Eukaryotic translation initiation factor 5A precursor [(ec-eIF-5A(Lys)] is the only cellular protein known to contain a specific lysine residue which is transformed into the unique amino acid hypusine [N^{ε}-(4-amino-2-hydroxybutyl)-lysine]. This amino acid is formed by a series of post-translational reactions starting with the transfer of the butylamine moiety from spermidine to the ε-amino group of one of the lysine residues in the eIF-5A precursor protein, thus forming peptide-bound deoxyhypusine (Park et al., 1982). This intermediate is not accumulated in cells but is immediately hydroxylated at C-2 of the incoming 4-aminobutyl moiety to form hypusine (Abbruzzese et al., 1985, 1986, 1988b). More recently it was found that eIF-5A undergoes a post-translational modification catalyzed by transglutaminase (Beninati et al., 1995).

eIF-5A promotes the formation of the first peptide bond during the initial stage of protein synthesis (Hershey, 1991). A series of observations suggests that eIF-5A plays a role in cell growth and differentiation. In fact, the ec-eIF-5A(Lys) modification is correlated with cell proliferation (Abbruzzese, 1988; Abbruzzese et al., 1988a; Beninati et al., 1990; Caraglia et al., 1997) and is vital for *Saccharomyces cerevisiae* growth (Schnier et al., 1991). Moreover, agents that block the lys/hyp transformation (Abbruzzese et al., 1989, 1991; Jakus et al., 1993; Park et al., 1994) inhibit the growth of mammalian cells (Park et al., 1993).

The protein, of M.W.18-kD, is highly conserved from yeast to mammalian cells (Gordon et al., 1987; Park et al., 1984). The human cDNA that encodes the eIF-5A precursor has been cloned and sequenced (Smit-McBride et al., 1989). Subsequent studies showed that the Lys50 residue is modified to hypusine and that the Lys50 $\rightarrow$ Arg variant is unable to stimulate methionyl-puromycin synthesis *in vitro* (Hershey et al., 1990; Park et al., 1991). The genes encoding the eIF-5A precursor have been cloned in yeast (Schnier et al., 1991) and the expression of at least one of them is required for viability. Moreover, the Lys50 $\rightarrow$ Arg variant is inactive *in vivo* (Smit-McBride et al., 1989). These data suggest that hypusine synthesis is required for the biological activity of the protein and for interaction with the ribosome.

Different isoforms of the protein have been isolated and structurally characterized (Joao et al., 1995; Klier et al., 1995). The main form (approximately 95% of the total) has a well-defined secondary structure (i.e. a predominantly β-sheet structure). Minor isoforms are characterized by acetylation of the ε-amino group of Lys-47. Two models of the protein have been postulated, each constituted by β-sheet structure packed into a two-domain organization (Joao et al., 1995; Klier et al., 1995).

Surface mutations seem to play a significant role in controlling the equilibrium between the native and denatured state (Dill et al., 1991; Shortle et al., 1992) and thus are ideal tools to study the effects exerted by mutations on

protein structure and function. We have studied the structural properties of the eIF-5A precursor protein (and of its Lys50 → Arg variant) in an attempt to shed light on its biochemical role. We used spectroscopic techniques to compare the mammalian native protein with its Lys50 → Arg variant obtained by site-directed mutagenesis. Our data strongly suggest that ec-eIF-5A (Lys) and its Lys50 → Arg variant, although structurally organized in similar folds, have different unfolding pathways.

Material and methods

Protein preparation

eIF-5A precursor(Lys) was prepared by overexpression of human eIF-5A-cDNA in E. coli. The variant form Lys50 → Arg of eIF-5A precursor was expressed in E. coli. The variant protein does not show functional activity (Smit-McBride et al., 1989). Proteins were homogeneous when tested by SDS-PAGE.

Site-directed mutagenesis

Standard recombinant DNA methods (Sambrook et al., 1989) were used unless stated otherwise. Oligonucleotide-directed construction of mutations was performed with the gapped duplex DNA method (Fritz et al., 1988) using pMa/c plasmid vectors. To obtain mutation Lys50 → Arg, a 24-mer oligonucleotide (5′-C GTG GCC GTG CCG GCC AGT CTT CG-3′) was made complementary to the Lys50 region, thus mutating the AAG codon for Lys to the CGG codon for Arg and creating a new NaeI restriction site. To confirm the mutation, the variant fragment was subcloned from pMa/c vectors into phages M13 mp 18 and mp 19. Sanger's dideoxynucleotide termination method was used for DNA sequencing (Sequenase kit, USBA).

Preparation of I-AEDANS derivative

eIF5A precursor-AEDANS was prepared according to the method described by Kang et al. (1979). The reaction was performed in 0.01 M phosphate buffer, pH 7.5, containing 0.1 M KCl. The reaction yield was controlled by varying the molar ratio between reagents and/or the reaction times. About 10% of the total protein molecules was labelled under our experimental conditions. The reaction was terminated by adding an excess of 2-mercaptoethanol. Unreacted I-AEDANS was removed by repeated dialyses against 0.05 M phosphate buffer, pH 7.5, or by gel filtration.

