Mutations at the Dimer, Hexamer, and Receptor-Binding Surfaces of Insulin Independently Affect Insulin–Insulin and Insulin–Receptor Interactions†

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ABSTRACT: Mutagenesis of the dimer- and hexamer-forming surfaces of insulin yields analogues with reduced tendencies to aggregate and dramatically altered pharmacokinetic properties. We recently showed that one such analogue, HisB10 → Asp, ProB28 → Lys, LysB29 → Pro human insulin (DKP-insulin), has enhanced affinity for the insulin receptor and is useful for studying the structure of the insulin monomer under physiologic solvent conditions [Weiss, M. A., Hua, Q. X., Lynch, C. S., Frank, B. H., & Shoelson, S. E. (1991) *Biochemistry 30*, 7373–7389]. DKP-insulin retains native secondary and tertiary structure in solution and may therefore provide an appropriate baseline for further studies of related analogues containing additional substitutions within the receptor-binding surface of insulin. To test this, we prepared a family of DKP analogues having potency-altering substitutions at the B24 and B25 positions using a streamlined approach to enzymatic semisynthesis which negates the need for amino-group protection. For comparison, similar analogues of native human insulin were prepared by standard semisynthetic methods. The DKP analogues show a reduced tendency to self-associate, as indicated by ^{1}H-NMR resonance line widths. In addition, CD spectra indicate that (with one exception) the native insulin fold is retained in each analogue; the exception, PheB24 → Gly, induces similar perturbations in both native insulin and DKP-insulin backgrounds. Notably, analogous substitutions exhibit parallel trends in receptor-binding potency over a wide range of affinities: D-PheB24 > unsubstituted > GlyB24 > SerB24 > AlaB25 > LeuB25 > SerB25, whether the substitution was in a native human or DKP-insulin background. Such "template independence" reflects an absence of functional interactions between the B24 and B25 sites and additional substitutions in DKP-insulin and demonstrates that mutations in discrete surfaces of insulin have independent effects on protein structure and function. In particular, the respective receptor-recognition (PheB24, PheB25), hexamer-forming (HisB10), and dimer-forming (ProB28, LysB29) surfaces of insulin may be regarded as independent targets for protein design. DKP-insulin provides an appropriate biophysical model for defining structure–function relationships in a monomeric template.

Insulin self-association provides a model for specific protein-protein recognition with application to drug design (Brange et al., 1988, 1990). Two surfaces of insulin are involved in such interactions, corresponding to crystallographic dimer and hexamer contacts (Adams et al., 1969; Peking Insulin Structure Group, 1971; Blundell et al., 1971; Baker et al., 1988). Substitutions in either contact surface alter self-association. The ability to engineer the oligomeric state of insulin in vitro—coupled with the hypothesis that self-association state might influence absorption rates following percutaneous insulin administration—has led to clinical trials of such analogues as rapid-acting insulin preparations (Brange et al., 1988, 1990; Kang et al., 1990, 1991a,b). Compared to standard mammalian insulin formulations, the pharmacokinetics of monomeric insulin analogues better match postprandial excursions in circulating glucose concentrations.

In addition to their promise as therapeutic agents, monomeric insulins provide an important new tool for biochemical and ^{1}H-NMR[1] studies of insulin structure and dynamics in solution. Such studies have been limited in the past by aggregation, which leads to broad ^{1}H-NMR resonances in the physiologic pH range. Accordingly, 2D-NMR studies of native insulin have been conducted at the extremes of pH [>10 (Ramesh & Bradbury, 1985), <2 (Boelens et al., 1990)] or in the presence of organic cosolvents (Weiss et al., 1989; Kline & Justice, 1990; Hua & Weiss, 1991). However, it has been difficult under such conditions to evaluate possible pH- or solvent-induced perturbations. To analyze the solution structure of insulin under physiologic conditions, we and others have initiated NMR studies of monomeric insulin analogues (Roy et al., 1990; Kristensen et al., 1991; Weiss et al., 1991).

In this study we investigate the effects of mutations within the receptor-binding surface of a monomeric analogue, and we compare these effects to those of analogous mutations in native insulin. The monomer under study, designated DKP-insulin, contains substitutions within both the hexamer

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[1] Abbreviations: CD, circular dichroism; CHO, Chinese hamster ovary; DKP-insulin, HisB10 → Asp, ProB28 → Lys, LysB29 → Pro human insulin; DOI, des[B23–B30]octapeptide insulin; AspB10-DOI, des[B23–B30]octapeptide HisB10 → Asp insulin; DPA, des[B26–B30]pentapeptide insulin amide; NMR, nuclear magnetic resonance; Tfa, trifluoroacetyl.

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(HisB10 → Asp) and dimer (ProB28 → Lys, LysB29 → Pro) surfaces of insulin; biochemical and biophysical properties of DKP-insulin have been described (Weiss et al., 1991; DiMarchi et al., 1992). The present comparative study tests whether DKP-insulin is an appropriate model for the native insulin monomer, i.e., whether the perturbing effects of mutations in native insulin have similar effects on the structure and function of DKP analogues. Such test mutations are introduced into the conserved PheB24 and PheB25 positions, which contribute to a nonpolar surface of insulin (Adams et al., 1969; Peking Insulin Structure Group, 1971; Blundell et al., 1971) proposed to contact the insulin receptor (Pullen et al., 1976; Gammeltoft, 1984; Baker et al., 1988). The special importance of PheB24 and PheB25 is underscored by the altered potencies of diabetes-associated human insulin mutations at these sites (Tager et al., 1980; Wollmer et al., 1981; Inouye et al., 1981; Shoelson et al., 1983a,b; Kobayashi et al., 1984; Tager, 1990; Steiner et al., 1990) and of related synthetic analogues (Inouye et al., 1982; Kobayashi et al., 1982b; Nakagawa & Tager, 1986, 1987; Mirmira & Tager, 1989; Mirmira et al., 1991).

The functional properties of some analogues are difficult to rationalize on the basis of static crystal structures of insulin. Therefore, a better understanding of the receptor "recognition code" requires additional biophysical analyses to investigate whether changes in receptor affinity result from (a) local loss or obstruction of necessary side-chain contacts within the insulin–receptor complex, (b) nonlocal perturbations in insulin structure that influence the ability to form important contacts, or (c) variations in protein dynamics relevant to postulated "induced fit" mechanisms (Baker et al., 1988; Mirmira & Tager, 1989; Derewenda et al., 1991; Hua et al., 1991). The present study represents a first step toward establishing a monomeric model for analysis of such alterations in insulin's receptor-binding surface. The results demonstrate that three distinct surfaces of insulin involved in protein–protein interaction—the hexamer, dimer, and receptor-binding surfaces—can be treated as independent design features.

Experimental Procedures

Peptide Synthesis. α-(Trifluoroacetyl)glycine (Tfa-Gly) was prepared by treatment of glycine with trifluoracetic anhydride (Aldrich) as described (Shoelson et al., 1983); Boc-D-Phe was from Bachem. Additional amino acids with standard Boc/Bzl protecting groups and reagents were purchased from Applied Biosystems; solvents for peptide synthesis were of the highest purity available (usually from EM Science). Automated syntheses were performed on an Applied Biosystems Model 430A synthesizer using 0.5 mmol of Boc-Thr(Bzl)-Pam resin (0.62 mmol/g) and standard DCC-mediated preformed symmetrical anhydride coupling protocols. Tfa-Gly was incorporated as a preformed HOBt ester. For semisyntheses of substituted native insulin analogues, peptides with the general sequence Tfa-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-OH were synthesized with the appropriate substitutions (D-Phe, Gly, Ser, Ala, or Leu) in place of one of the underlined Phe residues. Similarly, for semisyntheses of monomeric insulin analogues octapeptides with the general sequence H-Gly-Phe-Phe-Tyr-Thr-*Lys-Pro*-Thr-OH were synthesized with the appropriate substitutions; note the Pro-Lys to Lys-Pro switch of residues near the carboxyl terminus of the peptides (italics).

The final peptide products were cleaved from the resin using an anhydrous mixture of 20% trimethylsilyl trifluoromethanesulfonate, 12% thioanisole, 6% ethanedithiol, and 2% *m*-cresol in trifluoroacetic acid (Yajima et al., 1988), filtered, precipitated with ice-cold diethyl ether, desalted on a column (2.6 × 100 cm) of Bio-Gel P-2 equilibrated in 3 M acetic acid, and lyophilized. When necessary, isolated peptides were purified by reversed-phase HPLC (Waters' Prep 4000) on a Dynamax-300A 12-μm C8 column (41.4 × 250 mm) equipped with a matched guard column; peptides were eluted with a mobile phase composed of acetonitrile in 0.05% aqueous trifluoroacetic acid (80 mL/min). Following an initial 3.0-min isocratic elution at 26% acetonitrile, the acetonitrile composition was increased linearly at a rate of 1.0% every 5.0 min.

Semisynthesis of Native Insulin Analogues. Semisyntheses of native human insulin analogues were essentially as described (Shoelson et al., 1983; Nakagawa & Tager, 1986). Substituted Tfa-Gly-Xxx-Xxx-Tyr-Thr-Pro-Lys-Thr-OH peptides (25 mg, 0.022 mmol) were dissolved in 1.6 mL of dimethylformamide and reacted with 106 mg (0.43 mmol, 20-fold molar excess) of 2-(((*tert*-butoxycarbonyl)oxy)imino)-2-phenylacetonitrile (Boc-ON, Aldrich). The pH_{app} of the mixture was adjusted to 7.0 with *N*-methylmorpholine, and the reaction was allowed to proceed for 2 h at 22 °C to block the ε-amino group of lysine. The product was separated from reagents by gel filtration on an LH-20 (Pharmacia) column (1.6 × 90 cm) in methanol and dried under reduced pressure. The α-trifluoroacetyl protecting groups on Gly were then removed by treatment of the α-Tfa,ε-Boc-peptides with 3.0 mL of 10% aqueous piperidine for 1 h at 22 °C; products were separated from reagents by a second chromatographic purification on LH-20. Both reactions were judged to be complete by TLC analysis (silica gel 60, EM Science; developed in *n*-butanol/acetic acid/H_2O, 4:1:1); following exposure to HCl vapor, peptides were visualized by ninhydrin spray (Stewart & Young, 1984).

