Assembly of Dimeric Myeloperoxidase during Posttranslational Maturation in Human Leukemic HL-60 Cells†

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ABSTRACT: Myeloperoxidase is a major protein component of the azurophilic granules (specialized lysosomes) of normal human neutrophils and serves as part of a potent bactericidal system in the host defense function of these cells. In normal, mature cells, myeloperoxidase occurs exclusively as a dimer of M_r 150 000 while in immature leukemia cells, there are both monomeric (M_r 80 000) as well as dimeric species. Like other lysosomal enzymes, myeloperoxidase is synthesized as a larger glycosylated precursor (M_r 91 000) that undergoes processing through single-chain intermediates (M_r 81 000 and 74 000) to yield mature heavy (M_r 60 000) and light (M_r 15 000) subunits. To study the assembly of dimeric myeloperoxidase, azurophilic granules were isolated from either unlabeled or pulse-labeled ([^{35}S]methionine/cysteine) HL-60 cells, and myeloperoxidase was extracted and separated into monomeric and dimeric forms by FPLC gel filtration chromatography. Steady-state levels of dimeric and monomeric myeloperoxidase were found to account for 67% and 33%, respectively, of the total peroxidase activity and were correlated with the levels of associated heme as measured by absorption at 430 nm. Labeled myeloperoxidase polypeptides were immunoprecipitated using a monospecific rabbit antibody and were identified and quantitated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis/fluorography and liquid scintillation counting. After a 2-h pulse, labeled myeloperoxidase species of M_r 74 000 and 60 000 were found in fractions coeluting with the monomeric form of myeloperoxidase. Following chase periods from 6 to 68 h, 18–70%, respectively, of the total immunoprecipitable, labeled myeloperoxidase moved out of the monomer pool and was found to be associated with a M_r 60 000 subunit that coeluted with the dimeric form of myeloperoxidase. Quantitation of the time course of the conversion of monomeric to dimeric myeloperoxidase indicated a precursor–product relationship at the level of the mature M_r 60 000 subunit. The assembly of dimeric enzyme is a relatively late event in maturation with a $t_{1/2}$ of 36 h, implying that this process occurs in more mature, dense azurophilic granules.

The enzyme myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) or MPO[1] is a major protein component of the azurophilic granules of normal human neutrophils (Bretz & Baggiolini, 1974; Spitznagel et al., 1974; Wright & Gallin, 1977). These subcellular organelles also contain many lysosomal hydrolases and therefore are considered to be a type of specialized lysosome. In the presence of chloride ion and H_2O_2 (produced during the respiratory burst of activated neutrophils), MPO catalyzes the formation of hypochlorous acid, a powerful microbicidal agent (Klebanoff & Clark, 1978). Thus, MPO plays a central role in the host defense function of these phagocytic cells.

Myeloperoxidase is a glycoprotein of M_r 130 000–160 000 (Harrison et al., 1977; Andrews & Krinsky, 1981; Anderson et al., 1982) with a prosthetic group that appears to be a chlorin type of heme, although the exact structure is uncertain (Eglinton et al., 1982; Sibbett & Hurst, 1984; Babcock et al., 1985; Ikeda-Saito et al., 1985). In normal human peripheral blood neutrophils, the enzyme appears to occur exclusively as a dimer[2] composed of two heavy (M_r 55 000–63 000) and two light (M_r 10 000–15 000) subunits (Andrews & Krinsky, 1981; Olsen & Little, 1984). We and others (Yamada et al., 1981b; Pember et al., 1982; Miyasaki et al., 1986; Svensson et al., 1987) have shown that there are three major chromatographic forms of dimeric MPO, but the structural basis for this heterogeneity is not well understood. Monomeric MPO does not appear to occur in normal neutrophils, although the dimeric protein may be chemically reduced and alkylated to produce an active "hemi-myeloperoxidase" (Andrews & Krinsky, 1981). This is thought to involve the selective reduction of a single disulfide bond between the two heavy subunits. In contrast, human leukemia cells have been found to contain enzymatically active monomeric MPO (M_r 70 000–79 000) as well as the dimeric form (Yamada et al., 1981b; Morishita et al., 1984; Svensson et al., 1987). The amount of monomeric MPO varies with the cell source but has been reported to account for as much as 80% of the total enzyme in cultured human leukemic HL-60 cells (Yamada et al., 1981a).

Little is known about structure/activity relationships between the naturally occurring monomeric and dimeric forms of MPO. Ouchterlony double-diffusion analysis using rabbit antibodies was unable to distinguish between monomeric and dimeric forms of MPO from HL-60 cells, but the monomer could be distinguished from the larger species by microcomplement fixation (Yamada et al., 1981b). On the other hand, kinetic studies of the peroxidase and peroxidase–oxidase activities of MPO from normal and leukemic cells have revealed no significant differences between the monomeric and dimeric

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[1] Abbreviations: BSA, bovine serum albumin; CTAB, cetyltrimethylammonium bromide; Endo H, endo-β-*N*-acetylglucosaminidase H; MPO, myeloperoxidase; PBS, 10 mM potassium phosphate containing 150 mM NaCl, pH 7.4; PMSF, phenylmethanesulfonyl fluoride; PNS, postnuclear supernatant; Rz, rheinheit zahl or A_{430nm}/A_{280nm}; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, 10 mM Tris containing 150 mM NaCl, pH 7.6.

