



## Identification and Biophysical Characterization of Acid Induced Conformational State of A Single Chain Antibody Fragment

Ganesh Patil<sup>1</sup>, Hauke Lillie<sup>2</sup>, Rainer Rudolph<sup>2</sup>, Christian Lange<sup>2</sup>

<sup>1</sup>Vidya Pratishthan's School of Biotechnology, Baramati, Vidyanagari, 413 133, Maharashtra, India.

<sup>2</sup>Institute for Biotechnology, Martin Luther University, Halle, Kurt Mothes Strasse 3, D-06120 Halle, Germany.

\*Corresponding author's E-mail: [ganeshprotein@gmail.com](mailto:ganeshprotein@gmail.com)

Accepted on: 15-10-2013; Finalized on: 31-01-2014.

### ABSTRACT

Exposure to acidic conditions leads to varying conformational changes in wide range of biomolecules. Alternatively folded or molten globule-like conformational states have been reported for a series of proteins, including immunoglobulins and their derivatives. During assessment of renaturation properties of scFvOx and impact of pH on the stability, a conformation with distinct blue shift at acidic conditions was identified. This state was different from native and denatured species of the protein. Moreover, this conformation was found to be reasonably stable upon denaturant induced unfolding studies. However, CD spectroscopy and analytical ultracentrifugation studies revealed aggregation prone tendency of this antibody fragment construct. In reports available so far this was not the case. A thermodynamically compromised acid-induced conformation was reported.

**Keywords:** Antigen binding agent, Fluorescence spectroscopy, Single chain antibody fragment.

### INTRODUCTION

Immunoglobulin fold proteins and their recombinant derivatives are widely emerging as future age medicaments. Therefore, it is useful to have more detailed possible information about their structural stability properties under varying variety of experimental conditions. An alternatively folded state (AFS) is a clearly different from the native state with intact disulfide bonds.<sup>1-3</sup> This conformational state is formed upon exposure of protein molecule at deep acidic pH, such as 2-3. AFS is characterized by major changes in the peptide region of CD and fluorescence spectrum reflecting large structural rearrangements. These alternatively folded conformations show reasonable protein stability of the low-pH structure of antibodies is comparable to that of the native protein at pH 7.0.<sup>1,2</sup>

Several reports have discussed occurrence of acid induced structures for full length antibodies and their derivatives. Initially, studies were focused on full length antibodies' constant fragment of the light chain and the murine monoclonal antibody MAK33.<sup>1,2</sup> Subsequently, this observation was found to be applicable to smaller domains such as Fab fragment.<sup>4</sup> Welfle, *et al.* extrapolated pH induced conformational change findings to set of various immunoglobulin formats simultaneously *i.e.* monoclonal antibody, Fab and its Fc fragments.<sup>3</sup> Same observations were found to be applicable for the dimeric C<sub>H</sub>3 domain of the Fc fragment.<sup>5</sup> Summarily, IgG derivatives (Fab, V<sub>L</sub>, V<sub>H</sub>, C<sub>H</sub>1 and C<sub>H</sub>3) have been shown to adopt the AFS.

Martsev, *et al.* were the first to report differently structured state of an anti-ferritin scFv antibody fragment F11 under acidic conditions. In this report a partially structured state with a distorted  $\beta$ -sheet secondary structure resembling to the AFS adopted by an

immunoglobulin at acidic pH was described.<sup>6</sup> However, these scFv constructs were found to be reasonably stable during as by differential scanning calorimetric analysis. Recently, Feige *et al.*, reported striking differences in the biophysical properties of the alternatively folded of individual antibody domains that reflect the variation possible for domains of highly homologous native structures.<sup>7</sup> Interestingly, Kanmert, *et al.*, reported for the first time AFS could also be induced by heating human IgG4-Fc to 75°C at neutral pH and at a physiological salt concentration.<sup>8</sup>

