Characterization of Recombinant HIV-1 Tat and Its Interaction with TAR RNA†

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ABSTRACT: Recombinant HIV-1 Tat (Tat 1–86) has been purified from the cytoplasmic fraction of *Escherichia coli* without the use of protein denaturants or chaotropic agents. Chloroquine-mediated uptake of the purified protein into cells resulted in transactivation of the HIV LTR promoter. Tat retains 1.64 mol of Zn^{2+}/mol of protein by atomic absorption spectroscopy. Circular dichroism measurements indicated that the structure of recombinant Tat contains 15–20% α-helix. Filter binding assays showed that Tat binds to a 63-nucleotide target TAR RNA with a dissociation constant (K_d) of 10 nM at 25 °C, 0.05 M ionic strength, pH 7.5, in a 1:1 Tat–TAR RNA stoichiometry. Nonelectrostatic interactions provide the principal source of free energy of association. While the pH optimum occurs over a wide H^+ concentration, the salt dependence of K_d indicates formation of a single ion pair. UV-induced protein–RNA cross-linking produced a labeled Tat–TAR RNA adduct, indicating that direct contact occurred between the Tat protein and TAR RNA.

The Tat protein of human immunodeficiency virus (HIV)[1] type 1 is a virally encoded protein expressed in infected cells. The protein is essential for viral replication (Arya et al., 1985; Sodroski et al., 1985; Fisher et al., 1986; Dayton et al., 1986; Sadai et al., 1988). Tat is a transcription transactivator for the HIV-1 LTR promoter which contains the cis-acting responsive sequence TAR downstream from the transcription start site (Cullen, 1986; Peterlin et al., 1986; Rice & Mathews, 1988; Jakobovits et al., 1988; Feng & Holland, 1988; Hauber & Cullen, 1988). The protein up-regulates HIV-1 viral production many thousandfold (Feinberg et al., 1991) and is considered an excellent target for therapeutic intervention (Hsu et al., 1991). Tat has been shown to bind specifically to TAR RNA, which forms a stable stem–loop structure with a three-base bulge on the stem (Dingwall et al., 1989, 1990; Weeks et al., 1990; Roy et al., 1990). While Tat–TAR RNA interactions are an important focal point for the study of HIV gene expression, information regarding Tat protein structure might reveal its mechanism of action and also be useful in the design of inhibitors.

Methods for purification of recombinant HIV Tat have been developed; however, these procedures rely on techniques that lead to protein unfolding (Dingwall et al., 1990; Frankel et al., 1988; Muller et al., 1989, 1990). Structural analysis of Tat made by these methods would require that protein be refolded. While several groups have reported that refolded protein is functional in RNA binding, little information about Tat structure could be derived from these preparations. Generation of homogeneous, refolded Tat has been problematic due to its high cysteine content and susceptibility to oxidation (Frankel et al., 1988).

In this report, we describe a method for expression and purification of the protein that can be easily adapted for large-scale purification without the use of denaturants or chaotropic agents. Protein purified by this method is active in cell-based assays and is suitable for structural analysis. A stoichiometry of Tat–TAR RNA interaction and the physical characteristics of the interaction have been determined.

MATERIALS AND METHODS

Expression of Tat in Escherichia coli. A 825-base-pair *Eco*RI–*Nar*I cDNA fragment from pEV-TAT86 that includes the complete coding sequence for the 86 amino acid HIV-1 Tat (kindly provided by Dr. M. C. Graves, Hoffmann-La Roche, Inc.) was subcloned into the *Eco*RI–*Cla*I sites of pGEM-7Zf(+) (Promega). Site-directed mutagenesis was used to engineer an *Nde*I restriction site at the ATG start codon of the *tat* gene. The *Nde*I–*Bam*HI fragment was then cloned into the same restriction sites of pET-3a (Novagen) to produce pLWSTAT86, in which Tat expression is controlled by the T7 promoter. *E. coli* BL21DE3 (Novagen), carrying an integrated T7 RNA polymerase sequence under the control of the *lacUV5* promoter, was transfected with pLWSTAT86. Cells were grown in TB broth with ampicillin (50 μg/mL). Medium was supplemented with 1 mM $ZnSO_4$ at the time of induction with 0.5 mM IPTG. Following induction, cells were grown for 3 h at 30 °C, harvested, and stored at −20 °C until needed.

Protein Purification. E. coli BL21DE3 cells (10 g wet weight) expressing Tat were sonicated in 40 mL of lysis buffer (20 mM sodium phosphate, pH 7.8, 2.5% glycerol, 0.5 mM PMSF, 50 mM mannitol, 10 mM ascorbic acid, and 1 M KCl) and the lysate was clarified by centrifugation. Supernatant containing 90% of the expressed Tat was diluted with

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[1] Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; IPTG, isopropyl thioglactoside; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; DEPC, diethyl pyrocarbonate; NTP, nucleoside triphosphate; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamie gel electrophoresis; TAR, transactivation response element; TB, "terrific broth" [2.4% (w/v) yeast extract, 1.2% (w/v) tryptone, 0.4% (v/v) glycerol, 90 mM potassium phosphate (pH 7.0)].

