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Use of site-directed mutagenesis to enhance the epitope-shielding effect of covalent modification of proteins with polyethylene glycol

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ABSTRACT **Modification by covalent attachment of polyethylene glycol (PEG) can reduce the immunogenicity and prolong the circulating life of proteins, but the utility of this approach for any protein is restricted by the number and distribution of PEG attachment sites (e.g., ε-amino groups of lysine residues). We have developed a strategy for introducing additional sites for PEG attachment by using site-directed mutagenesis to selectively replace arginine with lysine codons and tested it with purine nucleoside phosphorylase (PNP) from *Escherichia coli*, an extremely stable but immunogenic enzyme, that could potentially be used to treat an inherited deficiency of PNP. A triple mutant, RK3, possessing three Arg → Lys substitutions was constructed that increased the number of lysines per PNP subunit from 14 to 17, providing an additional 18 potential PEG attachment sites per hexameric enzyme molecule. The wild-type and RK3 enzymes had similar catalytic activity, antigenicity, and immunogenicity. After PEG modification, both enzymes retained catalytic activity, the plasma half-life of both enzymes in mice increased from ≈4 hr to 4 days, and the binding of both enzymes by antisera raised against each unmodified enzyme was markedly diminished. However, antibody raised against wild-type PEG-PNP did not bind the PEG-RK3 enzyme. PEG-RK3 PNP was also substantially less immunogenic than wild-type PEG-PNP. Accelerated antibody-mediated clearance of PEG-PNP occurred in 2 of 12 mice treated with PEG-RK3 PNP, compared with 10 of 16 mice treated with the modified wild-type enzyme. This combined use of directed mutagenesis and PEG modification is aimed at permitting the widest choice of proteins, including products of genetic and chemical "engineering," to be used for therapy of inherited and acquired disorders.**

Enzyme replacement therapy for inherited metabolic diseases has been difficult to achieve due to the rapid elimination, inefficient cellular uptake, and potential immunogenicity of purified enzymes. Some approaches to solving these problems have shown promise in clinical trials—e.g., the treatment of adenosine deaminase (ADA) deficiency with ADA modified by covalent addition of polyethylene glycol (PEG) to prolong circulating life (1) and treatment of Gaucher disease with glucocerebrosidase bearing modified carbohydrate chains to promote uptake by hepatic kupffer cells (2). In addition to chemical modification, there is much interest in using recombinant genetic techniques to improve the therapeutic characteristics of proteins—e.g., modified forms of plasminogen activator (3), interleukin 2 (4), and soluble CD4 (5). Changes in protein structure may simultaneously create new epitopes and increase the risk of immunogenicity, as when a protein with otherwise desirable properties must be derived from a nonhuman source. The problem of immune recognition is not restricted to nonhuman or genetically modified proteins, as indicated by the development of inhibitory antibody to both purified plasma (6) and recombinant (7) human factor VIII in patients with hemophilia.

Attachment of PEG can diminish immunogenicity as well as prolong the circulating life of proteins (8–10). In >5 years of treating ADA deficiency with PEG-modified bovine ADA, there have been no allergic or hypersensitivity reactions; in a few patients anti-ADA antibody has accelerated clearance of PEG-ADA, but induction of tolerance or increasing the frequency of injections has allowed effective enzyme levels to be maintained (11, 12). For any protein the effects of PEG modification depend on the number and distribution of PEG attachment sites (e.g., lysyl ε-amino groups) relative to epitopes and structural elements that determine function and clearance. The ability to "mask" epitopes could theoretically be extended by selectively introducing additional sites for PEG addition. We have developed a strategy for using directed mutagenesis to achieve this goal and tested it with purine nucleoside phosphorylase (PNP)¶ from *Escherichia coli*, an extremely stable, but immunogenic enzyme that, because of its broad substrate range, could potentially be used to treat inherited deficiencies of both PNP and ADA.

