The Interaction of HIV-1 Tat(32-72) with its Target RNA: A Fluorescence and Nuclear Magnetic Resonance Study

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We performed intrinsic peptide fluorescence experiments to characterize the interaction between variants of the HIV-1 Tat(32-72) peptide BP1 and TAR RNA. K_d values for wild-type BP1 and cysteine-modified BP1 were found to be in the range of 60 to 70 nM for both peptides, indicating that free sulfhydryl groups of the cysteines within the peptide are not required for high affinity TAR binding. Thus, the mutant peptide BP1 (C34S, C37W) ($BP1_{SW}$) was used to further investigate peptide RNA interaction by fluorescence studies. Titration of $BP1_{SW}$ with TAR resulted in a dissociation constant ($K_d = 9$ nM) nearly an order of magnitude lower than that of the wild-type peptide. The change of the $BP1_{SW}$ fluorescence intensity on TAR binding was used to investigate the kinetics of this interaction by stopped-flow experiments. The results can be explained in terms of a two-step binding model, with a rapid diffusion-limited initial formation of a tight, but unspecific peptide RNA complex, followed by a relatively slow structural rearrangement step ($k \approx 60$ s^{-1}) in order to form the specific $BP1_{SW}$-TAR complex. Comparison of heteronuclear two-dimensional NMR spectra of $BP1_{SW}$ and $BP1_{SW}$ bound to TAR shows that only resonances from amino acid residues of the core and basic sequence regions are shifted on TAR binding. © 1997 Academic Press

The *Trans*-activator protein (Tat) of human immunodeficiency virus type 1 (HIV-1) is essential for virus replication (1-4) and acts as a transcription activator enhancing viral transcription several hundred fold (5-8). Tat binds to a uridine-rich bulge of a stem-loop structure at the 5′-end of the transcribed mRNA, the TAR (*trans*-activation responsive element) region, and is thus tethered close to the transcription complex (9).

The core and the basic regions of Tat are the key elements for recognition of TAR as shown by biochemical studies (10). Recently, structures of the complex between a 17 amino acid peptide identical to the basic region of the bovine immunodeficiency virus (BIV) Tat and the BIV TAR have been determined (11,12). From these studies, it could be clearly inferred that both components undergo severe structural reorganizations on complex formation. For HIV-1, structures of both components separately have been solved (13,14). In addition, the structure of HIV-1 TAR RNA has been determined in the presence of arginine (15) and a peptide analog of the basic region and part of the core region (16). Thus, detailed knowledge of the recognition process of HIV-1 Tat and TAR as would be required for a thorough understanding of the protein-RNA interaction on an atomic scale is not available. Spectroscopic and cross-link studies on interaction between a Tat peptide and TAR (17,18) have been reported, but the structure of TAR bound HIV-1 Tat or the even more desirable structure of the entire complex is still unknown.

Knowledge of kinetic parameters for the peptide - RNA complex formation is a prerequisite for understanding the recognition mechanism. In an effort, first, to understand the kinetics of HIV-1 Tat-TAR interaction and, secondly, to find a peptide model suitable for structural studies of the complex by NMR or X-ray crystallography, we performed intrinsic fluorescence spectroscopy, stopped-flow experiments, and heteronuclear nuclear magnetic resonance (NMR) spectroscopy to study the interaction of variants of HIV-1 Tat(32-72) peptides (BP1) with TAR RNA.

MATERIALS AND METHODS

Synthesis of wt HIV-1 Tat (32-72) peptide BP1. Wt BP1 was chemically synthesized as described before (19). The product was purified

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by reversed-phase high-performance liquid chromatography (HPLC) to more than 95% purity based on mass spectroscopic analysis (Shimadzu Compact MALDI). The synthetic peptide was freeze-dried and stored at −20°C.