We report herein acid induced conformational state of recombinant origin single chain variable fragment of an antibody fragment (scFv) against hapten oxazolone (scFvOx). These observations were reported for the first time for this scFv construct. On the way to understand refolding of antibody fragments and effect of pH on its renaturation yield these well discussed pH induced conformations were identified. To gain further insight into the underlying mechanisms of the formation of the acid induced conformation, we performed incubation of scFvOx at pH 2-3 and was characterized in further detail. pH dependent transition and occurrence blue shift transition followed by acid induced denaturation has been reported for scFvOx. CD spectroscopy and analytical ultracentrifugation studies reflected aggregation prone tendency of this protein.

### MATERIALS AND METHODS

#### Expression and preparation of scFvOx

The coding DNA sequence of recombinant anti-oxazolone single-chain antibody fragment (scFvOx), fused to sequences coding for an N-terminal hexahistidine tag and a C-terminal *myc* tag, was obtained from the expression

construct scFvOx/pHEN-1,<sup>9</sup> kindly provided by Dr. U. Fiedler (IPK, Gatersleben, Germany). The coding sequence was inserted between the unique *NdeI* and *BamHI* restriction sites of the bacterial expression vector pET15b (+). ScFvOx protein was expressed in *E. coli* BL21 (DE3) cells that had been co transformed with the helper plasmid pUBS520.<sup>5</sup> Procedure for expression, isolation of inclusion antibodies, renaturation and quantification of scFvOx has been described earlier in Lange, et al.<sup>10</sup>

### Purification of scFvOx

After production, preparative isolation and renaturation of scFvOx, the protein was purified by ion exchange chromatography, using a pre-packed 1mL Hi-Trap SP-Sepharose column (GE Healthcare). The renatured protein applied for purification was found to be relatively pure (data not shown, reported in 8). 45 mg of the protein was loaded on the column. No scFvOx was detected in the unbound and washing fractions. Bound protein was eluted with a gradient of increasing NaCl concentration. The purity of homogeneous pure protein with a relative molecular weight of 29kDa as observed by silver stained SDS-poly-acrylamide gel. Pure fractions corresponding to the size of scFvOx based on SDS-PAGE were pooled together. The biological activity of purified and refolded scFvOx was accessed by ELISA i.e. scFvOx antigen binding activity. Further the purified protein was characterized by CD spectroscopy, analytical size exclusion chromatography, reverse phase high performance chromatography and mass spectroscopy (data not shown, reported in 12).

The purified protein was quantified by using the extinction coefficient 280 for denatured scFvOx ( $49,740 \text{ M}^{-1} \text{ cm}^{-1}$ , corresponding to  $1.731 \text{ mL mg}^{-1} \text{ cm}^{-1}$ ) was calculated from the amino acid composition.<sup>11</sup> The concentration of a sample of purified refolded scFvOx was determined using the calculated extinction coefficient, and this sample served as a standard for the calibration of the antigen-binding assay.

### pH-dependent stability

The pH-dependent stability analysis was performed by monitoring changes in intrinsic tryptophan fluorescence. The native protein concentration used for this experiment was  $7.5 \mu\text{g mL}^{-1}$ . Buffers contained 0.1 M citric acid, 0.1 M phosphoric acid and 0.1 M boric acid, and were adjusted to the indicated pH values by addition of NaOH. The samples were incubated for 24 h at 20°C. Measurements were performed as discussed below.

### Fluorescence spectroscopy

Fluorescence spectroscopy measurements were performed with a FluoroMax-3 spectrophotometer. Spectroscopic analyses were carried out at a temperature of 20°C in a 1 cm quartz cuvette. Slit widths for excitation and emission were set to 5 nm. The excitation wavelength was set to 280 nm, and emission spectra were recorded in 1 nm intervals from 290 to 440 nm.