MATERIALS AND METHODS

Wild-type and RK3 mutant *E. coli* PNP (see below) were isolated at >95% purity with an overall yield of ≈30% from extracts of an overexpressing strain by chromatography on Q-Sepharose fast flow and phenyl-Sepharose. PNP assays (50 μl) contained 0.2 M potassium phosphate (pH 7.0) and 100 μM [8-^{14}C]inosine (8.3 μCi/μmol; 1 Ci = 37 GBq) (Moravek Biochemicals, Brea, CA). After incubation at 37°C, 5 μl was applied to cellulose TLC plates (PolygramCel 400 UV_{250}, Macherey & Nagel) prespotted with 5 μl of 3 mM inosine/3 mM hypoxanthine. Plates were developed in water and hypoxanthine spots were excised for liquid scintillation counting. One PNP unit catalyzes the conversion of 1 μmol of inosine to hypoxanthine per min. Protein was determined by the BCA method (Pierce) as described by the manufacturer.

PEG Modification. Disuccinyl monomethoxypolyethylene glycol (M_r 5000; SS-PEG) (13) was generously provided by Enzon (South Plainfield, NJ). After dialysis against 0.15 M NaCl/0.05 M sodium phosphate, pH 7.2 (PBS), PNP (1–10 mg/ml) was mixed with SS-PEG (30 mg/mg of PNP). After 1–3 hr at 23°C, unreacted SS-PEG was removed by dialysis at 4°C against PBS; gel filtration over Superose 12 confirmed the complete shift of enzyme to a higher molecular weight. PEG-modified PNP (PEG-PNP) was filter sterilized and

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Abbreviations: PNP, purine nucleoside phosphorylase; ADA, adenosine deaminase; SS-PEG, disuccinyl monomethoxypolyethylene glycol.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M60917).

stored at 0–4°C with <20% loss of activity over 6 months. Extent of modification was assessed by loss of fluorescamine-reactive amino groups (14), except that PNP samples were treated with 0.5% SDS for 30 min prior to adding the fluorescamine reagent. The percent modification was determined by comparing the slopes of plots of relative fluorescence (average of duplicates) vs. μg of enzyme protein obtained with the unmodified and PEG-modified enzymes.

Vascular Clearance of PNP and PEG-PNP. BALB/c mice were injected i.p. with 0.2 ml of PNP or PEG-PNP in PBS containing 0.5% BALB/c plasma (to stabilize the diluted enzyme). Heparinized blood obtained from the orbital plexus was centrifuged at 4°C for 10 min at 1000 rpm in an Eppendorf 5415 microcentrifuge. Plasma was recentrifuged to ensure absence of erythrocytes and care was taken to avoid release of erythrocyte PNP; plasma from uninjected mice was assayed as a control for hemolysis. Plasma was stored at 4°C and assayed within a week.

Detection of Anti-PNP Antibody. Murine IgG antibody to PNP was detected by ELISA, using a mouse hybridoma immunoglobulin screening kit (Calbiochem). In the standard format unmodified wild-type *E. coli* PNP was used as the immobilized antigen and absorbance of antigen-free wells was subtracted. Similar procedures were used to detect rabbit anti-PNP antibody, using the appropriate reagents. Since PEG modification might alter binding of antigen to plastic, a competition format was used to detect antibody to PEG-PNP. Samples of a predetermined dilution of an anti-PNP plasma or serum were mixed with increasing amounts of unmodified or PEG-modified PNP. After overnight incubation at 4°C, these mixtures were tested in the standard ELISA. Inhibition indicated binding of antibody by the antigen in solution.

Genetic Manipulations. Plasmid pSS322-5 harbors the tetracistronic *deo* operon, of which *deoD*, encoding *E. coli* PNP, is the most promoter-distal cistron (15). For determination of the *deoD* nucleotide sequence (Fig. 1), a 1500-base-pair *Eco*RV–*Pvu* II fragment was cloned from pSS322-5 into M13mp19 in both orientations. Ordered overlapping deletions within the *deoD* insert of each M13mp19 derivative were prepared (16) and sequenced (17) using deoxyadenosine 5'-[α-[^{35}S]thio]triphosphate and Sequenase version 2.0 (United States Biochemical). Oligonucleotide-directed mutagenesis of the *deoD* gene was carried out using the Amersham *in vitro* mutagenesis kit and verified by complete sequencing of the PNP coding region of mutant products. Wild-type and modified enzymes were overexpressed in *E. coli* BL21 (DE3) (18) in which the appropriate *deoD* gene was inserted at a *Nde* I site downstream of the T7 promoter (introduced by directed mutagenesis, inserting a thymine after nucleotide 122 of Fig. 1). Plasmid DNA was isolated, digested, and subcloned by standard methods (19, 20). Analysis of nucleotide and deduced protein sequences was performed with the University of Wisconsin Genetics Computer Group software package (21).

