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Volume 202, number 2 FEBS 3787 July 1986

On the analogy in the structure of the spleen green heme protein and granulocyte myeloperoxidase

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Received 30 April 1986

The molecular structure of the spleen green heme protein was reinvestigated by gel-permeation, SDS-polyacrylamide gel electrophoresis, and amino acid analysis. The results showed that the enzyme is a tetramer (M_r 1.5×10^5) with two heavy subunits (M_r 6×10^4 with a single prosthetic group per subunit) and two light subunits (M_r 1.5×10^4), and that the tetramer structure is maintained by disulfide bond(s). The amino acid composition of the spleen green heme protein is similar to that of granulocyte myeloperoxidase. The present results contradict the data of Davis and Averill [(1981) J. Biol. Chem. 256, 5992–5996], who reported the enzyme as a monomeric peroxidase with an M_r of 57 000.

Myeloperoxidase *Heme protein* *(Bovine spleen)*

1. INTRODUCTION

The presence of a monomeric peroxidase in bovine spleen, with optical absorption properties similar to those of granulocyte myeloperoxidase, was first reported by Davis and Averill [1]. Subsequently, these authors reported the presence of the same enzyme in human spleen [2]. Spectroscopic comparison of the spleen green heme protein and myeloperoxidase of polymorphonuclear leukocyte has indicated that both enzymes have identical prosthetic groups and iron coordination structure [3–5]. The spleen enzyme also exhibits catalytic properties very similar to those of myeloperoxidase [4]. Spleen contains significant numbers of macrophages/monocytes, which have been previously shown to contain myeloperoxidase [6]. The relationship between these two enzymes has, however, been ambiguous. Davis and Averill [1] proposed that the spleen enzyme is closely related to the heavy subunits of myeloperoxidase possibly as a degradation product or as a precursor. Studies on the biosynthesis of myeloperoxidase, using human myeloid leukemia HL-60 cells, have indicated that myeloperoxidase synthesis ceases as the cells differentiate to granulocytes or macrophages [7,8]. Although spleen is an additional site of hematopoiesis, granulocytes and monocytes are chiefly generated and matured in bone marrow. Thus, it is unlikely that large amounts of precursors of myeloperoxidase might be found in spleen. Furthermore, the M_r of the precursor of the heavy subunit of myeloperoxidase was reported as 77 000 or 81 000 [7,8]. Together, these findings seem to eliminate the possibility that the spleen green heme protein is a precursor of myeloperoxidase. On the other hand, the spleen enzyme preparation, which is the only green fraction obtained during the preparation procedure, is homogeneous. Proteolytic degradation usually produces inhomogeneous preparations of myeloperoxidase [9]. Thus, the possibility that the spleen enzyme is a degradation product of myeloperoxidase also seems remote. These considerations have prompted me to reinvestigate the M_r and subunit structure of the spleen green heme protein. In contrast to the original report of Davis and Averill [1], I find that the spleen green heme

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protein consists of two heavy and light subunits forming a tetramer with an M_r of approx. 1.5×10^5, indistinguishable from myeloperoxidase. The amino acid composition of the enzyme is also similar to that of granulocyte myeloperoxidases.

2. EXPERIMENTAL

The green heme protein was extracted and partially purified from bovine spleens obtained from a local abattoir as described by Davis and Averill [1]. A detailed description of the further purification procedure has been given in [4]. The A_{428nm}/A_{280nm} values of the preparation used here were between 0.82 and 0.83. Myeloperoxidase was purified from outdated granulocyte concentrate (supplied by the Penn-Jersey Blood Program of the American Red Cross, Philadelphia) as described in [10]. The A_{428nm}/A_{280nm} value was 0.83.

The half-enzyme of myeloperoxidase was prepared by reductive cleavage of the enzyme as in [11]. Dithiothreitol (DTT) was added to the enzyme preparations (enzyme concentration was approx. 0.3 mM iron chlorin in 0.2 M phosphate buffer, pH 7.0) to a final concentration of 0.1 M, and the mixture incubated for 80 min at room temperature. Iodoacetamide (IAA) was added to a final concentration of 0.2 M followed by incubation on ice for 40 min. The reaction mixture was gel filtered on a Sephadex G-25 column equilibrated with 0.2 M phosphate buffer, pH 7. This DTT-IAA treatment converted about 90% of myeloperoxidase into the half-enzyme with the remainder as unreacted tetramer, in agreement with the reported result [11]. The mixture was not further purified, since gel permeation on a Superose-12 column could readily distinguish the half-enzyme and the unreacted tetramer enzyme by the difference in their M_r (plots C in fig.1). The same procedure was also applied to the spleen green heme protein to prepare the DTT-IAA-treated enzyme.