Des-[B23–B30]octapeptide insulin (DOI), kindly provided by Dr. Ron Chance of Eli Lilly & Co., was reacted with Boc-ON reagent to yield Al,Bl-Boc_2-DOI (Shoelson et al., 1983). Al,Bl-Boc_2-DOI (12 mg) and a 5-fold molar excess of ε-Boc-octapeptide (12 mg) were dissolved in 0.12 mL of a mixture of 35% dimethylformamide/35% 1,4-butanediol/30% aqueous 0.25 M Tris, 2.0 mM $CaCl_2$ (pH 7.5) (Inouye et al., 1981; Nakagawa & Tager, 1986). The pH(app) of the peptide solution was adjusted to 7.0 with *N*-methylmorpholine, using the wet pH paper method described by Rees and Offord (1976). Addition of TPCK-treated trypsin (1.0 mg, Cooper) initiated the coupling reaction, which was allowed to proceed at room temperature (≈22 °C) and was monitored by HPLC. When peptide bond formation between the ArgB22 carboxyl group of A1,B1-Boc_2-DOI and the glycyl α-amino group of the octapeptide had progressed sufficiently (>60%), the reaction was terminated by precipitation with a 10-fold excess of acetone. After additional washes of the precipitate with acetone and ether and drying, Boc protecting groups were removed by treatment with trifluoroacetic acid in the presence of 5% anisole (30 min at 22 °C). The volume of trifluoroacetic acid was reduced under a stream of argon, and the residue was precipitated and washed with diethyl ether and dissolved in 3 M acetic acid. The substituted analogues were separated from unreacted DOI, octapeptide, and other reagents and products by gel filtration on a column (2.6 × 100 cm) of Bio-Gel P-6 equilibrated in 3 M acetic acid. Fractions corresponding to the pure insulin analogues were pooled and lyophilized.

Semisynthesis of Monomeric Insulin Analogues. Des-[B23–B30]octapeptide HisB10 → Asp insulin (AspB10-DOI) was prepared by treating biosynthetic HisB10 → Asp insulin (generously provied by Jens Brange, Novo) with trypsin as

described (Bromer & Chance, 1968); additional AspB10-DOI was obtained as a generous gift from Dr. Ron Chance, Eli Lilly & Co. AspB10-DOI (12 mg) and a 5-fold molar excess of the unprotected octapeptide (12 mg) were dissolved in 0.12 mL of a mixture of dimethylformamide/1,4-butanediol/aqueous Tris and reacted with trypsin as described above. At appropriate times, reactions were terminated by the direct addition of 3 M acetic acid. The substituted DKP analogues were separated from unreacted AspB10-DOI, octapeptide, and other reagents and products by gel filtration on a column (2.6 × 100 cm) of Bio-Gel P-6 equilibrated in 3 M acetic acid. Fractions corresponding to pure insulin analogues were pooled and lyophilized.

Insulin Receptor Preparation. Chinese hamster ovary (CHO) cells, transfected with human insulin receptor constructs and expressing 10^6 receptors/cell (Ebina et al., 1985), were grown in suspension in a 10-L spinner flask in modified McCoy's 5A media (Gibco) containing 0.0345 mg/mL L-proline and no $CaCl_2$ (Shymko et al., 1989; J. Backer, personal communication). Cells were harvested by centrifugation at 1000 rpm and solubilized at 4 °C in a solution of 1.0% Triton X-100/50 mM Hepes (pH 7.6) containing 2 mM PMSF and 0.1 mg/mL aprotinin (0.1 mL of cells/mL of solubilizing solution). Following centrifugation to remove insoluble material, the cell extract was passed over wheat germ agglutinin–agarose (Vector). The column (1.0 × 5.0 cm) was washed extensively, and the lectin-bound proteins were eluted with 0.3 M *N*-acetylglucosamine in Hepes buffer (50 mM, pH 7.4) containing 0.1% Triton X-100 as described (Shoelson et al., 1988). WGA-purified protein was stored at −70 °C until needed.

Equilibrium Binding with the Insulin Receptor. Insulin concentrations were determined using absorbance at 276 nm, assuming that for all samples a 1.0 mg/mL solution has an absorbance of 1.04 (Pekar & Frank, 1972). WGA-purified receptor, [^{125}I]iodo-TyrA14-insulin, and varying concentrations of native human insulin or unlabeled analogues were incubated together at 4 °C for 16 h in 0.20 mL of a pH 7.4 buffer solution containing 50 mM Hepes, 0.1% Triton X-100, 0.1% bovine serum albumin, and 0.11 M NaCl. [^{125}I]Insulin–receptor complexes were precipitated in the presence of 0.1% bovine γ-globulin and 12.5% poly(ethylene glycol) (PEG 8000) (final concentrations) at 4 °C. Sufficient WGA-purified protein was used to specifically precipitate 25–30% of total [^{125}I]iodo-TyrA14-insulin in the absence of unlabeled hormone.

Circular Dichroism Spectroscopy. Ultraviolet CD spectra were recorded on an Aviv Model 60H spectropolarimeter at 25 °C using a 0.1-cm path length cuvette. Samples (25 μM) were dissolved either in aqueous HCl at pH 2.0 or in 20 mM sodium phosphate/140 mM sodium chloride, pH 7.0 (PBS). The solutions were centrifuged, and protein concentrations were determined by UV absorbance (Pekar & Frank, 1972). Individual data points were collected at each wavelength (in nanometers) for 10 s, and the data were smoothed using the fit-in-program.

^{1}H-NMR Spectroscopy. Spectra were recorded at 500 MHz at Harvard Medical School as described (Weiss et al., 1991). Samples were prepared in 50 mM aqueous potassium phosphate buffer, pD 7.4 (direct meter reading), and spectra were obtained at 25 °C.

Results

Insulin Analogue Semisynthesis

Trypsin-Catalyzed Semisynthesis. Utilizing methods of trypsin-catalyzed semisynthesis adapted to insulin by Inouye et al. (1979, 1981a), many interesting analogues have previously been prepared that are modified within the carboxyl terminus (B23–B30) of the B-chain (Tager et al., 1980; Inouye et al., 1981a,b, 1982; Wollmer et al., 1981; Keefer et al., 1981; Assoian et al., 1982; Kobayashi et al., 1982a,b, 1984; Shoelson et al., 1983; Fischer et al., 1985, 1986; Nakagawa & Tager, 1986, 1987; Cassaretto et al., 1987; Mirmira & Tager, 1989; Mirmira et al., 1991). This method involves trypsin-catalyzed peptide bond formation between the ArgB22 α-carboxyl group of des-octapeptide insulin (DOI) and the α-amino group of a synthetic peptide (Figure 1). The α-amino groups of DOI (GlyA1, PheB1) and the octapeptide ε-amino group corresponding to LysB29 are reversibly blocked, usually with acid-labile Boc protecting groups. In this fashion peptide bond formation is directed to the desired position between ArgB22

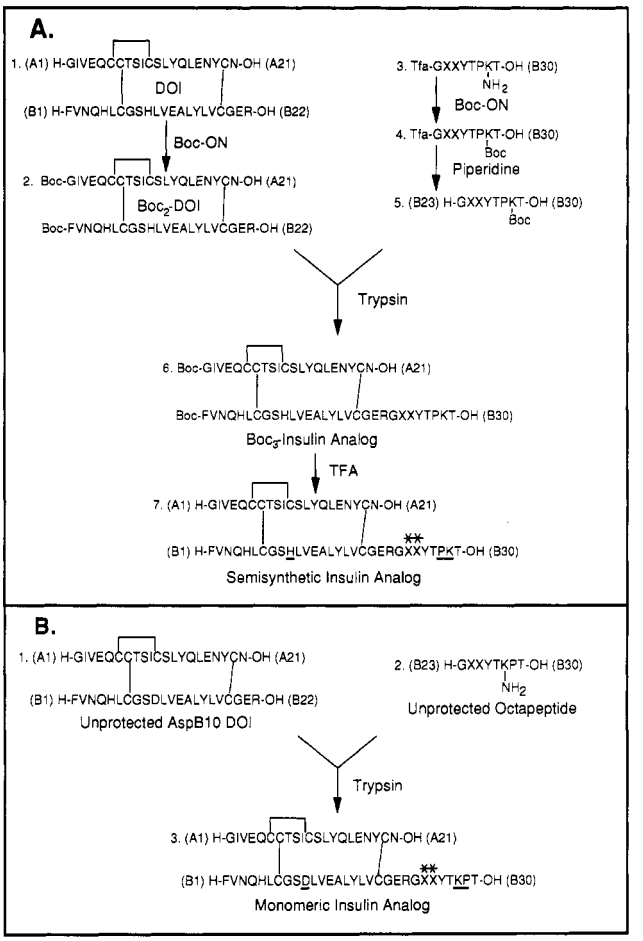

FIGURE 1: Semisynthetic strategies for preparing modified insulin analogues. (A) Standard method for insulin semisynthesis. The α-amino groups of des-[B23–B30]octapeptide insulin (1; DOI) are reversibly protected with *tert*-butyloxycarbonyl (Boc) groups to yield A1,B1-Boc_2-DOI (2). Substituted octapeptides corresponding to insulin residues B23–B30 are synthesized with the α-amino groups protected (3). The ε-amino group of the peptide lysine is first blocked with a Boc group (4), and the trifluoroacetyl (Tfa) protecting group on the N-terminal Gly is then removed to yield an octapeptide protected at the ε-position of lysine only (5). The precursors for semisynthesis, A1,B1-Boc_2-DOI (2) and ε-Boc octapeptide (5), are condensed in the presence of trypsin to form $A1^{\alpha}$,$B1^{\alpha}$,$B29^{\epsilon}$-Boc_3 insulin analogues (6). These are treated with trifluoracetic acid (TFA) to yield the corresponding analogues of native insulin (7). (B) A "streamlined" method for semisynthesis of monomeric insulin analogues. AspB10-DOI (1) and the appropriate octapeptides (2) are condensed with the assistance of trypsin in the complete absence of protecting groups. Underlines denote positions within the hexamer- (D^{B10}) and dimer- ($P^{B28} \leftrightarrow K^{B29}$) forming surfaces of insulin that have been substituted to prevent insulin–insulin aggregation. Asterisks denote residues B24 and B25, positions within insulin's receptor-binding surface that were substituted in this study.

