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Physicochemical basis for the rapid time-action of $Lys^{B28}Pro^{B29}$-insulin: Dissociation of a protein–ligand complex

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Abstract

The rate-limiting step for the absorption of insulin solutions after subcutaneous injection is considered to be the dissociation of self-associated hexamers to monomers. To accelerate this absorption process, insulin analogues have been designed that possess full biological activity and yet have greatly diminished tendencies to self-associate. Sedimentation velocity and static light scattering results show that the presence of zinc and phenolic ligands (*m*-cresol and/or phenol) cause one such insulin analogue, $Lys^{B28}Pro^{B29}$-human insulin (LysPro), to associate into a hexameric complex. Most importantly, this ligand-bound hexamer retains its rapid-acting pharmacokinetics and pharmacodynamics. The dissociation of the stabilized hexameric analogue has been studied in vitro using static light scattering as well as in vivo using a female pig pharmacodynamic model. Retention of rapid time-action is hypothesized to be due to altered subunit packing within the hexamer. Evidence for modified monomer–monomer interactions has been observed in the X-ray crystal structure of a zinc LysPro hexamer (Ciszak E et al., 1995, *Structure 3*:615–622). The solution state behavior of LysPro, reported here, has been interpreted with respect to the crystal structure results. In addition, the phenolic ligand binding differences between LysPro and insulin have been compared using isothermal titrating calorimetry and visible absorption spectroscopy of cobalt-containing hexamers. These studies establish that rapid-acting insulin analogues of this type can be stabilized in solution via the formation of hexamer complexes with altered dissociation properties.

Keywords: allosteric transition; in vivo activity; ligand binding; oligomer dissociation; protein structure

Since the introduction of insulin in the 1920s as a treatment for diabetes (Banting & Best, 1922), continuous strides have been made to improve overall therapy. Major advances in insulin purity, availability, and therapeutic efficacy have taken place (Brange et al., 1990). Despite these improvements, subcutaneous injection therapy still falls short of providing the patient with convenient regulation and normalized glycemic control. Specifically, frequent excursions from normal glycemic levels over a patient's lifetime result in several degenerative complications for diabetics (The Diabetes Control and Complications Trial Research Group, 1993). To help avoid these extreme glycemic levels, diabetics often practice multiple-injection therapy whereby insulin is administered with each meal. However, this therapy has not been optimized; the most rapid-acting insulin commercially available exhibits maximal activity too late after injection and lasts too long to control glucose levels optimally. Therefore, much effort has been devoted recently to creating novel insulin analogues that have a more rapid onset and shorter duration of activity (Brange et al., 1991; DiMarchi et al., 1992); related efforts to develop long-acting insulin preparations, typically crystalline suspensions, are also being pursued by other researchers.

One example of the short-acting insulin analogue class, and the subject of these studies, is $Lys^{B28}Pro^{B29}$-human insulin (LysPro) where the human insulin sequence at residues B28 and B29 has been inverted. Insulin analogues, such as LysPro, are designed to increase the kinetics of the rate-limiting steps of the subcutaneous absorption process (Binder, 1983) for insulin solutions (i.e., allow for the rapid dissociation of hexamers and dimers into monomers).

The absorption of soluble insulin after subcutaneous injection is a complex pharmacokinetic process that is governed by several

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variables. One factor that plays a pronounced role in insulin absorption is the association state (Binder, 1983). Regular insulin, the most rapid-acting insulin commercially available, is a solution of zinc-insulin hexamers. Zinc is added to promote association to hexamers and increase chemical stability (Brange, 1987). Upon injection of this solution in vivo, an initial rate-limiting lag phase occurs. The transport of insulin hexamers from the subcutaneous depot into the capillary membrane is thought to be restricted by steric hindrance (Binder, 1969; Pramming et al., 1984). Dilution within the interstitial space promotes dissociation of the hexamer complex into monomeric units, resulting in less restrained uptake (Brange et al., 1990). Any means by which this dissociation process could be expedited would be expected to accelerate the onset of activity. For instance, altering either the association state of insulin or ease with which an insulin hexamer dissociates to monomeric units after injection would result in faster diffusion and less restricted transport through the capillary membrane. This altered dissociation process makes insulin analogues such as LysPro of unique therapeutic value.

Historically, phenolic preservatives (*m*-cresol, phenol, and mixtures of *m*-cresol and phenol) are added to insulin solutions to reduce microbiological contamination. However, it has been shown that these phenolic ligands also affect insulin conformation and association (Brange, 1987) by binding to the insulin hexamer and inducing an allosteric conformational change. The ligand-bound state is known as the R-state, and the apo-form is known as the T-state after the nomenclature of Monod et al. (1965). Because the binding constant for phenolic ligands is weaker than the binding constant for zinc (Goldman & Carpenter, 1974; McGraw & Lindenbaum, 1990), it is thought that injection of a solution of R-state insulin hexamers must first convert to T-state hexamers, followed by dissociation.

In addition to the solution state studies of insulin association discussed above, single crystal X-ray diffraction studies have provided insight into the protein interactions that are critical to insulin self-association. Studies of insulin dimerization indicate that hydrophobic interactions and hydrogen bonding are responsible for stabilizing association (Fredericq, 1956; Jeffrey & Coates, 1966; Ciszak et al., 1995). The hexamer is mainly stabilized through zinc coordination, but additional polar and nonpolar residues are buried between the dimers as a result of hexamer assembly (Baker et al., 1988). The predominant nonpolar dimer contacts involve the C-terminal end of the B-chain, with B23–B26 and B28 being the most significant (Blundell et al., 1972; Baker et al., 1988). Association of the dimer is secured by the small antiparallel β-sheet of hydrogen bonds involving residues B24 and B26 (Baker et al., 1988). In addition, the importance of the C-terminus of the B-chain to dimerization is further evidenced by removal of B26–B30 in despentapeptide insulin, which does not alter the rest of the molecule significantly but does prevent dimer formation (Bi et al., 1984).

To manipulate insulin association, a series of analogues were synthesized having the lysine at B29 replaced with proline and the amino acid at B28 varied (Brems et al., 1992a). The LysPro analogue ($Lys^{B28}Pro^{B29}$-human insulin) was shown to be fully potent, exhibited decreased association in solution and displayed faster onset of action compared to human insulin (DiMarchi et al., 1992).

