Synchrotron Radiation-Induced X-Ray Emission to Identify Metal Ions in Preparations of Purified Protein

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ABSTRACT

The suitability of synchrotron radiation-induced X-ray emission (SRIXE) for the detection and identification of metal ions in preparations of purified, soluble proteins was tested. Glutathione S-transferase fused to the proximal zinc finger motif of human transcription factor IIIA or to the cysteine-rich motif of poliovirus protein 2C was expressed in bacteria and purified by affinity chromatography. Aqueous samples containing the purified proteins were analyzed with SRIXE, and trace amounts of zinc and iron were detected. Mutation of the zinc-coordinating residues in the cysteine-rich motif of poliovirus protein 2C resulted in the loss of the zinc-binding ability. Relative quantities of metal in the protein preparations as determined by SRIXE corresponded well with the metal:protein ratios calculated by using a 4-(2-pyridylazo)resorcinol-based assay. We conclude that SRIXE is an accurate, sensitive, and simple method for the detection and identification of protein-bound metal ions in small amounts of sample. Thus, SRIXE may have wide use as a particularly effective method for rapidly determining trace metals in microarray samples.

INTRODUCTION

Metal ions play important roles in protein structure and function. In metalloenzymes, the metal ion is directly involved in the catalysis of a chemical reaction (6). Other proteins require metal ions for the stabilization of the tertiary structure. Zinc finger proteins contain one or several small zinc-binding domains that mediate specific protein-nucleic acid or protein-protein interactions (2). The zinc ion is crucial for the proper folding and structure of the zinc finger. Other metal-binding proteins are involved in the homeostasis of metal concentrations in the living cell (6). The failure of such proteins to function properly results in serious disease syndromes such as Wilson's disease (3,25), Menkes disease (4,17,26), and hereditary hemochromatosis (8,14).

The study of protein function benefits from a detailed analysis of the protein's biochemical and biophysical properties. Metal-binding capabilities of a protein or of a protein domain can be indicative for a potential protein function. We tested the suitability of synchrotron radiation-induced X-ray emission (SRIXE) (5,10) for the detection and identification of protein-bound metal ions in preparations of purified, soluble protein. We used recombinant proteins consisting of glutathione S-transferase (GST) fused to zinc-binding motifs. The study shows that the minimal procedures for sample preparation and the small mass of protein needed make SRIXE an excellent tool for the detection and identification of protein-bound metal ions.

MATERIALS AND METHODS

Engineering of Expression Plasmids

Plasmids for the expression of recombinant fusion proteins in *E. coli* strain BL21(DE3) were derivatives of pGEX-KG (11). pGEX-CR^{wt} contained the coding sequence of the cysteine-rich motif of poliovirus protein 2C fused in frame to the distal end of the coding sequence of GST (Table 1) (19). Similarly, pGEX-CR^{mut} contained the coding sequence of the mutated cysteine-rich motif, in which the codons for cysteine and histidine had been replaced by those for serine and glutamine, respectively (19). pGEX-ZF3A contained the coding sequence for the proximal zinc finger of the human transcription factor IIIA [hTFIIIA; GenBank® accession no. D32257 (1)]. The cDNA of the zinc finger coding region was obtained by RT of total RNA from HeLa cells in the presence of random nonameric primers and subsequent PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and the specific positive sense primer 5'-CGGAATTCTCCGCGCCCCGCGCTTCC-3' and negative sense primer 5'-GGAGAGCTCTGCTCTCTCCCCCGTGTGC-3'. The RT-PCR product was digested with the restriction enzymes *Eco*RI and *Sac*I (Roche Molecular Biochemicals, Indianapolis, IN, USA) and inserted between the corresponding sites of pGEX-KG using T4 DNA ligase (Roche Molecular Biochemicals). *E. coli* DH5 α cells transformed with the ligated plasmid were used for plasmid propagation. The in-

