Structural properties of the Ectodomain of Hepatitis C Virus E2 Envelope Protein

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Abstract

We describe the structural and antigenic properties of a soluble form of hepatitis C virus E2 envelope protein ectodomain ending at residue 661 ($E2_{661}$) which is obtained in large quantities in a baculovirus/insect cell system**.** The protein is secreted to the cellular medium by virus-infected cells. $E2_{661}$ is glycosylated and possesses a high tendency to self-associate. In fact, analytical ultracentrifugation and size exclusion chromatography studies show that the purified protein is mainly composed of dimers, trimers and tetramers being the dimer the smallest species present in solution. The secondary structure was determined by deconvolution of the far-UV circular dichroism spectrum yielding 8% α-helix structure, 47% extended structure and 45% non-ordered structure. The near-UV CD spectrum is indicative of a folded structure. The fluorescence emission spectrum indicates that Trp residues occupy a relatively low hydrophobic environment. Finally, $E2_{661}$ binds to a monoclonal conformation specific antibody and to antibodies present in human sera from HCV-positive patients. All these features suggest that the secreted protein possesses a native-like conformation. The use of this independent folding domain may contribute to shed light on the biology of HCV and could also be used as a vaccine in the prevention of HCV infection.

Keywords: Hepatitis C Virus, envelope protein, E2, baculovirus, glycosylation

1. Introduction

Hepatitis C virus (HCV) is an enveloped, positive-stranded RNA virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family (Lindenbach and Rice, 2001). HCV is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (Major et al., 2001). There is no vaccine for HCV, and current antiviral therapy is based on the use of polyethylene glycol-modified interferon in combination with ribavirin. However, this treatment is expensive, relatively toxic, and effective in only half of the treated patients (Feld and Hoofnagle, 2005).

HCV encodes two envelope glycoproteins, E1 and E2. They are classified as type I integral transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic domain anchor. The ectodomains contain 5 or 6 and 11 N-glycosylation sites, respectively. During their synthesis, the ectodomains of HCV envelope glycoproteins are targeted to the endoplasmic reticulum lumen, where they are highly modified by N-linked glycosylation (Goffard and Dubuisson, 2003). *In vitro* expression studies have shown that both glycoproteins associate to form heterodimers, which accumulate in the endoplasmic reticulum, the proposed site for HCV assembly and budding (Op De Beeck et al., 2001).

The E2 glycoprotein extends from amino acid 384 to 746 of the polyprotein from which it derives. The transmembrane domain has been shown to encompass residues 718-746 (Cocquerel et al., 1998) and it has been demonstrated that only polypeptide chains ending before or at residue 661 are properly folded (Michalak et al., 1997). The E2 glycoprotein carries regions of extreme hypervariability (HVR1 and HVR2) (Hijikata et al., 1991; Kato et al., 1992). The most variable region, HVR-1, is located within the N-terminal 27 residues (384-

411) of E2, while HVR-2 resides in the 476-480 fragment. E2 protein has been described to interact with the large extracellular loop of human CD81, its putative cellular receptor (Flint et al., 1999; Owsianka et al., 2001; Pileri et al., 1998). It also elicits production of neutralizing antibodies against the virus, and is involved in viral morphogenesis (Bartosch and Cosset, 2006; Bartosch et al., 2005). The E2 protein is considered as a major candidate for anti-HCV vaccine. Antibodies specific for epitopes within HVR-1 have been reported to inhibit binding of E2 to cells and to block HCV infectivity *in vitro* and *in vivo* (Habersetzer et al., 1998; Shimizu et al., 1996; Zibert et al., 1999). Despite this, relatively little is known about this protein.

Because of the difficulties in propagating HCV in cell culture, many aspects of HCV life cycle remain unclear. A major advance in the investigation of HCV entry was the development of pseudoparticles (HCVpp), consisting of native HCV envelope glycoproteins E1 and E2 assembled into retroviral core particles (Bartosch et al., 2003; Hsu et al., 2003). This system is potentially powerful to identify and characterize molecules that block HCV entry. Furthermore, data obtained with HCVpp can also now be confirmed with the help of the recently developed cell culture system that allows efficient amplification of HCV (Wakita et al., 2005; Zhong et al., 2005).

Previous reports suggest that E1 and E2 interact to form a complex, which has been proposed as a functional subunit of HCV virions (Dubuisson et al., 1994; Ralston et al., 1993). Purified HCV glycoprotein complexes expressed by using vaccinia virus are non-covalently associated. However, in heterologous expression systems, E1 and E2 have a high tendency to form heterogeneous disulfide-linked aggregates, which could represent misfolded complexes (Cocquerel et al., 2003). Knowledge of the three-dimensional structure of HCV envelope protein E2 will

be of great value in the quest for a vaccine, in explaining existing data and in designing novel experiments. Current understanding of HCV envelope proteins is based on mammalian cell culture transient expression assays with viral and non-viral vectors. These systems produce very low levels of heterogeneous protein due to glycosylation and aggregation, and it is difficult to distinguish between molecules that undergo productive and non-productive folding (Flint et al., 2000). In the absence of high levels of native E2, some other complexes, such as E1E2 glycoproteins reconstituted into liposomes (Lambot et al., 2002) or virus-like particles expressed in insect cell systems (Clayton et al., 2002; Wellnitz et al., 2002), have been used to study virus–cell interactions. In this paper, we describe the cloning, expression and purification of the ectodomain of E2 in a baculovirus/insect cell system, as well as the characterization of its structural and antigenic properties. It behaves as an independent folding domain with native-like properties. Several criteria indicate that it is correctly folded and processed. The protein is secreted to the cell supernatant, it is glycosylated and contains carbohydrates bound at least through N-glycosidic bonds. Furthermore, it self-associates into dimeric and higher order soluble forms which are recognized by a conformation specific antibody and by antibodies present in sera from HCV-positive patients. The structural properties of the recombinant protein derived from circular dichroism and fluorescence spectroscopic studies are described.

