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In vitro Folding of Recombinant Hepatitis B Virus X-Protein Produced in *Escherichia coli*: Formation of Folding Intermediates

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The folding of recombinant hepatitis B virus X-protein (rHBx) solubilized from *Escherichia coli* inclusion bodies was investigated. By sequential dialysis of urea, rHBx was folded into its native structure, which was demonstrated by the efficacy of its transcriptional activation of the adenovirus major late promoter (MLP), fluorescence spectroscopy, and circular dichroism (CD) analysis. The decrease in CD values at 220 nm and a corresponding blue shift of the intrinsic fluorescence emission confirmed the ability of rHBx to refold in lower concentrations of urea, yielding the active protein. Equilibrium and kinetic studies of the refolding of rHBx were carried out by tryptophan fluorescence measurements. From the biphasic nature of the fluorescence curves, the existence of stable intermediate states in the renaturation process was inferred. Reverse phase-high performance liquid chromatography (RP-HPLC) analysis further demonstrated the existence of these intermediates and their apparent compactness.

Keywords: Circular dichroism, Fluorescence emission maximum, Hepatitis B virus X-protein, Refolding, Reverse phase-high performance liquid chromatography.

Introduction

The mechanistic study of protein folding is important for understanding the structure and function of proteins (Kim and Baldwin, 1982). Typically, denaturation and folding of small globular proteins is approximated by a two-state equilibrium because folding intermediates are usually short-lived (Pace, 1986). Experimental identification and structural characterization of intermediates appear to be important for the understanding of the folding process of proteins. In fact, intermediates have been detected during the course of renaturation of several small globular proteins including proteins with disulfide bonds (Mitchinson and Pain, 1985; Lu *et al.*, 1992; Zerovinick *et al.*, 1992; Ward *et al.*, 1995; Jeoung *et al.*, 1999).

Hepatitis B virus (HBV) contains an open reading frame, referred to as X, which encodes a protein of 154 amino acids. The precise role of hepatitis B virus X protein (HBx) during HBV infection is not clear. However, HBx has been shown to activate gene expression of diverse viral and cellular transcriptional control elements (Spandau and Lee, 1988; Twu *et al.*, 1989; Rossner, 1992), which may play important roles in the pathogenesis of hepatocellular carcinoma (Robinson, 1994). HBx is the least understood among the HBV proteins in terms of structure and function. It is produced in *E. coli* as a 16-kDa protein with a single tryptophan residue at position 120, located in the region important for its transactivating activity (Ritter *et al.*, 1991; Kumar *et al.*, 1996). HBx also contains 9 cysteines, whose disulfide arrangement has been established: one free cysteine at position 148 and four intramolecular disulfide bonds, cys7-cys78, cys17-cys115, cys61-cys137, and cys19-cys143 (Gupta *et al.*, 1995). However, the structural and functional relevance of these intramolecular linkages remains to be established. Mutational analyses of rHBx to localize the regions important for transactivation have been attempted by several other groups and ours. Ritter *et al.* (1991) and Kumar *et al.* (1996) identified amino acid sequences 32–148 and 58–140 to be important for its transactivating activity, respectively. The fully-conserved C-terminal

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region of HBx (residues 132 to 141) and three conserved *cysteines* (Cys-61, Cys-69, and Cys-137) were found to be crucial for the *trans*-activation function (Arii *et al.*, 1992; Kim *et al.*, 1993; Kumar *et al.*, 1996).

Detailed structure-function analysis of HBx should elucidate additional domains important for its activity. For this, rHBx was refolded into its active form through an oxidative folding procedure. To gain further insight into the conformational states of HBx, we undertook equilibrium renaturation studies of rHBx using techniques such as CD and fluorescence spectroscopy, which demonstrated the presence of intermediates in the folding pathway.

Materials and Methods

Construction of a HBx expression plasmid For expression of recombinant HBx, a 500 bp *Sal*I fragment from the pHBV-D vector containing the X-gene of HBV Korean type adr (Kim *et al.*, 1993) was treated with Klenow and inserted into the *Nco*I site of plamid pET-3d. The resulting expression vector, pET-3d-X, was used for the expression of HBx in *E. coli* for further experiments (Fig. 1A).

