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Sortase-catalyzed transformations that improve the properties of cytokines

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Recombinant protein therapeutics often suffer from short circulating half-life and poor stability, necessitating multiple injections and resulting in limited shelf-life. Conjugation to polyethylene glycol chains (PEG) extends the circulatory half-life of many proteins, but the methods for attachment often lack specificity, resulting in loss of biological activity. Using four-helix bundle cytokines as an example, we present a general platform that uses sortase-mediated transpeptidation to facilitate site-specific attachment of PEG to extend cytokine half-life with full retention of biological activity. Covalently joining the N and C termini of proteins to obtain circular polypeptides, again executed using sortase, increases thermal stability. We combined both PEGylation and circularization by exploiting two distinct sortase enzymes and the use of a molecular suture that allows both site-specific PEGylation and covalent closure. The method developed is general, uses a set of easily accessible reagents, and should be applicable to a wide variety of proteins, provided that their termini are not involved in receptor binding or function.

Many clinically relevant cytokines share a four-helix bundle structure, typified by IFNα, Granulocyte colony-stimulating factor 3 (GCSF-3), Erythropoietin (EPO), IL-2, IL-4, IL-7, IL-9, and IL-15. Crystal structures of cytokines with receptor fragments and biochemical studies that map residues critical for interaction of a cytokine with its receptor show that the receptor contacts the sides of the helical bundles (1). This mode of interaction positions the N and C termini of the cytokine away from the receptor.

Polyethylene glycol chains attached to therapeutically important proteins increase circulatory half-life, reduce clearance by kidney filtration, reduce proteolysis, and reduce the generation of neutralizing antibodies (2–4). The attachment of PEG commonly employs standard chemistries that target reactive amino acid side chains (e.g., cysteine and lysine). This strategy often generates a heterogeneous mixture in which multiple amino acids in the target are modified with a PEG chain, necessitating cumbersome separations and characterization (5). Such PEGylated molecules often show decreased biological activity, likely due to attachment of a PEG chain to a residue important for interaction with the receptor (6)–this problem may be overcome by site-specific PEGylation. Although engineering of a carefully placed unpaired cysteine residue allows site-specific PEGylation (7, 8), this method must be tailored to the specific protein target.

Sortase A from Staphylococcus aureus (SrtAStaph) is a thiol-containing transpeptidase that recognizes an LPXTG motif in multiple structurally unrelated substrates (9). SrtAStaph cleaves the peptide bond between the threonine and glycine residues with concomitant formation of a thioacyl enzyme intermediate that involves the catalytic cysteine and the substrate threonine. This acyl-enzyme is resolved by nucleophilic attack by the N terminus of an oligoglycine peptide, resulting in formation of an amide bond between the substrate protein and the incoming nucleophile (10). Sortase A tolerates C-terminal extensions of the oligoglycine nucleophile, allowing diverse functionalized nucleophiles to be installed site specifically onto proteins equipped with an LPXTG motif (11, 12). The related Streptococcus pyogenes SrtAstrep contains an additional threonine residue that is critical for interaction of a cytokine with its receptor show that the receptor contacts the sides of the helical bundles (1). This mode of interaction positions the N and C termini of the cytokine away from the receptor.

Sortase A from Staphylococcus aureus (SrtAStaph) is a thiol-containing transpeptidase that recognizes an LPXTG motif in multiple structurally unrelated substrates (9). SrtAStaph cleaves the peptide bond between the threonine and glycine residues with concomitant formation of a thioacyl enzyme intermediate that involves the catalytic cysteine and the substrate threonine. This acyl-enzyme is resolved by nucleophilic attack by the N terminus of an oligoglycine peptide, resulting in formation of an amide bond between the substrate protein and the incoming nucleophile (10). Sortase A tolerates C-terminal extensions of the oligoglycine nucleophile, allowing diverse functionalized nucleophiles to be installed site specifically onto proteins equipped with an LPXTG motif (11, 12). The related Streptococcus pyogenes SrtAstrep accepts di-alanine based nucleophiles, which the S. aureus enzyme does not. This sortase (SrtAStaph) cleaves the LPXTA motif between threonine and alanine and allows installation of modified alanine-based nucleophiles. SrtAStaph also recognizes and cleaves LPXTG motifs, albeit with reduced efficiency, however the LPXTA motif is refractory to cleavage by SrtAStaph (13, 14).

