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Improvement in the Solution Stability of Porcine Somatotropin by Chemical Modification of Cysteine Residues[†]

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A sustained release formulation is essential to commercialize porcine somatotropin (PST). Poor solution thermostability has hindered its development. Concentrated solutions of PST at elevated temperatures produced a physiologically nonreversible thixotropic gel. Disulfide exchange was noted during this aggregation. Two forms of dimeric PST, one of which was reducible to the monomer, were observed. Selective reductive alkylation of the $Cys^{181,189}$ disulfide suppressed this exchange and improved PST solution stability up to 3.4 times during incubation at ca. 250 mg/mL in phosphate-buffered saline (pH 7.4) for 14 days at 39 °C. Several cysteine derivatives were prepared which were bioactive in hypophysectomized rats, although the solution stability was variable. Carboxymethylation was used to link poly(ethylene glycol) chains. The results suggest that chemical modification of cysteine residues in PST can improve solution thermostability and is a general strategy for protein modification of either natural or mutated proteins.

INTRODUCTION

The somatotropins are protein hormones produced by the anterior pituitary which stimulate growth in virtually all vertebrate species. In mammals, these effects are the result of direct receptor stimulation and stimulation of the secretion of IGF-1, a related anabolic protein hormone (Hart and Johnsson, 1986). The administration of somatotropins to adult animals has been shown to offer a dual advantage: increased feed efficiency and an increase in the lean/fat ratio (McLaren et al., 1990; Campbell et al., 1991). Thus, finishing swine treated with daily injections of porcine somatotropin (PST) are less costly to feed and convert more of their nutrients into muscle protein, producing a more desirable product (Figure 1). Modern swine husbandry, however, renders such injections impractical. Commercial application of PST will necessarily require a device that, once implanted, delivers the protein at a steady rate over a period of 4–6 weeks. Packaging even a 3-week dose in an implant sufficiently small to facilitate administration will result in extremely concentrated PST solutions at swine body temperatures of ca. 39 °C. Unfortunately, we have observed that PST rapidly degenerates under these conditions, resulting in irreversible aggregates that severely limit release of monomeric, biologically active protein from the controlled release device.

To appreciate the nature of aggregate formation, detailed structural features of the protein needed to be understood. PST is a 191 amino acid protein containing two disulfide linkages. Crystallographic data indicate the tertiary structure consists of four antiparallel α-helicies (Abdel-Meguid et al., 1987). Bovine somatotropin (BST) has a high degree of homology to PST and is therefore expected to behave in a similar manner. Brems and coworkers have extensively studied the aggregation of BST and concluded that the aggregate forms as a result of a complex multistep process initiated by hydrophobic bonding at the interior surface of the third helix (Brems et al., 1985, 1986, 1988; Brems and Havel, 1989; Havel et al., 1986; Holzman et al., 1986). Our studies indicate that a disulfide-exchange reaction is involved in this aggregate formation under the conditions of our assay, which simulates a sustained-release device. Elimination of this process by chemical modification of the small loop cysteines in a recombinant version of PST (rPST) significantly enhances the solution stability of rPST, although other aggregation pathways remain. Reduction of the small loop disulfide should increase the conformational mobility in this region of the protein. The effect of this added flexibility on these other processes which also may contribute to the aggregation, e.g., the hydrophobic bonding described by Brems et al., is unclear. On balance, cysteine modification extends the useful half-life of the protein in concentrated solutions with no deleterious effect on the biological activity.

MATERIALS AND METHODS

Chemicals. Iodoacetic acid, iodoacetamide, sodium metabisulfite, bromosuccinic acid, methylthiomethanesulfonate, 1,3-propanesultone, 2-nitro-5-thiosulfobenzene, and ω-methoxypolyethylene glycols were purchased from Aldrich Chemical Co., Milwaukee, WI. *N*-Ethylmaleimide and *N*-(iodoethyl)trifluoroacetamide hydrochloride were purchased from Pierce Chemical Co., Rockford, IL. Dithiothreitol was obtained from Sigma (St. Louis, MO). Recombinant porcine somatotropin (rPST) was provided by the Biochemistry Group, Fermentation Development, Medical Research Division, American Cyanamid Co.

Methods. *General Procedure For Derivatization of rPST.* To a stirred solution of rPST (100 mg, 4.5 μmol) in 100 mL of aqueous ammonium acetate (0.5 M, pH 8.4) was added tricarboxymethylphosphine (2.59 mg, 9 μmol; Levinson et al., 1969). The resulting solution was stirred at room temperature for 0.5 h under a N_2 atmosphere. Four equivalents of the derivatizing reagent were then added to the reduced rPST. The reaction was monitored by titration for free thiol using the Ellman reagent (Creighton, 1989) or the Scheraga reagent (Thannhauser et al., 1987). After no free thiol groups were detected (generally about 2 h), the resulting solution was diluted and ultrafiltered through a YM10 membrane (10-kDa cutoff, Amicon Diaflor) to remove excess reagents and salts. The resulting aqueous solution was