2. Materials and methods

2.1. Construction of recombinant transfer vector

DNA encoding the ectodomain of E2 protein, residues 384-661 ($E2_{661}$), was inserted into a baculovirus transfer vector pAcGP67A (Pharmingen) with the addition of a six-histidine tag (His tag) immediately 3′ of the multiple cloning site. The cDNA encoding $E2_{661}$ was obtained by RT-PCR from the viral RNA of a strain 1HCV-PT, genotype 1a, using the following primers:

5′- cgc gga tcc c cat cac cat cac cat cac GAA ACC CAC GTC ACC GGG – 3′ (forward)

5′- ggg gaa ttc a CTC GGA CCT GTC CCT GTC – 3′ (reverse)

Two restriction sites, *BamH*I and *EcoR*I, were created at the 5′ and 3′ ends of $E2_{661}$ gene, respectively. The amplified reaction product was subcloned into pCR2.1 plasmid (Invitrogen) digested with *BamH*I and *EcoR*I. The resulting plasmid was digested with these restriction enzymes and, finally, the $E2_{661}$ cDNA was cloned into the pAcGP67A baculovirus transfer vector downstream of the strong *polh* promoter to create pAcGP67A-$E2_{661}$.

2.2. Insect cell culture and transfections

The insect cell line *Spodoptera frugiperda* (*Sf9*) was cultured in Insect X-Press serum-free media (BioWhittaker) at 27 ºC. *Sf9* cells were cotransfected with Baculogold™ DNA (Pharmingen) and the recombinant transfer vector pAcGP67A-$E2_{661}$ as indicated by the manufacturer. Several rounds of culture amplified the recombinant virus, and a high titer virus stock solution was harvested. To express the protein on a larger scale, High Five™ insect cells

(Invitrogen) were grown in Insect X-Press serum-free media prior to infection with high titer virus (>10^8 pfu/ml) at a multiplicity of infection of 5-10.

2.3. Purification of $E2_{661}$

Typically, 500 ml of recombinant baculovirus-infected insect cell cultures were harvested approximately 120 h postinfection and the cells pelleted by centrifugation at 5000 g for 10 min. The supernatant was dialyzed against 50 mM Tris-HCl, pH 8.0, 0.3 M NaCl and loaded onto a Ni^{2+}-Nitrilotriacetic acid agarose (Ni-NTA agarose) column (Qiagen) equilibrated with the same buffer. It was then washed with dialysis buffer containing 10 mM imidazole and later 30 mM imidazole. The recombinant $E2_{661}$ protein was eluted with 200 mM imidazole in dialysis buffer. The presence of $E2_{661}$ was monitored by SDS-PAGE throughout the purification.

2.4. Protein analysis

Protein concentrations were determined spectrophotometrically from the absorbance at 280 nm and the extinction coefficient calculated from the amino acid analysis. The absorption spectra were recorded on a Beckman DU-640 spectrophotometer. The amino acid analysis of hydrolyzed aliquots was performed on a Beckman 6300 automatic analyzer. Automated Edman protein degradation of $E2_{661}$ was performed using an Applied gas-phase sequencer (model 494).

2.5. Protein Deglycosylation

Protein samples were digested with N-glycosidase F (PNGase F, Roche) for 16 h at 37 ºC in 20 mM sodium phosphate, pH 7.0, 50 mM EDTA, and 1%

(p/v) octylglucoside. Ole e 1 protein was used as a control of deglycosylation (van Ree et al., 2000). Digested samples were mixed with 3X Laemmli sample buffer and analyzed by SDS-PAGE. The proteins were stained with Coomassie brilliant blue R 250 and also transferred to nitrocellulose membranes that were subsequently incubated with the lectin concanavalin A conjugated to biotin (Pierce). The glycoproteins were detected using HRP-streptavidin at a 1:1000 dilution.

2.6. Mass spectrometry analysis

The molecular mass of $E2_{661}$ was determined by Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-TOF) mass spectroscopy (MS) using a MALDI-TOF Bruker REFLEX IV (Bruker-Francen Analytic GmbH, Bremen, Germany) equipped with reflectron analyzer, delayed extraction capabilities and AutoXecute software for automated spectra acquisition. The matrix was a saturated solution of sinapinic acid (Sigma).

In order to identify $E2_{661}$, the protein was digested with trypsin. The resulting peptides were analyzed by MALDI-TOF MS and Surface-Enhanced Laser Desorption/Ionization-Time-of-Flight (SELDI-TOF) MS using a SELDI-TOF PBS-II mass spectrometer (Ciphergen ProteinChip System). For protein identification, tryptic peptide masses were transferred to BioTools 2.0 interface (Bruker Daltonics) to search in the NCBInr database using Mascot software (www.matrixscience.com; Matrix Science, London, UK).

2.7. Circular Dichroism

CD measurements were carried out on a Jasco spectropolarimeter, model J-715. All the measurements were carried out at 25 ºC with cells thermostated

with a Neslab RTE-111 water bath. Far-UV CD and near-UV CD spectra were measured at a protein concentration of 0.15 mg/ml and 0.5 mg/ml respectively, using protein dialyzed against 30 mM MOPS, pH 7.0, 0.1 M NaCl. The pathlengths were 1 and 5 mm, respectively. The contribution of the buffer was always subtracted. The spectra were calculated by using 110 as the mean residue molecular mass and the results are expressed in terms of residue molar ellipticity in $deg \cdot cm^2 \cdot dmol^{-1}$. The secondary structure of the protein was evaluated by computer fit of the dichroism spectra according to Convex Constraint Analysis (CCA) (Perczel et al., 1991). This method relies on an algorithm that calculates the contribution of the secondary structure elements that give rise to the original spectral curve without referring to spectra from model systems. The secondary structure was also predicted by the GOR IV method (Garnier et al., 1996).

2.8. Fluorescence spectroscopy

Emission spectra were obtained at 25 ºC using an SLM AMINCO 8000C spectrofluorimeter. Excitation and emission slit widths were set at 4 nm. The protein concentration was 0.05 mg/ml and a 0.4 x 1 cm cuvette was used. Buffer was 30 mM MOPS, pH 7.0, 100 mM NaCl. Excitation was performed at 275 or 295 nm, and the emission spectra were recorded over the range 285-450 nm. The contribution of the buffer was always subtracted. The tyrosine contribution to the emission spectra was calculated by subtracting the emission spectrum measured at λ_{exc} = 295 nm multiplied by a factor from that measured at λ_{exc} = 275 nm. The factor was obtained from the ratio between the fluorescence intensities measured with λ_{exc} = 275 and λ_{exc} = 295 nm at wavelengths above 380 nm, where there is no tyrosine contribution.

