Identification of a Lymphocyte Enzyme That Catalyzes Pentamer Immunoglobulin M Assembly*

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A protein with immunoglobulin M-polymerizing activity was isolated from the membrane fraction of mouse plasmacytoma cells secreting pentamer IgM. The isolation was achieved by taking advantage of the solubility of the protein in 50% ammonium sulfate, its relatively high net negative charge, and its sedimentation at 4.2 S. Analyses of the purified preparations showed that the polymerizing protein catalyzes the assembly of pentamer IgM in vitro; less than 1 mol of enzyme/10 mol of monomer IgM and 2 mol of J chain were found to promote 50% polymerization. Evidence that the enzyme also plays an essential role in the in vitro assembly process was obtained from the reaction rates of the polymerization catalyzed in vitro, the similarity between the pentamer IgM molecules synthesized in vitro and in vivo, and the finding that polymerizing enzyme is a specific product of B lymphocytes. Analyses of the mechanism of polymerization suggested that polymerizing enzyme is a sulfhydryl oxidase; it was found to be inactivated by chelating agents and to resemble Cu²⁺ in catalyzing the formation of IgM intersubunit disulfide bonds. These results raise the possibility that the assembly of pentamer IgM does not involve disulfide interchange as previously thought, but proceeds by the direct oxidation of monomer IgM and J chain sulfhydryls.

The polymerization of immunoglobulin M is known to play an important role in the differentiation of the B lymphocyte to antibody production. The unstimulated cell synthesizes only monomeric IgM which is incorporated into the plasma membrane and serves as an antigen receptor (1). After contact with the appropriate antigen or mitogen, however, the B lymphocyte differentiates into a blast cell that assembles and secretes pentameric IgM (2, 3). The polymerization of IgM appears to be required for its secretion since the monomeric form is not found extracellularly in significant amounts (4). Moreover, the polymerization reaction appears to be a prerequisite for the further differentiation of the blast lymphocyte since blockage of pentamer IgM formation results in the suppression of all subsequent IgG and IgA synthesis (5).

Despite the critical role of polymerization in B lymphocyte differentiation, the mechanism of the reaction remains to be established. Structural studies have shown that the pentamer is assembled by linking five IgM monomers and a single J chain through disulfide bonds (6). Analyses of the reductive cleavage products suggested that polymerization proceeds by a series of disulfide interchanges beginning with the formation of a J chain-containing dimer (7). This hypothesis was supported by the finding that disulfide interchange enzyme (thiol:protein oxidoreductase, EC 1.8.4.2) promotes the in vitro assembly of pentamer IgM (8). However, subsequent investigations have ruled out a catalytic function for disulfide interchange enzyme (9, 10).

The enzymatic requirements for IgM polymerization have been pursued in our laboratory by assaying extracts of pentamer IgM-secreting tumor cells for their ability to promote IgM assembly in vitro. By use of this system, it has been possible to identify a likely candidate for catalysis of IgM intersubunit bonds. A crude membrane-solubilized fraction from the IgM-secreting plasmacytomas was found to effect IgM polymerization at one-twentieth the protein concentration required for disulfide interchange enzyme (10). Studies were undertaken, therefore, to investigate the polymerizing component, (b) determine its catalytic properties and the fidelity of the pentamer IgM product, and (c) elucidate the mechanism of polymerizing action. The present paper describes the results of these studies.