Estimation of the M_r of the enzyme preparations by gel permeation was performed by use of an analytical Superose-12 (Pharmacia) column with a Pharmacia FPLC system in 0.2 M phosphate buffer, pH 7, operating at a flow rate of 0.4 ml per min at 23°C. Measurements were performed in triplicate.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run in triplicate using a Bio-Rad model 360 vertical slab-cell system. The running gel of 0.75 mm thickness consisted of a 12% acrylamide with 0.375 M Tris, pH 8.8, and 0.1% SDS. The stacking gel was 6% acrylamide with 0.1% SDS. The running buffer was 0.025 M Tris-glycine, pH 8.3, containing 0.1% SDS, and the sample buffer was 0.025 M Tris-glycine, pH 8.3, with 1% SDS, 2.5% glycerol and 2.5% monothioglycerol. Before loading, the samples were boiled for 3 min. Bromophenol blue was used as the tracking dye. After electrophoresis at 130 V for about 90 min, the gel was stained with Coomassie blue in 20% methanol/7% acetic acid for 3 h and destained with the same solution without dye.

Amino acid analyses were performed either at the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, using a Waters PicoTag system with precolumn derivatization by phenyl isothiocyanate, after 24, 36 and 72 h hydrolysis by 6 N HCl, or at the Department of Pathology and Experimental Medicine, University of Pennsylvania, using a Beckman 6300 amino acid analyzer with the ninhydrin method after 24 h hydrolysis. Both methods gave essentially the same results. For amino acid analysis of the DTT-IAA-treated spleen green heme protein, the unreacted native form of the enzyme was removed by gel permeation on Superose-12 as described above.

3. RESULTS

The elution profiles of the spleen green heme protein (A) and human granulocyte myeloperoxidase (B) on a gel-permeation column of Superose-12 are plotted in the left panel of fig.1 as monitored at 280 nm (solid line) and at 428 nm (broken line). It is apparent that the elution volume for the spleen green heme protein agrees well with that for myeloperoxidase, indicating that the M_r values of these two enzymes are the same. The calibration of the gel-filtration system with M_r standards is shown in the right panel of fig.1, from which the M_r values of the spleen green heme protein and myeloperoxidase were calculated as about 1.5×10^5. The M_r of 1.5×10^5 for myeloperoxidase is in good agreement with the reported values [11–13]. The apparent M_r for the spleen enzyme is considerably larger than 57000 reported by Davis

