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Circular dichroism and fluorescence studies on five mutant forms of protein synthesis initiation factor eIF-4E, from the yeast *Saccharomyces cerevisiae*

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CD studies have shown that five tryptophan to phenylalanine (W→F) mutants of eukaryotic initiation factor-4E (eIF-4E) contain low amounts of α-helix, the main elements of secondary structure being β-sheets/turns and aperiodic regions. Interactions with the cap analog m^7GpppG are accompanied by changes in overall secondary structure which include reductions, and in one case an increase in α-helix content, as well as increases in total β-structure (3 mutant forms) and decreases in total β-structure (2 mutant forms). These changes may also involve more significant perturbations of localized regions containing phenylalanine residues either involved in nucleotide binding, or close to the nucleotide-binding site. Measurements of intrinsic Trp fluorescence have shown different quantum yields and reduced m^7GpppG-induced quenching (with one exception). Acrylamide quenching studies yielded similar parameters for 4 of the mutants but 1 form displayed significantly reduced values. Melting experiments showed that the Trp fluorescence of 4 of the mutants decreased as the temperature was increased, this effect being reduced in 3 cases in the presence of m^7GpppG. W 58 F showed an increase in fluorescence as the temperature was raised and this effect was accentuated in the presence of nucleotide. A preliminary attempt has been made to correlate the spectroscopic data with the known biological importance of the individual Trp residues.

Tryptophan; CD; Fluorescence spectroscopy; Eukaryotic initiation factor-4E; (*S. cerevisiae*)

1. INTRODUCTION

A striking structural feature of all cellular eukaryotic mRNAs (except organelle mRNAs) analyzed to date is the 5′-terminal m^7GpppN (where N is any nucleotide) which was termed cap structure or 'cap' (reviews [1,2]). The cap structure has been shown in numerous studies to facilitate formation of a stable complex between 40 S ribosomal subunits and mRNA during translation initiation (recent reviews [3,4]).

Several studies were conducted in order to elucidate the function of the cap structure in the different processes in which it is involved. One approach was to identify and purify proteins that specifically interact with the mRNA 5′ cap structure. Using this approach, a 24 kDa polypeptide was initially identified in a high-salt wash fraction from rabbit reticulocyte ribosomes [5] and subsequently isolated by affinity chromatography [6]. This polypeptide was termed 24K-CBP, CBPI or eIF (eukaryotic initiation factor)-4E.

Recently, several laboratories have reported the

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Abbreviations: eIF-4E, eukaryotic initiation factor-4E; $K_{SV,eff}$, effective Stern-Volmer constant; W *n* F, tryptophan to phenylalanine mutant of eIF-4E at position *n* in the polypeptide chain; m^7GpppG, 7-methylguanosine-5′-triphosphate 5′-guanosine

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cloning and nucleotide sequencing of eIF-4E cDNA of yeast [7], human [8] and mouse [9]. The amino acid sequence as deduced from the cDNA clones predicts the presence of 8 tryptophans which are remarkably evolutionarily conserved in number and position between mammals and yeast [7–9]. This might be of particular significance in light of the report that tryptophans (particularly those flanked by negatively charged amino acids) might be involved in cap recognition [10]. We have overexpressed the yeast eIF-4E in *E. coli* and purified milligram quantities of this protein. In addition, we have overexpressed and purified five mutant eIF-4E proteins in which Trp residues 58, 75, 104, 115 and 130 were individually mutated to phenylalanine. Here, we present a study of the CD and fluorescence properties of the native and mutant proteins aimed at enhancing our understanding of the structure-function relationship of eIF-4E.

2. EXPERIMENTAL

E. coli-expressed yeast eIF-4E and the five W→F mutant forms of the protein were obtained as described by Edery et al. [11]. The crude protein solutions were applied to an m^7GDP-agarose matrix and the wild-type or mutant forms of eIF-4E were purified by affinity chromatography essentially as in [11]. Protein concentrations were determined either by using an extinction coefficient of 1.903 at 280 nm, or by amino acid analysis. CD measurements were made on a Jasco J-500C instrument interfaced with a DP500N data processor, as described [12]. The secondary structure of eIF-4E and the five mutants has been predicted using the program CONTIN which analyses CD spectra as a sum of data collected from 16 proteins whose structures are known from X-ray crystallography [13]. Fluorescence measurements were also made as in [12]. Fluorescence quenching was measured at the emission maxima of the proteins and was initiated by addition of 10-μl aliquots of 8 M acrylamide (Bio-Rad) solution. Protein solutions of 2 ml were used. The F values were corrected for the acrylamide absorption using:

