Alanine Screening Mutagenesis Establishes Tyrosine 60 of Bovine Insulin-like Growth Factor Binding Protein-2 as a Determinant of Insulin-like Growth Factor Binding*

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The determinants of insulin-like growth factor (IGF) binding to its binding proteins (IGFBPs) are poorly characterized in terms of important residues in the IGFBP molecule. We have previously used tyrosine iodination to implicate Tyr-60 in the IGF-binding site of bovine IGFBP-3 (Hobba, G. D., Forbes, B. E., Parkinson, E. J., Francis, G. L., and Wallace, J. C. (1996) J. Biol. Chem. 271, 30529–30536). In this report, we show that the mutagenic replacement of Tyr-60 with either Ala or Phe reduced the affinity of bIGFBP-2 for IGF-I (4.0- and 8.4-fold, respectively) and for IGF-II (3.5- and 4.0-fold, respectively). Although adjacent residues Val-59, Thr-61, Pro-62, and Arg-63 are well conserved in IGFBP family members, Ala substitution for these residues did not reduce the IGF affinity of bIGFBP-2. Kinetic analysis of the bIGFBP-2 mutants on IGF biosensor chips in the BIAcore instrument revealed that Tyr-60 → Phe bIGFBP-2 bound to the IGF-I surface 3.0-fold more slowly than bIGFBP-2 and was released 2.6-fold more rapidly than bIGFBP-2. We therefore propose that the hydroxyl group of Tyr-60 participates in a hydrogen bond that is important for the initial complex formation with IGF-I and the stabilization of this complex. In contrast, Tyr-60 → Ala bIGFBP-2 associated with the IGF-I surface 5.0-fold more rapidly than bIGFBP-2 but exhibited an 18.4-fold more rapid release from this surface compared with bIGFBP-2. Thus both the aromatic nature and the hydrogen bonding potential of the tyrosyl side chain of Tyr-60 are important structural determinants of the IGF-binding site of bIGFBP-2.

The insulin-like growth factors (IGF-I and IGF-II) are polypeptides that play a central role in vertebrate growth and development by stimulating cellular proliferation and differentiation (recently reviewed in Ref. 1). The biological activities of the IGFs are mediated mainly through the type 1 IGF receptor which is found on the surface of most cell types (1, 2). In turn, the bioavailability of the IGFs is regulated by a family of IGF-specific binding proteins (IGFBPs). The IGFBP family consists of six high affinity IGFBPs (IGFBP-1 to 6) (reviewed in Refs. 3 and 4) and possibly an additional four proteins that can associate with IGFs with lower affinity (5, 6). Conserved gene structures and the high degree of sequence identity between the high affinity IGFBPs suggest that these proteins possess three domains and a common IGF binding motif (3, 4, 7). The sequences of two of these putative domains, the N- and C-terminal cysteine-rich domains, are highly conserved. Where the IGFBP family members differ is in the middle domain and in the possession of phosphorylation and glycosylation sites or sites of association with other biomolecules such as heparin or the integrin receptor (3, 4).

At the molecular level, the IGFs have been well characterized. High resolution NMR structures of both IGF-I (8) and IGF-II (9) have been determined, and the overlapping regions that are responsible for IGFBP and receptor interactions have been identified by chemical modification (10, 11), epitope mapping (12), and by mutagenesis (13–18). However, insight into the overall structure of the IGFBPs has been restricted to multiple sequence alignment and secondary structure prediction (19). Furthermore, the growing number of IGFBP mutagenic studies described to date has focused on aspects of IGFBP biology such as heparin binding (20), integrin receptor binding (21), extracellular matrix binding (22), specific proteolysis (23, 24) or phosphorylation (25) rather than the systematic identification and characterization of a common IGF binding motif.

In terms of the IGF-binding site, both the N- and C-terminal cysteine-rich domains of IGFBPs are believed to participate. This is suggested by the observations that N-terminal cysteine-rich domains of IGFBP-1 (26), IGFBP-3 (27), IGFBP-4 (24), and IGFBP-5 (28) and C-terminal cysteine-rich domains of IGFBP-2 (29, 30) and IGFBP-3 (31) all possess residual IGF binding affinity. Yet, the specific residues of IGFBPs that are directly involved in IGF binding have not been identified.

