2) Structural characterization of the TAP-N/CTE-B complex

Overview

In collaboration with Katia Zanier and Michael Sattler at the EMBL-Heidelberg and Dmitry Svergun at the EMBL-Hamburg, I have used multidimensional NMR spectroscopy and solution SAXS experiments, respectively, to investigate the surface of interaction and conformational arrangement of the TAP-N/CTE-B complex. Atomic information of the complex by means of X-ray crystallography was elusive, given the difficulty I faced in obtaining well-diffracting crystals of the TAP-N/CTE-B complex.

2.1) Crystallization of TAP-N/CTE-B

Even if I managed to repeatedly reconstitute a stable and homogeneous protein/RNA complex, crystallographic success remained elusive. An extensive screening of crystallographic conditions was performed, based on several commercially available sparse-matrix kits. Screens were set-up at 4°C and room temperature (12°C) for various concentrations of TAP-N/CTE-B complex and the free CTE-B. Initially, no crystals were obtained in any of the cases. A sample prepared for SAXS experiments gave however, pyramidal crystals that diffracted only weakly (6-7 Å) and could not be reproduced (figure 29). Further, the data collected with synchrotron radiation was of poor quality and could not be indexed. These results set however, the basis for future efforts in trying to obtain well diffracting crystals.

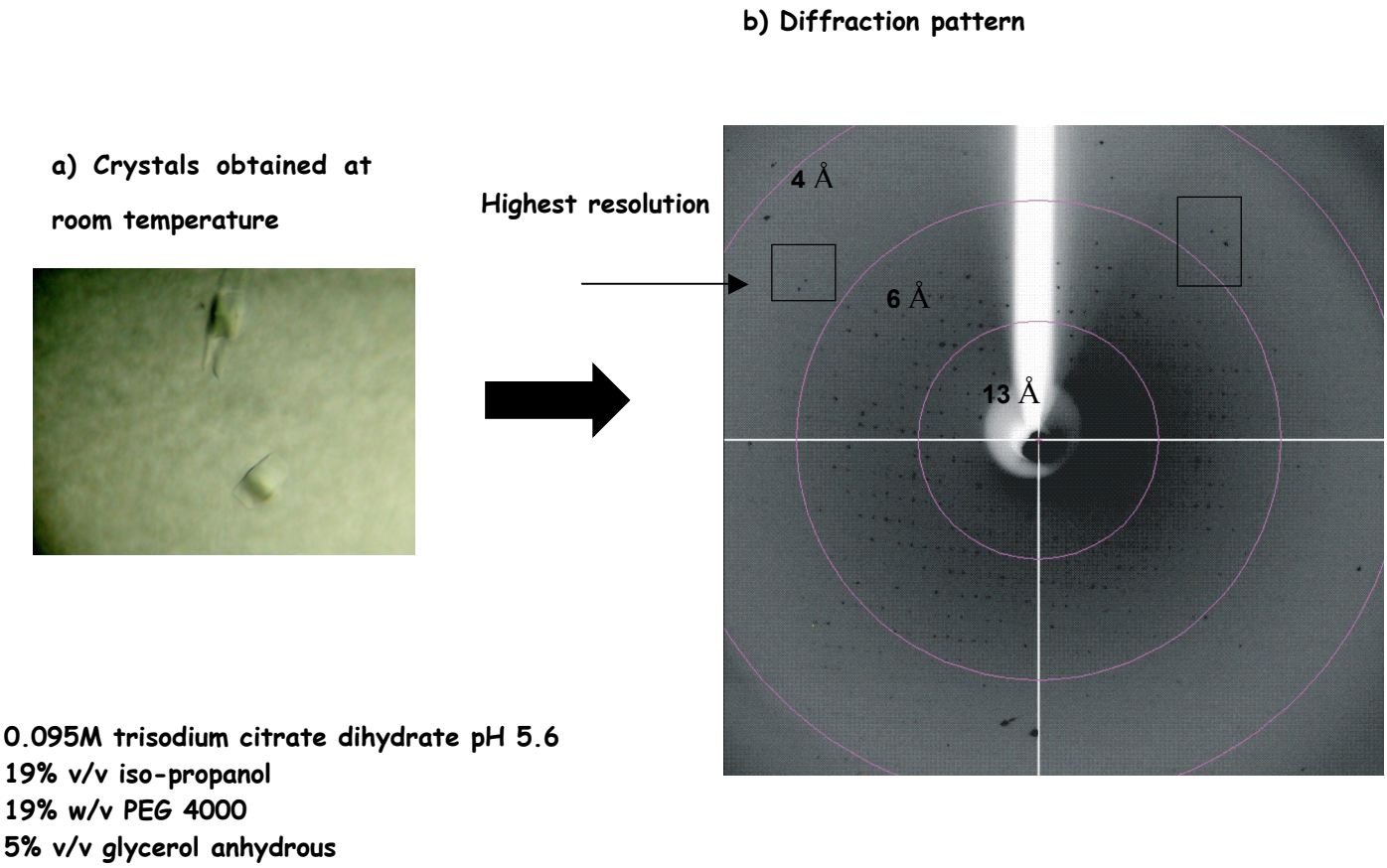

Figure 29. Crystallographic trials for a homogeneous TAP-N/CTE-B sample.

2.2) Multidimensional nuclear magnetic resonance (NMR): Mapping the surface of TAP-N that interacts with the CTE-B RNA

Overview

NMR-based chemical shift perturbation and backbone assignment experiments were performed to map the surface of TAP-N that interacts with the CTE-B RNA, on the basis of a "labelled receptor *vs* labelled receptor/unlabelled ligand" experimental strategy (figure 30).

