4 Experimental Procedures

4.1 NMR Experiments

A common approach to solve the protein structures reported in this thesis was used and a minimal set of spectra was recorded on the fully ^{13}C-^{15}N-labelled protein samples in all three cases, as described in detail in the following paragraph. Experiments specifically recorded for only one of the proteins are covered in the respective subsections.

For all proteins, triple resonance CBCA(CO)NH / CBCANH, (Grzesiek et al., 1992a) and HNCO/HN(CA)CO spectra (Grzesiek et al., 1992b) were recorded for the assignment of the backbone resonances. To identify sidechain resonances, HBHA(CO)NH, H(CCCO)NH-TOCSY, CC(CO)NH-TOCSY (Montelione et al., 1992) experiments in H_2O and H(C)CH-COSY and CCH-COSY (Kay et al., 2002) experiments in 100% D_2O were recorded. All spectra were processed using the XWINNMR software (Bruker Biospin GmbH).

All NOESY spectra were acquired using 80 ms mixing time. In addition to a 3D-^{15}N-NOESY-HSQC in H_2O and a 3D-^{13}C-NOESY-HMQC in D_2O, a variety of other NOESY-type spectra were recorded that provide better resolution in crucial parts of the standard spectra. These additional NOESY-experiments are also covered in the specific subsections.

In general, the SPARKY software (Goddard et al., 2002) was used to handle all spectral information. Protein resonances and all NOESY-crosspeaks were assigned using this software. Also the evaluation of relaxation data and integration was achieved through algorithms implemented in SPARKY.

4.1.1 NMR Spectra Used in Chapter 2

The samples used in this study were produced in the same way as for the structural studies by Castellani et al. only they were kept in solution at acidic pH. Both glycerol labelled samples were used. In principle it is advisable to have a fully labelled sample on which an HN(CO)CA can be recorded to obtain information on the sequential identity of the HNCA peaks. All samples were of roughly the same protein concentration, which makes it easier to compare the peak volumes obtained from the spectra.

Spectra were recorded on Bruker DRX600 (^{15}N-HSQCs 2D-HNCOs) equipped with a 5 mm TXI Cryoprobe and DMX750 (3D HNCAs) spectrometers. ^{15}HSQC-spectra were recorded with 16 scans per complex point (256 x 1024 complex points). The HSQC data was processed using a squared sinebell in f1 and a gaussian in f2 shifted by 90° as a window function dimension and zero filled to 512 (f1) x 1024 (f2) real points using linear forward prediction in the indirect dimension. 2D HNCO-spectra (Grzesiek et al., 1992a) were recorded in constant time with 128 scans per complex point (128 x 1024 complex points). The HNCO data was processed using a squared sinebell in f1 and a gaussian in f2 shifted by 90° as a window function dimension and zero filled to 1024 (f1) x 2048 (f2) real points using linear forward prediction in the indirect dimension. 3D HNCA-spectra (Grzesiek et al., 1992a) were recorded in constant time with 8 scans per complex point (160 x 74 x 1024 complex points). The data was processed using a squared sinebell shifted by 90° as a window function in all dimensions and zero filled to 256 (f1) x 256 (f2) x 1024 (f3) real points using linear forward prediction in the indirect dimensions. All spectra were processed using XWINNMR from Bruker. All spectra were integrated in Sparky 3.11 (Goddard et al., 2002) assuming gaussian peakshapes.

4.1.2 Sample and NMR Experiments SODD

NMR-Spectra were acquired at 37°C on a Bruker DRX600 spectrometer using a uniformly ^{15}N and ^{13}C labelled sample containing 1.3 mM protein cosisting of amino acids N358 – G456, 20 mM KH_2PO_4, 50 mM NaCl and 0.02 % NaN_3 at pH 6.0 in 90 % H_2O 10 % D_2O. For backbone resonance assignments a complete series of sidechain selective 15N-HSQC-experiments (Schubert et al., 1999; Schubert et al., 2001a; Schubert et al., 2001b) and a third 3D set of HA(CACO)NH/HA(CA)NH experiments were additionally recorded. Sidechains of Q and N were assigned using a series of sidechain-NH2 selective COSY-Spectra (Schmieder et al., 1998). Interproton distance information for structure calculation was derived from ^{15}N-NOESY-HSQC (Clore et al., 1992), a methyl-group filtered ^{13}C-NOESY-HQQC a 2D-1H-1H-NOESY (Jenner et al., 1972) and a ^{13}C-NOESY-HMQC (Clore et al., 1992) spectrum.

