Micromolar protein concentrations and metalloprotein stoichiometries obtained by inductively coupled plasma. Atomic emission spectrometric determination of sulfur

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The use of low-pressure vaporization for the Cu samples greatly reduced the residence time of Pb within the system and promoted the definition of distinct peaks as well as the removal of the Pb from the molten Cu droplet. Present work is aimed at enhancing the surface analysis capabilities of the technique by determining the origin of these peaks. Furthermore, elucidating mechanisms for low-pressure vaporization should facilitate the application of this technique to refractory analytes and solid matrices.

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Micromolar Protein Concentrations and Metalloprotein Stoichiometries Obtained by Inductively Coupled Plasma Atomic Emission Spectrometric Determination of Sulfur

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The concentrations of several micromolar solutions of proteins with known sulfur contents were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) of sulfur in the vacuum ultraviolet. These values are compared to concentrations obtained by using spectrophotometric measurements and published specific and molar absorptivities based on various conventional methods of protein determination. The two sets of values are in close agreement, indicating that ICP-AES of sulfur is an accurate means of determining microgram quantities of proteins. Standard deviations are within 2% of the mean values obtained for data sets of six replicate measurements. Dilute buffered protein solutions may be directly pumped into the nebulizer; sample digestion and other special sample preparations are not necessary. It is also demonstrated for several metalloproteins that multielement ICP-AES is an excellent means of determining stoichiometries of bound metal ions as both protein and metal assays may be rapidly performed on a single sample.

Many investigators in biochemistry and related areas are unaware that proteins may be quantified by inductively coupled plasma atomic emission spectrometry (ICP-AES) even if the protein in question contains no metallic elements. Temperatures in an ICP torch are sufficiently high to excite many nonmetallic elements, and several analytically useful emission lines in the vacuum ultraviolet have been observed (*1*). Of particular interest in this study is the prominent sulfur line at 180.73 nm, which has an estimated detection limit of 15 μg/L (*2*). Proteins with known contents of the sulfur-bearing residues cysteine and methionine can thus be quantified at the microgram level by ICP-AES provided the instrument is equipped with evacuated or otherwise oxygen-free optics.

ICP-AES determinations of total sulfur in tissue samples (*3*, *4*) and the use of an ICP-AES instrument as a sulfur detector in the high-performance liquid chromatography (HPLC) of tissue extracts (*5*) have been reported. The purpose of this report is to demonstrate the viability of pumping micromolar purified protein solutions directly into the nebulizer of the ICP-AES instrument and determining protein concentrations based on the emission of sulfur at 180.73 nm. It is also shown for several metalloproteins that this method permits the precise stoichiometries of bound metal ions to be determined by multielement ICP-AES of both sulfur and metals.

EXPERIMENTAL SECTION

Preparation of Samples and Standards. Horse kidney and rat liver metallothioneins were isolated and purified by a literature method (*6*). Cd_7metallothionein was prepared by adding excess $CdCl_2$ to a solution of native equine metallothionein. An excess of the reducing agent dithioerythritol was added to ensure complete reduction of cysteine sidechains. After 24 h the reaction mixture was fractionated on a Sephadex G-50 column. Horse heart cytochrome *c* was obtained from Sigma Chemical Co. and purified by carboxymethylcellulose ion-exchange chromatography. The other proteins used in this investigation were obtained as lyophilized powders from Sigma and used without further purification.

The ribonuclease sample was dissolved in 10 mM Tris/HCl buffer at pH 7.8. All other protein samples were dissolved in 5 mM phosphate buffer at pH 7.2. All samples were concentrated

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Table I. Compromise Operating Parameters

rf power	1200 W
viewing height	12 mm above coil
sample delivery rate	1 mL/min
argon flow rates	
plasma	15 L/min
auxiliary	1 L/min
nebulizer	1 L/min

by ultrafiltration (YM5 membrane, Amicon Corp.) and then passed through a 0.2-μm filter (Gelman Sciences Inc.). The ultrafiltrates were used as blanks for both ICP-AES and spectrophotometric measurements. The metallothionein samples used for ICP-AES measurements had concentrations between 0.75 and 1.6 mg/L in sulfur. All other protein samples used were about 5 mg/L in sulfur.