2.2.1) Backbone chemical shift assignments

To assign the sequential backbone chemical shifts of the protein, a set of optimized multidimensional NMR experiments were performed, which are described in the section of materials and methods (section 4.1), and schematized in figure 30. The backbone chemical shifts of 85% of TAP-N's amino acid sequence were assigned unambiguously (figure 31) and annotated to its corresponding ^{1}H-^{15}N correlation peak on a reference $^{1}H^{N}$-^{15}N HSQC TROSY spectra of ^{2}H-, ^{15}N- TAP-N (figure 32a, 32b). A plot of the secondary chemical shifts ($\Delta\delta$) versus the primary sequence of TAP-N is shown in figure 31. This plot provides a qualitative means for evaluating the accuracy of the assignments, as its values correlate with the secondary structure of the corresponding protein. So to speak, $\Delta\delta$ has a positive value on regions of the protein sequence that correspond to α-helices, negative values where β-strands are present, and a value close to cero, where loops and disordered regions are located.

2.2.2) Chemical shift perturbation experiments

A reference $^{1}H^{N}$-^{15}N HSQC TROSY spectra of a ^{2}H-, ^{15}N- labelled TAP-N sample was recorded (figure 32a) and compared to the $^{1}H^{N}$-^{15}N HSQC TROSY spectra of a ^{2}H-, ^{15}N- labelled TAP-N sample, which had been fully titrated with unlabelled CTE-B RNA (figure 32b). The ^{1}H-^{15}N correlation peaks of the reference spectra that shift upon CTE-B RNA-binding were assigned to the amino acid sequence of TAP-N, and the corresponding backbone amides were docked onto the available crystal structure of TAP102-372 (figure 33a) (Liker *et al*., 2000). TAP-N samples used during these experiments were deuterated, in order to improve the signal to noise ratio and to reduce the signal overlap of the NMR experiments.