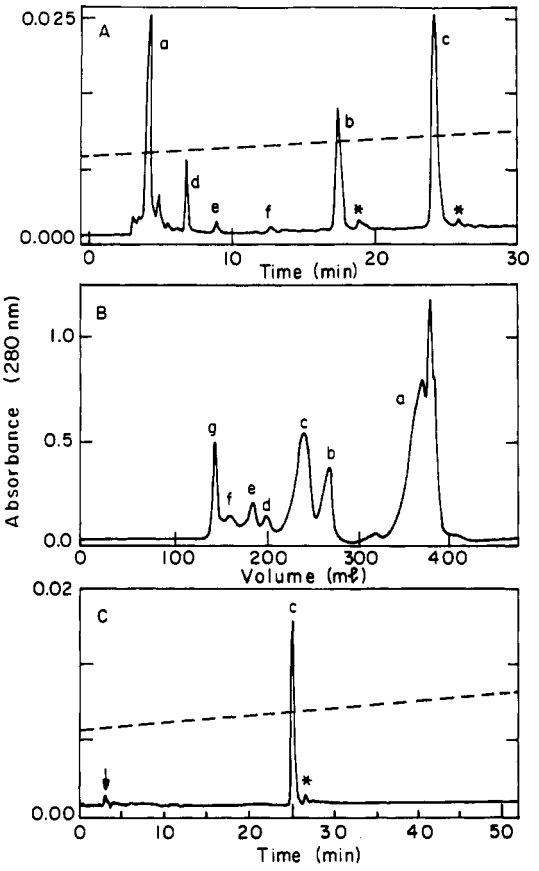

FIGURE 2: Chromatographic analysis and purification of labeled monomeric analogues. (A) HPLC analysis of an ongoing semisynthesis reaction; (B) Gel filtration (Bio-Gel P6; 3 M acetic acid) of the semisynthetic reaction mixture; (C) HPLC analysis of the isolated insulin following gel filtration. In all panels, peaks lettered a, b, and c correspond to octapeptide, DOI, and insulin, respectively; asterisks denote small amounts of deamidated forms. In panel B, additional peaks labeled d and e represent DOI–DOI and DOI–insulin dimers, respectively; peaks f and h are higher molecular weight covalent adducts and trypsin. HPLC analyses utilized a Bio-Rad RP-318 column (4.6 × 250 mm) equipped with a matched guard column. Following a 3.0-min isocratic elution with 27% acetonitrile in 0.05% aqueous trifluoroacetic acid, a linear gradient was initiated which increased the acetonitrile concentration by 1% every 5.0 min. Following isolated insulin analyses (panel C), stepping the acetonitrile concentration to 70% did not elute significant amounts of additional peptides.

and GlyB23, and spurious side reactions are suppressed.

Unprotected Octapeptides. When attempts are made to semisynthesize analogues of native insulin without the use of amino-group protection, mixtures of products result with low yields of the desired analogues (S. E. Shoelson, unpublished observation). However, for monomeric DKP-insulin analogues the ProB28-LysB29 sequence is reversed, which results in a Lys–Pro bond rather than the usual Lys–Thr bond of human insulin. Lys–Pro bonds are resistant to trypsin-catalyzed proteolysis in aqueous buffers, suggesting that this bond might also be resistant to hydrolysis and transpeptidation reactions in the mixed organic solvent mixtures used for semisynthesis (Ron Chance, personal communication). In fact, when Lys-Pro octapeptides were left unprotected and coupled with Boc_2-DOI under standard conditions for semisynthesis, no side reactions were observed (data not shown), confirming the resistance of the Lys–Pro bond to trypsin's action in 70% organic solvent.

Unprotected Des-Octapeptide Insulin. Des-pentapeptide insulin, a truncated analogue lacking residues B26–B30 (including the trypsin-sensitive LysB29) has been prepared

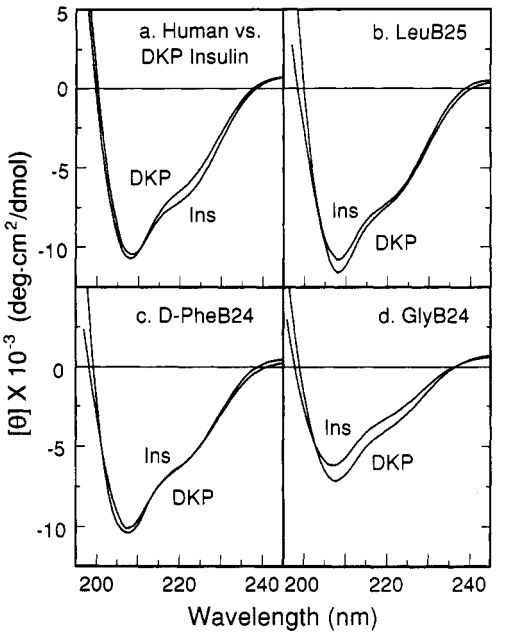

FIGURE 3: CD spectra of native insulin and DKP-insulin analogues at pH 2.0. Spectra were obtained in 10 mM HCl as described under Experimental Procedures. CD spectra: (a) unsubstituted human and DKP-insulin; (b) LeuB25; (c) D-PheB24; (d) GlyB24 analogues of human insulin (Ins) and DKP-insulin (DKP).

previously by trypsin-catalyzed semisynthesis with unprotected DOI (Kubrick & Coburn, 1986). We now extend this approach to the semisynthesis of full-length DKP-insulin analogues using unprotected Lys-Pro octapeptides and unprotected AspB10-DOI. Under standard conditions of solvent composition, pH, and protein concentration (Inouye et al., 1981; Shoelson et al., 1983; Nakagawa & Tager, 1986), we have typically obtained 50–70% yields of the desired insulin products (Figure 2A); unreacted DOI and low levels of covalent aggregates account for the remainder of recovered insulin-related peptides. Undesired tryptic impurities, products, and reactants were readily separated from the insulin analogues by gel filtration (Figure 2B). After lyophilization, the insulin analogue products were found to be >97% pure by analytical HPLC (Figure 2C). Amino acid analyses of all semisynthetic analogues and the results from fast atom bombardment mass spectrometry of selected analogues were as predicted. Therefore, synthetic steps involving addition and removal of protecting groups were avoided, circumventing the associated losses in yield and side reactions that give rise to impurities in the final product.

Biophysical Characterization of Analogues

Circular Dichroism Studies. Far-UV CD spectra were obtained to assess overall differences in structure between related analogues of native and DKP-insulin (Goldman & Carpenter, 1974; Strickland & Mercola, 1976; Wollmer et al., 1977, 1981; Inouye et al., 1981; Lu, 1981). Such spectra primarily reflect elements of secondary structure, although contributions from aromatic residues and cystines may be observed. CD spectra were obtained at pH 2.0 to suppress dimer formation and allow direct structural comparisons between analogues of human insulin and DKP-insulin. In addition, CD studies of DKP analogues were conducted at pH 7.0 to evaluate potential pH-dependent differences in structure.

CD spectra of unsubstituted human insulin and DKP-insulin are remarkably similar in aqueous HCl at pH 2.0. Nearly identical local minima are observed at 208 nm, and a slight attenuation is observed in the 222-nm band of DKP-insulin

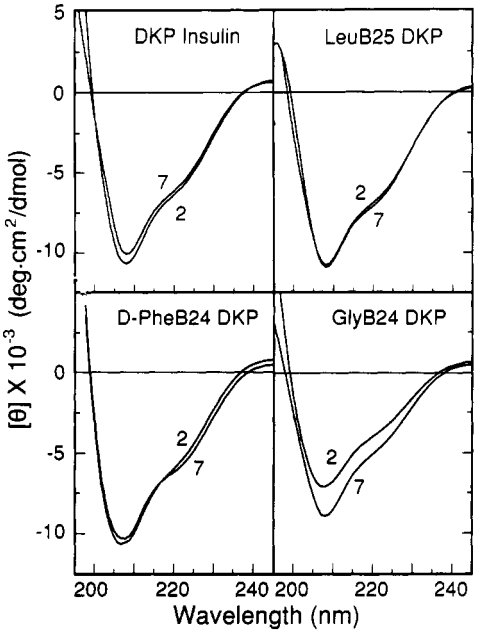

FIGURE 4: CD spectra of DKP analogues at pH 2.0 and pH 7.0. Spectra, obtained in aqueous HCl (pH 2.0) or phosphate buffer (pH 7.0) as described under Experimental Procedures, are labeled with the number 2 or 7 to reflect the pH.

relative to native insulin (Figure 3, panel a). These findings are consistent with previous NMR findings suggesting similar solution structures for human insulin and DKP-insulin (Weiss et al., 1989, 1991; Kline & Justice, 1990; Hua & Weiss, 1991).