### Circular dichroism spectroscopy

CD spectroscopy was performed with a Jasco J710 CD spectrometer. Measurements were performed at 20°C in a 0.5 mm quartz cuvette. Far UV CD spectra were recorded between 190 and 260 nm in 1 nm intervals, and 10 recordings were averaged.

### Analytical ultra-centrifugation

Sedimentation velocity scans were measured at 20°C in a Beckman XL-A ultra-centrifuge using double sector cells and an An Ti 50 rotor. The protein concentration was  $0.15 \text{ mg mL}^{-1}$ . The experiments were performed at 40,000 rpm, and optical scans were recorded every 10 minutes at 280 nm. The sedimentation equilibrium was established by centrifugation at 16,000 rpm for 45 h.

## RESULTS

### Expression and analysis of scFvOx

Detailed studies on expression, analysis and characterization of scFvOx have been documented earlier.<sup>12</sup>

### pH dependent stability of scFvOx

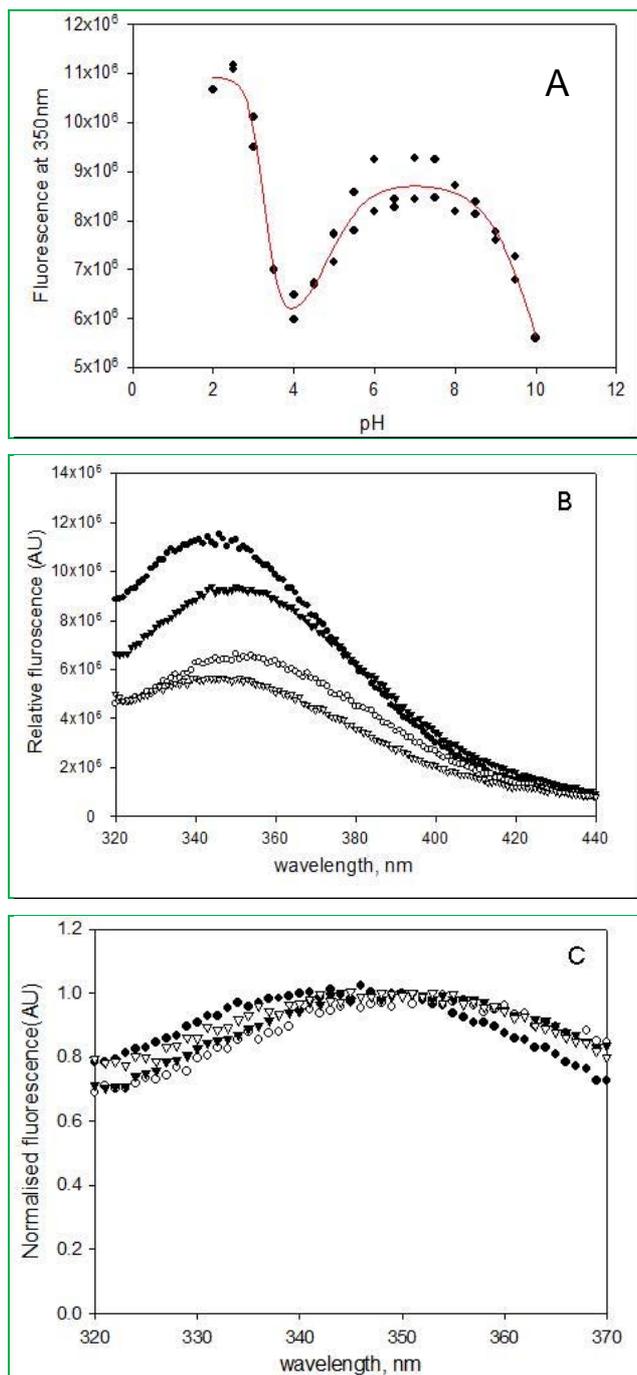
The influence of pH on the structural stability of scFvOx was investigated at pH between 2.0 and 10.0 (Figure 1A). For this purpose, purified scFvOx samples were incubated in a combined buffer system at the desired pH values for 24 h. In order to assess pH-induced structural changes, the fluorescence emission of the aromatic residues was measured at 350nm (Figure 1A).