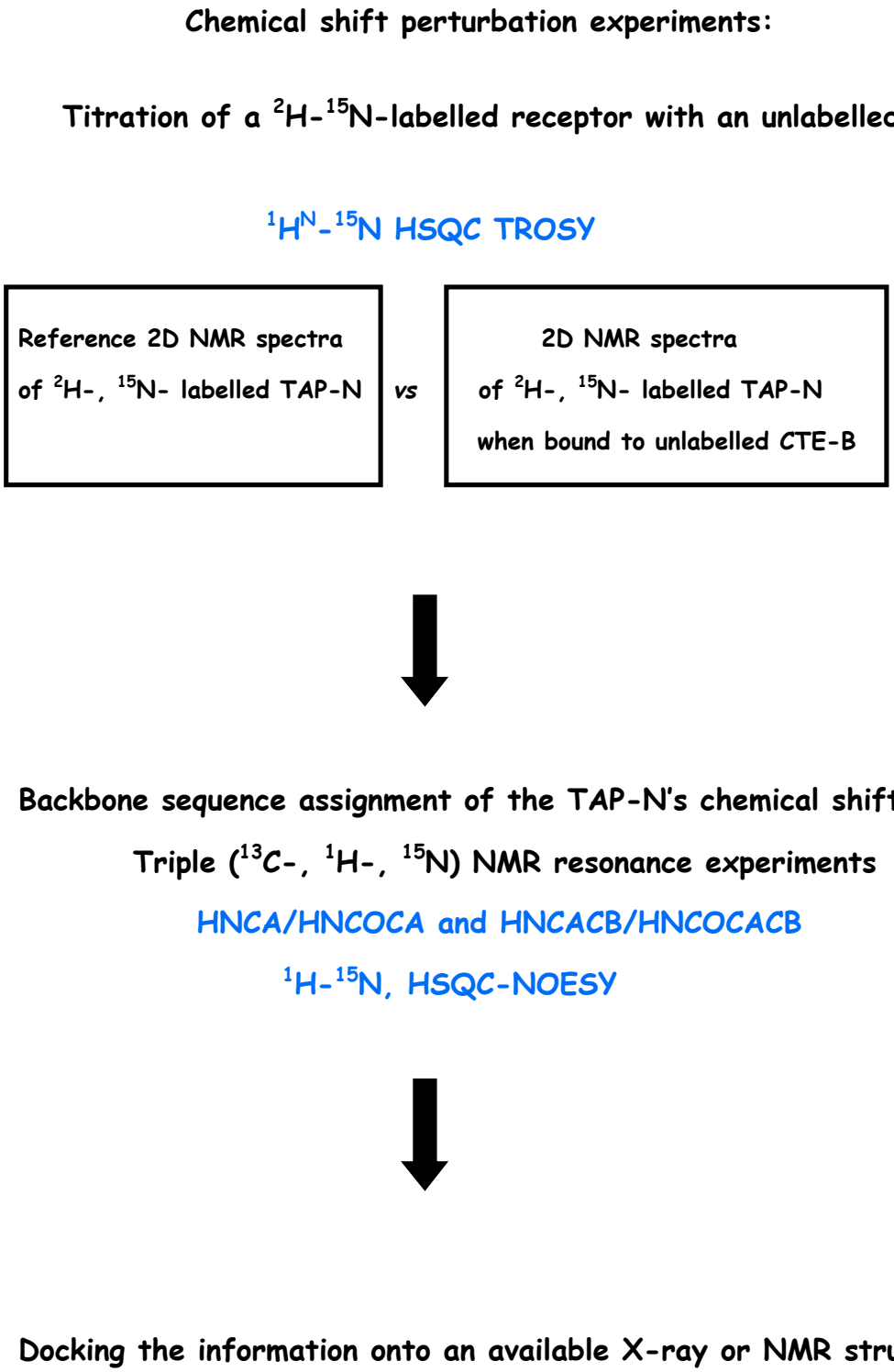

Figure 30. Diagram of the strategy used, for mapping the surface of interaction of TAP-N with the CTE-B RNA.