2.9. Fluorescence quenching

In order to determine the average accessibility of Trp residues, fluorescence quenching experiments with acrylamide and KI were performed at 25 ºC. Protein fluorescence was quenched by the presence of increasing amounts of acrylamide (0-600 mM) or KI (0-1M). The KI stock solution also contained 0.18 M sodium thiosulfate in order to prevent the formation of I_3^-. The fluorescence measurements were determined as continuous emission spectra between 300 and 450 nm with excitation at 290 nm. The emission spectra were corrected for dilution. The protein concentration was 0.1 mg/ml. Fluorescence intensities at the emission maximum were used for calculations. The fluorescence quenching data were analyzed according to the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}\,[Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence of quenchers, respectively, K_{SV} is the collisional Stern-Volmer constant, and $[Q]$ is the quencher concentration. When the plot was not linear, the fluorescence quenching data were analyzed according to the modified Stern-Volmer equation:

$$\frac{F_0}{(F_0 - F)} = \frac{1}{f_a} + \frac{1}{f_a \cdot K_{SV} \cdot [Q]}$$

where f_a is the fraction of fluorescence accessible to the quenching agent (Lehrer, 1971).

2.10. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli using 15% polyacrylamide gels (Laemmli, 1970). Samples were subjected to gel electrophoresis under either nonreducing or

reducing conditions (with 5% (v/v) β-mercaptoethanol) and the proteins were stained with Coomassie brilliant blue R-250. The molecular mass of the protein bands was estimated by comparison with protein markers of known molecular mass (Prestained SDS-PAGE Standards, Bio-Rad).

2.11. Western blotting

After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham) in 48 mM Tris/HCl, pH 9.0, containing 39 mM glycine, 0.0375% SDS and 20% (v/v) methanol, for 1 h at 1 mA/cm^2, by using a V20-SDB apparatus (Scie-Plas). To detect $E2_{661}$, membranes were incubated with a HRP-conjugated monoclonal anti-His (Sigma) at a 1:3000 dilution. The peroxidase reaction was developed with 3, 3'-diaminobenzidine tetrahydrochloride/H_2O_2. When the membranes were incubated with individual sera from HCV-positive human patients, $E2_{661}$ was detected via Enhanced Chemiluminiscence (ECL). After blotting, the membrane was incubated with the human sera diluted to 1:20, washed and incubated with HRP-conjugated anti-human IgG (Fc) (Sigma) diluted at 1:1000. $E2_{661}$ was detected by incubating the membranes with ECL reagents (Amersham Life Sciences) and exposure to photographic film.

2.12. Analytical ultracentrifugation

The sedimentation velocity experiments were carried out on a Beckman Optima XL-A analytical ultracentrifuge equipped with UV-VIS optics detection system, using an An60Ti rotor and 12 mm double-sector centerpieces. The experiments were performed at 20 ºC with a protein concentration of 5 μM. The buffer employed was 50 mM Tris-HCl, 300 mM NaCl, pH 7.0. The sedimentation

coefficient distributions were calculated by modelling of the sedimentation velocity data using the *c(s)* method (Schuck, 2000), as implemented in the SEDFIT program, from which the corresponding sedimentation coefficients (s-values) were obtained.

2.13. Size Exclusion Chromatography

The purified $E2_{661}$ was chromatographed on a FPLC (Pharmacia) system by using a Superdex G-200 column equilibrated with 50 mM Tris-HCl, 300 mM NaCl, pH 8.0. The separation was monitored at 280 nm. The elution volume of each peak was compared with that of molecular mass markers.

2.14. Enzyme Linked Immunosorbent Assay (ELISA)

96 wells microtitre plates (Costar 3690) were coated overnight at 4 ºC with 100 ng/well of purified recombinant $E2_{661}$ diluted to 1 μg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Unbound antigen was discarded, and the wells were blocked with 3% non-fat dry milk in PBS for 60 min at room temperature. After washing, the wells were incubated at 37ºC for 2 h with human serum. Eight HCV-positive human sera and ten HCV-negative human sera were used at a dilution of 1:200. The plates were washed three times with PBS/0.05% Tween 20 and incubated at 37ºC for 1 h with HRP-conjugated anti-human IgG (Fc) diluted at 1:10.000. Bound antibodies were detected by adding 100 mM sodium citrate, pH 5.0, 4% Methanol buffer containing H_2O_2 and the substrate *o*-phenylenediamine dihydrochloride (Merck). The optical density at 492 nm was measured using an ELISA Expert 96 microplate reader (ASYS Hitech). Sera from infected and control patients were provided by Dr. Fernando Vivanco (Fundación Jiménez Díaz, Madrid, Spain).

2.15. Immunoprecipitation

A 50 µl aliquot of rabbit anti-mouse immunoglobulin G bound to Sepharose beads (Pharmacia-LKB) was incubated with 2 µl of anti-E2 monoclonal antibody H53 or 2 µl of rabbit serum for 1 h at 4 ºC in 10 mM Tris-Cl, pH 7.5, containing 0.2% NP-40, 150 mM NaCl and 2 mM EDTA (TBS-NP-40). The MAb H53 is conformation-dependent and was a generous gift of Dr. Jean Dubuisson. Beads were then incubated with 2 µg of purified $E2_{661}$ for1 h at 4ºC. Between each step, the beads were washed twice with TBS–NP-40. After the last step, they were washed three times with this buffer and once with distilled water. The precipitates were then boiled for 5 min in SDS-PAGE sample buffer and analyzed on a 12% polyacrylamide gel. After electrophoresis and transfer to nitrocellulose membranes, protein $E2_{661}$ was detected by incubating the membranes with a polyclonal goat anti-E2 antibody (USBiological) at a 1:500 dilution followed with a rabbit anti-goat antibody conjugated to HRP diluted at 1:3000. The peroxidase reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride/H_2O_2.

3. Results and Discussion

Previous attempts have been made to obtain either full length E2 or E2 ectodomain. Expression of $E2_{661}$ in *Escherichia coli* led to the production of inclusion bodies which were solubilized with chaotropic agents. The non-glycosylated recombinant protein thus obtained was able to interact with the virus receptor CD81 and it was recognized by a number of anti-E2 antibodies (Hüssy et al., 1997; Xiang et al., 2006; Yurkova et al., 2004). The fact that $E2_{661}$ produced in *E. coli* is insoluble, points to glycosylation as an indispensable factor for the global folding of E2. In fact, it has been reported that the glycans of HCV envelope glycoproteins play a major role in protein folding and/or in HCV entry (Dubuisson and Rice, 1996; Goffard et al., 2005; Helenius and Aebi, 2001). The glycosylation also modulates the neutralizing activity of anti-HCV antibodies. At least three glycans in positions N1, N6 and N11 of E2 reduce the sensitivity of HCV pseudoparticles to antibody neutralization (Helle et al., 2007). In contrast, the glycosylation does not seem to be necessary for the binding to its receptor CD81 (Xiang et al., 2006; Yurkova et al., 2004).