Here we present a general strategy for site-specific modification of therapeutic recombinant proteins at their termini, an approach that is particularly well suited to the four-helix bundle cytokines, where the termini are distant from the receptor interaction site. We show improvements in thermal stability by cova-
lently joining the N and C termini of these proteins (15). We present a general method for combining both site-specific PEGylation and improved thermal stability in a single molecule by using a molecular suture produced in two successive rounds of sortase-catalyzed transeptidation (Fig. 1).

**Results**

**Modification of IFNα2.** We first applied the sortase reaction to human interferon alpha (IFNα2), a four-helix bundle cytokine where chemical PEGylation results in multiple positional isomers and a ~90% decrease in biological potency (16). We expressed in *Escherichia coli* two versions of IFNα2, both with an LPETG sortase motif immediately followed by a hexahistidine tag for ease of purification. At the amino terminus, one of the two proteins possesses a diglycine motif for cyclization. This material can either be cyclized or PEGylated at the C terminus (Fig. 2A). IFNα2 with an N-terminal diglycine motif and a C-terminal LPETG was successfully cyclized by incubation with SrtAStaph and purified to homogeneity by ion-exchange chromatography. The mass of the circularized product in a crude sortase reaction differs from a linear hydrolysis product by ~18 Da, consistent with an intramolecular transeptidation reaction (Fig. 2B). We identified the unique junction peptide that arises from intramolecular cyclization by tandem mass spectrometry (MS/MS) analysis of trypic digests (Fig. 2C). Site-specific PEGylation of the IFNα2 variant that lacks the N-terminal diglycine motif (and thus unable to undergo intramolecular cyclization) was achieved by incubation with sortase and a GGGK peptide to which a 10 kDa PEG moiety was affixed via the lysine ε-amine (Probe 1, Fig. 2A and Fig. S1A). We confirmed equipotent biological activity of all four interferon alpha variants in a Daudi cell proliferation inhibition assay when compared to a commercially available, non-PEGylated preparation (Fig. 2D and Table S1). Addition of a protein-sized PEG module to the C terminus of IFNα2 does not appreciably perturb receptor binding and biological potency.

We hypothesized that the circular IFNα2 would be more resistant to thermal denaturation, because the termini are clamped shut and thus should not “fray” (15, 17). We measured thermal denaturation with a Thermofluor assay, using the commercially available Sypro Orange dye (18). Indeed, the circular form of IFNα2 had a significantly elevated melting temperature (Tm) compared to the other three variants, all of which exhibited nearly identical Tm’s (Fig. 2F, Table S1, and Fig. S2A). Finally, we injected mice with either the linear IFNα2 variant used to construct the PEGylated form, or the IFNα2 variant bearing a 10 kDa PEG chain (Probe 1) at its C terminus and measured the serum levels over time by ELISA. The PEGylated form decayed with slower kinetics than its non-PEGylated counterpart (Fig. 2F and Table S1).

**Modification of a Single Protein Preparation by Multiple Nucleophiles.** The sortase platform offers the ability to install nearly any

![Synthesis and characterization of human IFNα2 conjugates. (A) GG-IFNα-LPETGHHGHis6 was used to obtain backbone-cyclized IFNα2, and GG-LEPTGHHGHis6 was used to make IFNα2-PEG (10 kDa), bearing a C-terminal PEG chain. Purified proteins were resolved by 12.5% SDS-PAGE and stained with Coomassie. (B) ESI-MS characterization of IFNα species in crude sortase reactions either before (linear GG-IFNα-LPETGHHGHis6) or after (cyclic IFNα2) overnight incubation at 25°C. (C) MS/MS identification of a trypic peptide comprising the IFNα2 C terminus, followed by the SrtAStaph cleavage site, joined to the N-terminal glycines in the IFNα2 precursor. (D) In vitro bioactivity of IFNα conjugates in a Daudi cell proliferation inhibition assay. Dose-response curves for Daudi cell proliferation inhibition were measured by [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay, in triplicate, with the standard deviation displayed. (E) Resistance of backbone-cyclized IFNα to thermal denaturation. Change in Tm for circular and PEGylated IFNα conjugates, relative to their precursor proteins, were measured by thermal shift assay, with n = 4. (F) Change in serum IFNα levels following tail vein injection of site-specifically PEGylated IFNα as well as its precursor protein. 10 μg of each protein was injected and, at the indicated time points, blood was withdrawn and cytokine concentration in serum was determined by ELISA assay. Data are means ± standard deviation for three mice in each group.
nongenetically encoded entity onto the C terminus of a single preparation of recombinant protein. We demonstrate this ability by synthesizing a nucleophile that bears a 20 kDa PEG moiety (Probe 2 and Fig. S14), as well as the previously described 10 kDa PEG probe (Probe 1), and appended them to the identical preparation of IFNα2 (Fig. 3A). Both conjugates were tested for biological activity (Fig. 3B) and are highly potent (Table S2).