The rationale for this study is based on our observation that Tyr-60 was protected from iodination in the IGF-bIGFBP-2 complex (32). Furthermore, when Tyr-60 was iodinated, it caused a reduction in the binding affinity of bIGFBP-2 for the IGF ligand. However, the residues Val-59, Thr-61, Pro-62, and Arg-63 could also conceivably play a role in IGF binding that is disrupted when Tyr-60 is iodinated. These latter residues are...
highly conserved across the whole IGFBP family, whereas all of the described IGFBP-1 sequences from various species possess an alanyl rather than tyrosyl residue at the position corresponding to Tyr-60 in bIGFBP-2, as shown in Fig. 1. Therefore, in order to determine which residues in the Tyr-60 region of bIGFBP-2 do influence IGF binding, alanine-scanning mutagenesis has been performed across residues 59 and 63 inclusive. Tyr-60 has also been substituted with Phe to distinguish between the hydrogen bonding properties and the hydrophobic and aromatic properties of the tyrosyl side chain with respect to IGF binding.

In this report, the IGF binding characteristics of Val-59→Ala, Tyr-60→Ala, Tyr-60→Phe, Thr-61→Ala, Pro-62→Ala, and Arg-63→Ala bIGFBP-2 have been compared with bIGFBP-2 by Western ligand blot, solution binding assays, and in 3 regions is shown. The table 1 indicates species variation within an IGFBP type, and complete sequence identity with the bIGFBP-2 sequence is denoted by a dash (–). Percent identity relative to bIGFBP-2, at each amino acid position is as follows: Cys-57 (100), Gly-58 (100), Val-59 (96), Tyr-60 (85), Thr-61 (93), Pro-62 (56), Arg-63 (82), and Cys-64 (100).

**Fig. 1. Sequence homology of the IGFBP family across the “Tyr-60 region.”** The Tyr-60 region lies in the N-terminal cysteine-rich region of the IGFBP family sequences and is found between the 9th and 10th cysteine residues from the N terminus (corresponding to Cys-57 and Cys-64 of bIGFBP-2, respectively). The sequences shown are a summary of 27 aligned IGFBPs using the single letter amino acid code, representing a range of species including bovine (b), chicken (c), human (h), murine (m), ovine (o), porcine (p), and rat (r). All of the observed sequence variation in this region is shown. The square open brackets ([) indicate species variation within an IGFBP type, and complete sequence identity with the bIGFBP-2 sequence is denoted by a dash (–). Percent identity relative to bIGFBP-2, at each amino acid position is as follows: Cys-57 (100), Gly-58 (100), Val-59 (96), Tyr-60 (85), Thr-61 (93), Pro-62 (56), Arg-63 (82), and Cys-64 (100).

**EXPERIMENTAL PROCEDURES**

**Materials—**Recombinant bIGFBP-2 was transiently expressed in the COS-1 (ATCC.CRL 1650) monkey kidney cell line and purified from medium conditioned by the transfected cells as described previously (33). Receptor grade IGF-I and IGF-II were the kind gifts of GroPep Pty. Ltd. (Adelaide, Australia). Radiolabeled 125I-IGF-I and 125I-IGF-II peptides were prepared to a specific activity of approximately 3 kBq/mol as described previously (34). Carrier-free Na125I was purchased from Amrad (Sydney, Australia). Six mutagenic oligonucleotides were synthesized by Bre-satec Ltd., Thebarton, South Australia, Australia. A summary of the mutagenic oligonucleotide sequences and the resulting amino acid sequence changes in bIGFBP-2 are shown in Fig. 2. For screening purposes, the mismatches in the bIGFBP-2 region were subcloned into the Phagemid vector Bluescript pBKS(--) Phagemid In Vitro Mutagenesis Version 2 kit (Bio-Rad, Regents Park, New South Wales, Australia). Six mutagenic oligonucleotides were synthesized by Bresatec Ltd., Thebarton, South Australia, Australia. A summary of the mutagenic amino acid sequences and the resulting amino acid sequence changes in bIGFBP-2 are shown in Fig. 2. For screening purposes, the mismatches in the bIGFBP-2 region were subcloned into the Phagemid vector Bluescript pBKS(--) Phagemid In Vitro Mutagenesis Version 2 kit (Bio-Rad, Regents Park, New South Wales, Australia). Six mutagenic oligonucleotides were synthesized by Bresatec Ltd., Thebarton, South Australia, Australia. A summary of the mutagenic oligonucleotide sequences and the resulting amino acid sequence changes in bIGFBP-2 are shown in Fig. 2. For screening purposes, the mismatches in the bIGFBP-2 region were subcloned into the Phagemid vector Bluescript pBKS(--) Phagemid In Vitro Mutagenesis Version 2 kit (Bio-Rad, Regents Park, New South Wales, Australia).