The physicochemical basis for the time action of LysPro has not been explored in detail. However, an X-ray structure of a LysPro hexamer has been solved recently (Ciszak et al., 1995). In this structure, the LysPro hexamer is found to have an analogous conformation

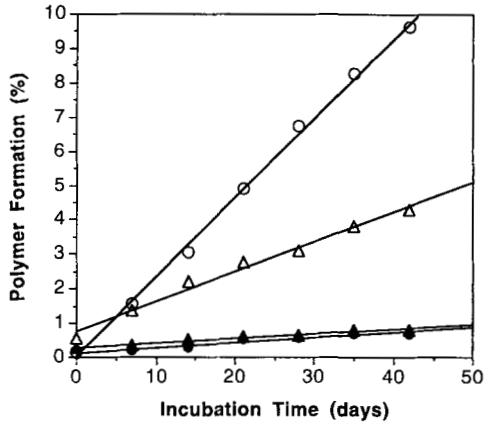

Fig. 1. Chemical degradation as monitored by percent polymer formation after incubation at 30 °C for various times: hexameric LysPro (+ zinc, + phenolic ligand, ●), hexameric insulin (+ zinc, + phenolic ligand, ▲), monomeric LysPro (− zinc, + phenolic ligand, ○) and zinc-free insulin (− zinc, + phenolic ligand, △). All samples contained 3.5 mg/mL protein, 1.25 mg/mL *m*-cresol, 1.09 mg/mL phenol, 16 mg/mL glycerol, and 7 mM phosphate, pH 7.4. The zinc-containing samples had 19.7 μg/mL zinc.

to the $T_3R_3^f$ structure[5] observed in insulin (Smith & Ciszak, 1994). The inversion of the amino acids at B28 and B29 causes disruptions of hydrophobic interactions that occur at the monomer–monomer interface. In addition, the hydrogen bonds at both ends of the β-sheet structure near the C-terminus of the B chain are weakened.

Although LysPro has proven to be fast-acting upon injection, soluble zinc-free preparations require stabilization with respect to chemical degradation to be of practical use (Brems et al., 1992b). The results presented here demonstrate that the addition of both zinc and phenolic ligands stabilize LysPro by promoting hexamer formation. Furthermore, the results were largely independent of the identity of the phenolic ligand (*m*-cresol, phenol, or a mixture of *m*-cresol and phenol). In the hexamer state, LysPro has chemical stability equivalent to that of human insulin while retaining its increased propensity to dissociate. The altered dissociation property preserves the desired, accelerated time-action of LysPro.

Results

Chemical stability

The chemical degradation of various laboratory-prepared insulin and LysPro solutions at 30 °C was studied using size-exclusion chromatography to measure the formation of high molecular weight polymers. The formation of disulfide-linked polymers has been shown to be the principal chemical degradation pathway for insulin (Brange, 1992; Brange et al., 1992) and chemical degradation results for LysPro are consistent with this mechanism. As shown in Figure 1, zinc-free LysPro shows the greatest rate of degradation of all the solutions studied, with a 1.6% per week increase in polymer formation over the six-week study. Zinc-free insulin undergoes a slower rate of polymer formation (0.6% per week), although even this rate of degradation is not acceptable for pharmaceutical use.

[5]The nomenclature used has been described in Ciszak et al. (1995), where "f" is used to indicate that the N-terminal helix is frayed, i.e., does not extend completely to the end of the N-terminus.

When zinc and phenolic ligands are added to insulin or LysPro solutions, however, the rate of high molecular weight polymer formation is reduced significantly (0.1% per week for insulin and LysPro).

Sedimentation velocity

To explore the association state of insulin and LysPro under different solution conditions, sedimentation velocity was utilized. The solvent- and temperature-corrected sedimentation coefficient, $s_{20,w}$, obtained for human insulin in the presence of zinc and phenolic ligands, was 3.4 S and for LysPro was 3.5 S. In the absence of these additives, human insulin exhibited a reduced sedimentation coefficient of 3.0 S (see Discussion). The sedimentation coefficient of LysPro in the absence of zinc and phenolic ligands was quite distinct from the other values reported (1.2 S). The association state of insulin with zinc and phenolic ligands is that of a hexamer because the sedimentation coefficient values are in agreement with those values obtained for other proteins of comparable molecular weight (Creighton, 1993). The observation that LysPro with zinc and phenolic ligands sediments in a very similar fashion suggests that the analogue is also in a hexameric state under these solution conditions. The results from static light scattering (SLS) experiments (see below) confirm the hexameric state of LysPro.

The sedimentation coefficients for the samples that do not contain zinc and phenolic ligands provide important information about their association states as well. The association state of zinc-free insulin was shown previously to be a heterogeneous mixture containing a variety of molecular weight species including hexamer (Mark & Jeffrey, 1990; Brems et al., 1992a). The heterogeneity in molecular weight species in the zinc-free human insulin sample explains the slightly smaller sedimentation coefficient compared to the homogeneous hexamer state found when zinc and phenolic ligand are present. It is important to emphasize that the sedimentation behavior of zinc- and phenolic ligand-free LysPro is strikingly different from all the other samples. This sedimentation coefficient is consistent with the analogue existing primarily in a monomeric state based on agreement with values obtained for other proteins of similar molecular weights (Creighton, 1993).

Figure 2 shows the distinctly different sedimentation velocity traces for LysPro in the presence and absence of zinc and phenolic ligands. The curves illustrate the different association states of the molecule under these conditions after 100.4 min and 93.5 min of sedimentation, respectively. Note that, after an extended period of centrifugation, there is an obvious difference in the amount of sedimentation and the shape of the profiles for the two preparations. In addition, the LysPro sample which contains zinc and phenolic ligands exhibits a pattern that is comparable to that for insulin in the presence of zinc, with or without phenolic ligand (data not shown).

Visible absorption spectroscopy

Figure 3A shows the extinction of the 574 nm absorbance signal upon titration of Co(II)-insulin and Co(II)-LysPro with a 1:1 molar mixture of *m*-cresol and phenol. The intense visible absorption spectrum that has been reported for the Co(II)-insulin hexamer in the presence of phenolic ligands and SCN^- ions exhibits a wavelength maximum at 574 nm and has been assigned to the pseudotetrahedral $Co(II)(His)_3(SCN^-)$ centers of the Co(II) R-state insulin hexamer (Roy et al., 1989). The wavelength maximum and spectral profile of the Co(II)-LysPro hexamer recorded under the same conditions appear identical to those of the Co(II)-insulin hexamer (Fig. 3B). Under saturating conditions of phenol and SCN^- (100 mM phenol, 50 mM SCN^-), both the Co(II)-LysPro and Co(II)-insulin hexamers give spectra possessing extinction coefficients near 850 M^{-1} cm^{-1} at 574 nm (data not shown). The visible spectrum of the R-state Co(II)-insulin hexamer has been shown to be extremely sensitive to perturbations in the Co(II) coordination sphere. Consequently, it is reasonable to conclude that the visible absorption spectrum observed for the Co(II)-LysPro hexamer in the presence of phenolic ligands and SCN^- ions is attributable to the same $Co(II)(His)_3(SCN^-)$ chromophore that has been characterized for insulin. The Co(II) extinction axis of Figure 3A thus quantitates the fraction of tetrahedral Co(II) centers corresponding to the given phenolic concentrations. Clearly the titration profiles of Co(II)-insulin and Co(II)-LysPro upon addition of phenolic ligands are different, illustrating the altered nature of the interaction of LysPro with phenolic ligands. Understanding the origin of this difference requires additional study.

