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# Simian Virus 40 Small-t Antigen Binds Two Zinc Ions

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**Six cysteine residues of the simian virus 40 small-t antigen (small-t) are important for stability of the protein. Stability has been shown to be related to the ability of small-t to bind zinc ions in vitro. Purified small-t expressed either in bacteria or from baculovirus vectors binds two molecules of zinc per molecule of protein. Thus, small-t may resemble GAL4, which contains a $Zn(II)_2Cys_6$ binuclear cluster.**

The simian virus 40 small-t antigen (small-t) is a small, cysteine-rich protein which enhances both productive and transforming infections by simian virus 40 (2, 4, 10, 17, 18). Several early efforts to express and isolate small-t from bacteria resulted in the observations that this protein was highly aggregated and that aggregation could not be reversed by the inclusion of reducing agents (1, 19). We have shown recently that small-t in monomeric form can be purified from bacteria when insoluble fractions which contain small-t are solubilized in the presence of zinc ions in addition to urea and reducing agents (6). These preparations have been used in studies of the binding of small-t to cellular proteins, now known to be the A and C subunits of protein phosphatase 2A (12, 14, 21). These studies showed that small-t inhibited the activity of protein phosphatase 2A against substrates such as myosin light chain and myelin basic protein (22), as well as against phosphorylated simian virus 40 large-T antigen and p53 isolated from transformed cell lines (15). Purified small-t was also shown to inhibit simian virus 40 DNA replication in vitro (3), a result predicted from the finding that in vitro viral DNA replication is stimulated by protein phosphatase 2A (20).

Purified preparations of small-t were used to assay associated zinc ions and to determine the stoichiometry of the binding. Small-t was purified by using Sephadex G200, DEAE-cellulose, and hydroxylapatite chromatography as described previously (6), with the exception that the protein was recovered from hydroxyapatite by using 0.1% *n*-octylglucopyranoside (Sigma Chemicals). *n*-Octylglucopyranoside was found to be an adequate substitute for other nonionic detergents, and it was readily removed by dialysis. This eliminated UV absorption by detergents, which interfered with zinc and protein determinations.

In initial experiments, zinc was found to be present in small-t preparations by atomic absorption spectroscopy with a Hitachi atomic absorption spectrometer equipped with a graphite electrothermal furnace. For these analyses, small-t was dialyzed overnight against 10 mM Tris-HCl, pH 8, containing 10 mM NaCl. Proteins were diluted 1:100 into metal-free water before analysis. The buffer showed an absorption of 0.0480, and small-t showed an absorption of 0.2025. These assays also showed that zinc ions were tightly associated and were not removed by this dialysis, dialysis against EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid] (absorption, 0.1607), dialysis against EDTA, or treatment with Chelex resin. Significant levels of zinc ions remained following dialysis against EGTA, which also slightly reduced the protein concentration. Although results are not shown here, dialysis against either EGTA or EDTA did not affect the monomeric behavior of small-t, as determined by chromatography on Sephadex G200. This is of interest because mutant proteins which fail to bind zinc ions also fail to fractionate as monomeric proteins in gel filtration (6).

Atomic absorption analyses were particularly useful because this procedure is specific for given ions, and they showed that small-t was bound to zinc ions. On the basis of these initial observations, the stoichiometry between zinc ions and small-t was measured. For this, reliable methods of protein determination were required. Initially, protein concentrations were determined by using BioRad assay kits, but this method yields concentrations relative to a marker protein, such as albumin, and the assay is influenced by relative concentrations of individual amino acids. For accurate protein measurements, we used a recently described UV-spectrophotometric assay (5) which is based on extinction coefficients calculated from known amino acid sequences. That report also showed that absorption by native proteins did not differ significantly from absorption by the proteins' denatured counterparts.

