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The fMet-tRNA binding domain of translational initiation factor IF2: role and environment of its two Cys residues

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Abstract Mutations of the cysteines (positions 668 and 714) were generated in the IF2 C domain of *Bacillus stearothermophilus* translation initiation factor IF2. The corresponding proteins were characterized functionally and structurally. Most (yet not all) amino acid replacements at both positions resulted in severe reduction of the fMet-tRNA binding activity of IF2 C without grossly altering its structure. Our work demonstrates that: (a) both Cys residues are buried within an hydrophobic core and not accessible to protonation or chemical substitution, (b) neither Cys is functionally essential and (c) both Cys residues are located near the active site, probably without participating directly in fMet-tRNA binding.

Key words: Protein synthesis; fMet-tRNA binding; Protein domain; Site-directed mutagenesis; Protein unfolding; Raman spectroscopy

1. Introduction

Initiation factor IF2 is involved in the initiation step of eubacterial translation, its main recognized function being the correct positioning of initiator fMet-tRNA^{fMet} in the ribosomal P site [1,2]. Additional roles of IF2 in other cellular processes such as transcription [3] and protein secretion [4] have been proposed. To perform its function in translation, IF2 interacts with fMet-tRNA^{fMet} through its C-terminal (24.5 kDa) domain (IF2 C) and with GTP/GDP and 50S ribosomal subunits through its central (40 kDa) G-domain [5,6]. Interaction with 30S is also essential, yet the localization of the active site responsible for this function remains elusive. Both G- and C-domains were obtained as recombinant proteins by genetic manipulation of *B. stearothermophilus infB* and expression in *Escherichia coli* [6]. Recently, our efforts have been concentrated on the attempt to elucidate the structure of the fMet-tRNA^{fMet} binding domain of IF2 (IF2 C) and the molecular nature of its interaction with the initiator tRNA [7,8]; insofar as this interaction is eubacteria-specific and vitally important for cell survival, it could also represent an ideal target for the rational design of a new class of antibiotics which are in high demand.

After obtaining some hints from chemical modification experiments that at least one of the two Cys residues of IF2 C might be involved in the interaction with fMet-tRNA, in this study we have investigated a possible functional role and the environment of the two Cys residues of IF2 C, also to verify the possibility of inserting a fluorescent reporter at a specific site of the molecule which could help the further characterization of its interactions.

We present data on the conformation, stability, unfolding behavior and initiator tRNA binding ability of these IF2 C variants and demonstrate that both Cys residues are buried within the structure of the native molecule in the proximity of the active site responsible for the IF2-fMet-tRNA interaction.

2. Materials and methods

2.1. Chemicals

Ultrapure guanidine hydrochloride (Gdn/HCl) and 1,4 dithiothreitol (DTT) were from ICN Biomedicals (Cleveland, OH, USA). Sodium cacodylate, iodoacetamide, 5,5′-dithionitrobenzoic acid (DTNB) and *N*-iodoacetylaminoethyl-8-naphthylamine-1-sulfonic acid (1,8-IAEDANS) were from Serva (Heidelberg, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Buffers

Buffer I: 2 mM sodium cacodylate, pH 7.0, 0.5 M NaCl, 5 mM $MgCl_2$. Buffer II: 100 mM Tris-HCl, pH 8.0, 160 mM NH_4Cl, 6 mM magnesiumacetate and 6 mM 2-mercaptoethanol.

2.3. Cloning, expression and purification methods

The *B. stearothermophilus* IF2 C domain was prepared as previously described [6,7]. Site-directed mutagenesis of Cys-668 and Cys-714 were performed by oligonucleotide-directed mutagenesis using the 'altered sites in vitro mutagenesis' protocol (Promega) and the mutations confirmed by DNA sequencing.

The overexpression and purification of the IF2 C variants were carried out essentially as described (Spurio et al., submitted for publication).

2.4. Sample preparation

Prior to spectroscopic measurements and chemical modification experiments, the protein samples were dialyzed exhaustively against Buffer I and subjected to gel filtration on a Superose 12 column (Pharmacia, Sweden) equilibrated against the same buffer. For Raman measurements the protein solutions were concentrated in Microcon tubes (Amicon, Germany) with 10 kDa cutoff. Protein concentrations were determined spectrophotometrically at 276 nm using the absorption coefficient of 0.424 ml mg^{-1} cm^{-1} calculated from the amino acid composition as described previously [9].