$$F_{corr} = f10^{A/2}$$

where A is the absorbance in a 1 cm cell at 295 nm. Fluorescence quenching data are most often described in terms of the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = \frac{\tau_0}{\tau} \quad (1)$$

where τ_0 and τ respectively denote the fluorescence lifetimes in the absence and presence of a collisional quencher [Q], and F_0 and F represent the fluorescence intensities at the emission maximum in the absence and presence of Q. The collisional quenching constant K_{SV} may be obtained from the initial slope of a plot of F_0/F vs [Q]. In [14] the Stern-Volmer equation is modified as:

$$\frac{F_0}{F_0-F} = \frac{1}{f_a} + \frac{1}{f_a K_q[Q]} \quad (2)$$

where f_a is the fraction of the fluorescence to which the quencher [Q] has access, and where each accessible chromophore has the same Stern-Volmer quenching constant K_q. A plot of $F_0/(F_0-F)$ vs $[Q]^{-1}$ in this special case will be linear with f_a and K_q being obtained by linear regression analysis from the slope and intercept, respectively.

3. RESULTS

3.1. *Far-UV CD*

The far-UV CD spectra of native eIF-4E and the five W → F mutants were measured in the absence and presence of a 5-fold molar excess of m^7GpppG. The results are shown in table 1. The mutant forms have approximately the same amount of α-helix as the wild-type protein (14–19%), with the exception of W 75 F which has a significantly lower amount (9%). Addition of excess m^7GpppG produces a reduction in α-helix which is maximally demonstrated with W 130 F. The exceptional case is W 104 F which actually undergoes a slight increase in α-helix content upon nucleotide addition.

3.2. *Near-UV CD*

The spectra of the five W → F mutants in this

Table 1

The far-UV CD data for five mutant forms of eIF-4E[a]

Protein	α-Helix	β-Sheet	β-Turn	Remainder
W 58 F	0.14	0.52	0.09	0.25
+ m^7GpppG[b]	0.13	0.48	0.08	0.31
W 75 F	0.09	0.64	0.01	0.26
+ m^7GpppG	0.07	0.57	0.22	0.15
W 104 F	0.19	0.41	0.09	0.31
+ m^7GpppG	0.23	0.42	0.04	0.32
W 115 F	0.16	0.47	0.13	0.24
+ m^7GpppG	0.15	0.43	0.19	0.23
W 130 F	0.19	0.43	0.06	0.32
+ m^7GpppG	0.07	0.58	0.16	0.19
W 130 F[c]	0.16	0.45	0.09	0.30
+ m^7GpppG	0.06	0.58	0.18	0.19
eIF-4E	0.14	0.47	0.12	0.27
+ m^7GpppG	0.08	0.57	0.13	0.22

[a] Measurements were carried out in 0.1 M KCl, 20 mM Hepes (pH 7.5), 0.2 mM EDTA

[b] m^7GpppG was added in an approximate mole ratio of 5:1 to the protein

[c] Protein concentration was determined by amino acid analysis

region are of the same general form as that of the wild-type protein, viz. two positive peaks near 280 and 290 nm and a broad positive ellipticity centered near 260 nm. Differences are noted between the individual mutants in the areas 250–270 and 275–295 nm, eg. reduced magnitude of the 280 and 290 nm peaks in the case of W 130 F, and altered spectral shape for W 115 F. Addition of a 5-fold molar excess of m^7GpppG affects the spectra of all the mutants to a greater or lesser degree; a minimal effect is noted with W 104 F. These effects are most easily discerned from a delta spectrum, generated by subtracting the protein spectrum from that of the protein plus nucleotide in each case. These results are shown in fig.1. The minimal effect noted with W 104 F is probably again correlated with the surface nature of Trp 104 (now Phe 104).