4.1.3 Sample and NMR Experiments: CI-B8

NMR-Spectra were acquired at 27°C on Bruker DRX600 and DMX750 spectrometers in standard configuration. All experiments were performed on a uniformly ^{15}N and ^{13}C labelled sample containing 0.81 mM protein, 20 mM KH_2PO_4, 50 mM NaCl and 0.02 % NaN_3 at pH 6.0 in 90 % H_2O 10 % D_2O if not otherwise stated. Backbone resonance assignment was carried out based on sidechain selective 15N-HSQC-experiments for A,G,S and P (Schubert et al., 1999; Schubert et al., 2000; Schubert et al., 2001) and by the procedure described in the general sectionn. Sidechains of Q and N were assigned using a series of sidechain-NH2 selective COSY-Spectra (Schmieder et al., 1998). Interproton distance information for structure calculation was derived from ^{15}N-NOESY-HSQC (Clore et al., 1992) and a 13C-NOESY-HSQC in water. Furthermore, a methyl-group filtered ^{13}C-NOESY-HQQC, 2D-1H-1H-NOESY (Jenner et al., 1972) and ^{13}C-NOESY-HMQC (Clore et al., 1992) spectra were acquired in D2O.

4.1.4 Sample and NMR Experiments: An1 like Zinc Finger

NMR-Spectra were acquired at 27°C on Bruker DRX600 and DMX750 spectrometers in standard configuration. All experiments were performed on a uniformly ^{15}N and ^{13}C labeled sample containing 0.26 mM protein, 20 mM KH_2PO_4, 50 mM NaCl, 1 mM DTT and 0.02 % NaN_3 at pH 6.0 in 90 % H_2O 10 % D_2O if not otherwise stated. Resonances were assigned by the procedure described above supplemented by HCCH-TOCSY for the aromatic-region (Kay et al., 2002) in 100% D2O. Inter-proton distance information for structure calculation was derived from ^{15}N-NOESY-HSQC (Clore et al., 1992) and a ^{13}C-NOESY-HSQC in water and ^{13}C-NOESY-HMQC (Clore et al., 1992) spectra acquired in D_2O. Furthermore, a methyl-group filtered ^{13}C-NOESY-HSQC was recorded for that the ^{13}C center was set to the aromatic carbon frequencies.

4.1.5 Relaxation rate measurements

Relaxation rate measurements were performed as a series of ^{1}H-^{15}N-HSQC type spectra, into which a delay or a CPMG sequence was introduced to measure exclusively T_1 or T_2 relaxation (Farrow et al., 1994). Both T_1 and T_2 relaxation times were extracted from two series comprising each of eleven spectra with delays of 12, 52, 102, 152, 202, 302, 402, 602, 902, 2002, and 5002 ms for T1 and 6, 10, 18, 26, 34, 42, 82, 122, 162, 202, and 242 ms for T2. Rates and errors were fitted as implemented in the SPARKY software.

4.2 Resonance Assignments

For all proteins the backbone resonances were obtained from the pairs CBCA(CO)NH and HN(CA)CO 3D heteronuclear triple resonance spectra. This allowed the identification of most of the backbone amide cross-peaks in the ^{15}N-HSQC. Sidechain resonances were assigned using 3D H(CCCO)NH-TOCSY and CC(CO)NH-TOCSY spectra building on the assigned C^{α} and C^{β} resonances. Connectivities were

established with a 3D HCCH-COSY. This procedure leads to an nearly complete assignment of all ^{1}H,^{13}C and ^{15}N resonances.