Cloning, expression, and purification of recombinant BP1$_{SW}$. BP1$_{SW}$ was expressed in *E. coli.* Plasmid pET16b/BP1, which is derived from pTK1 (20) via PCR cloning (Hoffmann et al., unpublished data) and carries the wt BP1 gene, was used as a template for PCR mutagenesis. The PCR reaction was primed by the 3′ oligonucleotide 5′-TAAGTAACATATGTACCACTCCCAAGTTTGGTTTATCAC-3′ replacing cysteine 34 by serine and 37 by tryptophan, and a commercially available T7-terminator primer as the 5′ oligonucleotide. The PCR fragment was digested and inserted between *Nde*I and *Bpu*1102I sites of pET16b (Novagen, Madison, USA) resulting in plasmid pET16b/BP1Trp. pET16b/BP1Trp was transformed into the *E. coli* strain DH5α for dideoxy-sequencing (21). Overexpression of BP1$_{SW}$ was performed in *E. coli* BL21(DE3). Purification of the BP1$_{SW}$ peptide was carried out as described for full-length Tat (20). Highly homogeneous preparations of the BP1$_{SW}$ were used as confirmed by mass spectroscopic analysis (Shimadzu Compact MALDI). For ^{15}N-labeled samples, cells were grown on M9 minimal medium with [^{15}N]-NH$_4$Cl as the sole nitrogen source.

Preparation of HIV-1 TAR RNA. The 29-nucleotide TAR RNA was synthesized by *in vitro* transcription using T7 RNA polymerase and synthetic DNA templates (MWG-BioTech, Ebersberg, Germany; 22). TAR RNA was ethanol precipitated and purified by 20% polyacrylamid gel electrophoresis under denaturing conditions (8.3M urea). The RNA product of correct length was eluted from the gel by using a Schleicher and Schuell electroelution apparatus and subsequent ethanol precipitation. The TAR RNA was dialyzed to remove multivalent ions and other low weight impurities against a high EDTA buffer (10 mM phosphate, 5 mM EDTA, 100mM NaCl, pH 6.4), a low EDTA buffer (10 mM phosphate, 0.1 mM EDTA, 100mM NaCl, pH 6.4), and finally against water. The pure TAR RNA was then freeze-dried and stored at −20°C.

Preparation of the cysteine-modified BP1. Wt BP1 peptide (10 mg/ml) was incubated for three hours in a reaction buffer containing 300 mM Tris/HCl, 4 mM EDTA, 50mM DTT, pH 8.5. Addition of 300 mM iodoacetamide started the modification reaction. After one hour the reaction mixture was dialyzed against 10mM phosphate, pH 6.4, freeze-dried, and stored at −20° C. The extent of the cysteine-modification was determined by the method of Ellman (23).

Fluorescence titrations. Fluorescence titrations were performed using a SLM Smart 8000 spectrofluorometer (Colora, Lorch) equipped with a PH-PC9635 photomultiplier. The buffer contained 10 mM phosphate, 50 mM NaCl, pH 6.4 in a total volume of 1.0 ml. For tyrosine / tryptophan fluorescence the samples were excited at 272 nm/ 295 nm and the emission intensity was measured at 303 nm and 348 nm, respectively (slit width of 1 and 16 nm, respectively). All data were corrected for the background intensity of the buffer, for dilution, and for the inner filter effect. Measurements were made in a 0.5 cm pathlength cuvette to minimize the inner filter effect, and the residual effect was estimated from control experiments using free tyrosine and *N*-acetyltryptophanamide. Data were evaluated using the program Grafit (Erithacus Software) by fitting a quadratic equation analogous to the one given by Müller et al. (24) describing the binding equilibrium. Values for the dissociation constant (K$_d$), the amplitude of the fluorescence change, and the peptide concentration were allowed to vary during the fitting procedure. All measurements were performed at a temperature of 25° C.