RESULTS

E. coli and mammalian PNP show little amino acid sequence homology and differ in important catalytic and physical properties. *E. coli* PNP has a much broader substrate range (22, 23) and is a hexamer with 14 potentially derivatizable lysines per subunit ($M_r \approx 26{,}000$) (24), whereas the human enzyme is trimeric with 12 lysines per subunit ($M_r \approx 32{,}000$) (24, 25); the bacterial enzyme is remarkably heat stable (23). From these properties we anticipated that the circulating life of *E. coli* PNP after PEG modification might be limited by residual immunogenicity rather than lability.

This expectation was verified by preliminary experiments comparing the stability at 37°C and clearance in mice of *E. coli* and bovine spleen PNPs after each was treated with a 30-fold excess by weight of SS-PEG. During incubation *in vitro* at 37°C, the activity of bovine PEG-PNP declined with a half-life of 20–24 hr whereas *E. coli* PEG-PNP lost no activity in 96 hr. PEG modification increased the plasma half-life of bovine PNP from 1–2 hr to ≈16 hr, whereas the plasma half-life of *E. coli* PEG-PNP was ≈4 days. To evaluate the immunogenicity of PEG-modified *E. coli* PNP, BALB/c mice were treated with i.p. injections of the enzyme (1.8 units) every 1–2 weeks and clearance was assessed by measuring plasma PNP activity 24 hr after injection. Decreased plasma PNP levels were evident by the fourth dose, and by the seventh dose only two of six mice still showed detectable plasma PNP activity 24 hr after injection (Fig. 2*A*). Rapid enzyme clearance correlated directly with the development of antibody to *E. coli* PNP (Fig. 2*B*). These experiments provided a baseline for studies aimed at diminishing the immunogenicity of PEG-derivatized *E. coli* PNP.

Preparation and PEG Modification of Wild-Type and Mutant *E. coli* PNP. In the absence of x-ray crystal structure data for *E. coli* PNP, we adopted an empirical strategy for creating PEG attachment sites by using site-directed mutagenesis of the *deoD* gene to substitute lysine for existing arginine codons. From inspection of the wild-type *deoD* gene sequence (Fig. 1) and from computer-assisted analysis of PNP secondary structure, candidate arginine codons were identified that were relatively distant from neighboring lysine codons or that were located in regions of the protein predicted to be exposed to solvent or to have a high antigenic index. This strategy resulted in selection of PNP Arg-38, -44, -136, and -208 for replacement.

No PNP activity was detected in strains expressing genes encoding the R44K substitution, either alone or as an R38K/