The HCV structural E2 protein has also been produced in mammalian expression systems (Owsianka et al., 2001; Patel et al., 2000) and yeast cells (Martinez-Donato et al., 2006). In all cases, the majority of the secreted $E2_{661}$ exhibited a molecular mass much higher than that expected because of the hyperglycosylation of the protein. Moreover, the levels of expression are so low that the recombinant proteins can only be used to detect HCV-specific antibodies in human sera. The expression of different polypeptide forms of E2 in recombinant baculovirus-infected insect or human hepatoma cells has also been

reported although no structural information is given (Cerino et al., 1997; Hsu et al., 1993; Hüssy et al., 1997; Hüssy et al., 1996; Matsuo et al., 2006; Matsuura et al., 1992). Thus, by using different recombinant constructs containing the E2 protein terminating at residue 662, several E2 related proteins were observed (Hsu et al., 1993). These were partially purified by cutting the protein bands from preparative SDS-PAGE and electroeluting them in PAGE running buffer. Their immunoreactivity was tested using sera from patients chronically infected with HCV and only 10% reacted with the E2 protein (Hsu et al., 1993). On the other hand, a protein comprising residues 406-660 of E2 was secreted by *Sf9* cells infected with recombinant baculovirus. The protein was purified under native conditions and used to show that it was glycosylated and that it reacted with antibodies from HCV-seropositive patients (Hüssy et al., 1996). Finally, a truncated soluble E2 protein, spanning residues 390-683, expressed in insect cells using a baculovirus vector was purified under native conditions from the culture supernatant. It was used to study the human antibody response to HCV infection (Cerino et al., 1997).

We describe herein the expression and purification of large quantities of a soluble form of E2 ectodomain ($E2_{661}$) which has allowed us to determine its structural and antigenic properties. To overcome the hyperglycosilation problems encountered in yeast, we have expressed $E2_{661}$ protein using a baculovirus expression system.

3.1. Expression and purification of $E2_{661}$ from High Five™ cells

To produce the recombinant virus that expresses the $E2_{661}$ protein with a 6x His tag, the gene was first cloned into the pAcGP67A transfer vector that uses the gp67 secretory sequence of the baculovirus envelope protein. Next, the

recombinant vector was transfected along with wild-type viral DNA into *Sf9* cells and in a homologous recombination event, the $E2_{661}$ gene was inserted into the viral genome. The protein was expressed by infecting a new batch of insect cells using amplified recombinant virus. Cells were grown and protocols were carried out as described in Materials and methods.

Recombinant $E2_{661}$ would have 287 amino acids, 278 corresponding to positions 384 to 661 of E2 and 9 extra amino acids at the N-terminal end, the sequence ADP due to cloning plus the 6xHis. After 48 h post-infection, virus-infected cells produced several proteins which were recognized by an anti-His antibody, a major one with a molecular mass of 50 kDa and other minors that could correspond to less glycosylated products and/or polypeptide chains truncated at their C-terminal (data not shown). Of all these proteins, a single one with a molecular mass of about 48 kDa was partially (60%) secreted to the cell supernatant.

When High Five™ cells were infected, this protein was almost completely secreted to the extracellular medium. The accumulated amount of secreted protein was monitored directly at various post-infection times by Western blot analysis (data not shown). The highest amount was obtained 5 days post-infection. From day 5, the amount decreased because insect cell lysosomal proteases were released from lysed cells.

The protein secreted to the extracellular medium was purified by a Ni-NTA-agarose column as described in Materials and methods. The fractions eluted with 200 mM imidazole contained a protein which behaves as a single band in SDS-PAGE with a molecular mass of 48.3 kDa (Fig. 1, lane 1). Its purity was assessed to be higher than 95% in the presence of reducing agents (Fig. 1, lane 1). The yield of the process was 5-6 mg of protein per liter of media. The molecular

mass of the purified protein is similar to that provided by other authors when $E2_{661}$ is obtained by transient expression in mammalian cells (Flint et al., 2000).

3.2. Biochemical characterization of $E2_{661}$

Several studies indicated that the secreted protein was indeed $E2_{661}$. When analyzed by UV spectroscopy, purified $E2_{661}$ gave an absorption spectrum characteristic of a soluble protein. It exhibited a maximum at 278 nm and a shoulder at 290 nm (data not shown). A value of 2.73 for $E^{0.1\%}$ at 280 nm was experimentally calculated. This specified extinction coefficient together with the absorbance at 280 nm were used to determine the protein concentration in all subsequent experiments. The amino acid composition of the recombinant protein was determined by amino acid analysis, being almost identical to that predicted from the DNA sequence. On the other hand, the Edman degradation of purified $E2_{661}$ confirmed that the amino-terminal sequence was ADPH, indicating that the gp67 signal sequence had been cleaved. Finally, the identity of $E2_{661}$ was checked by mass spectrometry after trypsin hydrolysis. The analysis of the tryptic peptides was carried out by MALDI-TOF and SELDI-TOF. In both cases, experimentally measured peptide masses were compared with the theoretical ones derived from the $E2_{661}$ sequence, using FindMond software package from Expasy Proteomics Server (www.expasy.org). This analysis assigned measured masses to peptides which cover almost 70% of the entire sequence (Table 1). However, some of the Asn residues in the putatively identified peptides are predicted to be N-glycosylation sites (Goffard and Dubuisson, 2003), specifically those which correspond to positions 44, 50, 57, 74, 202, 249 and 272, and the carbohydrate moiety would modify their masses. Hence, assigned masses correspond to either peptides which are not glycosylated or to other regions of the protein. In any case,

it seems clear that the isolated protein really corresponds to the ectodomain of E2. On the other hand, the fact that the peptides containing the Cys residues 55, 223, 233, 246, 270 and 278 are observed in the non-reduced sample, would indicate that at least 6 out of the 17 cysteines of E2 ectodomain are not forming inter or intramolecular disulphide bridges.