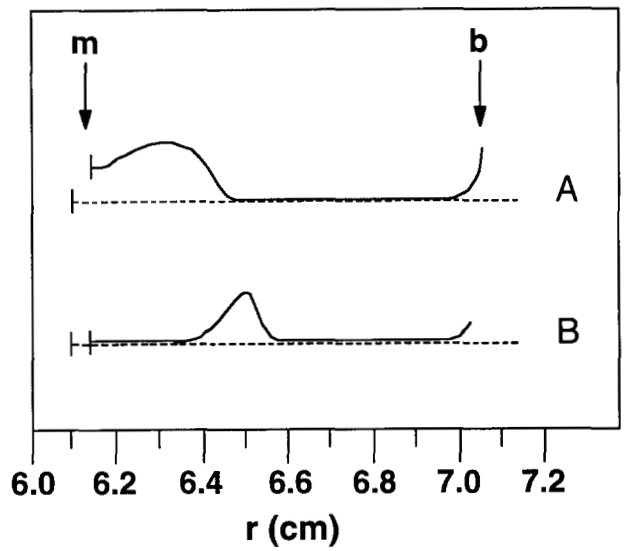

Fig. 2. Sedimentation velocity traces for monomeric and hexameric LysPro. **A:** Monomeric LysPro (− zinc, − phenolic ligand) after 100.4 min centrifugation. **B:** Hexameric LysPro (+ zinc, + phenolic ligand) after 93.5 min centrifugation. The LysPro sample with zinc and phenolic ligands had 19.7 μg/mL zinc, 1.25 mg/mL *m*-cresol, 1.09 mg/mL phenol, and 16 mg/mL glycerol. Both samples contained 3.5 mg/mL protein and 7 mM phosphate, pH 7.4.

CD

Far-UV CD spectra were collected (Fig. 4A) in order to explore changes in secondary structure between LysPro in the presence and absence of zinc and phenolic ligands (spectra could not be collected below 200 nm due to phenolic ligand absorption). The increase in negative ellipticity at 224 nm seen as phenol and *m*-cresol are added is consistent with an increase in α-helix. For insulin, phenolic ligands induce the T_6-state hexamer to convert to an R_6-state, whereby the eight N-terminal residues of the B-chain convert from an extended conformation to an α-helix (Renscheidt et al., 1984; Wollmer et al., 1987; Brader & Dunn, 1991). The ligand-induced change in ellipticity for LysPro, although smaller in

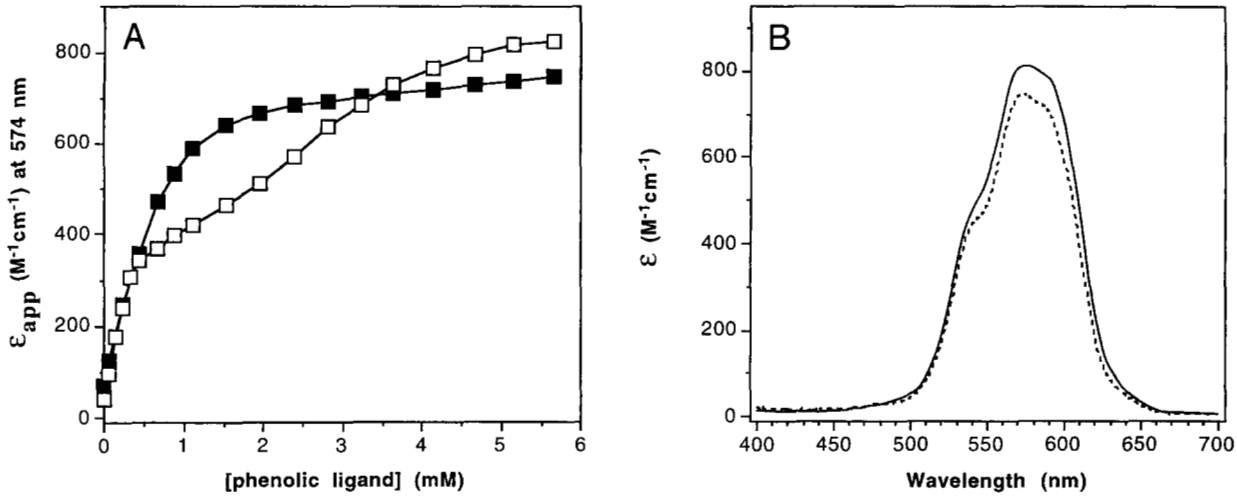

Fig. 3. Ligand-induced conformational transitions for Co(II)-LysPro and Co(II)-insulin as monitored by visible absorption spectroscopy. **A:** Apparent extinction at 574 nm for Co(II)-LysPro (■) and Co(II)-insulin (□) when titrated with 1:1 molar mixture of phenol and *m*-cresol. **B:** Visible absorption spectra at 5.5 mM phenolic ligand concentration for Co(II)-LysPro (– – – –) and Co(II)-insulin (——). Cobalt ion concentrations were used to determine extinction coefficients. Samples incorporated two cobalt ions per protein hexamer. The protein concentration was 11.6 mg/mL in 20 mM KSCN and 50 mM Tris-ClO_4, pH 8.0.

magnitude than that observed for insulin (data not shown), is indicative of an increase in α-helix. By analogy to the conformational change known to occur in insulin, this increase likely represents an extension of the B chain (B9–B19) α-helix into the N-terminal segment of LysPro.

To gain insight into changes in the environment of disulfide bonds and aromatic residues between LysPro samples with and without zinc and phenolic ligands, near-UV CD spectra were also collected (Fig. 4B). Significant changes are seen primarily at 265 nm and 251 nm. These differences probably indicate changes in the environment of aromatic residues and disulfide bonds, respectively, as has been observed for human insulin (Krüger et al., 1990). Zinc- and phenolic ligand-free LysPro is in a monomeric state (Brems et al., 1992a), whereas LysPro in the presence of zinc and phenolic ligands associates to hexamer (Fig. 2). When ligands such as phenol and *m*-cresol are added to zinc LysPro, hexamers are formed and a structural transition occurs that is similar to the T → R transition of insulin.

To explore this conformational transition of LysPro versus that of insulin in more detail, CD data were collected for various con-