An aliquot (0.2 mg) of the AEDANS labelled protein was suspended in 0.5 mL 0.1 M ammonium bicarbonate, supplemented with trypsin (molar ratio 1:100) and incubated for 4 hours at 37°C. The precipitate obtained after the incubation was separated from the supernatant by centrifugation. The soluble tryptic peptides were purified using a Beckman System Gold HPLC on a reverse-phase column eluted with a linear gradient over 0–70% acetonitrile in 0.2% (by volume) trifluoracetic acid at a flow rate of 1.5 mL/min. The elution was monitored with a Beckman 168 Diode Array Spectrophotometer (at 215 or 280 nm) and with a Shimadzu RF 535 fluorescence monitor (at 335 and 480 nm for excitation and emission, respectively). Only two peaks were detected by both fluorescence and absorption at 215 nm. One contained about 93% of the total fluorescence. The peaks were separately collected, dried under vacuum and resolubilized in small amounts (50 μL) of 1% trifluoroacetic acid solution. When monitored for fluorescence the two peptides showed emission bands with peaks equally centred at 495 nm (excitation: 335 nm). Since both peaks have similar emission properties, their surface was proportional to the concentration of the labelled molecules. The two peptides were put through an automated amino acid sequencer in an attempt to obtain information

on the sequence location of the reactive cysteines. The first five residues from the N-terminus of the major peptide are: KYEDI, while the minor peptide showed the sequence: MADDLDFE. These two short sequences are present only at positions 67–71 and 1–8, respectively. Therefore, under our experimental conditions, Cys 73 appears to be the main target of the labelling procedure.

Cysteine and pH titration

The DTNB method was used to titrate the sulphydryl group of native and denatured protein. To measure the total content of the sulphydryl groups, the protein sample was denatured in 5 M GdnHCl for 24 h. Measurements were made spectrophotometrically using $\varepsilon_{412} = 13{,}600\,M^{-1}cm^{-1}$ for the DTNB-sulphur adduct. Spectrophotometric measurements of native and of denatured protein solutions were performed at constant intervals (5 min) after each titratant addition to avoid a time dependence of the native protein. For pH titration, the samples were placed in a 2-mL glass cell equipped with a magnetic micro-stirrer under a pure N_2 flow to avoid the effects of CO_2. Temperature was kept at 20°C by a circulating water bath. Microamounts (5 μL or more) of NaOH 1 M were added using an Agla syringe equipped with a micrometric control system. Tyrosine content was determined spectrophotometrically with the formula: $\Delta\varepsilon_{295} = 2{,}330\,M^{-1} \times cm^{-1}$ (Donovan, 1969).

Spectral measurements

Fluorescence measurements were made in the range of fluorescence linearity. The absorbance of all solutions was between 0.01 and 0.05 ODU at the excitation wavelength. A thermostated bath was used to change the temperature of the solution cell. Fluorescence and polarization measurements were made with a Perkin-Elmer MPF 66B computer-driven spectrofluorometer. The polarization, p, was calculated by:

$$p = \left(I_{vv} - G \cdot I_{vh}\right) / \left(I_{vv} + G \cdot I_{vh}\right),$$

where $G = I_{hv}/I_{hh}$, I is the intensity, and the first and the second subscript refer to the plane of polarization of the excitation and emission beams, respectively, i.e. v, vertical, and h, horizontal. The computer calculated an average intensity value by accumulating 128 intensities for each measurement. Circular dichroism measurements were made with a Jobin Yvon Mark III spectropolarimeter equipped with a temperature-controlled cell holder. Mean residue ellipticities were calculated by:

$$[\theta]_\lambda = \frac{MRW\, q_{obs}}{10\, l\, c}$$

where $[\theta]_\lambda$ is the mean residue ellipticity in units of degrees centimetre squared per decmole at a particular wavelength, q_{obs} is the observed ellipticity, MRW is the mean residue molecular weight calculated from the sequence, l is the optical path length (cm), and c is the concentration in grams per mL. Cells between 0.01 and 1 cm were used in the far UV and CD spectra were accumulated by computer to yield reliable tracings. The circular dichroism spectra were analyzed in the region between 200 and 250 nm to evaluate the amount of secondary structure. A computerized program with different methods of analysis (Menendez-Arias et al., 1988) was used. Normal and second derivative spectra were recorded on both a Perkin Elmer Lambda Array 3840 and a Cary 219 spectrophotometer. The protein samples under spectroscopic investigation were always dialyzed against numerous changes of buffer, which was used as a blank for the measurements.

Denaturation studies

Chemical denaturation studies at a high protein concentration were complicated by the protein's tendency to aggregate. Sample solutions were routinely left to equilibrate at each GdnHCl concentration for at least 24 h at room temperature before measurements. The equilibrium was confirmed by verifying that the values of the fluorescence parameters remained constant in time. The final protein concentration at each GdnHCl concentration was 0.05 mg/mL. Since unfolding by GdnHCl was accompanied by changes of fluorescence and polarization, the reversibility of both phenomena was monitored in each case. During temperature measurements (between about 30 and 70°C) the protein samples under study were left at each temperature until an apparent equilibrium was reached. The structural transition was always reversible between 30 and 70°C. At temperatures above 70°C proteins showed a slow time-dependent tendency to aggregate.

Data analysis

Data analysis was performed with computer programs specifically designed for non linear curve fitting. GraphPad, version 4.0, by Harvey Motulsky (GraphPad Software) and FitAll, version 4.0, by MTR Software were extensively used for the statistical analysis of curve fitting to experimental data.