MATERIALS AND METHODS

Purification of Polymerizing Activity—Polymerizing material was isolated from two pentamer IgM-secreting mouse plasmacytomas, MOPC 104E and HPC 76. The MOPC 104E tumor was obtained from Dr. Anne Good, University of California, Berkeley, and the HPC 76 tumor from the laboratory of Dr. Leonard Herzenberg, Stanford University. In a typical preparation, 75 tumors (60 g) grown in Balb/c mice to a diameter of about 1 cm were excised and washed in ice-cold TS buffer (20 mM Tris-HCl, 0.25 M sucrose, pH 8.0). The tumors, several at a time, were disrupted by 5 strokes in a 15-ml Dounce homogenizer in 10 ml of TS buffer. The cells were then homogenized by 10 strokes in a 40-ml TenBroeck tissue grinder and the lysates were centrifuged 20 min at 1,900 rpm in an IEC 269 rotor. The pelleted material was re-extracted by homogenization with 5 strokes of the TenBroeck tissue grinder in half the original volume of TS buffer. After centrifugation as described above, the supernatants from this and the previous centrifugation were pooled (about 150 ml), centrifuged for 20 min at 10,000 rpm in a Sorvall SS34 rotor, and then recentrifuged for 2.5 h at 38,000 rpm in a Beckman type 40 rotor to separate the membrane fraction. All the above procedures were performed at 4 °C.

The pelleted membranes were resuspended in approximately 50 ml of TS buffer by homogenization in a 15-ml TenBroeck tissue grinder and then dissolved by the slow addition of 5% deoxycholate to a final

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1 R. A. Roth and M. E. Koshland, unpublished data.
concentration of 0.25% deoxycholate (w/v). The preparation was made 50% in ammonium sulfate, stirred for 60 min, and allowed to stand overnight. After the precipitate was removed by centrifugation for 30 min at 17,000 in a SS34 rotor, the supernatant was dialyzed exhaustively against 30 mm Tris- HCl, pH 8, and the entire solution (approximately 100 ml) was applied to a 50-ml column of DEAE-Sephadex A-50 in 0.1 M NaCl, pH 7.5. The column was washed with 100 ml of the same buffer and then with 300 to 500 ml of 10 mM Tris- HCl, pH 8, or until the absorbance at 278 nm of the effluent was below 0.04. The protein bound to the resin was eluted either by the use of a linear gradient of 0 to 0.5 M NaCl in 100 mM Tris-HCl buffer or by use of a stepwise gradient consisting of 150 ml each of 0.05, 0.1, 0.15, 0.2, and 0.25 M NaCl. In each case, 5-ml fractions were collected and analyzed directly for protein content and disulfide interchange activity. Analyses of polymerizing activity were performed after the fractions were pooled, concentrated approximately 10-fold by Amicon ultrafiltration on a FM 10 membrane, and dialyzed against 50 mM Tris-HCl, 0.1 M NaCl, pH 8.

For some experiments the polymerizing material was used at this stage of purification. For others, it was further purified by sucrose gradient centrifugation. The chromatographic fractions rich in polymerizing activity were layered on a 12-ml linear sucrose gradient (5 to 30%) and centrifuged with 20 mM Tris- HCl, pH 7.5, 0.1 M NaCl. The gradients were centrifuged for 4 hr at 39,000 rpm in a Beckman SW41 rotor and then collected in 0.35-ml aliquots. Disulfide interchange activity was measured directly on each aliquot; polymerizing activity was determined on pools of three aliquots that were concentrated 5-fold and dialyzed against 30 mM Tris-HCl, pH 7.5, 0.1 M NaCl.

Enzyme Assays—Disulfide interchange activity was measured either by insulin degradation (10) or by the conversion of randomly reoxidized ribonuclease to active enzyme (9).

Polymerization Assay—Pentamer IgM was isolated from the sera of mice bearing MOPC 104E or HPC 76 tumors by successive precipitation at ammonium sulfate concentrations of 50, 45, and 40%. The final precipitate was dissolved in 0.15 M NaCl, 20 mM Tris- HCl, pH 8, 1 mM EDTA, 0.02% sodium azide and dialyzed against the same buffer. After dialysis the sample was clarified by centrifugation at 40,000 × g and the supernatant was filtered through a Sepharose 6B column (5 × 110 cm) equilibrated in the sample buffer. Pentamer IgM was recovered at an elution volume of 300 ml and the pooled material gave a single band in SDS-agarose gel electrophoresis. For the polymerization assay, 1.3 mg of purified pentamer were reduced with 3 mM dithioerythritol for 1 hr at 22 °C and the free reducing agent was removed by passage of the solution over a Sephadex G-25 column (1.5 × 5 cm) equilibrated in 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5, 0.02% sodium azide. Fifteen-μl aliquots containing 25 μg of the reduced IgM were then incubated for 1 hr at 37 °C with 50 μl of the enzyme or cell extract sample to be tested. The reaction was terminated by dilution with 50 mM iodoacetamide and the extent of polymerization was determined by SDS-agarose-acrylamide gel electrophoresis as described previously (9). One polymerizing unit was defined as the amount of material capable of polymerizing 10 μg of IgM under the conditions described for the assay.