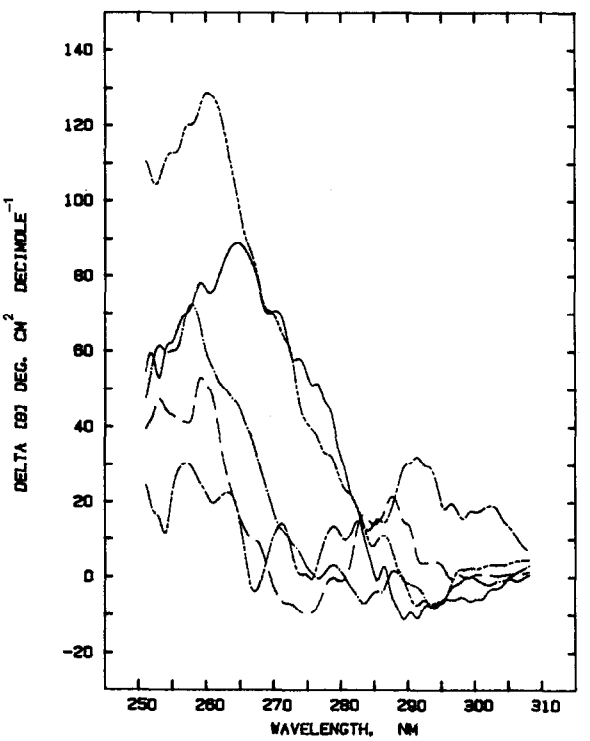

Fig.1. Near-UV CD difference spectra for five W → F mutant forms of eIF-4E in 0.1 M KCl, 20 mM Hepes (pH 7.5), 0.2 mM EDTA. Cell path length, 1 cm; temperature, 25°C. In all cases, spectra were generated by computer subtraction of the protein spectrum from that of the protein plus 5-fold molar excess of m^7GpppG. (——) W 58 F, (- -——) W 75 F, (-——) W 104 F, (● ——) W 115 F, (– – –) W 130 F. Protein concentrations ranged from 0.15 to 0.52 mg/ml.

3.3. *Fluorescence quantum yield*

Upon excitation at 295 nm three of the mutant forms of eIF-4E show emission maxima near 335 nm. The value for W 75 F and W 58 F is found at 332 nm. The quantum yield, in comparison with a solution of the amino acid L-tryptophan of known quantum yield 0.13 [15], has been determined for the five mutant forms. Addition of m^7GpppG to solutions of wild-type or mutant eIF-4E produces extensive quenching of the tryptophan fluorescence. These data are presented in table 2. With the exception of W 58 F and W 75 F which show a 38% reduction and 10% enhancement in quantum yield respectively, the other three mutant forms display reductions in quantum yield up to 14% for W 130 F. The nucleotide-induced quenching of Trp fluorescence is probably a consequence of stacking of key Trp residues with the nucleic acid bases as discussed earlier [12]. The varied extent of quenching may be an indicator of the importance of a particular Trp residue in determining the biological activity of the particular mutant form.

3.4. *Acrylamide quenching of fluorescence*

The results of typical acrylamide quenching experiments for wild-type eIF-4E and the five W → F mutant forms are compiled in table 3. The original Stern-Volmer plots from which the parameter K_{SV} is extracted in all cases display negative deviation from linearity, indicating that the fluorescence of certain tryptophan residues is selectively quenched before others, in the protein (not shown) [16]. The average degree of accessibility of the Trp fluorophores (f_a) is little altered in either the wild-type or

Table 2

Fluorescence parameters for eIF-4E and five W → F forms

Sample	Emission maximum (nm)	Q	Extent of quenching[a] (%) m^7GpppG
eIF-4E	335	0.105	48
W 58 F	332	0.065	38
W 75 F	332	0.115	40
W 104 F	335	0.094	28
W 115 F	335	0.104	48
W 130 F	335	0.090	41

Q, quantum yield of the protein in the absence of nucleotide; extent of quenching, values represent the percentage of nucleotide-induced fluorescence quenching at the end of titration experiments; nucleotide to protein mole ratio ≅5:1

Table 3

Parameters of Trp fluorescence quenching by acrylamide

Protein	Emission maximum (nm)		f_a	$K_q(M^{+1})$	$K_{SV,eff}$
	− quencher	+ quencher			
eIF-4E	335	325	0.86	8.01	6.70
W 58 F	332	324	0.82	5.35	3.17
W 75 F	332	325	0.83	8.12	6.17
W 104 F	335	325	0.87	7.76	6.17
W 115 F	335	332	0.89	7.72	6.60
W 130 F	335	330	0.85	8.24	6.88
W 130 F + m^7GpppG	335	330	0.91	6.60	5.47