Stopped-flow kinetics. Experiments on the kinetics of association of the BP1$_{SW}$ peptide with HIV-1 TAR RNA were performed using a stopped-flow apparatus (High Tech Scientific, Salisbury, England) under the same conditions as pointed out for the titration experiments. Excitation of tryptophan fluorescence was at 295 nm using a Hg-Xe high-pressure arc lamp (100 W), and detection was through
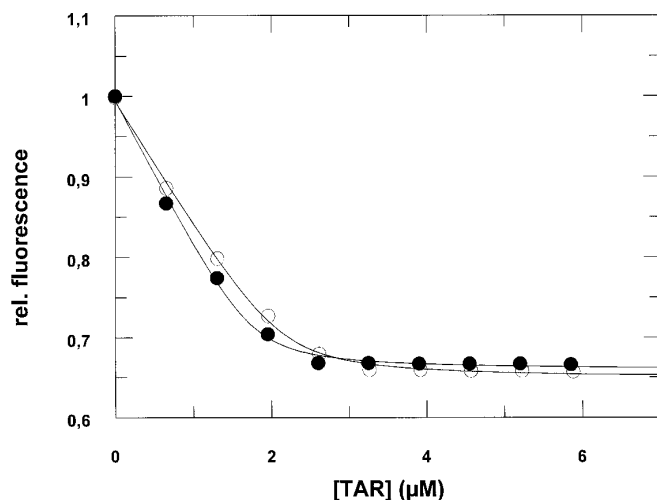
FIG. 1. Titration of reduced wild-type BP1 (●, 2 μM) and of cysteine-modified BP1 (○, 2 μM) with the 29-nucleotide HIV-1 TAR RNA using tyrosine fluorescence as an indicator of binding. The curve shows the best fit of the data to the quadratic equation describing BP1 TAR binding. Data evaluation results in K$_d$ = 57.5 nM and 35% as the relative maximal quenching of fluorescence induced by binding for the reduced wild-type BP1 and in K$_d$ = 71.6 nM and 35% as the relative maximal quenching of fluorescence induced by binding for the cysteine-modified BP1.

a filter with a cutoff at 342 nm. Data were converted using an analog to digital converter in an IBM PC-compatible computer and analyzed off-line using Grafit by fitting of second order reactions.

NMR spectroscopy. All NMR experiments were conducted on a Bruker AMX 600 spectrometer operating at 600 MHz for ^{1}H. Phase-sensitive detection of the indirectly observed frequencies was achieved by the TPPI method (25). Correlated ^{1}H-^{15}N HSQC spectra (26) in H$_2$O were obtained on a 1.2 mM totally ^{15}N-labeled peptide at 25 °C in 10 mM phosphate buffer, pH 6.4, with 50 mM NaCl. Unlabeled HIV-1 TAR, when used, was added in 1.2 fold excess over the peptide to ensure that virtually all peptide molecules are bound to TAR.

RESULTS AND DISCUSSION

The wild-type HIV-1 Tat (32-72) peptide BP1 contains no tryptophan residue and is thus not very well suited for intrinsic fluorescence measurements. However, it contains two tyrosine residues in the so called core domain that is responsible for specific high affinity binding to TAR. Although the fluorescence quantum yield of tyrosine is much less than that of tryptophan and also the inner filter effect caused by the TAR RNA is significant at an excitation wavelength of 272 nm, tyrosine fluorescence titration should lead to a rough guess of the peptide stoichiometry and affinity to its cognate RNA. Tyrosine fluorescence of wild-type BP1 is quenched upon addition of TAR RNA (Fig. 1). The change in fluorescence (about 35% quenching) can be used to determine the affinity of TAR RNA for the peptide. Data fitting by nonlinear regression to a quadratic equation describing the binding equilibrium resulted

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                  32         42         52         62         72

wild-type BP1    YHCQVCFITK GLGISYGRKK RRQRRRPSQG GQTHQDPIPK Q

BP1_SW             YHSQVWFITK GLGISYGRKK RRQRRRPSQG GQTHQDPIPK Q
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FIG. 2. Peptide sequence of wild-type BP1 comprising the RNA binding region of HIV-1 Tat compared to mutant $BP1_{SW}$. Mutations are highlighted in the sequence.

in a K_d value of approximately 60 nM. The change in intrinsic fluorescence may also be used to determine the binding stoichiometry and thus the fraction of synthesized BP1 active in binding to TAR RNA (active site-titration). The maximum fluorescence change should be reached when all BP1 molecules are bound to TAR. The fluorescence titration curve decreases linearly to nearly the minimum value, and the breakpoint indicates a stoichiometry of 1:1. This confirmed that all BP1 molecules were active in binding and interacted equally well with the TAR RNA.