**Superior Properties Endowed upon Sortase Substrates Are Applicable to Multiple Proteins.** Sortase allows the installation of the same nonnatural group onto different proteins, unrelated in sequence or amino acid composition. Accordingly, we applied the sortase reaction to a different four-helix bundle cytokine, GCSF-3, with similar results. We first made the linear precursor GCSF variants and then cyclized or PEGylated them with probe 1 using sortase (Fig. 4A). All variants possess equal or superior in vitro bioactivity relative to a commercial non-PEGylated preparation, as assessed by a standard cell proliferation assay with the NFS-60 murine myeloblastic cell line (19) (Fig. 4B and Table S3). Like cyclic IFNα2, cyclic GCSF-3 shows increased thermostability (Fig. 4C; Fig. S2B, and Table S3) and PEGylated GCSF-3 has a significantly extended circulatory half-life relative to its non-PEGylated precursor (Fig. 4D and Table S3) upon injection into mice. PEGylated GCSF-3 also led to a more robust and prolonged proliferation of granulocytes in injected mice than its non-PEGylated analog (Fig. 4E).

**Modification of Glycoproteins.** Many important therapeutics are glycosylated proteins that traverse the secretory pathway and are produced in mammalian cell culture. Are such proteins equally amenable to engineering using a sortase-mediated transpeptidation reaction? We chose human erythropoietin (EPO) because ~40% of its mass is composed of bulky, charged N-linked glycans, and cyclization provides a stringent test of the sortase method, as it requires (minimal) modifications at both termini. We replaced the endogenous EPO signal sequence with the murine H-2Kb signal sequence, followed by two glycine residues. This signal peptide is cleaved in mammalian cells with concomitant exposure of an N-terminal glycan residue. We added the requisite sortase motif followed by a hexahistidine tag at the C terminus of EPO for ease of purification. Human embryonic kidney (HEK)-293T cell lines stably transduced with this construct yielded preparations that were <50% pure after a single round of Nickel-nitri-tri-acid immobilized metal affinity chromatography (Ni-NTA IMAC) of conditioned medium. Material from such preparations can be cyclized successfully upon incubation with sortase (Fig. 5A). Again, we identified the unique junction peptide that arises from covalent ligation of the N and C termini by MS/MS analysis of cyclized EPO, shorn of all glycans by incubation with peptide: N-glycosidase F (PNGaseF), and digested with trypsin (Fig. 5B). Both the linear preparations as well as the sortase reaction containing cyclic EPO were tested in a cell proliferation assay with Ba/F3 cells that stably express the erythropoietin receptor (20) and these compared favorably with commercial EPO preparations. Most importantly, the cyclic form is as potent as its linear counterpart (Fig. 5C and Table S4), indicating that modification of the EPO termini does not affect its biological activity.