Pentamer IgM Assay—The sedimentation coefficient of the IgM product was determined by sucrose gradient centrifugation. For these experiments, the in vitro polymerization reaction was scaled up 3- to 4-fold. Samples of the reaction mixture were layered on 12-ml linear sucrose gradients (6 to 25% sucrose in 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.2 mM EDTA) and centrifuged for 16 hr at 38,000 rpm in a Beckman SW41 rotor. Fractions of 0.3 ml were collected, diluted 1:2 with Tris buffer (29 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.02% NaN3), and assayed for their absorbance at 278 nm. The sedimentation coefficients were calculated (11) by comparison to control gradients containing purified 7 S IgG and 19 S pentamer IgM.

The IgM products isolated by sucrose gradient centrifugation were also analyzed for their J chain content and their ability to fix complement. For the J chain determinations, the IgM was reduced by incubation in 100 mM dithiothreitol for 1 hr at 37 °C and the reduction was terminated by addition of a 1.5 molar excess of N-ethylmaleimide. The J chain released was quantitated by radioimmunoassay as described previously (12). For the complement fixation measurement, advantage was taken of the dextran binding properties of the MOPC 104E IgM. The procedure of Wasserman and Levine (13) was used as modified by Brown and Koshland (14). 2 μg of MOPC 104E IgM were incubated with 1 μg of a solution of guinea pig complement (15). The mixture was incubated with 1 μg of Leucocyte mesenteroides NRR (B-1355) in 1 ml of buffer containing 0.02 M triethanolamine-HCl, 0.128 × NaCl, 5 × 10⁻⁴ M MgCl², 1.5 × 10⁻⁴ M CaCl², 0.1% bovine serum albumin, pH 7.4. After 20 hr at 4 °C a series of 2-fold dilutions were rapidly made at 0 °C and an amount of whole guinea pig complement in 0.5 ml that would give 90% lysis was added to each dilution. The tubes were incubated 60 min at 37 °C, 0.2 ml of a 0.25% suspension of sensitized sheep red blood cells was added at 0 °C, and the tubes were reincubated for 60 min at 37 °C. The solutions were then centrifuged and the absorbance at 418 nm of the supernatants was read.

RESULTS

Isolation and Characterization of IgM-polymerizing Material—IgM-polymerizing component was first detected in the course of purifying disulfide interchange enzyme from IgM-secreting plasmacytoma cells. Extracts of the plasmacytoma cells were prepared by solubilizing the membrane fraction in detergent and removing the protein precipitable in 50% ammonium sulfate. The remaining protein solution was then chromatographed on a column of DEAE-Sephadex and the eluate was monitored for both IgM-polymerizing and disulfide interchange capacity. As the chromatographic patterns in Fig. 14 illustrate, the two activities were not coincident; polymerizing activity was eluted from the column with 0.1 M NaCl whereas disulfide interchange enzyme required higher salt concentrations. Although some enzyme was recovered in the 0.1 M fraction, the bulk eluted in the 0.15 M fraction. These findings indicated that the polymerizing activity of the plasmacytoma extracts was not due to disulfide interchange enzyme, but to a second component with a lower net negative charge.

