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PROPERTIES OF TWO URATE OXIDASES MODIFIED BY THE COVALENT ATTACHMENT OF POLY(ETHYLENE GLYCOL)

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Poly(ethylene glycol) of 5 000 daltons has been attached covalently to preparations of urate oxidase (urate oxygen oxidoreductase, EC 1 7 3 3) from hog liver and *Candida utilis* Attachment of sufficient poly(ethylene glycol) to either urate oxidase renders the enzyme incapable of eliciting antibody production in mice, or of reacting with antibodies to the unmodified enzyme The poly(ethylene glycol) urate oxidase conjugates exhibit higher K_m and lower V values than the unmodified urate oxidases Optimal pH values are increased for the poly(ethylene glycol) urate oxidases, and optimal temperatures are decreased The blood circulating lives of the modified urate oxidases following intravenous injection are much longer than those of the unmodified urate oxidases, repetitive injections over a period of 90 days did not alter the blood circulating lives of the poly(ethylene glycol) urate oxidases The unmodified enzymes, on the other hand, were cleared from the blood with extreme rapidity after a few intravenous injections

Introduction

The covalent attachment of increasing amounts of poly(ethylene glycol) to bovine serum albumin [1], bovine liver catalase [2] and bovine liver arginase [3] produces in each instance a decreasing response of the poly(ethylene glycol) protein conjugate to antibodies to the unmodified protein When sufficient poly(ethylene glycol) is attached, the conjugates no longer interact with these antibodies, and on repetitive intramuscular or intravenous injections fail to elicit immune responses The blood circulating lives of poly(ethylene glycol) enzymes are greatly extended over those of the unmodified enzymes, the conjugates retain adequate enzymic activities

These findings suggest a use for poly(ethylene glycol) enzyme conjugates in therapeutic applications Major drawbacks at this time to enzyme therapy are the cost and limited availability of human enzymes which are used to avoid the problem of immunogenicity, the short circulating lives of unmodified enzymes, whether of human origin or not [4], and the almost invariable development of antibodies against exogenous enzymes [5], which enhances their clearance rates and may produce adverse immune responses Various methods for circumventing the immune response have been devised These include enclosure of the enzyme in semipermeable microcapsules that can either be injected [6] or incorporated in an extracorporeal shunt [7], the trapping of enzymes in membranes for use in extracorporeal shunts [8], the enclosure of enzymes in erythrocytes [9] and liposomes [10], and the attachment of other hydrophilic polymers such as dextran [11], poly(vinylpyrrolidone) [12] and glycopeptides [13]

Urate oxidase (urate oxygen oxidoreductase, EC 1 7 3 3) catalyzes the oxidation of urate to

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allantoin urate + O_2 → CO_2 + allantoin In this paper, we report the properties of urate oxidase preparations from hog liver and *Candida utilis* that have been modified by covalent attachment of poly(ethylene glycol) Our objectives are to extend our studies on the general applicability of the modification procedure, and to determine if the same enzyme from different sources can be successfully modified by poly(ethylene glycol) attachment Urate oxidase has been employed therapeutically in the treatment of hyperuricemia and gout [14–17]

Experimental procedure

Uric acid was obtained from Fisher Scientific Company Trinitrobenzenesulfonic acid was purchased from the Nutritional Biochemical Corporation Monomethoxypoly(ethylene glycol) of 5 000 daltons was obtained from Union Carbide 2-*O*-Methoxypoly(ethylene glycol)-4,6-dichloro-*s*-triazine (activated poly(ethylene glycol)) was synthesized as described earlier [1] Hog liver urate oxidase was purchased from United States Biochemical Corporation (catalog no 23095) *C utilis* urate oxidase was a gift from the Toyobo Company, Ltd , Osaka, Japan

The urate oxidases were examined for heterogeneity by polyacrylamide disc-gel electrophoresis in the buffer system of Davis [18] Hog liver urate oxidase showed one major band and one minor band and was estimated to be 93% pure *C utilis* urate oxidase was a highly purified preparation that showed a single band

Blood urate oxidase 4 mg uric acid in 100 ml 0 1 M $NaBO_2/H_3BO_3$ (pH 8 5) was prepared fresh daily 0 10–0 15 ml blood was collected from the eye orbit of the mouse and the serum diluted 1 5 (v/v) with water The assay mixture contained 0 2 ml urate/0 05 ml 0 2 M borate buffer (pH 8 5)/0 05 ml diluted serum The mixture was swirled rapidly in a small test tube for a few seconds, then pipetted quickly into a microcuvette maintained at 37°C The decrease in absorbance at 290 nm was followed in a Gilford spectrophotometer equipped with a recorder The conversion of 1 μmol/min of urate to allantoin constitutes 1 unit of activity The assay is linear and proportional to enzyme concentration