SDS-PAGE in the presence of reducing agents showed a single band at 48.3 kDa that could correspond to the monomer (Fig. 1, lane 1). However, in the absence of reducing agents four main bands were observed (Fig. 1, lane 2). These have molecular masses that could correspond to monomeric, dimeric, trimeric and tetrameric forms of $E2_{661}$. Also, higher order structures which do not enter the gel were observed. Therefore, $E2_{661}$ would tend to self-associate through hydrophobic interactions and/or disulfide bridges. Mass spectrometry analysis of the intact protein confirmed the oligomeric nature of $E2_{661}$. MALDI-TOF yielded four peaks with molecular masses of 43.4, 81.1, 121.1 and 160.8 kDa (data not shown), which could also correspond to the same species observed by SDS-PAGE. The lower mass of the dimer, trimer and tetramer, as expected from that of the monomer, 43.4 kDa, could be due to the fact that the spectrometer is calibrated at the mass of the monomer and the error for the massess of the high order structures is considerable higher. On the other hand, the difference between the molecular mass values obtained by MS and electrophoresis could be due to the presence of the 6x His tag which promotes a lower migration on SDS-PAGE.

The oligomeric nature of $E2_{661}$ was also assessed by analytical ultracentrifugation. Sedimentation equilibrium experiments showed that the soluble protein was present as a mixture of molecular species. The polydispersity of the protein prompted us to use sedimentation velocity to determine the nature of the main species present in solution. Using this method, the presence of several

oligomeric species of $E2_{661}$ was detected (Fig. 2). The calculated molecular mass of the most abundant species was 96±12 kDa with a sedimentation coefficient of 4.3 S. Considering that the molecular mass of the $E2_{661}$ monomer determined by SDS-PAGE is 48.3 kDa, the major form found by analytical ultracentrifugation is compatible with a dimer. The area under the peaks allowed us to estimate that the dimer accounts for 30-40% of the total species present. The sedimentation coefficients of the other molecular forms observed are compatible with tetramers (20%), hexamers (15%) and higher forms (25%). A similar behaviour was observed when the purified protein was chromatographed on a Superdex G-200 FPLC column. Moreover, when the peak which eluted at the dimer volume was subjected to SDS-PAGE in the absence of reducing agents, only a band with the molecular mass of the monomer was observed (data not shown).

Therefore, as indicated by sedimentation velocity and size exclusion chromatography experiments, $E2_{661}$ has a high tendency to self-assocciate, being the dimer the smallest form present. The presence of dimers had also been proposed previously by other workers (Yagnik et al., 2000). The observed dimer is maintained by hydrophobic interactions which are disrupted by SDS when the protein is analyzed in SDS-PAGE in the absence of reducing agents. The dimeric forms which are observed by SDS-PAGE in the absence of reducing agents should come from the higher order oligomers which are disrupted by SDS, being the disulfide bridges somehow involved in the formation of some of the oligomeric species detected. However, it should be pointed out that the oligomers must be different from mere aggregates of misfolded forms of the protein since the spectroscopic properties of the dimer isolated from a Superdex G-200 column are practically indistinguishable from those of the $E2_{661}$ purified from the cellular medium and the far-UV CD spectrum is maintained in the presence of the

reducing agent tris-(2-carboxyethyl)-phosphine (data not shown). Thus, as previously proposed, the high tendency to form oligomers must be just an intrinsic property of E2 sequence (Dubuisson, 2000).

On the other hand, as mentioned above, E2 is predicted to contain several glycosylation sites (Goffard and Dubuisson, 2003). A global sequence analysis indicates that it has 11 potential glycosylation sites, most of which are well-conserved (Goffard and Dubuisson, 2003; Helle et al., 2007; Zhang et al., 2004a; Zhang et al., 2004b). The purified $E2_{661}$ behaves in SDS-PAGE in the presence of reducing agents as a single band with a molecular mass of 48.3 kDa while the theoretical mass based on the amino acid sequence is 31.65 kDa. Therefore, $E2_{661}$ must be glycosylated. In fact, $E2_{661}$ is recognized by concanavalin A (Fig. 3B, lane 1). Moreover, the enzymatic deglycosylation with PNGase, which removes carbohydrates bound through N-glycosydic bonds, rendered a double band at 35-37 kDa (Fig. 3A, lane 2) which must correspond to partially deglycosylated forms since both bands were recognized by concanavalin A (Fig. 3B, lane 2). Thus, $E2_{661}$ must contain some N-glycosidic bonds which are not accessible to the PNGase although the existence of O-glycosidic bonds can not be precluded. As a control of deglycosylation the allergen Ole e1 was used (van Ree et al., 2000). The results obtained indicated that deglycosylation was working properly (Fig. 3A, lanes 3-4).

3.3. Spectroscopic analysis of $E2_{661}$

The spectroscopic characterization of $E2_{661}$ was carried out by means of circular dichroism and fluorescence spectroscopy. Far-UV CD spectrum of $E2_{661}$ showed a minimum at 207 (Fig. 4A), indicative of a high percentage of non-regular structure. This spectrum is maintained in the presence of a 1 mM

concentration of the reducing agent tris-(2-carboxyethyl)-phosphine. Deconvolution of this spectrum using the program Convex Constraint Analysis (CCA) (Perczel et al., 1991) yielded the percentages of secondary structure given in Table 2. The predictive GOR IV method (Garnier et al., 1996), which is based upon the propensity of each amino acid to adopt a particular secondary structure, also indicates that helical structures are almost absent and that the main ordered structures are β-sheets (Table 2). The near-UV CD spectrum of $E2_{661}$ has two minima at 268 and 300 nm whose ellipticity values were –37 and –30 degree·cm^2·dmol^{-1}, respectively (Fig. 4B). These two dichroic bands reveal the asymmetric nature of the microenvironment of Trp and Tyr residues and, hence, the folded character of the purified protein.

The fluorescence emission spectrum of $E2_{661}$ showed a maximum at 334 nm (Fig. 5), which is typical of Trp residues in relatively low hydrophobic environments. The shape of the spectrum indicates that Tyr fluorescence is quenched by near residues or by energy transfer to Trp residues. The difference between the fluorescence spectra obtained upon excitation at 275 and 295 nm provides the low contribution of Tyr residues to the protein fluorescence (Fig. 5).

Acrylamide and iodide quenching of protein fluorescence were used to gain information on the degree of exposure of the fluorophores. As dynamic quenchers, both agents exert their action through collision with the fluorophores (Lehrer, 1971). Stern-Volmer plot of acrylamide quenching of $E2_{661}$ fluorescence was linear indicating that there is only one population of Trp residues accessible to the quencher (data not shown). Moreover, the value of f_a = 0.98 indicates that all fluorophores were accessible to acrylamide.