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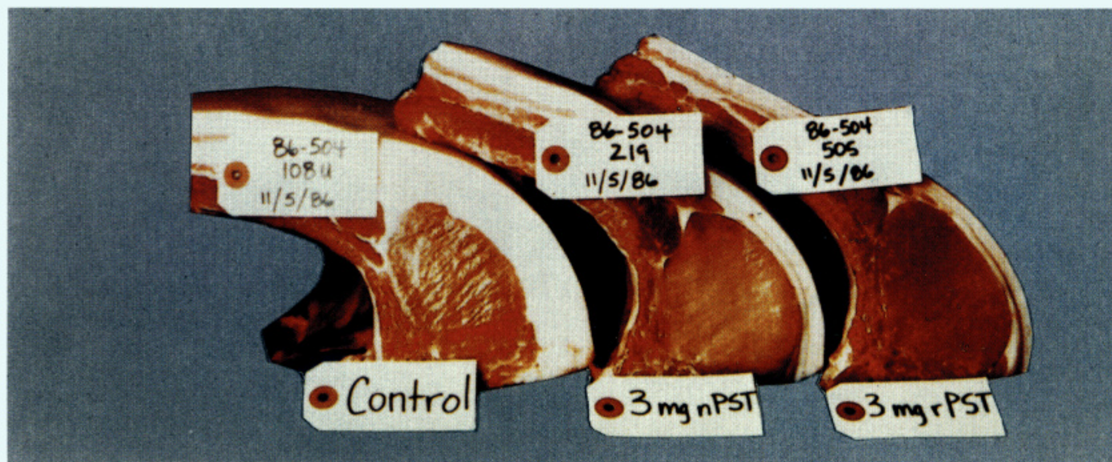


Figure 1. Pork chops from control (left, PBS) and PST-treated (middle, pituitary PST; right, recombinant PST) finishing swine. Swine were injected daily for 6 weeks prior to slaughter with PST or PBS vehicle. In the PST-treated animals, the loin muscle area is 14% larger and the subcutaneous fat is 50% less than in the control group. The repartitioning effect occurs throughout the animal.

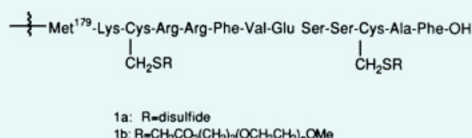


Figure 2. Sequence of C terminus of PST. The Rs represent the location of alkylations described in the text. Unmodified rPST retains a disulfide linkage at Cys[181,189].

lyophilized to yield the derivatized protein. The extent of derivatization was verified by amino acid analysis for (carboxymethyl)cysteine and by Scheraga titration for residual disulfide. Alternative procedures for derivatization are described under Results and Discussion.

Preparation of Iodoacetic Acid Derivatives. Esters and amides of iodoacetic acid were prepared from the appropriate alcohol or amine precursors using standard protocols for peptide coupling (dicyclohexylcarbodiimide). *N*-Aspartyliodoacetamide derivatives were prepared from suitably protected aspartic acid derivatives, which are readily available from commercial sources.

Amino Acid Analyses and Peptide Synthesis. Analyses were performed on a Beckman Model 7600 amino acid analyzer. Derivatized proteins were hydrolyzed in 6 M HCl for 2 days at 110 °C (Moore and Stein, 1963). Cyanogen bromide cleavage of CAM-PST was carried out in 70% formic acid with a 20 M excess of CNBr for 4 h at room temperature (Gross and Witkop, 1961). The carbon terminal dodecamer (Figure 2) was constructed by solid-phase peptide synthesis using Merrifield's (1963) *t*-Boc protocol in a Biosearch 9600 peptide synthesizer. Side-chain protecting groups were removed by HF, and oxidation of the cysteines was effected by air to yield the disulfide (Figure 2). The dodecamer was identified and isolated by reverse-phase HPLC using a Dynamax 300A C_{18} column (Rainin Co.) and eluted with a gradient starting with 100% H_2O (0.1% TFA) and progressing to 100% CH_3CN (0.1% TFA) linearly over a 30-min period with a flow rate of 1.0 mL/min.

Solution Stability Assays. Protein stability assays were conducted by dissolving rPST or derivatized PST in pH 7.4 phosphate-buffered saline (PBS) containing 0.05% sodium azide at concentrations between 10 and 300 mg/mL. The solutions were clarified by centrifugation at 10000*g* for 5 min. The supernatant was then distributed in 100-μL aliquots into 50-mL polypropylene centrifuge tubes which were hermetically sealed. These samples were incubated for varying lengths of time at 39 or 43 °C. At appropriate times, duplicate samples were removed and charged with 20 mL of PBS to suspend any precipitated material. The samples were then incubated at room temperature for 2 h with intermittent stirring. Aliquots were removed and clarified as above and the soluble protein (dimer and monomer) quantitated by gel filtration using size exclusion HPLC on Superose 12 (Pharmacia LKB). The protein was eluted with an aqueous solution containing 10% propanol, 0.6% NaCl, and 0.2% Tris-HCl adjusted to pH 11.0 with concentrated NaOH at a flow rate of 0.6 mL/min. Other samples from these stability studies were taken for SDS–PAGE according to the method of Laemmli (1970). Dimers of rPST, formed during the stability studies, were purified on a 2.5 × 100-cm Sephacryl 200HR column (Pharmacia LKB) in 0.1 M ammonium bicarbonate and freeze-dried. Quantitation of PST was also accomplished by extinction at 280 nm: a solution at 1 mg/mL in PBS has an optical density of ca. 0.68. For stability results, variations between duplicate samples were less than 5%. Most derivatives were assayed at least twice, and the stabilities observed were consistent.

