

Dimerization of the tat protein from human immunodeficiency virus: A cysteine-rich peptide mimics the normal metal-linked dimer interface

(acquired immunodeficiency syndrome/trans-activation/drug design/mass spectrometry)

ALAN D. FRANKEL*, LING CHEN†, ROBERT J. COTTER†, AND CARL O. PABO*‡

Departments of *Molecular Biology and Genetics and of †Pharmacology and Molecular Sciences, and the ‡Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21205

Communicated by Hamilton O. Smith, May 16, 1988

ABSTRACT We have synthesized an 18-amino acid peptide that contains the cysteine-rich region of the tat protein from human immunodeficiency virus. Previous experiments *in vitro* with the intact tat protein have shown that these cysteines serve as metal ligands, causing tat to form metal-linked dimers. Ultraviolet absorption spectra show that the synthetic peptide (tat₂₁₋₃₈) binds two Cd²⁺ or two Zn²⁺ ions per peptide monomer, and some changes in the circular dichroism spectra are seen as the metals bind. The peptide-metal complexes are completely resistant to proteolytic digestion, and mass spectrometry demonstrates that this peptide forms metal-linked dimers. The peptide can also combine with the intact tat protein to form metal-linked heterodimers. If these heterodimers are unable to trans-activate viral transcription, tat₂₁₋₃₈ could be a lead compound for designing drugs to treat acquired immunodeficiency syndrome.

The tat protein from human immunodeficiency virus is a viral trans-activator (1) and is an attractive target for drug design because the protein is essential for viral replication (2, 3). Tat is 86 amino acids long and contains a highly basic region (two lysines and six arginines within 9 residues) and a cysteine-rich region (seven cysteines within 16 residues) (Fig. 1) (4, 5). We have recently shown that purified tat can form metal-linked dimers (6). This dimer structure contains four metal ions, and it appears that two or more of the metal ions bridge the cysteine-rich regions from each monomer. tat-Cd²⁺ dimers are very stable (the protein dimerizes at low pH or in the presence of NaDodSO₄) and dimers can also be formed with Zn²⁺. From proteolytic digestion studies and circular dichroism spectra, we concluded that the structural effects of metal binding were localized primarily, if not exclusively, to the cysteine-rich region.

Although there is no direct evidence yet for the biological role of the metal-linked dimers, the reducing environment and relatively high metal concentrations in eukaryotic cells may allow tat to form similar dimers *in vivo*. If dimerization is required for trans-activation, one might inactivate tat by forming heterodimers with truncated or inactive subunits. We imagine these mutant subunits would be trans-dominant. [Many DNA-binding proteins can be inactivated by adding subunits that contain a normal dimer contact site but a defective DNA-binding site (7).] To explore this possibility and to simplify structural studies of the dimer interface, we have synthesized an 18-amino acid peptide containing just the cysteine-rich region of tat (residues 21-38; Fig. 1). We show that this peptide (tat₂₁₋₃₈) has metal-binding properties similar to intact tat. The peptide can form stable metal-linked dimers and also can form heterodimers with intact tat.

MATERIALS AND METHODS

Synthesis and Purification of tat₂₁₋₃₈. tat₂₁₋₃₈ was synthesized on an Applied Biosystems (Foster City, CA) model 430A peptide synthesizer and cleaved from the resin by using a low HF procedure (8). After ether extraction of the scavengers, the peptide was solubilized in 2% (vol/vol) 2-mercaptoethanol in water and then lyophilized. tat₂₁₋₃₈ was resuspended in 0.5 M dithiothreitol and heated at 90°C for 30 min to ensure complete reduction. The peptide was loaded onto a C₄ reverse-phase HPLC column in 0.1% trifluoroacetic acid, eluted with an acetonitrile gradient, and immediately lyophilized. All manipulations with the purified peptide were carried out in a nitrogen-purged anaerobic chamber. Peptide sequencing and amino acid analysis confirmed the identity of tat₂₁₋₃₈, and the amino acid analysis was used to quantitate the amount of peptide. tat₂₁₋₃₈ has an extinction coefficient of 2.33 optical density units per mg of peptide at 278 nm in 20 mM Tris-HCl (pH 7.2). Plasma desorption mass spectrometry (see below) showed that the purified peptide had the proper molecular weight (2104 atomic mass units), and titration with 5,5'-dithiobis(2-nitrobenzoic acid) (9) indicated that there were 7 mol of free thiol per mol of peptide.