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an equal volume of lysis buffer and passed over a column of chelating Sepharose Fast Flow (Pharmacia) that had been preequilibrated with zinc. The column was washed extensively with lysis buffer (minimum of 15 column volumes). Bound proteins were eluted with 100 mM sodium phosphate, pH 6.0, 2.5% glycerol, 50 mM mannitol, 10 mM ascorbic acid, 5 mM DTT, 0.5 mM PMSF, and 1 M NaCl. Tat-containing fractions were pooled, diluted with 10 mM diethanolamine, pH 9.0, 2.5% glycerol, 5 mM DTT, 0.5 mM PMSF, 50 mM mannitol, and 10 mM ascorbic acid and loaded on a Sepharose Fast Q column and a Sepharose Fast S column connected in series. A portion of the Tat binds to the Fast Q column along with major contaminants. Tat which flows through Fast Q binds to the Fast S column. Tat protein was eluted from the Fast S column in a 0.5–1.0 M NaCl gradient in 100 mM HEPES, pH 7.5, 2.5% glycerol, 5 mM DTT, 0.5 mM PMSF, 50 mM mannitol, and 10 mM ascorbic acid. Fractions containing Tat were pooled, concentrated, and stored at 4 °C or at –80 °C until needed.

Transactivation Assay. Purified Tat protein was assayed for its biological activity with HL3T1 cells, a HeLa cell line containing an integrated chloramphenicol acetyltransferase (CAT) gene under the control of the HIV LTR (Felber & Pavlakis, 1988). Cells were plated on 6-well plates at 3×10^5 cells/well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1 mM glutamine, and 50 μg/mL gentamicin. The following day, medium was replenished and supplemented with recombinant Tat and 100 μM chloroquine (Frankel & Pabo, 1988). After 24 h, the culture medium was exchanged with fresh medium without Tat and chloroquine. Cells were harvested 48 h after Tat addition and CAT assays were performed according to published procedures (Kingston & Sheen, 1990).

Protein Analysis. Protein concentrations were determined by colorimetric assay (Pierce) or by amino acid composition. Zinc content was determined by atomic absorption, and the ratio of metal to protein was determined using 9784 as the molecular weight for Tat.

Circular dichroism measurements (CD) were performed on a Jasco J-500A spectropolarimeter at room temperature. The instrument was calibrated with an aqueous solution of d-10-camphorsulfonic acid. Calculation of helical content was based on the 221- and 208-nm Cotton effects as described (Jibson & Li, 1981).

Computer predications of protein secondary structure were determined using the Peptide Structure program of the GCG Sequence Analysis software (Genetics Computer, Inc.) employing the algorithm of Chou and Fasman (Devereux et al., 1984).

Preparation of TAR RNA. The plasmid pTZ19-TAR was constructed by inserting complementary synthetic oligonucleotides coding for nucleotide +1 to +63 of HIV-1 TAR (HBX-3 strain) into the *Hin*dIII and *Bam*HI sites of a modified pTZ19 vector (Pharmacia), which lacks a *Sac*I site in the polylinker. TAR RNA fragments were prepared by in vitro transcription of a linearized pTZ19-TAR (cleaved at the *Bam*HI site of the polylinker) by T7 RNA polymerase. Transcription reactions contained 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 25 mM NaCl, 2 mM spermidine tris-(hydrochloride), 5 mM dithiothreitol, 50 μg/mL bovine serum albumin, 500 μM each ATP, CTP, and GTP, 100 μM UTP, 10 μCi of [α-^{32}P]UTP (3000 Ci/mmol), 1–2 μg of template, and 50–100 units of T7 RNA polymerase in a total 50-μL volume. Unlabeled RNAs were prepared in a 100-μL reaction with 1 mM each NTP, 10 μg of template, and 250 units of T7 RNA polymerase. Transcriptions were carried out at 37 °C for 90 min. DNA templates were then digested with 1 unit of RNase-free DNase (RQ-1 grade, Promega)/μg of DNA template for 10 min at 37 °C. RNA was purified by phenol–chloroform extraction, and unincorporated nucleotides were removed by gel filtration on a Bio-Spin-6 column (Bio-Rad). Labeled RNAs were quantitated on the basis of the specific activity of [α-^{32}P]UTP in the transcription reaction. RNAs were precipitated with ethanol and stored at –20 °C in sterile DEPC-treated water. RNAs were judged to be homogeneous and of the correct size by electrophoretic resolution on 12% denaturing polyacrylamide gels.