All studies were performed at 20°C in 0.1 M KCl, 20 mM Hepes (pH 7.5), 0.2 mM EDTA with an excitation wavelength of 295 nm. (− quencher) In the absence of quencher; (+ quencher) in the presence of the maximum amount of the quenchers used (0.43 M acrylamide)

mutant forms of eIF-4E. With the exception of W 58 F the values of the quenching constants K_q and $K_{SV,eff}$ for the other four mutants do not differ greatly from those noted for the wild-type protein and indicate only minor differences in the rate or effectiveness of quenching. The corresponding values noted for W 58 F are considerably reduced and imply that in this mutant form the rate and effectiveness of acrylamide quenching of the remaining Trp fluorescence is drastically diminished. It is also of interest to note that in the case of W 130 F + m^7GpppG although the apparent degree of accessibility is slightly enhanced (0.91 vs 0.86) the efficiency of quenching is reduced by 20% from the value in the absence of nucleotide.

3.5. *Fluorescence melting profiles*

The effect of temperature on the fluorescence of wild-type and mutant forms of eIF-4E was studied over the range 4–45°C. There is a considerable difference in melting behaviour among the mutants. Basically, for the wild-type protein, W 75 F, W 104 F, W 115 F and W 130 F the loss of fluorescence with increase in temperature appears to be a smooth continuous function with no apparent break points that would suggest dramatic conformational changes occurring in these proteins (not shown). W 58 F, on the other hand, shows the anomalous behavior of an increase in fluorescence as the temperature is increased. In the presence of a 5-fold molar excess of m^7GpppG the fluorescence is quenched to the extent listed in table 2. The remaining fluorescence changes with temperature as follows. With W 75 F the loss in fluorescence is reduced. For W 58 F there is an enhancement of the fluorescence increase effect. In the case of W 104 F the melting profiles are quite similar with or

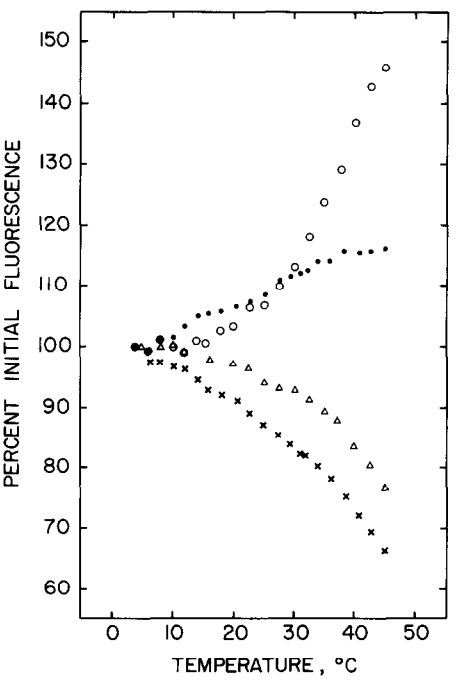

Fig.2. Fluorescence melting profiles for W 58 F (●,○) and W 75 F (×,△) in the absence (●,×) or presence (○,△) of a 5-fold molar excess of m^7GpppG, in 0.1 M KCl, 20 mM Hepes (pH 7.5), 0.2 mM EDTA over the temperature range 4–45°C. Both emission and excitation slits were set at 5 nm bandpass. Excitation was at 295 nm. Protein concentrations were 0.0097 and 0.0077 mg/ml, respectively.

Table 4

Fluorescence melting parameters for eIF-4E and five W → F mutant forms

Sample	Emission maximum (nm) at:		F^4_{45}	F^4_{30}	F^4_{20}	F^4_{10}
	4°C	45°C				
eIF-4E	335	335	0.57	0.74	0.84	0.96
+ m^7GpppG[a]	335	335	0.80	0.84	0.87	0.95
W 58 F	332	335	1.16	1.12	1.07	1.02
+ m^7GpppG	332	335	1.46	1.13	1.03	1.00
W 75 F	329	335	0.66	0.83	0.91	0.97
+ m^7GpppG	329	335	0.77	0.93	0.97	1.00
W 104 F	335	335	0.55	0.77	0.88	0.97
+ m^7GpppG	335	335	0.64	0.83	0.90	0.97
W 115 F	335	335	0.67	0.86	0.94	1.00
+ m^7GpppG	335	335	0.86	0.96	0.96	0.98
W 130 F	335	335	0.74	0.95	0.99	0.99
+ m^7GpppG	335	335	1.14	1.01	0.92	0.96