[2] Monomeric forms contain one heavy (H) and one light (L) subunit, either within a single polypeptide or processed to individual H and L subunits; dimeric forms have two processed heavy–light protomers.

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forms (Suzuki et al., 1986; Svensson et al., 1987). A monomeric M_r 70 000 species isolated from the cells of a patient with untreated acute myeloid leukemia was reported to exhibit decreased heat stability, and the molecular weight of both heavy and light subunits was slightly smaller compared to those of dimeric MPO from normal neutrophils (Morishita et al., 1984).

Studies on the biosynthesis of MPO in HL-60 cells have shown that, like other lysosomal enzymes, MPO is initially synthesized as a glycosylated preproprotein of approximate M_r 91 000 (Yamada, 1982; Hasilik et al., 1984; Olsson et al., 1984). This initial precursor is then sequentially processed in a lower density prelysosomal compartment(s) to single-chain intermediates of M_r ~81 000 and 74 000 (Olsson et al., 1984; Akin & Kinkade, 1986; Nauseef & Clark, 1986; Akin et al., 1987). Using an in vitro granule preparation, we have recently shown that the M_r 74 000 intermediate undergoes terminal proteolytic cleavage to form the heavy and light subunits of mature MPO (Akin & Kinkade, 1986). Unlike the processing of other lysosomal enzymes, this step occurred optimally in a neutral rather than an acidic environment and exhibited a $t_{1/2}$ of about 7 h.

To date, all studies on the biosynthesis of MPO have identified precursor and processed polypeptides using SDS–PAGE/fluorography under reducing conditions. For this reason, it is not known which intermediates are involved in the assembly of mature, dimeric MPO. We have addressed this question and herein present evidence that MPO is first processed to an enzymatically active monomeric species composed of a single heavy and light subunit. Subsequently, the mature monomer undergoes dimerization with a $t_{1/2}$ of 36 h, indicating that this process is a relatively late event in MPO maturation. A preliminary account of this work has been reported (Taylor et al., 1988).

Materials and Methods

Materials. Trans-^{35}S-labeled methionine/cysteine (specific activity 1267 mCi/mmol) was from ICN Radiochemicals, Irvine, CA. PMSF, CTAB, guaiacol, CM-Sepharose CL-6B, and Sephacryl S-300 were from Sigma, St. Louis, MO, and Endo H was from Boehringer-Mannheim, Indianapolis, IN. IgGsorb was purchased from The Enzyme Center, Malden, MA, and suspended to 10% w/v in TBS, pH 7.6, containing 1% BSA and 0.5% Triton X-100 (buffer A). Sephacryl S-200 HR was from Pharmacia-LKB, Piscataway, NJ, and Fluoro-Hance was from Research Products International, Mt. Prospect, IL.

Cell Culture. Human leukemia HL-60 cells were grown at 37 °C in liquid culture in 7.5% CO_2 in air using RPMI-1640 medium (Sigma) supplemented with 10% defined bovine calf serum (HyClone Laboratories, Logan, UT), penicillin (50 units/mL), and streptomycin (50 μg/mL) as previously reported (Akin & Kinkade, 1986).

Purification of MPO. HL-60 cells were lysed in hypotonic medium, and a PNS was obtained as previously described (Rice et al., 1986). Granules were obtained by centrifugation of the PNS at 38000*g* for 15 min. The granule pellet was extracted by freeze–thawing in TBS, pH 7.4, containing 1% CTAB until devoid of green color, and MPO was purified from the extract as previously described (Svensson et al., 1987) with the following modifications. The extract was centrifuged to remove debris and dialyzed against 20 mM potassium phosphate, pH 4.7. After removal of precipitated protein by centrifugation, the extract was adjusted to pH 8.0 and loaded on a CM-Sepharose CL-6B column (1.2 × 20 cm) equilibrated in 25 mM potassium phosphate, pH 8.0. The column was washed with buffer until the absorbance at 280 nm was less than 0.02, and the MPO was eluted with a linear gradient of potassium phosphate, pH 8.0 (25–500 mM, 240 mL). Fractions with an Rz > 0.70 were combined, concentrated, and chromatographed on a Sephacryl S-300 column equilibrated and run in 0.2 M potassium phosphate, pH 8.0, containing 0.1% CTAB. Fractions with an Rz > 0.81 which appeared homogeneous on SDS–PAGE were combined, concentrated, and resuspended at 1 mg/mL in PBS.