Solution structures of the substituted analogues were also compared, as exemplified in panels b–d of Figure 3. CD spectra obtained under these conditions are closely matched between each analogue pair (human vs DKP-insulin). These results suggest that in each case the substitutions have similar effects on global structure. In fact, spectra of most of the analogues were quite similar to spectra of the unsubstituted parent insulins (e.g., compare panels a–c of Figure 3). The only marked differences were observed for the GlyB24 analogues, both of which exhibited substantially less intensity than all other analogues (Figure 3, panel d). In addition, GlyB24 analogues showed the greatest differences between analogue pairs, with minima at both 208 and 222 nm having lower intensities for the native analogue (Figure 3, panel d).

CD spectra for DKP analogues were also obtained at pH 7.0 to determine whether changes in secondary structure accompany the transition to neutral pH (Figure 4). No significant differences were observed between pH values (2.0 vs 7.0) for any DKP analogue except GlyB24 DKP-insulin. These results suggest that the structures of the analogues (with the exception of GlyB24 DKP-insulin) are similar at the two pH values. This is consistent with ^{1}H-NMR studies of DKP-insulin, which similarly show no significant differences in core structure at the two pH values (data not shown).

GlyB24 DKP-insulin does exhibit pH-dependent spectral changes, with increases in intensity for both minima (208 and 220 nm) at pH 7.0 (Figure 4). Taken together, the variability of CD spectra observed for GlyB24 analogues—(a) lower intensities of both minima at pH 2.0, compared to the unsubstituted parent insulins, (b) the greatest difference between analogue pairs at pH 2.0, and (c) the greatest pH-dependent spectral shift, compared to other DKP-analogues—suggests that the PheB24 → Gly substitution destabilizes elements of insulin structure. In fact, the C-terminus of the B-chain of GlyB24 insulin is disordered in solution, and NMR evidence for the B20–B23 β-turn is lacking (Hua et al., 1991, 1992).

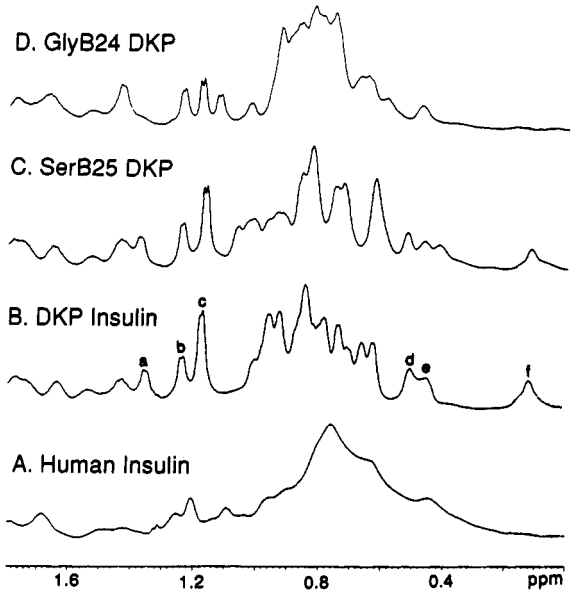

FIGURE 5: Aliphatic regions of the 500-MHz ^{1}H-NMR spectra of (A) native human insulin, (B) DKP-insulin, (C) PheB25 → Ser DKP-insulin, and (D) PheB24 → Gly DKP-insulin. Samples were in 50 mM aqueous potassium phosphate buffer, pH 7.4, and spectra were accumulated at 22 °C. Spectra, which are the sum of 128 scans, were apodized with a 2-Hz exponential multiplication prior to the Fourier transformation. All protein concentrations were approximately 1.0 mM. Resonances a–f are assigned as follows: (a) AlaB14 βCH_3; (b) ThrA8 γCH_3; (c) ThrB27 and ThrB30 γCH_3; (d) LeuB15 $\delta_1 CH_3$; (e) IleA10 δCH_3; (f) LeuB15 $\delta_2 CH_3$ and βCH.

Therefore, we propose that the observed variability in CD spectral amplitude for GlyB24 analogues reflects inherent differences in the time-averaged content of structural elements that may accompany alterations in overall molecular flexibility.

^{1}H-NMR of Human Insulin. The aliphatic region of the 1D ^{1}H-NMR spectrum of human insulin (Figure 5A), obtained at neutral pH (50 mM potassium phosphate, pH 7.4), consists of an overlapping set of broad resonances. This is due to insulin self-association (Jeffery & Coates, 1966a,b; Blundell et al., 1972; Goldman & Carpenter, 1974), which causes broadening of ^{1}H-NMR resonances as the result of intermediate-exchange mechanisms and the longer correlation times of the aggregates. 2D-NMR experiments do not help to resolve the poor spectral quality observed for insulin under these conditions as few cross-peaks are observed due to antiphase cancellation of 2D multiplets (COSY) or rapid longitudinal relaxation (NOESY) (Weiss et al., 1991).

^{1}H-NMR of DKP-Insulin. The 1D ^{1}H-NMR spectrum of DKP-insulin (Figure 5B) was observed under identical buffer conditions. In this case, line widths are appropriate for a protein the size of insulin, with no changes in line width observed over a broad concentration range (0.015–3.0 mM; data not shown). DKP-insulin remains monomeric even up to 10 mM protein concentrations (B. H. Frank, personal communication), so the line-broadening mechanisms operative in the case of native insulin do not apply. The 1D spectrum exhibits dispersion of chemical shifts characteristic of a stably folded structure. This is supported by 2D-NMR DQF-COSY and TOCSY spectra, which contain the expected number of amino acid spin systems, and 2D NOESY spectra, which reveal extensive interresidue contacts. Detailed analysis of such contacts indicate that DKP-insulin adopts a well-defined structure in solution, in general accord with crystal models (Weiss et al., 1991).

^{1}H-NMR of DKP-Insulin Analogues. 1D ^{1}H-NMR spectra of PheB25 → Ser and PheB24 → Gly analogues of DKP-

insulin are shown as panels C and D of Figure 5, respectively. Like DKP-insulin, the substituted DKP analogues exhibit spectral qualities expected of stably folded proteins the size of insulin, with appropriately narrow line widths and dispersion of chemical shifts. However, relative to DKP-insulin, the spectrum of the GlyB24 DKP analogue exhibits substantially less chemical shift dispersion in a selected subset of resonances. Presumably, this reflects in part loss of the PheB24 ring-current field. In particular, the methyl resonances of LeuB15, which are shifted far upfield in the spectrum of DKP-insulin (≈0.12 ppm, Figure 5, resonance f in panel B), are observed within the general methyl envelope (0.7–1.0 ppm) in the spectrum of GlyB24 DKP-insulin (panel D). LeuB15 is adjacent to PheB24 in insulin crystals, and a large upfield shift would be expected by this geometry. Additional loss of chemical shift dispersion can in principle result from a more flexible overall structure, a possibility that will be addressed in a future study.

In contrast, the extent of dispersion in the spectrum of SerB25 DKP-insulin is as great as or greater than that observed for DKP-insulin. Less significant diamagnetic effects are due to loss of the PheB25 ring current, consistent with its flexibility as a surface residue (Weiss et al., 1991). It is likely that the multiple small changes in chemical shifts observed in the SerB25 spectrum have a structural origin, at least in part (i.e., nonlocal changes in structure mediated by the SerB25 side chain). This hypothesis will be tested in the future by detailed comparisons of interproton distances (NOEs) in the unsubstituted and mutant proteins.

Insulin Receptor-Binding Affinities

Native Insulin and DKP Substitutions. Equilibrium binding assays were conducted with WGA-purified human insulin receptor, [^{125}I]iodo-TyrA14-insulin, and varying concentrations of either native human insulin or the unlabeled analogues. Values for K_D were calculated as the peptide concentration causing half-maximal inhibition of binding of [^{125}I]iodo-TyrA14-insulin to receptor; relative potencies were determined as (analogue potency/human insulin potency) × 100%.

