# Self-Association Properties of Monomeric Insulin Analogs Under Formulation Conditions

**Jane P. Richards,[1,2] Mary P. Stickelmeyer,[1] David B. Flora,[1] Ronald E. Chance,[1] Bruce H. Frank,[1] and Michael R. DeFelippis[1,3]**

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***Purpose.*** The purpose of the current study was to investigate the effects of two important excipients, zinc and *m*-cresol, on the self-association properties of a series of monomeric insulin analogs. In this way, the effects on formulation behavior of individual amino acid substitutions in the C-terminal region of the insulin B-chain could be compared.

***Methods.*** The self-association of ten insulin analogs was monitored by equilibrium and velocity analytical ultracentrifugation under three different conditions: (i) in neutral buffer alone; (ii) in neutral buffer containing zinc ion; and (iii) in neutral buffer containing both zinc ion and phenolic preservative (a typical condition for insulin formulations). The self-association properties of these analogs were compared to those of human insulin and the rapid-acting insulin analog $Lys^{B28}Pro^{B29}$-human insulin.

***Results.*** The analogs in the current study exhibited a wide range of association properties when examined in neutral buffer alone or in neutral buffer containing zinc ion. However, all of these analogs had association properties similar to human insulin in the presence of both zinc and *m*-cresol. Under these formulation conditions each analog had an apparent sedimentation coefficient of $s^* = 2.9–3.1$ *S*, which corresponds to the insulin hexamer.

***Conclusions.*** Analogs with changes in the B27–B29 region of human insulin form soluble hexamers in the presence of both zinc and *m*-cresol, and *m*-cresol binding overrides the otherwise destabilizing effects of these mutations on self assembly.

**KEY WORDS:** monomeric insulin analogs; analytical ultracentrifugation; peptide formulation; insulin association.

## INTRODUCTION

Insulin association is a complex phenomenon. In the absence of zinc, insulin forms dimers and higher order aggregates (1,2). X-ray crystallography has shown the dimer interface to include an anti-parallel beta strand between the C-terminal ends of the two B-chains (3). In the presence of zinc, three insulin dimers form a hexamer by coordinating two zinc ions through the histidine side chains of the B10 residues. The association of insulin in pharmaceutical preparations is further influenced by the addition of phenolic preservatives included to protect against microbial contamination. These phenolic compounds bind to a hydrophobic pocket formed between dimers within the insulin hexamer and facilitate a conformational change in the N-terminal region of the B-chain (4,5). This conformational transition further stabilizes the insulin hexamer and improves pharmaceutical solubility and stability (6–8).

Although hexamer formation is important for stabilizing insulin formulations, there are some pharmacological drawbacks associated with the dissolution of hexamers into active monomers following injection. There is an approximately 30–60 minute lag phase following injection of regular human insulin and onset of activity, necessitating careful timing of dose administration and food consumption. In addition, there is a slow clearance in which extended periods of elevated insulin can lead to hypoglycemia. As a result of these limitations, "monomeric insulin analogs" have been designed and characterized (9,10) and the relationship between reduced self-association and rapid onset of action established (11–14). One monomeric insulin analog, $Lys^{B28}Pro^{B29}$-human insulin (Humalog®), has been fully developed and is commercially available. $Lys^{B28}Pro^{B29}$-insulin was created by inverting the two amino acids in the B28 and B29 position of the C-terminal region of the B-chain. This sequence disrupts dimer formation and yields an insulin molecule that cannot form a specific population of hexamers, even in the presence of zinc ion (10).

The lack of dimerization and higher order association poses a general formulation problem for monomeric insulin analogs. The insulin hexamer possesses less solvent exposed surface area than in the monomer or dimer and is therefore more stable to physical and chemical degradation (6,7,15,16). Monomeric or dimeric insulin solutions have a greater tendency toward deamidation, degradation and fibril formation (7,16). Surprisingly, in the presence of both zinc and phenolic preservative, $Lys^{B28}Pro^{B29}$-insulin does form stable hexamers and exhibits the increased stability of human insulin hexamers while maintaining its rapid-action (17–20). Upon injection, $Lys^{B28}Pro^{B29}$-insulin hexamers are proposed to dissociate more rapidly than human insulin as preservative and zinc ions diffuse away and the hexamer falls apart (18,19). Therefore, this "monomeric," rapid-acting insulin analog retains its desirable pharmaceutical properties despite being formulated as a hexamer.

$Lys^{B28}Pro^{B29}$-insulin is just one of several analogs with changes in the C-terminal region of the B-chain that were synthesized to create a rapid-acting insulin analog (9,10). However, the association properties of only $Lys^{B28}Pro^{B29}$-insulin have been characterized under formulation conditions in any depth (19,20). The purpose of the current study was to determine the self-association properties of additional insulin analogs with modifications in the C-terminal region of the B-chain in an effort to understand better the properties governing insulin association and to expand our ability to design and formulate insulin analogs with specific functions.

## MATERIALS AND METHODS

### Preparation of Insulin Analogs

The insulin analogs (except $Asp^{B10}$ and $desPro^{B28}$) were constructed by trypsin-catalyzed reverse proteolysis essentially as described by Kubiak and Cowburn (21). Briefly, desoctapeptide insulin ($desB^{23-30}$) was prepared by tryptic digestion of

[1] Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

[2] Current address: Baxter Hemoglobin Therapeutics, Inc., Boulder, Colorado 80301.

[3] To whom correspondence should be addressed. (e-mail: defelippis_michael_r@lilly.com)

**ABBREVIATIONS:** DPI, despentapeptide insulin ($des^{B26-30}$); $s^*$, apparent sedimentation coefficient; *S*, Svedberg unit.