The titration experiment was performed with a completely reduced BP1 peptide as determined by the method of Ellman (23). To investigate whether the two free sulfhydryl groups of the BP1 cysteines are necessary for the high-affinity binding to TAR, cysteine 34 and cysteine 37 were covalently modified by iodoacetamide and a fluorescence titration experiment for this peptide was performed. The titration curve of modified BP1 with TAR is of nearly identical shape compared to the unmodified (Fig. 1). The data showed a quenching of 35% of tyrosine fluorescence and resulted in a dissociation constant (K_d) of approximately 70 nM. Since the two K_d-values of the wild-type and modified BP1 are nearly identical, we conclude that the sulfhydryl groups of Cys34 and Cys37 within the core domain are not involved in high-affinity binding to HIV-1 TAR RNA.

Because of the low fluorescence quantum yield of tyrosine, the BP1 concentration used here was chosen to be 100 times higher than the determined K_d. To gain more insight into the BP1-TAR interaction by fluorescence spectroscopy we accordingly designed the mutant peptide BP1(C37S,C37W) ($BP1_{SW}$; Fig. 2) as presence of a tryptophan residue in the peptide offers a potential signal for further fluorescence studies. Thus, $BP1_{SW}$ can be used in nanomolar concentrations which is in the range of K_d, and its tryptophan allows excitation at 295 nm, at which wavelength the inner filter effect of the TAR RNA is minimized. The fluorescence emission spectrum of $BP1_{SW}$ exhibits a maximum at $\lambda_{max} = 348$ nm, which is nearly identical to the emission maximum of N-acetyltryptophanamide, indicating that the tryptophan residue is solvent exposed. Addition of saturating concentrations of TAR RNA did not change the emission maximum, but resulted in an enhancement of the intrinsic fluorescence by 80%. The increase in

amplitude of $BP1_{SW}$ by addition of TAR is larger than the quenching seen for wt BP1. This is probably due to the different fluorescence properties of tryptophan compared to tyrosine, but could be also caused by a stronger interaction of the tryptophan indol group with the TAR RNA in comparison to the phenol groups of the tyrosines. The changes in tryptophan fluorescence can be used to determine the affinity of $BP1_{SW}$ to TAR RNA. From these experiments, $K_d = 9$ nM is obtained for the dissociation constant (Fig. 3). This K_d is 6 to 7 times lower than that determined for wt BP1 and is in the range of the K_d determined for the full-length HIV-1 Tat (10; 27). This experiment also supports the finding that the two cysteines within the core domain are not required for high-affinity binding of BP1 to TAR RNA. Thus, the two cysteines within the core sequence region may required for a function not related to TAR binding. Analysis of the binding stoichiometry as described before resulted in a 1:1 complex. Thus, a recombinant mutant Tat (32-72) peptide was designed that binds to TAR in a wild-type manner, at least with respect to its affinity. The presence of the tryptophan within the core region allowed further fluorescence experiments to be used to study the $BP1_{SW}$ TAR interac-
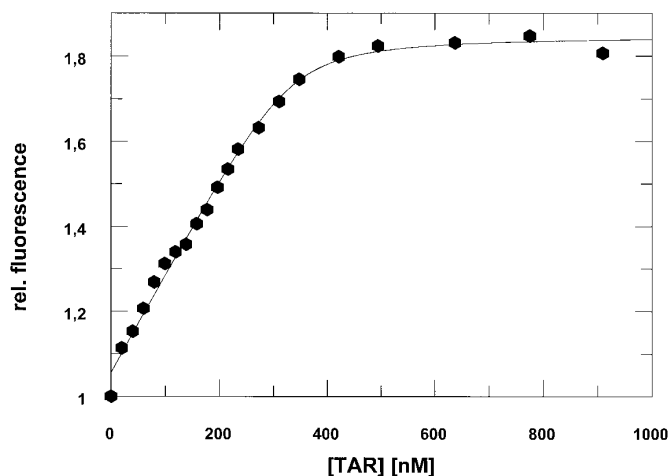
FIG. 3. Titration of $BP1_{SW}$, 300 nM, with the 29-nucleotide HIV-1 TAR RNA using tryptophan fluorescence as an indicator of complex formation. The curve shows the best fit to the data. K_d of 9.4 nM and a relative maximal amplitude of the fluorescence change of 80% was obtained.
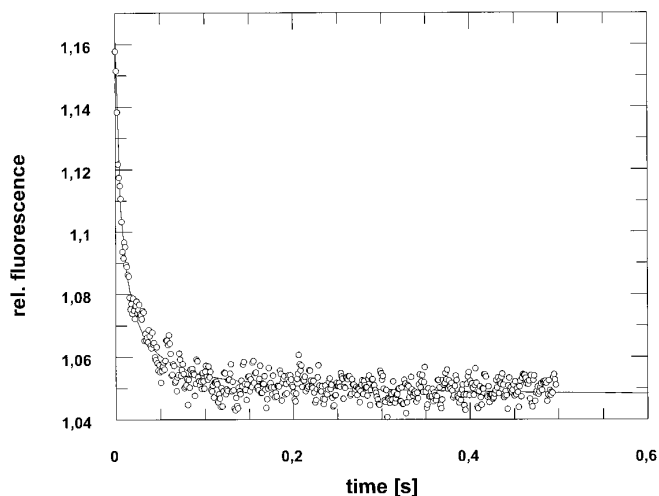
FIG. 4. Kinetics of binding of HIV-1 BP1$_{SW}$, 4 μM, to TAR, 4 μM, monitored by intrinsic tryptophan fluorescence. The two components were mixed in a stopped-flow apparatus. The data were best fitted using a second order equation with a rate constant of $3.8 \cdot 10^7$ M^{-1}s^{-1}.