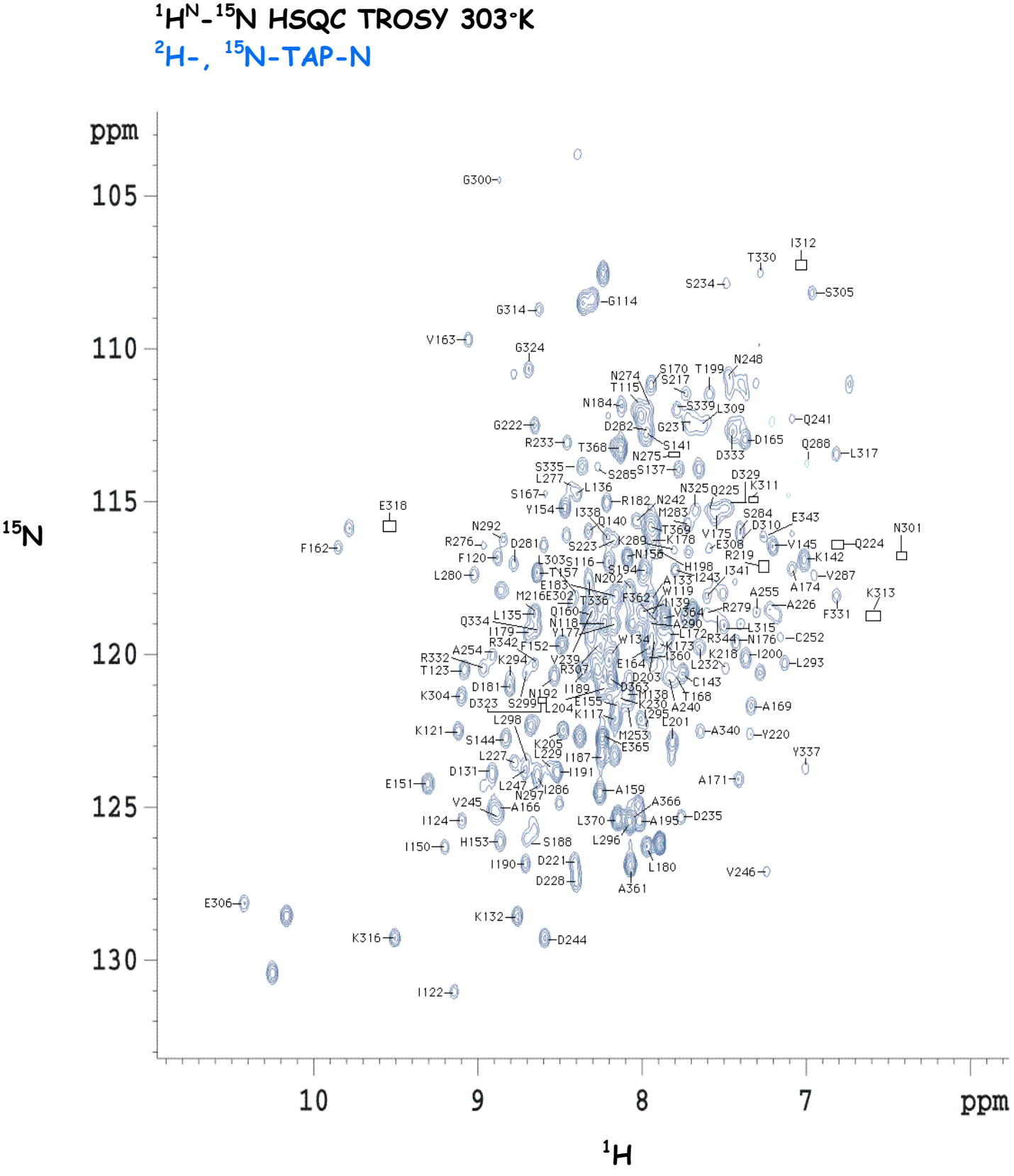

Figure 32a. Reference $^{1}H^{N}$-^{15}N HSQC TROSY of ^{2}H-, ^{15}N- TAP-N.