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porcine insulin (22). An octapeptide containing the new analog sequence ($B^{23-30}$) was synthesized by conventional solid phase synthesis using tert-Butoxycarbonyl (Boc) amino acids (Applied Biosystems). The peptide was condensed to desoctapeptide insulin by a trypsin-catalyzed semisynthesis reaction to form the full-length insulin analog ($A^{1-21}$ $B^{1-30}$). The analogs were purified by a series of gel permeation and reversed phase chromatography steps to at least 98% purity as measured by analytical HPLC. The calculated physical properties for the analogs are given in Table I. The $Asp^{B10}$ and $desPro^{B28}$ analogs were constructed by chain combination of recombinant A chain and the respective synthetic B chain (23).

The purified and lyophilized analogs were dissolved at 10–20 mg/ml in 0.01 M HCl and then diluted into the respective sedimentation buffer. For the samples run in buffer alone, the analogs were diluted into a buffer such that the final concentrations were 0.5–3.5 mg/ml analog, 7 mM sodium phosphate (pH = 7.2), 16 mg/ml glycerol, 50 mM NaCl and 1 mM EDTA. The final pH was adjusted to pH = 7.2–7.4 with 0.1 N NaOH. For the samples run in the presence of zinc ion, the protein was diluted into a buffer such that the final concentrations were 3.5 mg/ml analog (~100 U/ml), 7 mM sodium phosphate (pH = 7.2), 16 mg/ml glycerol, 50 mM NaCl, and 0.0175 mg/ml zinc ion (= 0.5% wt zinc ion per wt analog or approximately 2.7 molar equivalents of zinc per hexamer). The final pH was adjusted to pH = 7.2–7.4 with 0.1 N NaOH. Sodium chloride was included to minimize non-ideality artifacts that occurred during equilibrium sedimentation performed at high analog concentration and low ionic strength (data not shown). For the samples run in formulation buffer containing zinc ion and preservative, 3.15 μl of *m*-cresol (final concentration ~ 3.15 mg/ml *m*-cresol) were added to 1 ml of a solution containing 3.5 mg/ml analog, 7 mM sodium phosphate (pH = 7.2), 16 mg/ml glycerol, 50 mM NaCl and 0.0175 mg/ml zinc ion.

## Equilibrium Sedimentation

The association properties of the insulin analogs in the presence of buffer alone or in the presence of buffer containing zinc ion but no preservative were characterized by equilibrium sedimentation using a Beckman XL-A or XL-I analytical ultracentrifuge. The samples were loaded into 1.2 cm or 0.3 cm double sector cells (depending on protein concentration) for an initial UV absorbance at 276 nm of 0.5–1.0. The experiments were run at 16,000 rpm (for associating analogs) or 22,000 rpm (for predominantly monomeric analogs) and 22°C. Equilibrium was reached in 16–20 hours and was ascertained by a satisfactory overlap of two scans taken 4 h apart. Absorbance was monitored at 276 nm. The data were collected with Optima software provided by Beckman. The data were then used to calculate the relative molecular weight per monomer molecular weight (MW/M1) versus analog concentration (mg/ml) according to the following formula (2): $MW = RT/(1-V\rho)\omega^2 * 1/r * 1/C * dC/dr$, where MW is the analog weight average molecular weight, R is the gas constant, T is the absolute temperature, V is the partial specific volume, ρ is the solvent density, r is the radial position and *C* is the analog concentration. The calculated extinction coefficients for the analogs are given in Table I, the partial specific volume was assumed to be the same as for insulin, 0.73 ml/g (10), and the solvent density was measured to be 1.006 g/ml.

**Table I.** Calculated Physical Properties of Insulin Analogs

| Analog | MW (Da) | Ext. Coefficient[a] | pI |
|---|---|---|---|
| Human Insulin | 5808 | 1.051 | 5.51 |
| $Lys^{B28}Pro^{B29}$ | 5808 | 1.051 | 5.51 |
| $Ala^{B28}$ | 5782 | 1.055 | 5.51 |
| $Asp^{B28}Pro^{B29}$ | 5795 | 1.053 | 4.54 |
| $desLys^{B29}$ | 5679 | 1.074 | 4.93 |
| $desPro^{B28}$ | 5711 | 1.068 | 5.51 |
| $Ile^{B28}Pro^{B29}$ | 5793 | 1.053 | 4.93 |
| $Pro^{B29}$ | 5777 | 1.056 | 4.93 |
| $Pro^{B27}Thr^{B28}$ | 5808 | 1.051 | 5.51 |
| $Asp^{B10}$ | 5786 | 1.055 | 4.55 |
| DPI ($des^{B26-30}$) | 5217 | 0.900 | 4.93 |

[a] Extinction Coefficient at 276 nm for a 1 mg/ml solution in a 1 cm pathlength cell.

## Velocity Sedimentation

The association properties of the insulin analog in buffer containing zinc or in buffer containing both zinc and preservative were characterized by velocity sedimentation and monitored by absorbance (for buffer containing zinc) or interference (for buffer containing zinc and *m*-cresol) optics in an XL-I analytical ultracentrifuge. The samples were loaded into 1.2 cm double sector cells with the respective formulation buffer in the reference sector. Quartz windows were employed for the absorbance experiments and sapphire windows were used with the interference optics. The experiments were performed at 50,000 rpm and at 22°C. The data were analyzed by the whole boundary time derivative method developed by Stafford (24) and provided with the Beckman Optima software package version 3.01 h. The apparent differential sedimentation coefficient distribution functions, $g(s^*)$ versus $s^*$, were calculated from the time derivative as described by Stafford (25). The reported apparent sedimentation coefficient, $s^*$, for each analog (Table II) represents the peak of this profile as determined using a computer data reader tool. Similar sedimentation coefficients were obtained for the samples in the presence of zinc and *m*-cresol by manual calculation from photographic plates using a Beckman Model E ultracentrifuge equipped with Schlieren optics system (data not shown).