Table 1. Peptide Sequences of Purified Proteins

Protein Designation	Peptide Sequences Fused to the Carboxy Terminus of GST ^a	Calculated Molecular Weight (kDa)
GST-CR ^{wt}	PSRDGKLNAMATEMCKNC ^u HQPANFKRC ^u CPLVCGKAIQLMDKSSKLNSS	32.4
GST-CR ^{mut}	PSRDGKLNAMATEMSKNSQQPANFKRSSPLVSGKAIQLMDKSSKLNSS	32.3
GST-ZF3A	PPRPALPRRFIC ^u SFPDC ^u SANYSKAWKLD ^u HLCK ^u HTGERPELKLNSS	32.3
GST	<i>LDSMGRLELKLNSS</i>	28.6

^aGST expressed from pGEX-KG included a thrombin cleavage site and a glycine linker (11). Additional vector-derived sequences are depicted in italics. Potential zinc-coordinating amino acid residues are underlined.

tegrity of the RT-PCR-derived sequences in pGEX-ZF3A was confirmed by the sequencing of purified plasmid using Sequenase® (USB, Cleveland, OH, USA).

Protein Expression and Purification

GST-fusion proteins were expressed and purified as previously described (19) with modifications. *E. coli* strain BL21 (DE3) (24) transformed with the plasmids pGEX-KG, pGEX-CR^{wt}, pGEX-CR^{mut}, or pGEX-ZF3A were grown in 250 mL 2× YT broth (21) at 37°C in the presence of 100 µg/mL ampicillin (Sigma, St. Louis, MO, USA) until an absorbance at 600 nm (A_{600}) of between 0.9 and 1.0 was reached. IPTG was added to a final concentration of 0.1 mM, and the culture was incubated at 30°C for an additional 3 h. The pelleted cells were resuspended in 12.5 mL lysis buffer (20 mM HEPES/KOH, pH 7.5, 140 mM NaCl, 10 mM 2-mercaptoethanol) and lysed in a French pressure cell. The lysate was clarified by centrifugation at 12 000× g at 4°C for 10 min. The supernatant was split in two aliquots. To each, 125 µL (bed volume) glutathione Sepharose® (Amersham Biosciences, Piscataway, NJ, USA), equilibrated in lysis buffer, was added, and the GST-fusion proteins were allowed to bind to the Sepharose at 4°C for 45 min. The collected Sepharose beads were resuspended in 12.5 mL lysis buffer containing either 1 mM EDTA or 0.1 mM zinc acetate and incubated at 4°C for 10 min. Subsequently, the beads were washed three times with lysis buffer and once with elution buffer (40 mM HEPES/KOH, pH 8.0). GST fusion

proteins were eluted stepwise in a total volume of 450 µL elution buffer containing 10 mM reduced glutathione (Sigma). Glycerol (50 µL) was added to the eluates, which were subsequently stored at -80°C. These protein preparations were used in all experiments of this study. Protein purity was monitored by SDS-PAGE (15). Protein concentration was determined according to the method described by Gill and Hip-pel (9). All buffer solutions were prepared with ultra-pure water generated by an ELGA UHQ PS water purification system (Elga Labwater, Lowell, MA, USA).

Estimation of Metal Content Using 4-(2-pyridylazo)resorcinol (PAR)

To increase the accessibility of protein-bound metal ions for chelation by PAR (13,16) (Sigma), protein samples were digested with 50 µg/mL proteinase K (Sigma) in 50 µL HBS 200

(50 mM HEPES/KOH, pH 7.5, 0.2 M NaCl) at 56°C for 30 min (23). Subsequently, 50 µL HBS 200 containing 5 mM iodoacetamide (IAM; Sigma) and 0.2 mM PAR were added. Extinction at 490 nm was measured in an MRX® microplate reader (Thermo Labsystems, Helsinki, Finland). Various concentrations of zinc acetate ranging from 5 to 30 µM in HBS 200 served to create a standard curve for the estimation of the amount of metal present in the protein preparations (19). The amount of protein analyzed was adjusted so that the amount of released metal was within the linear range of the standard curve.