**Analysis of the Mutant bIGFBP-2 Analogs—**Lyophilized samples of approximately 10 µg of each bIGFBP-2 mutant were submitted for electrospray mass spectrometry. The analysis was carried out on a Perkin-Elmer SCI-EX API-300 triple quadrupole mass spectrometer at the Australian Research Council electrospray mass spectrometry unit, Adelaide. Peptide concentrations were accurately quantified by both electrospray mass spectrometry. The analysis was carried out on a Perkin-Elmer SCI-EX API-300 triple quadrupole mass spectrometer at the Australian Research Council electrospray mass spectrometry unit, Adelaide. Peptide concentrations were accurately quantified by both electrospray mass spectrometry and amino acid analysis on a Perkin-Elmer SCI-EX API-300 triple quadrupole mass spectrometer at the Australian Research Council electrospray mass spectrometry unit, Adelaide. Peptide concentrations were accurately quantified by both electrospray mass spectrometry and amino acid analysis on a Perkin-Elmer SCI-EX API-300 triple quadrupole mass spectrometer at the Australian Research Council electrospray mass spectrometry unit, Adelaide. Peptide concentrations were accurately quantified by both electrospray mass spectrometry and amino acid analysis on a Perkin-Elmer SCI-EX API-300 triple quadrupole mass spectrometer at the Australian Research Council electrospray mass spectrometry unit, Adelaide. Peptide concentrations were accurately quantified by both electrospray mass spectrometry and amino acid analysis on a Perkin-Elmer SCI-EX API-300 triple quadrupole mass spectrometer at the Australian Research Council electrospray mass spectrometry unit, Adelaide. Peptide concentrations were accurately quantified by both electrospray mass spectrometry and amino acid analysis on a Perkin-Elmer SCI-EX API-300 triple quadrupole mass spectrometer at the Australian Research Council electrospray mass spectrometry unit, Adelaide.
spectrum, the observed mass of each mutant with the exception of Tyr-60 (lane 4), with 200 ng of purified bIGFBP-2 (lane 7), and [Arg-63 → Ala]bIGFBP-2 (lane 8) were separated on a 12% acrylamide gel under nonreducing conditions, and the gel was silver-stained. Molecular weight markers are shown in lane 1, and their molecular weights are indicated on the left, b, the 125I-IGF-II ligand blot of a duplicate gel to (a), with 200 ng of purified bIGFBP-2 (lane 1), [Val-59 → Ala]bIGFBP-2 (lane 2), [Tyr-60 → Phe]bIGFBP-2 (lane 5), [Thr-61 → Ala]bIGFBP-2 (lane 6), [Pro-62 → Ala]bIGFBP-2 (lane 7), [Arg-63 → Ala]bIGFBP-2 (lane 8) and [Gly-64 → Ala]bIGFBP-2 (lane 9). An estimate of the sequence-predicted mass to within 3 mass units. Tyr-60 → Ala bIGFBP-2 possessed a mass that was 18 mass units greater than expected, possibly due to methionine oxidation. Electrospray mass spectrometry also indicated that in each of the bIGFBP-2 and bIGFBP-2 mutant samples there was a small but consistent contamination (approximately 5%) with a species that was 355 mass units greater than the predicted mass. When the N-terminal sequence of the minor contaminant of wild-type bIGFBP-2 was determined, the larger species was identified as a mis-processed form of bIGFBP-2. The N-terminus of the contaminant was Gly-Ala-Arg-Ala, corresponding to the last four residues of the leader peptide prior to the normal cleavage site (43) of mature bIGFBP-2.