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ATCGGCCAGACTCTGGCAAAATATTTTGGTACTTCTGATATGGAATATGGCAAAGCCATGTTCTGATGGATTTGGGCGGAGCGTTGACTCCGCCTTTGTTATGTCACAAAAAGGATAAAA   120
                                                                                                        R→K38
  M  A  T  P  H  I  N  A  E  M  G  D  F  A  D  V  V  L  M  P  G  D  P  L  R  A  K  Y  I  A  E  T  F  L  E  D  A  R  E  V   240
CAATGGCTACCCCACACATTAATGCAGAAATGGGCGATTTCGCTGACGTAGTTTTGATGCCAGGCGACCCGCTGCGTGCGAAGTATATTGCTGAAACTTTCCTTGAAGATGCCCGTGAAG
  N  N  V  R  G  M  L  G  F  T  G  T  Y  K  G  R  K  I  S  V  M  G  H  G  M  G  I  P  S  C  S  I  Y  T  K  E  L  I  T  D
TGAACAACGTTCGCGGTATGCTGGGCTTCACCGGTACTTACAAAGGCCGCAAAATTTCCGTAATGGGTCACGGTATGGGTATCCCGTCCTGCTCCATCTACACCAAAGAACTGATCACCG   360
  F  G  V  K  K  I  I  R  V  G  S  C  G  A  V  L  P  H  V  K  L  R  D  V  V  I  G  M  G  A  C  T  D  S  K  V  N  R  I  R
ATTTCGGCGTGAAGAAAATTATCCGCGTGGGTTCCTGTGGCGCAGTTCTGCCGCACGTAAAACTGCGCGACGTCGTTATCGGTATGGGTGCCTGCACCGATTCCAAAGTTAACCGCATCC   480
                                                R→K136
  F  K  D  H  D  F  A  A  I  A  D  F  D  M  V  R  N  A  V  D  A  A  K  A  L  G  I  D  A  R  V  G  N  L  F  S  A  D  L  F
GTTTTAAAGACCATGACTTTGCCGCTATCGCTGACTTCGACATGGTGCGTAACGCAGTAGATGCAGCTAAAGCACTGGGTATTGATGCTCGCGTGGGTAACCTGTTCTCCGCTGACCTGT   600
  Y  S  P  D  G  E  M  F  D  V  M  E  K  Y  G  I  L  G  V  E  M  E  A  A  G  I  Y  G  V  A  A  E  F  G  A  K  A  L  T  I
TCTACTCTCCGGACGGCGAAATGTTCGACGTGATGGAAAAATACGGCATTCTCGGCGTGGAAATGGAAGCGGCTGGTATCTACGGCGTCGCTGCAGAATTTGGCGCGAAAGCCCTGACCA   720
                R→K208
  C  T  V  S  D  H  I  R  T  H  E  Q  T  T  A  A  E  R  Q  T  T  F  N  D  M  I  K  I  A  L  E  S  V  L  L  G  D  K  E  •
TCTGCACCGTATCTGACCACATCCGCACTCACGAGCAGACCACTGCCGCTGAGCGTCAGACTACCTTCAACGACATGATCAAAATCGCACTGGAATCCGTTCTGCTGGGCGATAAAGAGT   840

AATTGTGTTTCGCTGCAAGGCGATTGCCTTGTGAAGCCGGAGCGGGAGACTGCTCCGGCTTTTTAGTATCTATTCATTTTTCTCTCCAGCTTGAATATTTTCGCTATACTTTTCAGTGAA   960
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FIG. 1. Nucleotide and deduced amino acid sequence of the *E. coli deoD* gene, showing the sequences for wild-type *E. coli* PNP and the locations of Arg → Lys replacements in the RK3 triple mutant.