Production of Antiserum to MPO. Purified MPO (200 μg/mL PBS) was filter-sterilized, mixed with 1 mL of complete Freund's adjuvant, and injected subcutaneously into the haunch of young adult, female New Zealand White rabbits. Booster injections of the same antigen were administered after 2 and 4 weeks, and blood was collected beginning the following week. Serum was obtained and shown to be monospecific by Western blot and immunoprecipitation analyses; the serum was used for experiments without further fractionation.

Pulse-Chase Experiments. Cells were harvested at a density of approximately 1 × 10^6 cells/mL, washed once in methionine-free medium containing 4% dialyzed calf serum, and resuspended at 5 × 10^6 cells/mL in the same methionine-free medium. One flask containing 10^8 cells was used for each time point. After a 1-h depletion at 37 °C, 5 μCi/mL trans-^{35}S-labeled methionine/cysteine was added, and the cells were pulsed for 2 h. Chase periods were begun by diluting the cell suspension to 1 × 10^6 cells/mL with complete RPMI-1640 medium as described previously (Akin & Kinkade, 1986). This cell concentration was maintained by further dilution when necessary during chase periods longer than 24 h.

Granule Isolation and Extraction. The cells were harvested by centrifugation and washed with PBS, pH 7.4, containing 1 mM PMSF. The cells (10^8 cells/mL) were lysed in hypotonic medium without β-mercaptoethanol at 4 °C and centrifuged at 900 rpm for 3 min in an IEC Model DPR-6000 centrifuge to obtain a PNS. A granule-containing pellet was then obtained by centrifugation of the PNS at 48400*g* for 10 min. The granule pellet was extracted by freeze–thawing 3 times in 1 mL of PBS, pH 7.4, containing 0.3% CTAB and 1 mM PMSF with vigorous vortexing for 1 min after each thaw. Insoluble material was removed by centrifugation and the resulting supernatant concentrated to about 100 μL using a Centricon 30 microconcentrator (Amicon).

Gel Filtration Chromatography. The extract was adjusted to a total volume of 300 μL with PBS and 200 μL chromatographed on two Sephacryl S-200 HR 10/30 columns (each 1 × 30 cm) connected in tandem using a Pharmacia FPLC system. The columns were equilibrated and run at 0.15 mL/min in 0.25 M potassium phosphate, pH 7.5, containing 0.1% CTAB. The absorbance of the column effluent was monitored at 430 nm using an ISCO V-4 detector, and fractions (0.3 mL) were collected. A size calibration curve was obtained using β-amylase (M_r 200 000), alcohol dehydrogenase (M_r 150 000), BSA (M_r 66 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 29 000), and cytochrome *c* (M_r 12 400) as standards.

Detection of Peroxidase Activity. The peroxidase activity in 25 μL of each column fraction was assayed using guaiacol as the electron donor by measuring the absorbance change at 470 nm as previously described (Pember et al., 1983).

Immunoprecipitation. The column fractions were diluted to a final volume of 1 mL with TBS, pH 7.6, containing 1 mg/mL BSA and 0.5% Triton X-100. The samples were preabsorbed for 1 h with 40 μL of IgGsorb suspension which was then removed by centrifugation at high speed for 3 min