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Abbreviations: CD, circular dichroism; $[\Theta]_{220}$, molar mean residue ellipticity at 220 nm; ΔG_{NI} and ΔG_{IU}, free energy of unfolding of native (N→I) and intermediate (I→U) states, respectively

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2.5. Raman spectroscopy

Raman spectra were measured with a T64000 (Jobin Yvon, France) spectrometer equipped with an Innova 90-5 argon laser (Coherent, USA) for excitation and with a charge coupled device for detection. Samples (12 μl) in quartz cuvettes, positioned in the macro chamber with a 90° setup, were excited at 488 nm with an intensity of about 100 mW; 16 spectral scans of 20 s and 32 of 30 s were averaged for the 480–1760 cm^{-1} and for the 2275–2815 cm^{-1} regions, respectively. After subtracting buffer and background contributions all spectra were normalized to the intensity of the phenylalanine vibration at 1003 cm^{-1}. Since a precipitate formed during dialysis of the IF2 C samples from pH 7.0 to pH 4.0, the Raman spectra were measured on the supernatants obtained after centrifugation.

2.6. Equilibrium unfolding and refolding studies

Denaturant-dependent conformational transitions were monitored measuring (at 25°C), with a Jasco J-720 spectropolarimeter interfaced to a Neslab water bath [10], ellipticity changes at 220 nm. Separate samples were prepared, each containing 0.080 mg ml^{-1} protein and the indicated concentration of the denaturing agent, and placed in a 0.10 cm cuvette.

In the refolding experiments the proteins were first unfolded by at least 6 h incubation at room temperature in 7 M Gdn/HCl and then refolded by addition of renaturation buffer followed by overnight incubation at room temperature. Concentrations of Gdn/HCl stock solution in the buffers were determined from refractive index measurements [11].

2.7. Data analysis

The fraction of unfolded protein f_u was calculated as described by Pace et al. [11]. Assuming two separate 'two-state' transitions of unfolding, the free energy changes ΔG_{NI} between native and intermediate state ($N \rightarrow I$) and ΔG_{IU} between intermediate and unfolded state ($I \rightarrow U$) were calculated as described [7].

2.8. IF2 C activity test

The interaction of IF2 C (wild-type (wt) or variants) with fMet-tRNA was measured as the protection of the latter from spontaneous hydrolysis. Reaction mixtures (50 μl) containing 22 μM f[^{35}S]Met-tRNA and increasing amounts of proteins in Buffer II were incubated at 37°C and samples (20 μl), withdrawn after 0 and 60 min, were spotted on Whatman 3MM paper discs to determine the cold trichloroacetic acid insoluble radioactivity.

3. Results and discussion

3.1. Functional implication of the Cys residues

The 24.5 kDa fMet-tRNA binding domain (IF2 C) of *B. stearothermophilus* IF2 contains two Cys residues at positions 668 and 714. Preliminary experiments with SH reagents in the presence of moderate concentrations of urea had indicated the possibility of modifying at least one of these residues. Since at least partial inactivation of IF2 C ensued, these experiments further suggested that one or both Cys residues of IF2 C might be located in the fMet-tRNA binding site of this domain.

To verify this hypothesis, identify the Cys residue functionally implicated and possibly introduce a single fluorescent tag at a specific site of the molecule without disturbing its function, we proceeded to mutagenize the two Cys residues by site-directed mutagenesis. Thus, both Cys-668 and -714 were individually substituted with Val, Ser, Tyr, Arg and Asp. Additional substitutions introduced at position 714 were Lys and Glu. After overexpression and purification, the resulting variant proteins were tested for their residual capacity to interact with fMet-tRNA. As seen from Fig. 1, the majority of the replacements at both positions resulted in severe reduction of the fMet-tRNA binding activity. In line with the more extensive conservation of Cys-714 (i.e. in 28 of 31 known primary structures of IF2) compared to Cys-668 (i.e. only in 17 of 31 cases) [NCBI-GenBank] the inactivations were, in general, slightly more severe for the replacements of Cys-714 than for Cys-668 suggesting that the first residue plays a more crucial structural and/or functional role. In spite of the inactivation caused by most of the Cys substitutions, we can conclude from these experiments that a Cys residue is not by itself