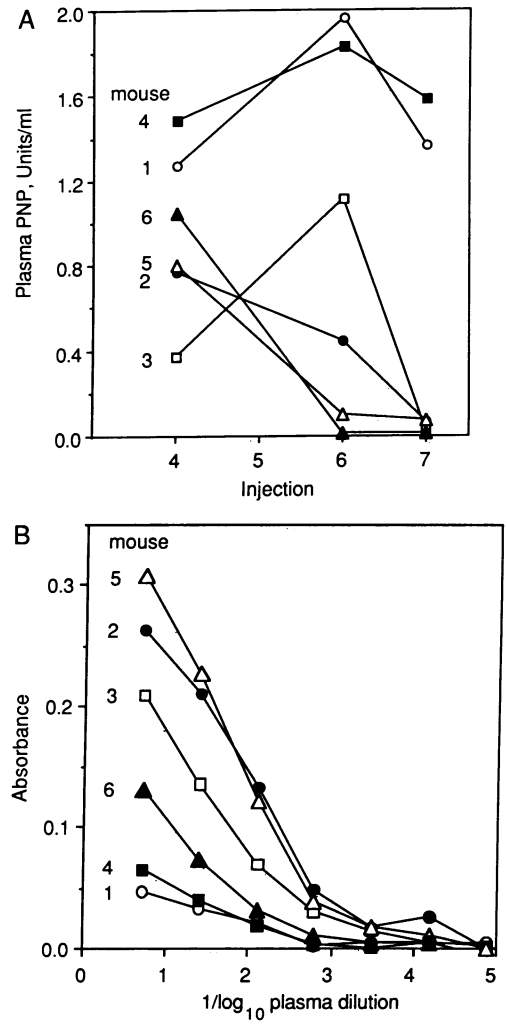

FIG. 2. Development of antibody-mediated clearance of PEG-modified *E. coli* PNP. Six BALB/c mice received weekly or biweekly i.p. injections of 1.75 units of PEG-modified *E. coli* PNP. (*A*) Plasma PNP activity in samples obtained from each mouse 24 hr after injections 4, 6, and 7. (*B*) ELISA titration of antibody to immobilized unmodified *E. coli* PNP in plasma samples of the same mice obtained after injection 9.

R44K double mutant, despite near wild-type amounts of PNP protein (data not shown). However, extracts of strains expressing PNP(R38K) alone had wild-type PNP specific activity. Arg → Lys substitutions at residues 136 and 208 were constructed on the *deoD* R38K background to create a series of double and triple mutants, all of which yielded wild-type levels of PNP activity. The triple mutant PNP(R38K/R136K/R208K), designated RK3, was selected for all epitope shielding studies. Computer-predicted secondary structure and antigenicity of RK3 and wild-type *E. coli* PNP were indistinguishable.

Comparison of Wild-Type and RK3 *E. coli* PNP and PEG-PNP. The purified wild-type and RK3 enzymes had the same specific activity and the expected reciprocal difference in arginine and lysine content (Table 1). After SDS denaturation and treatment with fluorescamine, RK3 PNP gave ≈23% higher relative fluorescence per μg of protein than wild-type PNP (19.5 ± 1.8 vs. 15.8 ± 1.5, respectively), consistent with the presence of three more lysine residues per subunit of the mutant enzyme. Studies of fluorescence quenching by iodide revealed no differences between the enzymes in the surface accessibility of tyrosine fluorophores (data not shown), confirming the absence of secondary structure perturbations by

Table 1. Properties of wild-type and RK3 mutant *E. coli* PNPs

Enzyme	Specific activity, units/mg	Residues per mol of subunit, no. Arg	Lys	% modification
Wild type	182	12.3	14.2	—
Mutant	214	9.2	17.2	—
PEG-wild type	330	—	—	74.1 ± 6.1
PEG-mutant	317	—	—	68.2 ± 5.0

Number of residues was determined from amino acid analysis. For percent modification, data are mean ± SD, based on three determinations.

the Arg → Lys replacements. PEG-modified wild-type and RK3 enzymes (prepared in parallel with a 30-fold excess SS-PEG) showed a slight apparent increase in specific activity and loss of 74% and 68% of fluorescamine-reactive primary amino groups (Table 1). Since fluorescence of fluorescamine adducts is influenced by neighboring amino acids (26, 27), these studies suggest, but do not prove, that the mutant enzyme could accept one more PEG strand(s) per subunit than the wild-type enzyme (or six per mutant hexamer).

After i.p. injection of mice with both unmodified enzymes, plasma PNP activity peaked in 1–2 hr and then decayed with a half-life of ≈4 hr; attachment of PEG did not affect rate of absorption but increased the half-life of each enzyme to ≈4 days (Fig. 3). The antigenicity (antibody-binding capacity) of each protein was examined by a competition ELISA using antisera from rabbits immunized with the unmodified wild-type and mutant *E. coli* enzymes (Fig. 4) and using pooled plasma from mice that had developed antibodies to the PEG-modified wild-type enzyme (Fig. 5). The unmodified enzymes were equally effective in binding both these antibody preparations, suggesting that the three Arg → Lys substitutions did not appreciably alter antigenicity. Both PEG-modified enzymes showed markedly reduced binding of the rabbit antisera (Fig. 4), indicating blocking by PEG of the major antigenic determinants of the unmodified enzymes. Notably, however, the pooled mouse antibody raised against wild-type PEG-PNP did not bind the PEG-modified RK3 enzyme (Fig. 5). The most straightforward interpretation of this result is that modification of the mutant protein with PEG masked a residual antigenic determinant (or determinants) exposed on the surface of PEG-modified wild-type *E. coli* PNP.