Hypox Rat Assay. The biological potency of the PST derivatives was determined in the hypox rat assay as described by Groesbeck and Parlow (1987) with the following modifications: rats were housed for 1 week prior to the initiation of the test; daily body weights were not determined, but animals growing faster than the mean were eliminated; no animals were eliminated on the basis of absolute body weight or daily gain at the completion of the experiment; and a single daily sc dose of drug was administered instead of two to four daily doses.

RESULTS AND DISCUSSION

The solution thermostability of rPST is an important consideration in the development of a commercial formulation since any practical device must be small (<2 cm^3) and contain a relatively large amount of protein. Upon hydration of such a device (implant), which occurs shortly after insertion into the animal's tissues, the protein will exist at high concentrations (>500 mg/mL) and be exposed to elevated temperatures (39 °C for swine). A practical device will have to deliver biologically effective levels of rPST (>3 mg/day) for at least 28 days. Consequently, we initially looked at the solution stability of rPST dissolved in PBS at 39 °C over extended periods and analyzed the soluble portion by size exclusion HPLC. When rPST samples were incubated at increasing concentrations for 14 days, the fraction of soluble monomeric protein decreased precipitously (Figure 3). The samples were characterized by a transition from a clear solution after dissolution to a translucent, thixotropic gel by day 14. This aggregated protein, which was not redissolved by dilution into PBS, was responsible for a considerable loss of the monomeric protein at the higher concentrations employed. Analysis of these soluble protein fractions (day 14) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) revealed that depletion of monomeric rPST also resulted from the formation of a covalent dimeric species with a molecular weight of ca. 44 000, a

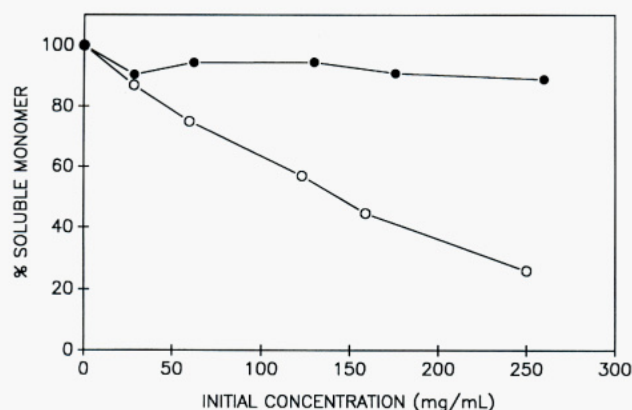


Figure 3. Concentration-dependent solution thermostability of CM-PST and rPST at 39 °C. The proteins were dissolved in PBS at increasing concentrations, incubated in duplicate for 14 days, and analyzed as described under Methods. The soluble monomer is plotted as a percentage of the starting protein at zero time. (○) rPST; (●) CM-PST.

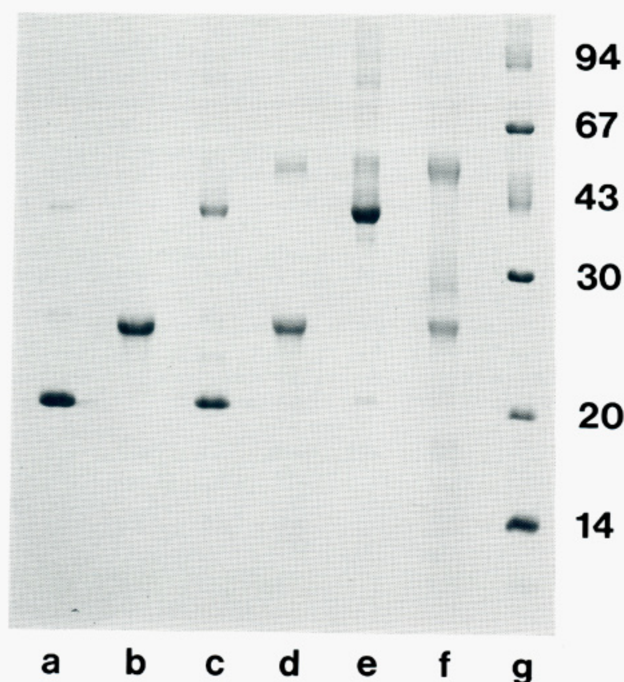


Figure 4. SDS–PAGE of samples of recombinant PST. A 15% acrylamide gel was used according to the method of Laemmli (1970). Samples (10 μg each) were (a) technical recombinant PST, (b) as (a) with β-mercaptoethanol, (c) the soluble fraction of recombinant PST after 14 days at 43 °C in PBS, (d) as (c) with β-mercaptoethanol, (e) PST dimer purified by gel filtration, (f) as (e) with β-mercaptoethanol, and (g) low molecular mass standards (approximate masses in kilodaltons are shown to the right of the gel).

fraction of which was clearly reducible (Figure 4). Thus, dimerization contributed to the formation of protein aggregates resulting, at least in part, from a disulfide-exchange reaction. We anticipated that suppression of the dimerization might enhance the overall solution stability of the protein.