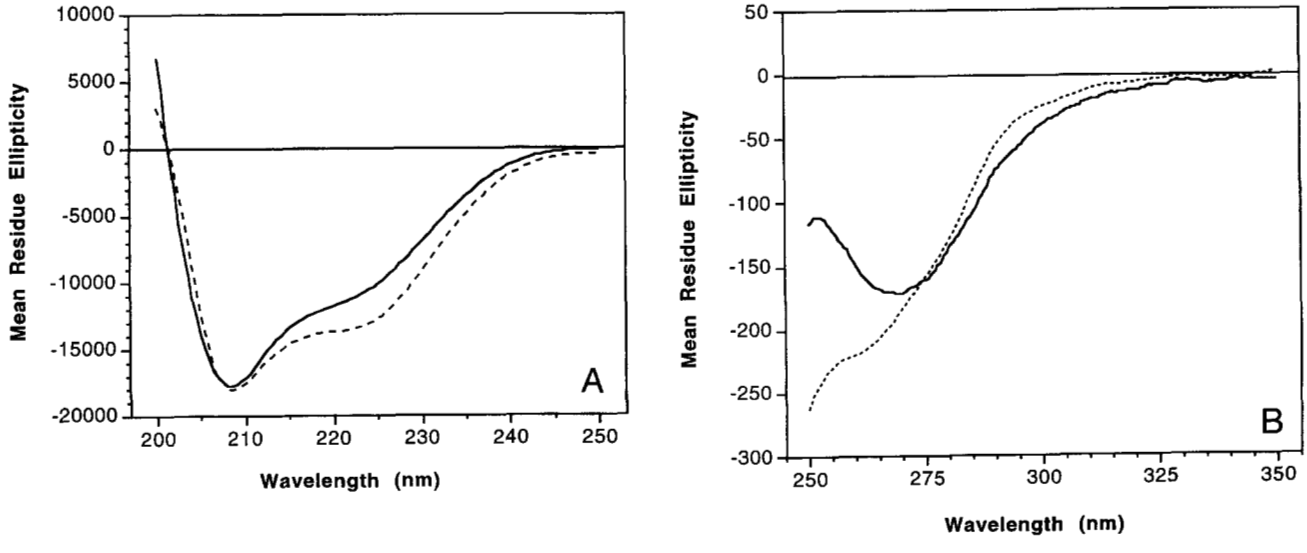

Fig. 4. CD spectra of LysPro. **A:** Far-UV CD spectra of monomeric LysPro (– zinc, – phenolic ligand, ——) and hexameric LysPro (+ zinc, + phenolic ligand, – – – –). **B:** Near-UV CD spectra of monomeric LysPro (– zinc, – phenolic ligand, ——) and hexameric LysPro (+ zinc, + phenolic ligand, – – – –). For LysPro samples that contained zinc and phenolic ligands, the zinc concentration was 19.7 μg/mL, *m*-cresol was 0.76 mg/mL, and phenol was 0.66 mg/mL phenol. All samples contained 3.5 mg/mL protein and 7 mM phosphate, pH 7.4. The units of the ordinate are deg·cm^2·$dmol^{-1}$.

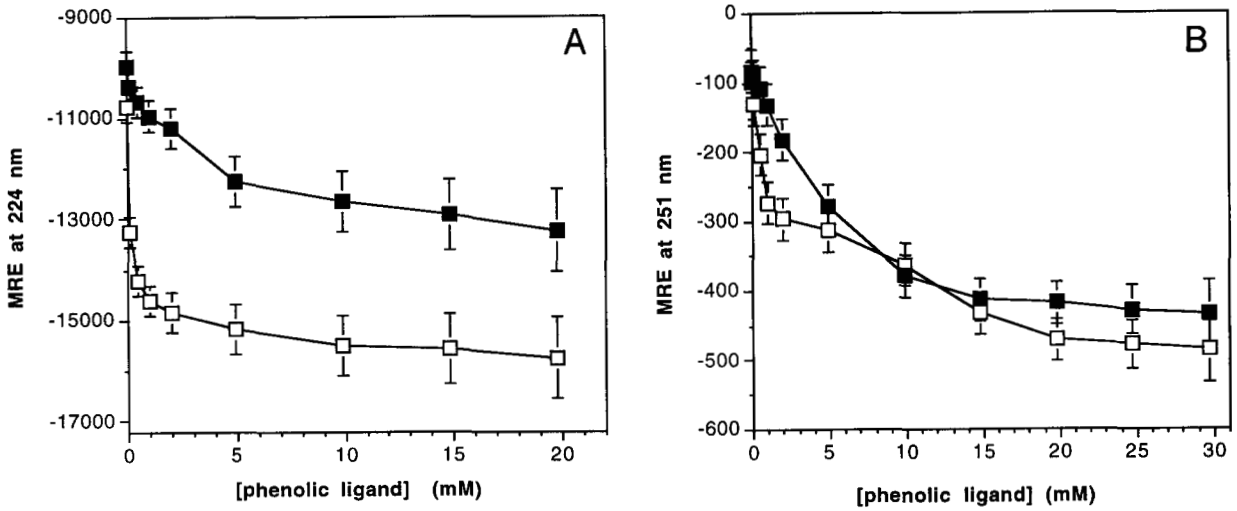

Fig. 5. CD monitoring of ligand-induced conformational transitions for insulin and LysPro. **A:** Far-UV CD results from titration of a 1:1 molar mixture of *m*-cresol and phenol into LysPro (■) and insulin (□) monitored at 224 nm. **B:** Near-UV CD results from titration of a 1:1 molar mixture of *m*-cresol and phenol into LysPro (■) and insulin (□) monitored at 251 nm. All samples contained 3.5 mg/mL protein, 19.7 μg/mL zinc, and 7 mM phosphate, pH 7.4. The units of the ordinate are deg·cm^2·dmol^{-1}.

centrations of a 1:1 molar mix of *m*-cresol and phenol at both 224 nm and 251 nm; although the data are not shown, similar results were obtained using phenol alone or *m*-cresol alone. The presence of residual anions such as chloride did not affect the titration profile significantly when phenolic ligand titrations were conducted in the presence and absence of 100 mM NaCl (data not shown). Figure 5A shows that insulin and LysPro possess different degrees of α-helix, particularly at low phenolic ligand concentrations. The dissimilarity between insulin and LysPro is also seen in the titrations monitored at 251 nm. The near- and far-UV CD titrations support the results from other methods (i.e., ligand–LysPro interactions are modified when compared to insulin under identical solution conditions).

Isothermal titrating calorimetry (ITC)

ITC was utilized as an alternate, nonspectroscopic, technique to monitor phenolic ligand binding to human insulin and LysPro hexamers. This technique monitors the heat evolved or consumed during the titration of zinc-containing solutions of either LysPro or human insulin with small aliquots of phenolic ligand; this calorimetric method is a function of multiple processes, e.g., ligand binding, helix-formation, hydration layer perturbation, etc. However, by measuring the heat associated with ligand binding, the signal is not subjected to the reduced signal/noise ratios observed with CD titrations (due to increasing absorption contribution of the phenolic ligands with increasing concentrations).

A comparison of the response profiles obtained at 25 °C, presented as heat per mole of injectant (Q_{inj}) versus total *m*-cresol concentration, for zinc-LysPro and zinc-insulin is shown in Figure 6; although the data are not shown, similar results were obtained using phenol alone or *m*-cresol alone. An initial inspection reveals that both titration curves exhibit two binding transitions composed of an exotherm and an endotherm; but clearly the two transitions do not overlap in either the vertical or the horizontal axis. The two transitions observed in zinc-insulin hexamers have been explained by considering the hexamer as a dimer of trimers. The initial exothermic transition has been assigned to intratrimer phenolic ligand binding that leads to the primary formation of a T_3R_3 intermediate and the latter endothermic transition has been attributed to the intratrimer phenolic ligand binding that leads to the primary formation of the fully saturated R_6 hexamer (Birnbaum et al., 1996). The endothermic nature of the second transition is modulated to a large degree by the strong endothermic contributions associated with negative intertrimer cooperativity (Birnbaum et al., 1996). Comparing the two titration curves clearly reveals that a similar binding pattern exists with both proteins; however, the magnitudes of both the exothermic and exothermic

