Proc. Nat. Acad. Sci. USA
Vol. 73, No. 4, pp. 1193–1195, April 1976
Biophysics

Electron probe x-ray analysis of single ferritin molecules

(electron microscopy/ionization cross section/analytic chemistry)

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Communicated by Robert E. Forster, January 12, 1976

ABSTRACT **Single molecules and groups of two or three ferritin molecules were subjected to electron probe x-ray microanalysis in a transmission electron microscope equipped with a liquid nitrogen cooled stage. Significant Fe K_α peaks were generated during 100-sec counts when single ferritin molecules were excited with a probe current of 0.35 nA/60 nm spot, less than the maximal current available in a thermionic gun. There was a linear relationship between the number of ferritin molecules analyzed and count rates. The experimental results are compared to the theoretically calculated Fe K_α yields and to the results of Isaacson and Johnson [(1975) *Ultramicroscopy* 1, 33–52] with electron energy loss analysis. We conclude that current state of the art electron probe x-ray analysis can realize the theoretically predicted sensitivity of the method, and estimate 0.9×10^{-19} g of Fe as the minimal mass detectable with maximal (thermionic) probe current during a 100-sec count and with 95% confidence.**

Electron probe analysis combined with cryoultramicrotomy permits the analysis of cell constituents ($Z \geq 11$) with a spatial resolution and sensitivity unavailable through conventional techniques (1, 2). It has been shown that it is feasible to detect with commercially available instruments calcium and other cations in single (50–80 nm diameter) mitochondrial granules (3, 4). Some years ago Hall (5) estimated that the minimal mass detectable (during a 20 sec count and with 20% error) with an energy dispersive detector in an ultrathin section would be approximately 2×10^{-18} g. Recently there has also been a revival of interest in characteristic electron energy loss analysis as an alternative means of determining the composition of biological materials at high spatial resolution (6–8); with this technique Isaacson and Johnson (6) have been able to detect (using a high vacuum microscope with field emission gun) the approximately 5000 Fe atoms in single ferritin molecules. To indicate the current state of the art of electron probe analysis and as a means of comparing its sensitivity with electron energy loss analysis, we explored the feasibility of detecting the iron core of single ferritin molecules (9) by recording the characteristic K x-rays generated through electron bombardment in a transmission electron microscope equipped with a conventional thermionic gun and a pole piece configuration permitting somewhat higher than usual current densities. The experimental results are compared to and found in good agreement with the theoretically calculated Fe K_α yields.

METHODS

Our instrumentation, described in detail elsewhere (10), consists of a Philips EM 301 transmission electron microscope with goniometer stage modified to accept a 30 mm^2 Si(Li) dispersive x-ray detector (Kevex) at a solid angle of approximately 0.005 (4π) steradians and interfaced with a multichannel analyzer (Kevex 5100) and a computer system (Tracor Northern NS 880). The microscope is equipped with a conventional thermionic emitter (tungsten filament). Currents were measured with a Faraday cup supplied by Philips. The current densities (J) attainable at 80 kV for a 10 nm diameter probe size are approximately $J = 43.3$ A/cm^2 in the scanning mode (using the upper objective pole piece of a Riecke-Ruska lens as the probe forming system). The average current density in 60–200 nm diameter spots (operating in the conventional mode) is $J = 18.0$ A/cm^2.

To realize the optimal minimal detectable mass attainable with this system it is essential to reduce to a minimum the background radiation (x-ray continuum) generated by extraneous sources. Contamination, the major source of extraneous continuum with small diameter (50 nm or less) probes in our system, can be eliminated or at least substantially reduced by operating at low temperatures (10). Therefore, the suspensions of ferritin molecules placed on thin (about 4 nm) carbon films were analyzed at −110°, using a Philips liquid nitrogen cooled specimen stage modified to permit the exit of generated x-rays towards the detector. Analysis at even lower temperatures was not feasible due to icing of the specimen. The stage drift of 1.5 nm/sec (specified by the manufacturer) is evident in the electron micrographs obtained and required continuous vigilance by the operator for maintaining the ferritin target in the center of the probe.