Disulfide linkages frequently contribute to the stabilization of protein secondary and tertiary structure. Therefore, the effect of each disulfide on both stability and activity, as well as the chemical selectivity of the derivatization, had to be considered. The disulfide bonds of somatotropins have been studied since the late 1960s when Li's group showed that reduced and derivatized human somatotropin (HST) retained its biological activity (Bewley et al., 1968a,b). HST was shown to form a tetracarboxymethyl derivative at cysteine positions when reduced and alkylated with iodoacetic acid under nondenaturing conditions. This modified form was claimed to exhibit growth-promoting properties comparable to those of the native protein. In contrast, BST forms a dicarboxymethyl derivative when reductively alkylated under nondenaturing conditions (Graf et al., 1975). Derivatization of BST likewise does not alter the biological activity.

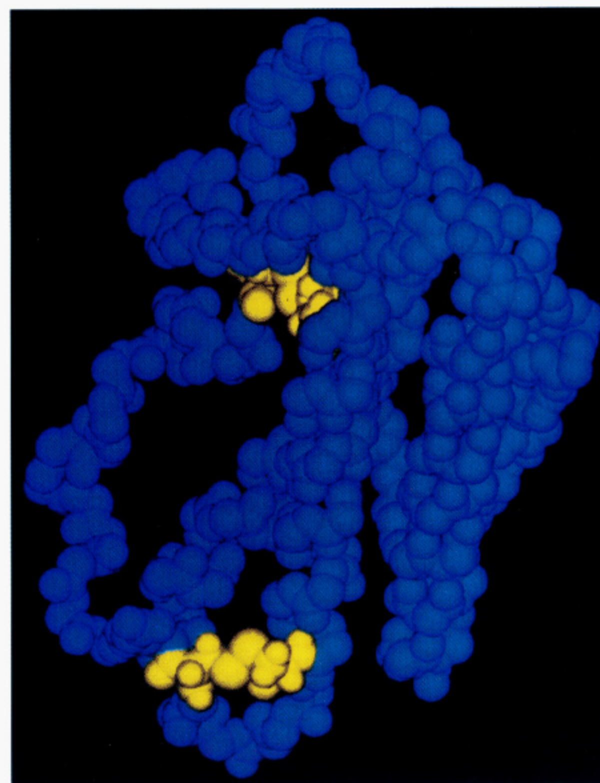
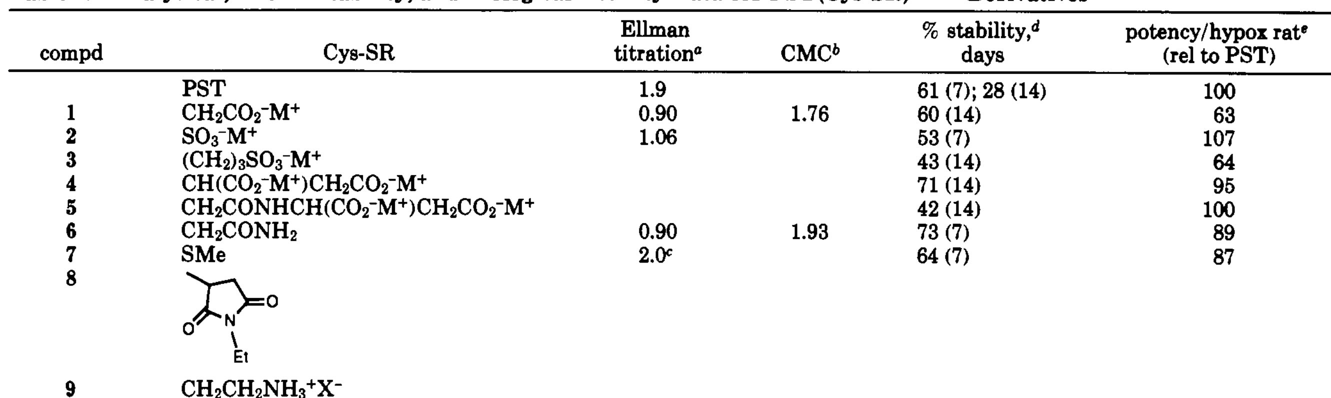


Figure 5. Space-filling model of PST backbone. The two disulfide linkages are yellow. The more accessible disulfide is the Cys^{181}–Cys^{189}.