Expression and purification of rHBx *E. coli* strain BL-21 containing pET-3d-X was grown overnight in LB medium containing ampicillin at a concentration of 20 μg/ml. Five ml of overnight culture was inoculated into 200 ml LB medium and mixed with 4 L of fresh LB medium after 2–3 h culture. Isopropyl-1-thio-beta-D-galactoside (IPTG) (0.2 mM) was added to the culture when cell growth had reached early log phase ($Abs_{600\ nm}$ = 0.4). Cells were then harvested after 6–7 h culture at 37°C. Inducibility of the fused gene was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Most of the induced rHBx protein existed in inclusion bodies and was easily collected by centrifugation at 5000 × g after sonication. Pellets containing inclusion bodies were washed with 3 M urea in 50 mM Tris·HCl (pH 7.0) to reduce nonspecific association of bacterial proteins (Park *et al.*, 1999). Pellets collected by simple centrifugation were solubilized in buffer A (8 M urea, 50 mM Tris·HCl, pH 8.0, 1 mM EDTA, 10 mM DTT) and loaded onto a CM-Sepharose-CL-6B column. Stepwise gradients of NaCl (0, 50, 100, 250 mM) were applied and rHBx was eluted at 0.1 M NaCl. The purification procedure described here is very simple and rapid, and the average yield of purified rHBx was approximately 4 mg/l of culture medium.

Renaturation of rHBx The purified rHBx dissolved in an 8 M urea solution containing 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, and 10 mM DTT was dialyzed sequentially against 4, 2, 1, and 0.1 M urea solutions containing 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, and 1 mM DTT, and finally against a solution of 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, and 1 mM DTT at 4°C. The refolding mixtures were subsequently analyzed by RP-HPLC, CD, and fluorescence spectroscopy. For RP-HPLC analysis, a C8 (4.6 × 250 mm, YMC Cor.) column with 7 μm particles was employed. A linear gradient of 25–60% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 40 min followed by 100% acetonitrile in 0.1% TFA over the next 10 min was used with a flow rate of 1 ml/min. The RP-HPLC analysis of the renatured rHBx suggested that more than 90% of the rHBx was successfully refolded. The renatured protein was stored in aliquots in liquid nitrogen until use.

***In vitro* transcription assay** The *in vitro* transcription assay was carried out using a HeLa nuclear extract *in vitro* transcription system (Promega) according to the manufacturer's directions. Adenovirus major late promoter (MLP)-containing plasmid (pDA-CAT, 100 ng) linearized with *Nco*I, was incubated at 30°C for 60 min in the presence or absence of the renatured rHBx (20, 50 ng) in a solution containing 10 mM HEPES (pH 7.9), 6 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 0.2 mM DTT, 10% glycerol, 400 μM of each ATP, CTP, and UTP, 16 μM GTP, 10 μCi [α^{32}P] GTP (3000 Ci/mmol), and the nuclear extract of HeLa cells. The transcription products were analyzed on a 16 × 18 cm denaturing polyacrylamide gel containing 5% acrylamide, 7 M urea, and TBE buffer and quantitated by scanning densitometry after gel electrophoresis and autoradiography. Size markers were prepared by kinasing dephosphorylated ΦX174 *Hin*fI DNA with [γ^{32}P] ATP.

Refolding studies In the folding experiments, rHBx, first completely reduced by DTT in the presence of 8 M urea at pH 8.0, was diluted into buffer containing urea as indicated and allowed to stand for ~20 h at 4°C. Solutions were incubated at 25°C for at least 1 h before the fluorescence spectra were recorded. Transition curves were constructed by plotting the shift in maximum wavelength versus the denaturant concentration. The kinetic experiments were initiated by a concentration jump of urea from 8 M to 0.08 M in 50 mM Tris·HCl buffer (pH 8.0) containing 1 mM EDTA and 1 mM DTT at 25°C. The concentrations of rHBx were ~0.05 mg/ml.

Fluorescence and circular dichroism Fluorescence emission spectra of refolding mixtures were recorded at 25°C from 300 to 450 nm with an excitation wavelength of 280 nm on a Shimadzu RF-5000 spectrofluorometer, using 0.5 cm pathlength cells. A step size of 0.5 mm was used with an average scan time of 2 s/nm. Spectra were background-corrected and λ_{max} values were estimated from the emission spectra. Fluorescence intensities were determined by measuring the emission at 350 nm. In all cases, the excitation wavelength was 280 nm and slit widths of both monochromators were 5 nm. CD measurements were conducted using a Jasco J-720 spectrometer with temperature controlled at 25°C. A quartz cell of 0.2 cm pathlength was used for measurements in the far-UV region of 210–260 nm. All spectra are the average of two scans. CD spectra were corrected for light scattering by subtracting the data of blank samples lacking protein, and converted to mean residue ellipticity. The protein concentration was ~0.05 mg/ml in 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, and 1 mM DTT with respective urea concentrations.