RESULTS

Electron micrographs of a single ferritin molecule before and after electron probe x-ray microanalysis with 60 nm diameter probe for 100 sec and of a group of three molecules are shown in Fig. 1. The hole formed in the carbon support is evidence of the continuous mass loss under the electron beam even at this low temperature. It may be possible to reduce or eliminate mass loss, at comparable current densities, by operating at liquid He temperatures (11) or by using lower current densities (ref. 12; Shuman and Somlyo, unpublished observations). The x-ray spectra obtained from a single ferritin molecule and from a group of three are shown in Fig. 2, and the results of analyses of 1, 2, and 3 ferritin molecules are shown in Table 1. The latter shows the linear increase in Fe K_α count rate with the number of ferritin molecules analyzed.

The theoretical count rate can be estimated from the x-ray production cross section, absolute detector efficiency, and measured probe current. The data in Table 1 were obtained with a measured probe current of 0.35 nA in a 60 nm diameter probe. The average current density is $J = 6.2$ A/cm^2 and, assuming a gaussian probe, the current density at the center of the probe is $J_0 = 12.5$ A/cm^2. The x-ray production cross section is composed of two factors, the ionization cross section q and the x-ray yield ω. For Fe K x-rays, $\omega = 0.347$ and $q = 3.95 \times 10^{-22}$ cm^2 from Durup and Platzman's expression (7, 13) for ionization cross section. The predicted count rate, $I = \omega q \epsilon J_0$ (electrons/cm^2-sec) is, where $\epsilon = 0.005$, the detector efficiency