Circular Dichroism—The secondary structure composition of the bIGFBP-2 mutants were compared with wild-type bIGFBP-2 using CD spectroscopy. An overlay of the far UV spectra of Val-59 → Ala, Tyr-60 → Ala, Tyr-60 → Phe bIGFBP-2, and wild-type bIGFBP-2 (Fig. 4) shows that the spectra of these engineered bIGFBP-2 analogs were essentially the same as wild-type bIGFBP-2. Any slight deviation from the CD spectra of bIGFBP-2 could be explained by minor differences in protein concentration. The CD spectra of Thr-61 → Ala, Pro-62 → Ala, and Arg-63 → Ala bIGFBP-2 were also very similar to wild-type bIGFBP-2 (not shown; these spectra were omitted for clarity).

Western Ligand Blots—The 125I-IGF-II binding by the bIGFBP-2 mutants was analyzed by Western ligand blot (Fig. 3b). Val-59 → Ala, Tyr-60 → Ala, and Tyr-60 → Phe bIGFBP-2 (Fig. 3b, lanes 2, 3 and 4) showed reduced 125I-IGF-II binding compared with wild-type bIGFBP-2 (Fig. 3b, lane 1). An estimate of the relative binding of each bIGFBP-2 mutant was provided by direct comparison of the band intensities from the ligand blot and the silver-stained gel. After the bound 125I-IGF-II radioac-
tivity corresponding to each bIGFBP-2 species was quantified by PhosphorImager analysis (Fig. 3b) and corrected for the amount of peptide present on the silver-stained gel (Fig. 3a), it was estimated that Val-59 → Ala, Tyr-60 → Ala, and Tyr-60 → Phe bIGFBP-2 retained approximately 40, 9, and 15% of the 125I-IGF-II bound by bIGFBP-2 respectively. In contrast, Thr-61 → Ala, Pro-62 → Ala, and Arg-63 → Ala bIGFBP-2 (Fig. 3b, lanes 5–7) all bound 125I-IGF-II to a similar extent to bIGFBP-2 (Fig. 3b, lane 1).

Solution IGF Binding Assay—The IGF binding abilities of the bIGFBP-2 mutants were characterized in solution binding assays (Fig. 5, Table I). Alanine substitution for Val-59, Thr-61, Pro-62, and Arg-63 of bIGFBP-2 did not significantly alter the half-maximal binding concentration (EC50) of bIGFBP-2 for either 125I-IGF-I (Fig. 5a) or 125I-IGF-II (Fig. 5b). In contrast, both Ala and Phe substitution for Tyr-60 of bIGFBP-2 resulted in a significant increase in the EC50 for both 125I-IGF-I (Tyr-60 → Ala = 3.4-fold and Tyr-60 → Phe = 4.7-fold) and 125I-IGF-II (Tyr-60 → Ala = 2.2-fold and Tyr-60 → Phe = 3.3-fold). The maximal 125I-IGF binding of the bIGFBP-2 mutants was similar to wild-type bIGFBP-2 with 60% of added IGF tracer for 125I-IGF-I and 47% of added IGF tracer for 125I-IGF-II. An exception was 125I-IGF-II tracer binding to Tyr-60 → Phe bIGFBP-2, where the maximal binding was 37%, a reduction by 1⁄5 with respect to wild-type bIGFBP-2 (Table I).