PST possesses a high degree of homology to BST (approximately 67%) and would be expected to behave similarly. To assess the potential for selective alkylation of one of the two disulfides, we examined a model of the protein. The low-resolution X-ray diffraction structure of rPST has been solved and reveals a globular form consisting of four antiparallel helices. (Abdel-Meguid et al., 1987). The atomic coordinates, however, have not been published. The crystal structure of HST has also been solved recently (Pavlovskii et al., 1989). They, however, published only the Cα coordinates. We built a model of rPST by superimposing the published Cα coordinates upon an idealized α-helix. The helices were rotated to maximize the amphiphilic interactions. The loop conformations were constructed and refined with stereodiagram programs. Finally, the resulting model was subjected to molecular mechanics minimization. The result of these manipulations is shown in Figure 5.

The model clearly shows the differences between the two disulfides. One disulfide links Cys^{53} and Cys^{164} (large loop) and is partially buried in the core of the protein. The second disulfide links Cys^{181} and Cys^{189} in the C terminus (small loop) and is exposed to the external milieu. On the basis of both literature precedents and our evaluation of this model, we believed that selective modification of this "small loop" disulfide was feasible.

Table I. Analytical, Thermostability, and Biological Activity Data for PST(Cys-SR)[181,189] Derivatives



compd	Cys-SR	Ellman titration[a]	CMC[b]	% stability,[d] days	potency/hypox rat[e] (rel to PST)
	PST	1.9		61 (7); 28 (14)	100
1	$CH_2CO_2^-M^+$	0.90	1.76	60 (14)	63
2	$SO_3^-M^+$	1.06		53 (7)	107
3	$(CH_2)_3SO_3^-M^+$			43 (14)	64
4	$CH(CO_2^-M^+)CH_2CO_2^-M^+$			71 (14)	95
5	$CH_2CONHCH(CO_2^-M^+)CH_2CO_2^-M^+$			42 (14)	100
6	CH_2CONH_2	0.90	1.93	73 (7)	89
7	SMe	2.0[c]		64 (7)	87
8	(N-ethylsuccinimide structure: O, N, Et)				
9	$CH_2CH_2NH_3^+X^-$				

[a] Ellman titration for disulfide bonds (theoretical = 1.0 or 2.0 (PST)). [b] Amino acid analysis for (carboxymethyl)cysteine (theoretical = 2.0). [c] Theoretical = 3.0 disulfides. [d] Data in parentheses represent days on incubation at 43 °C. [e] Single measurement precision determined from 10 pooled rBST positive control assays indicate that ±42% from the positive control defines the 95% confidence limits.

Initially, rPST was reduced with dithiothreitol in PBS and the reduced cysteines alkylated with iodoacetic acid under the conditions described by Bewley et al. (1968b). The resulting carboxymethylated protein (CM-PST) proved substantially more robust; after 14 days in the stability assay, less than 5% dimerization was detected compared to approximately 30% produced by the recombinant protein (data not shown). The solution stability of the monomeric form was also improved (Figure 3). At ca. 250 mg/mL, 89% of the CM-PST remained in solution compared with only 26% of rPST, a 3.4-fold improvement. Similar results were obtained when the protein was alkylated using iodoacetamide (CAM-PST). Furthermore, daily injections of the CM-PST into the hypophysectomized rats (Groesbeck and Parlow, 1987), which were used to assess the intrinsic biological activity, elicited growth similar to that of rPST (Table I). No significant alteration of the CD spectra of CM-PST was noted (data not shown), suggesting the conformation of the protein was not significantly perturbed by the derivatization. This is consistent with observations made on CAM-BST (Holzman et al., 1986).

Several approaches were taken to characterize the derivatized product. While the homology with bovine somatotropin and the model suggests reduction should proceed selectively at the C-terminal disulfide, the earlier papers on total reduction of HST raised lingering doubts. Titration of CAM-PST with Ellman's reagent (Creighton, 1989) indicated one disulfide remained in the product (see Table I). This conclusion was further buttressed by amino acid analysis of the modified protein which revealed the existence of two residues of (carboxymethyl)cysteine in the hydrolysate. (Under the hydrolysis conditions, unmodified cysteine suffered sufficient degradation to prevent quantitation.)

The presence of a methionine (Met) residue at position 179, along with the relative scarcity of Met at other sites, aided the identification of the modified disulfide. Cyanogen bromide (CNBr) cleaves proteins selectively adjacent to Met residues. CNBr-mediated cleavage of CAM-PST produced a 12-residue C-terminal fragment that contained both derivatized cysteines of the small loop disulfide (Figure 2). The terminal dodecapeptide was one of only two small fragments produced and hence was easily separated by reverse-phase HPLC from the large molecular weight fragments. The authentic dodecamer was prepared by solid-phase peptide synthesis and the disulfide produced by air oxidation. A sample of the authentic dodecamer was then reduced and alkylated with iodoacetic acid. Reverse-phase HPLC of the purified CNBr