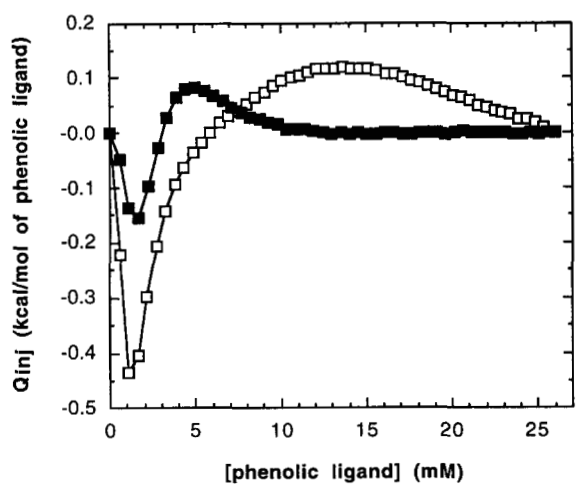

Fig. 6. ITC profiles of LysPro (■) and insulin (□) titrated with *m*-cresol. All samples contained 3.5 mg/mL protein, 19.7 μg/mL zinc, 16 mg/mL glycerol, and 7 mM sodium phosphate, pH 7.4. Concentrated stock *m*-cresol solutions used for the titrations were 194.2 mM for insulin and 199.7 mM for LysPro and also contained 16 mg/mL glycerol and 7 mM sodium phosphate, pH 7.4. Standard deviations are plotted but do not show because they are smaller than the symbols used.

transitions are reduced in the LysPro binding isotherm. In addition, the LysPro curve saturates at ~15 mM *m*-cresol, whereas the insulin curve does not saturate until *m*-cresol concentrations reach >25 mM.

Our interpretation of the LysPro ITC profiles is conditioned by other results presented in this manuscript, additional unpublished data (Birnbaum et al., 1995), and the published literature (Ciszak et al., 1995). The biphasic binding profiles suggest that the zinc-LysPro may bind phenolic ligands in a two-stage process that is analogous to the $T_6 \leftrightarrow T_3R_3 \leftrightarrow R_6$ observed in zinc-insulin hexamers. Evidence of such a two-stage process is consistent with the formation of an intermediate species, similar to the crystallographic $T_3R_3^f$ species identified previously (Ciszak et al., 1995), as well as the formation of an R_6-type species that is inferred from monophasic metal ion extraction studies (Birnbaum et al., 1995), the maximal cobalt extinction values obtained (Fig. 3A), and the maximal CD signal observed at 251 nm (Fig. 5B). However, the differences in the ligand binding energies are distinct for LysPro and human insulin and are most likely due to two factors.

1. The initial association state of LysPro in the presence of metal and absence of phenolic ligand (Birnbaum et al., 1995) is not exclusively T_6, consequently the heats associated with hexamer assembly are likely convoluted in the energetics of the titration profile.

2. The intertrimer interactions mediated through the monomer–monomer interfaces of the dimers within the formed hexameric complex of LysPro are altered due to the sequence inversion (Ciszak et al., 1995), consequently the energetics associated with the intertrimer cooperative events in the LysPro hexamer are likely altered.

This latter observation may also explain why the negative intertrimer cooperativity is reduced in LysPro, allowing for hexameric saturation with phenolic ligands at approximately half the concentration levels needed for zinc-insulin hexamers (Fig. 6). These results are also consistent with saturation behavior observed in the cobalt (Fig. 3A) and CD (Fig. 5) studies discussed earlier.

SLS

The in vitro dissociation properties of several LysPro and insulin preparations were probed using SLS. To gain insight into the origin of the analogue's fast action, the average molecular weight was measured as the hexamers and associated species dissociate upon dilution. As seen in Figure 7, the dissociation profiles of LysPro and insulin in the presence of zinc and phenolic ligands are quite different. Even though both preparations contain protein in a hexameric state, LysPro dissociates much more readily than insulin. Over the concentration range probed, the insulin hexamer remains significantly intact, whereas that of LysPro more easily dissociates from an average molecular weight of a hexamer to that of approximately a dimer at the lowest concentration studied. The results from samples without zinc and phenolic ligands are included to illustrate the monomeric nature of LysPro and the more associated state of zinc-free insulin and its subsequent ability to dissociate in the absence of zinc and phenolic ligands. These in vitro observations support the results described previously, including the spectroscopic differences between LysPro and insulin. In particular,

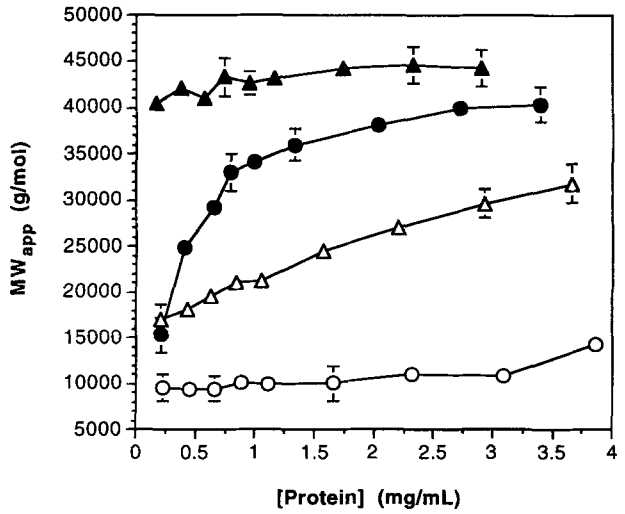

Fig. 7. Static light scattering results to monitor in vitro dissociation: hexameric LysPro (+ zinc, + phenolic ligand, ●), hexameric insulin (+ zinc, + phenolic ligand, ▲), monomeric LysPro (− zinc, − phenolic ligand, ○), and zinc-free insulin (− zinc, − phenolic ligand, △). See text for details of sample preparation. Scattered light was measured using 488 nm excitation and a 90° scattering angle. Zinc-containing samples also contained phenolic ligands and were identical to those described in Figure 1 (19.7 μg/mL zinc, 1.25 mg/mL *m*-cresol, 1.09 mg/mL phenol, and 16 mg/mL glycerol). All samples contained 7 mM phosphate, pH 7.4.

these observations indicate that the ligand-induced complexation of LysPro hexamer is more easily dissociated by dilution.

In vivo time-action

The in vivo rate of absorption upon injection into female pigs was studied for several LysPro and insulin preparations (Fig. 8). LysPro was examined in both the monomeric and hexameric states, whereas insulin was examined in the hexameric state only. These results elucidate two important points: first, both LysPro preparations have essentially identical rates of absorption and second, they both have faster rates of absorption than insulin. Thus, the association state for LysPro did not affect its time-action, but, in comparison, the insulin hexamer was delayed in transport to the bloodstream. It should also be noted that the biological effectiveness of both LysPro preparations is equivalent with respect to dose and to the pattern of glucose removal (data not shown).

Discussion

When designing insulin analogues that exist as monomers in solution, one must consider the fact that such solutions generally possess inadequate chemical stability to be therapeutically useful. Thus, it has been necessary to establish an approach whereby appropriate resistance to chemical degradation could be achieved and the rapid absorption associated with the monomeric analogue could still be retained. The present studies have utilized a series of biophysical techniques to elucidate the characteristics needed to reach such a goal.

The Discussion is divided into two parts: the first discusses chemical stability and structural results; the second discusses how the structural results contribute to an understanding of the rapid time-action of LysPro in the hexameric state.