Since iodide is charged and highly hydrated, it will only interact with those fluorophores at or near the surface. Stern-Volmer plot of quenching of

$E2_{661}$ fluorescence by iodide was linear up to 0.4 M, with a marked upward curvature above this concentration (data not shown). At iodide concentrations higher than 0.4 M the maximum of the emission spectrum undergoes a red shift which indicates that the protein is denatured, with two populations of Trp residues, accessible and non-accessible to iodide. Moreover, the population of fluorophores accessible to quencher is homogeneous, since when quenching of $E2_{661}$ fluorescence data were plotted according to the modified Stern-Volmer relationship, a linear plot was observed (Fig. 6). The value of f_a = 0.83 calculated from this plot indicates that eight of the ten Trp residues were accessible to iodide. The low value of K_{SV}, 5.2 M^{-1}, indicated that the accessible Trp residues are not at the surface since K_{SV} would otherwise be higher (K_{SV} for free N-acetyl-L-tryptophanamide in aqueous solution is 17.5 M^{-1}) (Lehrer, 1971). Taken together, all these results indicate that $E2_{661}$ recombinant protein has a somehow open conformation, but different from a denatured state.

3.4. Antigenic properties of $E2_{661}$

A panel of eight HCV-positive human sera and ten HCV-negative human sera was used to assess the antigenic properties of the $E2_{661}$ recombinant protein. All sera were tested by ELISA for E2-specific antibodies as described in Material and methods. $E2_{661}$ recombinant protein was able to bind to antibodies present in all HCV-positive human sera at a dilution of 1:200. The optical density values reached with the positive sera (Fig. 7, sera 1-8) were much higher than those of the negative controls (Fig. 7, sera 9 and 10; only two of the ten negative sera are depicted). The observed differences in the value of the optical density at 492 nm are consistent with the HCV titer as determined by COBAS TaqMan HCV test (Hu et al., 2005) (data not shown).

The structural properties of the different epitopes were assessed by Western blot analysis. The effect of the denaturation with SDS and β-mercaptoethanol on the reactivity of E2 against the human sera was tested. As depicted in Fig. 7, sera 1, 2, 3, 4 and 8 reacted to a different extent with denatured and reduced $E2_{661}$ while sera 5, 6 and 7 gave almost no reaction (Fig. 7). This suggests that some of the antibodies present in HCV-positive human sera bind to conformation-dependent epitopes. Therefore, $E2_{661}$ should contain both continuous and discontinuous antigenic determinants. Reduction would destroy the antigenic reactivity of discontinuous epitopes thus indicating the role of disulfide bridges in stabilizing their conformation.

Finally, the conformation of $E2_{661}$ was examined by immunoprecipitation with an anti-E2 conformation-dependent monoclonal antibody, H53. The $E2_{661}$ protein was efficiently immunoprecipitated by the antibody, suggesting that the purified protein possesses a native-like conformation. Moreover, all the oligomeric forms were able to react with the antibody.

HCV envelope glycoproteins, E1 and E2, have been shown to form noncovalent heterodimers as well as heterogeneous disulfide-linked aggregates (Dubuisson et al., 1994; Ralston et al., 1993). Characterization of the noncovalent heterodimer with conformation dependent monoclonal antibodies has suggested that this oligomer is likely the prebudding form of the functional complex (Deleersnyder et al., 1997). Michalak and coworkers (1997) have shown that the folding of E1 is helped by the coexpression of E2 (Michalak et al., 1997). Moreover, two earlier studies indicate that HCV envelope glycoproteins cooperate for the formation of a functional complex and that both glycoproteins have to be co-expressed to analyze their functional properties (Brazzoli et al., 2005;

Cocquerel et al., 2003). Taking into account the results described in this paper it seems reasonable to asume that $E2_{661}$ recombinant protein is folded in a native conformation and presents antigenic properties similar to E2 assembled in the HCV virion. Therefore, E2 ectodomain would not need the presence of E1 to achieve its native structure, it represents the structural core of functional E2, and it behaves as an independent folding domain. Now that high levels of properly folded $E2_{661}$ can be obtained, further studies of its three-dimensional structure as well as its role in receptor binding and in the fusion mechanism of the HCV can be carried out. Also, and since $E2_{661}$ containing different sequences of the HVR1 can be obtained (M. Rodríguez-Rodríguez and F. Gavilanes, unpublished data), a mixture of polypeptide chains corresponding to the ectodomain of E2 envelope protein could be used as a vaccine in the prevention of HCV infection.

Acknowledgments

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Figure captions

Fig. 1. Oligomeric nature of $E2_{661}$. The purified $E2_{661}$ was subjected to SDS-PAGE in the presence (lane 1) and in the absence (lane 2) of reducing agent. (P) Protein size markers. (a) monomer, (b) dimer, (c) trimer and (d) tetramer. The gel was stained with Coomassie Brilliant blue R-250.

Fig. 2. Sedimentation velocity analysis of $E2_{661}$. The results are shown as the sedimentation coefficient distribution *c(s)*. The experiment was carried out at 5 μM native $E2_{661}$.

Fig. 3. Analysis of the deglycosylation of $E2_{661}$ by PNGase. (A) SDS-PAGE stained with Coomassie blue. (1) $E2_{661}$ untreated; (2) $E2_{661}$ treated with PNGase; (3 and 4) Ole e 1 protein used as control before and after treatment with PNGase, respectively (van Ree et al., 2000); (P) Protein size markers. (B) SDS-PAGE stained with concanavalin A. (1) $E2_{661}$ untreated; (2) $E2_{661}$ treated with PNGase. After transferring, the nitrocellulose membranes were incubated with biotinylated concanavalin A and the proteins were detected using peroxidase-conjugated streptavidin as described in the Materials and methods section. The digestion with PNGase was carried out for 16 h at 37 ºC in 20 mM sodium phosphate, pH 7.0, 50 mM EDTA, and 1% (v/v) octylglucoside.

Fig. 4. Circular dichroism spectra of $E2_{661}$. (A) Far-UV CD spectrum of $E2_{661}$ at pH 7.0. The spectrum was recorded between 190 and 250 nm with a protein concentration of 0.15 mg/ml in a cylindrical cuvette of 0.1 cm pathlength. (B)

Near-UV CD spectrum of $E2_{661}$ at pH 7.0. The pathlength of the cuvette was 0.5 cm and the protein concentration was 0.5 mg/ml. The buffer was MOPS 30 mM, pH 7.0, 100 mM NaCl. Both spectra were recorded five times, averaged and corrected for buffer contributions. Data were collected at 25 ºC and are expressed as residue molar ellipticity.