Sulfur standards were solutions made from anhydrous Na_2SO_4. Standard solutions of the metallic elements were purchased from Aldrich Chemical Co.

Instrumentation. Elemental analyses were made by using a Perkin-Elmer Plasma II emission spectrometer equipped with a Perkin-Elmer Series 7000 computer. The 1-m Ebert monochromator (1800 lines/mm holographically ruled grating) is sealed in a heavy-walled aluminum tube maintained at 5 mTorr. The path from the argon ICP source to the optical components was purged with a 5 L/min flow of nitrogen. The sample introduction system is comprised of a peristaltic pump (12 rollers, silicone rubber pump tubing) and a cross-flow nebulizer (*7*). Argon flow to the nebulizer is regulated by a mass flow controller. The compromise operating parameters employed are shown in Table I.

Absorbance measurements were recorded on an IBM 9430 UV–vis spectrophotometer. Samples were diluted to give absorbance values between 0.5 and 1.0 in a 1 cm path length quartz cell.

Measurements. Individual ICP-AES measurements were obtained by scanning a 0.020-nm window centered on the emission line of interest and recording intensity counts at the wavelength of maximum emission. For multielement analyses the monochromator slews from one emission line to the next in a sequential fashion. The emission lines scanned were S I 180.73, Fe II 259.94, Cd II 214.44, Zn I 213.86, and Cu I 324.75 nm. Six replicate measurements were recorded for each element of each standard, sample, and blank. Linear calibrations were obtained immediately prior to running each protein sample and corresponding ultrafiltrate blank.

RESULTS AND DISCUSSION

Accuracy. Elemental analysis of proteins by ICP-AES has traditionally been restricted to metals in acid-digested samples of metalloproteins. We initially sought to demonstrate that accurate absolute concentrations of proteins, regardless of the presence or absence of bound metals, can be obtained based on sulfur content by pumping buffered micromolar solutions directly into the nebulizer without any prior sample preparation and using aqueous solutions of Na_2SO_4 as sulfur standards. The protein solutions, although relatively dilute, do have slightly different physical properties than the Na_2SO_4 standards, such as viscosity and surface tension, which could lead to differences in aerosol formation in the nebulizer and systematic errors in the results. One test of accuracy is to compare concentrations experimentally determined by ICP-AES of sulfur with concentrations determined spectrophotometrically by using published absorptivities based on traditional methods of protein determination (Table II).

While the list of published molar absorptivities in Table II is not an exhaustive one, it is an attempt to list as many literature values as possible in a nonselective manner. The spread in the distribution of literature values for a particular protein is to be expected as the measurements were done by different workers using the various analytical methods listed.

In general, very close agreement is found between the experimental ICP-AES concentrations and the central tendencies of the values obtained by using published spectrophotometric absorptivities. According to these comparisons the ICP-AES method of protein quantification via determination of sulfur is an accurate one, at least within the bounds of the concentration range and conditions described in the Experimental Section.

Bound Metal Stoichiometries and Metal Contents. Bound metal stoichiometries are traditionally obtained by determination of the metallic component by atomic absorption or ICP-AES, and determination of the polypeptide component by independent means such as dry weight or total nitrogen determinations. These same stoichiometries, however, can be obtained by an ICP-AES determination of both components using a single sample. Bound metal stoichiometries for three homogeneous proteins and metal contents for two heterogeneous native metallothioneins, as determined by multielement ICP-AES of sulfur and metals, are shown in Table III. Metallothionein chelates a diversity of metal ions, primarily Cd and Zn ions, both in vivo and in vitro (*8*). Thus samples of the "native" protein have variable metal compositions depending on the source and method of isolation as illustrated by the rat liver and equine kidney metallothioneins in Table III.

Because of this variability in metal content, concentrations of native metallothionein cannot be satisfactorily determined by a metals assay. Concentrations can be accurately determined by a sulfur assay, however, as all mammalian metallothioneins have identical sulfur contents (21 mol of S/mol of polypeptide). The sum of the Cd, Zn and Cu contents in Table III, for either of the native metallothioneins, is slightly below the maximum binding capacity (seven divalent metal ions), but this probably results from the binding of minor amounts of other metal ions. ICP-AES analysis of the homogeneous Cd_7metallothionein sample gave the stoichiometric seven Cd ions within the limits of experimental error. Multielement ICP-AES has been useful in studying synthetic Pt(II) adducts of metallothionein (*9*).