To evaluate immunogenicity, parallel groups of mice were treated with 10 serial i.p. injections of the native and PEG-modified wild-type and RK3 enzymes. Circulating life of PNP activity and antibody to PNP, detected by ELISA using

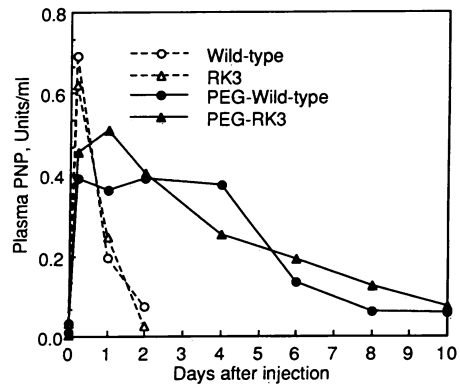

FIG. 3. Circulating life of unmodified and PEG-modified wild-type and RK3 mutant *E. coli* PNP. Mice received i.p. injections of 1.5 units of unmodified or 4 units of PEG-modified enzymes. Plasma PNP was determined at the indicated times after injection. Each point is the average value for two mice for the unmodified enzymes or four mice for the PEG-modified enzymes.

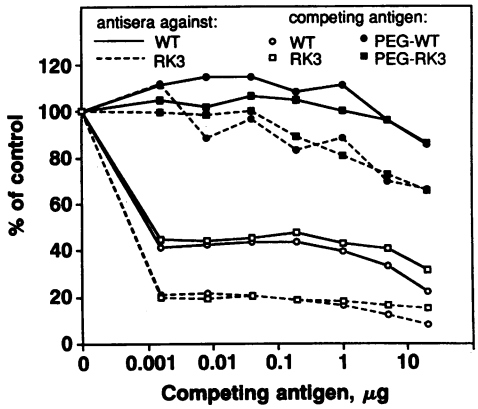

FIG. 4. Binding of rabbit anti-*E. coli* PNP antisera to unmodified and PEG-modified wild-type and RK3 mutant *E. coli* PNPs. Samples of rabbit antisera raised against wild-type *E. coli* PNP (solid lines) or against RK3 mutant *E. coli* PNP (dashed lines) were mixed with various amounts of unmodified (open symbols) or PEG-modified (solid symbols) wild-type (circles) or RK3 (squares) *E. coli* PNP. After overnight incubation at 4°C, these mixtures were tested in an ELISA for reaction of rabbit IgG with immobilized unmodified *E. coli* PNP. Control was absorbance obtained in the absence of competing antigen.

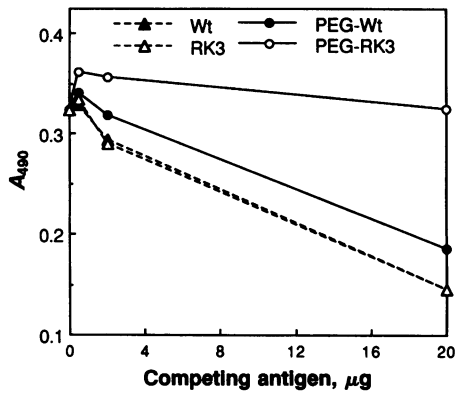

FIG. 5. Ability of mouse antibody raised against PEG-modified wild-type *E. coli* PNP to bind unmodified and PEG-modified wild-type and RK3 mutant *E. coli* PNP. Samples of pooled plasma from four mice immunized with nine injections of wild-type *E. coli* PEG-PNP (mice 2, 3, 5, and 6 in Fig. 2) were mixed with the indicated amounts of unmodified or PEG-modified wild-type or RK3 *E. coli* PNP. After overnight incubation at 4°C, these mixtures were tested in an ELISA for reaction of mouse IgG with immobilized unmodified wild-type *E. coli* PNP.