Results

Purification and renaturation of rHBx Full-length HBx (154 amino acids) was expressed in *E. coli* (BL21) using the T7 expression system (pKM-X; Fig. 1A). The recombinant HBx found in inclusion bodies was further

purified to homogeneity by washing of the inclusion body proteins, which were then dissolved and subjected to CM-sepharose ion-exchange column chromatography. Figure 1B shows a chromatogram of CM-sepharose ion-exchange chromatography and HBx on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained by Coomassie Blue. The final rHBx was 98–99% pure as assessed by SDS-PAGE (Fig. 1B) and RP-HPLC analysis (data not shown). The identity of the purified rHBx was confirmed by N-terminal amino acids sequence analysis and immunoblotting with rabbit polyclonal antibody raised against synthetic peptides of HBx sequence and monoclonal antibody raised against purified rHBx (data not shown). The purified protein was renatured by sequential dialysis. To test that the purified and renatured rHBx was functional, we assayed the efficacy of the renatured rHBx on transcriptional activation of the adenovirus major late promoter using nuclear extracts of HeLa cells. A specific RNA polymerase II transcript corresponding to 550 bp was detected (Fig. 2). The synthesis of this transcript was enhanced by rHBx in a dose-dependent manner. In the presence of 20 and 50 ng of renatured rHBx, the transcription level was increased by 4- and 7-fold, respectively. These results suggest that the purified rHBx was refolded to a functional protein which exhibits *in vitro* biological activity.

Refolding of rHBx followed by spectroscopy

Renaturation of rHBx at pH 8.0 was followed by two spectroscopic probes: far-UV CD spectroscopy and fluorescence emission spectroscopy (Jeon and Kim, 1999). The far-UV CD spectra of rHBx at different urea concentrations are shown in Fig. 3A. Purified rHBx in 8 M urea is unfolded as demonstrated by its CD spectrum (Fig. 3A, 8 M). When the urea concentration was decreased by sequential dialysis, CD values at 220 nm decreased, which reflects the increase in ordered structure with decreasing concentrations of urea.

The renaturation of rHBx was also monitored by fluorescence spectroscopy (Fig. 3B). The analysis of fluorescence emission spectra at different denaturant concentrations shows a shift in the maximum emission fluorescence wavelength during refolding. Dialysis of rHBx against decreasing concentration of urea was accompanied by a corresponding blue shift of the intrinsic fluorescence emission at λ_{max} from 352 to 338 nm. This is