The responsible component was identified as a protein by several criteria. First, the polymerizing activity was nondialyzable. When the 0.1 M salt fractions from the DEAE-Sephadex column or the fractions obtained in subsequent purification steps were extensively dialyzed, their capacity to promote IgM-polymerization was quantitatively retained. Second, the polymerizing activity of the preparations could be abolished by protease treatment. Digestion with subtilisin BPN’ or subtilisin Carlsberg at concentrations of 0.6 mg/ml of enzyme reduced the polymerizing activity by 80%.

![Fig. 1. DEAE-Sephadex chromatography of membrane protein extracts from MOPC 104E plasmacytoma (A) and mouse liver (B). The preparation of the extracts, the column chromatography, and the analyses of the fractions are described under “Materials and Methods.”](https://example.com/fig1.png)

2 The abbreviation used is; SDS, sodium dodecyl sulfate.
caused a progressive loss in polymerizing activity that paralleled the decrease in protein content and the inactivation was complete after 1 h at 37°C. The activity was retained, however, if proteolysis was inhibited by making the reaction mixture 1 mM in phenylmethanesulfonyl fluoride.

The polymerizing protein was found to differ from disulfide interchange enzyme in immunological properties as well as in net charge. The disulfide interchange enzyme that co-eluted with polymerizing activity in the 0.1 M salt fraction was removed by absorption with rabbit antibody prepared against a purified form of the liver enzyme (10). Radioimmunoassays showed that the enzyme concentration was reduced from 0.1 mg/ml to undetectable levels, i.e., less than 0.5 ng/ml. Despite this depletion the polymerizing capacity of the fraction remained unchanged, averaging 30 units/ml before and after absorption. The polymerizing activity could be removed, however, by absorption with antibody prepared against the crude 0.1 M salt fraction.

The polymerizing protein was also found to have a more limited tissue distribution than disulfide interchange enzyme. When mouse liver cells were fractionated by the procedures described above and the extracts were chromatographed on DEAE-Sephadex, only disulfide interchange enzyme was recovered in the eluate. No peak of polymerizing activity could be detected (Fig. 1B). Similar results were obtained with extracts from a B lymphoma line representative of an unstimulated B cell (15). In a stepwise elution off DEAE-Sephadex, the 0.05-0.1 M salt fraction lacked polymerizing activity, the 0.1-0.15 M fraction exhibited a low level, and the 0.15-0.2 M fraction contained the expected disulfide interchange enzyme. These findings suggested that polymerizing component is a specific product of B lymphocytes, particularly associated with differentiation to antibody secretion.

The yield of polymerizing component from IgM-secreting plasmacytomas varied considerably. For 18 preparations the activity recovered after DEAE-Sephadex chromatography ranged from 20 to 200 polymerizing units/mg of protein with a typical preparation yielding a total of 2 mg of protein. This lack of reproducibility could be explained in several ways. It is possible that the extent to which polymerizing component was denatured during isolation varied from one preparation to another, as has been observed for other membrane proteins. It is also possible that the preparations contained varying amounts of an inhibitor or varying amounts of one or more cofactors required for polymerization. These latter possibilities appeared less likely because the addition of a preparation of low specific activity to one of high specific activity did not decrease the polymerizing capacity more than that expected by the dilution involved.

Analyses of the 0.1 M salt fractions rich in polymerizing activity showed that they contained a number of different proteins. In SDS-polyacrylamide gel electrophoresis at least eight bands were obtained with mobilities corresponding to Mw = 10,000 to 200,000 (Fig. 2, gel a). The major 60,000 dalton band was identified as disulfide interchange enzyme by comparison of the gel patterns obtained before and after the fractions were absorbed with anti-enzyme antibody. Various methods were applied to isolate polymerizing material from these fractions and thus identify the responsible protein among the remaining components. Most of the procedures were unsuccessful. Polymerizing activity could not be selectively eluted by ion exchange chromatography on SE-Sephadex at pH 6 or DEAE-Sephadex at pH 6.5. It was not bound to Sepharose coupled with the lectins concanavalin A, rcin, soybean agglutinin, peanut agglutinin, wheat germ agglutinin, horseshoe crab agglutinin, or Ulex europaeus agglutinin. The activity was bound by the hydrophobic resin phenyl-Sepha-