Chemicals and solutions

Spectroscopic grade guanidinium chloride was from Schwarz-Mann. All chemicals were reagent grade and were purchased from BDH (British Drug Houses, Great Britain). In denaturation experiments the protein was added to buffered solutions of GdnHCl; 0.15 M KCl was present in all solutions. The spectroscopic measurements were then monitored until an apparent equilibrium was reached. pH measurements were carried out by using combination electrodes with a pH meter.

Results

Absorbance, derivative spectra, circular dichroism and SH- residues titration

The spectrum of the eIF-5A precursor protein at 25°C was dominated by the absorption of the three tyrosines; the maximum was centred at 275 nm because the protein lacks tryptophan (data not shown). The shoulder at 286.6 nm can be attributed to the 0-0 transition band of tyrosine (Donovan, 1973; Sears and Beychock, 1973). It was clearly detectable as a derivative peak centred at 286.6 nm (Ragone et al., 1984, 1987). The ripples over 250–260 nm can be attributed to phenylalanines (Donovan, 1973) and their contribution was clearly evident in the second derivative spectrum (Ragone et al., 1984, 1987).

We have analysed the far-ultraviolet CD spectra of the native protein at 25 and 70°C. The spectrum at 25°C had a pronounced minimum centred at about 205 nm, and an evident shoulder at 215–216 nm (data not shown). The latter feature suggested the presence of β-sheet structure that was characterized by a dichroic band with a minimum at 216 nm. The spectrum at 70°C did not show any appreciable temperature effect (data not shown). This result suggested that temperature had a limited effect on the protein structure. The titration of the sulphydryl group of the wild type protein by DTNB has been also performed. Only three SH groups out of four were detected in the native protein

suggesting that one cysteine residue was too deeply embedded in the protein structure to be available to solvent (data not shown). Moreover, mapping data indicated that Cys 73 was the main target of the labelling reaction (data not shown).

ec-eIF-5A(Lys) fluorescence and GdnHCl effect

ec-eIF-5A(Lys) fluorescence is the result of tyrosine excitation by UV radiation. We used this technique to probe the structural effects produced by the structure perturbing agents, guanidinium hydrochloride, pH and temperature. Figure 1 shows the fluorescence emission band of native and denatured protein. The maximum of the native protein was centred at about 304 nm, while that of the denatured protein was at about 305–306 nm. The two spectra at pH 7.5 showed a significant difference when their fluorescence peaks were normalized by maxima. The fluorescence intensity at longer wavelengths was higher for the native protein. The protein lost this feature when denatured in 6 M GdnHCl suggesting that the spectral effect may be related to a structural feature in the native protein that involves the tyrosine residue. We found that the difference spectrum between the fluorescence of the native and denatured protein had a maximum at 337–338 nm (Fig. 1, inset). Because tryptophan is absent, the spectrum coincided with that of the tyrosinate emission band (Lakovicz, 1983).

Figure 2 shows the dependence of emission maximum (upper panel) and quantum yield (lower panel) on GdnHCl concentrations at 25°C of a 0.02 mg/mL concentration of eIF-5A precursor. At about 2 M GdnHCl the protein was

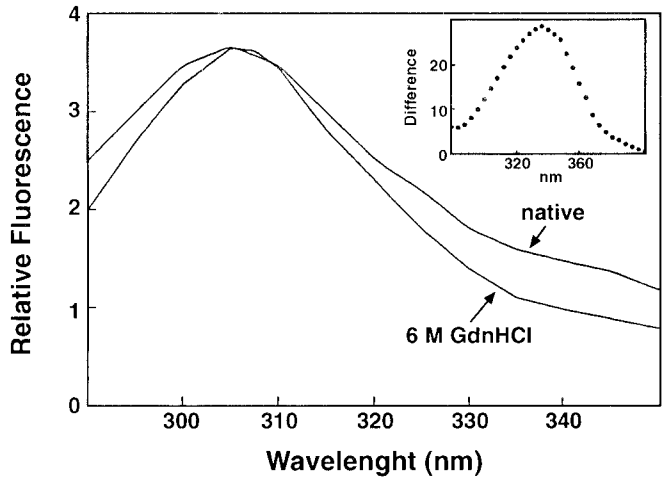

Fig. 1. Fluorescence emission of the eIF-5A precursor (excitation at 276 nm). The spectrum of the native protein was obtained at pH 7.5 in 0.01 M phosphate containing 0.15 M KCl. The spectrum of the denaturated protein was obtained using the same buffer plus 6 M GdnHCl. Protein concentration was 0.05 mg/mL for both experiments. The inset shows the fluorescence difference spectrum between 0 and 6 M GdnHCl

unfolded. In fact, the band width at that denaturant concentration was about 34 nm, a value typical of free tyrosine in neutral aqueous solution. The overlapping transition curves confirmed that the protein was indeed unfolded. We analysed the tyrosine fluorescence to obtain information about unfolding in the C-terminal region of the eIF-5A precursor, where these residues are prevalently located. We also used an extrinsic fluorophore, I-AEDANS, as a structural probe that indicated that Cys 73 should be located in a hinge segment between two structured regions, while Cys 22 is in an unordered segment. Therefore, perturbation of the microenvironments of these residues may provide structural information. We used a low (probe:protein) molar ratio to selectively label exposed and reactive cysteine(s). Cys 73 was the preferred target, while Cys 22 was only marginally labelled. Far UV CD spectra of the protein derivative did not show any appreciable difference as compared to the spectrum of the native protein. The effect of the denaturant concentration on the emission maximum shift of the labelled protein is reported in Fig. 2 (upper panel). The inset shows the fluorescence spectrum of the protein derivative. I-AEDANS reacted specifically with the cysteine. Table 1 shows the fluorescence properties of the labelled protein and of the