The rationale behind the design of monomeric DKP-insulin, developed at the Lilly Research Laboratories, is based on differences in primary structure between insulin and IGF-1 (see Discussion). The effects of these substitutions on insulin self-association have been presented elsewhere (Weiss et al., 1991; DiMarchi et al., 1992); the individual effects of each substitution on receptor binding are shown in Figure 6. Switching the positions of ProB28 and LysB29 to form [LysB28, ProB29]human insulin has no significant effect on receptor-binding potency. Although these positions are critical for dimerization, neither residue appears to play a direct role in receptor recognition. In contrast, the HisB10 → Asp substitution enhances binding potency ≈2-fold in equilibrium radioreceptor assays. The structural basis for this slight increase in affinity is unclear, from either ^{1}H-NMR (Weiss et al., 1991) or molecular modeling (data not shown) studies. The B10 position has not been thought to participate in binding directly (Pullen et al., 1976; Baker et al., 1988). Furthermore, no notable perturbations are seen by 2D-NMR in the orientations of additional residues that are likely to interact directly with the receptor (e.g., PheB25; Weiss et al., 1991). It is noteworthy, however, that the magnitude of this effect is thermodynamically small (a 2-fold difference in affinity represents ≤0.5 kcal of free energy, less than a single hydrogen bond). Simultaneous substitution at both positions (DKP-insulin) has an effect on receptor binding identical to that of the single HisB10 → Asp mutation. These data in conjunction

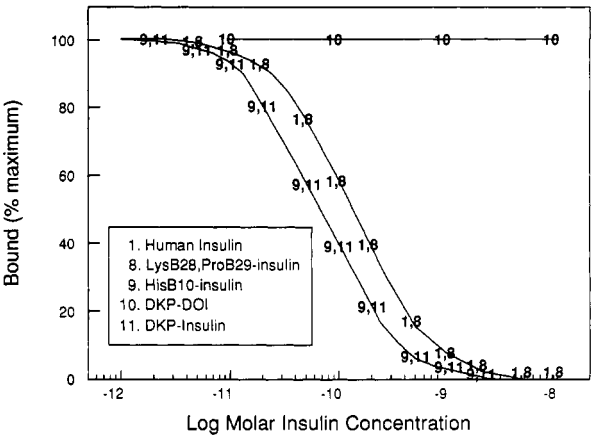

FIGURE 6: Binding of analogues substituted at B10, B28, and B29 positions to WGA-purified human insulin receptor. Analogues and [^{125}I]iodo-TyrA14-insulin were incubated with partially purified human insulin receptor as described under Experimental Procedures. Numbers (Table I) are used to indicate data points rather than symbols: (1) human insulin; (8) ProB24 → Lys, LysB29 → Pro human insulin; (9) HisB10 → Asp insulin; (10) des-[B23–B30]octapeptide HisB10 → Asp insulin (AspB10-DOI); (11) HisB10 → Asp, ProB28 → Lys, LysB29 → Pro insulin (DKP-insulin).

with results of Brange and co-workers (1988, 1990) support the notion that substitutions at the dimer and hexamer surfaces have independent effects on insulin structure and function.

PheB24 Substitutions. In insulin crystals the benzyl side chain of PheB24 packs against residues that contribute to insulin's core, including ValB12, LeuB15, and CysB19 (Blundell et al., 1971; Baker et al., 1988). A similar placement of the side chain has been found in solution (Weiss et al., 1989, 1991; Kline & Justice, 1990). Substitutions at this position with L-amino acids having nonaromatic side chains have been shown to reduce binding potency and perturb solution structure, as assessed by CD spectroscopy. These studies suggest a requirement for interresidue side chain and backbone contact involving PheB24 (Tager et al., 1980; Wollmer et al., 1981; Inuoye et al., 1981; Shoelson et al., 1983b; Kobayashi et al., 1984; Nakagawa & Tager, 1987; Mirmira & Tager, 1989; Mirmira et al., 1991). By contrast, substitutions of the B24 position with either residues having a D-configuration or glycine have little effect on binding potency (Kobayashi et al., 1982b; Mirmira & Tager, 1989; Mirmira et al., 1991), suggesting that the side chain of invariant PheB24 might not be important for receptor recognition, per se.

In the current study, we have reexamined the effects of certain interesting B24 substitutions on the structure and function of native insulin and monomeric DKP-insulin (Figure 7 and Table I). Replacement of the PheB24 residue of native human insulin with serine provides Insulin Los Angeles, an abnormal insulin associated with diabetes (Shoelson et al., 1983a,b, 1984; Haneda et al., 1984). In this study, we found a 7% relative binding potency for SerB24 insulin, in accord with previous findings (Shoelson et al., 1983b; Kobayashi et al., 1984). By contrast, substitution of L-PheB24 with D-phenylalanine enhances receptor-binding potency 180% (Figure 7, Table I) as previously shown (Inouye et al., 1982; Kobayashi et al., 1982b; Mirmira & Tager, 1989). In addition, in this study we prepared GlyB24 insulin to help assess the relative roles of D- and L-side chains in maintaining core structure and overall molecular flexibility. GlyB24 insulin exhibits 22% potency, again in reasonable agreement with the findings of Mirmira and Tager (1989).

Identical substitutions were made in DKP-insulin. In this case, potencies relative to human insulin were 14% for SerB24

Table I

identifying number	analogue	relative potency (%) this study[a]	relative potency (%) previous report
1	human insulin	100	
2	PheB24 → D-Phe insulin	180(3)	160[b,c]
3	PheB24 → Gly insulin	22(4)	78[c]
4	PheB24 → Ser insulin	7.0(3)	0.7–16[d,e]
5	PheB25 → Ala insulin	7.0(2)	10–15[f]
6	PheB25 → Leu insulin	2.0(2)	1–5[f-j]
7	PheB25 → Ser insulin	1.0(3)	0.5–8[d,e,j]
8	HisB10 → Asp insulin	200(3)	200–500[k,l]
9	ProB28 ↔ LysB29 insulin	100(3)	
10	HisB10 → Asp DOI	<0.1(2)	
11	DKP-insulin	200(3)	
12	PheB24 → D-Phe DKP-insulin	330(4)	
13	PheB24 → Gly DKP-insulin	50(3)	
14	PheB24 → Ser DKP-insulin	14(3)	
15	PheB25 → Ala DKP-insulin	11(3)	
16	PheB25 → Leu DKP-insulin	3.0(3)	
17	PheB25 → Ser DKP-insulin	1.6(3)	

[a] The number of times binding assays were repeated for each analogue are in parentheses. [b] Kobayashi et al. (1982b). [c] Mirmira and Tager (1989). [d] Shoelson et al. (1983). [e] Kobayashi et al. (1984). [f] Inouye et al. (1981). [g] Tager et al. (1980). [h] Wollmer et al. (1981). [i] Kobayashi et al. (1982a). [j] Nakagawa and Tager (1986). [k] Schwartz et al. (1987). [l] Brange et al. (1988).

DKP-insulin, 50% for GlyB24 DKP-insulin, and 330% for D-PheB24 DKP-insulin (Figure 7, Table I). The trend in relative potencies was the same in DKP-insulin as in native insulin (D-PheB24 > L-Phe > GlyB24 > SerB24), although in each case the potency of the substituted DKP analogue was slightly higher. To assess the isolated effects of the B24 substitutions, potencies of DKP analogues relative to DKP-insulin were determined as (DKP analogue potency/DKP-insulin potency) × 100%. Computed in this fashion, relative potencies were 8% for SerB24, 28% for GlyB24, and 183% for D-PheB24 analogues of DKP-insulin, remarkably similar to the relative potencies of the same substitutions in native insulin. The parallel trends and similar magnitudes in binding potency suggest that the effects of substitutions at the B24 position are independent of the effects of concomitant substitutions at the B10, B28, and B29 positions.

PheB25 Substitutions. The side chain of PheB25 adopts two asymmetric orientations in 2-zinc insulin crystals (Blundell, 1971; Peking, Insulin Structure Group, 1971; Baker et al., 1988). In molecule 1 (Peking Insulin Structure Group, 1971), the side chain turns inward to contact TyrA19, whereas the side chain of molecule 2 points outward from the molecule. Analyses by 2D-NMR suggest that in solution the orientation of this side chain resembles molecule 2 and that it is presumably flexible and surrounded by water molecules (Weiss et al., 1989, 1991). This model suggests that the PheB25 side chain would be free to interact with the receptor directly, and it rationalizes why substitutions at this position generally have dramatic effects on receptor-binding potency. In fact, any substitution that eliminates an L-configured β-aromatic side chain reduces binding potency substantially (Nakagawa & Tager, 1986; Mirmira et al., 1991). Other aromatic L-amino acids are tolerated, however, even those with more extended or substituted ring structures like tryptophan, naphthylalanine (Nakagawa & Tager, 1986), benzoylphenylalanine (Shoelson et al., 1992), and additional analogues substituted at the para position of PheB25 (Mirmira & Tager, 1991).

We have also investigated the effects of substitutions at the B25 position of human insulin and DKP-insulin. One such analogue, PheB25 → Leu insulin (Insulin Chicago), is a naturally occurring mutation associated with diabetes mellitus

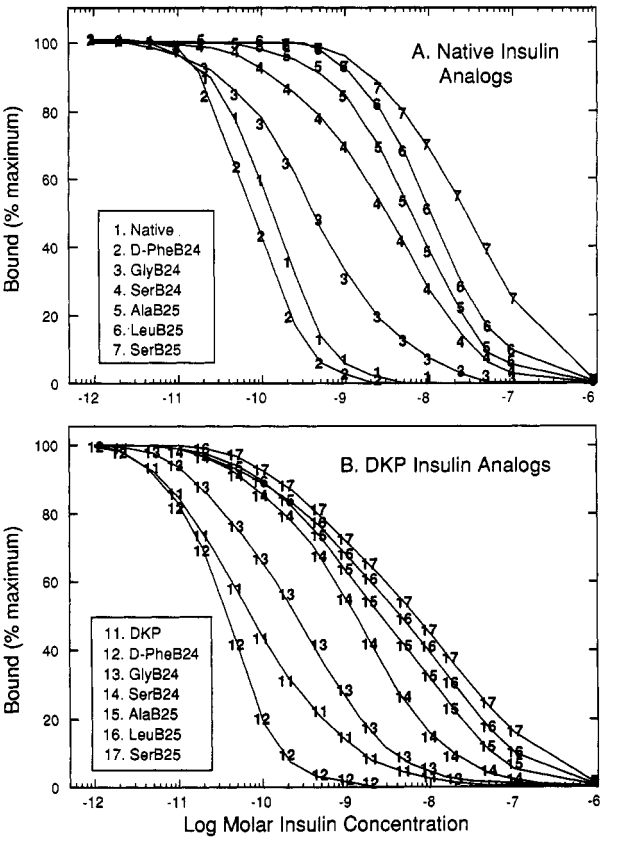

FIGURE 7: Binding potencies of (A) single point mutants of human insulin substituted at the B24 and B25 positions and (B) similar analogues of DKP-insulin substituted at the B24 and B25 positions. Conditions for equilibrium binding assays with WGA-purified insulin receptors were conducted at 4 °C as described under Materials and Methods. Numbers, identified in Table I, denote data points. Analogues 1–7 (panel A) are single mutants of human insulin: (1) human insulin; (2) PheB24 → D-Phe insulin; (3) PheB24 → Gly insulin; (4) PheB24 → Ser insulin; (5) PheB25 → Ala insulin; (6) PheB25 → Leu insulin; (7) PheB25 → Ser insulin. Analogues 11–17 (panel B) are the corresponding mutants of DKP-insulin: (11) DKP-insulin (HisB10 → Asp, ProB28 → Lys, LysB29 → Pro insulin); (12) PheB24 → D-Phe DKP-insulin; (13) PheB24 → Gly DKP-insulin; (14) PheB24 → Ser DKP-insulin; (15) PheB25 → Ala DKP-insulin; (16) PheB25 → Leu DKP-insulin; (17) PheB25 → Ser DKP-insulin.