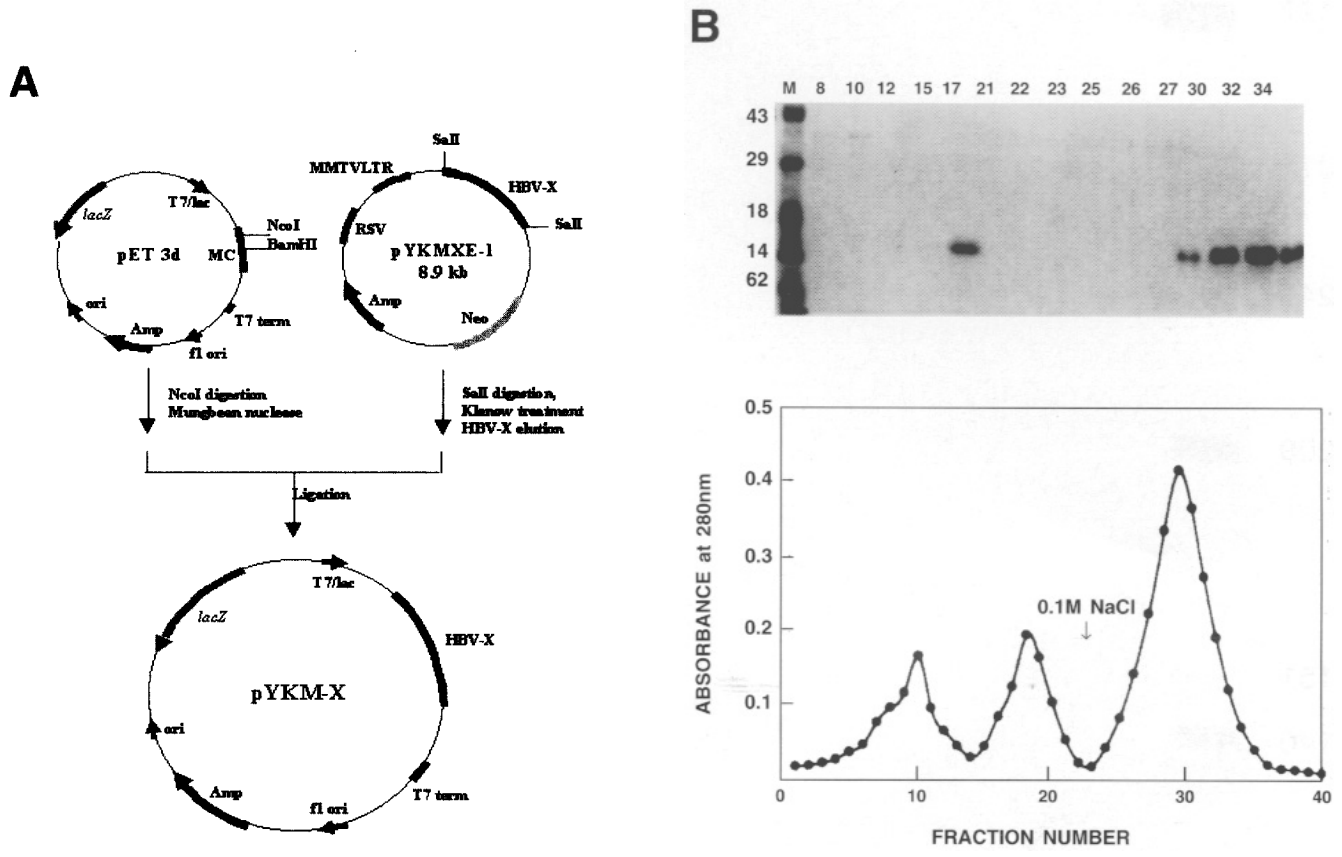

Fig. 1. Cloning and expression of rHBx. **A.** Map of expression plasmid pYKM-X. The HBx gene-containing DNA fragment (500 bp *Sal*I fragment) was inserted into the *Nco*I site of vector pET-3d for regulation by the T7 promoter. T7 terminator represents the transcription termination signal. The ampicillin resistance gene and the origin of replication are shown as amp and ori, respectively. **B.** SDS-PAGE analysis of different CM-Sepharose column chromatography purification steps. Fractions of each purification stage were prepared, subjected to 15% polyacrylamide gel electrophoresis (reducing conditions), and stained with Coomassie blue.

consistent with rHBx assuming a conformational state in which one tryptophan residue of rHBx protein is less solvent-exposed upon refolding. However, the fluorescence intensity of one tryptophan in rHBx is unusually decreased during the refolding process, suggesting that it is quenched by neighboring residues in the native protein. Taken together, the CD and fluorescence data indicate that, upon refolding by sequential dialysis of urea, rHBx adopted an ordered structure in which one tryptophan residue is less solvent-exposed.

RP-HPLC and fluorescence studies of the equilibrium refolding of rHBx for probing the folding intermediates rHBx contains a single tryptophan residue which is primarily responsible for the fluorescence of proteins. The tryptophan residue of rHBx is localized in a predicted α-helical structure (Chou and Fasman, 1978), the region important for its transactivating activity (Ritter *et al.*, 1991; Kumar *et al.*, 1996). Thus, the effect of urea on the fluorescence properties of rHBx reflects the conformational events that occur in this domain. For this reason, the equilibrium refolding of rHBx upon removal of denaturant was further investigated by fluorescence spectroscopy. The change in wavelength of the fluorescence emission maximum as a function of urea