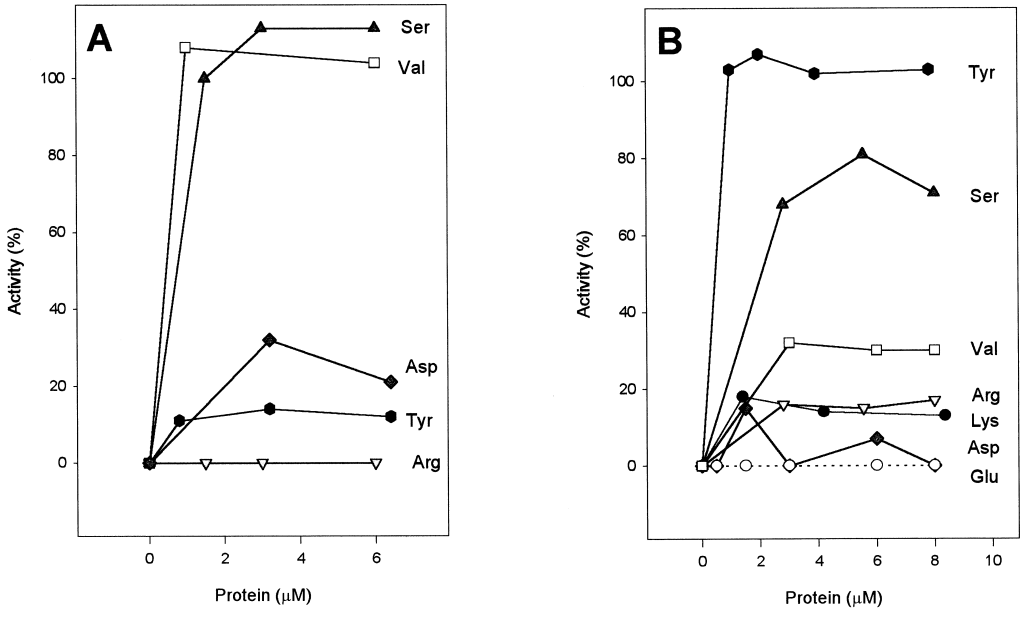

Fig. 1. Effect of the Cys→Val substitutions on the interaction of IF2 C with initiator tRNA. The interaction of the IF2 C molecules bearing the indicated mutations in Cys-668 (A) and Cys-714 (B) with f[^{35}S]Met-tRNA was measured from the protection of the latter from spontaneous hydrolysis as described in Section 2. The activity measured as a function of protein concentration (abscissa) is expressed as percent of the activity displayed, at the same concentrations, by wt IF2 C (ordinate),

required at either position since full preservation of the functional activity of IF2 C was observed after substitution of Cys-668 with Ser or Val and of Cys-714 with Tyr; furthermore, only partial loss of affinity for fMet-tRNA was observed after substitution of Tyr-714 with Ser. Finally, residual activity well above the background was found in the Asp-668 and Val-714 mutants.

3.2. Characterization of the environment of the Cys residues

To characterize the environment of the Cys residues and to investigate possible structural alterations induced by their substitutions, we selected the two mutant proteins with Cys → Val substitutions at either position. After showing by CD spectroscopy that these two mutants have the same type of fold and secondary structure content as the wt and had not undergone any structural change detectable by this method, we characterized these mutant domains by Raman spectroscopy comparing them to wt IF2 C.