Preparations of the 17-kDa small-t from bacteria also contain a 14-kDa derivative, which arises from internal initiation of translation (6). In general, the 17-kDa intact small-t accounted for over 75% of the total protein, but estimates of the proportion of 17-kDa small-t were made for each preparation. For protein determination by UV absorption, extinction coefficients were calculated on the basis of the known amino acid sequences of small-t and the 14-kDa derivative (14-kDa derivative, Trp = 5, Tyr = 6, Cys = 10; 17-kDa small-t, Trp = 5, Tyr = 7, Cys = 11). The extinction coefficients were calculated to be 44,420 and 37,330 $M^{-1}$ $cm^{-1}$ for the 17-kDa and 14-kDa proteins, respectively. In one preparation, it was confirmed that the UV absorption by the native protein was identical to that shown by the protein denatured with 7 M guanidine-HCl, as described for the original method (data not shown). In three separate preparations of small-t, protein concentrations were confirmed by total amino acid analysis of acid-hydrolyzed proteins. Calculations of protein concentration based on spectrophotometric analyses differed by less than 10% from the concentrations determined on the basis of amino acid analysis.

Using the buffers and conditions necessary to maintain active small-t, we found it difficult to obtain good calibration

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TABLE 1. Stoichiometry of zinc binding to small-t

| Preparation | Amt (μmol) of: | | Ratio |
|---|---|---|---|
| | Small-t | Zinc | |
| Bacterial | | | |
| 1 | 1.61 | 3.54 | 2.20 |
| 2 | 1.78 | 3.20 | 1.79 |
| 3 | 2.90 | 5.89 | 2.03 |
| 4 | 1.20 | 2.46 | 2.05 |
| Baculovirus | 2.52 | 4.15 | 1.65 |

of zinc concentrations by atomic absorption. More-reproducible results were obtained by using a colorimetric assay used previously to determine the stoichiometry of zinc ions bound to transcription factor IIIA. In this assay, zinc ions coordinated by cysteine residues are released from proteins by using *p*-(hydroxy-mercuri)-benzenesulfonate (PMPS). Free zinc ions are then detected by using the metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) (16). PAR (Sigma Chemicals) was maintained as a 5 mM stock solution at 4°C in a foil-wrapped polystyrene tube. New stock solutions were prepared when the molar absorptivity of standard zinc-PAR complex solutions began to deviate from the literature value ($6.6 \times 10^4$ $M^{-1}$ $cm^{-1}$). Stock solutions of zinc (Ricca Co.) were purchased from Baxter Healthcare, Inc. Water obtained from a Millipore MilliQ system was found to have zinc contamination of less than 1 ppb when tested by atomic absorption spectroscopy, and this water was used to prepare all the reagents used to dialyze small-t before analysis. All protein preparations and solutions were stored in polystyrene tubes to reduce the exogenous zinc contamination commonly found with other types of plastic. Dialysis buffer (20 mM Tris-HCl, pH 8, containing 10 mM NaCl) was prepared from Tris-HCl and NaCl stock solutions which had been passed through Chelex resin before use. Dialysis tubing was heated at 80°C with several changes of metal-free water and handled exclusively with powder-free gloves.

Four independent preparations of small-t were made from bacteria as previously described (6), except that cultures were grown for 4 h before induction and then small-t expression was induced for an additional 4 h. Also, for the final chromatography step on hydroxylapatite, the dialyzable detergent *n*-octylglucopyranoside (0.1%) was used in place of Triton X-100. Solutions of small-t (1 to 2 μM) were made in dialysis buffer containing 0.1 mM PAR. The amount of protein-bound zinc was determined by monitoring $A_{500}$ both before and after the addition of 10 mM PMPS to a final concentration of 0.2 mM. After an initial reading, additional PMPS was added to the solution to ensure that zinc release was complete (no further increase in absorbance). The concentration of zinc released was measured against a standard curve by using a calibrated solution of zinc chloride (Ricca Co.). Dilutions of the zinc stock solution were prepared in the same buffer used for the protein analysis with 0.1 mM PAR.