(Tager et al., 1979; Given et al., 1979; Shoelson et al., 1983a). This substitution reduces binding potency to 2% (Figure 7 and Table I), in accord with previous findings (Tager et al., 1980; Wollmer et al., 1981; Inouye et al., 1981; Kobayashi et al., 1982a). We have also studied SerB25 and AlaB25 analogues of human insulin which exhibit 1% and 7% potencies, respectively, also in reasonable agreement with previous findings (Inouye et al., 1981; Shoelson et al., 1983b; Kobayashi et al., 1984; Nakagawa & Tager, 1986).

Similar analogues of DKP-insulin were synthesized, and their relaive relative receptor-binding potencies were measured: 3% for LeuB25 DKP-insulin, 1.6% for SerB25 DKP-insulin, and 11% for AlaB25 DKP-insulin (relative to native human insulin). As observed for identical substitutions in human insulin, the rank order of relative potencies was PheB25 ≫ AlaB25 > LeuB25 > SerB25. In each case, DKP analogues were slightly more potent than related analogues of native insulin. When normalized relative to DKP-insulin, however, potencies of DKP analogues were 1.7% for LeuB25, 1% for SerB25, and 6% for AlaB25 DKP analogues. Therefore, substitutions at the B25 position have similar effects on the receptor-binding potencies of native insulin and DKP-insulin. These results provide further evidence for the hypothesis that functional consequences of substitutions within the receptor-

binding surface are independent of substitutions at the B10 and B28/B29 positions.

Discussion

The packing of protomers in crystals provides a model for insulin self-association in solution and has been well characterized in several distinct crystal forms (Adam et al., 1969; Peking Insulin Structure Group, 1971; Blundell et al., 1972; Bentley et al., 1978; Dodson et al., 1979; Smith et al., 1984; Bi et al., 1984; Baker et al., 1988; Derewenda et al., 1989). In 2-Zn and 4-Zn crystal structures, each unit cell consists of six insulin molecules in a hexameric array. The hexamer consists of three equivalent dimers, which in turn are composed of inequivalent protomers. There are two independent insulin–insulin interfaces in the crystal state: the hexamer- and dimer-contact surfaces. Recent 2D-NMR studies suggest that these surfaces are retained in the solution structure of the insulin monomer (Hua & Weiss, 1991), and the interactions between insulin molecules in dimers and hexamers in neutral solution are thought to be similar to those observed in crystals. Insulin analogues with substitutions in each surface have been described and exhibit altered in vitro self-association (Brange et al., 1988, 1990; Markussen et al., 1988; Weiss et al., 1991; DiMarchi et al., 1992) and in vivo pharmacokinetic behavior (Kang et al., 1990, 1991). In this Discussion section we first review the design of DKP-insulin and then consider its utility as a monomeric template for analysis of additional mutations in insulin's receptor-recognition surface.

DKP-Insulin: An Engineered Insulin Monomer

Dimer Contact. The dimer-forming surface of insulin includes contributions from residues A21, B8, B9, B12, B16, B20, and B21 and the B23–B28 segment. In particular, there are extensive contacts between adjacent B23–B26 segments of the two molecules which form a stretch of well-defined antiparallel β-pleated sheet (Baker et al., 1988). IGF-1 does not form such dimers. Although the amino acid contents of insulin and IGF-1 are similar in these domains, the order of residues is switched at two key positions: insulin is (B23)-GFFYTPKT(B30) and IGF-1 is (22)GFYFNKPT(29). The Phe $\leftrightarrow$ Tyr switch has little effect either on receptor binding affinity (Mirmira et al., 1991) or on insulin self-association (B. H. Frank, personal communication). The Pro $\leftrightarrow$ Lys swap similarly has little effect on receptor binding (Figure 6 and Table I) although it does dramatically reduce dimerization by a factor of at least 10^3 (DiMarchi et al., 1992).

Hexamer Contact. Residues of insulin that interact at the hexamer interface include A13, A14, A17, B1, B2, B4, B10, B13, B14, and B17–B20. Three HisB10 imidazole rings coordinate each zinc atom in 2-zinc crystals, forming a nucleus for hexamer formation. The corresponding residue of IGF-1, which neither chelates zinc nor forms hexamers, is glutamic acid. Hagfish insulin, which also does not form hexamers, has an aspartic acid at the corresponding position, suggesting that an acidic residue at the B10 position might destabilize associations involving this surface. As shown previously, AspB10 insulin exhibits enhanced receptor-binding potency and reduced self-association (Schwartz et al., 1987; Brange et al., 1988, 1990).

DKP-Insulin as a Monomeric Template for Studying Effects of Mutations in the Receptor-Binding Surface of Insulin

HisB10 $\rightarrow$ Asp, ProB28 $\rightarrow$ Lys, LysB29 $\rightarrow$ Pro insulin (DKP-insulin), an analogue substituted at both hexamer and dimer surfaces, remains essentially monomeric at neutral pH at concentrations $\leq$10 mM (DiMarchi et al., 1992; Weiss et al., 1991). Nevertheless, receptor-binding potency remains intact, presumably because residues involved in receptor recognition have not been altered. In a previous study we exploited the monomeric nature of DKP-insulin to assess the structure of an insulin monomer under physiologic pH and solvent conditions. We now extend this approach to the design of insulin analogues having additional substitutions within the receptor-binding domain.

Because detailed structural analyses of the insulin–receptor complex have not yet been possible, considerably less is known about the residues which interact at this protein–protein interface. Nevertheless, a putative receptor-binding surface of insulin has been deduced from comparison of the crystallographic structures of insulin, sequences of insulins from many animal species, and additional studies of insulin analogues (Pullen et al., 1976; Gammeltoft, 1984; Baker et al., 1988). Thus, GlyA1, GlnA5, TyrA19, AsnA21, ValB12, TyrB16, GlyB23, PheB24, and PheB25 are proposed to contribute to the surface of insulin that becomes buried within the insulin–receptor complex. Inspection of residues involved in dimer, hexamer, and receptor associations shows regions of overlap only between residues at the dimer and putative receptor interfaces; virtually no overlap is predicted between the receptor-binding surface and insulin's hexamer-forming surface. In DKP-insulin, none of the substitutions are involved directly in receptor recognition, and so receptor affinity is minimally altered (Figure 6 and Table I).

The conserved phenylalanine residues at the B24 and B25 positions are key elements in directing insulin–receptor interactions and in formation of insulin dimers. The contributions of these residues have been well studied by mutational analyses (Tager et al., 1980; Wollmer et al., 1981; Inouye et al., 1981; Kobayashi et al., 1982, 1984; Shoelson et al., 1983b; Nakagawa & Tager, 1986; Mirmira & Tager, 1989; Mirmira et al., 1991) and thus provide a general test of whether DKP-insulin is an appropriate monomeric template for future analogue design. This test case permits us to evaluate the consequences of simultaneous substitutions at all three interactive surfaces of insulin (dimer, hexamer, and receptor).

Mutagenesis of the PheB24 and PheB25 Positions

The benzyl side chains of PheB24 and PheB25 appear to have different roles in maintaining insulin structure and supporting intermolecular associations (Blundell et al., 1971; Wollmer et al., 1981; Nakagawa & Tager, 1986, 1987; Baker et al., 1988; Mirmira & Tager, 1989). PheB24 projects in toward the hydrophobic core of insulin, with its benzyl side chain packed against ValB12, LeuB15, and the A20–B19 disulfide. The PheB24 side chain helps stabilize the B20–B23 β-turn (Hua et al., 1991, 1992) and may not interact directly with the insulin receptor. By contrast, the PheB25 side chain projects outward in solution and is available to interact with the insulin receptor directly.

Insulin dimerization involves an extensive series of van der Waals' interactions between adjacent nonpolar surfaces. The PheB24-PheB25-TyrB26 segment of one monomer forms an antiparallel β-sheet with the contiguous TyrB26-PheB25-PheB24 segment of the opposing monomer. Main-chain hydrogen bonds are formed between PheB24(1) and TyrB26(2) and similarly between PheB24(2) and TyrB26(1). Therefore, substitutions within this domain affect dimer formation in addition to receptor recognition and usually reduce the stability of the dimer. The HisB10 $\rightarrow$ Asp and ProB28 $\leftrightarrow$ LysB29 substitutions of DKP-insulin so completely inhibit insulin association that we were unable to determine whether B24 and

B25 substitutions yielded additional effects on DKP-insulin self-association. CD and NMR analyses reveal no detectable self-association of DKP-analogues, validating its use as a monomeric template.