**Two-Step Transacylation Allows Combination of Enhanced Properties.** Having shown for multiple examples that sortase-mediated site-specific modification of the C terminus with PEG increases circulatory half-life with nearly no loss of biological activity, and that covalent closure of the N and C termini yield proteins stabilized against thermal denaturation, we executed a scheme that combines these desirable properties (Fig. 1). In the first step, we exploit a previously described sortase from *Streptococcus pyogenes* (13, 14) that accepts alanine-based nucleophiles to affix a peptide containing a nonnatural amino acid containing amino oxime at the place of suture. Next, the amino oxime group is used in a bioorthogonal oxime ligation reaction (21–23) with commercially available methoxy-capped PEG-propionaldehyde (Fig. S1B). We recombinantly expressed IFNα2 bearing two glycines at the N terminus, and at the C terminus the SrtAStaph cleavage site, followed by a hexahistidine tag. The purified protein was subjected to the two-step transacylation procedure to yield circular IFNα2 bearing the amino oxyc group. After ion exchange chromatography, this material was PEGylated and purified by cation exchange chromatography (Fig. 6A). Without optimization, the final yield of circular PEGylated protein was ~10%, because of the multiple separate chromatography and protein concentration steps involved in production and PEGylation. The mass of the material before oxime ligation was consistent with its cyclization (Fig. S3). Digestion with AspN and MS/MS analysis revealed the unique peptide that arises as a consequence of backbone cyclization consisting of the molecular suture probe stitched between the C and N termini of IFNα2 (Fig. 6B). All three variants yielded virtually indistinguishable IC50 values in an inhibition of cell proliferation assay (Fig. 6C and Table S5) and the circular versions were stabilized against thermal denaturation (Fig. 6D, Table S5, and Fig. S2C). The circular versions remained biologically potent after boiling when given the opportunity to refold, whereas we were unable to extract an IC50 value for the boiled linear preparation over the concentration range assayed (Fig. 6E and Table S6). We injected the linear and the circular, PEGylated forms of IFNα2 into the tail vein of mice and measured half-life by ELISA. The circular, PEGylated species was
cleared significantly more slowly than the linear form (Fig. 6F and Table S5).

**Discussion**

The sortase transpeptidase reaction allows facile site-specific PEGylation of multiple distinct proteins. In all cases tested, the site-specific C-terminal PEGylation proceeds efficiently (Fig. S3B) and yields adducts of known stoichiometry that are biologically equipotent to the non-PEGylated versions, but retain the increase in circulatory half life associated with PEG modification. We attribute these properties to the fact that all enzymatic transformations are performed on native proteins, procedures that should not affect overall conformation or exposure of functionally important side chains. Because our approach to PEGylation is site specific, all preparations are also homogenous and easily purified (5). Although we limit our examples to four-helix bundle cytokines, this platform should be readily extended to structurally distinct therapeutic proteins, with the singular requirement that the C terminus is not involved in receptor binding. A sortase-based approach requires the genetic fusion of a very small (five amino acid) tag to the protein of interest and all transformations occur under native conditions. The additional amino acids that result from fusion to the sortase recognition motif are close to the site of PEG attachment. Immunogenicity of this site is very likely reduced by the PEG moiety shielding this area (3). We and others have labeled proteins with an exposed N-terminal glycine using sortase (14, 24). This approach should therefore be readily extended to site-specific PEGylation at the N terminus as well.

Cyclization of the backbone of protein-based therapeutics also proceeds efficiently (Fig. S3B) and yields preparations that are more resistant to thermal denaturation. Cyclic proteins are also resistant to exoproteolytic attack (25, 26), a feature that may enhance utility of any therapeutic proteins exposed to exoproteases, for example upon receptor mediated internalization. In addition, cyclization of proteins and peptides has been shown to improve potency, stability, and oral bioavailability (27–30).

Finally, we have inserted a nontemplate encoded entity in what is topologically internal to a circular protein, a feat that cannot be accomplished genetically by intein-based methods or by any other currently known means. More generally, this dual transacylation scheme can be used to insert nonnatural groups what is topologically internal to a circular protein, a feat that cannot be accomplished genetically by intein-based methods or by any other currently known means. More generally, this dual transacylation scheme can be used to insert nonnatural groups between fully native proteins expressed separately, or between protein domains. We suspect that this approach will find application in the protein engineering field and so further extend the utility of sortases as tools.

**Experimental Procedures**

**Sortase Reactions.** Sortase reactions with SrtA<sub>Staph</sub> were performed as described previously (11). For either cyclization or PEGylation, reactions containing 50 μM substrate, 50 μM SrtA<sub>Staph</sub> and 1 mM probe (for PEGylation) were incubated overnight at 25 °C without agitation. Reactions were purified by cation exchange chromatography on a Mono-S (GE Healthcare). For IFNα2 reactions, continuous gradient chromatography was performed with 20 mM 2-(N-morpholino) ethane sulfonic acid (MES) pH 5.0 and 20 mM MES, 1 M NaCl pH5.0, as eluent. For GCSF reactions, continuous gradient chromatography was performed...
with 20 mM sodium acetate pH 4.5 and 20 mM sodium acetate, 1 M NaCl pH 4.5, as eluant. Peaks containing the desired product were pooled, concentrated with Vivaspin 50 centrifugal concentrators (Sigma) and protein concentration was determined by Bradford assay.