Tat–TAR Binding. Filter bindings assays were performed in binding buffer (10 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, and 5% glycerol) containing 100 μg/mL BSA, 100 nM [^{32}P]-TAR RNA [10^8 dpm/μg], and the specified amount of Tat protein in a 40-μL volume. Incubations were initiated by adding 20 μL each of Tat and [^{32}P]-TAR RNA (both in binding buffer), mixing gently, and incubating for 20 min at 25 °C. Duplicate aliquots of the reaction were then applied to prewetted nitrocellulose filters (0.45-μm Type HA, Millipore) under vacuum, and the filters were washed with 1 mL of binding buffer. Filters were dried at 80 °C and bound [^{32}P]-TAR RNA was quantitated by liquid scintillation counting. All assays were corrected for nonspecific binding (less than 5% of the total) by incubating TAR RNA without Tat protein. In RNA excess assays, Tat concentration was kept constant and [^{32}P]-TAR RNA (10^6 dpm/μg) was increased until no further binding was detected. Salt-dependence assays were performed by varying the concentration of NaCl in the binding and wash buffers. For pH-dependence assays, 10 mM sodium acetate, sodium phosphate, or Tris-HCl buffers were used as appropriate.

UV Cross Linking. Tat protein was cross-linked to [^{32}P]-TAR RNA by a published procedure with modifications (Marciniak et al., 1990). Various amounts of Tat protein were incubated with labeled wild-type or bulge-mutant TAR RNA (2 ng containing 1×10^6 cpm) in 20 mM HEPES, pH 7.9, 60 mM KCl, 2 mM $MgCl_2$, 150 μM DTT, and 5% glycerol for 10 min at 30 °C and then transferred to ice. To a duplicate set of incubations, yeast tRNA (5 μg) was added to the samples and the tubes were irradiated with UV light in a Stratalinker 1800 apparatus (Stratagene) for 15–20 min. Following irradiation, RNase A was added to a final concentration of 10 μg/mL and the samples were incubated at 37 °C for 15–30 min. SDS–PAGE sample buffer was added, followed by heating at 65 °C for 5 min or boiling. Proteins were separated by SDS–PAGE on a 15% gel, transferred to nitrocellulose, and analyzed by autoradiography. Tat protein on the blot was subsequently identified using a monoclonal antibody to Tat (Roy et al., 1990).

RESULTS

Tat Expression and Purification. HIV Tat expressed in *E. coli* strain BL21DE3 under the control of the inducible T7 polymerase accumulated in the cytoplasm as a soluble protein. When cells were broken by sonication, 90% of the expressed Tat remained in the soluble fraction. Since the majority of the protein was soluble, extraction with chaotropic agents was not needed prior to protein purification. Addition of $ZnCl_2$ to the growth medium immediately before induction allows incorporation of metal into expressed protein while avoiding metal toxicity to the cells during growth.

Tat was purified using affinity and ion-exchange chromatography. A representative SDS–PAGE gel through steps of

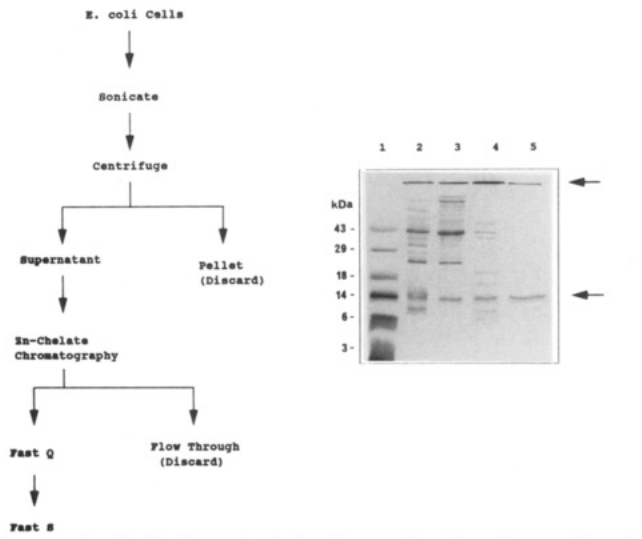

FIGURE 1: (Left) Flow chart showing purification of recombinant HIV-1 Tat. (Right) SDS–PAGE of HIV-1 Tat purification. Samples from each step were subjected to SDS–PAGE as described under Materials and Methods. Lane 1, molecular weight standards; lane 2, soluble fraction from centrifugation of *E. coli* cell break; lane 3, protein elution from Zn^{2+} chelate resin; lane 4, early fraction from Sepharose Fast S column containing Tat with other proteins; lane 5, purified protein eluted from Sepharose Fast S column; arrow, position of Tat protein identified using monoclonal antibodies to HIV-1 Tat.