**Table II.** Apparent Sedimentation Coefficients, s*, Obtained from the Peak of the g(s*) Function

| Analog | plus 0.5% zinc | plus zinc and m-cresol |
|---|---|---|
| Human insulin | 3.0 | 3.0 |
| $Lys^{B28}Pro^{B29}$ | ~3.9 heterogeneous | 3.0 |
| $Ala^{B28}$ | 3.2 | 3.1 |
| $Asp^{B28}Pro^{B29}$ | ~2.5 heterogeneous | 2.9 |
| $desLys^{B29}$ | 2.9 | 3.0 |
| $desPro^{B28}$ | 2.9 | 3.0 |
| $Ile^{B28}Pro^{B29}$ | ~2.7 heterogeneous | 2.9 |
| $Pro^{B29}$ | 2.8 | 3.0 |
| $Pro^{B27}Thr^{B28}$ | 3.0 | 3.0 |
| $Asp^{B10}$ | ~1.2 heterogeneous | 1.2; 4.3 |
| DPI ($des^{B26-30}$) | 5.0 | 3.4–5.4 |

## RESULTS

Several insulin analogs were studied to evaluate the contribution of the C-terminal region of the insulin B-chain to self-association and formulation behavior. These alterations focused around the B27, B28 and B29 positions, because these positions have been frequently targeted in efforts to design pharmaceutically useful insulin analogs (9,10). Changes in these residues can have a dramatic effect on insulin association and pharmacodynamics, while having little effect on receptor binding (26). The criteria used to select the analogs for this study were: (i) the analogs were amenable to construction by semisynthetic techniques; and (ii) the amino acid changes focused on elucidating the individual contributions of the lysine and proline in positions B28 and B29. Two other analogs, $Asp^{B10}$ and despentapeptide insulin ($des^{B26\text{-}30}$ or DPI), illustrate different approaches to reducing insulin association and were included in this study for comparison. Figure 1 illustrates the analog B-chain amino acid sequences. Human insulin and $Lys^{B28}$-$Pro^{B29}$-insulin served as the controls representing the two commercial standards.

### Neutral Buffer Alone

Equilibrium sedimentation was performed in the absence of zinc and preservative for at least three different analog concentrations and the composite of weight average molecular weight per monomer molecular weight (MW/M1) versus concentration is plotted in Figure 2. The analogs exhibited varying degrees of association in neutral buffer alone. Most of the analogs are considered "monomeric" in the sense that they have a significantly reduced tendency to self-associate at low concentrations (9,10). There are some notable exceptions. The $desLys^{B29}$ analog, which retains the Pro at position B28, shows probable dimer formation at low concentrations in a manner similar to human insulin, but $desLys^{B29}$ shows less aggregation at increasing concentrations. $Asp^{B10}$, which retains the native sequence in the dimerization domain, forms soluble dimers under these conditions but does not form higher order species. By characterization in neutral buffer alone, these analogs fall into three categories: (i) those that are soluble and relatively non-associated at concentrations up to 3–4 mg/ml ($Lys^{B28}Pro^{B29}$, $Asp^{B28}Pro^{B29}$, $Pro^{B29}$, $Ile^{B28}Pro^{B29}$ and $Asp^{B10}$); (ii) those that are soluble but aggregate (human insulin, $desLys^{B29}$, $desPro^{B28}$, $Ala^{B28}$, and $Pro^{B27}Thr^{B28}$); and (iii) those that are insoluble and precipitate, preventing sedimentation analysis (DPI, data not shown).

Velocity sedimentation was not performed on these analogs in the absence of zinc and preservative because the molecular weight of monomeric insulin (5808 Da) is too low and the solutions are too heterogeneous for obtaining sedimentation coefficients under these conditions.

### Neutral Buffer Containing Zinc Ion

The association properties of the insulin analogs in the neutral buffered solution containing 0.5% zinc ion (per wt analog) were first evaluated by equilibrium sedimentation (Figure 3). Calculation of the weight average molecular weight versus concentration (with an initial loading concentration of 3.5 mg/ml) shows a concentration distribution from approximately 1–5 mg/ml. Only $desLys^{B29}$ shows evidence of hexamer formation at lower concentrations in a manner similar to human insulin. However, unlike $Lys^{B28}Pro^{B29}$ which aggregates into high molecular weight species greater than hexamer in the presence of zinc, some of the other monomeric insulin analogs appear to form hexameric complexes at higher concentrations as the plot of MW/M1 versus concentration approaches six.