SRIXE

SRIXE analysis was performed at the X26A X-ray microprobe beamline of the Brookhaven National Laboratory Synchrotron Light Source. The X-ray beam had an energy of 9.6 keV and dimensions of 1000 µm in the horizontal direction and 150 µm in the vertical direction. Five microliters of purified protein were spotted onto a hydrophobic API membrane (Process Analytics, Orem, UT, USA), forming a stable droplet. The thickness of the membrane at the sample position was only 0.15 µm so that background from scattered X-rays was minimized. The membrane was immediately placed into the incident beam at a 45° angle. The sample was positioned in the path of the beam with the help of a microscope mounted perpendicularly to the sample supporting membrane, that is, at 135° to the incident X-ray beam. The optimal sample position was determined by SRIXE scanning of the liquid droplet along the z-axis. The position with the strongest

Figure 1. Analysis of purified zinc-treated protein preparations by SDS-PAGE and Coomassie® Blue staining. Lane 1, molecular weight marker (Bio-Rad Laboratories, Hercules, CA, USA). The relative molecular weights in kDa are indicated on the left. Lane 2, purified GST (see Table 1); lane 3, GST-CR^{wt}; lane 4, GST-CR^{mut}; and lane 5, GST-ZF3A.

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Table 2. Concentrations of Protein and Zinc in the Protein Preparations

Protein Designation	Treatment	Protein ^a (g/L)	Zinc ^b (mol/L)	Zinc (g/L)	Zinc (atoms/L)	Zinc (atoms/5 μ L ^c)
GST	Zinc	5.32	1.08×10^{-4}	7.00×10^{-3}	$6.46 \times 10^{+19}$	$3.23 \times 10^{+14}$
GST-CR ^{mut}	Zinc	3.48	5.52×10^{-5}	3.59×10^{-3}	$3.31 \times 10^{+19}$	$1.65 \times 10^{+14}$
GST-CR ^{wt}	Zinc	5.82	3.60×10^{-4}	2.34×10^{-2}	$2.16 \times 10^{+20}$	$1.08 \times 10^{+15}$
GST-ZF3A	Zinc	4.30	1.13×10^{-4}	7.38×10^{-3}	$6.81 \times 10^{+19}$	$3.40 \times 10^{+14}$
GST	EDTA	5.18	4.99×10^{-6}	3.25×10^{-4}	$3.00 \times 10^{+18}$	$1.50 \times 10^{+13}$
GST-CR ^{mut}	EDTA	3.32	2.03×10^{-6}	1.32×10^{-4}	$1.22 \times 10^{+18}$	$6.09 \times 10^{+12}$
GST-CR ^{wt}	EDTA	5.59	1.92×10^{-5}	1.25×10^{-3}	$1.15 \times 10^{+19}$	$5.76 \times 10^{+13}$
GST-ZF3A	EDTA	3.56	8.41×10^{-6}	5.46×10^{-4}	$5.04 \times 10^{+18}$	$2.52 \times 10^{+13}$

^aMeasured by extinction of light at 280 nm.
^bMeasured colorimetrically in the presence of PAR.
^cSample size used for SRIXE analyses.

total signal was chosen for data accumulation, which was typically allowed to proceed for 10 min. Fluorescent X-rays were detected using a 30-mm² energy-dispersive Si(Li) X-ray detector with an energy resolution of approximately 160 eV that was mounted at 90° 4 cm from the incident X-ray beam (5). X-ray spectra and peak area calculations were obtained using a Canberra ND9900 data-acquisition system (Canberra, Meriden, CT, USA). Emission intensity was normalized to the actual ion chamber and protein concentration.

RESULTS AND DISCUSSION

The use of SRIXE for the qualitative and quantitative determination of metal content in biological, geological, and environmental samples is well established (10). The versatility of the technique is based on its low detection limits, multi-element sensitivity, and high spatial resolution (5,10). Typically, thin sections of dried, embedded, or otherwise fixed materials are used as specimen. The purpose of this study was to evaluate SRIXE as a tool to detect and identify metal ions in preparations of purified, soluble protein. In contrast to other spectroscopic methods, SRIXE can analyze samples under atmospheric conditions, making laborious sample preparation unnecessary. In addition, SRIXE has the potential to be used for the analysis of samples arranged in microarrays because of the high spatial resolution of the microprobe, high

detection sensitivity, and minimal amounts of sample materials needed.