$$I_{D\&P} = 5.3 \times 10^{-5} \text{ counts/sec-atom.}$$

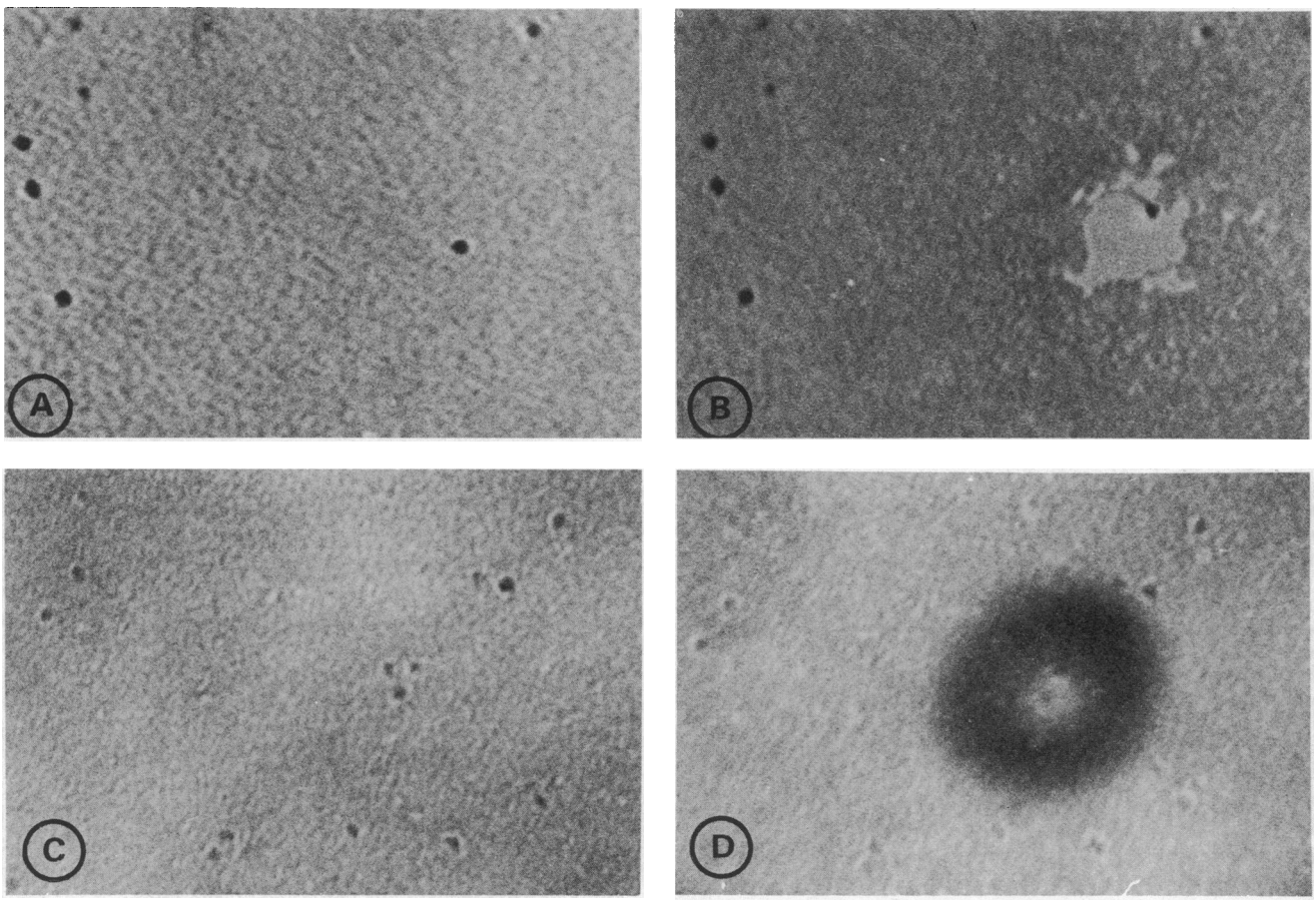

FIG. 1. Electron micrographs at 300,000 × of ferritin molecule(s) analyzed at two substrate temperatures. (A and B) Single ferritin molecule, before and after analysis at T = −110°. (C and D) Cluster of three molecules analyzed at T = −100°. Comparison of (B) and (D) shows strong variation of mass loss and contamination as a function of substrate temperature.

Considering that a fully saturated ferritin core contains 5000 iron atoms, the predicted count rate per ferritin molecule is

$$I_{D\&P} = 0.27 \text{ counts/sec-molecule.}$$

Experimentally, the count rate is observed to be

$$I_{exp} = 0.53 \pm 0.09 \text{ counts/sec-molecule}$$

or nearly twice the value predicted from the Durup and Platzman cross section. Although these results with a single ferritin molecule give an accurate measure of instrument sensitivity, the diameter and current density of a focused probe are not known well enough to give an accurate measure of the absolute x-ray production cross section for iron. Another set of analyses was performed on the same specimen with a large defocused probe. The probe current was measured with a Faraday cup to be 1.15 nA, and assumed to be uniformly distributed over a 930 nm ± 10% diameter. The number of molecules included under the beam was obtained from a pair of micrographs taken before and after analysis. In three trials, with an average of 514 molecules in the beam, there were an average of 2.26 counts per sec in the Fe $K_{\alpha,\beta}$ peak. The ionization cross section derived from these large spot analyses was $q = 4.67 \times 10^{-22}$ cm^2. The error in q, including counting statistics and current density error, is approximately 30%.

With a 162 ev resolution detector and the ML quantitation routine developed by F. Schamber (14) and described by Shuman and Somlyo (10), an isolated peak with 25 counts can be analyzed with an error of ± 12 counts (i.e., 95% confidence level). For a 100 sec collection time and the experimental conditions described, the minimum detectable mass is therefore about 2.3×10^{-19} g; using the maximum available probe current of 1.0 nA in a 60 nm probe, the minimum detectable mass should be reduced to 0.9×10^{-19} g.

DISCUSSION

Our results show that, as with characteristic energy loss analysis with a field emission source (6), it is also feasible to detect the iron core of a single ferritin molecule with electron probe x-ray microanalysis and using a thermionic source. Thus, present "state of the art" electron probe instrumentation, with some modifications, can realize the best sensitivity theoretically anticipated by Hall (5), and further improvements would have to come from the use of higher brightness (e.g., field emission) sources. In this regard our experience, showing significant mass loss even when using submaximal current densities available from a conventional gun and at low temperatures, suggests the desirability of using special stages at liquid helium temperatures and a high vacuum microscope to eliminate water vapor and icing. Optimization of the geometrical efficiency of the energy dispersive x-ray detectors, probably most likely to be obtained through multiplexing of several detectors, would seem to be the second feasible approach for improving the sensitivity of electron probe x-ray analysis.

According to the present results, electron probe x-ray analysis is as sensitive as characteristic energy loss analysis for the detection of the minimal mass of medium Z (i.e., Fe)