![Fig. 2. SDS-gel electrophoresis of polymerizing material. a, after DEAE-Sephadex chromatography; 20 μg of a fraction high in polymerizing activity were electrophoresed on a 7% polyacrylamide-SDS cylindrical gel; marker proteins (gel not shown) included IgG, Mw = 150,000, bovine serum albumin, Mw = 68,000, ovalbumin, Mw = 43,000; concanavalin A, Mw = 25,000, ε-h, after sucrose gradient centrifugation; 20 μl of the pooled fractions shown in Fig. 3 were electrophoresed on a 10% polyacrylamide-SDS slab gel. c, fractions 10–12; d, fractions 13–15; e, fractions 16–18; f, fractions 19–21; g, fractions 22–24; h, fractions 25–27. The micrograms of protein/track were 0.2, 0.6, 2.6, 2.8, 1.0, and 0.2, respectively. b, Control of disulfide interchange enzyme purified from mouse liver.rose, but strong detergent was required to release the activity and the elution was not reproducible.

A further purification could be achieved, however, by sucrose gradient centrifugation of the fractions high in polymerizing activity. As illustrated in Fig. 3, the polymerizing material sedimented more rapidly than disulfide interchange enzyme to give a clean separation of the two activities. It was calculated from these data that the polymerizing component had a sedimentation coefficient of 4.2 S compared to the value of 3.26 S determined for disulfide interchange enzyme (16). When the protein content of the sucrose gradient fractions was examined by SDS-polyacrylamide gel electrophoresis, the pooled fractions containing most of the polymerizing activity (fractions 22–24), exhibited only a single band which had an apparent Mw = 57,000 (Fig. 2, slot g), and thus ran slightly ahead of disulfide interchange enzyme (Fig. 2, slots b and e). It was unlikely that this band represented the polymerizing protein because a band of similar mobility and greater staining intensity was observed in the adjacent sucrose gradient fraction that had negligible polymerizing activity (Fig. 2, slot f).

Thus, the polymerizing component appeared to be present in such low concentration that it was not detected under the conditions used for the gel electrophoresis.

**Characterization of the Polymerization Reaction—**The polymerizing ability of the sucrose gradient-purified material was assessed by incubating increasing concentrations with reduced IgM and J chain and quantitating the reaction products by SDS-agarose-polyacrylamide gel electrophoresis. From densitometer tracings it was found that concentrations of 5 and 20 μg/ml converted 43% and 91%, respectively, of the IgM to pentamer. Similar results were obtained when the reaction mixtures were analyzed by sucrose gradient centrifugation (Fig. 4). In those mixtures containing 5 and 20 μg/ml of polymerizing fraction, 36% and 93%, respectively, of the IgM sedimented in the same position as a 19 S pentamer standard and the remainder sedimented as 8 S monomer. SDS
gel electrophoresis of these products (Fig. 5) confirmed that they were covalently linked structures. In contrast, in the absence of polymerizing material less than 10% of the IgM was in the polymer form and 91% sedimented in the 8 S position. Analyses of the latter peak on SDS gel electrophoresis revealed that the material was comprised predominantly of noncovalently associated half-monomers. From these measurements it was estimated that sucrose gradient centrifugation provided a 10-fold enrichment in polymerizing material. The concentration of crude extract required for 50% polymerization of IgM ranged from 50 to 100 μg/ml, whereas after sucrose gradient centrifugation the 50% endpoint was achieved with concentrations an order of magnitude lower.