Ultraviolet Absorption and Circular Dichroism Spectra. Peptide samples for absorption spectroscopy (10-50 μg/ml in 20 mM Tris-HCl, pH 7.2) were prepared in an anaerobic chamber, and spectra were recorded and analyzed on a Hewlett-Packard 8451A diode array spectrophotometer. For the pH titration, the preformed Cd²⁺ complex was acidified with HCl. Circular dichroism spectra were recorded at 27°C on an Aviv model 60DS spectropolarimeter. The sample compartment was purged with nitrogen and spectra were averaged over five scans.

Mass Spectrometry. Mass spectrometric analyses were carried out on a BIO-ION Nordic (Uppsala) BIN 10K plasma desorption mass spectrometer. To check the mass of the peptide, lyophilized apo-tat₂₁₋₃₈ was dissolved in water (7 nmol per 10 μl) and deposited on a sample foil coated with nitrocellulose. To study the peptide-metal complexes, 7 nmol of peptide was incubated with 3 equivalents of CdCl₂ in 20 mM Tris-HCl (pH 7.0). The complex was lyophilized and redissolved in water, deposited onto a nitrocellulose foil, and washed with 0.01% acetic acid to remove excess Cd ions before counting. Sample ions were collected to a preset value of 2 × 10⁶ counts. Masses were standardized by using the H⁺ and Na⁺ peaks from the same spectra.

Proteolysis. Apo-tat₂₁₋₃₈ and tat₂₁₋₃₈-Cd²⁺ complexes (4 μg) were digested with trypsin or chymotrypsin (2 μg) for 2 min at room temperature in 20 mM Tris-HCl, pH 7.0. Digestions were stopped by adding 25 μg of phenylmethylsulfonyl fluoride to each reaction mixture, and the products were analyzed on a native 20% polyacrylamide gel (30 mM sodium acetate, pH 4.5). The gel was extensively prerun to remove ammonium persulfate, and CdCl₂ was added to the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

ALA CYS THR ASN CYS TYR CYS LYS LYS
 CYS CYS PHE HIS CYS GLN VAL CYS PHE

Met Glu Pro Val Asp Pro Arg Leu Glu Pro
 Trp Lys His Pro Gly Ser Gln Pro Lys Thr
 Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys
 Cys Phe His Cys Gln Val Cys Phe Ile Thr
 Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys
 Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln
 Gly Ser Gln Thr His Gln Val Ser Leu Ser
 Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp
 Pro Thr Gly Pro Lys Glu

FIG. 1. Amino acid sequences of the tat₂₁₋₃₈ peptide (Upper) and the tat protein (4, 5). The peptide corresponds to residues 21-38 of tat (boxed).

apo-tat₂₁₋₃₈ digests prior to loading since the apo-peptide was not readily visible on native gels.

RESULTS AND DISCUSSION

Metal Binding of tat₂₁₋₃₈. Ultraviolet absorption spectra with CdCl₂ or ZnCl₂ indicated that tat₂₁₋₃₈ binds metals with properties similar to intact tat. Fig. 2a shows the spectra obtained as 0-2.0 molar equivalents of CdCl₂ were added to the peptide. There is a maximum in the difference spectra at 248 nm, indicating charge transfer between the metals and sulfur ligands. No further changes in the spectrum were observed as additional CdCl₂ was added, suggesting that 2