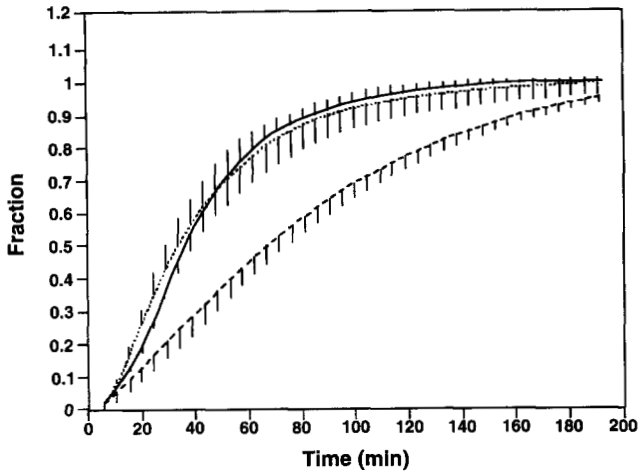

Fig. 8. LysPro and insulin in vivo cumulative fractional absorption in pigs: hexameric insulin (+ zinc, + phenolic ligand, – – – –), hexameric LysPro (+ zinc, + phenolic ligand, · · · · ·), and monomeric LysPro (− zinc, + phenolic ligand, ——). Hexameric insulin samples contained 19.7 μg/mL zinc, 2.5 mg/mL *m*-cresol, 16 mg/mL glycerol, and 14 mM sodium phosphate. Hexameric LysPro samples were identical to those described in Figure 1 (19.7 μg/mL zinc, 1.25 mg/mL *m*-cresol, 1.09 mg/mL phenol, 16 mg/mL glycerol, and 7 mM sodium phosphate). Monomeric LysPro samples contained 16 mg/mL glycerol, 2.5 mg/mL *m*-cresol, and 14.1 mM phosphate. All samples contained 3.5 mg/mL protein and had a pH of 7.4.

Stability and conformation of ligand-bound LysPro hexamer

Before discussing the LysPro hexamer results, and comparing them to those for insulin, a brief review of the insulin literature will be given. The human insulin hexamer complex formed in the presence of metal ions such as zinc or cobalt and the subsequent conformational changes that occur upon addition of phenolic ligands have been well documented (Bentley et al., 1976; Renscheidt et al., 1984; Wollmer et al., 1987; Baker et al., 1988; Derewenda et al., 1989; Roy et al., 1989; Thomas & Wollmer, 1989; Krüger et al., 1990; Brader & Dunn, 1991; Brader et al., 1991; Smith & Dodson, 1992). For human insulin, the presence of zinc promotes association to the hexameric state in what is referred to as the T_6-state conformation, where two zinc ions are bound by the His^{B10} residues of each subunit. An octahedral coordination geometry is completed by three water molecules (Blundell et al., 1972; Baker et al., 1988). The addition of phenolic ligands induces a conformational transition by binding to specific sites on the insulin hexamer, resulting in the N-terminal eight amino acids of the B-chain converting from an extended conformation to an α-helix. The two zinc atoms adopt a tetrahedral coordination geometry involving the His^{B10} residues of each subunit and a fourth exogenous small molecule ligand. This resulting R-state hexamer is more compact and less flexible, and its dissociation is retarded compared to that of the T-state (Kaarsholm et al., 1989). In addition, a stable intermediate species, $T_3R_3^f$, has been identified where one trimer of the hexamer is in the T-state and the other is in an R^f-state (Chothia et al., 1983). Both the hexamerization and the attainment of the R_6 conformation have been shown to stabilize insulin from chemical degradation.

In contrast to insulin, LysPro exhibits very weak association behavior in the absence of zinc and, furthermore, zinc ion alone does not cause LysPro to form a hexameric complex. The data shown demonstrate that the presence of a phenolic ligand is required for LysPro to associate into a hexamer complex that resembles that formed by insulin under the same conditions. However, other results (CD, visible absorption spectroscopy, ITC, and light scattering) suggest that the insulin hexamer complex with zinc and phenolic ligand is conformationally and energetically different from the LysPro hexamer complex formed under analogous solution conditions.

The resistance to chemical degradation for LysPro has been increased by implementing a strategy whereby the analogue solution is supplemented by the addition of zinc and phenolic ligands (Fig. 1). These ligands induce a hexamer complex whose conformation contains more α-helix than the phenolic ligand-free form (Fig. 4A), but measurably less α-helix than insulin under the same solution conditions (Fig. 5A). The presence of less α-helix in the LysPro hexamer complex than in the insulin R_6 complex can be explained as due to an altered structure for the LysPro hexamer such as T_3R_3, $T_3R_3^f$, or R_6^f complexes. In the following, each of these possibilities is further discussed with regard to consistency with all of the available results.

Published X-ray structures suggest insulin has sufficient flexibility that it can form a T_3R_3 complex or even a $T_3R_3^f$ complex (Smith & Ciszak, 1994), lending support to the proposal that the complex formed by LysPro with zinc and phenolic ligands could resemble a T_3R_3 or $T_3R_3^f$ structure in solution. Additional support for such a structure comes from the recent crystal structure work (Ciszak et al., 1995) on a crystalline complex of LysPro with zinc and phenol, which has shown that the hexamer consists of three TR^f dimers.

However, there are several experimental results that are not consistent with a T_3R_3 or $T_3R_3^f$ solution state structure and, instead, favor the interpretation that the LysPro hexamer more closely resembles R_6^f. These results include the cobalt hexamer extinction coefficient results (Fig. 3), the phenolic ligand titration results (Figs. 4, 5, 6), and unpublished data on the extraction of metal ions from LysPro and insulin hexamers (Birnbaum et al., 1995). Thus, considering all the experimental evidence, we would suggest that the solution state LysPro hexamer exists in the R_6^f conformation.

The solution state structure of LysPro hexamers cannot be determined conclusively from the data presented here and additional structural data, such as high-resolution ^1H NMR spectra, will be needed before the solution state structure is known at the atomic level. The conformational flexibility of insulin is well-known and undoubtedly contributes to the difficulty in making definitive structural interpretations for LysPro as well.

Conserving the rapid time-action of LysPro

One significant difference between insulin and LysPro is that the in vivo time-action of hexameric LysPro is faster than hexameric insulin (Fig. 8) even though they have equivalent chemical stability (Fig. 1). This more rapid time-action results from the increased dissociation constant of LysPro, as demonstrated in Figure 7, which illustrates that LysPro has a greater propensity to dissociate than insulin. Due to the ability of LysPro hexamers to dissociate more readily, a population of lower molecular weight species is attained within the interstitial space more quickly. This allows for more rapid passage and less restricted transport of LysPro through the capillary membrane compared to insulin, which does not dissociate as readily to the smaller species required for absorption. Figure 9