The fidelity of the pentamer IgM product was ascertained not only from its sedimentation behavior, but also from measurements of J chain content and complement-fixing activity. For the J chain determinations, the pentamer and monomer peaks isolated by sucrose gradient centrifugation were reduced and alkylated and the J chain released was quantitated by radioimmunoassay. The results are summarized in Table I. The pentamer product was found to have 1.5% of its protein as J chain in good agreement with the value of 1.6% obtained for IgM pentamer synthesized in vivo. As expected, the monomer IgM fractions off the sucrose gradient contained negligible amounts of J chain, less than 0.1% of the total protein present. For the complement fixation determinations, the polymerization reaction was carried out using the IgM monomers and J chain from the dextran binding MOPC 104E protein (17). The products of the reaction were then separated by sucrose gradient centrifugation and the isolated monomers and pentamers were assayed for their ability to fix complement in the presence of an optimal amount of B1355 dextran. As shown in Table I, the pentamer product was highly active; the amount required for 50% fixation, 80 ng, was equivalent to that of in vivo synthesized IgM. In contrast, the monomer IgM product was found to be incapable of fixing complement even when amounts as large as 2 μg were tested. This observation held

FIG. 3. Sucrose gradient centrifugation of polymerizing material and disulfide interchange enzyme. Five hundred μg of polymerizing material recovered from the DEAE-Sephadex column and 60 μg of purified disulfide interchange (DSI) enzyme were layered on separate 5-25% sucrose gradients and centrifuged for 44 h at 39,000 rpm. Fractions of 0.3 ml were analyzed individually for disulfide interchange activity and in pools of three for polymerizing activity (PE). 125I-Insulin was included in each gradient to verify that the gradients sedimented and fractionated in the same fashion.

FIG. 4. Sucrose gradient centrifugation of the products of in vitro polymerization. Reduced IgM and J chain, incubated with either (a) 0, (b) 5, or (c) 20 μg/ml of polymerizing component were layered on a sucrose gradient and centrifuged. The gradients were fractionated and the absorbance at 278 nm was determined. The amount of polymerizing component added did not contribute significantly to the absorbance. The positions of purified IgG and pentamer IgM are indicated by the arrows labeled 7 S and 19 S, respectively. ML, milliliters.

FIG. 5. SDS gel electrophoresis of the products of in vitro polymerization after separation by sucrose gradient centrifugation. a, IgM monomer fraction from gradient a in Fig. 4; b and e, IgM monomer and IgM pentamer fractions from gradient b in Fig. 4; c and d, ascending and descending limbs of IgM pentamer fraction from gradient c in Fig. 4; f, control pentamer.

| TABLE 1 | J chain content and complement fixing activity of repolymerized IgM |
|----------|---------------------------------------------------------------------|
|          | J chain content* | Complement fixing activity* |
| IgM      | % | ng |
| Control pentamer IgM | 1.6 | 87 |
| Repolymerized pentamer IgM | 1.5 | 80 |
| Monomer IgM | 0.1 | >1000 |

"J chain was measured by radioimmunoassay and the results are expressed as the percentage of protein which is J chain. The results are the averages of 3 separate determinations.

4 Complement fixing activity was measured as described under "Materials and Methods" and the results are expressed as the amount of protein required to fix 50% of the complement under the conditions of the assay. The results are the averages of 3 separate determinations.
true whether the IgM monomer was isolated from reaction mixtures with polymerizing protein or those without.

The kinetics of the polymerization reaction were investigated by incubating 20 \( \mu \)g/ml of polymerizing material with IgM monomers and J chain and then following the formation of polymer product as a function of time. After 2 min of reaction, no polymer could be detected by SDS-agarose-polyacrylamide gel electrophoresis (Fig. 6A); after 5 min dimer and trimer intermediates appeared; pentamer product was first observed after 10 min and the amount formed reached a plateau at 40 min. When the polymer yields were determined and plotted versus time, the curve in Fig. 6B was obtained. It can be seen that the percentage of polymerized IgM increased linearly from a value of 12 at 5 min to a value of 47 at 20 min.