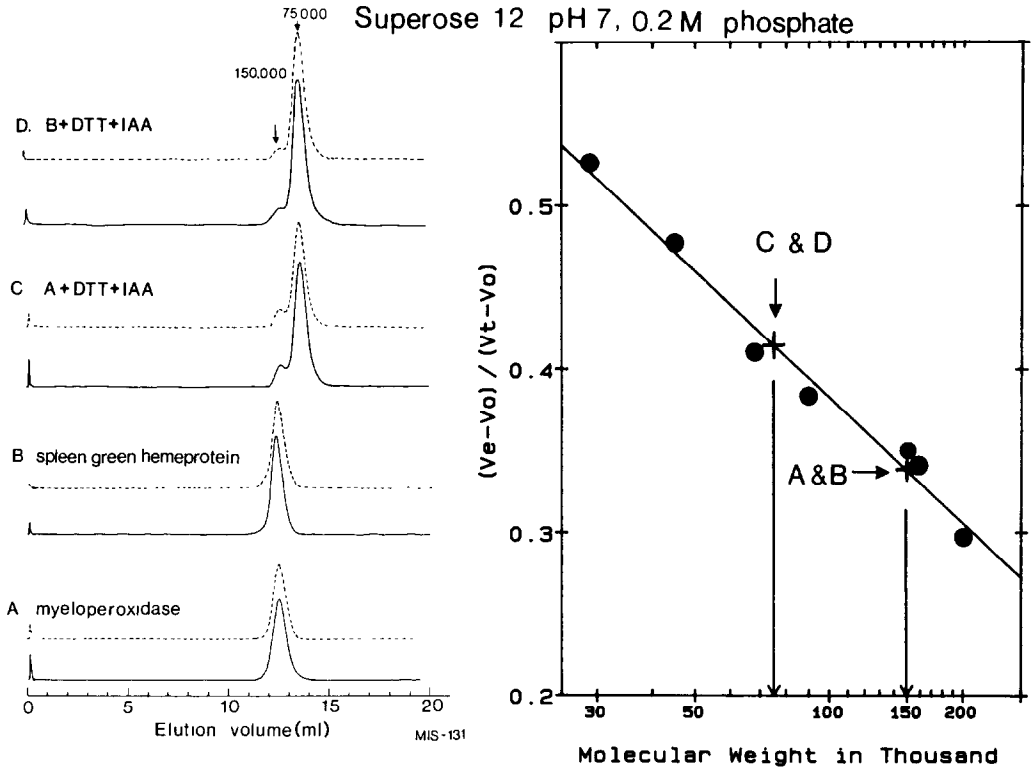

Fig.1. (Left) Elution profile on a column of Superose-12 using 0.2 M phosphate buffer. The solid and dotted curves represent the elution profile monitored at 280 and 428 nm, respectively. Flow rate, 0.4 ml/min. (A) Myeloperoxidase; (B) spleen green heme protein; (C) DTT-IAA-treated (half-enzyme) myeloperoxidase prepared as described in section 2 and (D) DTT-IAA-treated spleen green heme protein in the same manner. (Right) Determination of the M_r by Superose-12 gel-permeation chromatography. V_e, V_t and V_0 represent the elution volume, total volume of the packed gel, and void volume of the column, respectively. The cross marked as A & B corresponds to the $(V_e - V_0)/(V_t - V_0)$ of the peak in plots A and B of the left panel, respectively. C and D represent the major peak in the elution profile of C and D of the left panel, respectively. The M_r standards included carbonic anhydrase (M_r 29000), ovalbumin and its dimer (45000 and 90000), bovine serum albumin (68000), horse alcohol dehydrogenase (150000), aldolase (158000) and β-amylase (200000). Each of the standards was run separately under the same conditions used for the samples.

and Averill [1] from Sephadex G-75 gel-permeation chromatography.

SDS-PAGE of the spleen green heme protein is shown in fig.2. There are two major bands corresponding to M_r values of about 6×10^4 and 1.5×10^4, together with a faint band of M_r 4.5×10^4; this weak band must be a contaminant in the preparation, because its intensity is too weak to be a subunit of the enzyme. This electrophoretic pattern is essentially the same as those reported for myeloperoxidase of normal leukocytes [9,11]. Before staining the gel, a green band was seen at the position which corresponded to the high-M_r subunit. The chromophore group, an iron chlorin, is thus associated with the heavy subunits via covalent linkage(s), as in myeloperoxidase. Together with the result of gel permeation, it can be concluded that the spleen green heme protein is a tetramer with an M_r of about 1.5×10^5, which consists of two identical heavy subunits (M_r 6×10^4) and two light subunits (M_r 1.5×10^4).

Andrews and Krinsky [11] reported that one molecule of myeloperoxidase is cleaved into two identical molecules of the half-enzyme with an M_r of 7.5×10^4, each a dimer containing one heavy and one light subunit, by reduction with DTT and following alkylation with IAA. Fig.1 plots the elution profile for the DTT-IAA-treated myeloperox-