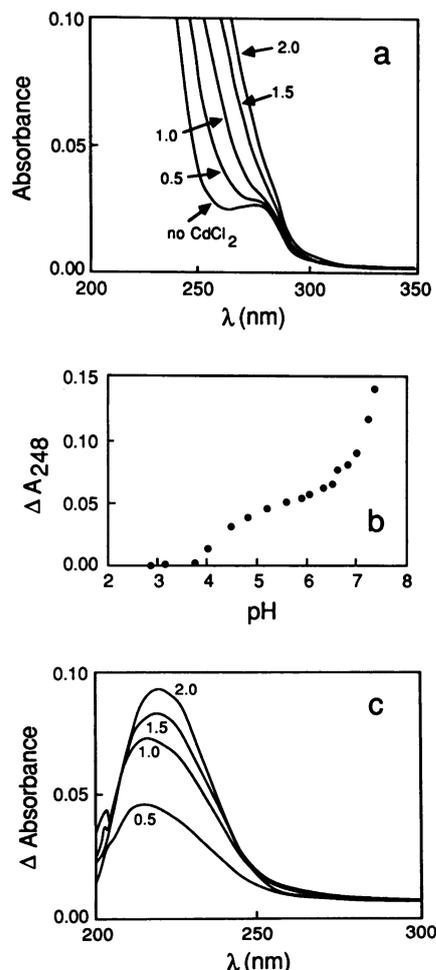


FIG. 2. (a) Ultraviolet absorption spectra of apo-tat₂₁₋₃₈ and tat₂₁₋₃₈ with 0.5-2.0 molar equivalents of CdCl₂. (b) pH dependence of CdCl₂ binding to tat₂₁₋₃₈. (c) Difference absorption spectra of tat₂₁₋₃₈ with 0.5-2.0 molar equivalents of ZnCl₂.

metal ions bind per peptide molecule. These spectra are virtually identical to those seen with intact tat (6). Difference spectra were used to monitor the effect of pH on the peptide-Cd²⁺ complex (Fig. 2b). Dissociation of the complex appears to be biphasic, with ≈60% of the metal dissociated when the pH is reduced to 6.0. Additional dissociation occurs between pH 3.8 and 4.5. In contrast, the intact protein showed a single dissociation occurring between pH 3.0 and pH 4.0. This result suggests that the peptide-Cd²⁺ complex is not as stable as the tat-Cd²⁺ complex, and it appears that the affinity of one of the binding sites is reduced. It is possible that this peptide is missing some residues from the normal binding site, or that the presence of charged termini in the vicinity of the metal binding sites may account for the reduced stability of the peptide-Cd²⁺ complex. However, the other binding site seems to be intact because the midpoint of the low pH transition is very similar to that measured with the intact protein (6). The stoichiometry of zinc binding was also determined. Fig. 2c shows the difference absorption spectra obtained as 0.5-2.0 equivalents of ZnCl₂ were added to the peptide. There is a maximum at 218 nm, and no further changes were seen as additional ZnCl₂ was added. Cd²⁺ easily displaced Zn²⁺ from the complex, while a 5000-fold excess of ZnCl₂ only partially displaced Cd²⁺. In summary, these difference spectra, metal stoichiometries, and relative affinities of Zn²⁺ and Cd²⁺ are all similar to those observed with intact tat (6).

Metal-Linked Dimerization of tat₂₁₋₃₈. Does tat₂₁₋₃₈, like the intact protein, form metal-linked dimers? Because of the small size of the peptide and problems resulting from oxidation, we were unable to readily monitor dimerization on native or NaDodSO₄ gels. However, the low molecular weight of the peptide made it possible to use plasma desorption mass spectrometry to monitor dimerization. Two broad peaks are observed for the tat₂₁₋₃₈-Cd²⁺ complex (Fig. 3), and these correspond roughly to the molecular weights of the monomer and dimer. Each peak exhibits some "fine structure" that indicates the stoichiometry of the peptide-metal complexes. Within the "monomer" peak, some singly charged complexes contain two Cd²⁺ ions and one peptide molecule (accompanied by the loss of an appropriate number of protons). For example, the major peak observed at 2325 atomic mass units probably corresponds to tat₂₁₋₃₈ + 2Cd²⁺ - 3H⁺ (2326.8 atomic mass units calculated), although this could correspond to a doubly charged dimer (10). The peak at 2223 atomic mass units probably contains a monomer with

