Effect of Covalent Attachment of Polyethylene Glycol on Immunogenicity and Circulating Life of Bovine Liver Catalase*

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Methoxypolyethylene glycols of 1900 daltons (PEG-1900) or 5000 daltons (PEG-5000) were covalently attached to bovine liver catalase using 2,4,6-trichloro-s-triazine as the coupling agent. Rabbits were immunized by the intravenous and intramuscular routes with catalase modified by covalent attachment of PEG-1900 to 43% of the amino groups (PEG-1900-catalase). The intravenous antiserum did not yield detectable antibodies against PEG-1900-catalase or native catalase, as determined by Ouchterlony and complement fixation methods, whereas the intramuscular antiserum contained antibodies to both PEG-1900-catalase and catalase. PEG-1900 did not react with either antiserum. Catalase was prepared in which PEG-5000 was attached to 40% of the amino groups (PEG-5000-catalase). This catalase preparation did not react with either antiserum. PEG-1900-catalase retained 93% of its enzymatic activity; PEG-5000-catalase retained 95%. PEG-5000-catalase exhibited enhanced circulating lives in the blood of acatalasemic mice during repetitive intravenous injections. No evidence was seen of an immune response to injections of the modified enzymes. Mice injected repetitively with PEG-5000-catalase remained immune competent for unmodified catalase, and no evidence of tissue or organ damage was seen.

We report in a previous paper (1) that covalent attachment of polyethylene glycol to bovine serum albumin renders the protein incapable of eliciting antibody to itself or unmodified albumin.† PEG-albumin shows extended circulating life in the blood. In this paper we report the covalent attachment of PEG to bovine liver catalase, and studies on the enzymic properties, immunogenicity, and blood circulating life of the modified enzyme.

EXPERIMENTAL PROCEDURES

Bovine liver catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) was obtained from Sigma Chemical Co. Cyanuric chloride was obtained from Aldrich Chemical Co. and was recrystallized twice from anhydrous benzene immediately before use. Monomethoxypolyethylene glycol systems of 1900 and 5000 daltons (Carbowax 2000 and 5000) were supplied by Union Carbide. Trypsin, chymotrypsin, and protease (from Streptomyces griseus) were obtained from Sigma.

Acatalasemic mice were the kind gift of Dr. Robert N. Feinstein, Argonne National Laboratory. The preparation of 2-O-methoxypolyethylene glycol-4,6-dichloro-s-triazine ("activated PEG") has been described (1). Protein was determined by the biuret procedure, and primary amino groups according to Habeeb (2).

Attachment of Activated PEG to Catalase – Reactions were carried out at 4°. To 500 mg of catalase in 50 ml of 0.1 m sodium tetraborate, pH 9.2, was added with constant stirring 4.3 g of activated PEG-1900, an amount 10-fold in excess of available amino groups. The solution was maintained at pH 9.2 by means of a pH-stat (Radiometer, Copenhagen). After 1 h, unattached PEG-1900 was removed by dialysis against 5.01 m phosphate, pH 7.3, using an Amicon ultrafiltration apparatus and the XM-50 membrane. Catalase was reacted in a similar manner with a 10-fold excess of activated PEG-5000. PEG-1900-catalase showed a 43% modification of amino groups by attachment of PEG, with retention of 95% of the original enzymic activity, while PEG-5000-catalase had a 40% modification of amino groups, and 95% activity.

A series of PEG-1900-catalase preparations was made in which increasing fractions of the amino groups were substituted with PEG-1900. Treatment of catalase with activated PEG-1900 in molar amounts equal to 1, 3, and 7 eq of the available amino groups resulted in products with 33, 19, and 37% of the amino groups substituted by PEG-1900, respectively.

Assay Methods – Catalase and PEG-catalase preparations were assayed according to the method of Beers and Sizer (3) in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. Blood catalase was assayed by the procedure of Feinstein (4). Activity is expressed as perborate units (millimoles of perborate oxidized in 5 min).

Immunological Procedures – Antisera were prepared against catalase and PEG-1900-catalase in New Zealand White, adult female rabbits as described earlier for native and modified bovine serum albumin. Activity is expressed as perborate units (millimoles of perborate oxidized in 5 min).

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The abbreviations used are: albumin, bovine serum albumin; PEG-1900, monomethoxypolyethylene glycol of 1900 daltons; PEG-5000, monomethoxypolyethylene glycol of 5000 daltons. PEG-1900-catalase, the catalase preparation with PEG-1900 attached covalently to 43% of its amino groups; PEG-5000-catalase, catalase with PEG-5000 attached to 40% of the available amino groups. When
Attachment of Polyethylene Glycol to Bovine Liver Catalase

The antisera were tested in gel diffusion slides for in vitro precipitating activity. Slides (1.0% agarose in 0.01 M phosphate-buffered saline, pH 7.3, with 1.0% sodium azide) were incubated overnight at room temperature.

Complement fixation (5) was performed using antisera dilutions of 1/100 and 1/200 and antigen concentrations of 100 to 0.39 pg/ml.