Covalent attachment of poly(ethylene glycol) to urate oxidase Reactions were carried out at 4°C The procedure described previously by Abuchowski et al [1], was followed for *C utilis* urate oxidase 450 mg activated-poly(ethylene glycol), an amount in 3-fold molar excess of amino groups, were added with rapid stirring to 50 mg urate oxidase which were dissolved in 10 ml 0 1 M borate buffer (pH 9 5) The pH was maintained at 9 5 by means of a pH-stat (Radiometer) for 0 5 h After dialysis the enzyme was stored at 4°C and showed little loss of activity after 1 month

Hog liver urate oxidase is relatively insoluble below pH 10 4 50 mg enzyme were dissolved in 10 ml 0 1 M borate buffer (pH 10 4) 750 mg activated-poly(ethylene glycol), a 3-fold molar excess over amino groups, were added and the pH was maintained at 10 4 for 0 5 h, followed by dialysis against 0 1 M phosphate buffer (pH 7 35) (attachment of poly(ethylene glycol) solubilized the urate oxidase) The modified enzyme was stable at 4°C for at least a month

The amount of poly(ethylene glycol) attached to each enzyme was estimated by measuring the reduction in primary amino groups by the method of Habeeb [19] Protein concentration was measured by the biuret method [20] Poly(ethylene glycol) was found to be attached to 57% of the amino groups of *C utilis* urate oxidase with retention of 23% of the original activity, and to 58% of the amino groups of hog liver urate oxidase with retention of 28% of the original activity These preparations were used for the bulk of the studies reported here They appear to be non-immunogenic Other preparations of poly(ethylene glycol) urate oxidase were made using smaller ratios of activated-poly(ethylene glycol) to amines Although more active, these preparations showed evidence of immunogenicity When studies are reported on urate oxidase preparations with lesser percentages of amino group modifications, the percentage of modification is given in parentheses, e g , hog liver poly(ethylene glycol) urate oxidase (37%)

Immunological procedures Antisera were prepared against urate oxidase and poly(ethylene glycol) urate oxidase preparations from both hog liver and *C utilis* in male white mice Urate oxidase or poly(ethylene glycol) urate oxidase (0 38 mg protein in 0 13 ml buffer) was homogenized with 0 13

ml complete Freund's adjuvant and injected intraperitoneally Injections were repeated twice at weekly intervals The mice were bled 1 week after the third injection Antisera were stored at −20°C Gel diffusion plates [21] were prepared with 1% agarose in phosphate-buffered saline (pH 7 3), with 1% sodium azide Plates were incubated overnight at room temperature

In vivo circulating studies Four groups of 21 mice each were injected via the tail vein on a thrice-weekly schedule (2-, 2- and 3-day intervals) with 0 2 unit urate oxidase or poly(ethylene glycol) urate oxidase from *C utilis* or hog liver Immediately following the 1st, 13th, 26th and 39th injection, blood urate oxidase levels were assayed over a period of 48 h Sets of three mice from each group were used to obtain each time value

Results

Effect of covalent attachment of poly(ethylene glycol) on urate oxidase activity and immunogenicity Each of the urate oxidases shows decreasing activity with the attachment of increasing amounts of poly(ethylene glycol) (Fig 1) As modification approaches 60% of available amino groups, each of the poly(ethylene glycol) urate oxidases becomes incapable of eliciting precipitating antibodies when tested in mice or rabbits, or of reacting with antibodies to the unmodified enzyme No evidence of cross-reactivity was seen between the liver and yeast enzymes Fitzpatrick and co-workers also failed to find evidence of cross-reactivity between mammalian and fungal urate oxidases [22]

Changes in properties following modification Poly(ethylene glycol) attachment to both urate oxidases results in the following changes (1) a shift of pH optima to slightly higher values, (2) downward shift in temperature optima of several degrees, the shift being somewhat more pronounced for hog liver urate oxidase

Kinetic studies Kinetic parameters were determined by the use of varying substrate levels in the assay, and an amount of enzyme that gave ΔA_{290}

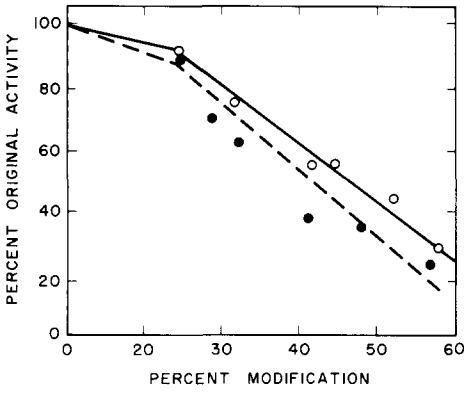

Fig 1 Effect on activities of attaching increasing amounts of poly(ethylene glycol) to hog liver and *C utilis* urate oxidase Enzymes were reacted with 0 5, 1 0, 1 5, 2 0, 2 5 or 3 0 mol activated-poly(ethylene glycol)/mol amino groups Percent modification increased with increasing ratios of activated poly(ethylene glycol) to amino groups Dashed line, filled circles, *C utilis* poly(ethylene glycol) urate oxidase, solid line, open circles, hog liver poly(ethylene glycol) urate oxidase