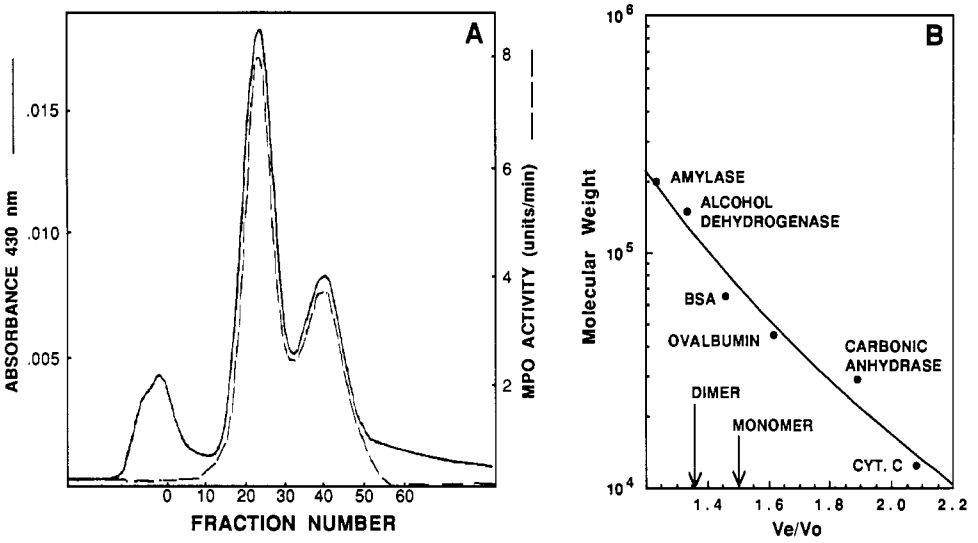

FIGURE 1: (A) Separation of monomeric and dimeric MPO by FPLC gel filtration chromatography. An HL-60 cell granule extract from 1.1×10^8 cells was subjected to chromatography using two Sephacryl S-200 HR columns (1×30 cm) connected in tandem. The column was eluted with 0.25 M potassium phosphate, pH 7.5, containing 0.1% CTAB at a flow rate of 0.15 mL/min. Fractions of 0.15 mL were collected starting at an elution volume of 16 mL (fraction 0). Absorbance at 430 nm (—); peroxidase activity (– –). (B) Calibration curve obtained by chromatographing standard proteins on the Sephacryl S-200 HR system described in (A) above using identical conditions except for the omission of CTAB. The elution positions of monomeric and dimeric MPO are indicated by arrows. The column void volume (V_0) of 14.25 mL was determined by using blue dextran.

in a Hermle Model Z230M microcentrifuge. Rabbit serum (10 μL) containing monospecific polyclonal antibodies prepared against HL-60 MPO was added to each fraction. After incubation at 4 °C overnight, 50 μL of IgGsorb was added, and the samples were agitated briskly for an additional hour. The IgGsorb was pelleted by centrifugation of the samples for 3 min on low speed in the microcentrifuge, and the pellets were washed twice with 0.5 mL of buffer A and once with TBS. The pellets were resuspended for 15 min at room temperature in 40 μL of sample buffer (buffer B) consisting of 125 mM Tris, pH 6.8, 100 mM dithiothreitol, 1% SDS, 10% glycerol, and 0.025% bromphenol blue and then placed in a boiling water bath for 10 min.

Endo H Digestion. Any insoluble material in the samples was pelleted for 3 min at high speed in the microcentrifuge. Ten-microliter aliquots of the supernatants were removed and incubated overnight at 37 °C with 30 μL of 10 mM $NaPO_4$, pH 6.0, containing 1 milliunit of Endo H. The reaction was stopped by the addition of 10 μL of 4× buffer B, and the samples were placed in a boiling water bath for 3 min.

SDS-PAGE Electrophoresis and Fluorography. The samples were electrophoresed on minigels (8 cm × 10 cm × 0.8 mm) using a modified Laemmli system (Laemmli, 1970). The running gel and stacking gel consisted of 12% and 5% acrylamide, respectively, with bis(acrylamide):acrylamide ratios of 1:130 and 1:37.5, respectively. Gels were stained with 0.15% Coomassie Brilliant Blue R-250. Following destaining, the gels were soaked for 5 min in distilled water followed by gentle agitation in Fluoro-Hance for 10 min. The gels were then dried and exposed to Kodak X-Omat AR film for several days at −80 °C.

Quantitation of Radiolabeled MPO Species on SDS-PAGE. Radioactive bands were excised from the dried gels, rehydrated in 200 μL of water, and digested in 2 mL of NCS tissue solubilizer (Amersham) at 50 °C for 16 h. Ten milliliters of aqueous scintillation fluor (Amersham) was added, and samples were counted using a Beckman LS-330 liquid scintillation counter.

Results

Our initial approach to investigating the assembly of dimeric MPO was to develop a highly reproducible procedure for the separation and quantitation of monomeric and dimeric forms of the enzyme. This was accomplished by using an FPLC gel filtration chromatography system employing a Sephacryl S-200 HR matrix (see Materials and Methods for details). Figure 1 shows a representative elution profile obtained when a granule extract from HL-60 cells was fractionated by using this system. Two major heme absorption peaks were observed, both of which correlated directly with peroxidase activity in collected fractions. Calibration of the column with proteins of known molecular weight indicated that the earlier eluting peak corresponded to dimeric species of MPO with a molecular weight of about 150 000 and the later eluting peak corresponded to monomeric species of MPO having a molecular weight of about 80 000. These values were consistent with the previously reported molecular weights of dimeric and monomeric forms of MPO from HL-60 cells as determined by using sucrose density gradient centrifugation and standard gel filtration chromatography (Yamada et al., 1981a; Andrews & Krinsky, 1981; Svensson et al., 1987).[3] Recovery of applied MPO based on enzyme activity was 90% ($N = 4$). The steady-state levels of dimeric and monomeric MPO were 67% and 33% of the total recovered peroxidase activity based on four independent determinations using cells from the same passage number (passage 17). A third peak having absorbancy at 430 nm (Figure 1) was frequently observed to be eluted in extremely variable amounts; this material was not associated with any detectable peroxidase activity and was not investigated further.

The conversion of monomeric to dimeric MPO was next investigated in pulse–chase experiments using HL-60 cells that had been labeled with [^{35}S]methionine/cysteine for 2 h and chased for different periods of time. Preliminary experiments indicated that assembly of the dimer involved only the more mature M_r 74 000 and 60 000 monomeric forms of MPO; therefore, we chose to use a neutral pH extraction procedure

[3] Examination of multiple MPO species using FPLC cation-exchange and gel filtration chromatographic systems has shown that monomeric MPO is associated exclusively with form I (Taylor et al., 1989).