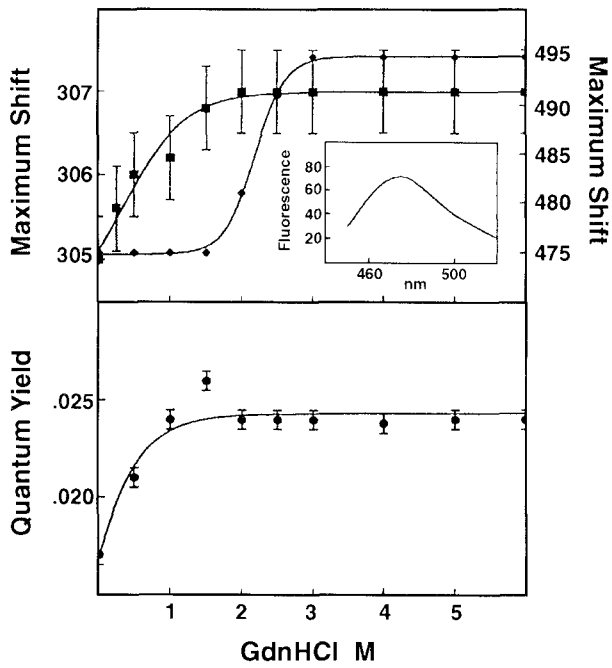

Fig. 2. Effect of GdnHCl on the fluorescence of the eIF-5A precursor. Upper panel: the maximum shift dependence of the eIF-5A precursor (■) (excitation at 276 nm, slits of 1 nm) and on its AEDANS-labeled derivative (◆) (excitation at 335 nm) in 0.01 M phosphate, 0.15 M KCl, pH 7.5. Protein concentration was 0.02 mg/mL. The inset shows the fluorescence emission of the eIF-5A precursor-AEDANS in the same solution (excitation at 335 nm). Lower panel: the quantum yield of the eIF-5A precursor in the same experimental conditions as reported for the upper panel. Bars on experimental points indicate error determined from four experiments

Table 1. Fluorescence properties of N-AcCysAEDANS and eIF-5A precursor-AEDANS in five solvents

Compound	Solvent	Max (nm)	Quantum yield
N-AcCys AEDANS	Phosphate pH 7	495	0.26 ± 0.02
N-AcCys AEDANS	6 M GdnHCl[a]	495	0.26 ± 0.03
N-AcCys AEDANS	Ethanol	460	0.70 ± 0.02
eIF-5A-AEDANS	Phosphate pH 7	478	0.55 ± 0.03
eIF-5A-AEDANS	6 M GdnHCl[a]	495	0.28 ± 0.04

[a] GdnHCl solutions were buffered by phosphate at pH 7.

model compound N-acetyl-cysteine-AEDANS monitored under our experimental conditions. The fluorescence maximum of the protein derivative was at 478 nm and its quantum yield was 0.55. These values, compared with those of the model compound, suggested that the probe was located partially in an apolar environment. Fluorescence polarization of the fluorophore was constantly around 0.185 ± 0.005 suggesting that the fluorophore was bound within a rigidly structured protein matrix. The transition curve (Fig. 2, upper panel) obtained with the labelled protein was different from that obtained with tyrosyl fluorescence. Transition started when the other was almost completed and the midpoint was at 2.2 ± 0.1 M GdnHCl. The fluorescence maximum at 3 M GdnHCl was shifted to 495 nm with a quantum yield of 0.28, thus indicating that the probe was normalized to the aqueous solvent consequent to a structural transition. Moreover, the steep transition curve suggested that the labelled Cys 22 had little effect on the fluorescence of the main probe. In fact, the steep transition intrinsically suggested that both cysteines followed the same structural event, i.e., their fluorescence emission trends were superimposable.

Effect of pH

The effect of pH on the fluorescence emission of the eIF-5A precursor is shown in Fig. 3A. At alkaline pH, the emission band was quenched but remained centred at 304–305 nm. At pH > 10, the emission peak was red-shifted to 345 nm and the emission intensity was significantly lowered. The maximum shift to 345 nm and the fluorescence intensity decrease can be explained by the ionization of tyrosine residues in alkaline solution, analogous to the ionization of the model compound (Lakovicz, 1983). Concomitantly to the ionization of titratable tyrosines the protein showed a pronounced shoulder at 306–307 nm on the blue side of the spectrum. The shoulder was probably due to tyrosyl residue(s) that were not ionized. The spectrophotometric titration of the native protein at alkaline pH is shown in Fig. 3B. The titration showed that only two residues (2.3 ± 0.2) were available in the native protein suggesting that one of tyrosines was not available to the titratant. The apparent pK of titratable tyrosines was about 9.9. Figure 4 illustrates the effect of