The Raman spectrum of IF2 C in the 480 to 1760 cm^{-1} region (Fig. 2A, tracing 1) is almost identical to those of the two mutants yielding essentially flat difference spectra with IF2 C Cys668Val (tracing 2) and with IF2 C Cys714Val (tracing 3). The lack of differences in this region of the spectrum, which includes the amide bands and the bands characteristic of amino acid side chains, indicates that the three proteins have essentially the same conformations; the identical intensity ratio of the tyrosine doublet ($I_{854}/I_{828} = 1.82$) further indicates a similar surface localization of the seven Tyr residues and a similar environment of their OH groups [12]. Raman bands in the spectral region between 2500 and 2620 cm^{-1}, on the other hand, are exclusively due to stretching vibrations of the SH groups whose frequency is diagnostic of the strength of the hydrogen bonds of the SH donors; thus, bands at 2525–2560 cm^{-1} correlate with strong hydrogen bonds, bands at 2560–2575 cm^{-1} with hydrogen bonds of moderate strength while bands of weakly hydrogen-bonded SH groups are found at 2575–2580 cm^{-1} [13]. As seen from Fig. 2B, the spectra of wt IF2 C at pH 10.0 (tracing 3), 7.0 (tracing 4), and 4.0 (tracing 5) display two bands at 2559 cm^{-1} and at 2571 cm^{-1} having different intensity. This indicates that the two Cys residues of this molecule have different vibrational properties and therefore different environments. In contrast to wt IF2 C, a single Cys band is visible in the spectrum of IF2 C Cys668Val at 2571 cm^{-1} (tracing 1) and of IF2 C Cys714Val at 2559 cm^{-1} (tracing 2). Thus, we conclude that the less intense, lower frequency band at 2559 cm^{-1} belongs to Cys-668 which is probably involved in a medium-strong hydrogen bond, while the more intense higher frequency band at 2571 cm^{-1} belongs to Cys-714 which forms a weaker hydrogen bond. The position of the SH band of Cys-668 in the IF2 C Cys714Val mutant is similar to that found in IF2 C, whereas in IF2 C Cys668Val the Cys-714 band is slightly shifted (5 cm^{-1}), suggesting a marginal weakening of its H-bond in this mutant.

Finally, since, as seen above, the intensities and the positions of both Cys bands of IF2 C are almost pH-independent, at least between pH 4.0 and 10, we conclude that both Cys residues are buried within a hydrophobic region of the protein and are inaccessible to protonation [14].