Protein was found to be stable in between pH 6.0 to 8.0. Apparently, acid and base-induced denaturation occurred below pH 5.0 and above pH 9.0, respectively (Figure 1A). Below pH 4.0, an apparent second acid-induced transition caused a strong increase in the fluorescence signal at 350 nm (Figure 1A). The maxima of the fluorescence emission spectra of scFvOx at pH 4.0 and pH 9.5, respectively, show a red shift, similar to the spectrum of GdnHCl denatured protein at pH 7.0 (Figure 1B and 1C).

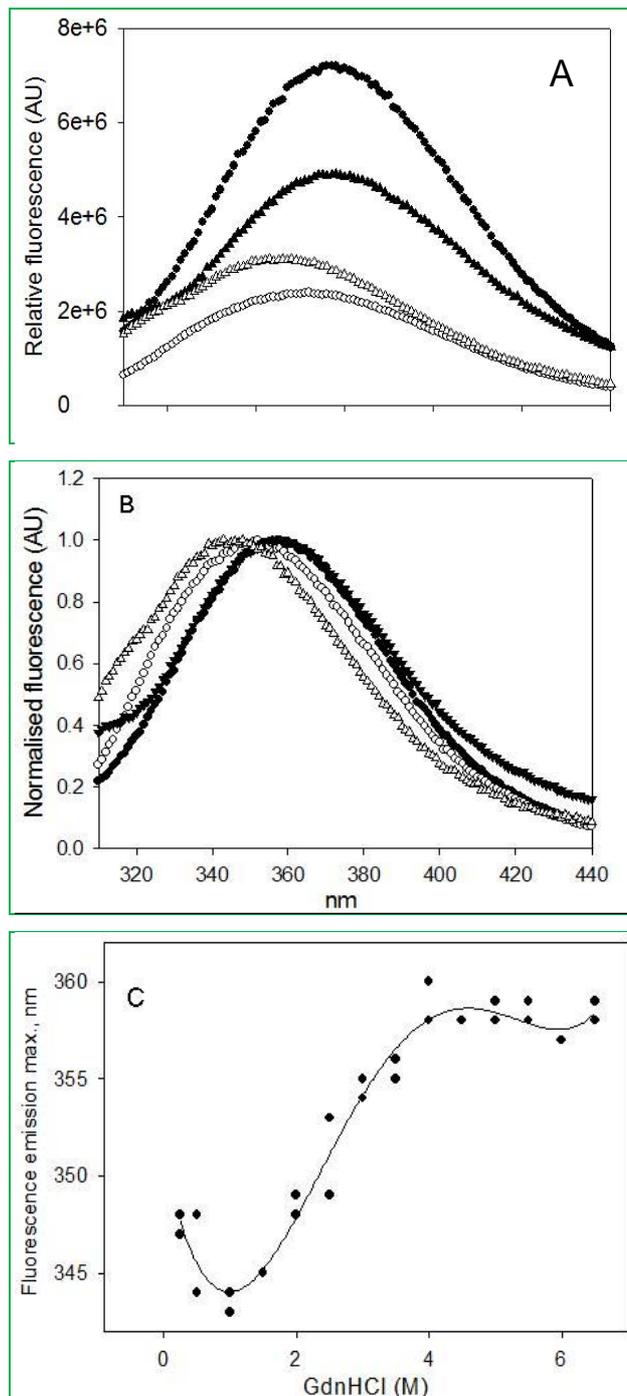
The overall fluorescence intensity of the acid- and base-denatured protein was decreased compared to that of the native state, while chemical denaturation (Figure 1.1, B and C) had led to a strong increase in maximum fluorescence intensity. In contrast, the fluorescence emission spectrum of scFvOx at pH 2.0 showed a significant blue shift of the emission maximum to 341 nm (Figure 1B and 1C). The maximum fluorescence emission intensity was approximately 30% higher than that of the native protein at pH 7.0 (Figure 1B and 1C). The data have been normalized in Figure 1C.