We have used short metal-binding peptides fused to GST (Table 1). GST-CR^{wt} contained the cysteine-rich motif of the polioviral nonstructural protein 2C. Using the metal chelator PAR, we have previously demonstrated that this motif is zinc binding (19). When the five cysteine residues and the histidine residue within the motif were replaced by serine and glutamine residues, respectively (GST-CR^{mut}; Table 1), zinc binding was lost (19). GST-ZF3A contained the proximal zinc finger of the nine zinc finger-containing domain of human transcription factor IIIA. Homologous zinc fingers have been shown to bind zinc (18). Finally, GST without the metal binding motif was expressed to determine the metal-binding properties of GST itself. All buffers used during purification contained the reducing agent 2-mercaptoethanol to keep the thiol groups of the cysteine side chains in a reduced form and, thus, competent for metal coordination. Of each protein two preparations were made. One preparation included a short incubation of the immobilized protein with zinc before protein purification to saturate the metal-binding sites with zinc. The other preparation included a short incubation with the chelating agent EDTA before protein purification to remove protein-bound metals. Protein preparations were analyzed for purity and correct size of the fusion proteins by SDS-PAGE (Figure 1). Each preparation contained one major band

that migrated according to its expected size (Table 1). Faster migrating bands represent GST and, most likely, other truncation products. Zinc and EDTA treatment did not alter the migration pattern or the relative quantities of the proteins (not shown). Overall, we estimated a purity of 90%, which was sufficiently pure to conduct the experiments.

PAR was used to determine the approximate metal content of the protein preparations. Two molecules of PAR chelate one metal ion, resulting in an increase of light extinction at 500 nm (13). A standard curve of known concentrations of zinc was used to estimate the concentration of metal ions in the protein preparations and to calculate the metal:protein stoichiometry (Figure 2). A stoichiometry of 0.6 was obtained with the GST preparation. Zinc binding of GST has been found earlier, using a ⁶⁵zinc-blotting assay (20). The molar metal:protein ratio obtained for GST was similar to that for GST-CR^{mut}, which indicates that the mutated cysteine-rich motif of protein 2C did not contribute to metal binding. In contrast, GST-CR^{wt} bound metal in a molar ratio of 2, indicating that the five cysteine residues and the single histidine residue of the wild-type sequence promote metal binding of the motif. GST fused to a classical zinc finger motif (GST-ZF3A) resulted in a higher metal-to-protein ratio than GST alone, but the ratio was surprisingly low if compared to that of GST-CR^{wt} (see below). EDTA treatment before protein purifi-

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cation reduced the metal content of the recombinant proteins dramatically (Figure 2). Table 2 lists the absolute concentrations of protein and metal in the protein preparations.

For SRIXE analyses, 5 μ L of the protein preparations described above were spotted onto the hydrophobic AP1 membrane. The hydrophobic surface of AP1 prevented the droplet from spreading and running down along the vertical mount during measurement. The diameter of the droplets, 3.3 mm, was determined by the volume of material used and should be constant to approximately 90%. This makes possible the accurate comparison of zinc content from sample to sample. In addition, the AP1 membranes contribute negligible background, which is important for obtaining the lowest possible detection limits. X-ray spectra were collected for 10 min. The sample was stable for at least 30 min because data collection could be repeated twice on the same sample without significant differences between the spectra (not shown). This implies that there were no radiation damage effects of consequence and/or loss by evaporation or change in sample shape. Typical X-ray spectra obtained for the various protein preparations are presented in Figure 3A. The spectra of the proteins charged with zinc show a peak at 8.63 keV, the energy of the zinc K_{α} X-ray. This peak was drastically reduced in samples that had been treated with EDTA, in agreement with the results obtained with PAR. Besides zinc, other elements show emission at their respective energy line. All protein preparations contained trace amounts of nickel evident by the presence of a minor peak at 7.5 keV. A distinct peak at 6.4 keV indicative for iron was found in the spectra of the zinc-binding proteins GST-ZF3A and GST-CR^{wt}, but not in those of GST and GST-CR^{mut} (Figure 3A). The peak for iron did not disappear after EDTA treatment. We suspect that iron ions were provided as trace contaminants in the buffer solutions used during protein purification after EDTA treatment because no special measures were taken to remove metal contaminants from the solutions. Thus, it appears that the zinc finger of hTFIIIA and the zinc-binding motif of protein 2C have some affinity to iron.