wild-type *E. coli* PNP as immobilized antigen, were evaluated in each mouse after the last injection. All mice (4 of 4 mice) treated with the unmodified wild-type or mutant enzymes developed rapid antibody-mediated clearance of the enzymes (data not shown). After injection 10 only 1 of 6 mice treated with wild-type PEG-PNP still showed prolonged PNP circulation; no increase in plasma PNP activity was detectable after injection of the other 5 mice (Fig. 6*A*); each of the latter had developed antibodies to *E. coli* PNP (Fig. 6*C*). By contrast, 4 of 6 mice treated with the PEG-modified mutant enzyme showed prolonged PNP circulation (Fig. 6*B*); three of these mice showed detectable but lower levels of anti-PNP antibody (Fig. 6*D*). By combining data from this and a second similar experiment with the data from Fig. 2, 10 of 16 mice (63%) injected with wild-type PEG-PNP developed significant levels of anti-PNP antibody (A_{490} > 0.1 unit/μl of plasma), associated with a marked decline in PNP circulating life. In contrast, only 2 of 12 mice (17%) treated with RK3 PEG-PNP developed rapid clearance; anti-PNP antibody at >0.1 A_{490} unit/μl of plasma occurred in 3 mice, but these levels did not correlate with clearance of enzyme.

DISCUSSION

Modification of primary amino groups, which are largely confined to the protein surface (28), with activated forms of PEG can be achieved under mild conditions. When successful, catalytic activity is retained but immunogenicity and

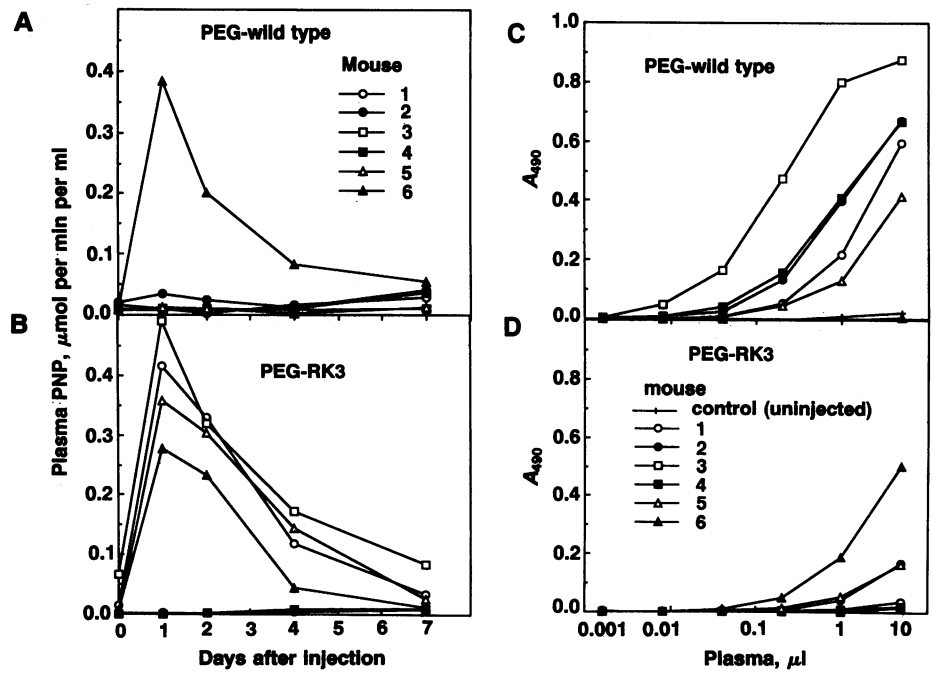

FIG. 6. Immunogenicity of PEG-modified wild-type and RK3 mutant *E. coli* PNPs. At 1- to 2-week intervals, parallel groups of six mice were treated with i.p. injections of 4 units of either PEG-modified wild-type (*A* and *C*) or PEG-modified RK3 mutant (*B* and *D*) *E. coli* PNP. After injection 10, samples were obtained to measure plasma PNP activity (*A* and *B*) and for determining antibody to immobilized unmodified wild-type *E. coli* PNP by ELISA (*C* and *D*).