$^{1}H^{N}$-^{15}N HSQC TROSY 303°K

^{2}H-, ^{15}N-TAP-N

^{2}H-, ^{15}N-TAP-N/CTE-B

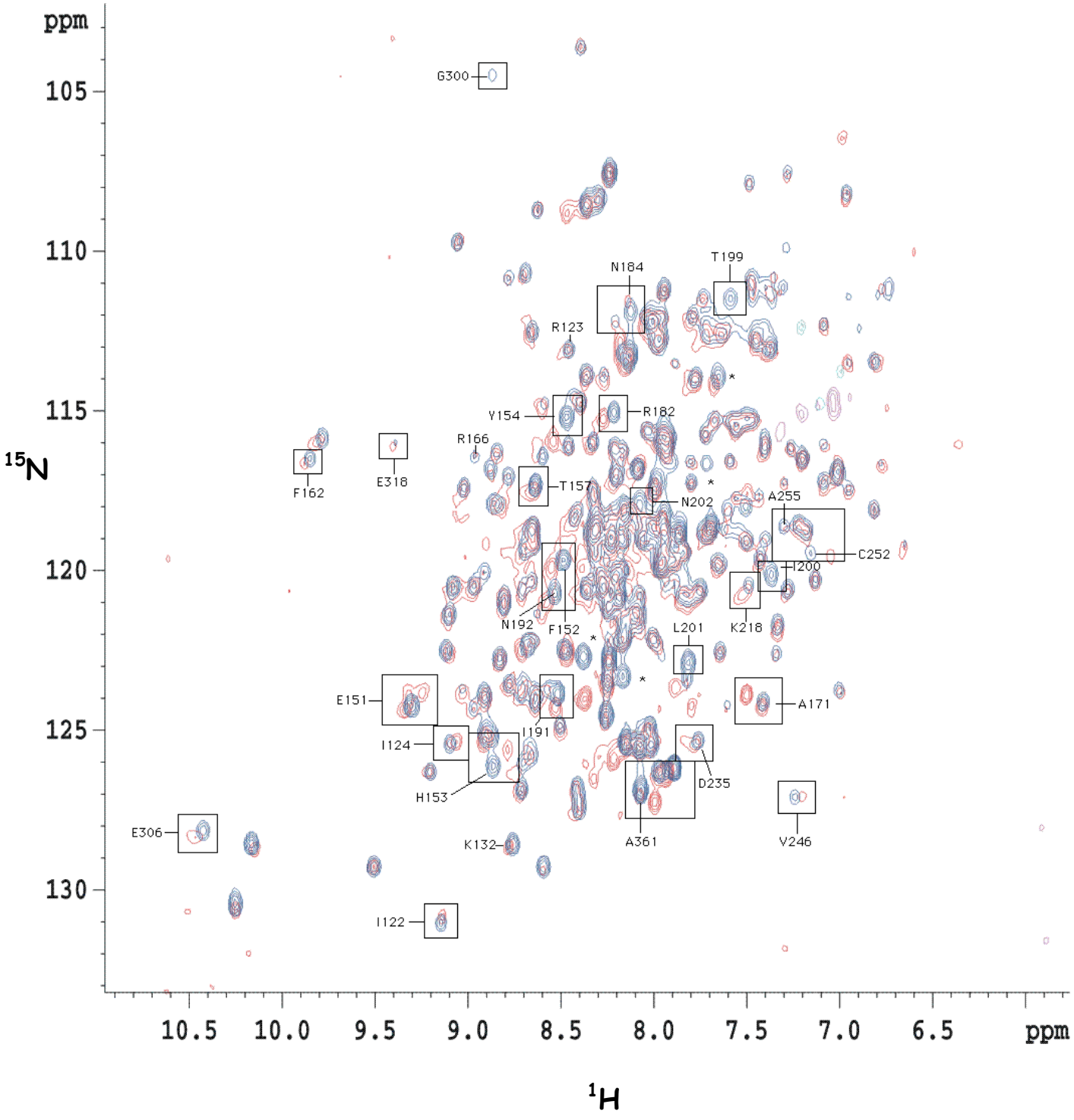

Figure 32b. Blue: Reference $^{1}H^{N}$-^{15}N HSQC TROSY of ^{2}H-, ^{15}N- TAP-N.
Red: $^{1}H^{N}$-^{15}N HSQC TROSY of ^{2}H-, ^{15}N- TAP-N bound to unlabelled CTE-B
* ^{1}H-^{15}N correlations that shift, but could not be assigned

Conclusions

In agreement with previous mutagenic studies (Liker E. *et al*., 2000; Coburn *et al*., 2001), most backbone amides perturbed, localize to the RBD of TAP-N and to the linker, with few scattered perturbations in the LRR domain, mostly on its convex side (table 1, figure 33a). We can conclude from these results that TAP-N binds to the CTE-B RNA through the β-sheet of the RBD and the C-terminal linker that connects to the LRR, on the side opposite to the β-sheet surface of the LRR domain (figure 33a, 33b). Whether or not the linker acquires conformation, and/or there is an induced-fit domain rearrangement, will be ruled out with NMR saturation transfer (ST) (Takahashi *et al*., 2000) and probably residual dipolar coupling (RDC) (Tjandra and Bax 1997) experiments. For this, the sequential backbone chemical shifts of the TAP-N/CTE-B complex are necessary.