BIAcore Analysis—Kinetic analyses of the association and dissociation of bIGFBP-2 and the bIGFBP-2 mutants with immobilized IGF-I and IGF-II were carried out in the BIAcore. Fig. 6, a and b, shows a representative subset of the sensorgram data (for qualitative comparison) that was used to generate the kinetic constants summarized in Table II. The interactions between all of the bIGFBP-2 peptides and both IGF-I and IGF-II were difficult to resolve to a single binding site model. In the case of IGF-I interactions, a single apparent association constant and two apparent dissociation constants produced the best fit of the sensorgram data. In contrast, two apparent association and two apparent dissociation constants were necessary for modeling bIGFBP-2 interactions on the IGF-II biosensor surface. The absolute values of the apparent kinetic constants for the IGBP/IGF interactions (k_on, k_off, and K_D, Table II) varied by up to 25% of the mean value between the two biosensor chips used in this study. However, the ranking of the bIGFBP-2 mutants relative to bIGFBP-2 (i.e. the fold differences in k_on, k_off, and K_D, Table II) were the same on both biosensor chips used in this study.

Mutagenesis in the Tyr-60 region of bIGFBP-2 produced a range of effects on the association rates, dissociation rates, and hence the overall affinity for IGFs that were clearly evident in the BIAcore experiments. On the IGF-I biosensor surface (Fig. 6a, Table II) Val-59 → Ala, Tyr-60 → Ala, and Thr-61 → Ala bIGFBP-2 exhibited association rates (k_on) that were 5.5-, 5.0-, and 2.2-fold more rapid than wild-type bIGFBP-2, respectively. In contrast, the association rates (k_on) of Pro-62 → Ala and Arg-63 → Ala bIGFBP-2 were slightly less than bIGFBP-2. The
slowest association rate ($k_{on}$) was observed for Tyr-60 → Phe bIgFBP-2 which bound 3.0-fold more slowly than bIgFBP-2. Although two dissociation components ($k_{off1}$ and $k_{off2}$) were necessary to model the behaviour of bIgFBP-2 and the bIgFBP-2 mutants on IGF-I biosensor surfaces, the rapid dissociation component ($k_{off1}$), which accounted for approximately 10% of the total interaction, was essentially the same for all of the bIgFBP-2 peptides. In contrast, the slow dissociation component ($k_{off2}$) was affected by mutagenesis in the Tyr-60 region. Therefore, the slow dissociation component ($k_{off2}$), which represented the major proportion of the dissociating population, was considered the more relevant component in this analysis. Three bIgFBP-2 mutants, Thr-61 → Ala, Pro-62 → Ala, and Arg-63 → Ala bIgFBP-2, exhibited apparent dissociation rates ($k_{off2}$) that were similar to bIgFBP-2 (Fig. 6a, Table II). The apparent dissociation rate ($k_{off2}$) of Tyr-60 → Ala bIgFBP-2 was 2.6-fold more rapid than bIgFBP-2. Large increases in the apparent rate of dissociation ($k_{off2}$) were observed for Val-59 → Ala and Tyr-60 → Ala bIgFBP-2 which were released from the IGF-I biosensor surface 9.4 and 18.4 times more rapidly than bIgFBP-2 (Fig. 6a, Table II). The apparent affinities of bIgFBP-2 and the Tyr-60 region bIgFBP-2 mutants for the IGF-I surface, expressed as the dissociation rate constant ($K_D$), were derived from the apparent association rate ($k_{on}$) and the slow dissociation rate ($k_{off2}$) according to the relationship $K_D = k_{off2}/k_{on}$. The only bIgFBP-2 mutants that exhibited apparent $K_D$ values that were significantly higher than bIgFBP-2 (0.5 nM) were Tyr-60 → Ala bIgFBP-2 (2.0 nM) and Tyr-60 → Phe bIgFBP-2 (4.2 nM) corresponding to a 4.0- and 8.4-fold drop in apparent affinity, respectively.