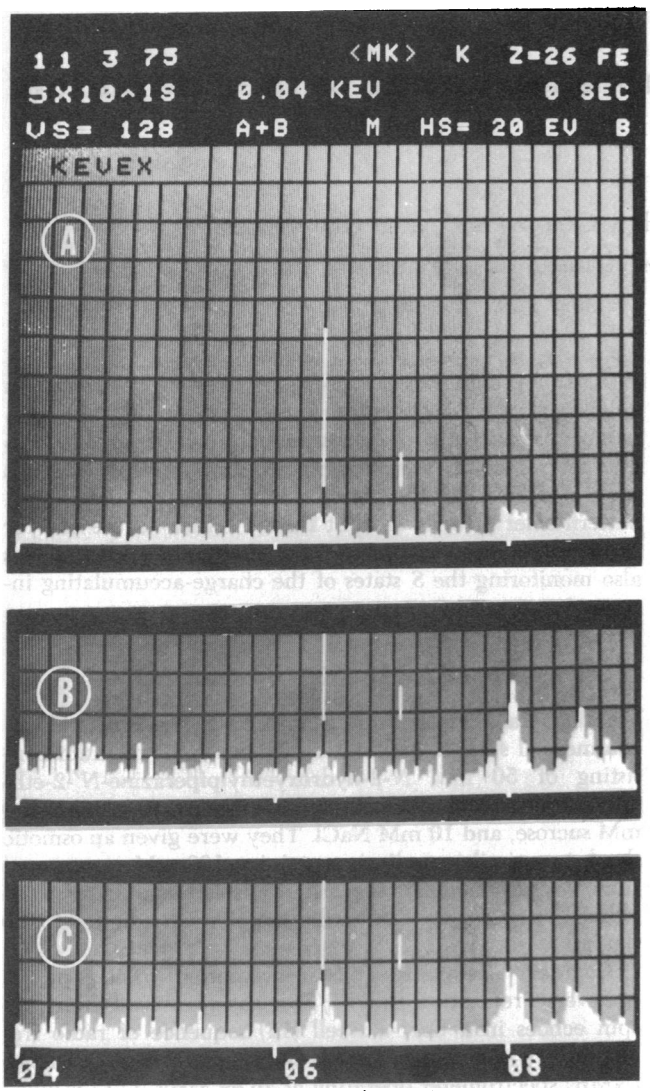

FIG. 2. X-ray spectra of ferritin molecule in energy region of iron K_α peak. Cu and Zn K lines are from grid holder. (A) Single ferritin at −110°, 54 sec count, trial no. 5. (B) Cluster of three molecules at −100°, 60 sec count, trial no. 1; the large continuum contribution is due to contamination. (C) Three molecules at −110°, 64 sec count, trial no. 10.

elements. Defining the minimal detectable mass as the amount of Fe detectable during a 100 sec count with the maximum emission current available with our thermionic source with 95% confidence, we find this to be 0.9×10^{-19} g.* With decreasing atomic number the advantages of electron energy loss analysis should be greater (6), although there may be some degradation of signal to noise by multiple inelastic scattering due to difficulties in preparing sufficiently thin biological specimens. The detection of several elements through characteristic electron energy loss analysis can also be time comsuming since, with current instrumentation, each energy band has to be scanned separately (6). We anticipate that the most sensitive and practical detection system for the entire range of "biological Z" elements will consist of an instrument equipped with both electron energy analyzer and energy dispersive x-ray detector in which specimens can be analyzed at high vacuum and at very low temperatures.

* Using Hall's more conservative definition of minimal detectable mass, this is equivalent to 1×10^{-18} g. The use of our definition has become more feasible with the availability of computerized spectrum fitting techniques.

Table 1. Count rate (counts/sec) in Fe K_α peak of ferritin molecule(s)

Trial no.	No. of ferritin molecules under probe[a]		
	1	2	3
1	0.56 ± 0.33	1.15 ± 0.26	1.20 ± 0.29
2	1.07 ± 0.40	1.46 ± 0.28	1.27 ± 0.27
3	1.18 ± 0.46	0.65 ± 0.24	2.17 ± 0.25
4	0.83 ± 0.31	1.41 ± 0.21	2.16 ± 0.80[b]
5	0.76 ± 0.25	0.87 ± 0.23	1.34 ± 0.32
6	0.56 ± 0.32	0.83 ± 0.28	2.63 ± 0.39
7	0.34 ± 0.17	0.89 ± 0.24	1.92 ± 0.23
8	0.37 ± 0.15	1.20 ± 0.29	1.49 ± 0.33
9			1.70 ± 0.45
10			1.57 ± 0.25
Mean ± SD	0.53 ± 0.09	1.06 ± 0.09	1.69 ± 0.10

[a] Molecule(s) in center of 60 nm diameter, 0.35 nA probe.
[b] Collection time of 11 sec; all others, 50–100 sec.

This work was supported by HL 15835 to the Pennsylvania Muscle Institute.

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