[a] Nucleotide to protein mole ratio ≅ 5:1

F^4_{45}, F^4_{30}, F^4_{20} and F^4_{10} indicate, respectively, the ratio of the fluorescence noted at four specific temperatures (45, 30, 20 and 10°C) to the initial fluorescence noted at 4°C

without nucleotide. The rate of loss of fluorescence for W 115 F is considerably slowed down (not shown). The fluorescence decrease noted for W 130 F is inverted in the presence of m^7GpppG (not shown). Representative data are shown in fig.2 for W 58 F and W 75 F in the absence and presence of m^7GpppG. Table 4 lists some additional parameters for all five mutant forms. For W 75 F and W 115 F in the presence of m^7GpppG the reduction in loss of fluorescence as the temperature is raised may be attributed to a stabilizing effect as a result of the binding of this nucleotide to these mutants. The inverted behaviour noted for W 130 F in the presence of bound nucleotide may be explained by a mechanism involving release of the nucleotide as the temperature is raised, thereby allowing a dequenching of the Trp fluorescence. W 58 F may be a cold-sensitive mutant and here also, dequenching may explain the elevated enhancement in fluorescence noted in the presence of m^7GpppG as the temperature is raised. For W 104 F there is apparently neither a stabilizing nor dequenching effect of bound nucleotide, hence the similarity noted in melting behaviour.

4. DISCUSSION

The nucleotide-induced changes in far-UV ellipticity were varied, ranging from little change in structure (W 58 F, W 115 F), through a normal loss of α-helix (W 130 F) to an increase in helical content (W 104 F). These responses are further reflections of differences in folding in these mutant proteins, presumably in regions involved in nucleotide binding. These differences are further demonstrated by the varied responses to the presence of nucleotide as monitored by changes in the aromatic CD spectra, probably due to increased optical activity of phenylalanine residues involved in stacking interactions with dinucleotide molecules, although it is not possible from these data to specify precisely which residues are involved.

Very recent work has divided the 8 Trp residues in eIF-4E into 3 groups with respect to mRNA crosslinking activity: (i) tryptophans strongly required for biological activity include residues 43, 46, 104 and 166; (ii) Trp residues that reduce the crosslinking ability include nos 58, 115 and 130. W 75 F is not required for cap recognition [17]. Is it possible to correlate these observations with the spectroscopic data? The answer seems to be that partial correlations can be made. In the first category, there is only one mutant form available for study, viz. W 104 F. In [14] it was suggested that Trp 104 lay on the surface of the protein and would most likely be the key residue in the cap-binding site located in an α-helix-turn-α-helix structural motif. The far-UV CD response to the presence of m^7GpppG was unique in that an increase in α-helix was noted. The degree of Trp

quenching induced by m^7GpppG (28 vs 48% for wild-type eIF-4E) fits well with the data from the crosslinking experiments reported in [17] where it was found that crosslinking of W 104 F to the mRNA cap structure is reduced more than 90% vs the wild type. The fluorescence melting profile for this form is also unique in that it is unaffected by the binding of nucleotide.

Modifications to residues in category (ii) produce a variety of results which include the very low quantum yield, reduced acrylamide quenching constants and anomalous fluorescence melting profiles noted for W 58 F, as well as the large reduction in α-helix noted for W 130 F. Two residues in this category, Trp 115 and 130, are located in the putative turn region (Trp 115) and the adjoining α-helix (Trp 130) and may tend to stabilize or add specificity to the interactions with nucleotides.

The mutant form involving the non-essential Trp, W 75 F, shows a low amount of α-helix, and maximum generation of a new ellipticity band near 260 nm in the presence of m^7GpppG as well as an elevated value for Trp quantum yield.

The CD and fluorescence results described here are important because they demonstrate quite clearly that although the replacements must certainly be considered conservative the resulting mutant forms show subtle alterations in folding as reflected by the differences noted in these spectroscopic properties in the absence or presence of nucleotide. These differences may underlie their different biological activities [17].

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