tion in more detail. Moreover, NMR work could be done to characterize structural properties of the peptide RNA complex. The possibility of isotopically labelling through expression of BP1$_{SW}$ in *E. coli* as well as the absence of the cysteines favors BP1$_{SW}$, which comprises the whole RNA binding region, for studying the interaction with TAR.

The changes in intrinsic peptide fluorescence described here can be used for transient experiments on the interaction of BP1$_{SW}$ with TAR. Quenching of the tryptophan fluorescence occurs in a single phase when the two components are mixed at equimolar ratio in a stopped-flow apparatus (Fig. 4). In a control experiment performed with BP1$_{SW}$ alone no fluorescence change was detected, indicating that the peptide's fluorescence signal was stable during the experiment (data not shown). The experiment was performed using equal concentrations of peptide and RNA at 1 μM, 2 μM and 4 μM. At all concentrations, good fits to a second order equation were obtained. The rate of the transient seen was concentration dependent, but fitted second order rate constants, which should be independent of concentration for a one step reaction, decreased with increasing concentration. The values obtained were $6.2 \cdot 10^7$ M^{-1}s^{-1}, $5.4 \cdot 10^7$ M^{-1}s^{-1}, $3.8 \cdot 10^7$ M^{-1}s^{-1} at 1, 2 and 4 μM respectively. This decreasing tendency of the apparent second order rate constant could be explained by a two-step mechanism in which the observed fluorescence change occurs in the second step. The fact that the fitted second order rate constant decreases with increasing concentration would be a result of beginning saturation of the first step. A further observation in this experiment was that the fluorescence was quenched when the two components were mixed in the

stopped-flow apparatus, whereas an increase of the tryptophan fluorescence was observed in the titration experiment. This could occur if a first rapid binding step occurs, which cannot be observed in the stopped flow machine because of the mixing dead time of ca. 2 ms, and in this step, a relatively large increase in fluorescence occurs which is followed by the decrease observed in the stopped flow experiments. We therefore conclude that the binding reaction is probably a two-step process, as shown in the scheme.

$$\text{BP1}_{SW} + \text{TAR} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{BP1}_{SW\cdot TAR^*} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{BP1SW}\cdot\text{TAR}$$