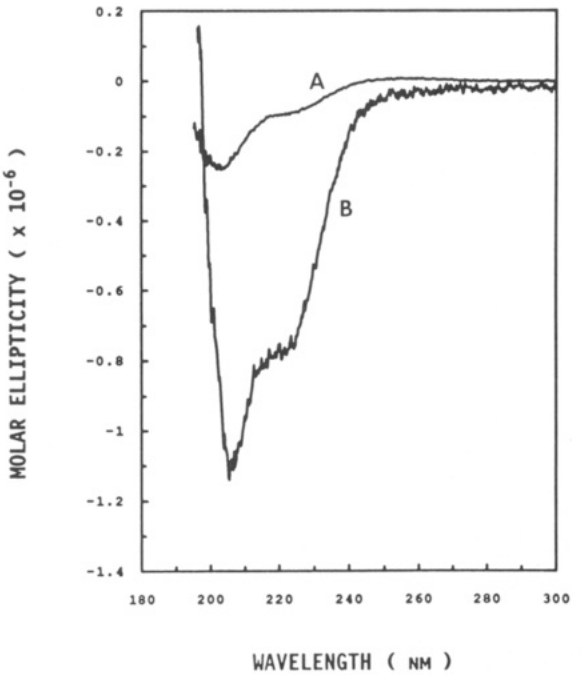

FIGURE 2: Circular dichroism spectra of HIV-1 Tat. Spectra of the protein at 0.4 mg of protein/mL were obtained at room temperature and corrected for contributions by the buffer components. (A) Spectrum in 100 mM sodium phosphate, pH 7.5, 200 mM NaCl, 5 mM dithiothreitol, 0.5 mM mannitol, and 0.3 mM ascorbic acid. (B) An equivalent sample diluted with an equal volume of trifluoroethanol.

the purification is shown in Figure 1. Note that on SDS–PAGE a portion of the Tat protein remains at the resolving gel interface. This is due to oxidation of the protein caused by ammonium persulfate used to polymerize the gels (Frankel et al., 1988). Buffers used throughout the purification contained mannitol and ascorbic acid to reduce protein oxidation. This appeared to be critical to the success of the purification procedure. The majority of the protein formed oxidized aggregates that could not be reduced by dithiothreitol when these reagents were omitted from the buffer. Following disruption of the cells by sonication, Tat was found to be bound nonspecifically to *E. coli* nucleic acids (as judged by absorption at 260 nm). It is this interaction that has made purification of the protein by standard ion-exchange chromatography difficult. Therefore, a procedure that separates the protein–nucleic acid aggregate was required in the initial phase of purification. While Tat in crude cellular extracts would not fractionate away from nucleic acids on either cation- or anion-exchange columns, the protein bound tightly to a Zn chelate resin (Figure 1, lane 3). While the aggregate was bound to the metal chelate column, a high-salt wash of the column disrupted protein–nucleic acid interactions, allowing removal of the nucleic acids. Following elution from the metal chelate column at low pH, the protein was further purified by ion-exchange chromatography on successive anion- and cation-exchange columns. Tat eluted from the Sepharose Fast S column was judged to be more than 95% pure on the basis of gel electrophoresis and N-terminal sequencing.

Protein Characterization. The presence of bound divalent metals in the purified protein was determined by atomic absorption spectroscopy. We found 1.64 mol of Zn^{2+}/mol of Tat protein, and the presence of Cu^{2+} or Fe^{2+} was not detected. Bound Zn^{2+} in native protein could be displaced by Cd^{2+}, as measured by the appearance of a characteristic 248-nm peak in difference spectra obtained in the presence of added $CdCl_2$ (Frankel et al., 1988). Previously, a model was proposed in which a protein dimer binds 4 mol of Zn^{2+} (Frankel et al., 1988). If this model is correct, our atomic absorption measurements indicates that some metal binding sites in the protein preparation are vacant; however, one cannot rule out the possibility that a stable protein can form with less than 2 metal ions/mol. More recently, Rice and Chan showed that monomeric Tat existed in mammalian cells (Rice & Chan, 1991). Gel-filtration chromatography indicates that our Tat preparation is a mixture of monomer and high molecular weight aggregate (Figure 1).

Structural studies of recombinant Tat protein purified using chaotropic agents have failed to identify any secondary structure in the purified protein (Frankel et al., 1988). Computer analysis suggests that Tat consists of approximately 15% helix, with β-sheet and turn structures also present (Devereux et al., 1984). We analyzed recombinant Tat using circular dichroism (Figure 2). In aqueous buffer (curve A), the protein has a small amount of helical content, which becomes more pronounced when measured in trifluoroethanol (curve B). Calculation of the helical content from the negative Cotton effect indicates the presence of approximately 15–20% helix. Analysis of Tat protein that was detergent-denatured revealed less than 3% helix in the denatured protein. Therefore, in contrast to published works (Frankel et al., 1988), our expression system and purification procedure appears to generate folded protein.