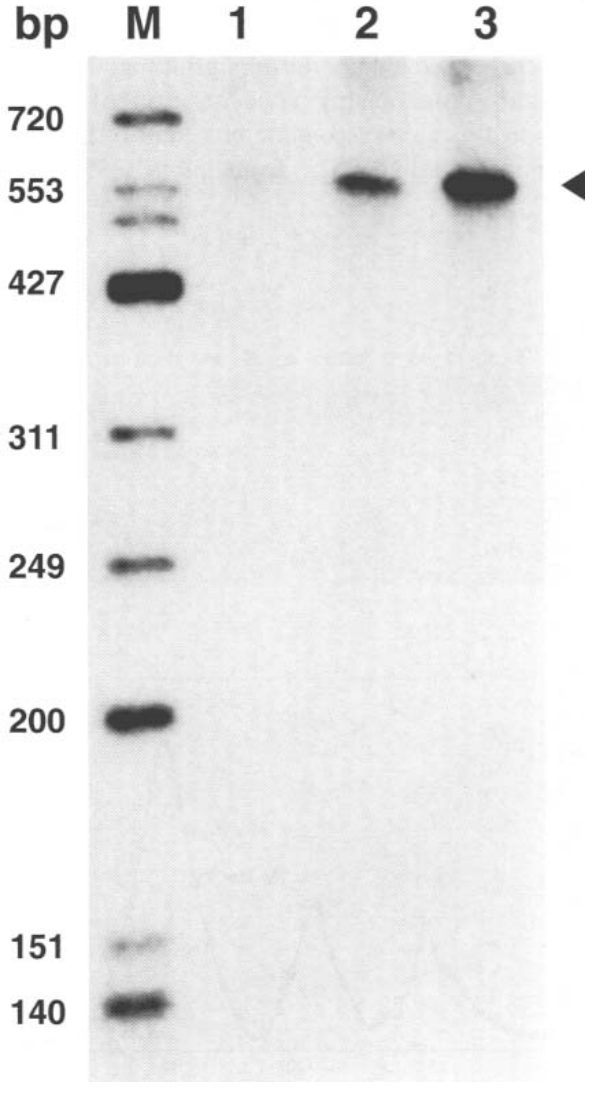

Fig. 2. *In vitro* transcriptional activation by rHBx. Gel electrophoresis of [^{32}P]GTP-labeled RNA of *in vitro* transcribed adenovirus major late promoter containing plasmid (pDA-CAT) in the absence (lane 1) or presence (lane 2, 20 ng; lane 3, 50 ng) of renatured rHBx. The molecular weight sizes of ΦX174 *Hin*fI size markers (M) are indicated on the left.

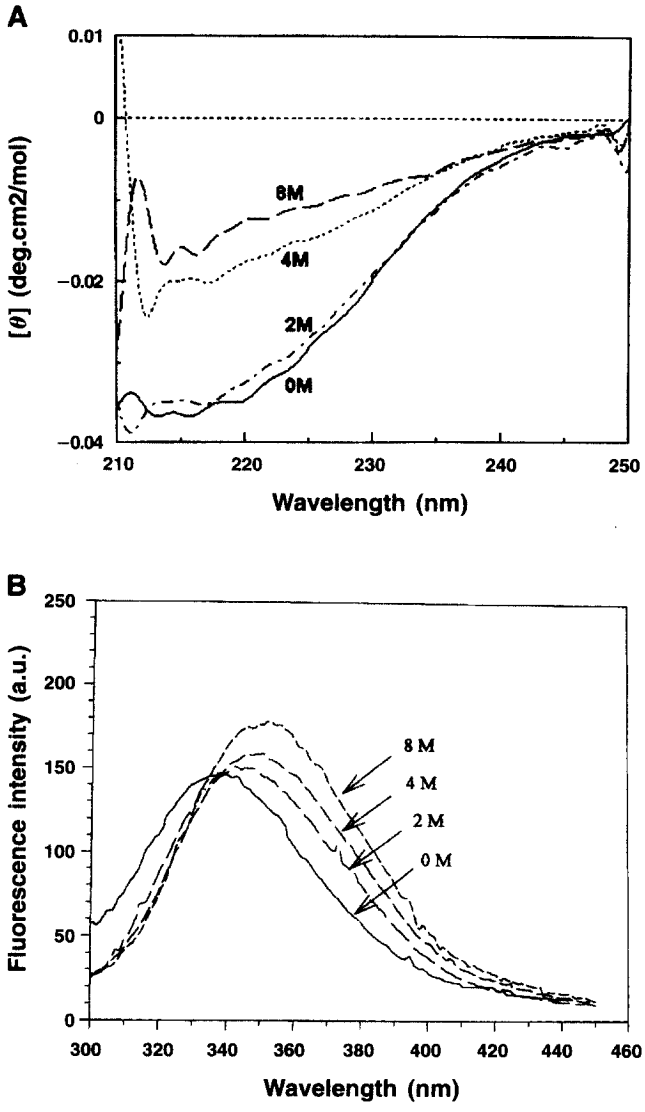

Fig. 3. Far-UV CD spectra and fluorescence emission spectra for the refolding of rHBx at different final concentrations of urea. Refolding was initiated by sequential dialysis of urea. **A.** At each stage, CD spectra were recorded, and converted to mean residue ellipticity using the protein concentration calibrated by the Bio-Rad protein assay. **B.** Fluorescence emission spectra of rHBx were acquired at the indicated urea concentrations. The temperature was 25°C and the excitation wavelength was 280 nm.

concentration at pH 8.0 was monitored, and the obtained transition curve is shown in Fig. 4. For the renaturation of rHBx, two transitions were observed in the fluorescence profile, one at 0.5 M urea and the second at 3 M urea. The biphasic nature of the fluorescence curves is indicative of intermediates in the folding pathway. The states of conformational intermediates between the native and fully-unfolded forms were then significantly populated at intermediate concentrations of denaturant.