3.3. Reactivity of the Cys residues of IF2 C

In agreement with the above finding that in native IF2 C

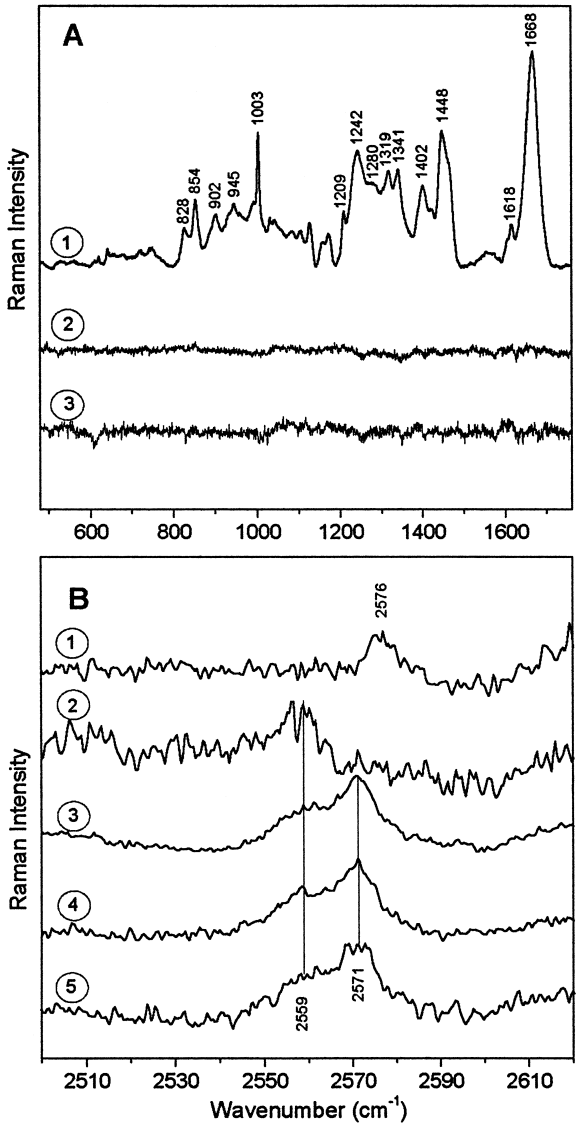

Fig. 2. Raman spectra of IF2 C, IF2 C Cys668Val and IF2 C Cys714Val. A: Wavenumber region 480–1760 cm^{-1}; 1: Spectrum of IF2 C in Buffer I. Difference spectra: (2) IF2 C minus IF2 C Cys668Val, (3) IF2 C minus IF2 C Cys714Val. B: Raman spectra in the region 2500–2620 cm^{-1} of variants (1) IF2 C Cys668Val, (2) IF2 C Cys714Val and of IF2 C at (3) pH 10.0, (4) pH 7.0 and (5) pH 4.0. IF2 C concentrations were 23 mg ml^{1} at pH 7.0; 27 mg ml^{-1} at pH 10.0; 6 mg ml^{-1} at pH 4.0; IF2 C Cys668Val and IF2 C Cys714Val at pH 7.0 were 17 mg ml^{-1} and 7 mg ml^{-1}, respectively.

neither Cys residue can be titrated in the pH interval from 4 to 10 (Fig. 2B), neither SH-group was found to be accessible to DTNB under non-denaturing conditions. Both thiol groups become reactive, however, in the presence of 2.5 M Gdn/HCl with approximately 85% and 60% of the reaction occurring almost instantaneously and reaching saturation (one thiol group substituted per protein molecule) within about 2 min and 10 min for Cys-668 and Cys-714, respectively. Results similar to those obtained with DTNB were also obtained with iodoacetamide. Furthermore, since the covalent introduction of a fluorescent tag at specific sites of the molecule could be useful to study the interaction of IF2 with fMet-tRNA and/or with other components of the translational ap-

paratus, an attempt was made to label the two IF2 C mutants with *N*-iodoaminoethyl-8-naphthylamine-1-sulfonic acid (1,8-IAEDANS) upon exposure of the SH-groups by unfolding these proteins with Gdn/HCl. Both Cys-668 and Cys-714 were found to react completely (i.e. molar ratio ≅ 1 in 6 M Gdn/HCl) but, upon renaturing the resulting labeled proteins, the CD spectra and the unfolding behavior of these molecules revealed that the protein structure cannot accept a bulky hydrophobic marker without undergoing a noticeable conformational change. These data explain the functional inactivation obtained in our preliminary experiment (mentioned above) and indicate that the investigation of the interactions of IF2 with its natural ligands must be pursued using other spectroscopic approaches.

3.4. Guanidine hydrochloride-induced unfolding and structural consequences of Cys mutagenesis

As mentioned above, neither CD nor Raman spectroscopy indicated the occurrence of relevant structural changes of the IF2 C-domain upon substitution of its two Cys residues with valine. Since unfolding studies are a sensitive tool for the identification of minute conformational changes in proteins, we used this approach to determine the stability of the same two IF2 C variants. For this purpose, the Gdn/HCl-induced unfolding of these molecules was followed monitoring the variation of ellipticity at 220 nm as a function of increasing Gdn/HCl concentrations in comparison to that of wt IF2 C. The normalized unfolding curves show a similar two-step unfolding for all protein samples (Fig. 3), the only significant difference being the slightly lower half transition concentration of denaturing agent found for IF2 C Cys714Val (3.7 M) compared to both IF2 C and IF2 C Cys668Val (4.1 M). The free energies of unfolding ($\Delta G_{NI}^{H_2O}$ and $\Delta G_{IU}^{H_2O}$), calculated from the unfolding curves assuming two 'two-state' transitions, are summarized in Table 1. In conclusion, these unfolding studies revealed a basically similar two-step unfolding behavior for both IF2 C variants and demonstrate that, even though the Cys714Val substitution has minor effects on its conformational stability, which is probably reflected in the reduced activity of this mutant (Fig. 1B), overall the structure of IF2 C is flexible enough to tolerate the substitution of both its Cys residues by Val.