All spectra showed a strong peak for the potassium K_{α} X-ray. Potassium was an ingredient in the buffer solutions (see Materials and Methods section). The concomitant detection of zinc, nickel, iron, and potassium demonstrates the benefit of the multi-element specificity of SRIXE. Under the conditions used in the present experiment, SRIXE has the potential to detect atoms with characteristic X-ray energies from 3 to 20 keV.

To quantitate the amount of zinc in the protein preparations by SRIXE, the areas under the zinc peaks were calculated, normalized to ion chamber and protein concentration, and plotted on a bar graph (Figure 3B). The relative amounts of zinc were in good agreement with the metal:protein ratios obtained with the PAR assay (Figure 2). The zinc content was lowest in the proteins GST and GST-CR^{mut}. GST-ZF3A and GST-CR^{wt} contained higher amounts of zinc. Again, GST-ZF3A appeared to bind zinc poorly if compared to GST-CR^{wt}. In contrast to protein 2C, which contains only one metal-binding site, hTF3A contains nine zinc fingers in a row, which may bind zinc cooperatively. In GST-ZF3A, only one zinc fin-

ger is expressed resulting in the loss of potential cooperativity. An alternative explanation is that not all of the nine zinc fingers in hTF3A have to be charged with zinc for hTF3A to be functional. Therefore, an individual zinc finger may afford to express a low zinc-binding capacity. Indeed, previous analyses of the homologous transcription factor IIIA of *Xenopus laevis* have revealed a zinc:protein stoichiometry of 2 rather than the theoretically expected stoichiometry of 9 (12,22).

A minimum detection limit for the SRIXE analysis was calculated using a spectrum (Figure 3A) obtained for the GST/Zn material, which had a zinc concentration of 7×10^{-6} g zinc/g solution based on the PAR determination shown in Table 2. The minimum detection limit was taken to be given by $3 \times (\text{background})^{1/2}$, as discussed by Currie (7). This relationship gave a value of 49×10^{-9} g/g for the minimum detection limit.

The volume of the sample material analyzed by SRIXE is determined both by the beam area and the absorption of the incident X-rays and the emerging zinc K_{α} X-rays. The production of X-rays at a sample thickness greater than

Figure 2. Colorimetric estimation of metal content in preparations of purified proteins by the PAR assay. The molar metal-to-protein ratio is plotted on the y-axis. The average and standard deviation of three assays are shown.

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1.5 mm is less than 10% of the production at the sample surface. This corresponds to a SRIXE analysis of a volume given by $0.150 \text{ mm} \times 1.0 \text{ mm} \times 1.5 \text{ mm}$ or 225 nL. The number of zinc atoms contained in this volume for the GST/Zn sample is then 1.45×10^{13} from the concentration shown in Table 1. For a sample with a zinc concentration corresponding to the minimum detection limit, the value is reduced by the ratio of the minimum detection limit to the concentration shown in Table 2 or 0.049/7.00 or 0.007 giving a value of 1.02×10^{11} zinc atoms. This is an up to 100-fold lower detection limit than that obtained with the more laborious techniques usually used to detect metal ions in proteins. An estimate was also made of the expected X-ray production based on estimates of the X-ray flux, the number of zinc atoms in the sample, the X-ray cross-section, and the detector geometry. This yield was compared to the measured background counts to estimate the detection limit. The result was consistent with the above concentration estimate using PAR.

Further developments may increase the sensitivity and versatility of SRIXE in the detection of protein-bound metal ions. Using dried samples will increase the concentration of metals and reduce scattering background. In our experience, drying of the protein solution on the API membrane resulted in a highly heterogeneous distribution of metal within the specimen and was thus not useful for analysis with the microprobe beam of beamline X26A. Ideally, the dried sample would be of a diameter smaller than that of the incident beam.