PheB24 Substitutions. As discussed above, receptor-binding potencies of SerB24 insulin (Insulin Los Angeles) and LeuB24 insulin are each ≈10% that of native insulin (this study; Tager et al., 1980; Wollmer et al., 1981; Inouye et al., 1981; Shoelson et al., 1983; Nakagawa & Tager, 1986). Interestingly, substitution at the B24 position with the D-isomer of either phenylalanine or tyrosine increases binding potency and substitution with glycine has but a slightly deleterious effect (Kobayashi et al., 1982; Mirmira & Tager, 1989; Mirmira et al., 1991; this study). These findings led Mirmira and Tager (1989) to propose that conformational adjustments occur in the structure of insulin upon interaction with the receptor. Our 2D-NMR studies of GlyB24 insulin have shown an unfolding of structure with separation between a disordered B20–B30 segment and a relatively normal core structure composed of the A-chain and B-chain residues B1–B20 (Hua et al., 1991, 1992). Within insulin crystals, this segment lies against hydrophobic core residues of the A-chain (IleA2 and ValA3). The ability of the B23–B30 segment of GlyB24 insulin to adopt alternative conformations while binding affinity is maintained suggests that underlying residues might be exposed for direct contact during receptor complex formation.

PheB25 Substitutions. Removal of the PheB25 ring by substitution with alanine reduces the affinity of the insulin–receptor interaction ≈7–12-fold (Table I). There is no evidence for either local or distant perturbations in AlaB25 insulin structure (this study and unpublished observations), suggesting that the reduction in affinity results from direct elimination of interactions between the PheB25 ring and residues within the receptor-binding pocket. Similarly, the PheB25 → Leu substitution (Insulin Chicago) has no direct effect on insulin's global structure, and the branched aliphatic side chain appears to project outward in solution (Weiss et al., 1989; unpublished observations). Therefore, the ≈50-fold reduction in receptor-binding affinity exhibited by this analogue appears to result from both loss of important contacts between the phenyl ring and the receptor and additional steric effects that further inhibit tight association.

From these observations, SerB25 insulin might be expected to exhibit an affinity similar to that of AlaB25 insulin. However, this analogue has been found to have ≤1% binding affinity [Shoelson et al., 1983; Nakagawa & Tager, 1986; this study; Kobayashi et al. (1984) noted a slightly higher affinity]. Preliminary NMR and molecular modeling studies reveal a potential structural explanation for the weaker than expected binding potency of SerB25 insulin. Upfield-shifted resonances of native insulin are shifted further upfield in SerB25 insulin, and downfield-shifted resonances are shifted further downfield. Enhancement of chemical shift dispersion appears to result from a global stabilization of structure. Molecular modeling studies suggest that this could result from an inward turning of the SerB25 side chain with the accompanying formation of a new hydrogen bond between the SerB25 hydroxyl group and the main-chain carbonyl of TyrA19 (D. T. Nguyen, M. A. Weiss, & M. Karplus, unpublished observations). The current study was prompted in part by the importance of performing a detailed structural analyses of these and additional analogues under physiologic solvent conditions.

The independence of effects observed for substitutions at the B10, B24, B25, and B28/B29 positions was not fully anticipated. For example, des-[B26–B30]pentapeptide insulin amide (DPA) is a truncated analogue with full affinity for the insulin receptor (Fischer et al., 1985; Nakagawa & Tager, 1986). DPA is missing a substantial portion of the residues involved in dimer formation and thus exhibits reduced self-association in solution (Jeffery, 1986). We have prepared AspB10-DPA, which, being substituted at the hexamer surface as well, has little potential for insulin–insulin interaction whatsoever. AspB10-DPA is a fully potent agonist (S. E. Shoelson, data not shown) which might be useful as a template (like DKP-insulin) for studying the effects of additional substitutions on structure and function. In fact, one such analogue, AspB10,TyrB25-DPA, exhibits additive effects on binding, suggesting that these substitutions may be independent (Schwartz et al., 1989). However, it has become clear that substituted DPA analogues do *not* exhibit parallel effects on receptor-binding affinity (Nakagawa & Tager, 1986, 1987; Casaretto et al., 1987). Mutagenesis of the B24 and B25 positions of DPA reveal dramatic differences (DPA = AlaB25-DPA > SerB25-DPA > LeuB25-DPA > D-PheB24-DPA) from the corresponding changes in native insulin (D-PheB24 > insulin ≫ AlaB25 > LeuB25 > SerB25). Therefore, although AspB10-DPA might be monomeric at high protein concentrations and easily substituted by trypsin-catalyzed semisynthesis, it would be a poor choice of template for studying structural and dynamic effects of substitutions in the receptor-binding surface of native insulin.

CONCLUSIONS

Mutagenesis of the dimer- (ProB28 ↔ LysB29) and hexamer- (HisB10 → Asp) forming surfaces of insulin provides a monomeric insulin analogue (designated DKP-insulin). The substituted residues of DKP-insulin are not involved in insulin–receptor interactions, and DKP-insulin retains full (slightly enhanced) receptor-binding potency. In this paper, the structural and functional consequences of additional substitutions within the receptor recognition surface of DKP-insulin were compared to those of analogous substitutions in native human insulin. We conclude that substitutions at three different surfaces of insulin involved in distinct protein–protein interactions—the hexamer (B10), dimer (ProB28, LysB29), and receptor-binding surfaces (PheB24, PheB25)—can be modeled as independent design features. Using this approach, self-association and receptor potency can be altered independently in the design of novel analogues with therapeutic potential. These results provide a foundation for comparative 2D-NMR studies of mutant insulins, which will facilitate evaluations of the effects of these mutations on insulin structure and dynamics.

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REFERENCES

Adams, M. J., Blundell, J. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Hardine, M. M., Hodgkin, D.

C., Rimer, B., & Sheet, S. (1969) *Nature (London) 224*, 491–495.

Assoian, R. K., Thomas, N. E., Kaiser, E. T., & Tager, H. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5147–5151.

Baker, E. N., Blundell, T. E., Cutfield, G. S., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. M. C., Hubbard, R. E., Iassac, M. W., Reynolds, D. C., Sakabe, K. S., Sakabe, N., & Vjayan, N. M. (1988) *Philos. Trans. R. Soc. London, B 319*, 389–456.

Bentley, G. A., Dodson, E. J., Dodson, G. G., & Levitova, A. (1978) *J. Mol. Biol. 125*, 387–396.

Bi, R. C., Dauter, Z., Dodson, E. J., Dodson, G. G., Giordano, F., & Reynolds, C. D. (1984) *Biopolymers 32*, 391–395.

Blundell, T. E., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., Mercola, D. A., & Vijayan, M. (1971) *Nature (London) 231*, 506–511.

Blundell, T., Dodson, G. G., Hodgkin, D. M. C., & Mercola, D. (1972) *Adv. Protein Chem. 26*, 279–402.

Bradbury, J. H., & Ramesh, V. (1985) *Biochem. J. 229*, 731–737.

Brange, J., Ribel, U., Hansen, J. F., Dodson, G., Hansen, M. T., Havelund, S., Melberg, S. G., Norris, F., Norris, K., Snel, L., Sorensen, A. R., & Voigt, H. O. (1988) *Nature (London) 333*, 679–682.

Brange, J., Owens, D. R., King, S., & Volund, A. (1990) *Diabetes Care 13*, 923–954.

Bromer, W. W., & Chance, R. E. (1967) *Biochim. Biophys. Acta 133*, 219–223.

Casaretto, M., Spoden, M., Diaconescu, C., Gattner, H.-G., Zahn, H., Brandenburg, D., & Wollmer, A. (1987) *Biol. Chem. Hoppe-Seyler 368*, 709–716.

Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Reynolds, C. D., Smith, G. D., Sparks, C., & Swenson, D. (1989) *Nature (London) 338*, 594–596.

Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., & Bing, X. (1991) *J. Mol. Biol. 220*, 425–433.

DiMarchi, R. D., Mayer, J. P., Fan, L., Long, H., Sundell, K., Slieker, L. J., Shaw, W. N., Howey, D., Frank, B. H., Shields, J. E., & Chance, R. E. (1992) in *Peptides: Chemistry and Biology* (Smith, J. A., & Rivier, J. E., Eds.) Escom Science Publishers, Leiden, The Netherlands (in press).

Dodson, E. J., Dodson, G. G., & Hodgkin, C. D. (1979) *Biochemistry 18*, 469–479.

Ebina, Y., Edery, M., Leland, E., Beaudoin, J., Roth, R. A., & Rutter, W. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8014–8018.

Fischer, W. H., Saunders, D., Brandenburg, D., Wollmer, A., & Zahn, H. (1985) *Biol. Chem. Hoppe-Seyler 366*, 521–525.

Fischer, W. H., Saunders, D., Brandenburg, D., Diaconescu, C., Wollmer, A., Dodson, G., De Meyts, P., & Zahn, H. (1986) *Biol. Chem. Hoppe-Seyler 367*, 999–1006.

Gameltoft, S. (1984) *Physiol. Rev. 64*, 1321–1377.

Goldman, J., & Carpenter, F. H. (1974) *Biochemistry 13*, 4566–4574.

Haneda, M., Polonsky, K. P., Bergenstal, R. M., Jaspan, J. B., Shoelson, S. E., Blix, P. M., Chan, S. J., Kwok, S. C. M., Wischner, W. B., Zeidler, A., Olefsky, J. M., Freidenberg, G., Tager, H. S., Steiner, D. S., & Rubenstein, A. H. (1984) *N. Engl. J. Med. 310*, 1288–1294.

Hua, Q. X., & Weiss, M. A. (1990) *Biochemistry 29*, 10545–10555.

Hua, Q. X., & Weiss, M. A. (1991) *Biochemistry 30*, 5055–5515.