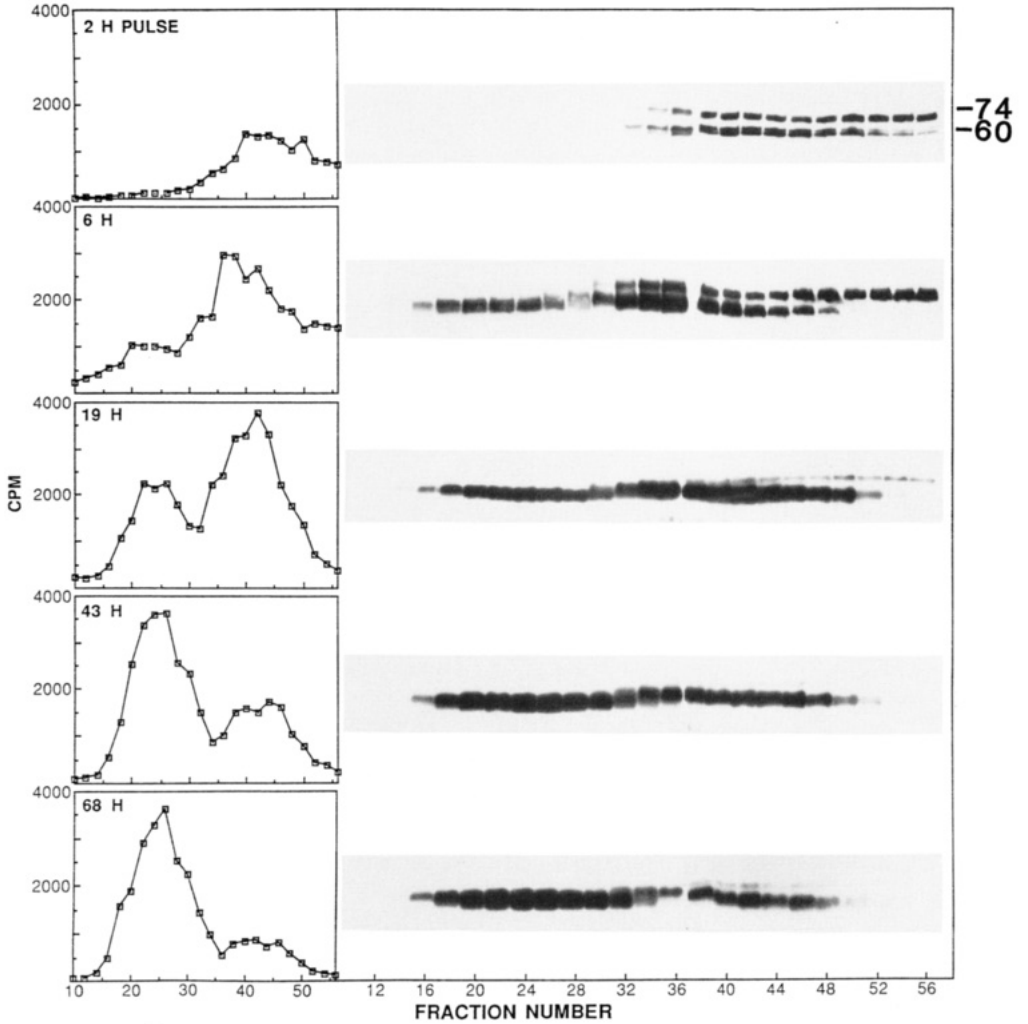

FIGURE 2: Incorporation of trans-^{35}S-labeled methionine/cysteine into monomeric and dimeric MPO species in HL-60 cells. Cells were pulse-labeled for 2 h and chased for the indicated periods of time. MPO was extracted from granules and fractionated into monomeric and dimeric species by FPLC gel filtration chromatography (cf. Figure 1). After determination of peroxidase activity, even-numbered column fractions were combined with the preceding odd-numbered fraction and then immunoprecipitated using a monospecific rabbit anti-MPO antibody (left panels). The MPO species were identified by SDS–PAGE and fluorography (right panels). Molecular weight standards were run simultaneously (not shown) to determine apparent molecular weight values of 74K and 60K as labeled on the right side of the figure. For clarity of presentation, the light subunit is not shown.

previously shown to selectively extract these two MPO species (Akin & Kinkade, 1987). In these experiments, granules were isolated after different chase periods, the labeled MPO was extracted, and the extracts were analyzed for their content of monomeric and dimeric species by using the FPLC method described earlier (cf. Figure 1). Radiolabeled MPO was quantitated after subjecting each of the collected fractions to immunoprecipitation using a monospecific rabbit polyclonal antibody. As shown in Figure 2 (top left panel), following a 2-h pulse and no chase, immunoprecipitable radiolabeled MPO was observed in column fractions that corresponded to the monomeric form of MPO (fractions ≥35). The data shown in the successive four left panels of Figure 2 (6–68-h chase periods) indicated that with time, label associated with the monomeric pool of MPO gradually shifted to the dimeric pool. After about 40–45 h, the ratio of labeled monomer and dimer pools was essentially the same as the ratio of peroxidase activities seen in a standard extract of unlabeled granules (cf. Figure 1).