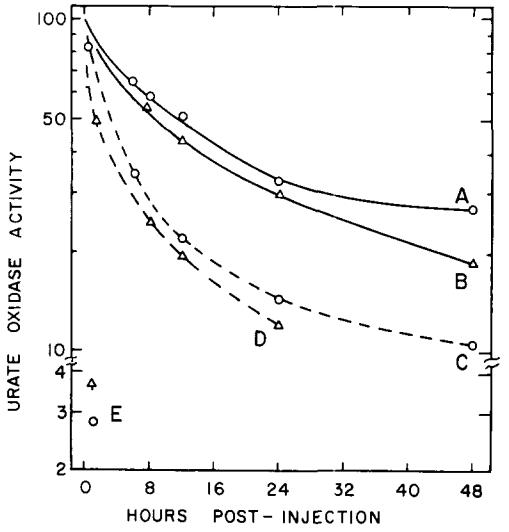

Fig 2 Blood circulating life, in the mouse, of hog liver poly(ethylene glycol) urate oxidase (37%) and poly(ethylene glycol) urate oxidase (47%) Six mice were used for each curve, and each point is the average of three mice Curves A and B are the circulating lives of poly(ethylene glycol) urate oxidase (37%, ○———○) and poly(ethylene glycol) urate oxidase (47%, △———△), respectively, following the first intravenous injection Curves C and D are the circulating lives of poly(ethylene glycol) urate oxidase (37%, ○———○) and poly(ethylene glycol) urate oxidase (47%, △———△), respectively, following the 8th injection on the 2-, 2-and 3-day schedule Curve E shows the rapid removal of poly(ethylene glycol) urate oxidase (37%, ○ ○) and poly(ethylene glycol) urate oxidase (47%, △ △) after 13 injections

of about 0 1/min under optimal substrate concentrations For hog liver urate oxidase, the native enzyme has a V of 8 2 units/mg and a K_m of 2 0 10^{-5} M The poly(ethylene glycol) urate oxidase has a V of 2 1 units/mg and a K_m of 6 9 10^{-5} M

For *C utilis* urate oxidase, the native enzyme has a V of 3 33 units/mg and a K_m of 5 0 10^{-5} M Poly(ethylene glycol) urate oxidase has a V of 0 47 units/mg and a K_m of 5 6 10^{-5} M Values were read from Eadie-Hofstee plots

Circulating life of poly(ethylene glycol) urate oxidases Fig 2 shows the blood circulating lives, in mice, of insufficiently modified poly(ethylene glycol) urate oxidase preparations from hog liver The two preparations (37 and 47% modified) show the characteristic extended blood circulating lives of poly(ethylene glycol) proteins following the first intravenous injection (curves A and B), but after a few additional injections on the thrice-weekly schedule the circulating lives show a drastic decrease as seen in curves C and D After a month of injections (curve E), the 37 and 47% modified-poly(ethylene glycol) urate oxidases are removed as rapidly as the unmodified hog liver urate oxidase (Fig 3B)

Completely different patterns of blood circulating life are seen with the most highly modified poly(ethylene glycol) urate oxidases from both hog liver and *C utilis* (Fig 3) Both the unmodified and modified enzymes circulate in the blood for many hours following the first injections, although the blood levels of the poly(ethylene glycol) urate oxidases are considerably higher (Fig 3A) However, with the standard injection schedule antibodies to the native urate oxidases appear within 2 weeks Fig 3B shows the circulating lives after 13 injections (30 days) The unmodified enzymes in each case are removed rapidly, while the highly-modified enzymes show circulating lives similar to those following the first injections Comparable results for poly(ethylene glycol) urate oxidases are seen after 60 days (Fig 3C) and 90 days (Fig 3D) Poly(ethylene glycol) urate oxidases from both sources appear to have approx identical circulating lives Antibodies to the unmodified or modified enzymes were not detectable after 90 days of injections of poly(ethylene glycol) urate oxidases