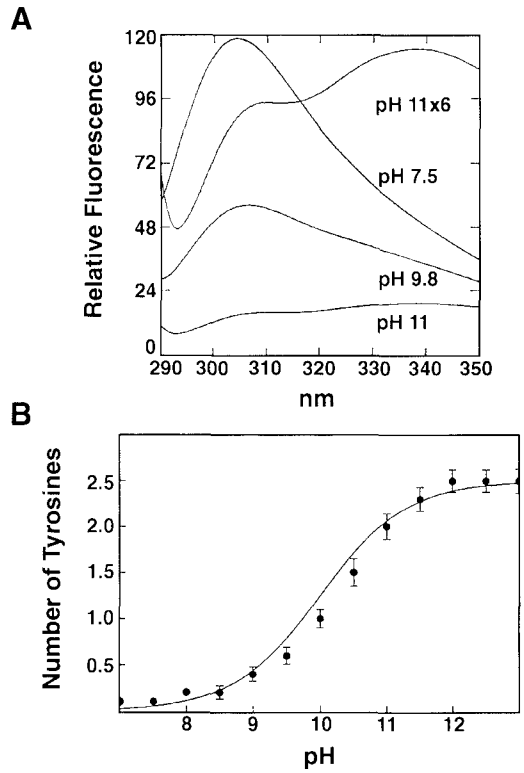

Fig. 3. **A** Fluorescence emission spectra of the eIF-5A precursor at various pH in Tris/phosphate 0.05 M, 0.15 M KCl. The spectrum at pH 11 has been enlarged 6 fold, to reveal specific spectral features. Excitation was at 276 nm. Protein concentration was 0.03 mg/mL. **B** Spectrophotometric titration of eIF-5A precursor tyrosines. The titration was followed by absorbance at 295 nm. Protein concentration was about 0.8 mg/mL

pH on the protein structure. A transition centred at about pH 10.5–10.6 was revealed by fluorescence polarization at 305 nm. The fluorescence polarization is a measure of the rotational freedom of a fluorophore (Lakovicz, 1983). The polarization decrease from 0.18 to 0.10, in a pH range where the fluorescence decrease is marginal, probably reflected a pH-dependent change in the structural microenvironment of the tyrosines. Tyrosine polarization still reflected structural constriction being its value far from that characteristic of the tyrosine in a fully unfolded polypeptide, i.e. 0.05 (Lakovicz, 1983). The non-titratable tyrosine residue might be trapped in an apolar environment of the compact residual structure. However, the alkaline transition was reversible only from pH 10.2–10.3. Above those pH values, the transition was irreversible because of the presence of aggregated proteins. The inset of Fig. 4 shows the effect of pH on the fluorescence emission at 305 nm. The fluorescence was quenched and the transition curve was similar to that obtained with spectrophotometry. Neither fluorescence quenching nor the spectrophotometric titration of tyrosines overlapped the polarization transition. This confirms that two out of three tyrosines were available to the

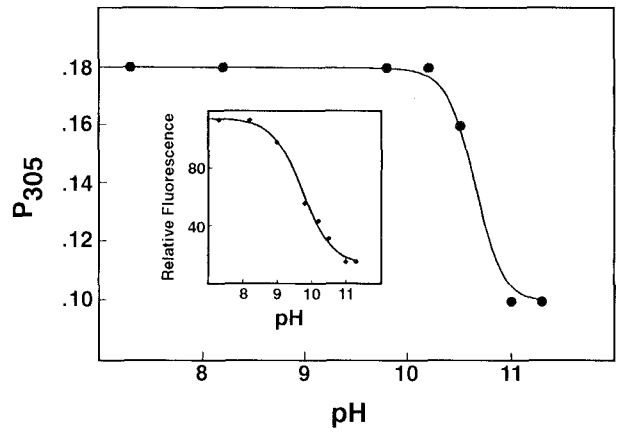

Fig. 4. pH effect on the fluorescence polarization of eIF-5A precursor at 305 nm (●) by excitation at 276 nm. Protein concentration was 0.05 mg/mL and experimental conditions are the same reported in the legend to Fig. 3. The inset shows the same experiment with fluorescence emission at 305 nm

titratant before denaturation and, therefore, they were probably more exposed to solvent in the native protein.

Structural comparison between wild-type and the Lys50 → Arg variant of the eIF-5A precursor

Lys 50 is a functionally important residue because of its post-translational modification into the unique amino acid hypusine. The site-directed variant Lys50 → Arg was obtained and purified. The Lys → Arg mutation is frequent in proteins from extreme thermophilic micro-organisms. Thus, it is a prime candidate for the molecular basis of thermostability of these unique proteins (Argos et al., 1983; Fontana, 1988), and could be involved in enhancing protein thermostability (Argos et al., 1983; Fontana, 1988; Mrabet et al., 1992). Furthermore, arginine is chemically and structurally similar to lysine. Far UV CD, fluorescence and absorption data showed that the Lys50 → Arg variant and the wild type protein were structurally indistinguishable. Structural perturbation curves obtained by using GdnHCl and various pH values were also similar. Thus, the general structural properties of the wild type protein and its behaviour in solution seemed to be retained by the site-directed variant. Nevertheless, we found that the two proteins reacted differently to thermal denaturation.