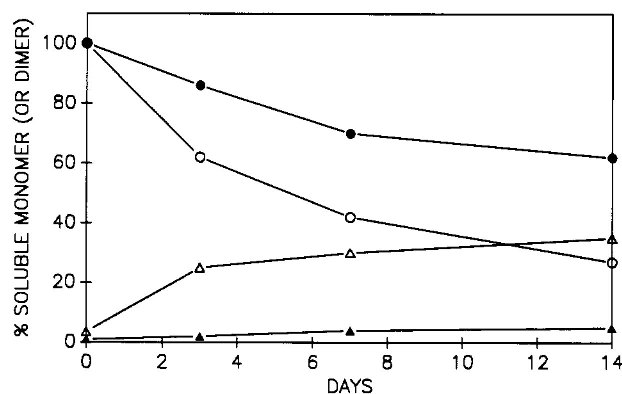


Figure 6. Solution thermostability of rPST and CM-PST initially dissolved at 100 mg/mL in PBS and then incubated at 43 °C. The soluble monomeric form of each species as the percentage of the original protein is represented by the upper curves: (○) rPST; (●) CM-PST. The extent of the dimerization of these species as a percentage of the protein remaining in solution is represented by the lower curves: (△) rPST; (▲) CM-PST. Data points were generated from duplicate analyses.

product exhibited a peak that coincided with the alkylated synthetic dodecamer (not shown). No peak coincident with the unmodified peptide could be detected. The combined results of these divergent approaches strongly point to Cys[181]–Cys[189] as the sole reactive site.

Since stability of the protein was important to establish beyond 14 days, and to limit the duration of stability studies, we developed a more demanding assay for solution stability. Protein was dissolved as before in PBS at ca. 100 mg/mL and incubated at 43 °C for various periods of time up to 14 days. The results of such a study comparing CM-PST and rPST are shown in Figure 6. The stability after 14 days at 43 °C was equivalent to 28 days at 39 °C (data not shown).

Carboxymethylation and carbamidomethylation of rPST, however, did not completely resolve the stability problem. Depletion of monomeric CM-PST was still evident (Figure 6), although dimer formation was essentially suppressed: approximately 60% of CM-PST remained in solution and monomeric after 14 days compared to <20% for rPST. This initial success prompted us to consider other cysteine modifications since selectivity has always been the major limitation on site-specific chemical modification of proteins. (Cysteine, with its highly nucleophilic thiomethyl side chain, has always been the exception.) Also, many cysteine-specific reagents have been identified and used in structural studies. However, their utilization for protein modification with retention of activity has ap-

parently been limited by concern for effects on secondary and tertiary structure. The results with CM-PST and CAM-PST suggested that such concerns may not always be justified in practice.

While dithiothreitol (DTT) is the most commonly used reagent for reduction of disulfides, large excesses of DTT are required. This, in turn, requires a correspondingly larger excess of derivatizing reagent, which hardly promotes selectivity. Tributylphosphine (Ruegg and Rudinger, 1977) is widely reported to be an excellent stoichiometric reagent for reduction of disulfides: however, low aqueous solubility limits its use. Levinson et al. (1969) reported the reduction of disulfides with tris(hydroxymethyl)phosphine and tris(carboxyethyl)phosphine, two water-soluble phosphine derivatives. We have found both are excellent reducing agents which could be used in almost stoichiometric quantities.

Having established that CM- and CAM-PST (Table I; 1 and 6) would likely be more stable than rPST in a controlled-release device and that these modified proteins retain their biological activity, a representative sampling of the known cysteine-selective reagents was investigated (Kenyon and Bruice, 1977; Lundblad and Noyes, 1984; Means and Feeney, 1971). We hoped first to identify alkylating agents which maximized stability and ultimately to optimize their effect. Aside from their susceptibility to nucleophilic attack by thiolate anions, these reagents have little in common. Our initial strategy was to examine a variety of them and attempt to identify some element which helped suppress rPST aggregation. While it seemed unlikely that a small perturbation of the macromolecule could significantly alter its stability in solution, the charge on the derivatization reagent would clearly influence the surface charge of the protein. A second approach exploited the efficient transformation of α-haloacids with cysteine while utilizing the carboxylic acid moiety as a tether (vide infra).

The results are compiled in Table I. Compounds 1–3 and 6 were produced by reaction with iodoacetic acid, sodium tetrathionate, 1,3-propanesultone, and iodoacetamide, respectively. Compound 7 is a mixed disulfide produced by reaction of the reduced disulfide with methylthiomethanesulfonate. Compound 8 was readily obtained from the well-known reaction of *N*-alkylmaleimides with the thiomethyl side chain. Finally, the basic 2-aminoethyl side chain was appended with *N*-(2-iodoethyl)-trifluoroacetamide (compound 9). The latter two adducts could not be isolated. In all cases, derivatization was limited to the small loop disulfide. Attempts to produce tetraalkylated PST derivatives by incorporation of a denaturing agent into the reaction produced only insoluble intractable material.