In order to determine whether these analogs were forming discrete hexamers or a heterogeneous mixture of aggregates, velocity sedimentation was also performed on the analogs in the presence of neutral buffered solution containing zinc. Whole boundary analysis by the time derivative method (24,25) yields a differential sedimentation coefficient distribution function, $g(s^*)$ versus $s^*$, which gives insight into the association state and the degree of heterogeneity of these analogs (Figure 4 and Table II). At 3.5 mg/ml (~100 U/ml) and in the presence of 0.5% zinc ion, several of the analogs ($desLys^{B29}$, $desPro^{B28}$, $Pro^{B27}Thr^{B28}$, $Pro^{B29}$, and $Ala^{B28}$) have sedimentation coeffi-

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human Insulin | F | V | N | Q | H | L | C | G | S | H | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | Y | T | P | K | T |
| $Lys^{B28}Pro^{B29}$ | F | V | N | Q | H | L | C | G | S | H | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | Y | T | K | P | T |
| $Ala^{B28}$ | F | V | N | Q | H | L | C | G | S | H | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | Y | T | A | K | T |
| $Asp^{B28}Pro^{B29}$ | F | V | N | Q | H | L | C | G | S | H | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | Y | T | D | P | T |
| $desLys^{B29}$ | F | V | N | Q | H | L | C | G | S | H | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | Y | T | P | T | |
| $desPro^{B28}$ | F | V | N | Q | H | L | C | G | S | H | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | Y | T | K | T | |
| $Ile^{B28}Pro^{B29}$ | F | V | N | Q | H | L | C | G | S | H | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | Y | T | I | P | T |
| $Pro^{B29}$ | F | V | N | Q | H | L | C | G | S | H | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | Y | T | P | P | T |
| $Pro^{B27}Thr^{B28}$ | F | V | N | Q | H | L | C | G | S | H | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | Y | P | T | K | T |
| $Asp^{B10}$ | F | V | N | Q | H | L | C | G | S | D | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | Y | T | P | K | T |
| DPI ($des^{B26\text{-}30}$) | F | V | N | Q | H | L | C | G | S | H | L | V | E | A | L | Y | L | V | C | G | E | R | G | F | F | | | | | |

**Fig. 1.** Amino acid sequence of the insulin analog B-chains. The A-chain amino acid sequence is unchanged from human insulin (not shown). The altered residues are boxed.

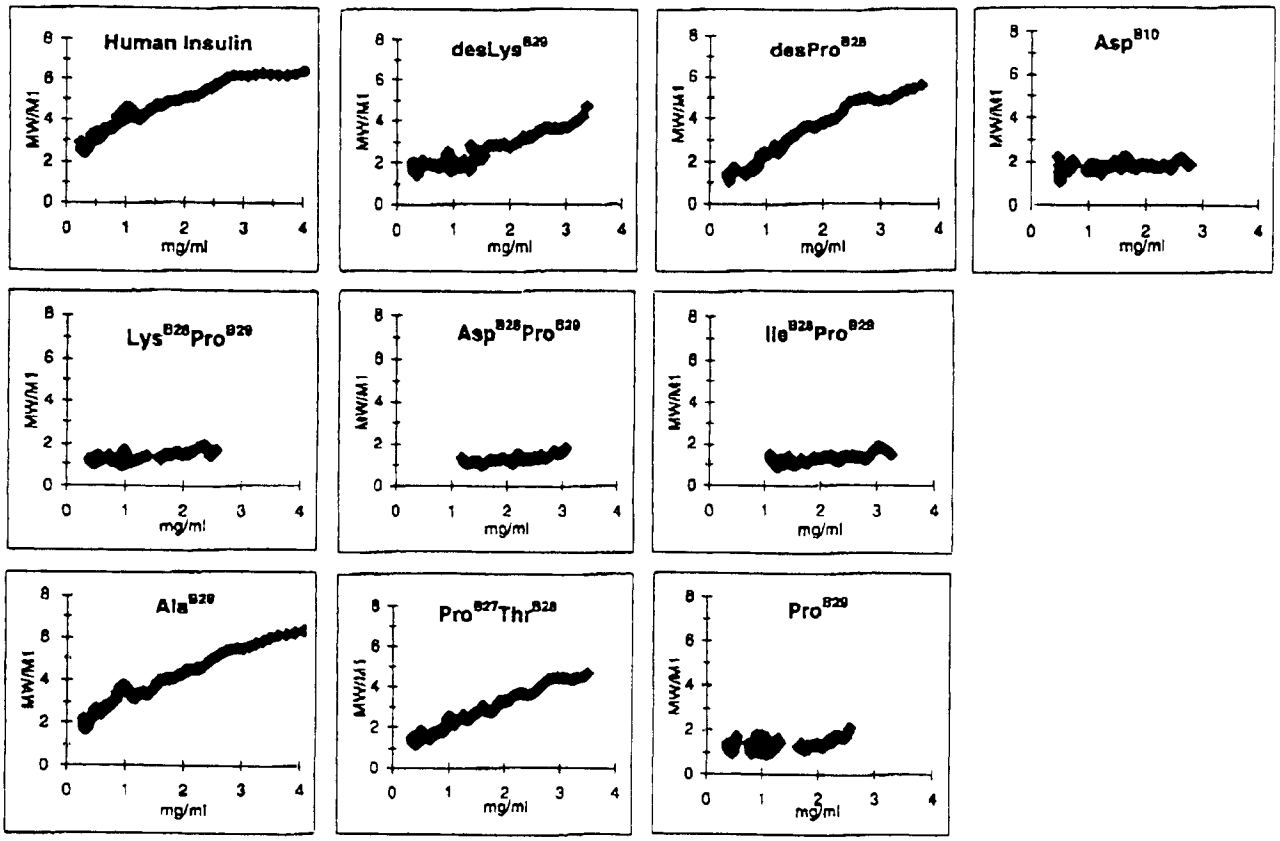


**Fig. 2.** Equilibrium sedimentation of insulin analogs in neutral buffer. The apparent weight average molecular weight per monomer molecular weight (MW/M1) is plotted against analog concentration (mg/ml). A composite of at least three different loading concentrations is shown for each analog.