In Vivo Circulation Studies—A catalesemic mouse was injected three times weekly at 2-, 2-, and 3-day intervals in the tail vein with PEG-1900-catalase, or PEG-5000-catalase, for a period of 90 days. The circulating life of injected catalase was measured at 1, 30, 60, and 90 days (immediately following the 1st, 13th, 26th, and 39th injection).

Proteolytic Digestion—Catalase or PEG-5000-catalase, 1.6 mg of either, was digested at room temperature with 5 mg of trypsin, 5 mg of chymotrypsin, or 10 mg of protease in a total volume of 1 ml. Aliquots were taken at various time intervals and assayed spectrophotometrically for activity. The relatively large amounts of enzymes were used because of resistance of PEG-5000-catalase to digestion.

RESULTS

pH Optima of Catalase and PEG-1900-Catalase—The activity of PEG-1900-catalase appears to be quite similar to that of native catalase (Fig. 1). At the extremes of pH (4.5 to 6, 8 to 9.5), PEG-1900-catalase retains somewhat greater activity, which suggests a slight resistance to denaturation. Exposure to pH 12 for 1 min at 20°C caused complete inactivation of both catalase and PEG-1900-catalase. At this pH, catalase dissociates into subunits (6).

Effect of Modification of Thermal Stability—The stabilities of catalase and PEG-catalase were tested by two methods. The first method involved holding the enzymes at specific temperatures for 5 min, followed by rapid cooling to room temperature, and assay. The results, shown in Fig. 2, indicate that both catalase and PEG-1900-catalase begin to denature at about 65°C. PEG appears to have no effect on stabilization to high temperatures. By contrast, Marshall and Rabinowitz (7) report that covalent attachment of dextran to catalase yields a dextran-catalase conjugate that is more resistant to heat denaturation than native catalase.

In the second method, catalase and PEG-5000-catalase were assayed at various temperatures by the perborate method (Fig. 3). Catalase shows maximum activity at a temperature of about 40°C, which is several degrees higher than that shown by PEG-5000-catalase. This suggests that PEG attachment causes some destabilization of the catalase structure. A second point of interest is the increase in activity of both catalase and PEG-5000-catalase as the temperature approaches zero. Such behavior may be characteristic of the perborate assay. Published rate constants for the decomposition of hydrogen peroxide as a function of temperature do not show this change (8).

Proteolytic Digestion of Catalase and PEG-Catalase—Catalase incubated with trypsin showed a rapid decrease in activity, with total loss of activity after 40 min (Fig. 4A). This is in agreement with published results (9). PEG-5000-catalase, conversely, lost activity very slowly. After 150 min, 90% of the original activity remained. Chymotrypsin did not inactivate catalase as rapidly as trypsin (Fig. 4B). After 60 min, 30% activity was lost. PEG-5000-catalase was virtually unaffected, retaining 98% activity. Catalase lost 90% of its activity upon digestion by Streptomyces griseus protease for 60 min (Fig. 4C), while PEG-5000-catalase lost 20% of its activity. This protease has a wide specificity (10), which may account for its comparatively greater activity against PEG-5000-catalase than trypsin and chymotrypsin.

Effect on Antibody Production of Covalent Attachment of Polyethylene Glycol to Catalase—Initial experiments were performed using PEG-1900. Catalase was modified to varying degrees (13, 19, 37, and 43%) with PEG-1900. These preparations were used to immunize rabbits by either intravenous or intramuscular injection.

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**Fig. 1 (left).** Effect of pH on the activity of catalase and PEG-1900-catalase. Five microliters of a 3 mg/ml solution of either catalase (open symbols) or PEG-1900-catalase (closed symbols) was diluted in 100 ml of the appropriate buffer and immediately assayed by the spectrophotometric method. O or ●, 0.05 M sodium acetate; △ or △, 0.05 M sodium phosphate; □ or ■, 0.05 M sodium borate.

**Fig. 2 (right).** Thermal stability of catalase and PEG-1900-catalase. Samples (1 mg of protein/ml) of catalase or PEG-1900-catalase in 0.01 M phosphate, pH 7.3, were placed in a water bath of specific temperature. After 10 min, the samples were cooled in an ice bath to room temperature and assayed spectrophotometrically. ○, catalase; △, PEG-1900-catalase; ●, values coincide.
By the intravenous route, native catalase elicited a strong immune response (Table I). Anticatalase antiserum reacted with all of the PEG-1900-catalase preparations. Greatest reaction was seen with native catalase and the least with PEG-1900-catalase (45%). Evidently a 45% modification of the amino groups of catalase with PEG-1900 was not sufficient to inhibit the antigen-antibody reaction.

Anti-PEG-1900-catalase (13%) antiserum showed a decrease in antibody production, and the antibody present reacted only with native catalase and 13% and 19% modified PEG-1900-catalase. Antiserum to PEG-1900-catalase (19%) was quite similar to antiserum to PEG-1900-catalase (37%). The reaction with native catalase was weak and one animal from either group did not produce antibody capable of reacting with the modified catalases. There was no detectable reaction between antiserum to PEG-1900-catalase (43%) and any of the antigens.