Table 1

Mutagenesis data	NMR data
R 128	I 122 T 199
K 129	I 124 I 201
K 132	K 132 N 202
R 233	F 152 K 218
R 276	H 153 D 235
Y 278	Y 154 V 246
E 306	T 157 C 252
K 347	F 162 A 255
	A 166 G 300
	A 171 E 306
	R 182 E 318
	N 184 A 361

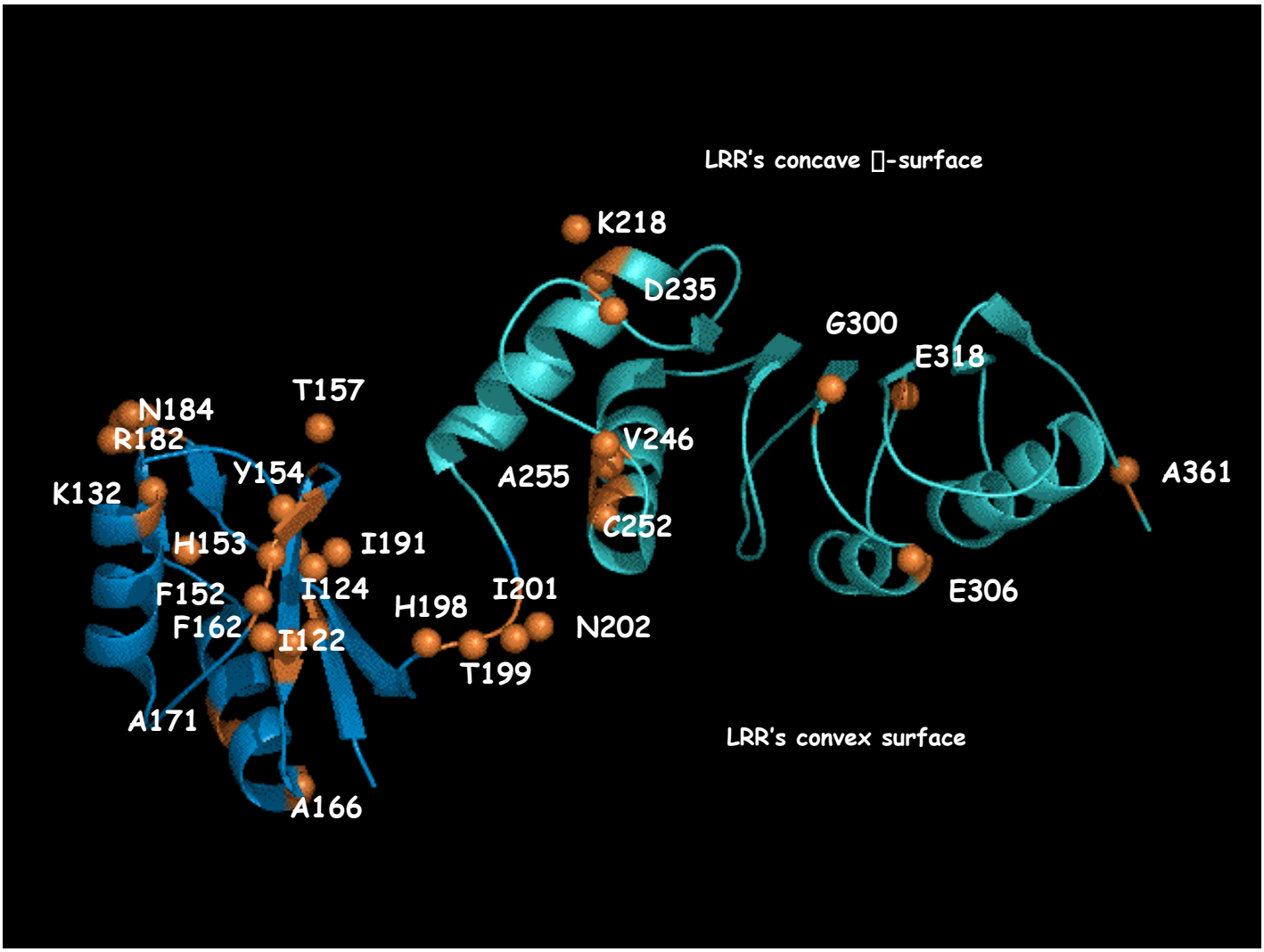

Figure 33a. The surface of TAP-N, where backbone amides are perturbed upon CTE-B RNA binding. Each orange sphere represents and amide on the backbone of TAP96-372.