cients similar to human insulin (2.9–3.1 *S*) which forms soluble hexamers (19,20). The visible shoulder on the left side of some of these curves ($Pro^{B29}$, $desPro^{B28}$, $Ala^{B28}$) indicates some heterogeneity and suggests the presence of species smaller than hexamers, and the right shoulder on $Ala^{B28}$ suggests the presence of higher molecular weight species larger than hexamers. $Lys^{B28}Pro^{B29}$ is highly heterogeneous and exhibits aggregates larger than hexamers under these conditions, while $Asp^{B28}Pro^{B29}$ and $Ile^{B29}Pro^{B29}$, also heterogeneous under these conditions, tend toward lower molecular weight species smaller than hexamers. The $Asp^{B10}$ analog appears predominantly dimeric (Figure 3) and does not form higher associated species under these conditions, since it is unable to coordinate zinc through the histidine residue normally found at position B10. DPI was highly aggregated with one discernible sedimentation coefficient around 5.0 and a significant shoulder of low molecular weight species. DPI precipitated at higher concentrations (> 4 mg/ml).

### Neutral Buffer Containing Zinc Ion and *m*-Cresol

The association properties of the analogs in the presence of both zinc ion and the phenolic preservative *m*-cresol were measured by velocity sedimentation using interference optics. The equilibrium properties of the analogs under these conditions were not assessed since the presence of *m*-cresol prevented the use of the absorbance optics and thus prevented calculation of MW/M1 versus concentration. The apparent sedimentation coefficients, $s^*$, are listed in Table II and are reported as the peak of the sedimentation coefficient distribution function, $g(s^*)$ vs. $s^*$ (Figure 5). All of the analogs characterized with alterations in the B27–B29 were soluble and had sedimentation coefficients similar to human insulin (2.9–3.1 *S*). The analogs typically showed less heterogeneity in the presence of zinc and *m*-cresol than in the presence of zinc but no *m*-cresol.

The $Asp^{B10}$ analog forms stable dimers, but is unable to form zinc-coordinated hexamers because it lacks the histidine side chain required for zinc binding which is normally found in position B10 (Figure 4). Nevertheless, the association properties of $Asp^{B10}$ change dramatically in the presence of zinc and *m*-cresol (Figure 5). At least two sedimentation peaks can be observed in the $g(s^*)$ vs. $s^*$ functions with $s^*_1 = 1.2$ and $s^*_2 = 4.3$. We also characterized the effect of zinc and preservative on the association of the truncated despentapeptide insulin (DPI). DPI has a very broad, heterogeneous peak. However, it is interesting to note that the overall solubility of DPI is improved in the presence of *m*-cresol (data not shown).

## DISCUSSION

Over the past decade a number of insulin analogs with reduced self-association properties have been constructed in an effort to create an insulin therapy with a faster onset of action and more rapid clearance than regular human insulin. The com-