Heat did not affect dichroic activity at 220 nm between 35 and 65°C. Dichroic trends were similar although the variant protein seemed to show a more sloping temperature curve (data not shown). On the contrary, tyrosine polarization was temperature-dependent between 30 and 65°C. Figure 5 shows that the two proteins reacted differently to change in temperature. The wild-type eIF-5A showed a structural transition centred between 35 and 50°C, while the variant showed a deeper transition between

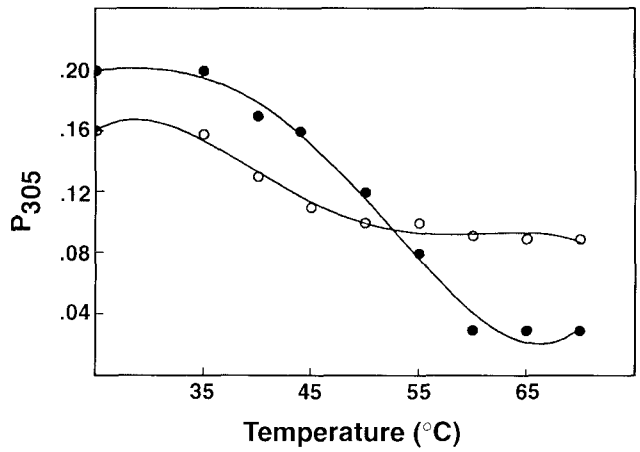

Fig. 5. Effect of temperature on fluorescence polarization of the eIF-5A precursor (○) and its Lys50 → Arg variant (●) detected at 305 nm (excitation at 276 nm) in 0.01 M phosphate, 0.15 M KCl, pH 7.5. Protein concentration was 0.05 mg/mL

35 and 60°C. The magnitude of the two polarizations was significantly different. Higher temperatures increased the mobility of tyrosines of the wild-type protein indicating a conformational change, but fluorophores still reflected structural rigidity at 60°C. In fact, polarization was only 0.10 upon completion of transition suggesting the presence of partially unfolded intermediate forms and/or aggregation. The polarization of the variant protein, instead, decreased to 0.05 at 60°C, thereby indicating freely rotating fluorophores and unfolding of the tyrosine microenvironments. However, quite constant CD signals at 220 nm still indicated the presence of organized structure. The only explanation of these observations is that in solution both proteins contained partially unfolded molecules, i.e., intermediate forms, even though the proteins seemed to reach their final states through different pathways. This fact indirectly suggests that the microenvironment of at least one of the tyrosines was influenced in some way by the mutation. Such a structural difference coincides with the finding that the wild-type protein polarization at pH 7 was 0.161 ± 0.002, a value which was significantly lower than that of the variant form and indicates that the tyrosine microenvironments in the native protein were unlike those of the variant form. The enhanced fluorescence polarization of the variant form of eIF-5A (0.198 ± 0.003) indicates a decrease in fluorophore mobility or an enhancement of structural rigidity of the molecular regions containing the tyrosine residue(s).

Because rigidity and tyrosine burial are closely related, we performed fluorescence-quenching studies with acrylamide and KI. An unchanged quenching constant (K_{SV}) with an increased accessibility fraction (f_a) for acrylamide (Table 2) accounted for a major accessibility to the quencher of the inner tyrosine residues in the native protein. Differently, the unchanged K_{sv} and f_a values for the polar quencher, KI, demonstrated that the tyrosine

Table 2. Fluorescence quenching by acrylamide and iodide

	K_{sv} (M^{-1})		f_a	
eIF-5A	Acrylamide	KI	Acrylamide	KI
Native	2.4 (±0.1)	1.4 (±0.1)	0.4 (±0.1)	0.2 (±0.1)
Variant (Lys50 → Arg)	2.5 (±0.1)	1.3 (±0.1)	0.9 (±0.1)	0.3 (±0.1)

Stern-Volmer constants, K_{sv}, and accessibility fractions, f_a, for tyrosine residues in *native* and *variant* form of eIF-5A. Results are means of four separate experiments performed in triplicate. K_{sv} was measured as described by Eftink and Ghiron (1976); f_a was measured as described by Lehrer (1971).

residues located close to the protein surface are not modified. These results clearly demonstrated that the Lys50 → Arg mutation perturbed the structural organization of the tyrosine(s). Because tyrosines are clustered in the C-terminal part of the molecule, it seemed that temperature perturbs locally that domain. These intermediates were probably responsible for aggregation as a consequence of hydrophobic patches exposed on their surface.

Discussion

Our study on the human eukaryotic initiation factor 5A precursor has revealed various features of its structural organization and behaviour in solution. The protein possessed only three tyrosines and lacks tryptophan as an intrinsic structural probe. The protein had a complex structure, and our data confirm the two independent folding domains that were predicted on theoretical grounds (Joao et al., 1995; Klier et al., 1995).

The different location of tyrosines and cysteines identified in this study demonstrated that the unfolding of the two structural regions was independent. It is feasible that the two perturbation curves obtained with tyrosine and I-AEDANS fluorescence reflected the independent unfolding of different structural regions of the protein. In fact, since two tyrosines were prevalently clustered in the C-terminal region and their transition was not superimposable to that of the I-AEDANS derivative, we can conclude that the extrinsic fluorophore followed the structural fate of a different structural region. Consequently, the less stable folding centre was probably located in the C-terminal region.

Circular dichroism (not shown) was uninformative in this study because it monitored a broad transition (Klier et al., 1995). At 3 M GdnHCl the structure was completely lost. However, broad transition curves (and not superimposable denaturation curves) generally indicate that such overlapping structural phenomena result from the denaturation of multiple domains. Denaturation transitions of the native eIF-5A precursor and its labelled derivative were not reversible under our experimental conditions.