Cellular Transactivation by Tat Protein. Biological activity of purified Tat protein is measured in a cell-based assay in which Tat induces expression of CAT placed under the control of the HIV LTR in HL3T1 cells. In the absence of HIV Tat, HL3T1 cells expressed very low basal CAT activity. Addition of purified recombinant Tat to these cells stimulated CAT expression in a dose-dependent manner (Figure 3). As little as 10 ng of purified protein/3×10^5 cells was sufficient to stimulate CAT expression, indicating that the purified protein was highly competent for transactivation in vivo, and a 135-fold increase in CAT activity was observed when Tat was increased to 50 ng. Up to 1000-fold stimulation of CAT activity has been observed with larger amounts of Tat before the protein caused cytotoxicity (data not shown).

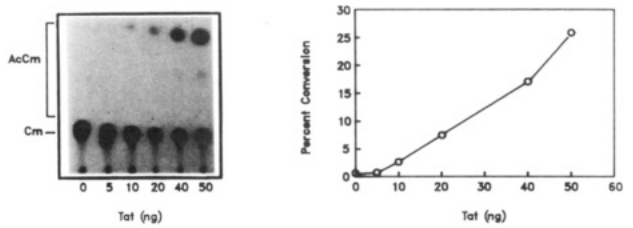

FIGURE 3: Biological activity of purified HIV-1 Tat. Recombinant HIV-1 Tat was added to HL3T1 cells containing an integrated chloramphenicol acetyltransferase (CAT) gene under the control of the HIV LTR (Felber & Pavakis, 1988)). One day after cells were plated (3×10^5 cells/well for 6-well plates), fresh medium supplemented with 100 μM chloroquine and purified Tat was added to each well and the cells were incubated for 24 h. Medium containing chloroquine and Tat was removed after 24 h, and cells were harvested 48 h after Tat addition. CAT assays were performed according to published procedures (Kingston & Sheen, 1990). Percent conversions represent the amount of [^{14}C]chloramphenical converted to the acetylated derivative by CAT. (A) Autoradiogram of the CAT assay. (B) Data in panel A was quantitated by densitometric scanning.

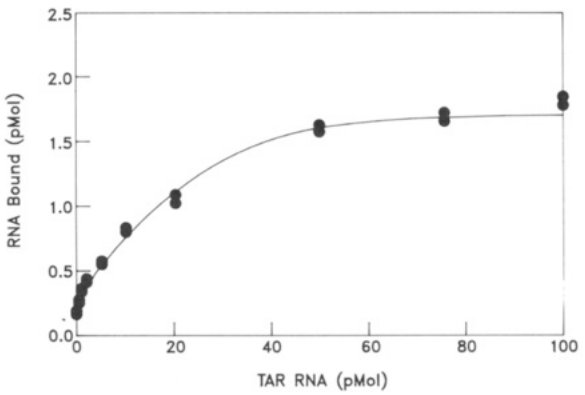

FIGURE 4: RNA binding to recombinant HIV Tat. Tat monomer (2 pmol, equivalent to 50 ng of protein) was incubated at 25 °C with 0–100 pmol of [^{32}P]-TAR RNA for 20 min. The amount of RNA bound was determined from filter binding as described under Materials and Methods. Results of duplicate samples are shown.

Tat–TAR RNA Binding. A two-part procedure was used to determine the affinity and stoichiometry of Tat–TAR interaction. To derive a dissociation constant K_d for the Tat–TAR RNA complex, Tat and TAR RNA were incubated for 20 min and binding was detected by protein retention on nitrocellulose filters. Bound RNA was retained on the nitrocellulose filters with the protein while unbound RNA was removed. Assuming that the Tat preparation was fully active, a $K_d = 35$ nM for the Tat–TAR interaction was obtained by Scatchard analysis (data not shown). To prove that the recombinant Tat protein was fully competent for RNA binding, we employed an RNA excess assay. While the concentration of Tat was held constant at 2 pmol, TAR RNA was added at increasing concentrations until no additional RNA was bound (Figure 4). At saturation, 2 pmol of Tat bound 1.75 pmol of TAR RNA. When corrected for the efficiency of protein binding to nitrocellulose (80%, data not shown), 2.1 pmol of TAR RNA was bound. These data suggest that despite the existence of monomer and higher aggregates as judged by gel filtration, recombinant Tat was fully competent for RNA binding and the stoichiometry of Tat–TAR RNA interaction is 1:1.

Specificity of Tat binding to TAR RNA was examined using nonlabeled TAR RNA and yeast tRNA as competitors in filter binding assays. TAR RNA competes 10-fold more efficiently than tRNA for binding to Tat, although competition by a 200-fold excess of tRNA could be observed, indicating some nonspecific interaction with RNA as measured by the filter binding assay. Other single-stranded competitor RNAs such as poly(A), poly(U), and poly(C) and double stranded RNAs such as poly(C-G) and poly(A–U) did not significantly compete for Tat binding with TAR RNA (data not shown).