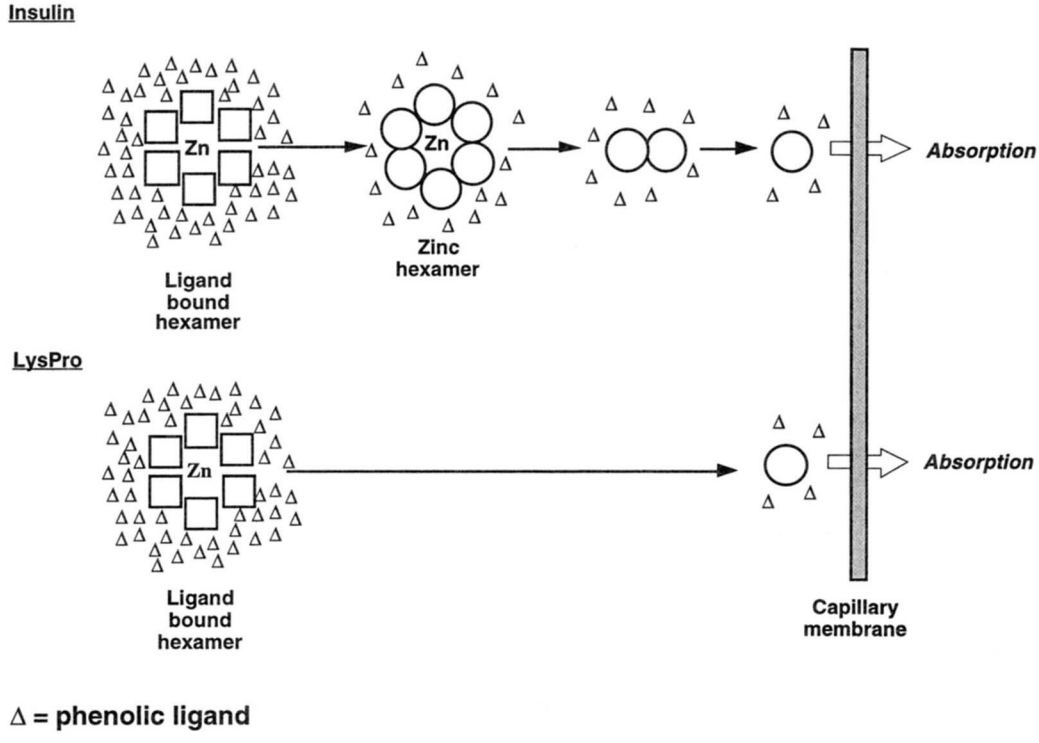

Fig. 9. Schematic diagram illustrating how the differences in physicochemical properties between insulin and LysPro are related to their different in vivo behaviors.

contains a simplified depiction of these concepts, which relate physicochemical properties with in vivo results.

The origin of the enhanced dissociation constant of LysPro compared to insulin is reflected in the significant differences observed using several biophysical techniques. For example, such a difference is evident by the divergent near- and far-UV CD spectra for LysPro and insulin under identical conditions (Fig. 4A and B; Wollmer et al., 1987). Further CD results examine these differences in more detail and reveal the noncoincident titration profiles for both zinc-insulin and zinc-LysPro in various amounts of phenolic ligand as depicted in Figure 5A and B. The same noncoincidence is detected in the phenolic ligand binding curves generated in Figure 6 by ITC and demonstrates that the thermal signatures for zinc-insulin and zinc-LysPro to bind phenolic ligands are quite distinct. The differences seen between zinc-insulin and zinc-LysPro are also present in the cobalt complexes as evidenced by the titration profiles monitored in the visible spectrum as depicted in Figure 3A. Overall, these results show that the formation of a ligand–protein complex for LysPro is unique when compared to insulin.

The results reported here support the concept that the size of the insulin unit is a determining factor in the transfer of insulin to the bloodstream. Numerous insulin analogue molecules including $Asp^{B9}Glu^{B27}$-insulin, Asp^{B28}-insulin, Asp^{B9}-insulin, and Glu^{B12}-insulin have been investigated previously and were shown to be absorbed faster from subcutis than wild-type insulin (Brange et al., 1988). Like LysPro, these analogues were found to be monomeric under therapeutically useful concentrations. However, to exploit the therapeutic benefit of improved glucose control and convenience possessed by these insulin analogues, it is necessary to stabilize them against shelf-life chemical degradation and bacterial growth.

Knowledge that the presence of certain additives enables a monomeric analogue such as LysPro to associate into stable hexamers with an increased tendency to dissociate provides a framework that can aid in future synthetic modifications for other therapeutic uses. In addition, a more thorough understanding of the structure and dynamics of LysPro within the hexamer and the effect on the binding of phenolic ligands, including the ligand-induced conformational changes, would certainly further our understanding of protein structure.

Although the chemical stability of LysPro can be enhanced by adding zinc and phenolic ligands, and the resultant hexamer retains the same rapid time-action profile as the monomeric species, the precise molecular origin of the modified hexamer dissociation behavior is not yet fully understood. More detailed probing into the structure of the LysPro hexamer by high resolution structural methods such as NMR and X-ray crystallography is needed in order to fully elucidate the role subunit interactions within the hexamer may play in relation to the in vivo biological response.

Materials and methods

Materials

Human insulin and LysPro were obtained from Eli Lilly and Company. Both proteins were derived biosynthetically through heterologous expression of the appropriate gene vector in *Escherichia coli*. Zinc-free human insulin was supplied as a lyophilized powder and zinc-free LysPro was supplied as sodium crystals. Zinc-LysPro and zinc-human insulin were supplied as crystals. The purity of the proteins was 98% or greater. All other chemicals were of analytical grade or higher.

Preparation of protein solutions

Zinc-free protein solutions were prepared from zinc-free insulin or zinc-free LysPro at 3.5 mg/mL in 7 mM sodium phosphate, pH 7.4. Where specified, these samples also incorporated phenolic ligands, and/or 16 mg/mL glycerol, and/or 14.1 mM sodium phosphate, depending on the experiment performed.

Protein solutions containing zinc and phenolic ligands were prepared from zinc-free insulin or zinc-free LysPro at 3.5 mg/mL and contained 7 mM or 14 mM sodium phosphate, pH 7.4, 1.25 mg/mL *m*-cresol, 1.09 mg/mL phenol, 16 mg/mL glycerol, and 19.7 μg/mL zinc. Where specified, the human insulin solutions contained 2.5 mg/mL *m*-cresol rather than the mixture of phenol and *m*-cresol.

Prior to the addition of phenolic ligands, protein concentrations were determined by UV absorption spectroscopy using an AVIV model 14 DS double-beam spectrophotometer. Protein concentrations were calculated based on an extinction coefficient of 1.05 $(mg/mL)^{-1} \cdot cm^{-1}$ at 276 nm for both human insulin (Frank et al., 1972) and LysPro.

Chemical stability

For chemical stability studies, solutions were prepared as described above (see Preparation of protein solutions) although the zinc-free solutions also incorporated 1.25 mg/mL *m*-cresol, 1.09 mg/mL phenol, and 16 mg/mL glycerol. Degradation was initiated by incubating these solutions of insulin and LysPro at 30 °C while maintaining control samples at 5 °C. At seven-day intervals, samples were removed and assayed for high molecular weight species using size-exclusion HPLC. Analyses were performed by injecting 20-μL samples onto a Dupont Zorbax GF-250 Special (9.4 × 250 mm) column using a mixture of 69% 0.4 M ammonium bicarbonate, pH 7.8, and 31% acetonitrile as the eluting solution (flow rate of 0.5 mL/min at ambient temperature and detection at 214 nm) (Farid et al., 1989). The percent polymer formation was determined from the ratio of the high molecular weight peak to the total area of the monomer and high molecular weight peaks.