Fig. 5. Synthesis and characterization of cyclic human erythropoietin. (A) GG-EPO-LPETGHis$_6$, bearing the H-2Kb signal sequence was expressed in HEK-293T cells. Conditioned media was subjected to Ni-NTA IMAC purification. Eluted material was supplemented with 100 μM SrtA$_{Staph}$ and incubated at 25°C. Aliquots were removed at the indicated time points, subjected to 12.5% SDS-PAGE and protein was visualized by silver staining. (B) MS/MS identification of a peptide generated by trypsic digestion containing the C terminus of EPO, followed by the SrtA$_{Staph}$ cleavage site and joined to the N terminus of EPO. (C) In vitro bioactivity of linear and cyclic EPO conjugates in a cell proliferation assay using BaF3 cells stably expressing the erythropoietin receptor (BaF3-EPO). Eluted material from Ni-NTA IMAC purification was incubated in the presence or absence of sortase for 16 h at 25°C and these crude reactions were measured for in vitro EPO bioactivity. Dose response curves for BaF3-EPO cell proliferation were measured by MTT assay, in triplicate, with the standard deviation displayed. Concentrations were not adjusted for impurities in the EPO preparations.

Fig. 6. Synthesis and characterization of cyclic, PEGylated IFNα2. (A) GG-IFNα2-LPETGHis$_6$ was used to make backbone-cyclized IFNα2 with the amino o xo moiety stitched between the site of closure. This material was then used to generate cyclic, PEGylated IFNα2 by aniline catalyzed oxime ligation. Purified proteins were resolved by 12.5% SDS-PAGE and stained with Coomassie. (B) MS/MS identification of a peptide generated by AspN digestion containing the C terminus of IFNα2, followed by the alanine-based probe joined to the N terminus of IFNα2 by SrtA$_{Staph}$. Note that the amino o xo group was likely lost during MS/MS analysis and ESI-MS reconstructions of unfragmented protein revealed a mass consistent with installation of the amino o xo group (Fig. S3). (C) In vitro bioactivity of linear and cyclic IFNα2 conjugates in a Daudi cell proliferation inhibition assay. Dose response curves for Daudi cell proliferation inhibition were measured by MTT assay, in triplicate, with the standard deviation displayed. (D) Resistance of cyclic-AOAA and circular-PEGylated IFNα2 to thermal denaturation. Change in Tm for circular-AOAA and circular-PEGylated IFNα2 conjugates, relative to their precursor protein, were measured by thermal shift assay (n = 4). (E) Circular cytokines remain biologically active after boiling. The indicated variants were diluted to 1 μg/mL in RPMI/10% serum, boiled for 4 min, allowed to cool at room temperature for 16 h, and assayed for inhibition of Daudi cell proliferation. (F) Change in serum IFNα levels following tail vein injection of circular, site-specifically PEGylated IFNα2 as well as its linear precursor protein. 10 μg of each protein was injected and, at the indicated time points, blood was withdrawn and cytokine concentration in serum was determined by ELISA assay. Data are means from three mice in each group, with the standard deviation displayed.

Propionaldehyde, and 100 mM aniline (JT Baker) and incubated at 30°C for 3 h without agitation. This reaction mixture was again purified by cation exchange chromatography, concentrated, and protein concentration was determined by Bradford assay.

**Thermal Denaturation Assays.** Thermal shift assays were performed in a Roche 480 lightcycler and fluorescence was measured with Excitation = 533 nm, Emission = 610 nm. For GCSF-3, 1 μg of each variant was mixed with 1 μL of 100x Sypro orange in 25 μL total volume of 50 mM acetate pH 4.0, 150 mM NaCl, n = 4 for all samples with buffer as a blank control and lysozyme as a positive control. For IFNα2, 1 μg of each variant was mixed with
1 µL of 100x Sypro orange (Invitrogen) in 25 µL total volume of 50 mM MES ph 5.0, 150 mM NaCl n = 4 for all samples with buffer as a blank control and lysozyme as a positive control. Samples were heated from ambient temperature to 95°C at 0.01°C/s with 6 acquisitions/°C. The included Roche software was used to calculate the negative first derivative of the temperature change as a function of time and the minima were used as the $T_m$. Because of the impurities in the cyclic EPO preparations, $T_m$ was not determined.