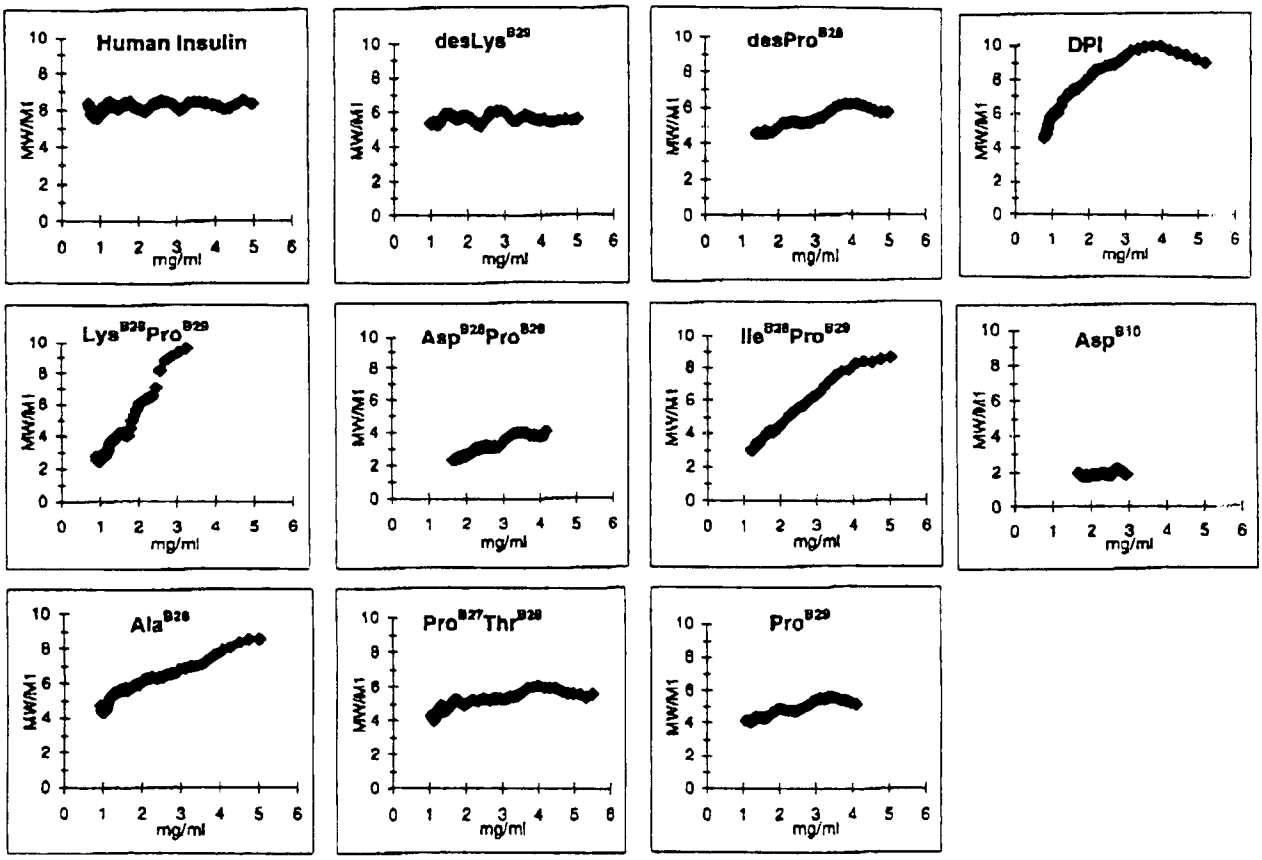


**Fig. 3.** Equilibrium sedimentation of insulin analogs in neutral buffer containing 0.5% per weight zinc ion. The axes are as in Figure 2. A single loading concentration of 3.5 mg/ml analog is presented.

mercial availability of one such analog (Lys$^{B28}$Pro$^{B29}$-insulin) and the clinical testing of other analogs (*i.e.* Asp$^{B28}$ and Asp$^{B10}$ insulin) (11,12) are testimony to the success of this approach. A look back at development of Lys$^{B28}$Pro$^{B29}$-insulin, which was formulated as a stable hexamer yet maintained the rapid-action of a monomeric insulin preparation, underscores the importance of coordinating protein design and formulation development in the production of protein pharmaceuticals. However, few other monomeric insulin analogs have undergone the rigorous biophysical and structural characterization of Lys$^{B28}$Pro$^{B29}$-insulin (18–20), and rarely has a whole series of analogs been systematically characterized under the same formulation conditions.

The self-association and formulation behavior of both human insulin and Lys$^{B28}$Pro$^{B29}$-insulin have been extensively characterized by a wide range of biophysical techniques in addition to analytical ultracentrifugation. These techniques include light scattering (19,27), spectroscopy (20), circular dichroism (10,19), and X-ray crystallography (3,18). Human insulin and Lys$^{B28}$Pro$^{B29}$-insulin are therefore two well-characterized systems which provide a context for interpreting the analytical ultracentrifuge data presented in the current study. For example, a sedimentation coefficient of approximately 3.0 *S* corresponds to the insulin hexameric species as previously determined by light scattering in conjunction with analytical ultracentrifugation (19). For the following discussion, we consider it reasonable to assume that other insulin analogs with sedimentation coefficients in this range also exist as hexamers.

In the presence of neutral buffered phosphate containing glycerol and sodium chloride, and in the absence of zinc or phenolic preservative, the insulin analogs showed a wide range of solubility and self-association behavior. Of the analogs studied, only Asp$^{B10}$ and desLys$^{B29}$ appeared to retain a propensity to form dimers at concentrations similar to human insulin (lower limit of our presented experiments using absorbance optics was approximately 0.3 mg/ml). Asp$^{B10}$ has been previously shown to form stable dimers but loses the ability to coordinate zinc through the His$^{B10}$ residue and thus cannot form zinc-coordinated hexamers (9). Asp$^{B10}$ serves as a control to test our ability to distinguish between monomer and dimer in this assay. The ability of desLys$^{B29}$, in which the B29 lysine is removed and the B30 threonine is effectively moved to position B29, to self-associate is consistent with an earlier study where des$^{B29-30}$ insulin was shown to retain its self-association properties (10). Together these findings indicate that residues B29 and B30 can be removed without affecting insulin self-association. On the other hand, desPro$^{B28}$, in which the B28 proline is removed resulting in a shift to Lys$^{B28}$Thr$^{B29}$, shows reduced self-association in the absence of zinc and preservative. The properties of desPro$^{B28}$ are consistent with those of des$^{B28-30}$ (10), which also exhibits disrupted self-association, and underscore the importance of the proline at position B28 in normal insulin dimerization.

Further evidence of the importance of the B28 proline comes from studying the Xaa$^{B28}$Pro$^{B29}$ insulin analogs. The