Another unique feature of this protein was the presence of a fluorescence band at neutral pH, attributable to the presence of a tyrosinate. The weak tyrosinate band obtained at neutral pH was visible because this protein contains only tyrosine. In fact, in the proteins that contain tryptophan this peculiar feature of tyrosine fluorescence is masked by the strong tryptophan fluorescence. However, this phenomenon is rare because tyrosine proteins are scarcely represented and because the presence of tyrosinate at neutral pH is generally a rare occurrence. Viewed in the light of the spectral features of the native and denatured protein at neutral pH (i.e., only two tyrosines residues were on average spectrophotometrically titratable at alkaline pH, and the third residue was available only in 6M GdnHCl when the protein can be considered totally unfolded), it is conceivable that one of the eIF-5A precursor tyrosines might form a hydrogen bond with a close acceptor at neutral pH. X-ray or NMR studies will be able to shed light on this intriguing point.

The comparative structural analysis of ec-eIF-5A(Lys) and of its site-directed Lys50 → Arg variant has clarified some aspects of the structural role played by lysine 50. This lysine is important because it is modified into hypusine as a consequence of several post-translational modifications. The two proteins compared in this study had a similar structural organization. This is not surprising because the change of a single surface residue is not expected to alter greatly the general structural properties of a protein (Alber, 1989). Thermal stress revealed local individual differences between the two structures.

The general structural response of the two proteins to temperature was similar. Neither protein unfolded according to a two-state process. In fact, far UV CD did not reveal an appreciable unfolding, while the fluorescence polarization showed only local changes. The C-terminal region seemed to be structurally less thermo-resistant and, thus, unfolded locally, while the N-terminal region appeared to be more stable. However, none of the probes we used yielded direct structural data on thermal unfolding of the N-terminal region. Tyrosine polarization indicated the presence of rigidity in the thermally unfolded wild-type protein, while the same fluorophores were more freely rotating in the variant. It is feasible that the single residue change causes lysine to interact differently with the surrounding protein matrix thus altering the behaviour of local tyrosine(s). The different structural intermediates detected during thermal unfolding of the two proteins reflected structural differences. These structural differences were indirect evidence that the unfolding of the native protein and its variant followed different pathways.

In conclusion, the stability and structural properties of the variant protein were similar to those of ec-eIF-5A(lys), but the two proteins had a different unfolding transition and thus a different conformation. This means that the polypeptide chain had several energy minima, probably of similar magnitudes, on the protein energy surface. These minima were separated by energy barriers. Therefore, our data suggest that we are dealing with a kinetically controlled process rather than a thermodynamically controlled

reaction. Lastly, we suggest that the structural organization of the eIF-5A precursor protein governs its function possibly by regulating the interaction with other macromolecular components.

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References

Abbruzzese A (1988) Developmental pattern for deoxyhypusine hydroxylase in rat brain. J Neurochem 50: 695–699

Abbruzzese A, Park MH, Folk JE (1985) Deoxyhypusine hydroxylase: distribution and partial purification from rat testis. Fed Proc 44: 1487

Abbruzzese A, Park MH, Folk JE (1986) Deoxhypusine hydroxylase from rat testis. Partial purification and characterization. J Biol Chem 261: 3085–3089

Abbruzzese A, Isernia T, Liguori V, Beninati S (1988a) Polyamine-dependent post-translational modification of protein and cell proliferation. In: Perin A et al (eds) Perspective in polyamine research. Wichtig ED, Milan, pp 79–84

Abbruzzese A, Liguori V, Park MH (1988b) Deoxyhypusine hydroxylase. Adv Exp Med Biol 250: 459–466

Abbruzzese A, Park MH, Beninati S, Folk JE (1989) Inhibition of deoxyhypusine hydroxylase by polyamines and by a deoxyhypusine peptide. Biochem Biophys Acta 997: 248–255

Abbruzzese A, Hanauske-Abel HM, Park MH, Henke S, Folk JE (1991) The active site of deoxyhypusyl hydroxylase: use of catecholpeptides and their component chelator and peptide moieties as molecular probes. Biochem Biophys Acta 1077: 159–166

Alber TJ (1989) Mutational effects on protein stability. Annu Rev Biochem 58: 765–798

Argos P, Siezen BJ (1983) Structural homology of lens crystallins. A method to detect protein structural homology from primary sequences. Eur J Biochem 131: 143–148

Beninati S, Abbruzzese A, Folk JE (1990) High-performance liquid chromatographic method for determination of hypusine and deoxyhypusine. Anal Biochem 184: 16–20

Beninati S, Nicolini L, Jakus J, Passeggio, Abbruzzese A (1995) Identification of a substrate site for transglutaminases on the human protein synthesis initiation factor 5A. Biochem J 305: 725–728

Caraglia M, Passeggio A, Beninati S, Leardi A, Nicolini L, Improta S, Pinto A, Bianco AR, Tagliaferri P, Abbruzzese A (1997) Interferon $\alpha 2$ recombinant and epidermal growth factor modulate proliferation and hypusine synthesis in human epidermoid cancer KB cells. Biochem J 324: 737–741

Dill KA, Shortle D (1991) Denaturated states of proteins. Annu Rev Biochem 60: 795–825

Donovan JW (1969) In: Leach SJ (ed) Physical principles and techniques of protein chemistry. Part A. Academic Press, New York, pp 101–170

Donovan JW (1973) Spectrophotometric titration of the functional groups of proteins. Methods Enzymol 27: 525–548