As shown in Table 1, the results obtained are consistent with the binding of two zinc ions by each molecule of small-t. This method also proved that metal ions were coordinated by cysteine residues (i.e., the ions were PMPS releasable). It was possible that zinc binding was an artifact of the bacterial preparations, because the protein in these preparations is isolated in insoluble form, solubilized in urea, and then allowed to renature in the presence of zinc ions. As an alternate approach, small-t purified from baculovirus vectors was analyzed. Small-t was expressed in insect cells at levels of about 1% of the total cellular protein. In contrast to small-t expressed from bacteria, the small-t expressed from baculovirus vectors is soluble (10a, 11). Small-t was recovered from soluble fractions by passage through a DEAE-cellulose column, to which it does not bind in 100 mM NaCl (1, 6), and then it was isolated on PAb419-Sepharose (7) affinity columns. Small-t was eluted from antibody columns with buffer containing 20 mM triethylamine, pH 11.3. After neutralization, small amounts of the eluted antibody were removed by using protein A-Sepharose. Analysis by gel electrophoresis showed that small-t was >95% pure. As shown in Table 1, PMPS released metal ions from small-t purified from baculovirus-infected insect cells, indicating that metal binding by bacterially expressed small-t was not simply a reflection of the methods used to purify the protein.

The finding that slightly less than 2 mol of zinc was present per mol of small-t from insect cells may result from the isolation procedure. In contrast to the method used with small-t preparations from bacteria, gel filtration columns were not used during the purification of small-t from insect cells. Although the majority of the baculovirus-expressed small-t was monomeric, as determined by gel filtration after purification, it is possible that a small fraction of small-t molecules remained aggregated in dilute solution or that some zinc was released by the high-pH buffer used for elution from the antibody affinity columns. However, the calculated ratio of 1.65 molecules of zinc per molecule of protein is nearly the same as the ratios for several of the small-t bacterial preparations, which ranged from 1.8 to 2.2. It is also possible that some of the small-t isolated from insect cells was associated with ions other than zinc. The colorimetric assay used here detects a variety of metal ions, and each metal complex has a slightly different extinction coefficient. To determine specifically whether zinc was present, a second preparation of small-t expressed from baculovirus vectors was analyzed by atomic absorption spectroscopy. Strong signals were obtained, confirming the presence of zinc ions in at least a portion of the small-t molecules isolated from baculovirus-infected insect cells (data not shown).

Unfortunately, several attempts to isolate sufficient quantities of highly purified small-t from animal cells were unsuccessful because of the low levels of small-t expressed in natural infections. Although small-t could be obtained in a highly enriched form (20 to 30% pure), parallel fractions from uninfected cells contained detectable levels of metal ions that interfered with the assays.

In summary, these results suggest that the binding of zinc ions by small-t is not unique to small-t purified from bacterial cells and that small-t can bind two molecules of zinc per molecule of protein. Thus, small-t resembles the GAL4 transcription factor, in which six Cys residues are involved in the binding of two zinc ions in a $Zn(II)_2Cys_6$ binuclear cluster (13). Direct proof of this would require analyses such as nuclear magnetic resonance, for which small-t derivatives bound to analyzable metals such as cadmium would be needed. Unfortunately, it has not been possible as yet to obtain soluble small-t bound to any ion other than zinc. The likely coordinate binding of two zinc ions by six Cys residues contrasts with the binding in more commonly found zinc finger structures, such as that of the viral large-T antigen (9), in which four Cys residues, or Cys-His combinations, are involved in the binding of a single zinc ion. Mutational

analyses of small-t are consistent with this interpretation, as mutation of any one of the Cys residues found in the two CysXCysXXCys clusters leads to instability of the protein in animal cells (8) and failure of the mutant protein to form monomers when solubilized from bacterial fractions in the presence of zinc ions (6). The instability of the mutant proteins in animal cells suggests that the ability of small-t to bind zinc ions may provide structural integrity to the intracellular protein.

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