Cell-Based Bioactivity Assays. Daudi cell proliferation inhibition assays to measure the activity of IFNα2 conjugates were performed as described (8) and compared to commercial IFNα2 (PBL Interferon Source) with low passage Daudi cells (ATCC), cultured in RPMI medium 1640/10% Heat inactivated fetal calf serum (FCS)/50 units/mL penicillin, 50 µg/mL streptomycin sulfate. Cell proliferation was measured by MTT assay according to manufacturer’s directions (ATCC). For the denaturation assay, samples diluted to 1 µg/mL in RPMI medium 1640/10% IFS were boiled for 4 min, allowed to cool at room temperature for 16 h, and assayed for Daudi cell proliferation inhibition. NFS-60 cell proliferation assays to measure G-CSF conjugate activity were performed as follows. NFS-60 cells (a kind gift from James Ihle, St. Jude Children’s Research Hospital) were cultured in RPMI medium 1640/10%FCS/50 units/mL penicillin, 50 µg/mL streptomycin sulfate supplemented with murine IL-3 (20 µU/mL, R&D Systems). Cells were washed extensively in complete RPMI medium 1640 medium lacking IL-3 and resuspended at 1 x 10^5 cells/mL in complete RPMI medium 1640 lacking IL-3. Titrated GCSF conjugates (50 µL) in complete RPMI medium 1640 were aliquoted into a flat bottom 96 well plate and 50 µL of cells (0.5 x 10^5 cells/well) were added to each well. Cells were cultured for 3 d and an MTT assay (ATTC) was performed according to manufacturer’s directions. Each plate contained a titration of commercial GCSF3 (Peprotech) and readings were blanked against wells containing only NFS-60 cells. The activity of each conjugate was measured a minimum of $n = 3$ times. BaF3 cells stably expressing the EPO receptor (BaF3 EPO-R cells, a kind gift from Harvey Lodish, Whitehead Institute) were used to measure EPO activity. BaF3 EPO-R cells were cultured as described for NFS-60 cells and assays were performed essentially as described for G-CSF, except EPO circularization reactions incubated with or without sortase for 16 h at 37°C were used. The total protein concentration of the EPO input material (measured by the Bradford method) was used and was not adjusted for impurities. Commercial preparations of EPO (eBioscience) were used as standards and readings were blanked against BaF3 EPO-R cells cultured with no cytokines. Activities were measured a minimum of $n = 3$ times.

Circulatory Half-Life Assays. For circulatory half-life assays, mice were injected in the tail vein with each protein (10 µg per mouse for IFNα2 conjugates, 5 µg per mouse for G-CSF conjugates) and subjected to retroorbital eye bleed at the indicated time points. Blood was harvested, centrifuged at 1,957xg in a tabletop centrifuge, and serum was collected and snap-frozen. Elisa assays to measure the quantity of cytokine in serum samples were performed according to the manufacturer’s directions (IFNα from PBL Interferon Source, G-CSF from Invitrogen). Each conjugate was injected into $n = 3$ mice. Data was fit to a two phase exponential decay in GraphPad Prism.

Granulocyte Proliferation Assays. Peripheral blood was collected retroorbitally into EDTA collection tubes. Red blood cells were lysed in hypotonic lysis buffer and the remaining peripheral blood mononuclear cells were stained with anti-myeloid differentiation antigen-1 (Gr-1) antibody labeled with phycoerythrin (PE) (BD Pharmingen), anti-cluster of differentiation 11b protein antibody labeled with FITC (anti-CD11b-FITC) (BD Pharmingen), and 7-Aminoactinomycin D (7-AAD) (ViaProbe, BD Pharmingen). Cells were analyzed using a FACS Calibur flow cytometer (BD).

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3. Hershfield MS, et al. (1991) Use of site-directed mutagenesis to enhance the epitope attenuation as a function of time and the minima were used as the $T_m$. Because of the impurities in the cyclic EPO preparations, $T_m$ was not determined.