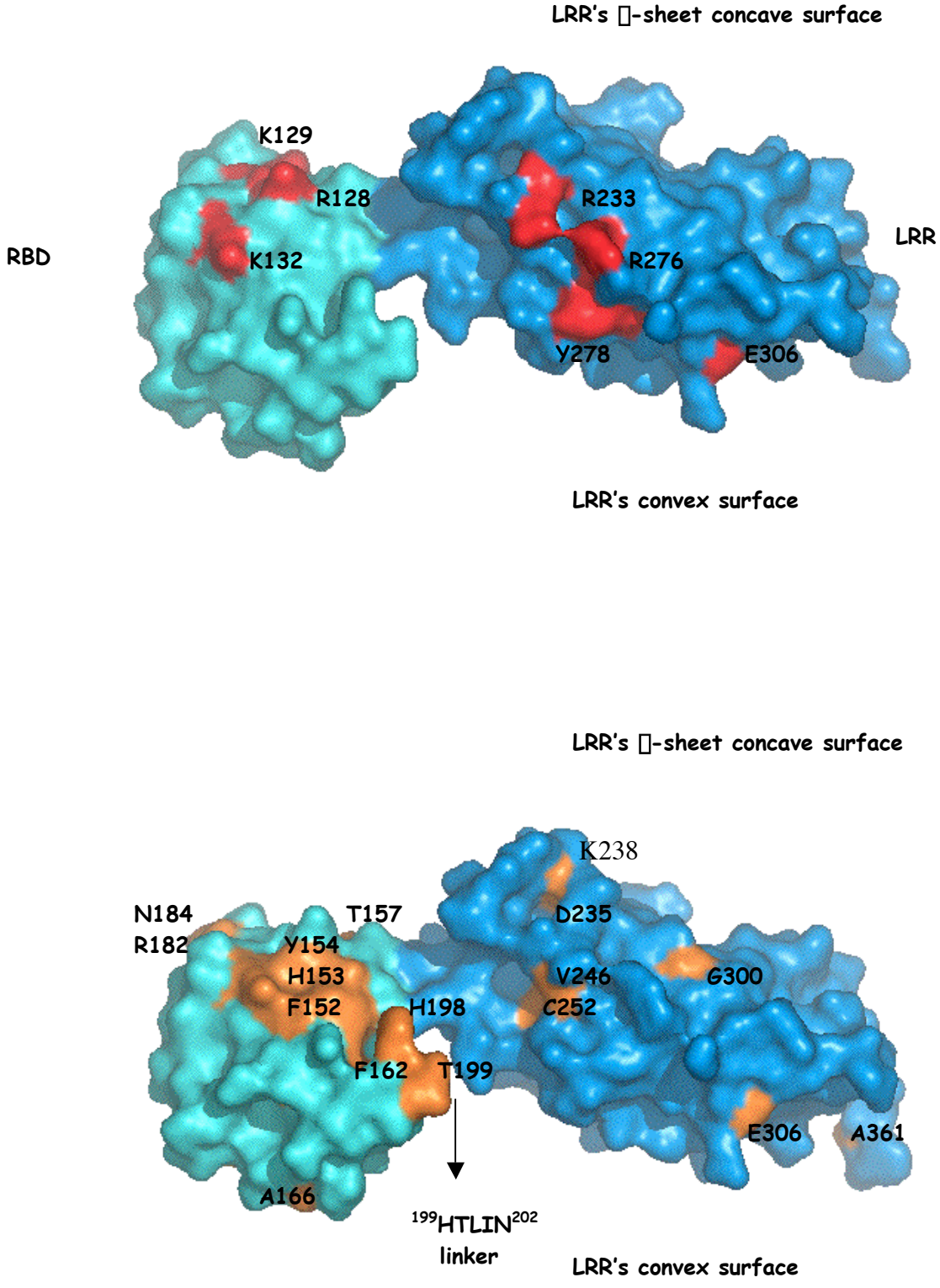

Figure 33b. Surface representation of TAP96-372.
red: surface of TAP-N identified by mutagenesis, orange: surface of TAP-N identified by chemical shift perturbation assays.