Eftink MR, Ghiron CA (1976) Exposure of tryptophanyl residues in proteins. Biochemistry 15: 672–680

Fontana A (1988) Structure and stability of thermophilic enzymes. Studies on thermolysin. Biophys Chem 29: 181–193

Fritz HJ, Hohlmaier J, Kramer W, Ohmayer A, Wippler J (1988) Oligonucleotide-directed constructions of mutations: a gapped duplex DNA procedure without enzymatic reactions in vitro. Nucleic Acid Res 16: 6987–6999

Gordon ED, Mora R, Meredith SC, Lee C, Lindquist SL (1987) Eukaryotic initiation factor 4D, the hypusine containing protein, is conserved among eukaryotes. J Biol Chem 262: 16585–16589

Hershey JWB (1991) Translational control in mammalian cells. Annu Rev Biochem 60: 717–755

Hershey JWB, Smit-McBride Z, Schnier J (1990) The role of mammalian initiation factor eIF-4D and its hypusine modification in translation. Biochim Biophys Acta 1050: 160–162

Jakus J, Wolff EC, Park MH, Folk JE (1993) Features of the spermidine-binding site of deoxyhypusine synthase as derived from inhibition studies. J Biol Chem 268: 13151–13159

Joao HC, Csonga R, Klier H, Koettnitz K, Auer M, Eder J (1995) The polypeptide chain of eukaryotic initiation factor 5A occurs in two distinct conformations in the absence of the hypusine modification. Biochemistry 34: 14703–14711

Kang C, Wells B, Cantor CR (1979) A fluorescence derivative of ribosomal protein S18 which permits direct observation of messenger RNA binding. J Biol Chem 254: 6667–6672

Klier H, Csonga R, Joao HC, Eckerskorn C, Auer M, Lottspeich F, Eder J (1995) Isolation and structural characterization of different isoforms of the hypusine-containing protein eIF5A from HeLa cells. Biochemistry 34: 14693–14702

Lakowicz JR (1983) Principles of fluorescence spectroscopy. Plenum Press, New York London

Lehrer SS (1971) Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence of model-compounds and of lysozyme by iodide ion. Biochemistry 10: 3254

Menendez-Arias L, Gomez-Gutierrez J, Garcia-Fernandez M, Garcia-Tejedor A, Moran F (1988) A basic microcomputer program to calculate the secondary structure of proteins from their circular dichroism spectrum. Comput Appli Biosci 4: 479–482

Mrabet NT, Van der Broeck A, Van den Brande I, Stanssens P, Laroche Y, Lambeir A, Matthijssens G, Jenkins J, Chiadmi M, van Tilbeurgh H, Rey F, Janin J, Quax WJ, Lasters I, De Maeyer M, Wodak SJ (1992) Arginine residues as stabilizing elements in proteins. Biochemistry 31: 2239–2253

Park MH, Cooper HL, Folk JE (1982) The biosynthesis of protein-bound hypusine (N^{ε}-(4-amino-2-hydroxybutyl)lysine): lysine as the amino acid precursor and the intermediate role of deoxhypusine (N^{ε}-(4-amino-butyl)lysine). J Biol Chem 257: 7219–7222

Park MH, Chung SI, Cooper HL, Folk JE (1984) The mammalian hypusine-containing protein, eukaryotic initiation factor 4D. J Biol Chem 259: 4563–4565

Park MH, Wolff EC, Smith-McBride Z, Hershey JWB, Folk JE (1991) Comparison of the activities of variant forms of eIF-4D: the requirement for hypusine or deoxyhypusine. J Biol Chem 266: 7988–7994

Park MH, Wolff EC, Folk JE (1993) Is hypusine essential for eukaryotic cell proliferation? Trends Biochem Sci 18: 475–479

Park MH, Wolff EC, Lee YB, Folk JE (1994) Antiproliferative effects of inhibitors of deoxyhypusine synthase. J Biol Chem 269: 27827–27832

Ragone R, Colonna G, Balestrieri C, Servillo L, Irace G (1984) Determination of tyrosine exposure in proteins by second-derivative spectroscopy. Biochemistry 23: 1871–1875

Ragone R, Colonna G, Bismuto E, Irace G (1987) Unfolding pathway of myoglobin: effect of denaturants in solvent accessibility to tyrosyl residues detected by second-derivative spectroscopy. Biochemistry 26: 2130–2134

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Arbor, NY

Schnier J, Schwelbeerger HG, Smit-McBride Z, Kang HA, Hershey JWB (1991) Translation initiation factor 5A and its hypusine modification are essential for cell viability in the yeast Saccharomyces cerevisiae. Mol Cell Biol 11: 3105–3114
Sears DW, Beychock S (1973) In: Leach SJ (ed) Physical principles and techniques of protein chemistry. Part C. Academic Press, New York London, pp 445–593
Shortle D, Chan HS, Dill KA (1992) Modeling the effects of mutations on the denaturated states of protein. Protein Sci 1: 210–215
Smit-McBride Z, Dever TE, Hershey JWB, Merrick WC (1989) Sequence determination and cDNA cloning of eukaryotic initiation factor 4D, the hypusine-containing protein. J Biol Chem 264: 1578–1583
Willis KJ, Szabo AG (1991) Fluorescence decay kinetics of tyrosinate and tyrosine hydrogen-bonded complexes. J Phys Chem 95: 1585–1589

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