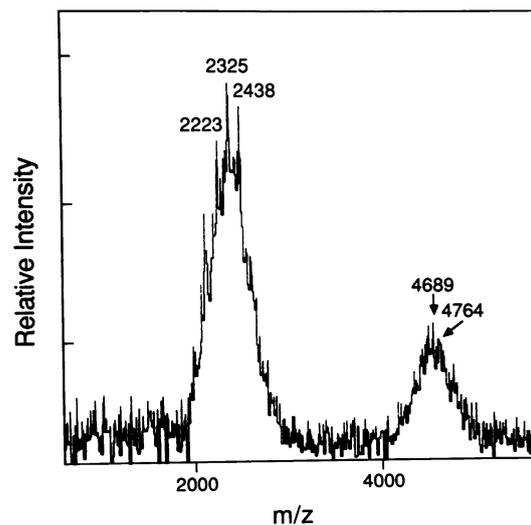


FIG. 3. Plasma desorption mass spectrometry of the tat₂₁₋₃₈-Cd²⁺ complex. The molecular weights of some major species are indicated.

one Cd²⁺ ion and the peak at 2438 atomic mass units probably contains a monomer with three Cd²⁺ ions.

The "dimer" peak is less well resolved than the monomer peak but we believe the major species are tat₂₁₋₃₈ dimers with four bound Cd²⁺ ions (adjusted by the addition of Cl⁻ anions, or subtraction of protons, to achieve a single charge). The major species do not differ by integral masses of cadmium. The peak at 4689 atomic mass units may correspond to 2 tat₂₁₋₃₈ + 4 Cd²⁺ + Cl⁻ - 6H⁺ (4689.1 atomic mass units calculated), and the peak at 4764 may correspond to 2 tat₂₁₋₃₈ + 4 Cd²⁺ + 3 Cl⁻ - 4H⁺ (4763.1 atomic mass units). The "codesorption" of Cl⁻ anions to produce singly charged species is reasonable since CdCl₂ was present in these samples. These results confirm that tat₂₁₋₃₈ binds two metal ions per monomer and demonstrate that the peptide forms stable metal-linked dimers.

Folding Monitored by Proteolysis and Circular Dichroism. The tat₂₁₋₃₈ peptide folds in the presence of Cd²⁺, and the peptide-Cd²⁺ complex is remarkably stable to proteolytic digestion. The complex was completely protected from proteolytic digestion even when a large amount of trypsin or chymotrypsin was used (2 μg of protease per 4 μg of tat₂₁₋₃₈ for 2 min; Fig. 4, lanes 4 and 6). In the absence of metal, the peptide was entirely digested by these proteases (lanes 3 and 5). (Since the apo-peptide was not readily visible on native gels, CdCl₂ was added to the apo-tat₂₁₋₃₈ digests just before loading the gel.) The peptide band seen with CdCl₂ (lane 2) was shown to contain Cd²⁺ by autoradiography of complexes formed with ^{115m}Cd. Zn²⁺ also gave a complex that was very stable to proteolytic digestion.

The circular dichroism spectrum of the peptide changes considerably when Zn²⁺ or Cd²⁺ is added (Fig. 5). In a previous study, we found that the CD spectrum of intact tat changed very little as the metals bound, leading us to suggest that metals do not mediate a global folding transition for tat (6). Apparently, the structural changes are concentrated in the cysteine-rich region since we see greater changes in the spectrum (on a wt/wt basis) as metals bind to the peptide.