In order to determine which of the different monomeric species of MPO were actually involved in dimer formation, we subjected each of the eluted and immunoprecipitated fractions to SDS–PAGE and fluorography. The data in the right panels of Figure 2 show the radioautograms obtained for each of the chromatographic fractions corresponding to the left panels of this same figure. It can be seen that following a 2-h pulse labeling period, both the M_r 74 000 polypeptide and the M_r 60 000 heavy subunit of MPO were associated exclusively with monomeric MPO (Figure 2, top right panel). Interestingly, both species appeared as closely spaced but distinct doublets on the gels. Furthermore, there was a gradual but distinct change in the pattern of these doublets in both the M_r 74 000 and M_r 60 000 species across the elution profile. In the earlier eluting fractions, the slower mobility band of both species predominated. However, with increasing elution volume, the relative amount of the slower mobility band decreased while the relative amount of the faster mobility band progressively increased (Figure 2, right panels, 2-h pulse and 6-h chase, respectively). These observations suggested a size heterogeneity between these two species that persisted when the M_r 74 000 polypeptide was processed to the M_r 60 000 heavy subunit. A variable, minor amount of radioactivity (<5%) was associated with several bands of approximate M_r 40 000–50 000 that are generally considered to be degradation products and/or artifacts of the gel sample preparation procedure (data not shown).

After a 6-h chase period, the labeled M_r 60 000 heavy subunit began to appear as a broad band on SDS–PAGE in

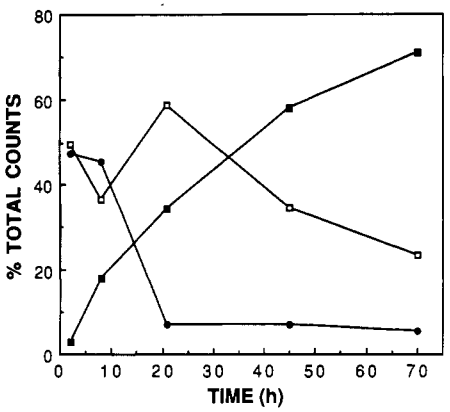

FIGURE 3: Time course of the processing of the M_r 74000 single-chain MPO precursor to monomeric and dimeric forms of the M_r 60000 heavy chain. Bands (cf. Figure 2, right panels) corresponding to the M_r 74000 precursor [(●) fractions 34–56], M_r 60000 monomer [(□) fractions 34–56], and M_r 60000 dimer [(■) fractions 12–32] were excised from the gels and quantitated by liquid scintillation counting.

the region of the elution profile associated with dimeric MPO (Figure 2, left and right panels, 6-h chase). This band appeared to have a slightly lower apparent molecular weight than the heavy subunit observed to coelute with the monomeric species. The absence of any appreciable M_r 74000 precursor in the dimeric region indicated that assembly of the dimer did not occur prior to processing of the monomeric M_r 74000 species to the more mature M_r 60000 heavy subunit. With increasing periods of chase (Figure 2, right panels, 19-, 43-, and 68-h chases, respectively), the processed, mature M_r 60000 heavy subunit continued to accumulate in the dimeric region, and by 19 h, most of the pool of monomeric M_r 74000 precursor was exhausted, accounting for less than 9% of the total labeled MPO.

The kinetics of the coversion of the monomeric M_r 60000 heavy subunit into a dimeric species was determined by excising the radioactive bands from the gels and quantitating those associated with monomeric MPO (M_r 74000 and 60000) as well as with dimeric MPO (M_r 60000) by liquid scintillation counting. These results are shown in Figure 3. Label associated with the monomeric M_r 74000 species was observed to decrease with time, and there was a corresponding increase in label associated with both the monomeric and dimeric M_r 60000 species. By 19 h of chase, the amount of label associated with the monomeric M_r 74000 species reached a minimum and remained constant during subsequent chase periods up to about 70 h. During this same period of time, there was a progressive decrease in the labeled monomeric M_r 60000 species that was accompanied by a corresponding increase in the dimeric form. Interestingly, as shown in Figure 4, the precursor–product relationship between the monomeric and dimeric forms of the M_r 60000 heavy subunit appeared to follow first-order kinetics with a half-life of approximately 36 h. This presumably reflects dilution of the labeled monomer species by the large pool of preexisting, unlabeled monomer.