Renaturation of rHBx was also monitored by RP-HPLC. The RP-HPLC traces plotted in Fig. 5 illustrate how the equilibrium between fully-denatured and native forms varied as a function of denaturant concentration. The fully-reduced denatured rHBx in 8 M urea was retained longer and eluted at R_t = 30 min (8 M) as a single peak, whereas the refolded native rHBx was eluted as a single sharp peak at R_t = 25 min (0 M). Their retention times differed by approximately 5 min. Upon refolding by reducing the urea concentration, the retention time was shifted to the earlier time points with a broad peak. Partially-folded intermediate forms were significantly populated in 2 M urea solution, which appears at R_t = 27.5 min (2 M). RP-HPLC data further confirmed that compact intermediate states exist in 2 M urea.

Refolding kinetics The kinetics of reactivation of rHBx following dilution of denaturant were investigated by fluorescence spectroscopy. Refolding reactions of rHBx were initiated by diluting urea to subdenaturing concentrations from 8 M to 0.08 M at 25°C. Changes in the fluorescence intensity were monitored at 350 nm where the fluorescence intensity of rHBx changed most dramatically with varying urea concentration. The biphasic transition was observed in the tryptophan fluorescence, the half-times lying around 6 and 60 min (Fig. 6). The faster phase was responsible for about 60–70% of the total amplitude change, with the remainder associated with the slower phase. The amplitude of the faster phase was larger than that of the slow phase, indicating that the fast phase predominated in the folding reaction. This temporal behavior indicates the existence of intermediates. The two phases were interpreted as a transition from denatured to partially-folded intermediates, I, and a transition from I to the native state, N. The model predicts a rapid loss of fluorescence intensity as the protein is converted from the unfolded state to the intermediate state, followed by a slower breakdown of the intermediate to the native state. Both equilibrium and kinetic folding studies suggest that intermediates accumulate on the refolding pathway.

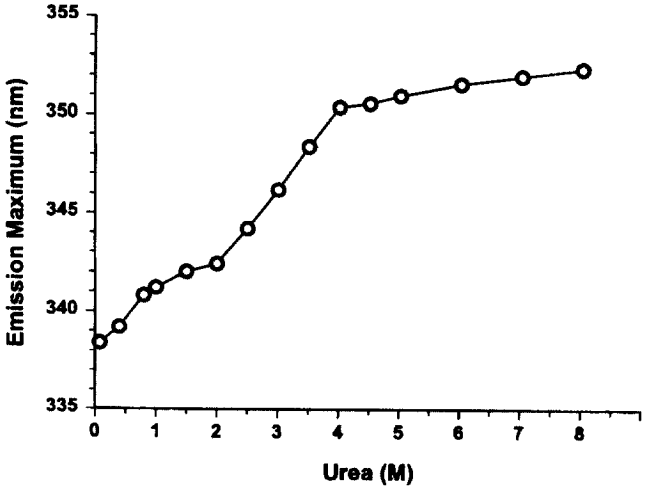

Fig. 4. Equilibrium refolding transitions of rHBx monitored by fluorescence spectroscopy. The denatured rHBx was folded by diluting urea from 8 M to the concentrations indicated on the ordinate. Refolding transition was assessed by variation of the maximum fluorescence emission wavelength (excitation wavelength, 280 nm).