antigenicity are diminished substantially (10). Presumably, flexible hydrophilic PEG strands interfere with antigen processing and antibody binding but not with access of small molecules to the active site located in a more "interior" PEG-free environment. We postulate that relatively lysyl-poor areas of the protein surface may remain immunogenic. We have adopted a strategy for introducing additional PEG attachment sites to more effectively mask these residual epitopes, by using site-directed mutagenesis to replace selected arginine codons with lysine codons. This approach, which preserves the mild chemistry and specificity of derivatization with SS-PEG, assumes that arginine residues are also likely to occur on the protein surface and that many Arg → Lys substitutions will not have severe adverse effects on enzyme folding, subunit assembly, stability, or function.

E. coli PNP has provided a reasonably stringent model to test this strategy. PEG modification reduced but did not eliminate its immunogenicity; its three-dimensional structure and the locations of the active site, subunit contacts (the enzyme is a hexamer), and major epitopes are unknown. Thus, the initial choice of four arginines as candidates for lysine replacement relied on inspection of the amino acid sequence and common methods of secondary structure analysis to predict regions of solvent accessibility and high antigenic index. Three of these substitutions were tolerated, and a mutant RK3 *deoD* gene encoding Arg → Lys substitutions at positions 38, 136, and 208 was constructed and overexpressed. The purified RK3 phosphorylase, possessing 18 new potential PEG attachment sites per hexameric enzyme molecule, was indistinguishable from wild-type *E. coli* PNP in terms of specific activity, antigenicity, and immunogenicity in mice and rabbits. Modification of ≈70% of primary amino groups by reaction with excess SS-PEG had similar effects on the wild-type and mutant enzymes: (*i*) catalytic activity was unaffected; (*ii*) the plasma half-life in mice of each enzyme increased from ≈4 hr to 4 days; and (*iii*) the binding of antisera raised against each unmodified enzyme was markedly diminished.

Two important differences were observed. (*i*) Rapid antibody-mediated enzyme clearance developed much more frequently in mice treated with the PEG-modified wild-type than with the mutant PEG-PNP (i.e., in 10 of 16 vs. 2 of 12 mice, respectively). (*ii*) Anti-PNP antibody that developed in mice treated with wild-type PEG-PNP did not bind PEG-modified RK3 PNP, although it was about equally reactive with both unmodified enzymes and with the PEG-modified wild-type enzyme. Thus, as tested under conditions resembling replacement therapy, we significantly reduced the residual immunogenicity and antigenicity of PEG-modified wild-type *E. coli* PNP. Whether immunogenicity was reduced sufficiently to allow treatment of human PNP deficiency with PEG-modified RK3 PNP is unclear. A strain of PNP-deficient mice has been developed (29, ||). In preliminary experiments, *E. coli* PEG-PNP lowered urinary levels of PNP substrates in these mice (F. F. Snyder, personal communication). Further studies with the RK3 mutant PEG-PNP may allow longer experiments necessary to determine whether abnormal T-cell development will respond to chronic enzyme replacement.

If immunogenicity were the only concern in developing enzyme or other protein-based therapies, then only human gene products would be used. However, other factors, such as stability, specific activity, or substrate range, may be of comparable or overriding importance, as illustrated by the contrasting properties of bacterial and mammalian PNP. The greater stability and longer circulating life of *E. coli* compared to bovine PEG-PNP would be advantageous for therapy, and the ability of the *E. coli* enzyme to catalyze the phosphorolysis of adenosine nucleosides (22, 23) would, in theory, allow its use to treat ADA as well as PNP deficiency. In attempting to minimize the immunogenicity of PEG-modified proteins, the present investigation is aimed at allowing the widest possible choice of proteins, including products of genetic or chemical engineering, to be used for achieving effective therapy. The application of our strategy to *E. coli* PNP indicates potential for general utility.

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