4.2.1 Assignment of the SODD-BAG domain

In addition to the approach described above a complete set of amino acid selective experiments was used to guide the backbone assignment of SODD. For Asparagines and Glutamines Sidechain selective experiments were used to assign the sidechain amino groups.

4.2.2 Assignment of the Zinc finger domain

The crucial assignment of the histidine sidechains of the zinc-finger domain was achieved using the information from the ^{13}C-NOESY spectra. Both the H^{β}-aromatic crosspeaks from the normal ^{13}C-NOESY-HMQC as well as the signals from the NH-groups of the sidechains in the ^{15}C-NOESY-HSQC were evaluated. Interestingly, all residues that are coordinating the zinc centers have H^{β}-C^{β} chemical shifts in he same range.

4.3 Secondary structure Prediction

Secondary structure predictions were made using the chemical shift based approaches of PLATON (Labudde et al., 2002) and TALOS (Cornilescu et al., 1999). Chemical shift distributions of C^{α} and C^{β} chemical shifts were additionally analyzed according equation 4.1 (Metzler et al., 1993).

$$\Delta\Delta\delta_i = \frac{1}{3}\sum_{i-1}^{i+1}(\Delta\delta_{C\alpha_n} - \Delta\delta_{C\beta_n}) \qquad \text{(eq. 4.1)}$$

where $\Delta\delta C^{\alpha}_{i}$ and $\Delta\delta C^{\beta}_{i}$ are the differences of the observed chemical shift for C^{α} and C^{β} of an individual amino acid in the proteins and the random coil chemical shifts as obtained from BMRB.

4.4 Structure Calculation

4.4.1 Structure Calculation: SODD BAG-Domain

Structures were calculated by the program CNS 1.01 (Brunger et al., 1998) using a standard simulated annealing protocol. Initial trials with automatic NOE assignment with ARIA failed due to severe overlap in the ^{1}H-resonances of the methyl-groups. Thus the NOESY spectra were assigned manually. A total of 731 NOE-derived inter-residue distance restraints were extracted from a ^{1}H-^{1}H 2D NOESY, ^{15}N- and ^{13}C-edited 3D NOESYs and a special ^{13}C-edited 3D NOESY that allowed to resolve the methyl groups. The distance restraints were categorized in four classes, I: >3.5 Å, II: > 4.7 Å, III: > 5.5 Å and IV: > 6.5 Å. Peak-volumes of each spectrum were calibrated to a set of known distances. In addition, 52 distance restraints mimicking H-bonds based on characteristic short range NOE patterns, and 70 pairs of F,Y angle restraints generated by TALOS were used (Table 3.1).

4.4.2 NOE-Assignment and Structure Calculation: CI-B8

Assignment of NOESY-spectra was obtained using the automatic NOESY-assignment and structure calculation software CANDID / DYANA (Herrmann et al., 2002). Distance restraints were then ported back into SPARKY 3.1 and manually refined by iterative assignment corrections/structure calculations using CNS 1.0. The assigned distance restraints were categorized in three classes, I: >3.2 Å, II: > 4.2 Å and III: > 6.5 Å, for these calculations. Final structure calculations were based on a total of 1166 NOE-derived inter-residue distance restraints, 37 distance restraints mimicking H-bonds based on characteristic short range NOE patterns, and 54 pairs of F,Y angle restraints generated by TALOS. A disulfide bridge between C24 and C58 was included. Nineteen structures selected by overall energy were then subjected to a refinement in

water using X-PLOR-NIH (Schwieters et al., 2003) and the parallhdg5.3 forcefields (Linge et al., 2003). For analysis of the final ensemble MOLMOL (Koradi et al., 1996), Procheck-NMR (Laskowski et al., 1996) and WHATIF (Vriend, 1990) were used.

4.4.3 NOE-Assignment and Structure Calculation: An1-like Zinc Finger Domain

The automated structure calculation using CANDID/DYANA led to an low quality ensemble that resembled the structure of the mouse homolog 1WFE. Therefore a model for our sequence was built by simple *in silico* mutagenesis and minimization based on the mouse structure. Since all residues that make up the core of 1WFE are conserved and the sequences have exactly the same length this conservative approach led to an model of the human protein that had no deviations in the structure of the backbone and the conserved sidechains. This model was then used a guide for the assignment of all NOE cross-peaks.