These observations suggest the sequestration of the aromatic amino acids within the interior of the protein and therefore some degree of structure formation of scFvOx polypeptide at pH below 3.0. Acid-induced alternatively folded or molten globule-like conformational states have been described for a series of proteins, including immunoglobulins and antibody fragments.<sup>1, 13</sup>

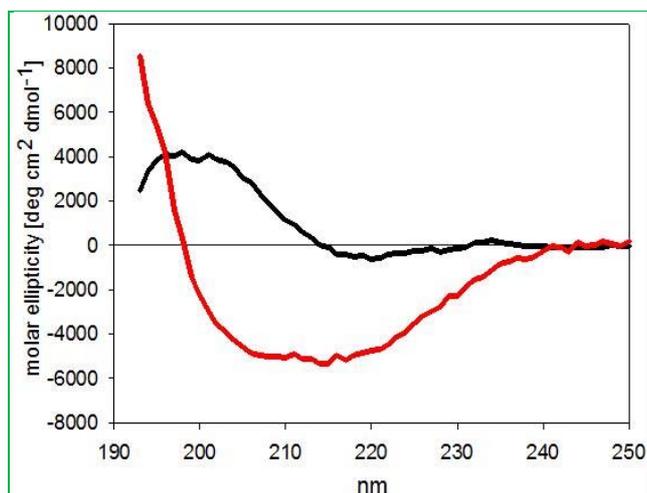
This is the first report on appearance of pH induced conformational state during an entire spectrum of a pH transition curve.



**Figure 1:** pH dependent stability of scFvOx; A. pH transition of scFvOx - For analysis,  $7.5 \mu\text{g mL}^{-1}$  protein were incubated at  $20^\circ\text{C}$  in a buffer containing 0.1 M citric acid, 0.1 M phosphoric acid and 0.1 M boric acid adjusted to the indicated pH values. Excitation was done at a wavelength 280 nm. Fluorescence emission at 350 nm was monitored. The solid line is meant to guide the eye. B. Fluorescence emission spectra of scFvOx at different pH values - at pH 2.0 (-●-), at pH 4.0 (-○-), at pH 7.0 (-▼-), and at pH 9.5 (▽-) respectively. C. Normalized fluorescence data- from figure B.



**Figure 2:** Spectroscopic characterization of acid-induced state; A. Fluorescence spectra and B. Normalized data of the fluorescence spectra A- For analysis native  $7.5 \mu\text{g mL}^{-1}$  of protein were incubated in 50 mM sodium phosphate, pH 7.0, containing 50 mM NaCl (-○-), 50 mM of phosphate, containing 6 M GdnHCl (-●-). 50 mM sodium phosphate, pH 2.0, containing 50 mM NaCl (▽), and 50 mM of sodium phosphate, pH 2.0, containing 6 M GdnHCl (-▼-). C. Denaturant-induced unfolding transition- For analysis  $7.5 \mu\text{g mL}^{-1}$  of protein was incubated in 50 mM sodium phosphate, pH 2.0, containing the indicated concentrations of GdnHCl. After incubation for 16 h at  $20^\circ\text{C}$ , fluorescence emission spectra were measured. The solid line is meant to guide the eye. Excitation wavelength was set at 280 nm.



**Figure 3:** CD spectra of scFvOx; Recorded in 25 mM sodium phosphate, containing 50 mM NaCl, adjusted to pH 2.0 (---), and pH 7.0 (---) respectively.