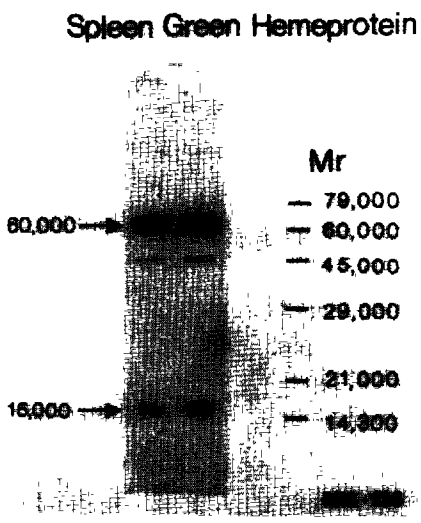

Fig.2. SDS-PAGE of the spleen green heme protein. The right track contains about twice the amount of the enzyme in the left track. The bands for the heavy and light subunits are indicated by the arrows. The lines and numbers listed at the right-hand side of the gel correspond to the positions for the M_r standards which were run in separate tracks. The standards used were transferrin (M_r 79000), catalase (60000), ovalbumin (45000), carbonic anhydrase (29000), soybean trypsin inhibitor (21000) and lysozyme (14300).

idase (curve C) and the DTT-IAA-treated spleen green heme protein (curve D) from a Superose-12 column monitored at 280 nm (solid line) and 428 nm (broken line). The DTT-IAA-treated myeloperoxidase showed a major peak, which accounted for about 90% of the total integrated area, with a small band representing the remaining 10% eluting at the same volume as the native myeloperoxidase. The M_r of the major band was 7.5×10^4, appropriate for the half-enzyme of myeloperoxidase, confirming the original report [11]. The DTT-IAA-treated spleen green heme protein exhibits the same elution profile as the DTT-IAA-treated myeloperoxidase. The apparent M_r of the DTT-IAA-treated spleen green heme protein is the same as that of the half-enzyme of myeloperoxidase, 7.5×10^4. The DTT-IAA-treated spleen enzyme is the half-enzyme of the spleen green heme protein. Formation of the half-enzyme of the spleen green heme protein by reduction and alkylation indicates the presence of a disulfide bond(s) in a manner similar to that in

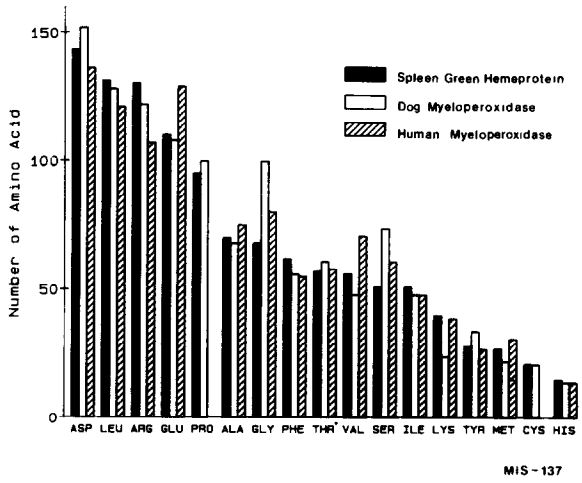

Fig.3. Comparison of the amino acid composition of the spleen green heme protein with that of myeloperoxidase of dog and human granulocytes, which are respectively taken from [12,14].

myeloperoxidase, as proposed by Andrews and Krinsky [11].

Fig.3 compares the amino acid composition of the spleen green heme protein with those of human and dog granulocyte myeloperoxidase [12,13]. The spleen green heme protein has an amino acid composition surprisingly similar to that of granulocyte myeloperoxidases of other origins. The number of each amino acid in the DTT-IAA-treated form, after normalization for an M_r of 75000, is almost half of that in the corresponding amino acid of the native enzyme. This further supports the idea that the DTT-IAA-treated enzyme is a half-enzyme, composed of one each of the heavy and the light subunits.

4. DISCUSSION

The present results, which indicate that a spleen green heme protein exhibits a molecular structure very similar to that of myeloperoxidase, argue strongly against the original reports by Davis and Averill [1,2] who described the spleen green heme protein as a monomeric protein with an M_r of 57000. Parallel experiments using human myeloperoxidase clearly show that the M_r of the spleen enzyme is the same as that of myeloperoxidase, the size of which has been established as