The interaction of Tat with TAR RNA was characterized by using the nitrocellulose filter binding assay as described above. In a series of papers, Record and collegues have presented a thermodynamic description of the interaction of proteins with deoxyribonucleic acids and oligonucleotides (Record et al., 1976; Lohman et al., 1980). The theory described in these papers provide a mathematical means of calculating the contribution to the overall free energy of protein–nucleic acid binding provided by entropic counterion release. The analysis makes several assumptions: (1) protein interaction with nucleic acid occurs with adjacent phosphate groups, (2) anion binding to the protein is neglected, and (3) the concentration of interacting components is sufficiently low to minimize any potential effect of differential hydration. Recently, Witherell and Uhlenbeck (1989) demonstrated that these principles could be applied to the interaction of protein with RNA. Thus, by analyzing the effects of pH, ionic strength, and temperature on the binding interaction, it is possible to determine the thermodynamic factors that contribute to Tat–TAR complex formation.

The ionic strength dependence of RNA binding to Tat was determined over a wide range of salt concentrations, from 50 to 800 mM. The results in panel A of Figure 5 demonstrate that K_a decreased with increasing ionic strength, indicating that some ionic interactions are involved in complex formation. From these data, a slope of 1.3 is obtained. On the basis of theoretic assumptions as described above, this suggests an upper limit of one ion pair between nucleoside phosphate groups on the RNA and a cationic group of the protein; however, contribution by zinc ions to RNA binding cannot be excluded. It is estimated that, at 1 M salt, a single lysine–phosphate ion pair contributes approximately +0.2 kcal/mol to binding energy (Lohman et al., 1980). From the K_a value, ΔG was calculated to be –9.3 kcal/mol of complex. Thus nonelectrostatic contribution to the free energy is –9.5 kcal/mol, or more than 90% of the total free energy for binding.

The temperature dependence of Tat–TAR RNA binding was also measured, and the data were analyzed in the form of a van't Hoff plot (Figure 5, panel B). The ΔH obtained from the slope of the line is –0.6 kcal/mol of complex, and ΔS is calculated to be +34.6 cal K^{-1} (mol of complex)$^{-1}$ at 25 °C. The results suggest that complex formation is only slightly favored by enthalpy and is favored by entropic factors.

The pH effect on binding was measured by substituting appropriate buffers for HEPES used in assays at pH 7.5 (Figure 5, panel C). Although complex formation was most efficient near physiological pH, binding did not change drastically above pH 8.0 or below pH 6.0. This suggests that titratible lysyl residues do not contribute significantly to the binding interaction. The susceptibility of the RNA to base-mediated cleavage made it impossible to test the pH dependence above pH 10.0. Thus the potential role of an arginine residue could not be determined. Recently, Calnan et al. (1991) have identified a novel binding motif, an arginine fork, which may be important in the interaction of Tat with RNA. The results of our titrations do not rule out such a model.

Formation of Covalent Tat–TAR Adducts by UV Cross-Linking. Contacts between amino acids residues on Tat and TAR RNA can be detected by UV cross-linking (Figure 6). SDS–PAGE shows radiolabeled protein is generated when