Sample sizes in the range of nanoliters are being used in microarray technology. The combination of SRIXE with microarray technology will make possible the efficient screening of numerous samples for protein-bound metal ions. Use of the smaller sample volumes will necessitate improvements in the sensitivity of the SRIXE approach.

The number of zinc atoms in an assumed microarray sample volume of 0.5 nL would be in the range from 6.1×10^8 to 1.1×10^{11} atoms. These values are below the detection limits for the present experimental apparatus. It will be possible to increase the sensitivity because the material will be concentrat-

ed in a smaller volume and drying of the sample in an area smaller than the beam size will be feasible. A smaller area will also make possible the use of existing Kirkpatrick-Baez focusing mirrors at the beam line used for this experiment to concentrate the beam over the sample area. Microarray analysis will also be easily attainable

by use of undulator sources at third-generation synchrotron X-ray sources such as the Advanced Photon Source at Argonne National Laboratory or the Advanced Light Source at Lawrence Berkeley National Laboratory where the photon flux is roughly 1000 times more intense than that used in the present experiment.

Figure 3. Detection of metal ions in preparations of purified proteins by SRIXE. (A) X-ray spectra of the protein preparations indicated. Relative intensities (counts) were measured from 3 to 9 keV at intervals of 12 eV (x-axis). The counts normalized to ion chamber and protein concentration were plotted logarithmically on the y-axis. The peaks for K, Fe, Ni, and Zn are indicated. (B) Relative amounts of zinc. Area calculation of the zinc K_{α} peaks in the spectra shown in panel A was performed using the ND9900 data acquisition software (Canberra). For the zinc-treated samples GST, GST-CR^{wt}, and GST-ZF3A, the zinc areas of three independent spectra were calculated. Their average values and standard deviations are displayed.

In conclusion, the multi-element sensitivity and other properties of SRIXE make it a highly suitable method for the identification of protein-bound metal ions. In conjunction with the expression of a potential metal-binding motif fused to GST or another protein for which a simple purification regimen exists, SRIXE can quickly identify metal-binding properties both qualitatively and quantitatively. Site-directed mutagenesis of the metal-binding motif allows the investigator to identify the residues involved in metal coordination. Although a relatively high purity of the protein to be analyzed is required, only small amounts of protein are needed because of the high sensitivity of SRIXE. Therefore, SRIXE is also suitable for the analysis of nonrecombinant proteins from cultured cells or fresh tissue. Very often, a protein of interest is of low abundance in the starting material or difficult to purify, resulting in small amounts of purified protein. However, the metal binding sites of nonrecombinant proteins are likely to be occupied with the biologically relevant metal ion, making metal-charging of the protein unnecessary. Therefore, SRIXE can be the method of choice to detect and identify protein-bound metal ions.

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REFERENCES

1. Arakawa, H., H. Nagase, N. Hayashi, M. Ogawa, M. Nagata, T. Fujiwara, E. Takahashi, S. Shin, and Y. Nakamura. 1995. Molecular cloning, characterization, and chromosomal mapping of a novel human gene (GTF3A) that is highly homologous to Xeno-