Sedimentation velocity

Samples for sedimentation velocity experiments were prepared as described above (see Preparation of protein solutions). Using a Model E Beckman/Spinco analytical ultracentrifuge, each sample was centrifuged in a double-sector cell at 60,000 rpm for at least 140 min. The observed sedimentation coefficients, s_{obs}, were determined as described by van Holde (1985). These values were then corrected to standard conditions of water at 20 °C ($s_{20,w}$) using the following equation:

$$s_{20,w} = s_{obs} \frac{(1 - \bar{v}\rho)_{20,w}\eta_{T,b}}{(1 - \bar{v}\rho)_{T,b}\eta_{20,w}}$$

where $\bar{v}$ is the partial specific volume of the protein, ρ is solution density, η is solution viscosity, and the subscripts refer to the experimental temperature (T = experiment temperature, 20 = 20 °C) and solvent conditions (b = buffer, w = water). The estimated error in $s_{20,w}$ values is ±10%.

SLS

SLS experiments were conducted by starting with 3.5 mg/mL protein solutions whose preparation was described above (see Preparation of protein solutions). From each solution, a series of dilutions was prepared spanning the protein concentration range of 0.2–3.5 mg/mL; dilutions were made with 7 mM sodium phosphate buffer, pH 7.4. These solutions were then filtered through 0.2-μm Gelman low protein binding filters before performing the SLS measurements. The protein concentration for each of these samples was determined using reversed-phase HPLC.

In order to compensate for the scattered light contributed by solvent, measurements were made of the scattered light from protein-free solutions that had been filtered through 0.2-μm Gelman low protein binding filters. These solutions contained all solution constituents, except protein, at the same concentration as the corresponding protein sample sets.

SLS experiments were performed using a Brookhaven Instruments 2030AT autocorrelator and goniometer. All measurements were made with a 1-mm pinhole at a 90° scattering angle using a Lexel Model 3500 argon ion laser set at 488 nm. The temperature was maintained at 25 °C by a Neslab RTE-110 temperature bath. The signal at the photomultiplier tube was calibrated using 0.1-μm filtered toluene.

Weight-average (apparent) molecular weights were calculated as described by Cantor and Schimmel (1982) using a refractive index increment of 0.183 mL/g (Hvidt, 1991). Error bars were determined using the standard deviation from the average apparent molecular weight for the three samples analyzed at each concentration.

In vivo time-action

The in vivo biological properties of insulin and LysPro were examined by measuring the absorption of various preparations after subcutaneous injection into young 15–25-kg female pigs. The solutions were prepared using the procedures described above (see Preparation of protein solutions). The composition of each sample is given in the legend for Figure 8. Each sample was analyzed using separate groups of four pigs that had surgically preimplanted jugular and arterial catheters. Two separate experiments were conducted using each pig, with both experiments preceded by a 20-h fast. In addition, somatostatin was infused (0.5 μg/kg-min) to suppress endogenous insulin secretion. On day one, an intravenous infusion of the appropriately prepared sample was administered at 8 mU/kg-min for 2 h (28.8 U/mg protein). The infusion was then discontinued. Blood samples were obtained at intervals during the infusion and more frequently during the decay following discontinuation of the infusion. Three days later, a second study was conducted using a subcutaneous injection of the sample at 0.3 U/kg (28.8 U/mg protein). Blood glucose was monitored frequently and clamped using a variable infusion of 20% glucose at individual fasting levels (approximately 5.0 mmol/L). Blood samples for radioimmunoassay were collected at intervals after injection for a total of 290 min. The response to a bolus injection of sample was inferred from the decay curve of sample concentrations. This was used, together with the concentrations following subcutaneous injection in the process of mathematical deconvolution, to calculate the rate of absorption of each separate sample. Cumulative absorption was calculated by integrating the resultant absorption curve.

CD

Two types of CD experiments were performed. In the first type, wavelength scans were recorded, whereas in the other experi-

ments, titrations with phenolic ligand were performed with detection at single wavelengths. For the wavelength scan experiments, the zinc-free protein solutions were prepared as described previously (see Preparation of protein solutions); the solutions containing zinc and phenolic ligands did not contain glycerol and the phenolic ligand concentrations were 0.76 mg/mL *m*-cresol and 0.66 mg/mL phenol. For the phenolic ligand titration experiments, the samples contained insulin or LysPro at 3.5 mg/mL in 7 mM sodium phosphate, pH 7.4, 19.7 μg/mL zinc. Stock solutions containing a 1:1 molar mixture of phenol and *m*-cresol in 7 mM sodium phosphate and pH 7.4 were added incrementally throughout the experiment. Total volume of phenolic ligand solution added was small and did not dilute the protein concentration significantly. All CD measurements were obtained using an AVIV Model 62 DS spectrometer with results reported as mean residue ellipticity (MRE) having units of $deg \cdot cm^2 \cdot dmol^{-1}$ as described by Cantor and Schimmel (1982). A mean residue weight of 113.9 g/mol was used for both human insulin and LysPro.

Visible absorption spectroscopy

Visible absorption spectra were collected on cobalt-insulin and cobalt-LysPro solutions as the solutions were titrated with phenolic ligands. To prepare the cobalt-containing proteins, stock solutions of metal-free insulin and metal-free LysPro were prepared at 11.6 mg/mL in 50 mM Tris-ClO_4, pH 8.0, and 20 mM KSCN. Co(II), in the form of cobalt sulfate, was then added to each stock solution in the ratio of two metal ions per hexamer. Quantities of a 1:1 molar mixture of *m*-cresol and phenol in ethanol were titrated into the cobalt-protein stock solutions and the absorbance at 574 nm was measured using an AVIV Model 14 DS double beam spectrophotometer. Extinction coefficients were calculated based on cobalt ion concentrations. Total volume of phenolic ligand solution added was small and did not dilute the protein concentration significantly.

ITC

ITC experiments were conducted by starting with 3.5 mg/mL protein solutions whose preparation was described above (see Preparation of protein solutions). Starting with either zinc-free human insulin or zinc LysPro, the solutions were prepared to contain 3.5 mg/mL protein, 19.7 μg/mL zinc, 16 mg/mL glycerol, and 7 mM sodium phosphate, pH 7.4. The titrating solution was a concentrated phenolic ligand solution containing 200 mM phenolic ligand, 16 mg/mL glycerol, and 7 mM sodium phosphate, pH 7.4.

ITC experiments were performed at 25 °C using a Microcal Omega titration calorimeter attached to a Lauda/Brinkmann RCS6 Bath/Circulator to maintain a constant temperature for all experiments. A single experiment consisted of a sequence of 50 injections. Each injection contained 3.5 μL of phenolic ligand solution, which was delivered over a 3.5-s period. A 5-min delay between injections was sufficient to allow the binding to reach equilibrium. Heats of dilution were determined from the last 10–15 injections, where the binding was complete and the measured heats were due only to titrant dilution. For LysPro, the heats of dilution over the course of the titration were determined by drawing a line through these latter points using a linear regression algorithm. The estimated heat of dilution profile was subtracted from the original data. For insulin, a separate heat of dilution titration was performed and subtracted from the original data because saturation was not achieved. Triplicate runs were performed for both LysPro and human insulin, and the heats of injection were averaged and a standard deviation was determined. The heats of injections were plotted relative to total phenolic ligand added.

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