Inhibition of tat Dimerization by tat₂₁₋₃₈. Can tat₂₁₋₃₈ "inhibit" tat dimer formation by forming metal-linked heterodimers? Since tat-Cd²⁺ dimers are stable during NaDodSO₄ gel electrophoresis (6), we used this method to look for heterodimers (Fig. 6). Apo-tat runs as a set of oligomeric bands resulting from oxidation during electrophoresis (lane 1), while the tat-Cd²⁺ complex runs predominantly as a dimer (lane 2). Addition of CdCl₂ to a mixture of tat₂₁₋₃₈ and intact tat (2 mol of peptide per mol of tat) results in the appearance of a band of intermediate molecular weight, which must be a Cd²⁺-linked heterodimer (lane 3). About 70% of the tat is complexed with the peptide, suggesting that the dimer interface formed by the peptide is about as stable

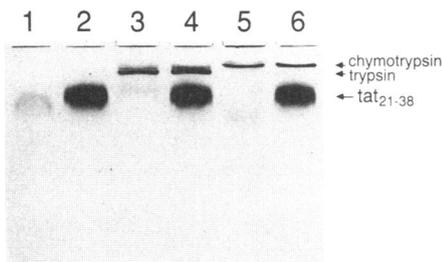


FIG. 4. Proteolytic digestion of tat₂₁₋₃₈. Apo-tat₂₁₋₃₈ (lane 3) and the tat₂₁₋₃₈-Cd²⁺ complex (lane 4) were treated with trypsin. The apo-peptide and tat₂₁₋₃₈-Cd²⁺ complexes were also treated with chymotrypsin (lanes 5 and 6, respectively). Lane 1 contains undigested apo-tat₂₁₋₃₈; lane 2 contains the undigested tat₂₁₋₃₈-Cd²⁺ complex. (As mentioned in the text, Cd²⁺ was added to samples 3 and 5 after digestion to ensure that any remaining peptide would be easy to visualize.)

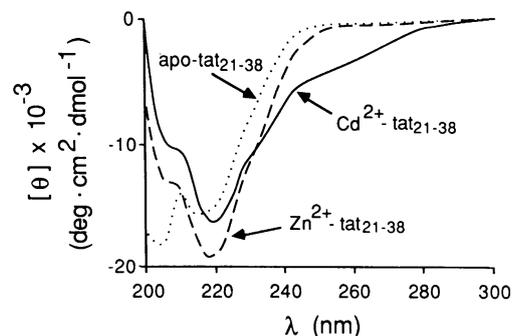


FIG. 5. Circular dichroism spectra of apo-tat₂₁₋₃₈ (· · ·), tat₂₁₋₃₈-Cd²⁺ complexes (—), or tat₂₁₋₃₈-Zn²⁺ complexes (---). tat₂₁₋₃₈ samples (300 μg/ml in 10 mM Tris·HCl, pH 7.2) were prepared in an anaerobic chamber and spectra were measured with a 0.05-cm demountable quartz cuvette.

as the dimer interface formed by the intact protein. In experiments with ^{115m}Cd, we have shown that the heterodimer band seen on NaDodSO₄ gels contains Cd, and we have also shown that incubating the heterodimer with EDTA dissociates the complex. Prior formation of the dimer does not kinetically block heterodimer formation. When the peptide was added to preformed tat-Cd²⁺ dimers, the peptide readily displaced the protein and formed heterodimers.

Conclusions. We have shown that an 18-amino acid peptide that contains the cysteine-rich region of tat (tat₂₁₋₃₈) can form metal-linked dimers. This finding supports our previous conclusion that this region forms the dimer interface for the protein (6). This peptide should simplify further structural studies of the metal-binding dimer interface since it may be readily amenable to analysis by x-ray crystallography or two-dimensional NMR. Furthermore, we have shown that the peptide can inhibit dimerization of the intact protein by forming heterodimers. Since it is likely that dimerization is important for the trans-activating activity of tat, this peptide or other fragments containing the cysteine-rich region may act as trans-dominant mutants and inactivate functional tat. Testing this prediction will test the importance of metal-linked dimerization *in vivo*.