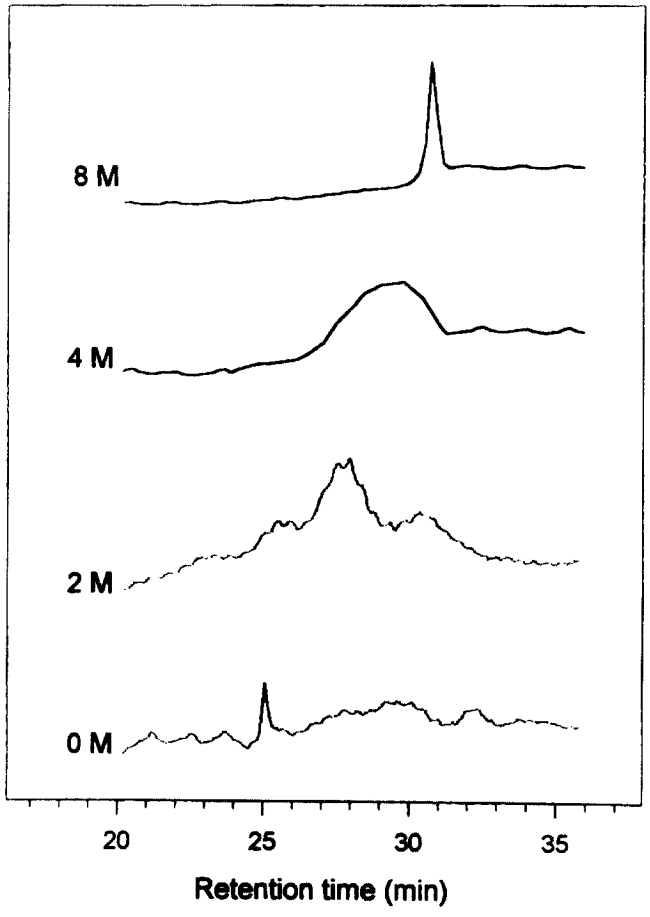

Fig. 5. RP-HPLC analysis of folding mixtures. Refolding was initiated by sequential dialysis of urea, and folding mixtures at the indicated urea concentrations were subjected to RP-HPLC (C8, 4.6 × 250 mm). A linear gradient from 25–60% acetonitrile in 0.1% TFA over 40 min followed by 100% acetonitrile over the next 10 min was used at a flow rate of 1 ml/min. The elution was monitored by UV absorbance at 220 nm.

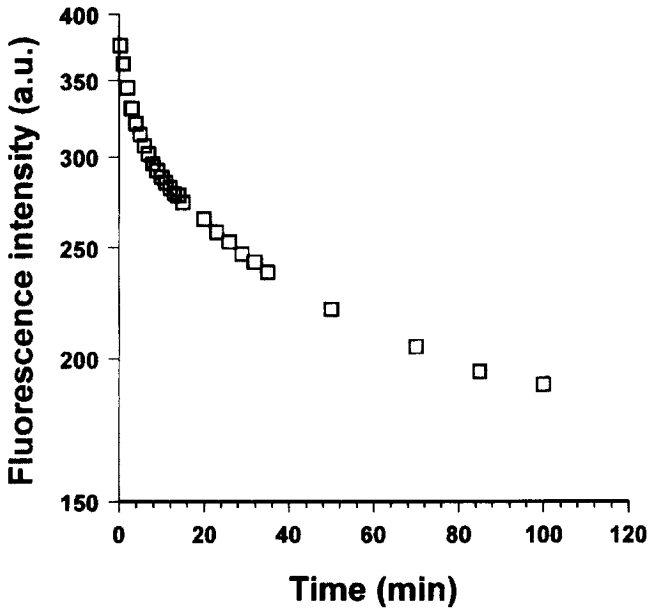

Fig. 6. Changes in protein fluorescence with time during the folding of rHBx. Refolding was initiated by mixing 1 part of unfolded protein (0.5 mg/ml) in buffer containing no urea (pH 8.0) with 9 parts of buffer solution containing 0 M urea. Excitation was at 280 nm, and emission was monitored at 350 nm.

All the unfolded molecules rapidly equilibrate under folding conditions with a few partially-folded, stable intermediates which are also in rapid equilibrium. All the molecules pass through a common slow step, which involves going through a transition state. The intermediates I would be considered as analogues of the molten globule of soluble proteins, implying that in I the global structure of rHBx would be established, but not yet the detailed structure.

Discussion

In this study, the oxidative folding pathway of rHBx was investigated using CD, fluorescence spectroscopy, and RP-HPLC. The most powerful methods applied in folding studies make use of the specific spectral properties of proteins. They represent the most reliable and sensitive criteria to characterize the native and denatured states, as well as sufficiently long-lived and highly-populated intermediates. HBx has a single tryptophan residue at position 120. This tryptophan residue is fully-conserved in all of the X-gene sequences (Lo *et al.*, 1998), and is also located in the protein domain that is important for the protein's various activities (Ritter *et al.*, 1991; Kumar *et al.*, 1996). In these regards, the unique tryptophan in HBx provides a probe of the local events during equilibrium and kinetic folding studies. These measurements of changes in the tryptophan environment during folding and unfolding can be combined with information on the formation and breakdown of secondary structure by far-UV CD measurements.

When rHBx was renatured by sequential dialysis of urea, it was folded into functional HBx, which was demonstrated by efficacy of its transcriptional activation of the adenovirus major late promoter. The refolding of rHBx to the native conformation was further demonstrated by fluorescence and CD analysis. The far-UV region of the CD spectrum is especially useful, since it provides a qualitative measure of the average secondary structure content. The decreased CD values of the refolded rHBx at 220 nm relative to the unfolded form demonstrated that rHBx adopts ordered structures with decreasing concentrations of urea. Also, the decreased fluorescence quantum yield and blue-shift of λ_{max} of the refolded form of rHBx relative to the unfolded molecule probably reflect an increase in the fluorescence quenching of one tryptophan residue and also transfer of the tryptophan residue to a more hydrophobic environment. One possible explanation for the unusual fluorescence quenching upon refolding could be the consequence of a decrease in flexibility of the protein, moving the tryptophan close to the charge and therefore facilitating the fluorescence quenching. From the amino acids sequence analysis of HBx, the best possible quencher appears to be the aspartate residues located in the acidic region (amino acids 106–127). Studies on the three-dimensional structure of the native HBx will be required for better understanding of the fluorescence quenching. Indeed, the lower fluorescence quantum yield of the native form was reported for some proteins (Brems *et al.*, 1985).