The first step is presumably diffusion controlled, which means we can assume that K$_{+1}$ is 10^9 M^{-1}s^{-1}. Since the stopped flow experiments showed signs of saturation at μM concentrations, we conclude the K$_d$ value for the first step (i.e. 1/K$_1$) is in the μM range. Assuming a value of 1 μM (probably an underestimate), k_{-1} would be 1000 s^{-1}. In this scheme, the apparent second order rate constant determined at low concentration is given approximately by K$_1 \cdot$k$_{+2}$ (28), and the value obtained in this work ($6.2 \cdot 10^7$ M^{-1}s^{-1}) would lead to a value of 62 s^{-1} for k$_{+2}$. While this is only an indirect estimate, it is likely that this value lies in the correct range, since fitting the data at 4 μM concentration as a single exponential gives a value of 54 s^{-1}. Not surprisingly, one fit in this procedure is not good, and it would only be expected to find single exponential behavior under the conditions used (i.e. equal concentrations of peptide and RNA) at saturating concentrations of reagents. However, the condition that this cannot be faster than the calculated value of k$_{+2}$ still holds. It should be pointed out that a more realistic value for the first step would be somewhat higher than 1 μM, and this would lead to a correspondingly higher value of k$_{+2}$. Staying with the value of 1 μM for 1/K$_1$, we can use the affinity of the complex (which is given by K$_1 \cdot (1 + K_2)$ for the model used) to calculate the value of K$_2$ (ca. 100), which in turn leads to a value of ca. 0.6 s^{-1} for k$_{-2}$, the rate-limiting step in dissociation of the complex. Thus, the stopped-flow experiments lead to first kinetic data of the Tat-TAR interaction.

The binding model could be interpreted in the following manner. The first step represents the formation of an unspecific peptide RNA complex BP1$_{SW}\cdot$TAR* due to electrostatic interactions between the positively charged basic amino acid residues of the peptide and the negatively charged phosphate backbone of the RNA. The tryptophan residue of the peptide approaches the RNA, which results in fluorescence enhancement. In the second step, a specific complex BP1$_{SW}\cdot$TAR is formed and involves a conformational reorganization in both components. Such structural rearrangements of various Tat peptides as well as of the
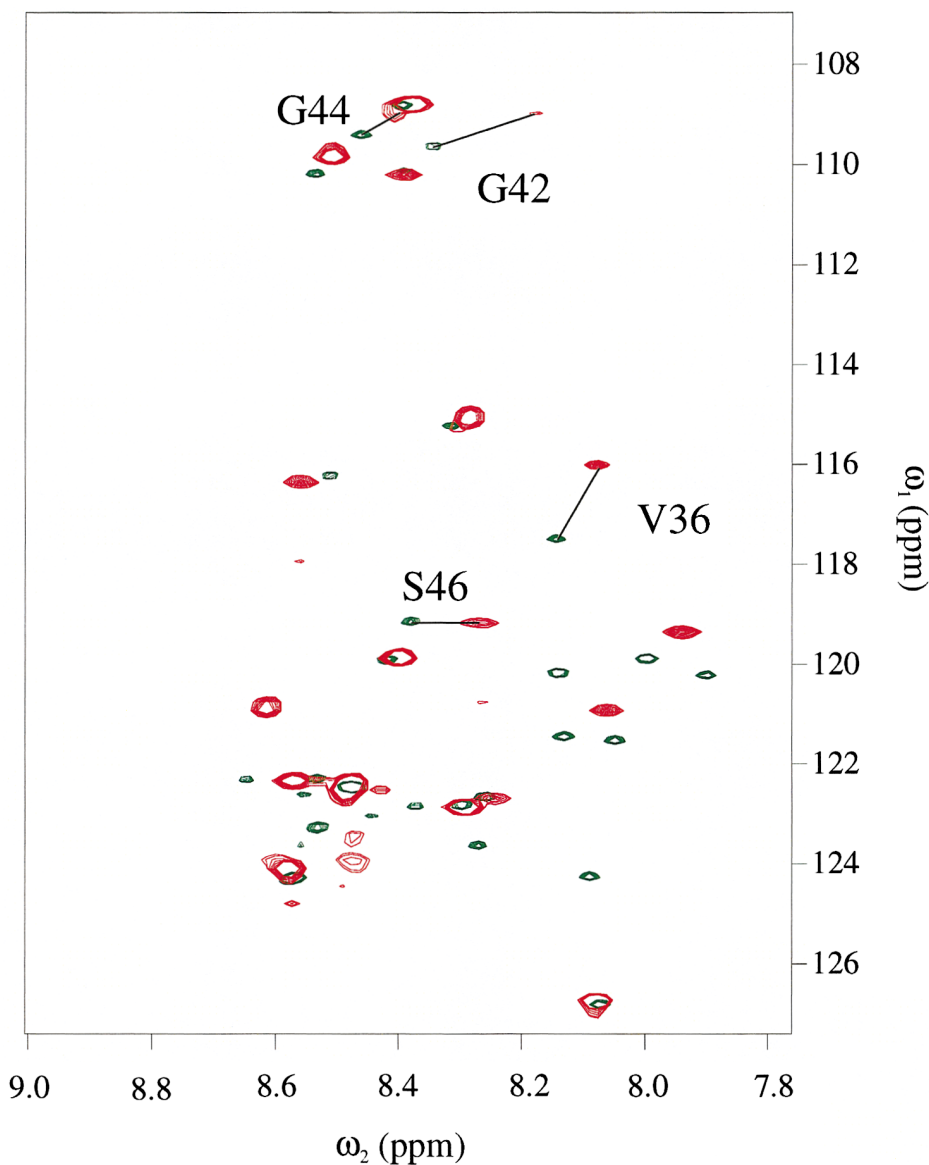
FIG. 5. Superposition of ^{15}N-HSQC spectra for ^{15}N labeled BP1$_{SW}$ (1.2 mM) in the absence (green contours) and presence (red contours) of the 29-nucleotide TAR RNA. Unlabeled HIV-1 TAR was added in 1.2 fold excess over the peptide to ensure virtually complete peptide binding to TAR. Several resonances of amino acids that show significant chemical shift changes are annotated.