tat is an attractive target for drug design, and the unusual metal-linked dimer interface may offer opportunities for rational drug design. Our results with this peptide are encouraging, but there are obvious problems with using peptides as drugs. Peptides are often degraded or do not cross cell membranes. However, it might eventually be possible to

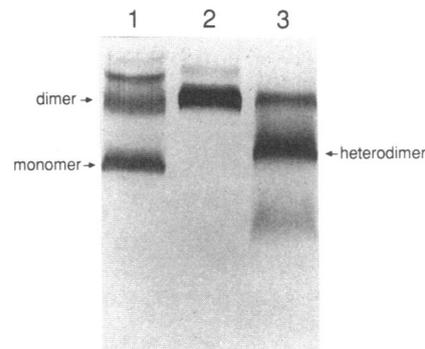


FIG. 6. NaDodSO₄ gel electrophoresis of tat dimers and tat₂₁₋₃₈-tat heterodimers. Lanes: 1, 3 μg of apo-tat; 2, 3 μg of tat-Cd²⁺ complex; 3, 3 μg of tat with 1 μg of tat₂₁₋₃₈ and Cd²⁺. Samples were electrophoresed on a 20% NaDodSO₄/polyacrylamide gel using the conditions of Laemmli (11), but with no stacking gel, and the gel was prerun for 30 min before loading. Positions of the protein monomer, protein dimer, and protein-peptide heterodimer are indicated with arrows.

use liposomes to deliver peptides or their genes to specific cells (12). It might also be possible to make hydrophobic variants of this peptide, which could be more easily taken up by cells. Alternatively, one might design chelators or other organic compounds that would bind to tat monomers and inhibit normal metal-linked dimerization.

We are especially grateful to Clark Riley and Van Vogel for peptide synthesis and sequencing. We thank Christine Debouck and Martin Rosenberg of Smith Kline & French Laboratories for supplying cells containing the tat protein, Peter Kim for advice on cleavage and extraction of the peptide, Peter Dervan for helpful suggestions, P. Shenbagamurthi for amino acid analysis, Howard Dintzis for the use of his Hewlett-Packard spectrophotometer, and David Shortle for the use of his Aviv spectropolarimeter. This work was supported by the Howard Hughes Medical Institute and by National Institutes of Health Grant GM31471.

1. Sodroski, J., Rosen, C., Wong-Staal, F., Salahuddin, S. Z., Popovic, M., Arya, S., Gallo, R. C. & Haseltine, W. A. (1985) *Science* **227**, 171-173.
2. Fisher, A. G., Feinberg, M. B., Josephs, S. F., Harper, M. E.,

- Marselle, L. M., Reyes, G., Gonda, M. A., Aldovini, A., Debouck, C., Gallo, R. C. & Wong-Staal, F. (1986) *Nature (London)* **320**, 367-371.
3. Dayton, A. I., Sodroski, J. G., Rosen, C. A., Goh, W. C. & Haseltine, W. A. (1986) *Cell* **44**, 941-947.
4. Arya, S., Guo, C., Josephs, S. F. & Wong-Staal, F. (1985) *Science* **229**, 69-73.
5. Sodroski, J., Patarca, R., Rosen, C., Wong-Staal, F. & Haseltine, W. A. (1985) *Science* **229**, 74-77.
6. Frankel, A. D., Bredt, D. S. & Pabo, C. O. (1988) *Science* **240**, 70-73.
7. Miller, J. H. & Reznikoff, W. S. (1980) *The Operon* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
8. Stewart, J. M. & Young, J. D. (1984) *Solid Phase Peptide Synthesis* (Pierce Chemical, Rockford, IL).
9. Means, G. E. & Feeney, R. E. (1971) *Chemical Modification of Proteins* (Holden-Day, San Francisco).
10. Alai, M., Demirev, P., Fenselau, C. & Cotter, R. J. (1986) *Anal. Chem.* **58**, 1303-1307.
11. Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685.
12. Wang, C.-Y. & Huang, L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7851-7855.