After initial trials with the approach successfully used for CI-B8, the NOE assignment was performed manually based on a model built from 1WFE. Structure calculations were performed using a slightly modified standard simulated annealing protocol in XPLOR-NIH (Schwieters et al., 2003). The modifications were introduced to allow more motion around the metal clusters in the high temperature stages. The clusters were built into the structure using colvalent bonds for that the bond energy was increasing from very low values to normal simulated annealing parameters during the progress of the protocol. This procedure is similar to the methods widely in use for disulfide bridges. After iterative assignment of the NOE peaks, a final ensemble was calculated that was then subsequently submitted to the water refinement protocol that was already used for CI-B8 (Linge et al., 2003).

4.5 Modelling of the SODD-BAG / HSP70 complex

The SODD BAG-domain was fitted into the hBag1 complex structure 1HX1 using the coordinates of the backbone atoms of amino acids 414, 420, 424, 438, 446 and 453. Sidechain conformations of these residues were manually optimized to achieve the same contacts of the corresponding residues in the hBag1/HSP70 complex. Missing atoms in the crystal structure were added and this starting structure was minimized with AMBER5 (Pearlman et al., 1995) prior to the low restrained molecular dynamics simulation.. For the minimization of the complex model all atoms of HSP70 and the C^{α}-atoms of SODD were kept fixed in space additionally restraints for the conserved contacts of the residues mentioned above were applied. Molecular dynamics of the system was simulated for 1 ns with AMBER5 using TP3 water. For the SODD restraints on C^{α}-atoms were released, allowing the domain itself to move, while additional HN-O restraints in a-helical regions and a few helix-helix restraints were introduced in this calculation to stabilize secondary and tertiary structure.

4.6 Confirmation of the Disulfide Bond in CI-B8

The oxidized state of the protein was confirmed by tryptic digestion of CI-B8 followed by mass-spectrometry. The expected fragments with m/z = 905.403 and 769.413 that contain C58 and C24 were missing entirely in the mass-spectrum. Instead, an additional fragment with m/z = 1674.816 that corresponds to the combined mass of the covalently linked fragments I21-R26 and E57-K64 was detected, showing clearly the existence of the disulfide bridge.

4.7 Surface Accessability of methyl-groups in CI-B8

To test the accessability of the surface to water simple T_1-measurements for the methyl-groups were performed using a slightly modified HMQC pulse sequence. In this sequence a 180° hard pulse on the protons and a adjustable delay were applied before the normal HMQC sequence. These experiments were performed with and

without adding the relaxation enhancing agent Gd^{3+}-DTPA-BMA to the solution. With each sample experiments with delays of 3 μs, 60 ms and 100 ms were recorded and the approximate T_1-relaxation rate was fitted as the slope of the volumes. The ratio of the relaxation rates with and without Gd^{3+}-DTPA-BMA was then mapped onto the methyl-groups of the protein

4.8 Determination of the Redox Potential of CI-B8

The redox potential of the oxidized CI-B8 was determined by a method described elsewhere (Mossner et al., 1998). Briefly, the protein was incubated with $[GSH]^2/[GSSG]$ redox buffers set to $[GSH]^2/[GSSG]$-ratios of 0.0004 to 1000 M at a constant GSSG concentration of 10 μM in the same buffer that was used for the NMR-measurements. The resulting structural changes were observed by tryptophane-fluorescence spectroscopy. Data from three measurements was normalized and averaged prior to the fitting of the model, which was modified to allow for cooperativity of the transition. For the calculation of the redox potential from the equilibration constant a standard potential of E0'= –240mV was used for the GSH/GSSG-couple at pH 7.0. To account for our different measurements conditions, the pH-dependency of the potentials was taken into account by the application of equation 4.2 (Wunderlich et al., 1993).

$$E_0^{pH} = E_0' - 60.2\ mV \times (pH - 7) \qquad (4.2)$$

4.9 References for the Experimental Section

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