The solution stability of these compounds varies greatly, and the variation is not readily correlated with their structures. The data in Table I, while not definitive, did suggest that incorporation of carboxylic acid side chains produced a significant improvement. The most dramatic difference was the effect of the acidic vs the basic side chain. The reasons for the failure of the structurally related anionic sulfonic acid derivatives (2 and 3) to afford comparable stability are not clear. The sulfur derivatives differ from carboxylic acids in charge density and the length of the linker between the charged atom and the protein backbone. The formation of the S-sulfite (2) is a potentially reversible reaction and we have no proof of its retention under the conditions of the stability assay. They were, however, both significantly more stable than rPST.

While the perturbation produced by the alkylating agent may seem small relative to reduction of the disulfide, conversion of the neutral disulfide to a bis(carboxylic acid) will alter the surface charge and thereby the isoelectric point of the protein. In addition, localized ion pairing could also be altered. Altered solubility, state of aggregation, or conformation is a known consequence of charge alteration. (Means and Feeney, 1971). The surface charge on the protein directly influences the isoelectric point of the protein. The isoelectric point, p*I*, is the pH which results in a net neutral charge on the protein. This property influences the solubility of the protein, which frequently is minimized at the p*I*. The rationale is that minimization of the surface charge also minimizes the electrostatic repulsion between protein molecules, enhancing their ability to approach each other and form an aggregate. Altering the pH results in a surplus of either positive or negative charges which increase repulsive forces between the molecules. If the newly incorporated charges are close to a contact point, the added repulsion might produce a stabilizing effect by hindering the approach of two proteins and thereby limit aggregation.

We attempted to ascertain whether additional carboxyl groups would further enhance the stabilization. The most straightforward approach to introducing a dicarboxylic acid in place of the carboxymethyl moiety is to use a halosuccinic derivative. For example, bromosuccinate reacted with reduced rPST; however, the rate was slow, and unreacted thiol groups could be detected after several days. The more reactive iodide, prepared by subjecting bromosuccinic acid to a Finkelstein reaction, was much more efficient. Although the reaction was slower than that with iodoacetic acid (primary vs secondary halide), the reaction did completely block the thiol groups in about 24 h (compound 4, Table I). Utilization of iodoacetic acid as a linker afforded an even more convenient approach. The highly reactive primary α-iodocarbonyl is retained, and the carboxylic group was exploited as a mooring for other functionalities. In this case di-*t*-Boc-aspartic acid and IAA were coupled with dicyclohexylcarbodiimide (DCC) and subsequently deprotected with trifluoroacetic acid (TFA). The resulting amide reacted rapidly with rPST to yield compound 5 (Table I). Both 4 and 5 exhibited substantially improved stability relative to rPST.

Neutral side-chain incorporation into rPST yielded unpredictable solution stability (compounds 6 and 7 vs 8, Table I). CAM-PST is virtually identical to CM-PST in stability, while the maleimide (8) could not be isolated. The neutral mixed disulfide (7) is intermediate in stability but also is potentially reversible if free thiols were formed during the stability test. Despite the lack of consistency, it was tempting to speculate that the anionic side chain was a stabilizing factor.

The attachment of polymeric surface modifiers to proteins has been an area of intensive research (Veronese, 1989; Inada et al., 1990). Modification of proteins often markedly alters the chemical and physical properties of the protein. Shielding hydrophilic or hydrophobic segments of the protein can change physical properties such as solubility, which subsequently can alter the pharmacokinetics of the compound in vivo. Masking a proteolytic site on the protein can slow degradation of the protein and extend the half-life of the compound. Similarly, undesirable immunological responses to foreign proteins can be attenuated if the relevant epitope is shielded by the polymer. The list of proteins that have been linked to polymers is long. One particularly relevant example is L-asparaginase, which is used clinically for the treatment

Table II. Thermostability and Biological Activity Data for Polyethylene Glycol Derivatives of PST

PST(Cys-SR)[181,189]	% stability,[a] days	% potency (hypox rat)[b] (rel to PST)
$CH_2CO_2(CH_2CH_2O)_nMe$		
1: $M_w \sim 2000$	52 (7); 39 (14)	107
2: $M_w \sim 5000$	19 (7)	
$CH_2CONH(CH_2CH_2O)_nMe$		
3: $M_w \sim 550$	55 (7); 42 (14)[c]	178[d]
4: $M_w \sim 2000$	78 (7); 63 (14)[c]	125
5: $M_w \sim 5000$	0 (7)[c]	82
rPST	61 (7); 28 (14)	100

[a] Data in parentheses represent days of incubation at 43 °C. [b] See Table I, footnote *e*. [c] Initial monomer concentration in these studies was only 50 mg/mL. [d] Significantly different from the positive control.

of lymphocytic leukemia and malignant lymphosarcoma. The polyethylene glycol (PEG) conjugate was found to decrease the prevalence of anti-L-asparaginase antibodies (Fuertges and Abuchowski, 1990).