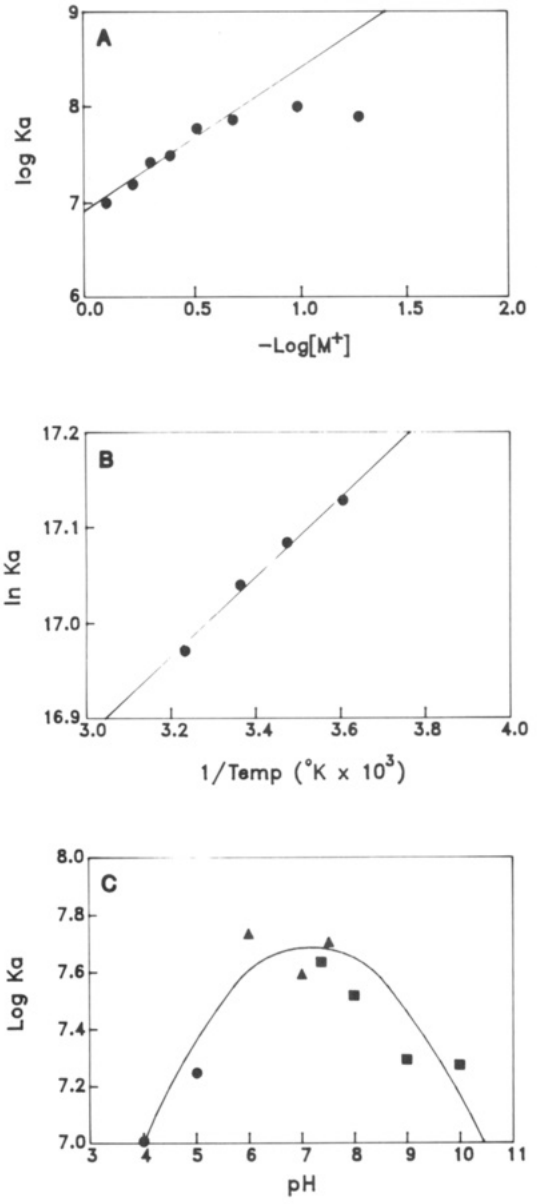

FIGURE 5: (A) Salt dependence of K_a for Tat–TAR RNA interaction. Filter binding assays were performed in standard binding buffer at 25 °C, with the NaCl concentrations adjusted to give the total cation concentration, [M+], indicated. The extrapolated slope of the regression (s = 1.3) corresponds to an upper limit of ion pairs formed between the protein and the RNA. (B) Temperature dependence of K_a for Tat–TAR RNA interaction. Filter binding assays were performed in standard binding buffer at the temperature indicated. The extrapolated slope of the regression line corresponds to ΔH = 0.6 kcal. (C) pH dependence of K_a for Tat–TAR RNA interaction. Filter binding assays were performed with TAR RNA at 25 °C, 0.05 M NaCl and 50 mM NaOAc (●), 50 mM sodium phosphate (▲), or 50 mM Tris-HCl (■) adjusted to the indicated pH.

purified Tat is incubated with radiolabeled TAR RNA followed by UV cross-linking and RNase digestion. Cross-linking requires Tat concentrations above 25 nM (approximately 250 ng of protein/mL), close to the K_d value determined. Protein–TAR cross-linking is specific, since this can occur in the presence of a large excess of tRNA, although some competition with tRNA is observed. The reduction in cross-linking is consistent with the competition observed in the filter binding assays (not shown). The migration of the radiolabeled protein band in SDS–PAGE is consistent with the labeling of the Tat protein. Moreover, Western blots indicate that the protein recognized by an anti-Tat antibody is the same 14-kDa band detected by autoradiography. The

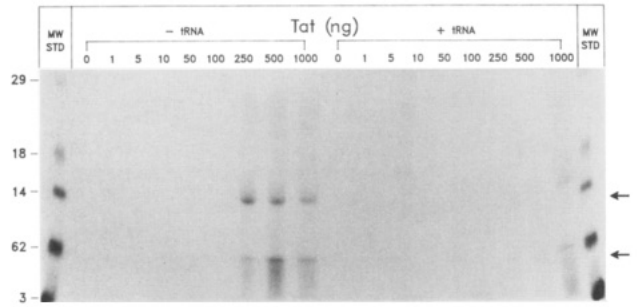

FIGURE 6: UV cross-linking of HIV Tat to TAR RNA. Radiolabeled [α-^{32}P]-TAR RNA was incubated with the indicated amounts of purified Tat without or with 5 μg of yeast tRNA for 20 min. Samples were irradiated with UV light as described by Marcianak et al. (1990). Following digestion with RNase A and SDS–PAGE, radiolabeled bands were identified by fluorography. Protein samples from a separate gel were transferred to nitrocellulose paper and probed with a monoclonal antibody to HIV Tat. The positions of the immunoreactive bands relative to molecular weight standards are noted by the arrows.

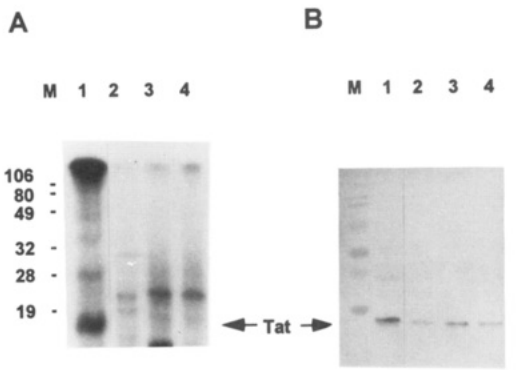

FIGURE 7: Cross-linking of Tat with TAR bulge mutants. Purified Tat was incubated with wild-type or mutant TAR RNA as described for Figure 6. Samples were UV cross-linked, subjected to SDS–PAGE, and transferred to nitrocellulose for autoradiography as described under Materials and Methods. Following autoradiography (panel A), the nitrocellulose was blotted with a monoclonal antibody to Tat (panel B). M, molecular weight standards; lane 1, wild-type TAR RNA; lane 2, bulge deletion mutant Δ23–25; lane 3, bulge complement insertion mutant +39–41; lane 4, bulge deletion mutant Δ17.

radiolabeled 5.5-kDa band observed represents a proteolytic fragment of Tat that is generated during cross-linking. This band is also detected by Western blotting with an anti-Tat antibody.