- pus transcription factor IIIA. *Cytogenet. Cell Genet.* 70:235-238.
2. Berg, J.M. and Y. Shi. 1996. The galvanization of biology: a growing appreciation for the roles of zinc. *Science* 271:1081-1085.
3. Bull, P.C., G.R. Thomas, J.M. Rommens, J.R. Forbes, and D.W. Cox. 1993. The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nat. Genet.* 5:327-337.
4. Chelly, J., Z. Tumer, T. Tonnesen, A. Pettersson, Y. Ishikawa-Brush, N. Tommerup, and A.P. Monaco. 1993. Isolation of a candidate gene for the Menkes disease that encodes a potential heavy metal binding protein. *Nat. Genet.* 3:14-19.
5. Cline, H.T., S. Witte, and K.W. Jones. 1996. Low lead levels stunt neuronal growth in a reversible manner. *Proc. Natl. Acad. Sci. USA.* 93:9915-9920.
6. Coleman, J.E. 1992. Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. *Annu. Rev. Biochem.* 61:897-946.
7. Currie, L.A. 1968. Limits for qualitative detection and quantitative determination. Application to radiochemistry. *Anal. Chem.* 40:586-593.
8. Feder, J.N. 1999. The hereditary hemochromatosis gene (HFE): a MHC class I-like gene that functions in the regulation of iron homeostasis. *Immunol. Res.* 20:175-185.
9. Gill, S.C. and P.H. Hippel. 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* 182:319-326.
10. Gordon, B.M. and K.W. Jones. 1993. X-ray emission imaging, p. 303-344. *In* M.D. Morris (Ed.), *Microscopic and Spectroscopic Imaging of the Chemical State*, vol. 16. Marcel Dekker, New York.
11. Guan, K.L. and J.E. Dixon. 1991. Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* 192:262-267.
12. Hanas, J.S., D.J. Hazuda, D.F. Bogenhagen, F.Y. Wu, and C.W. Wu. 1983. Xenopus transcription factor A requires zinc for binding to the 5 S RNA gene. *J. Biol. Chem.* 258:14120-14125.
13. Hunt, J.B., S.H. Neece, H.K. Schachman, and A. Ginsburg. 1984. Mercurial-promoted Zn^{2+} release from *Escherichia coli* aspartate transcarbamoylase. *J. Biol. Chem.* 259:14793-14803.
14. Jouanolle, A.M., P. Fergelot, G. Gandon, J. Yaouanq, J.V. Le Gall, and V. David. 1997. A candidate gene for hemochromatosis: frequency of the C282Y and H63D mutations. *Hum. Genet.* 100:544-547.
15. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacterial T4. *Nature* 227:680-685.
16. Liu, J., Y. Gagnon, J. Gauthier, L. Furenlid, P.-J. L'Heureux, M. Auger, O. Nureki, S. Yokoyama, and J. Lapointe. 1995. The zinc-binding site of *Escherichia coli* glutamyl-tRNA synthetase is located in the acceptor-binding domain. *J. Biol. Chem.* 270:15162-15169.
17. Mercer, J.F., J. Livingston, B. Hall, J.A. Paynter, C. Begy, S. Chandrasekharappa, P. Lockhart, A. Grimes et al. 1993. Isolation of a partial candidate gene for Menkes disease by positional cloning. *Nat. Genet.* 3:20-25.
18. Miller, J., A.D. McLachlan, and A. Klug. 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus oocytes*. *EMBO J.* 4:1609-1614.
19. Pfister, T., K.W. Jones, and E. Wimmer. 2000. A cysteine-rich motif in poliovirus protein 2CATPase is involved in RNA replication and binds zinc in vitro. *J. Virol.* 74:334-343.
20. Rose, P.E. and B.S. Schaffhausen. 1995. Zinc-binding and protein-protein interactions by the polyoma virus large T antigen zinc finger. *J. Virol.* 69:2842-2849.
21. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. CSH Laboratory Press, Cold Spring Harbor, NY.
22. Shang, Z., Y.D. Liao, F.Y. Wu, and C.W. Wu. 1989. Zinc release from *Xenopus* transcription factor IIIA induced by chemical modification. *Biochemistry* 28:9790-9795.
23. Sommergruber, W., G. Casari, F. Fessl, J. Seipelt, and T. Skern. 1994. The 2A proteinase of human rhinovirus is a zinc containing enzyme. *Virology* 204:815-818.
24. Studier, F.W., A.H. Rosenberg, J.J. Dunn, and J.W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60-89.
25. Tanzi, R.E., K. Petrukhin, I. Chernov, J.L. Pellequer, W. Wasco, B. Ross, D.M. Romano, E. Parano et al. 1993. The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. *Nat. Genet.* 5:344-350.
26. Vulpe, C., B. Levinson, S. Whitney, S. Packman, and J. Gitschier. 1993. Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. *Nat. Genet.* 3:7-13.

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