A major obstacle to utilization of this technology has been chemical selectivity. The free amine of lysine residues has been the primary site of attachment. A variety of strategies have been devised to link polymer chains to lysine residues. These include succinylation of the polymer and subsequent coupling of the nascent carboxylic acid to lysine utilizing methodology developed for polypeptide synthesis, e.g., coupling of active esters such as *N*-hydroxysuccinimide/DCC couplings, acyl azides, cyanuric chloride, and syn-triazine based reagents. The success of any of these procedures is critically dependent upon the level of substitution achieved, as overreaction can result in significant diminution of the biological activity. Since lysine is an abundant component of many proteins and the reactivities of most lysines will be similar, the only way to control the reaction is by careful optimization of variables such as reaction time.

Polyethylene glycol monomethyl ether has been the most extensively utilized polymer. It is commercially available in a variety of chain lengths, has only one chemically reactive site at the terminus of the chain, is biologically inert, and has unique physical properties by virtue of its amphiphilic character. Many other polymers have also been investigated and range from highly hydrophilic dextran to more hydrophobic polypropylene glycol. For our purposes, the carboxylic acid of (carboxymethyl)cysteine affords a very adaptable functional group to tether macromolecules. PEG iodoacetates were easily prepared by DCC-mediated coupling, and we initially investigated two PEG molecular weights. The resulting esters readily alkylated the reduced small loop cysteines as facilely as iodoacetic acid. The reaction was monitored by SDS–PAGE, which characterized the derivative by the increase in its molecular weight (not shown). The stability and biological activity data of these PEG derivatives are compiled in Table II (compounds 1 and 2). Neither of the two esters prepared for this study was substantially more stable than rPST. The higher molecular weight ester was, in fact, significantly less stable than rPST.

Although the failure of the PEG esters to enhance PST solution stability appears to eliminate their utility in a parenteral product, the modified proteins were substantially less soluble than PST, and this behavior might be exploited from a formulation perspective. As a result, the synthesis was repeated on a larger scale to produce material for implants. The only isolated product from this reaction migrated at the same rate as CM-PST on SDS–PAGE rather than the slower moving material observed previously. Since no unreacted cysteine was detected by Ellman titrations and the adduct exhibited equivalent optical extinction to rPST at 280 nm, the results indicated that the PEG ester was susceptible to rapid hydrolysis.

To further confirm this, the initial pilot-scale reaction was repeated with careful control of the pH during both the reaction and subsequent chromatographic workup. Again, the most diagnostic feature of this product, confirmed by SDS–PAGE, was a substantially higher molecular weight protein (ca. 80 000) and was identical to the initially prepared PEG derivative. When this was incubated at pH 8.0 in 0.02 M sodium phosphate and monitored by SDS–PAGE, the product was rapidly converted to a new form with electrophoretic mobility intermediate to those of the starting material and CM-PST. This intermediate underwent a slow transformation over a 5-day period to a product whose electrophoretic mobility was identical to that of CM-PST. The Cys[181] is surrounded by three basic residues (Figure 2), while Cys[189] has no charged neighbors. Either hydrolytic scission of the ester in this highly basic microenvironment or intramolecular lactam formation initiated by one of the basic side chains on the activated carbonyl moiety could easily account for the differential stability of the two PEG esters.

Such hydrolytic instability can be circumvented by replacing the ester linkage with the corresponding amide. The appropriate PEG amines were easily prepared via the corresponding azides (Zalipsky et al., 1983), and DCC-mediated coupling efficiently formed the required iodoacetamides. Derivatization of the cysteine residues with these agents proceeded normally, and the products showed no hydrolytic instability. The stability data and biological activity of these derivatives (compounds 3–5, Table II) were similar to the previous observations made with PEG esters. The solubility was substantially lower than that of the native protein, which necessitated lowering the initial concentration below that in the standard stability assay. While the derivative having a molecular weight of 2000 appeared to be more stable than the corresponding ester (it retained its high molecular weight throughout the stability assay), the lower starting concentration would be expected to decrease the aggregation rate. Also consistent with the ester results was the trend toward lower solution stability with the longer chain derivatives.

Although attachment of polyethylene glycol was not an effective solution to the PST stability problem, the utilization of iodoacetic acid derivatives of cysteine does afford a novel general approach for protein modification. The relative lack of abundance of cysteine compared to lysine and other amino acids and the high degree of selectivity possible with cysteine-selective reagents circumvent the problems associated with overderivatization of lysine. Without question, recombinant techniques have made a vast array of specifically altered proteins available. Chemical modification, however, has an inherent flexibility which is not limited by the structure of the 20 naturally occurring amino acids. The combination of unique polymeric modifiers and derivatization reagents affords a nearly unlimited variety of new proteins.

Even for proteins lacking a nonessential reactive cystine, a combination of recombinant techniques and chemical derivatization holds promise. Mutants containing cysteine at carefully planned surface-accessible sites can likely be engineered. This strategy recently was applied to IL-2 by a group of investigators at Cetus (Goodson and Katre, 1990). Our results with CM- and CAM-PST indicate that relatively simple derivatization at only two positions can produce a profound improvement in protein solution stability.

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