These results indicate that attachment of PEG-1900 to 43% of the amino groups yielded an adduct that did not elicit antibody production by the intravenous route of administration. When immunization was carried out by the intramuscular route, however, all of the PEG-1900-catalase preparations elicited an immune response. This may be due to denaturation of the enzyme during the homogenization of antigen with adjuvant, and exposure of antigenic determinants, or simply due to the use of adjuvant, which enhances the response. All of the intramuscularly derived antisera reacted with PEG-1900-catalase (43%). Intramuscularly derived antisera were tested against PEG-5000-catalase. As shown in Fig. 5, PEG-5000-catalase did not react with either anticalase antiserum or anti-PEG-1900-catalase (43%) antiserum.

**Enzyme Replacement Therapy in Acatalasemic Mice—**

**Table I**

*Antisera were prepared in rabbit pairs by the intravenous injection of catalase, or catalase to which increasing amounts of PEG-1900 were attached. Center wells contained 10 μl of antiserum and peripheral wells contained 10 μl of a 1 mg/ml solution of catalase or modified catalase in 0.01 M phosphate-buffered saline, pH 7.3. Results are expressed as estimated relative amount of precipitation for each antiserum-antigen pair. Tests were carried out in quadruplicate. ++ + indicates greatest precipitation; -- indicates no observable precipitation.*

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*Results checked by complement fixation.*
attachment of a linear, flexible, uncharged hydrophilic polymer to available but nonessential groups on an enzyme might provide a shell around the enzyme that covers antigenic determinants and, by presenting a flexible, unbranched, hydrophilic surface for inspection by the immune processes, prevent recognition of the interior enzyme as a foreign substance against which an immune response would be provoked. At the same time, the shell would be permeable to the smaller substrates so that enzymatic activity could continue.

PEG was selected for covalent attachment because of its nonimmunogenicity and compatibility with blood (13, 14) and because it best fits the criteria we selected. Dextran, another promising polymer, was rejected because of its known immunogenicity in humans (15, 16). The monomethoxypolyethylene glycols offer the additional advantage of having a single terminal hydroxyl group for activation or modification for coupling purposes.

Catalase has a molecular weight of 242,000 and contains 108 lysine residues (17). The attachment of PEG-1900 to 43% of the free amino groups yields an adduct of about 335,000 daltons. Attachment of PEG-5000 to 40% of catalase amino groups approximately doubles the size of the adduct. These are minimum values, as other groups on the enzyme also may have reacted with activated PEG during the coupling process. The slight decrease in enzymatic activity exhibited by the modified catalases indicates that small molecules have little difficulty penetrating the PEG layer that presumably surrounds the enzyme.

The ease with which experimental animals tolerate repetitive injections of PEG-catalase over extended periods suggests a future for PEG-enzymes in enzyme therapy. Preliminary work in our laboratory has shown that several other enzymes can be modified by PEG attachment without excessive loss of activity and, in the case of one enzyme, uricase, which is being used in clinical trials.

**DISCUSSION**

A major objective of the work reported in this study was to develop procedures for reducing or eliminating the immunogenicity of proteins and, in the case of enzymes, also to retain reasonable activity. Good immunogens typically have a rigid, complex surface structure to which antibodies can be made. We rationalized that the covalent attachment of a linear, flexible, uncharged hydrophilic polymer to available but nonessential groups on an enzyme might provide a shell around the enzyme that covers antigenic determinants and, by presenting a flexible, unbranched, hydrophilic surface for inspection by the immune processes, prevent recognition of the interior enzyme as a foreign substance against which an immune response would be provoked. At the same time, the shell would be permeable to the smaller substrates so that enzymatic activity could continue.

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**FIG. 7.** Blood circulating life of catalase (x) and PEG-5000-catalase (O). Twenty animals were used in each group; individual points represent the average of four animals. Otherwise, conditions are similar to those given in Fig. 6. Vertical bars show the range of values; circles give the mean.
tested by repetitive injections as described in this paper, we also see extended blood circulating life and absence of apparent immunological effects.

If, by PEG attachment, a substantial percentage of enzymes can be rendered apparently nonimmunogenic and capable of extended circulating life while retaining activity, the way seems opened for large scale expansion of enzyme therapy. For example, enzymes from diverse and inexpensive sources may be used. The investigator, free of concern for adverse immunological effects, designs experiments related to the particular metabolic aspects of the clinical problem with which he or she is dealing. Long term enzyme therapy would seem routine. For alteration of blood metabolites, PEG-enzymes might be injected directly into the blood stream; for storage diseases, incorporation of PEG-enzymes into liposomes (17, 18) or erythrocytes (19, 20) for eventual uptake into lysosomes would seem feasible. PEG-enzymes may prove to be resistant to degradation by lysosomal proteases, as PEG-catalase is to trypsin, chymotrypsin, and S. griseus protease.

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