Fig. 5. Fluorescence emission spectra of $E2_{661}$. The excitation wavelength was 275 nm (—) and 295 nm (---). The emission spectra were recorded between 300 and 450 nm. The spectrum obtained after excitation at 295 nm was normalized at wavelengths above 380 nm. The contribution of Tyr residues (•••) to the emission spectrum was calculated as described in the Materials and Methods section. Protein concentration was 0.05 mg/ml. The buffer was sodium phosphate 20 mM, pH 7.5, 100 mM NaCl. Spectra were collected at 25 ºC. The contribution of the buffer was always subtracted.

Fig. 6. Modified Stern-Volmer plot of iodide quenching of $E2_{661}$ fluorescence. The values of the fluorescence at 334 nm (F) were obtained from the emission spectra of $E2_{661}$ in the presence of increasing amounts of iodide (0-1 M). The emission spectra were recorded with an excitation of 290 nm. The protein concentration was 0.1 mg/ml. The value of F_0 was measured for the same $E2_{661}$ sample in the absence of iodide. The data were plotted according to the modified Stern-Volmer relationship as described in the Materials and Methods section and the parameters were deduced by linear regression.

Fig. 7. Reactivity of $E2_{661}$ against HCV-positive and negative human sera. The upper part shows the results obtained in an ELISA assay as described in the

Materials and Methods section. Eight HCV-positive human sera (1-8) and ten HCV-negative human sera were used at a dilution of 1:200. Only the results obtained with two negative controls (9 and 10) are shown since all of them gave virtually the same optical density. Bound antibodies were detected with peroxidase conjugated anti-human IgG (Fc) diluted at 1:10000. The lower part shows the results of the Western blot analysis after electrophoresis in the presence of SDS and β-mercaptoethanol. The nitrocellulose membranes which were incubated with individual sera from HCV-positive and negative patients at a dilution of 1:20, were developed with peroxidase-conjugate goat anti-human IgG diluted at 1:1000 and $E2_{661}$ was detected with ECL detection reagents.

Tables

Table 1

Tryptic peptides of native $E2_{661}$ recombinant protein identified by SELDI-TOF and MALDI-TOF MS.

Molecular Mass[a]	Peptide[b]	Position[c]
SELDI-TOF		
750.110	**C**NLEDR	278-283
961.520	HPEATYSR	215-222
1021.260	**C**NLEDRDR	278-285
1028.580	CLVNYPYR	233-240
1048.550	MYVGGVEHR	257-265
1064.540	LEAA**C**NWTR	266-274
1074.560	**C**GSGPWITPR	223-232
1089.850	KHPEATYSR	214-222
1800.030	LWHYP**C**TINYTIFK	241-254
2092.400	MYVGGVEHRLEAA**C**NWTR	257-274
2184.610	SV**C**GPVY**C**FTPSPVVVGTTDR	127-147
2531.800	V**C**GAPP**C**VIGGVGNNTLH**C**PTD**C**FR	189-213
2659.660	V**C**GAPP**C**VIGGVGNNTLH**C**PTD**C**FRK	189-214
2809.090	CLVNYPYRLWHYPCTINYTIFK	233-254
3474.510	ADPHHHHHHETHVTGGTAAQTTAGLVSLLSPGAK	1-34
4347.460	QDIQLINTNGSWHINSTALN**C**NDSLYTGWLAGLFYHHK	35-72
MALDI-TOF		
996.420	FNSSG**C**PER	73-81
1028.580	**C**LVNYPYR	233-240
1048.550	MYVGGVEHR	257-265
1064.540	LEAA**C**NWTR	266-274
1074.560	**C**GSGPWITPR	223-232
2092.400	MYVGGVEHRLEAA**C**NWTR	257-274
2184.610	SV**C**GPVY**C**FTPSPVVVGTTDR	127-147
2531.800	V**C**GAPP**C**VIGGVGNNTLH**C**PTD**C**FR	189-213

[a]The molecular mass corresponds to the experimental value. [b]The cysteine residues are shown in bold. [c]The numbers indicate the position of the aminoacid in the cloned protein.

Table 2

Secondary structure of $E2_{661}$.

%	Experimental[a]	Theoretical[b]
α-helix	8	2
β□-sheet	47	34
Non-ordered	45	64

[a]The experimental values were calculated from the far-UV CD spectrum by using the deconvolution program CCA (Perczel et al., 1991). [b]The theoretical values were calculated by GOR IV method (Garnier et al., 1996).