about 150000 [11,13–15]. The origin of this discrepancy in the M_r and subunit structure is not apparent, but the anomalous behavior of myeloperoxidase on gel-permeation chromatography has been reported; the apparent M_r of myeloperoxidase obtained by gel-permeation chromatography tends to be considerably smaller than that obtained by sedimentation equilibrium measurements or than the possible M_r estimated by SDS-PAGE. For example, Harrison et al. [12] reported that gel-permeation chromatography on either Biogel A 1.5 M or Biogel P 200 gave an apparent M_r of about 6×10^4 for canine myeloperoxidase, which exhibited an M_r of 142000 by sedimentation equilibrium. Andrews and Krinsky [11] obtained an apparent M_r of 88000 for native human myeloperoxidase by gel permeation on Sephadex G-150 (50 mM Tris, pH 7.0, containing 0.5% cetyltrimethylammonium bromide, CTAB) while a value of 153000 was given by sedimentation equilibrium. They also found a binding of myeloperoxidase to Sephadex G-150 matrix when the chromatography was performed at neutral pH without CTAB. Only Yamada et al. [13] obtained the same M_r of 150000 for the large components of myeloperoxidase fractions from human promyelic leukemia HL-60 cells by both Sephacryl S-200 gel-permeation chromatography (0.1 M phosphate buffer containing 0.02% CTAB) and sedimentation equilibrium. I found anomalous retardation in the elution of the spleen green heme protein from columns of Superose-12 and Sephacryl S-200 when they were run with 0.05 M Tris buffer, pH 7.0, containing 0.1 M KCl. The enzyme exhibited an elution volume larger than cytochrome *c* on Superose-12, and the elution volume for the enzyme was slightly larger than carbonic anhydrase on a column of Sephacryl S-200. It is conceivable that the enzymes might interact with the gel due to the basic nature of the enzymes. Such an interaction could increase the elution volume. When the spleen enzyme was subjected to gel permeation on a column of Sephacryl S-200 running with 0.2 M phosphate buffer at pH 7 without CTAB, the apparent M_r of the spleen green heme protein was estimated as 1.3×10^5. The choice of the gel and solvent system thus seems to be a crucial factor for the effective determination of the M_r of myeloperoxidase and the spleen green heme protein by gel-permeation chromatography. Here, the elution volume of the spleen enzyme was found to be the same as that for myeloperoxidase, indicating that the spleen green heme protein and myeloperoxidase have the same M_r values.

Another discrepancy lies in the result from SDS-PAGE. Davis and Averill [1] described the presence of only one band corresponding to an M_r of 57000 in their SDS-PAGE, from which they concluded that the enzyme consists of a single polypeptide. Since details of SDS-PAGE were not given in their paper, a direct comparison of the present SDS-PAGE and theirs cannot be made. The intensity of the band for the low-M_r subunit in my SDS-PAGE of the spleen green heme protein and that of myeloperoxidase is less than one-tenth of that of the heavy subunits band, in accordance with the previous report for myeloperoxidase [11]. It is conceivable that Davis and Averill [1] might have mistaken this faint light subunit band in their SDS-PAGE as the light subunit of purple acid phosphatase (M_r 15000) which is copurified with the spleen green heme protein. The separation of purple acid phosphatase from the green heme protein seemed to be difficult in the chromatographic procedures used by them according to their paper [1].

One criticism against the current results might be that the enzyme preparation I am using in this study differs from that used by Davis and Averill [1]. The methods for extraction and partial purification by cellulose phosphate are identical. My further purification procedure is based on the same principle of the original Davis and Averill procedure; ionic exchange chromatography followed by gel-permeation chromatography [1,4]. There was only one green fraction during the course of the preparative procedure. There is no doubt that the spleen green heme protein which I obtained is the same enzyme that was used for the determination of the M_r by Davis and Averill [1].

A definitive comparison of the structure of the spleen green heme protein with granulocyte myeloperoxidase requires further characterization of the molecular properties of these two enzymes, such as the amino acid sequence. Nevertheless, the present results on the similarity in the molecular structure, in terms of M_r, number and size of the subunits and amino acid composition, together with the earlier findings of identical spectroscopic and enzymatic properties [1,3–5], are strong in-

dications that the spleen green heme protein and myeloperoxidase are in fact the same.

ACKNOWLEDGEMENTS

I thank D. Safer for performing the SDS-PAGE, G.D. Markham for generous provision of the amino acid analysis, members of the Penn-Jersey Blood Service for providing the granulocyte concentrate, R.C. Prince for his comments, and T. Yonetani and B.R. Masters for their facilities. This work was supported by Research Grant AI-20463 from the National Institute of Allergy and Infectious Diseases.

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