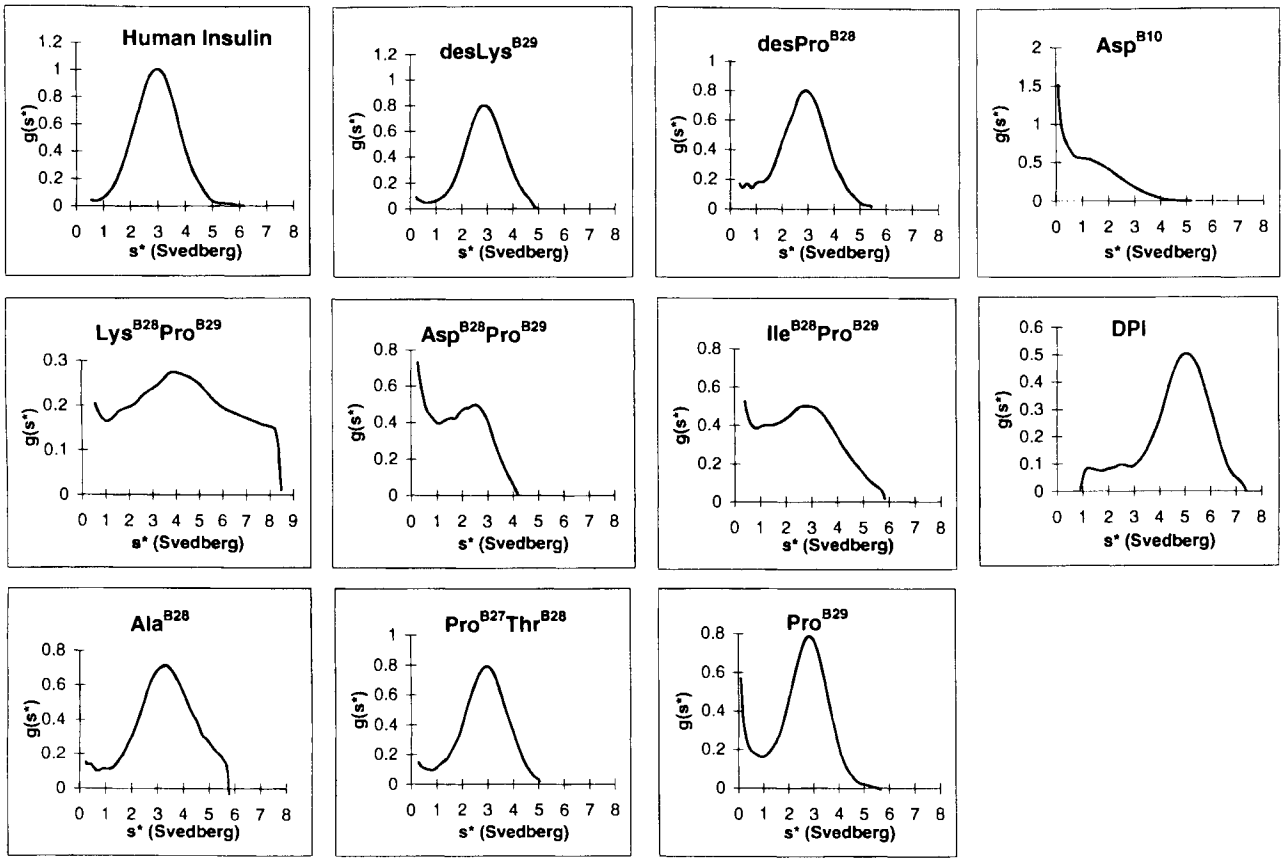


**Fig. 4.** Velocity sedimentation of insulin analogs in neutral buffer containing 0.5% per weight zinc ion. The sedimentation coefficient distribution function, g($s^*$) versus $s^*$, is shown. The loading concentration was 3.5 mg/ml analog.

Xaa$^{B28}$Pro$^{B29}$ series of analogs, in which a proline is introduced at position B29 and the residue in position B28 is varied, are particularly disrupted in their ability to dimerize and self-associate in the absence of zinc and preservative. Here we compared the effects of positive charge (Lys$^{B28}$Pro$^{B29}$), negative charge (Asp$^{B28}$Pro$^{B29}$), and a hydrophobic residue (Ile$^{B28}$Pro$^{B29}$). Regardless of the nature of the amino acid at position B28, no significant self-association was detected in the absence of zinc and preservative when the proline residue was introduced at B29. Furthermore, these three analogs showed little tendency toward specific, zinc-coordinated hexamer formation in the absence of preservative. Interestingly, the single amino acid substitution Pro$^{B29}$ and the inversion Pro$^{B27}$Thr$^{B28}$, while still disrupted in dimer formation in the absence of zinc, showed greater tendency toward hexamer formation in the presence of zinc, although in the case of Pro$^{B29}$ a significant shoulder of low molecular weight species was also present. Removing the proline from position B28 in addition to introducing a proline at position B29 has the greatest effect on disrupting insulin self-association. Introducing the proline at position B27 appears less disruptive.

Although the Lys$^{B28}$Pro$^{B29}$ rapid-acting insulin analog does not form a homogeneous population of soluble hexamers in the presence of zinc without preservative (19,20), several of the analogs in the current study (desLys$^{B29}$, desPro$^{B28}$, Pro$^{B27}$Thr$^{B28}$, Pro$^{B29}$, and Ala$^{B28}$) appear to form zinc-coordinated hexameric complexes, as deduced from their apparent sedimentation coefficients of $s^* = 2.9$–$3.1$ $S$ and homogeneity of the g($s^*$) distribution function in the absence of preservative.

All of the analogs with changes in the B27–B29 region, including the highly disrupted Xaa$^{B28}$Pro$^{B29}$ analogs, formed soluble complexes with sedimentation coefficients similar to human insulin in the presence of zinc and *m*-cresol. Plots of the g($s^*$) versus $s^*$ function indicate that the addition of *m*-cresol reduces the overall heterogeneity observed in the presence of zinc but without preservative (compare Figure 4 and Figure 5). Therefore, *m*-cresol appears to stabilize all hexameric insulin analog complexes.

For the purpose of this study, we explored the effects of only one phenolic preservative, *m*-cresol, on hexamer formation. Both phenol and *m*-cresol have been shown to induce hexamer formation in Lys$^{B28}$Pro$^{B29}$-insulin (19), and Lys$^{B28}$-Pro$^{B29}$ has been shown to exhibit the same order of phenolic ligand binding affinities as human insulin, resorcinol >> phenol >*m*-cresol (20). It is reasonable to suggest that other preservatives would also stabilize hexamer formation for the above monomeric insulin analogs, although relative binding affinities remain to be measured.