TAR RNA on complex formation have been reported previously (16, 17, 29, 30). The tryptophan residue within the core sequence region that is responsible for the peptide binding specificity adopts its correct position in the specific BP1$_{SW}$-TAR complex. This process can be observed as a decrease of the fluorescence in the stopped-flow experiment. The kinetics of this second step are relatively slow and can be characterized by the first-order rate constant of ca. 60 s^{-1}, or somewhat higher.

Summarizing, we showed that fluorescence techniques are well suited to monitor the interaction of Tat derived peptides comprising the whole RNA binding region with TAR. Similar studies were performed to investigate the HIV-1 Reverse Transcriptase-Primer/Template interaction (31).

In order to find evidence which amino acid residues of BP1$_{SW}$ interact with TAR, two-dimensional ^{15}N-heteronuclear single-quantum coherence (^{15}N-HSQC) spectra were recorded. Complex formation is indicated by the observation of ^{15}N- or ^{1}H- amide chemical shift changes upon addition of the TAR RNA to a totally ^{15}N-labeled. BP1$_{SW}$. From an analysis of these changes the sequence regions of BP1$_{SW}$ involved in TAR binding may be identified. Resonances of free and TAR bound BP1$_{SW}$ were assigned

by NOESY and TOCSY experiments. The superposition of ^{15}N-HSQC spectra for BP1$_{SW}$ in the absence and presence of TAR shows that most of the resonances shifted upon TAR addition (Fig. 5). This is in good agreement with previous circular dichroism experiments (17), where a structural reorganization from unfolded BP1 to an extended conformation on TAR addition could be observed. Interestingly, all resonances that are shifted in the NMR experiment belong to biochemically identified binding sites of Tat. The most significant chemical shift changes are observed for Gly42 and Gly44 (Fig. 5) within the COOH-terminal end of the core sequence region of BP1$_{SW}$, a sequence region known to be responsible for specific TAR binding (10). Thus, these residues might play a key role in the conformational reorganization of the peptide, probably towards an extended conformation like a β-sheet (17). Of the resonances whose chemical shifts are unaffected by TAR addition, most belong to amino acids representing the so-called Gln-rich sequence region forming the COOH-terminal part of the peptide (amino acid residues 60 to 72). This region was so far not shown to be involved in Tat - TAR interactions (10). Thus, our results from the present NMR experiments are in good agreement with previously reported biochemical data that show that BP1$_{SW}$ peptide binds to TAR under conditions suitable for NMR analysis.

ACKNOWLEDGMENTS

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