Figure 1

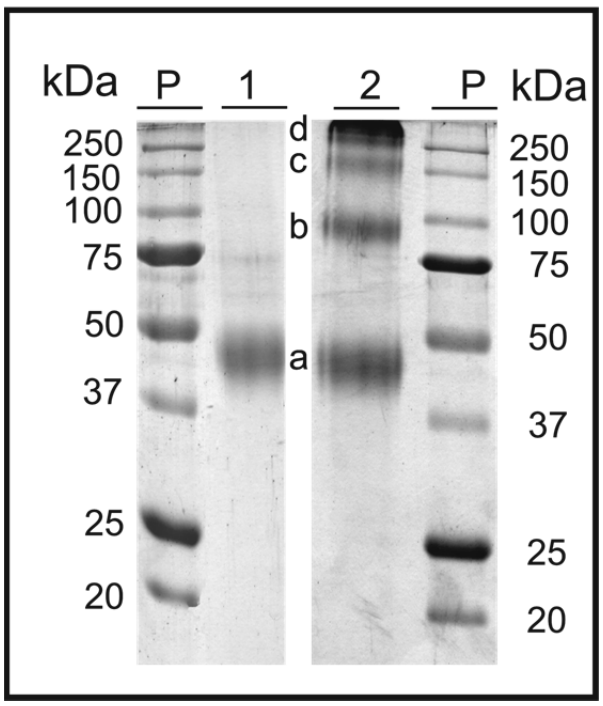

Figure 2

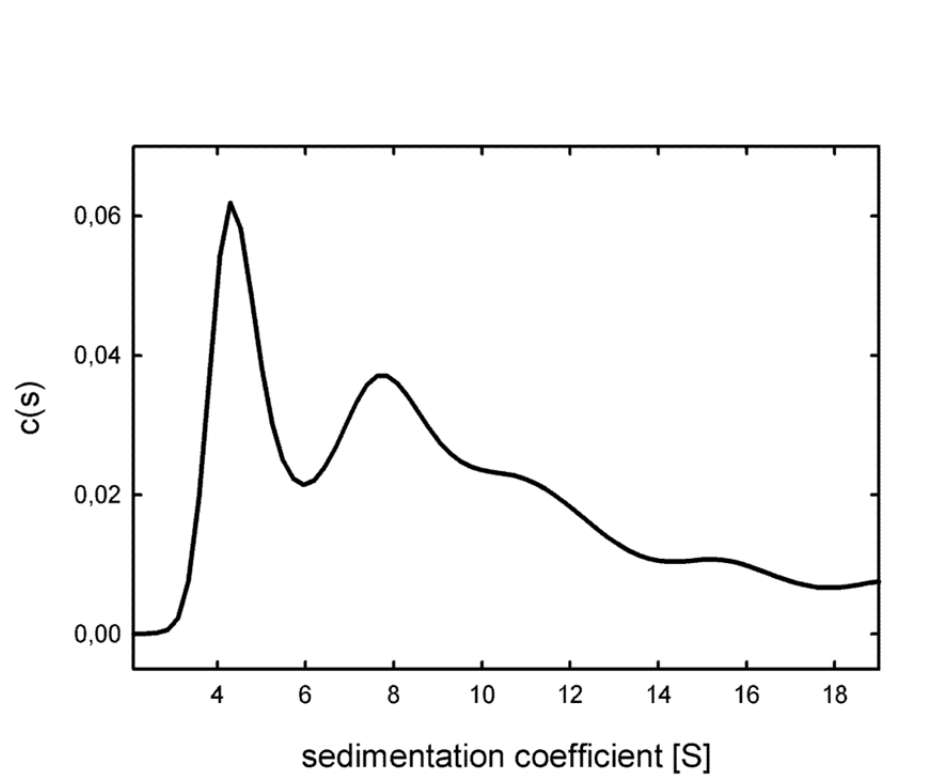

Figure 3

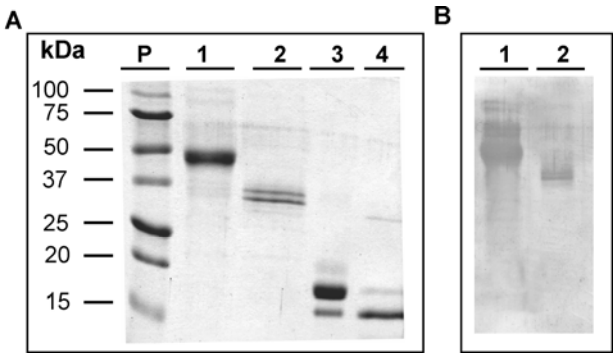

Figure 4

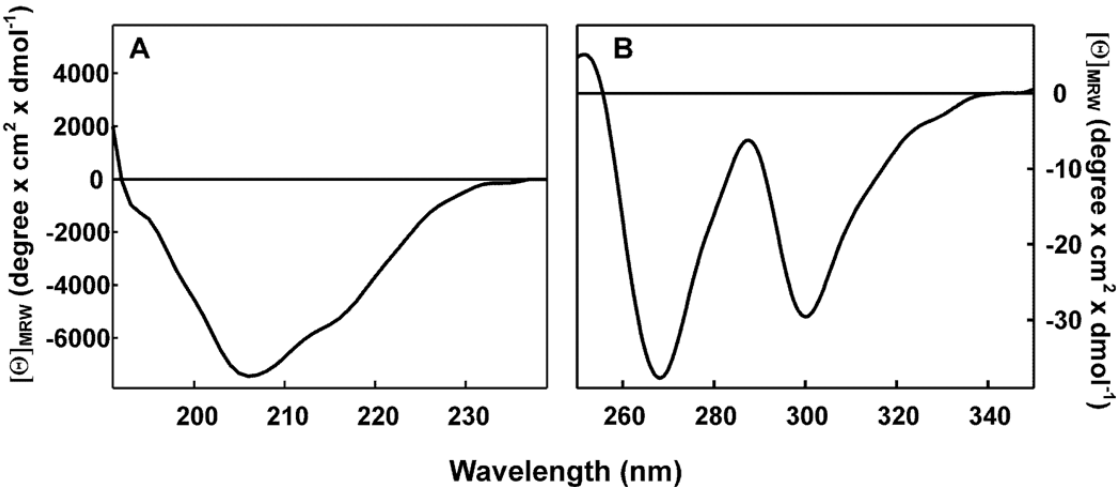

Figure 5

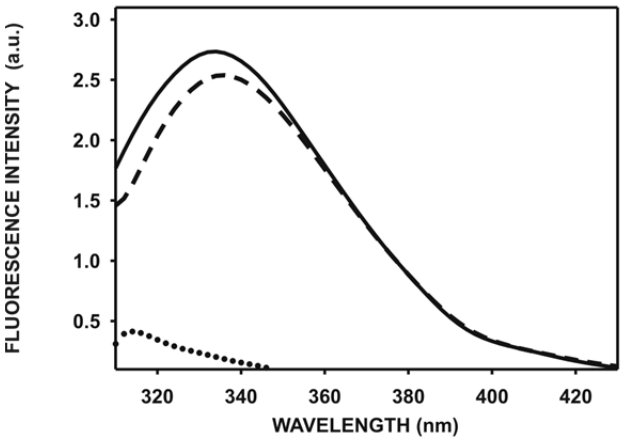

Figure 6

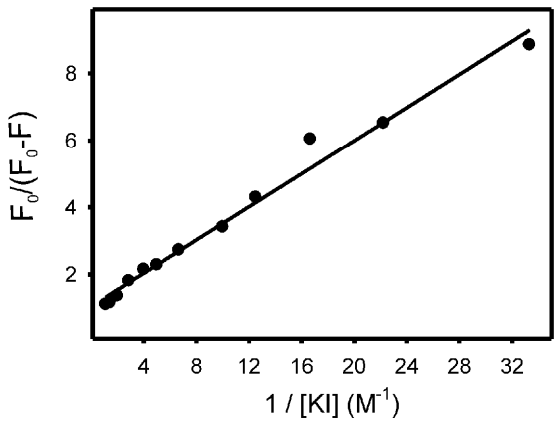

Figure 7