The Asp$^{B10}$ analog exhibited some unusual association behavior under the tested formulation conditions. Asp$^{B10}$ was primarily dimeric in neutral buffer alone or in neutral buffer containing zinc ion. However, with the addition of *m*-cresol as well as zinc, two sedimentation coefficients could be resolved. The lower value, $s^* = 1.2$, likely represents the dimer, whereas

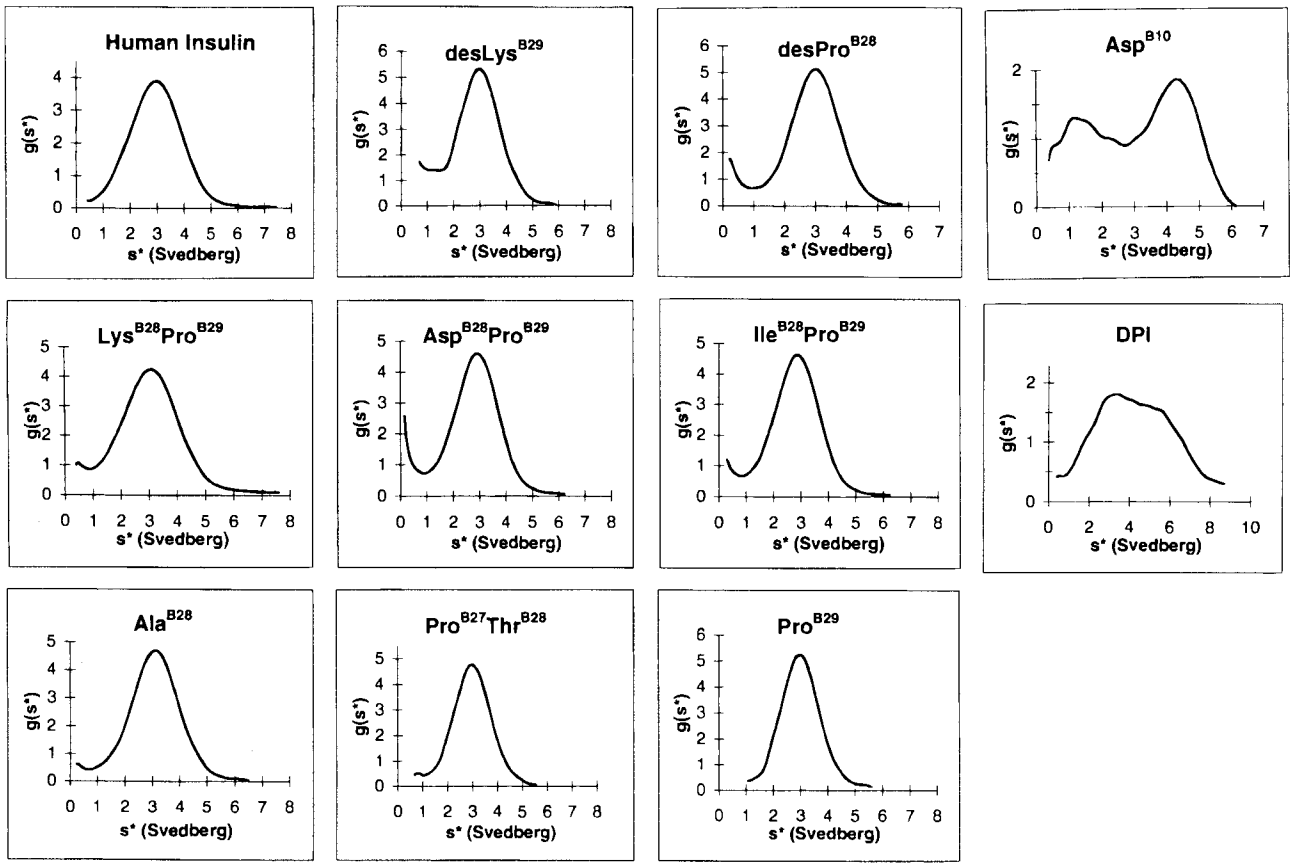


**Fig. 5.** Velocity sedimentation of insulin analogs in neutral buffer containing 0.5% per weight zinc ion and 3.15 mg/ml *m*-cresol. The sedimentation coefficient distribution function, g($s^*$) versus $s^*$, is shown. The loading concentration was 3.5 mg/ml analog.

the higher sedimentation coefficient, $s^* = 4.3$ may represent the Asp$^{B10}$ dodecamer observed in the crystal structure (28). Although the final crystal structure has not been published, the Asp$^{B10}$ dodecamer is proposed to be held together by zinc ions coordinated through the His$^{B5}$ residue (28). The addition of *m*-cresol in the absence of zinc did not induce formation of the higher molecular weight species (data not shown).

## Pharmaceutical Implications

An obvious question to raise when evaluating the formulation characteristics of protein analogs is: how do formulation characteristics correlate with *in vivo* activity? Unfortunately, for practical reasons, only a handful of insulin analogs have been tested in complex animal models or human trials. Nevertheless, a few observations can be made. Decreased self-association has been accompanied by a more rapid time action profile, and a correlation between the degree of self-association and time-action has been demonstrated (11). Data are not available to determine whether there is a sufficient difference in the self-association properties of the B27–B29 insulin analogs characterized in this study to result in a measurable difference in *in vivo* time-action within this series of analogs. With the exception of desLys$^{B29}$, we would predict all of the included analogs to be faster acting than human insulin.

In addition to the development of improved rapid-acting insulin therapies, an important application of characterizing the formulation characteristics of a wide range of insulin analogs is toward the development of improved insulin delivery systems. Significant effort is underway to improve and develop alternative delivery devices in order to meet the needs of an increasing population of diabetic patients (29). Current efforts include the development of transdermal delivery systems (which are accompanied by molecular size and charge limitations), oral formulations, nasal or bronchial administration, as well as improved constant delivery devices. Each new technology will require specially formulated insulin preparations that optimize delivery and availability, minimize interactions with device surfaces, and meet the usual requirements of long-term chemical and physical stability. Understanding the self-association properties of a variety of insulin analogs in the presence of important excipients is the first step toward developing new insulin analogs for improved therapies.

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