Discussion

The present study has confirmed earlier reports (Yamada et al., 1981a,b) of the occurrence of a small form of MPO in human leukemic cells (Figure 1). In addition, we have provided evidence for the in vivo assembly of dimeric MPO from a monomeric precursor. Because processing of MPO is relatively complex, the monomeric precursor could theoretically be any one of several intermediates thus far identified. These include single-chain precursors of M_r 91000, 81000, and 74000, as well as a species derived by proteolytic cleavage of

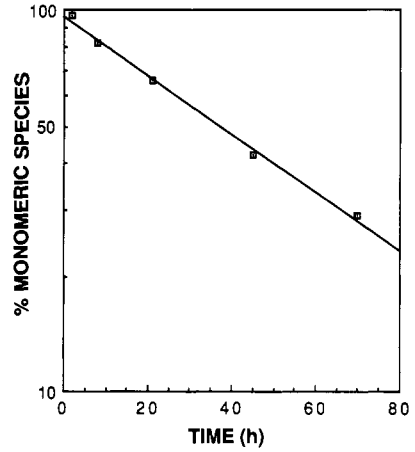

FIGURE 4: Kinetics of the conversion of ^{35}S-labeled monomeric MPO to a mature, dimeric species in HL-60 cells.

the M_r 74000 precursor which is composed of a single heavy and light subunit of M_r 60000 and 15000, respectively. This latter species exhibits full enzymatic activity (Yamada et al., 1981b), although it is still not known whether any of the larger, single-chain precursors are enzymatically active.

In pulse–chase experiments, the M_r 74000 polypeptide precursor was observed to coelute with monomeric MPO under nondenaturing conditions (Figure 2). These data clearly indicated that dimerization of this species did not occur and suggested that proteolytic cleavage to the heavy and light subunits took place prior to formation of a dimeric MPO species. These in vivo results also confirmed previous work from our laboratory showing that the heavy and light subunits were derived from the M_r 74000 single-chain precursor in an isolated granule system (Akin & Kinkade, 1986).

The observation that the M_r 74000 species occurred as a doublet on SDS–PAGE also has several interesting implications. As this polypeptide was processed to the heavy and light chains of the monomeric precursor species, the characteristic doublet was retained in the heavy subunit, and the differences in the relative mobilities of these two bands reflected the same differences seen across the elution profile of the M_r 74000 single-chain precursor (Figure 2). Furthermore, the heavy subunits in fractions coeluting with monomer had calculated molecular weight of 64000 and 61000 while the heavy subunit(s) coeluting in the dimeric region appeared on the SDS–PAGE gels as a broad band of apparent M_r 61000–58000. These data indicate there are distinct and reproducible size differences between these species that are conserved as processing proceeds and suggest that further processing of the heavy subunit of MPO takes place during or subsequent to the dimerization process.

Interestingly, the broad appearance of the band coeluting in the dimeric region suggested that more than one species of heavy subunit might be present in these samples. Although not clearly resolved by our SDS–PAGE system, the heterogeneity of dimeric MPO from HL-60 cell extracts has been demonstrated using cation-exchange chromatography (Yamada et al., 1981b; Taylor et al., 1989), and small differences in heavy subunit molecular weight from multiple forms of dimeric MPO have been reported (Pember et al., 1983; Morita et al., 1986). Whether or not these observations are related to the recent demonstration of amino- and carboxyl-terminal clipping of both the heavy and light subunits (Hashinaka et al., 1988; Iwamoto et al., 1988) is not yet known.

While the exact nature and timing of these events are not well understood, proteolytic processing associated with the conversion of monomer to dimer in later stages of maturation

may be at least partially responsible for the three major chromatographic forms of dimeric MPO that we and others have described (Yamada et al., 1981b; Pember et al., 1982; Miyasaki et al., 1986; Svensson et al., 1987). This would be consistent with reports of small but reproducible differences in the amino acid compositions of these three forms (Pember et al., 1983; Iwamoto et al., 1988). Also, the observation that this heterogeneity persists following treatment with the endoglycosidase *N*-glycanase (Wright et al., 1987) indicates that the multiplicity of MPO dimers is not due solely to differences in carbohydrate moieties. Another possible contributing factor to the heterogeneity of both monomeric and dimeric MPO species relates to the recent reports of multiple mRNAs for MPO (Chang et al., 1986; Johnson et al., 1987; Weil et al., 1987). These have been shown to arise as a result of alternative splicing, differential polyadenylation, and the use of an alternate transcriptional start site (Hashinaka et al., 1988; Johnson, 1989). Of possible significance in this regard is our observation that the M_r 91 000 and 81 000 single-chain precursors also occur as doublets when subjected to high-resolving SDS-PAGE.[4]

Heterogeneity at the level of the monomeric precursor raises the intriguing possibility that heterogeneity among dimeric MPO species might result from assembly of unlike monomers (Harrison et al., 1977; Nauseef & Malech, 1986). However, a definitive answer to this question will require the development of methods for the separation of the individual heavy and light subunits from each of the major forms of dimeric MPO. In addition, there is little information regarding other possible contributing factors to MPO heterogeneity such as the presence and nature of phosphate-containing and hybrid oligosaccharides (Hasilik et al., 1984; Strömberg et al., 1985) or the structural differences related to the reported inequivalence between the two prosthetic groups (Schultz, 1980). Clearly, more complete structural studies are necessary to determine the differences among the multiple forms of MPO and to understand the steps involved in MPO biosynthesis and processing.