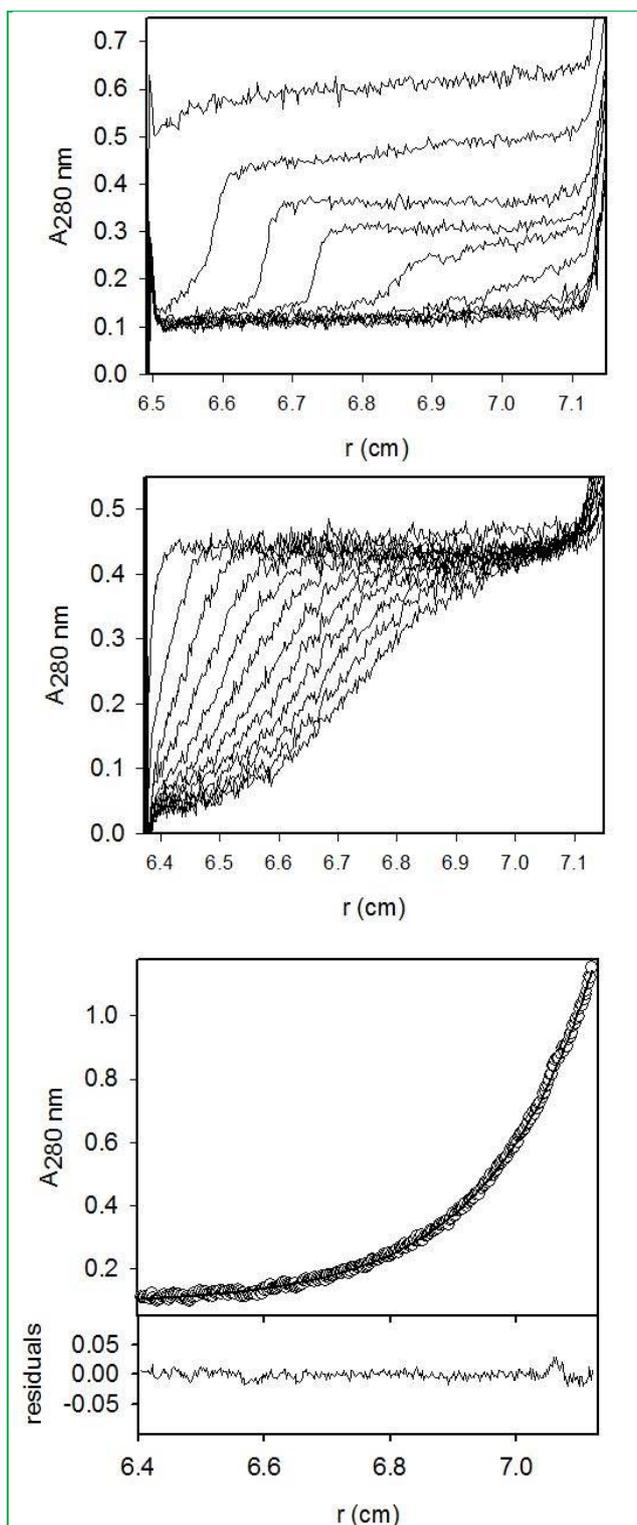
### Characterization of the acid-induced state

#### GdnHCl-induced unfolding of the acid-induced state

The putative acid-induced state of scFvOx was further characterized. For this purpose, the fluorescence spectra of scFvOx were recorded at pH 2.0 in the presence or absence of the denaturant GdnHCl and compared to the spectra of native and denatured protein at pH 7.0 (Figure 2A and 2B). As described above, scFvOx exhibited an increase in fluorescence emission intensity and a blue shift of the emission maximum to 343 nm (Figure 2A and 2B) at pH 2.0. This blue shifted fluorescence spectrum was clearly distinguishable from that of the native as well as from that of the GdnHCl-denatured spectrum at pH 7.0. After addition of 6 M GdnHCl at pH 2.0, a red shift in the fluorescence emission maximum of scFvOx to 359 nm was observed (Figure 2B), this corresponds to fluorescence maximum of the GdnHCl-denatured protein at pH 7.0. These findings suggest that the acid-induced conformation at pH 2.0 and the GdnHCl-induced conformations represent distinct protein states.