Hua, Q. X., Chen, Y. J., Wang, C. C., Wang, D. C., & Roberts, G. C. K. (1989) *Biochim. Biophys. Acta 994*, 114–120.

Hua, Q. X., Shoelson, S. E., Kochoyan, M., & Weiss, M. A. (1991) *Nature (London) 354*, 238–241.

Hua, Q. X., Shoelson, S. E., & Weiss, M. A. (1992) *J. Mol. Biol.* (submitted for publication).

Inouye, K., Watanabe, K., Morihara, K., Tochino, Y., Kanaya, T., Emura, J., & Sakakibara, S. (1979) *J. Am. Chem. Soc. 101*, 751–752.

Inouye, K., Watanabe, K., Tochino, Y., Kobayashi, M., & Shigeta, Y. (1981a) *Biopolymers 20*, 1845–1858.

Inouye, K., Watanabe, K., Tochino, Y., Kanaya, T., Kobayashi, M., & Shigeta, Y. (1981b) *Experientia 37*, 811–813.

Inouye, K., Watanabe, K., Tochino, Y., Kobayashi, M., & Shigeta, Y. (1983) in *Peptide Chemistry 1982* (Sakakibara, S., Ed.) pp 277–282, Protein Research Foundation, Osaka, Japan.

Jeffrey, P. D., & Coates, J. H. (1966a) *Biochemistry 5*, 489–498.

Jeffrey, P. D., & Coates, J. H. (1966b) *Biochemistry 5*, 3820–3825.

Jeffrey, P. D. (1986) *Hoppe-Seyler's Z. Biol. Chem. 367*, 363–369.

Kang, S., Owens, D. R., Vora, J. P., & Brange, J. (1990) *Lancet 330*, 303–306.

Kang, S., Creagh, F. M., Peters, J. R., Brange, J., Volund, A., & Owens, D. R. (1991a) *Diabetes Care 14*, 571–577.

Kang, S., Brange, J., Burch, A., Volund, A., & Owens, D. R. (1991b) *Diabetes Care 14*, 942–948.

Kline, A. D., & Justice, R. M., Jr. (1990) *Biochemistry 29*, 2906–2913.

Kobayashi, M., Ohgaku, S., Iwasaki, M., Maegawa, H., Shigeta, Y., & Inouye, K. (1982a) *Biochem. J. 206*, 597–603.

Kobayashi, M., Ohgaku, S., Iwasaki, M., Maegawa, H., Shigeta, Y., & Inouye, K. (1982b) *Biochem. Biophys. Res. Commun. 107*, 329–336.

Kobayashi, M., Haneda, M., Maegawa, H., Watanabe, N., Takada, Y., Shigeta, Y., & Inouye, K. (1984) *Biochem. Biophys. Res. Commun. 119*, 49–57.

Kristensen, S. M., Jorgensen, A. M., & Led, J. J. (1991) *J. Mol. Biol. 218*, 221–231.

Kubrick, T., & Coburn, D. (1986) *Int. J. Pept. Protein Res. 27*, 514–521.

Lu, Z.-X. (1981) *Sci. Sin. 24*, 1566–1574.

Markussen, J., Diers, I., Hougaard, P., Langkjar, L., Norris, K., Snel, L., Sorensen, A. R., Sorensen, E., & Voigt, H. O. (1988) *Protein Eng. 2*, 157–166.

Mirmira, R., & Tager, H. S. (1989) *J. Biol. Chem. 264*, 6349–6354.

Mirmira, R., & Tager, H. S. (1991) *Biochemistry 30*, 8222–8229.

Mirmira, R., Nakagawa, S. H., & Tager, H. S. (1991) *J. Biol. Chem. 264*, 1428–1436.

Nakagawa, S. H., & Tager, H. S. (1986) *J. Biol. Chem. 261*, 7332–7341.

Nakagawa, S. H., & Tager, H. S. (1987) *J. Biol. Chem. 262*, 1204–12058.

Pekar, A. H., & Frank, B. H. (1972) *Biochemistry 11*, 4013–4016.

Peking Insulin Structure Group (1971) *Peking Rev. 40*, 11–16.

Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, A., Brandenburg, D., Zahn, H., Gliemann, J., & Gammeltoft, S. (1976) *Nature (London) 259*, 369–373.
Rees, A. R., & Offord, R. E. (1976) *Biochem. J. 159*, 487–493.
Roy, M., Lee, R. W.-K., Brange, J., & Dunn, M. F. (1990) *J. Biol. Chem. 265*, 5448.
Schwartz, G. P., Burke, G. T., & Katsoyanis, P. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6408–6411.
Schwartz, G. P., Burke, G. T., & Katsoyanis, P. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 458–461.
Shoelson, S. E., Lynch, C. S., Chatterjee, S., Chaudhuri, M., & Feng, Y.-M. (1992) in *Peptides: Chemistry and Biology* (Smith, J. A., & Rivier, J. E., Eds.) Escom Science Publishers, Leiden, The Netherlands (in press).
Shoelson, S. E., Fickova, M., Haneda, M., Nahum, A., Musso, G., Kaiser, E. T., Rubenstein, A. H., & Tager, H. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7390–7394.
Shoelson, S. E., Haneda, M., Blix, P., Nanjo, K., Sanke, T., Inouye, K., Steiner, D., Rubenstein, A., & Tager, H. (1983b) *Nature (London) 302*, 540–542.
Shoelson, S. E., Polonsky, K. P., Zeidler, A., Rubenstein, A. H., & Tager, H. S. (1984) *J. Clin. Invest. 73*, 540–542.
Shoelson, S. E., White, M. F., & Kahn, C. R. (1988) *J. Biol. Chem. 263*, 4852–4860.
Shymko, R. M., Gonzales, N. S., Backer, J. M., White, M. F., & DeMeyts, P. (1988) *Biochem. Biophys. Res. Commun. 164*, 191–198.
Smith, G. D., Swenson, D. C., Dodson, E. J., Dodson, G. G., & Reynolds, C. D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7093–7097.
Steiner, D. F., Bell, G. I., & Tager, H. S. (1990) in *Endocrinology* (DeGroot, L. J., Ed.) pp 1263–1289, W. S. Saunders Co., Philadelphia, PA.
Strickland, E. H., & Mercola, D. (1976) *Biochemistry 15*, 3875–3884.
Tager, H., Given, B., Baldwin, D., Mako, M., Markese, J., Rubenstein, A., Olefsky, J., Kobayashi, M., Kolterman, O., & Poucher, R. (1979) *Nature (London) 281*, 122–125.
Tager, H., Thomas, N., Assoian, R., Rubenstein, A., Saekow, M., Olefsky, J., & Kaiser, E. T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3181–3185.
Weiss, M. A., Nguyen, D. T., Khait, I., Inouye, K., Frank, B. H., Beckage, M., O'Shea, E. K., Shoelson, S. E., Karplus, M., & Neuringer, L. J. (1989) *Biochemistry 29*, 9855–9873.
Weiss, M. A., Hua, Q. X., Lynch, C. S., Frank, B. H., & Shoelson, S. E. (1991) *Biochemistry 30*, 7373–7389.
Wollmer, A., Fleischhauer, J., Strassburger, W., Thiele, H., Bradenburg, D., Dodson, G., & Mercola, D. (1979) *Biochemistry 29*, 8389–8401.
Wollmer, A., Strassburger, W., Glatter, U., Dodson, G. G., McCall, M., & Rittel, W. (1981) *Z. Physiol. Chem. 362*, 581–591.
Yajima, H., Fujii, N., Funakoshi, S., Watanabe, T., Murayama, E., & Otaka, A. (1988) *Tetrahedron 44*, 805–819.

Binding of Basic Peptides to Acidic Lipids in Membranes: Effects of Inserting Alanine(s) between the Basic Residues†

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ABSTRACT: We studied the binding of peptides containing five basic residues to membranes containing acidic lipids. The peptides have five arginine or lysine residues and zero, one, or two alanines between the basic groups. The vesicles were formed from mixtures of a zwitterionic lipid, phosphatidylcholine, and an acidic lipid, either phosphatidylserine or phosphatidylglycerol. Measuring the binding using equilibrium dialysis, ultrafiltration, and electrophoretic mobility techniques, we found that all peptides bind to the membranes with a sigmoidal dependence on the mole fraction of acidic lipid. The sigmoidal dependence (Hill coefficient >1 or apparent cooperativity) is due to both electrostatics and reduction of dimensionality and can be described by a simple model that combines Gouy–Chapman–Stern theory with mass action formalism. The adjustable parameter in this model is the microscopic association constant k between a basic residue and an acidic lipid ($1 < k < 10$ M^{-1}). The addition of alanine residues decreases the affinity of the peptides for the membranes; two alanines inserted between the basic residues reduces k 2-fold. Equivalently, the affinity of the peptide for the membrane decreases 10-fold, probably due to a combination of local electrostatic effects and the increased loss of entropy that may occur when the more massive alanine-containing peptides bind to the membrane. The arginine peptides bind more strongly than the lysine peptides: k for an arginine residue is 2-fold higher than for a lysine residue. Our results imply that a cluster of arginine and lysine residues with interspersed electrically neutral amino acids can bind a significant fraction of a cytoplasmic protein to the plasma membrane if the cluster contains more than five basic residues.

Several classes of proteins contain clusters of positively charged amino acids that may bind to negatively charged phospholipids, which are located preferentially on the cytoplasmic surface of a plasma membrane (Op den Kamp, 1979; Bishop & Bell, 1988). The intrinsic or membrane-spanning proteins comprise one class. The cytoplasmic domain of these proteins often contains clusters of basic residues, which may help determine the orientation of these proteins in the mem-

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