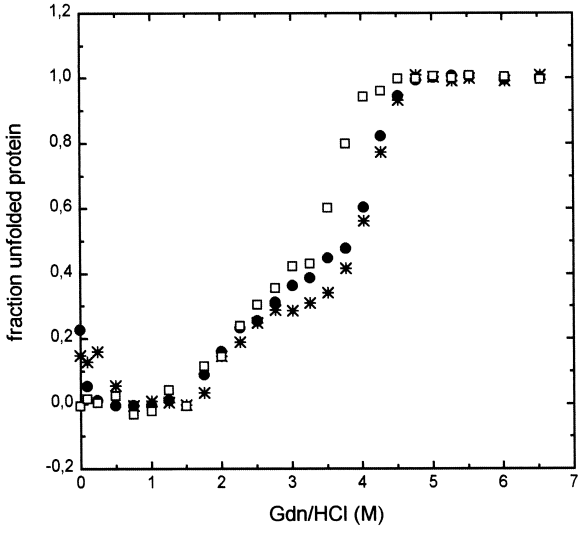

Fig. 3. Effect of the Cys→Val substitutions on the Gdn/HCl-induced unfolding of IF2 C. Normalized Gdn/HCl-induced unfolding curves of IF2 C (*) IF2 C Cys668Val (●) and IF2 C Cys714Val (□) monitored measuring changes of the ellipticity at 220 nm at 25°C at 0.080 mg ml^{-1} protein concentration in Buffer I.

Table 1
Gdn/HCl-induced unfolding of IF2 C Cys→Val variants[a]

Protein	$\Delta G_{NI}^{H_2O}$	m_{NI}	$c_{m,NI}$	$\Delta G_{IU}^{H_2O}$	m_{IU}	$c_{m,IU}$
IF2 C[b]	6.2	3.0	2.05	12.9	3.1	4.14
IF2 C Cys668Val	2.8	1.2	2.2	15.4	3.7	4.2
IF2 C Cys714Val	3.4	1.6	2.1	12.1	3.3	3.7
IF2 C cam[c]	4.8	2.3	2.1	12.3	3.0	4.1

Units are as follows: ΔG^{H_2O}, kcal mol^{-1}; m, kcal mol^{-1} M^{-1}; c_m, M; m_{NI} and m_{IU}, cooperativity parameter of unfolding transition; c_m, Gdn/HCl transition midpoint concentration.
[a]in Buffer I
[b]Values are taken from [7].
[c]IF2 C cam, *S*-carboxyaminomethylated IF2 C.

3.5. Conclusions

Unlike the interaction between elongation factor EF-Tu and aminoacyl-tRNAs, which requires the participation of all three domains (I, II and III) of the factor [15,16], the active site of IF2 interacting with the initiator tRNA is entirely confined within its C-terminal domain (IF2 C), whose affinity for fMet-tRNA is essentially the same as that of native IF2 [6]. Taking advantage of two IF2 C variants in which the two Cys residues of this domain had been substituted with Val, we have investigated the environment of each Cys, demonstrating that both of them are buried within the structure, involved in hydrogen bonding of medium-high (Cys-668) and of medium-low (Cys-714) strength and are therefore, at least in the native state of the protein, not amenable to react with SH reagents. Accordingly, we have demonstrated that placing a fluorescent reporter on either Cys is not possible without irreversibly denaturing the protein. On the other hand, the finding that Cys-668 and Cys-714 react only upon partial denaturation of the protein structure opens an interesting possibility for pursuing further the investigation on the relationship between structure and function of IF2 C as well as on the topographical localization of IF2 on the ribosome. In fact, one can envisage that the introduction of additional, exposed Cys residues in other positions of the molecule may result in proteins which would accept a fluorescent label or an Fe(II)-EDTA tether only at these new positions without disturbing the two naturally occurring Cys residues. As to the structural and functional role of Cys-668 and Cys-714, our results demonstrate that substitution of these residues does not result in any but small conformational changes detectable by CD and Raman spectroscopy and by unfolding studies. Most but not all the Cys substitutions introduced in IF2 C caused a severe inactivation of the fMet-tRNA binding capacity of the protein. Taken together, these results indicate that neither Cys-668 nor Cys-714 is strictly required for maintaining the structural and functional integrity of the protein. Nevertheless, though probably not directly participating in the interaction with initiator tRNA, these residues are part of a structural element which constitutes the fMet-tRNA binding site of the factor. It is likely that the majority of the amino acid replacements at position 668 and 714 alter somewhat the folding of this structural element and causes an adverse effect on the affinity of IF2 C for the initiator-tRNA.

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