2.3) Small angle X-ray scattering (SAXS):

The domain arrangement of TAP-N unbound and in complex with the CTE-B RNA

Overview

Solution SAXS measurements of mono-disperse macromolecules, provide a means for investigating the inter-domain arrangement and determining a low-resolution shape, of multidomain proteins and protein/protein or protein/RNA assemblages (Svergun and Koch 2002; Koch *et al.*, 2003). Also, solution SAXS is convenient for the localization of the interacting surfaces within macromolecular assemblages, especially when combined with available atomic models, biochemical data (e.g. mutagenesis) and other biophysical tools (e.g. chemical shift perturbation experiments).

The domain orientation of TAP-N free and when bound to the CTE-B RNA is currently unknown. In the X-ray structure of TAP102-372, the protein is extended, with the RBD and LRR domains, making no contacts with each other (Liker *et al.*, 2000). This domain orientation could be due to crystal contacts, and thus, not necessarily reflect the conformation of TAP-N in solution. Further, the existence of a flexible linker connecting these two domains, argues for a possible induced fit rearrangement of TAP-N, upon ligand-recognition. In order to calculate the low-resolution shape of TAP-N and the TAP-N/CTE-B complex, we have used synchrotron radiation to collect SAXS data of TAP-N, the CTE-B RNA, and the TAP-N/CTE-B complex. This information has been combined with the available X-ray structure of TAP102-372 to calculate a low-resolution shape of TAP-N and TAP-N/CTE-B in solution, by rigid body refinement. The surface of interaction, mapped by chemical shift perturbation experiments, was taken into account, to determine a reliable position of TAP-N and the CTE-B RNA in the protein/RNA complex. My role during this part of the project was to prepare mg amounts of TAP-N, CTE-B RNA and TAP-N/CTE-B complex. As mentioned in the section of materials and methods, a single batch of fresh sample was prepared and concentrated to 20 mg/ml. SAXS data was collected for a dilution series of each macromolecule (CTE-B, TAP-

N and TAP-N/CTE-B), ranging from 2 to 20 mg/ml. Data analysis is currently being performed by Dr. Dmitry Svergun, at the EMBL-Hamburg. Preliminary analysis of the information indicates that, in agreement with the X-ray structure, TAP-N is extended in solution (Svergun DI, personal communication). As for the TAP-N/CTE-B complex, the RNA seems to stand in parallel to the extended protein, on the convex side of the LRR repeat, contacting mainly the RBD domain and the linker. These results are in agreement with the NMR-based chemical shift perturbation experiments presented here and with the available mutagenesis data (Liker *et al.*, Coburn *et al.*, 2001).

2.4) Overall conclusions:

Taking together the data obtained from multidimensional NMR and SAXS experiments

The results I present here show that TAP-N binds to the CTE-B RNA in a different mode, as compared with its structural and biochemical homologue, the U2B"/U2A'-U2snRNA spliceosomal complex (figure 14). The RNA-binding surface of TAP-N that I have mapped by NMR spectroscopy corresponds mainly to the β-sheet platform of TAP's RBD domain and to the linker connecting the RBD to the LRR (figure 33b). Worth to mention is the fact that the concave β-sheet platform of the LRR domain of TAP-N, does not seem to be perturbed, upon RNA-binding. SAXS experiments of TAP-N unbound and in complex with the CTE-B RNA, have shed light on the domain-domain arrangement of TAP-N in solution and as mentioned above, corroborated the surface of interaction mapped by NMR. Dimitry Svergun at the EMBL-Hamburg is currently building a dummy residue-based model of the shape of TAP-N and TAP-N/CTE-B in solution. The resolution of this reconstruction will be of approximately 25 Å, which allows for the observation of long-range conformational changes. Finally, it is still not clear how does TAP-N, which features a single-stranded RNA-binding domain specifically recognize a double-stranded RNA moiety, such as the CTE-B. A clear picture of the TAP-N/CTE-B RNA complex will only become available with atomic data. To this point, we have managed to obtain crystals diffracting to 6-7 Å. More effort has to be made in the future, regarding this. An appealing approach is the systematic design of RNA constructs, with the intention of obtaining better packed, well diffracting crystals of the TAP-N/CTE-B complex.