The experimentally determined Fe content of ferrimyoglobin in Table III falls short of the true stoichiometry by about 10%. Spectrophotometric absorbance measurements of this sample taken at the Soret band at 409 nm arising from absorption by the iron containing heme moiety, and the band at 280 nm arising from absorption by tyrosine and tryptophan residues, also indicated the presence of 10% apoprotein, which lacks heme. The ferricytochrome *c* sample, which was purified extensively by ion-exchange chromatography, yielded an experimental result that is within 5% of the true stoichiometry.

Precision. The standard deviations of the sulfur analyses in Table II are within 2% of the mean for six replicate measurements. The metal binding stoichiometries in Table III fall within 4–5% of the mean due to the uncertainties associated with both sulfur and metal determinations. This level of precision is acceptable for most applications, particularly when one considers the rather poor reproducibility of many other existing methods of protein determination. If one wishes to make very fine distinctions (such as whether a certain protein binds 10 or only 9 metal ions) this level of precision becomes intolerable. Small gains in precision might be realized by using optimized instrumental parameters for each element instead of compromise parameters, a pulseless syringe pump, improvement of power supply tolerance, and other refinements. The careful use of an internal standard (*10*) to mitigate precision-limiting flicker noise in the nebulizer (*11*) may also improve precision.

Sensitivity. The sensitivity of any destructive technique of protein determination is a general concern. We routinely obtain satisfactory results for samples with sulfur concentrations of 1 mg/L and higher. The volume of sample required

Table II. Comparison of Protein Concentrations Determined by ICP-AES (S I 180.73 nm) with Concentrations Obtained by Using Published Spectrophotometric Molar Absorptivities

protein[a]	protein concn/μM ICP[c]	protein concn/μM UV-vis[d]	$\epsilon/10^4$ M^{-1} cm^{-1} (λ/nm)[b]	ref
Cd_7metallothionein equine kidney MW 6.88 kD, 21 mol S/mol	8.56 ± 0.12	8.19	*11.4* (250) H	16, 17
ferricytochrome *c* equine kidney MW 12.4 kD, 4 mol S/mol	47.3 ± 1.2	49.8	*11.2* (410) B	18, 19
		51.0	*10.95* (410) B	20
		51.7	2.40 (280) D	21
		52.6	*10.6* (410) B	22, 23
		53.4	*2.32* (280) B	22, 23
		58.5	2.12 (280) D	24
ferrimyoglobin equine MW 17.5 kD, 2 mol S/mol	78.4 ± 1.3	67.0	*18.8* (409) H	25, 26
		67.7	*18.6* (409) C	27
		73.6	*17.1* (409) C	28
		76	*3.2* (280) H	29
		78.5	*3.12* (280) C	28
		78.8	*16.0* (409) H	30, 23
ribonuclease A bovine pancreas MW 13.7 kD, 12 mol S/mol	14.8 ± 0.3	14.1	*1.19* (278) A	31, 32, 23
		14.7	*1.14* (278) D, E	33, 23
		14.9	*1.13* (278) H	34, 23
		15.8	*1.06* (278) H	35, 36
		16.2	1.04 (278) D	37, 36
		16.6	1.01 (278) H	38
		17	0.98 (278) H	39, 23
trypsin bovine pancreas MW 24.0 kD, 14 mol S/mol	18.1 ± 0.2	15	*4.4* (280) A	40, 41, 42
		16.3	4.14 (280) D	43, 23
		16.9	4.00 (280) F	44, 23
		16.9	3.98 (280) D	45, 23
		17.5	3.86 (280) A	46, 42
		17.5	3.86 (280) H	47, 42
		17.8	3.79 (280) H	48, 42
		18.0	3.74 (280) D, F	24, 42
		18.0	3.74 (280) D	49, 23
		18.1	3.72 (280) H	50, 23
		18.1	3.72 (280) H	51, 42
		18.3	3.69 (280) G	52, 42
		18.3	3.69 (280) H	53, 42
		18.4	3.67 (280) A, D	54, 42
		19.5	3.46 (280) H	55, 42
chymotrypsinogen A bovine MW 25.6 kD, 12 mol S/mol	10.2 ± 0.2	10.1	5.32 (280) D	56, 21
		10.2	5.30 (282) F	57
		10.4	5.20 (282) D	58, 23
		10.4	5.20 (282) D	59, 23
		10.4	5.17 (280) H	60
		10.5	5.15 (280) H	61
		10.6	5.12 (282) H	62
		10.6	5.12 (282) H	63
		10.6	5.11 (280) H	37, 23
		10.8	5.04 (282) D	64
		11.7	4.61 (280) F	65
		13.1	4.13 (282) D	66