The ability to obtain the fluorescence spectrum of protein folding intermediates will allow a better understanding of the structural nature of these intermediates. The wavelength of maximal emission for tryptophan provides a qualitative measure of the environments of the tryptophan in these structural changes. Hence, equilibrium refolding studies of rHBx were conducted using fluorescence spectroscopy. The transition curve was constructed by plotting the shift in maximum wavelength versus the denaturant concentration. The biphasic nature of the fluorescence curve implicates the presence of equilibrium intermediates at intermediate urea concentrations. These experiments support the view that equilibrium refolding of rHBx is not described adequately by a cooperative two-state system. A three-state model suggests that intermediates exist at a significant concentration between the unfolded state and the folded state.

Fluorescence of the tryptophan residue in the intermediates is different from either the fully- folded or the unfolded state. The position of the fluorescence maximum suggests that the tryptophan residue becomes less solvent-exposed in the intermediates. CD

measurements also show that the intermediates in the renaturation profile of rHBx adopt ordered structures. Since RP-HPLC analysis was successfully applied to detect the native and fully-denatured forms as well as intermediate forms (Lu *et al.*, 1992), refolding of rHBx was monitored by RP-HPLC to detect the folding intermediates. The fully-reduced (U), partially-folded (I), and the folded (N) forms could be separated by RP-HPLC. Moreover, HPLC analysis indicates that the intermediates were eluted between the unfolded state (U) and the folded state (N), suggesting that the intermediates have less hydrophobicity than the unfolded states. These results support that the intermediates exhibit ordered structure with a looser, more dynamic conformation, in which persistent tertiary interactions are minimized. Therefore, it is likely that the intermediates are the result of local losses of structure, which have molten globule characteristics (Brems *et al.*, 1985). The molten globule describes a conformational state with a hydrodynamic volume between that of native and unfolded states, which contains secondary but little tertiary structure. A considerable number of proteins have been shown to fold through molten globule intermediates, I, some exhibiting a stable molten globule under mild denaturing conditions. The molten globule model suggests that the transition to the fully-folded state should be non-cooperative.

The kinetic experiments were performed in order to characterize the folding pathway. They can be used to detect metastable intermediates and to characterize their structure and rate-determining transition state of folding. In refolding experiments, rHBx in the denaturating buffer is rapidly diluted into native buffers. The folding is monitored by the same probe as in the equilibrium experiments. The kinetic curves for rHBx could be fit to a bi-exponential with high quality, which is associated with the formation of folding intermediates, I. Kinetic complexities usually result from conformational heterogeneity of the folding state. In most cases, the heterogeneity rises from intrinsically slow conformational steps, such as isomerization of peptide bonds (Schmid, 1992) and disulfide bond formation or rearrangements (Creighton, 1990; Weissman and Kim, 1992). Disulfide formation is a kinetic liability, at least for *in vitro* folding. Folding from the denatured state is very fast (ms to min) for small proteins that do not contain disulfide bonds or in which the native disulfide bonds are intact. However, when folding requires disulfide formation, the renaturation rate is usually much slower (min to h) (Saxena and Wetlanfer, 1970). Among the nine cysteines of HBx, three conserved cysteines (Cys-61, Cys-69, and Cys-137) were found to be crucial for its *trans*-activation function (Arii *et al.*, 1992; Kim *et al.*, 1993; Kumar *et al.*, 1996), which could be involved in the formation of disulfide bonds. Hence, the complex folding kinetics of rHBx could be due to formation of the intramolecular disulfide linkages, which are essential to the structure and function of the folded rHBx. Determination of the free-SH groups from each refolding step will help to gain insight into the structure of intermediates. Further studies are thus required to characterize the structure of intermediates, including their disulfide bond status.

In summary, these studies demonstrate that the intermediate folded forms of rHBx accumulated upon refolding with removal of denaturant. At intermediate urea concentrations (i.e., 2 M), rHBx appears to exist in a partially-folded state. These partially-folded intermediates have been noted to possess many of the properties of the molten globule state. Further experiments are necessary for a better understanding of the nature of the intermediates that are formed during the folding of rHBx.

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