Data derived from longer chase periods (19–68 h) during the time course of mature MPO dimer assembly provide some insight into the nature of the subcellular compartment(s) within which this process is occurring. Earlier work, presenting a complete time course for MPO maturation in HL-60 cells, has shown that after a 2-h pulse-label, radioactivity associated with newly synthesized M_r 91 000 and 81 000 precursors first appears in a lower density prelysosomal compartment(s) and accounts for a significant fraction of the total radioactivity found in immunoprecipitable MPO species (Olsson et al., 1984; Akin & Kinkade, 1987; Akin et al., 1987). However, after a 16-h chase, greater than 87% of the total radioactivity is found in the heavy and light subunits of mature MPO which resides in the denser granule fractions (Akin et al., 1987). Therefore, at these longer chase periods (19–68 h), the granule isolation and selective extraction procedure used in this study optimizes recovery of radiolabeled MPO species in the denser granule fractions, since insignificant radioactivity remains in MPO precursors associated with the lower density fractions. We have observed in the present study that dimerization of MPO monomer occurs with a $t_{1/2}$ of 36 h (Figure 4). In contrast, we have reported that processing of the M_r 74 000 precursor to heavy and light subunits in an in vitro granule preparation occurred with a $t_{1/2}$ of 7 h (Akin & Kinkade, 1986). Taken together, these observations strongly suggest that the assembly of MPO dimer is taking place in more mature, dense azurophilic granules.

In summary, the present studies provide evidence for the intermediate nature of a more mature monomeric MPO in the synthesis of dimeric MPO and indicate that this event is a relatively late one in the maturation of the enzyme. Additional studies utilizing Percoll density gradient centrifugation to separate different subcellular compartments from both human leukemic HL-60 cells and normal human bone marrow cells should provide further insight into the sorting and processing of the multiple forms of MPO, as well as the origin and function of distinct types of azurophilic granules (Rice et al., 1986, 1987; Parmley et al., 1987).

[4] K. L. Taylor, I. Pinnix, and J. M. Kinkade, Jr., unpublished data.

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Multiple Replacements at Position 211 in the α Subunit of Tryptophan Synthase as a Probe of the Folding Unit Association Reaction†

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ABSTRACT: Equilibrium and kinetic studies on the folding of a series of amino acid replacements at position 211 in the α subunit of tryptophan synthase from *Escherichia coli* were performed in order to determine the role of this position in the rate-limiting step in folding. Previous studies [Beasty, A. M., Hurle, M. R., Manz, J. T., Stackhouse, T., Onuffer, J. J., & Matthews, C. R. (1986) *Biochemisty 25*, 2965–2974] have shown that the rate-limiting step corresponds to the association/dissociation of the amino (residues 1–188) and carboxy (residues 189–268) folding units. In terms of the secondary structure, the amino folding unit consists of the first six strands and five α helices of this α/β barrel protein. The carboxy folding unit comprises the remaining two strands and three α helices; position 211 is in strand 7. Replacement of the wild-type glycine at position 211 with serine, valine, and tryptophan at most alters the rate of dissociation of the folding units; association is not changed significantly. In contrast, glutamic acid and arginine dramatically decelerate and accelerate, respectively, both association and dissociation. The difference in effects is attributed to long-range electrostatic interactions for these charged side chains; steric effects and/or hydrogen bonding play lesser roles. When considered with previous data on replacements at other positions in the α subunit [Hurle, M. R., Tweedy, N. B., & Matthews, C. R. (1986) *Biochemistry 25*, 6356–6360], it is clear that β strands 6 (in the amino folding unit) and 7 (in the carboxy folding unit and containing position 211) dock late in the folding process.

The determination of the mechanism of protein folding requires detailed information on the structures of partially folded forms and the transition states which link these forms and the native conformation. Structures of folding intermediates are beginning to become available through advances in technology such as the recent combination of hydrogen-exchange and two-dimensional nuclear magnetic resonance (2D NMR)[1] spectroscopy (Udgaonkar & Baldwin, 1988; Roder et al., 1988) and peptide synthesis (Oas & Kim, 1988; Marqusee &

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[1] Abbreviations: 2D NMR, two-dimensional nuclear magnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; G211X (where X = D, E, R, S, V, and W), single amino acid code with the first letter designating the wild-type residue followed by the position and the second letter designating the mutation, i.e., G211D referes to the replacement of glycine by aspartic acid at position 211, and E, R, S, V, and W refer to glutamic acid, arginine, serine, valine, and tryptophan, respectively; C_{mxy}, urea concentration at the midpoint of the transition between species x and y.

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