The temperature dependence of the reaction was determined by allowing the polymerizing material to react with IgM monomers and J chain for 60 min at 0, 22, 37, and 46 °C. There was no detectable pentamer formed at 0 °C; at 22 °C the reaction was 90% complete, and at 37 and 46 °C the extent of polymerization was essentially the same.

Mechanism of Polymerization—Polymerizing component could induce the formation of IgM intersubunit bonds by either of two mechanisms, disulfide interchange or sulfhydryl oxidation. The possibility of an interchange mechanism appeared less likely in view of the results obtained with disulfide interchange enzyme. The enzyme is known to catalyze disulfide bond rearrangement in insulin and randomly oxidized ribonuclease (18); yet in IgM assembly it is active only as a nonspecific protein primer (9, 10). Moreover, a second interchange system, mixtures of reduced and oxidized glutathione (19), was found to be completely ineffective in IgM polymerization (Fig. 7). No intersubunit bond formation was detected although the ratio of oxidized to reduced glutathione in the polymerization mixture was varied from 1:5 to 1:80 and the total glutathione concentration was in 10⁴ molar excess over monomer.

The possibility of an oxidative mechanism was suggested by recent findings that a metalloprotein can catalyze the oxidation of sulfhydryl groups in proteins (20) and known oxidizing agents, such as Cu²⁺, can promote disulfide bond formation in IgM (21). This possibility was investigated by determining the effect of chelating agents on the activity of polymerizing component. As the data in Table II show, the assembly of pentamer IgM was inhibited when the reaction was carried out in the presence of EDTA; concentrations as
low as 100 µm blocked polymerization. As a control for non-specific effects, two additional chelating agents, 1,10-phenanthroline and 8-hydroxyquinoline, were also tested (Table II). The inhibition obtained by all three agents indicated that polymerizing component requires metal ion in order to function. Furthermore, polymerizing component was significantly more effective than free metal ions at promoting IgM polymerization (Table III). Of the ions tested, Cu²⁺ had the highest activity, but molar concentrations 10 times that of polymerizing component were required to achieve an equivalent extent of polymerization and the products of the reaction were more heterogeneous. Zinc and Fe²⁺ were found to be active only at much higher, nonphysiological concentrations and Ca²⁺, Mn²⁺, and Mg²⁺ were without effect. These results suggested that polymerizing component might be a copper-binding protein.

**Discussion**

On the basis of the data presented in this paper, polymerizing component was identified as a lymphocyte-specific enzyme that catalyzes the in vivo assembly of pentamer IgM antibody. The presence of polymerizing activity in lymphocyte extracts has been reported previously, but the activity was attributed to disulfide interchange enzyme (22). Our studies show that the formation of IgM intersubunit bonds is the property of a molecule distinct from disulfide interchange enzyme. Although polymerizing enzyme and disulfide interchange enzyme are both membrane-bound proteins soluble in 50% ammonium sulfate, polymerizing enzyme was found to have a lower net negative charge and a more rapid sedimentation rate. Advantage was taken of these differences to separate the two enzymes and thus obtain a highly enriched preparation of the polymerizing protein.

Our studies did not, however, succeed in completely purifying polymerizing enzyme from other cell constituents so that its molecular weight and amino acid composition could be determined. Similar difficulties have been encountered with other membrane-bound proteins present in low concentrations. From the protein content of the most highly purified enzyme preparation and the assumption of an overall yield of 10%, it was estimated that the plasmacytoma cell contains less than 10⁷ molecules of polymerizing enzyme. On this basis, the theoretical enzyme content of 100 tumors, each containing 10⁹ cells, would be only 100 µg. Thus further characterization of polymerizing enzyme will require a large scale preparation or the use of different approaches, such as cloning.

Although polymerizing enzyme has yet to be purified, its enzymatic behavior was established by analyses of the sucrose gradient-purified material. In the in vitro polymerization assay 0.3 to 0.5 µg of the enzyme preparation was found to effect 50% polymerization of 25 µg of monomer IgM and J chain. It was calculated from these data and an assumed M₀ = 70,000 for polymerizing enzyme that the 50% endpoint was achieved with less than 1 µmol of enzyme/10 µmol of monomer and 2 mol of J chain. This measure of catalytic activity represents a minimum value since all the protein present in the sucrose gradient fraction was assumed to be polymerizing enzyme.