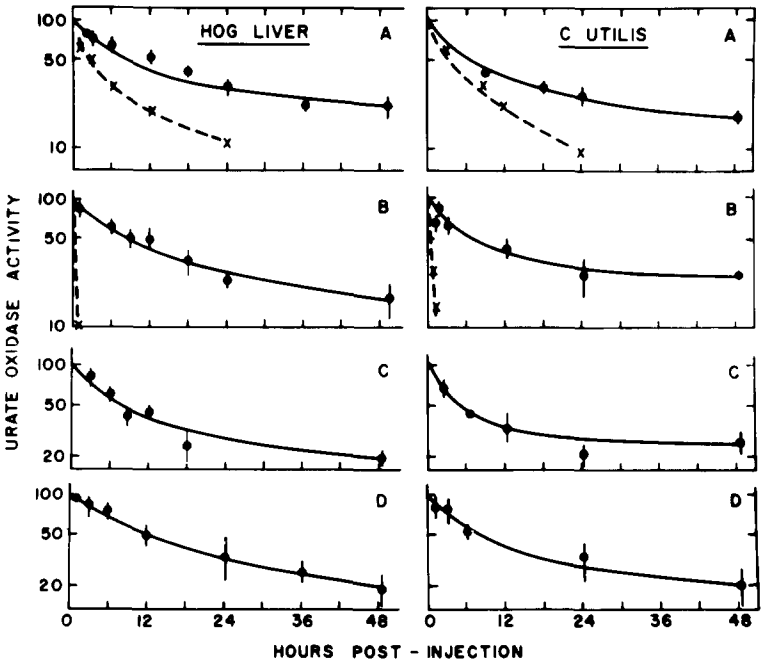

Fig 3 Blood circulating life in mice of unmodified urate oxidase and poly(ethylene glycol) urate oxidase from hog liver (left) and *C utilis* (right) Circulating life following the first (A), 13th (B), 26th (C) and 37th (D) injection •———•, poly(ethylene glycol) urate oxidase, X – – – X, unmodified urate oxidase

Repetitive injections of the poly(ethylene glycol) urate oxidases might have produced immunosuppression To test for this possibility, the mice previously injected with poly(ethylene glycol) urate oxidases for 90 days were injected intravenously with the respective unmodified urate oxidase for 1 month on the thrice-weekly schedule Antibodies to the urate oxidases were detectable within 2 weeks, accompanied by rapid removal from blood of the injected urate oxidases Immunosuppression appears to be ruled out by these findings

Discussion

The ability to modify both enzymes to the point of apparent non-immunogenicity while retaining reasonable activity and K_m values, encourages us to think that by using the poly(ethylene glycol) modification procedure it should be possible to select therapeutic enzymes on the basis of cost and desirable kinetic properties Microorganisms may prove to be the most practical source of many therapeutic enzymes because of the ease with which large quantities of a microorganism can be cultured, and the ability to increase enzyme yields by manipulation of culture conditions, selection of high-yielding mutant strains or recombinant DNA techniques

Our chief criterion for non-immunogenicity of poly(ethylene glycol) urate oxidases (and other poly(ethylene glycol) enzymes) is the unaltered blood circulating life of the modified enzyme following numerous injections over an extended period of time The gel immunodiffusion test as a criterion of non-immunogenicity may give misleading results, in that the enzymes can be modified with poly(ethylene glycol) to a point where they show little or no evidence of antigen-antibody reaction in the gel, but nevertheless elicit antibody production when injected intravenously

Poly(ethylene glycol) attachment increases the solubility of enzymes, and renders them soluble over an extended pH range Beef liver catalase, for example, is soluble at pH 1 after attachment of poly(ethylene glycol) [2] Similar effects have been observed with other enzymes and with poly(ethylene glycol) albumin [1] Solubilization by poly(ethylene glycol) attachment may find general use in the solubilization of enzymes or other proteins, whether for eventual therapeutic use or for other purposes where solubility is important

Urate oxidase was first used in animal studies in 1943 to decrease serum urate levels in the chicken [23] Numerous tests in humans have been conducted since 1956 using urate oxidase from both animal [14] and microbial [15–17] sources Extended treatment resulted in detectable immune responses

To overcome the immunogenicity problem, Venter et al [24] immobilized bacterial urate oxidase to glass beads which were incorporated in an extracorporeal shunt system in the dog Blood and urinary urate levels were reduced, and urinary allantoin was increased in short-term experiments There was little detectable loss of urate oxidase Ihler and co-workers [9] have incorporated hog liver urate oxidase into human erythrocytes and characterized some of the in vitro properties of the enzyme-loaded cells They foresee the possible use of urate oxidase-loaded erythrocytes in the treatment of urate-related diseases

The unchanged circulating lives of the poly(ethylene glycol) urate oxidases after many repetitive injections, and lack of detectable antibody formation, suggests a future for these enzyme conjugates in the treatment of hyperuricemia and gout Nishimura et al [25] report that attachment of poly(ethylene glycol) to 43% of the amino groups of *C utilis* urate oxidase yields a conjugate that has lost the ability to bind to anti-urate oxidase antiserum from rabbit This degree of modification would seem to be insufficient to prevent development of antibodies if the adduct were injected Experiments on the long-term effectiveness of poly(ethylene glycol) urate oxidases in lowering blood urate levels are currently underway

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