[a] Molecular weights and sulfur contents were taken from amino acid sequences. [b] Most of the ε values in this column were obtained from reported specific absorptivities ($A_{1cm}^{1\%}$) using the relationship: $\epsilon = MWA_{1cm}^{1\%}/10$. Those values reported in the original literature as molar absorptivities (ε) are italicized. Also shown is the analytical method upon which the published absorptivity is based: amino acid analysis, A; colorimetric Fe determination, B; colorimetric heme determination, C; dry weight, D; Nessler N determination, E; Kjeldahl N determination, F; refractometry, G; no explicit reference to analytical method given, H. [c] (Mean of sample–mean of blank) ± (standard deviation) for six replicate measurements of both sample and blank. [d] The concentrations in this column were obtained by using reported specific and molar absorptivities listed above and experimentally obtained absorbance measurements.

is between 1 and 4 mL, which corresponds to the consumption of ten to several hundred micrograms of protein depending on molar sulfur content of the protein. While the sample volumes required for a pneumatic nebulizer are about 2 orders of magnitude greater than those required for a graphite furnace, they are not at all prohibitive in terms of the milligram scale on which protein research is often conducted.

Flow injection analysis (FIA) coupled with emerging total-consumption (direct injection) sample introduction systems could, in principle, reduce sample volumes from several milliliters to several microliters and greatly increase the applicability of ICP-AES to proteins (*12*). FIA utilizes the transient signals generated by injecting small discrete sample "plugs" into a carrier stream and thus eliminates the wasteful dead-volume of the conventional continuous-flow mode of sample presentation. Experimental sample introduction systems with high transport efficiencies have been described and include a miniature concentric nebulizer installed in the injecter tube of a conventional ICP torch (*13*) and a thermospray vaporizer (*14*).

Relative Merits of the Method. The main advantages of protein determination by ICP-AES of sulfur, besides the high selectivity and multielement capability of atomic spectrometry in general, are the generality and rapidity of the method and the ease of sample preparation. The method is applicable to any protein having a known content of sulfur. The only significant spectral interferences with the sulfur line at 180.73 nm are wing-overlaps of Ca and Mn lines that have intensities about 2 orders of magnitude smaller on a molar basis (*15*). Perchloric acid digestion, dry ashing, and other time-consuming and error-prone preparatory steps are not necessary. A promising application of the method is the rapid and precise determination of metal binding stoichiometries in metalloproteins by determining both protein and metals for a single sample by a single method. In addition, ICP-AES is a useful method for determination of sulfur content of

Table III. Metal Binding Stoichiometries and Metal Contents of Metalloproteins Determined by ICP-AES of Sulfur and Metals

protein	metal	mol of metal/mol[a]
native metallothionein rat liver	Cd	3.02 ± 0.15
	Zn	3.18 ± 0.10
	Cu	0.26 ± 0.03
	total	6.46 ± 0.18
native metallothionein equine kidney	Cd	5.04 ± 0.36
	Zn	0.94 ± 0.08
	Cu	0.63 ± 0.23
	total	6.61 ± 0.41
Cd_7metallothionein equine kidney	Cd	6.95 ± 0.22
ferricytochrome *c* equine heart	Fe	0.955 ± 0.028
ferrimyoglobin equine	Fe	0.900 ± 0.045[b]

[a] (Mean of sample–mean of blank) ± (standard deviation) for six replicate measurements of both sample and blank. [b] Spectrophotometric measurements indicated that this sample contained 90% holoprotein and 10% apoprotein.

proteins following quantification of total protein by other methods.

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