In order to investigate the conformational shift from the acid-induced state to the GdnHCl induced state at pH 2.0, scFvOx was incubated for 6 h at pH 2.0 and subsequently transferred to buffers containing increasing concentrations of GdnHCl at pH 2.0. After additional incubation for 24 h, fluorescence spectra of the protein samples were measured. Formation of aggregates at pH 2.0 precluded a quantitative evaluation of the transition between acid induced and GdnHCl-induced state. However, when the maxima of the fluorescence emission spectra were plotted against the concentration of denaturant, a cooperative transition with a midpoint at approx. 2.6 M GdnHCl was observed (Figure 2C).

This suggests that the acid-induced state contains tertiary contacts that have to be disrupted simultaneously upon unfolding, and defines an alternative folded state of the protein.



**Figure 4:** Analytical ultra-centrifugation of scFvOx: A. pH 2.0 and B. pH 7.0: The analysis was performed in 25 mM sodium phosphate, pH 2.0, containing 50 mM NaCl and 25 mM sodium phosphate, pH 7.0, containing 50 mM NaCl. Protein concentration was  $110 \mu\text{g mL}^{-1}$ . The sedimentation velocity run was carried out at 40,000 rpm and  $20^\circ\text{C}$ . Scans were taken every 10 minutes. C. Equilibrium sedimentation experiment: The sedimentation equilibrium was established by centrifugation at 16,000 rpm for 45 h. The equilibrium distribution of a monomeric species was fit to the data (---). Lower panel: Residuals of the fit.

### Circular dichroism

The far-UV CD spectrum of the acid-induced state of scFvOx was recorded at pH 2.0 (Figure 3). Interestingly, the spectrum at 220 nm showed significantly higher amplitude than that of the native protein and indicated a significant content of ordered secondary structure elements. In addition more negative ellipticity is indicative of aggregation prone-tendency of the protein under experimental condition. The content of  $\beta$ -sheet, helix and turn was  $22.07\% \pm 7.2$ ,  $1.9\% (\pm 0.018)$  and  $23.21\% (\pm 0.019)$ , respectively. These observations support the idea that the acid-induced state of scFvOx assumes an alternative, ordered conformation with a high content of secondary structure elements.

### Ultra-centrifugation analysis of scFvOx

During the GdnHCl-induced unfolding of scFvOx at pH 2.0 (cf. sub-section above), a quantitative analysis of the results was precluded by loss of signal during the incubation of the samples, possibly due to protein aggregation. In order to evaluate oligomerisation of aggregation status, scFvOx was subjected to a sedimentation velocity ultra-centrifugation. In this an experiment was performed at two different conditions pH 2.0 and 7.0 (Figure 4). At pH 2.0 the protein rapidly settled down in the measurement cell, clearly indicating aggregation (Figure 4A). This observation clearly reflects aggregation tendency to scFvOx protein under these conditions. However, this observation was in contradiction to the several reports suggesting these structures are significantly stable under these conditions.<sup>4,1</sup> However, situation at pH 7.0 (Figure 4B) was in contrast. Under the conditions of the measurement, scFvOx was not found to form aggregates (Figure 4B and 4C). The sedimentation velocity of the native protein corresponded to a sedimentation coefficients (app) of 2.16 S. This value was consistent with monomeric scFvOx. The evaluation of the equilibrium sedimentation experiment (Figure 4B) gave a molecular mass of 27.4 kDa, in good agreement with the expected value of 29 kDa for the monomeric protein.

The very limited solubility of the acid-induced conformation of scFvOx also precluded its thorough biophysical characterization. Although, hallmarks of a proper AFS could be observed spectroscopically, definite proof for the existence of a distinct thermodynamic state remained elusive.