By comparing the activity of polymerizing enzyme with that of agents known to promote disulfide bonding, it was also possible to obtain information on the mechanism of polymerizing enzyme action. Of the agents tested in the in vitro polymerization assay, only Cu²⁺ exhibited a catalytic activity approaching that of polymerizing enzyme preparation. Other divalent cations and disulfide interchange systems either failed to induce pentamer IgM assembly or did so at very high noncatalytic concentrations. The similarity in the behavior of polymerizing enzyme and Cu²⁺ suggested that polymerizing enzyme might be a sulfhydryl oxidase. This deduction was supported by the finding that the activity of polymerizing enzyme is inhibited by three different metal-chelating agents. Both sulfhydryl oxidases previously described (20, 23) have been shown to be metalloproteins. On the basis of these preliminary data, it would appear that the polymerization of IgM proceeds by the direct oxidation of monomer and J chain sulfhydrs.

Evidence that the enzyme functions in the in vitro synthesis of pentamer IgM was obtained from analyses of the products of in vitro polymerization. The enzyme was shown to be capable of synthesizing a pentamer molecule that was indistinguishable from its in vivo counterpart. The in vitro product contained the correct amount of J chain, i.e. 1 mol/5 mol of monomer. It co-sedimented with pentamer synthesized in vivo on a sucrose gradient run under non-denaturing conditions, electrophoresed in the same position on a SDS-agarose-polyacrylamide gel, and could fix complement to the same extent. The ability of pentamer IgM to fix complement is a particularly sensitive indicator of the secondary, tertiary, and quaternary structure of the molecule.

The kinetics of polymerization were also consistent with a role for polymerizing enzyme in in vivo IgM synthesis. Analyses of the in vitro rate of polymerization showed that the reaction goes to 50% completion in 12 min. A comparable rate of polymerization in vivo would not be unreasonable considering that the transit time of IgM from the ribosome to the external medium has been shown to be 1 to 2 h (24). In addition it is likely that the reactants are present in higher concentration at the cellular sites of IgM assembly and thus polymerization would proceed more rapidly. When the molarity of intracellular monomer and J chain was calculated from measurements of the diameter of IgM-secreting plasmacytomas cells and their IgM and J chain contents (25), the concentration of monomer was found to be essentially equivalent to that used in the in vitro assay, 2 x 10⁻⁶ µM, and the concentration of J chain was 5-fold higher. This calculation underestimates the internal concentrations, perhaps as much as a factor of 10, because it does not take into account the limited distribution of the reactants within the cell and any effect of compartmentalization.

Finally, the tissue distribution of polymerizing enzyme supported its role in in vivo IgM assembly. The enzyme was found in significant quantities only in cells differentiated to Ig secretion. It was absent from liver cells and present at low levels, if at all, in a lymphoma line representative of an unstimulated B cell. These data indicate that the enzyme serves the specific function of catalyzing the disulfide cross-linking required for IgM and IgA polymerization. Moreover, the data suggest that the synthesis of polymerizing enzyme, like that of J chain (10, 25), may be induced during antigen triggering of B lymphocytes.

| Metal Ion | Concentration | IgM polymerization % |
|----------|---------------|----------------------|
| CuCl₂    | 1 mM          | 55                   |
| CuCl₂    | 2 µM          | 70                   |
| CuCl₂    | 0.5 µM        | 15                   |
| FeCl₃    | 1 mM          | 1.5                  |
| ZnCl₂    | 1 mM          | 5                    |
| MnCl₂    | 1 mM          | 0                    |
| MgCl₂    | 1 mM          | 0                    |
| CaCl₂    | 1 mM          | 0                    |
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