Specificity of Tat–TAR interaction was examined by comparing cross-linking of Tat with wild-type and mutant TAR RNAs (Figure 7). Previously, it has been demonstrated that the bulge structure at residues +23–25 is required for transactivation and Tat binding (Selby et al., 1989; Dingwall et al., 1990; Weeks et al., 1990). When cross-linking was performed with mutants that either deleted residues +23–25, added residues complimentary to the bulge at position +39, or eliminated the A at residue +17, no cross-linking to Tat protein was observed. This verifies our observation that interaction of Tat with TAR is specific and involves sufficiently close contact between the protein and nucleic acid for cross-linking to occur.

DISCUSSION

We have demonstrated that recombinant HIV-1 Tat can be purified from *E. coli* using a procedure that does not require the use of denaturants or protein unfolding. When Tat protein is expressed in *E. coli*, it binds tightly and nonspecifically to

nucleic acids. While bound to nucleic acids, the protein cannot bind to ion-exchange resins; however, it will bind to a metal chelate column charged with Zn(II). Following extensive washing of the bound protein with a high-salt buffer to remove nucleic acids, the resulting crude protein preparation will bind to ion-exchange columns and can be purified by conventional methods in the absence of denaturants. Using this procedure, protein refolding can be avoided, making structural studies with this preparation possible. Previously reported methods for Tat purification involving chromatography under denaturing conditions have resulted in Tat preparations that lack secondary structure, although some evidence for secondary structure has been obtained with synthetic peptides (Loret et al., 1991). Circular dichroism analysis of our Tat protein indicates the presence of 15–20% α-helical structure in the protein.

Atomic adsorption and amino acid analysis show 1.64 mol of Zn(II) bound/mol of Tat. A structural model of Tat has been suggested that describes Tat as a metal-linked dimer with 2 Zn(II)/mol of Tat (Frankel et al., 1988). This model was based on migration of renatured Tat through native polyacrylamide gels to measure protein size. We have found no evidence that our Tat protein exists as a dimer in solution. The protein in our preparation is a mixture of both monomeric protein and high molecular weight aggregate as evidenced by gel-filtration chromatography in the absence of detergents or chaotropic agents. It is unclear if this aggregation is concentration dependent or related to the physical state of the protein. Recently, Rice and Chan have shown that Tat expressed in COS cells at low concentrations exists as a monomer (Rice & Chan, 1991).

In addition to the structural studies that have been performed on Tat, we have demonstrated that the protein prepared by this method is functional in cell-based assays. It has previously been shown that mammalian cells can take up externally added Tat from the medium and the protein can transactivate HIV LTR-driven gene expression (Frankel & Pabo, 1988). Using HL3T1 cells, 180 pM recombinant Tat is sufficient for cellular uptake and transactivation of the integrated HIV LTR CAT construct. The specific activity of this protein is 10–100-fold higher than protein preparations that have previously been reported.

Binding studies by gel-shift analysis using renatured recombinant Tat protein or synthetic peptides of various domains of Tat have identified the region of the protein that is essential for TAR RNA recognition and a K_d for the complex has been determined (Dingwall et al., 1989; Weeks et al., 1990; Roy et al., 1990). Results from our filter binding assays confirm the reported K_d and further address the thermodynamic properties of complex formation. Measuring the K_a dependence on temperature reveals both an enthalpic and an entropic contribution to complex formation of 0.6 kcal mol^{-1} and 34.6 cal K^{-1} mol^{-1}, respectively. Studies using synthetic peptides and site-directed mutagenesis have narrowed the site of protein–RNA interaction to the basic domain of the protein (residues 49–57), which includes six arginine residues. Salt dependence of the binding affinity indicates that a maximum of one ion pair is involved with Tat binding to TAR RNA. It is possible that one of these arginine residues interacts with the phosphate backbone of TAR RNA. This would support the arginine fork model recently proposed by Calnan et al. (1991). Although a single ion pair is formed between the protein and TAR RNA upon binding, it appears that 80% of the binding energy is contributed by nonelectrostatic interactions. Identification of the contact points between TAT and TAR RNA will require high-resolution structural information that can be derived by NMR or X-ray crystallography of a Tat–TAR RNA complex.

UV cross-linking indicate that recombinant Tat binds directly to TAR RNA. Direct contact between the protein and nucleic acid is required for formation of a stable cross-linked complex. The specific cross-linking of Tat to wild-type TAR but not to the bulge mutants provides additional indication of the quality of protein that can be prepared by this method. Due to the low amount of cross-linked complex recovered, direct amino acid sequencing of the Tat–TAR cross-linked complex failed to identify the amino acid residue that formed the cross-link. Further studies will be needed to identify the amino acid residues involved in cross-linking.

Characterization of the interaction of HIV Tat with its target RNA is an important step in understanding the mechanism by which this essential protein functions in viral replication. The ability to prepare large amounts of recombinant protein that can function in both cellular and cell-free assays and is suitable for structural analysis will provide us with an important tool to further assess this interaction. In addition, structural information about the protein structure may provide additional targets for therapeutic intervention.

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