### DISCUSSION

Changes in physico-chemical parameters such as pH, temperature and salt concentration or addition of moderate amounts of denaturants to a protein solution are associated with the protein forming non-natively folded states. Immunoglobulin i.e. IgG and some of its derivatives having reported capable of forming alternative structures, induced by acidification.<sup>1,13</sup> This state is in many aspects related to the molten globule but

with distinguishing properties related mainly to chemical stability and formation of oligomeric structures.<sup>14, 15</sup>

Acid-induced state of scFv fragments, as observed for scFvOx for pH below 3.0 in this study (Figure 1, 2 and 3), has not been previously reported. This state was characterized by a blue shift in the fluorescence emission maximum, apparent cooperative folding and a pronounced tendency to aggregate (Figure 1, 2 and 3). CD spectroscopic observations suggested that the acid-induced state had higher content of  $\beta$ -sheet secondary structures than native scFvOx. This co-operative unfolding indicates an ordered conformation with tertiary contacts, which suggests the formation of an acid induced conformation of scFvOx similar to AFS. However, in contradiction to previous reports with this family of molecules under similar conditions scFvOx was found to be aggregation prone.

Incubation of proteins at low pH often leads to the loss of their native structure. The product of this denaturation process is often not a random coil but an assembly of conformations termed molten globule.<sup>15-17</sup> The molten globule, originally characterized for  $\alpha$ -lactalbumin.<sup>14-15, 18-19</sup> Exhibits a significant amount of secondary structure but only few stable tertiary contacts.<sup>15, 16</sup>

The stability of the molten globule is only marginal and unfolding is not a co-operative process. In the case of antibodies and antibody fragments (Fab, CH<sub>3</sub> domain) however, incubation of the protein at pH 2.0 appears to lead to a specific non-native structure and not a molten globule.<sup>2,3,13</sup> This conformation, termed the AFS, is characterized by co-operative unfolding transition indicating a stable tertiary fold. A similar conformation was described for a Fab fragment<sup>4</sup> and a dimeric CH<sub>3</sub> domain.<sup>5</sup> Domain interactions between the two polypeptide chains of the Fab fragment were found to stabilize the AFS.<sup>4</sup> In case of V<sub>H</sub>, variable domain of a heavy chain in another case, significant amount of tertiary structures were detected in case of acid induced conformation. Moreover, this least stable domain of immunoglobulin fold proteins under physiological conditions was found to be most stable in AFS. It is also the nearest structural format of scFv's. Thus, incubation of scFvOx at acidic pH leads to the formation of AFS like conformation. This phenomenon could be a common feature of the immunoglobulin fold.

Martsev *et al.*, reported certain unusual structural and functional properties of a scFv fragment F11. In this case a functional protein trapped under physiological conditions in a partially structured state was reported.<sup>6</sup> The scFv fragment F11 provides an unusual example of a functional partially structured state that, in contrast to previous observations, possesses long-term stability under physiological conditions. Despite structural similarity, differences in folding and stability exist between different domains.<sup>20</sup> pH induced transition and up shift in fluorescence are the direct evidences of structural rearrangements in scFvOx protein upon exposure to acidic



conditions. Recombinant antibodies, fragments and their derivatives are heading to towards important class of reagents for various biomedical applications. Therefore, stability properties of these molecules under various experimental conditions are a matter of scientific concern and interest.

### CONCLUSION

Incubation of scFvOx at acidic conditions showed characteristic blue shift in intrinsic fluorescence up-shift. ScFvOx showed precipitating tendency at acidic pH conditions. This observation might be applicable to other recombinant antibody fragment formats.

**Acknowledgement:** In fond memory of a great mentor Late Prof. Dr. Rainer Rudolph. This work was supported by the Federal State (Land) of Saxony-Anhalt within the framework of the research network "Wertschöpfung durch Proteineals Wirkstoffe und Werkzeuge" (Grant no. 3537 A/0903 L). GP received scholarship from German Ministry for Research and Development (BMBF).

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Source of Support: Nil, Conflict of Interest: None.