The interactions of bIgFBP-2 and the Tyr-60 region bIgFBP-2 mutants with the IGF-II biosensor surface are shown in Fig. 6b. Two association components were derived from the sensorgram data, and the relative contribution of each to the total binding profile was estimated using the BIASimulation program. Between the bIgFBP-2 mutants, the rapid binding component played a varying role in the interaction. Therefore, for each bIgFBP-2 mutant, the two association constants were

![Fig. 6. BIAcore analysis of bIgFBP-2 and selected bIgFBP-2 mutants.](image)

### Table I

Summary of IGF binding parameters derived from solution binding assays

|            | Maximum bound | Relative maximal binding | $EC_{50}$ | Relative $EC_{50}$ |
|------------|---------------|--------------------------|-----------|--------------------|
| $^{125}$I-IGF-I                   | %             | $nm$                     |           |                   |
| bIgFBP-2   | 61            | 1.0                      | 0.15      | 1.0               |
| Val-59 → Ala | 60            | 1.0                      | 0.12      | 0.8               |
| Tyr-60 → Ala | 57            | 0.9                      | 0.51      | 3.4               |
| Tyr-60 → Phe | 60            | 1.0                      | 0.70      | 4.7               |
| Thr-61 → Ala | 61            | 1.0                      | 0.13      | 0.9               |
| Pro-62 → Ala | 58            | 0.9                      | 0.16      | 1.1               |
| Arg-63 → Ala | 62            | 1.0                      | 0.19      | 1.3               |
| $^{125}$I-IGF-II                  | %             | $nm$                     |           |                   |
| bIgFBP-2   | 47            | 1.0                      | 0.11      | 1.0               |
| Val-59 → Ala | 47            | 1.0                      | 0.07      | 0.6               |
| Tyr-60 → Ala | 44            | 0.9                      | 0.24      | 2.2               |
| Tyr-60 → Phe | 37            | 0.8                      | 0.36      | 3.3               |
| Thr-61 → Ala | 45            | 1.0                      | 0.11      | 1.0               |
| Pro-62 → Ala | 44            | 0.9                      | 0.11      | 1.0               |
| Arg-63 → Ala | 44            | 0.9                      | 0.08      | 0.7               |

*The maximum level of $^{125}$I-IGF binding attained in the assay is expressed as a percentage of total $^{125}$I-IGF added.

*The concentration of bIgFBP-2 peptide required to bind 50% of the maximum $^{125}$I-IGF binding level attained in the assay. $R^2$ values for the dose/response curve fits were equal to or greater than 0.95.

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**Fig. 6.** BIAcore analysis of bIgFBP-2 and selected bIgFBP-2 mutants. Shown are representative sensorgrams for the interaction of bIgFBP-2, [Val-59 → Ala]bIgFBP-2, [Tyr-60 → Ala]bIgFBP-2, and [Tyr-60 → Phe]bIgFBP-2 with IGF-I biosensor surfaces (60–120 RU) (a) and IGF-II biosensor surfaces (60–120 RU) (b).
TABLE II

| IGF-I (60 RU) | $k_{on}$ $\times 10^5$ (1/Ms) | Relative $k_{on}$ | $k_{on(app)}$ $\times 10^{-5}$ (1/s) | Relative $k_{on(app)}$ | $K_D$ (nM) | Relative $K_D$ |
|---------------|------------------|-----------------|----------------|----------------|----------|----------------|
| bIGFBP-2      | 0.6$^b$           | 1.0$^b$         | 7.3            | 32             | 1.0      | 0.3           |
| Val-59 $\rightarrow$ Ala | 3.3 | 5.5 | 6.6 | 300 | 9.4 | 0.9 | 1.8 |
| Tyr-60 $\rightarrow$ Ala | 3.0 | 5.0 | 6.0 | 94 | 2.5 | 4.2 | 8.4 |
| Thr-61 $\rightarrow$ Ala | 3.2 | 2.2 | 3.0 | 45 | 1.4 | 0.4 | 0.8 |
| Pro-62 $\rightarrow$ Ala | 0.5 | 0.8 | 18.0 | 34 | 1.1 | 0.9 | 1.8 |
| Arg-63 $\rightarrow$ Ala | 0.4 | 0.7 | 18.0 | 34 | 1.1 | 0.9 | 1.8 |

| IGF-II (66 RU) | $k_{on}$ $\times 10^5$ (1/Ms) | Relative $k_{on}$ | $k_{on(app)}$ $\times 10^{-5}$ (1/s) | Relative $k_{on(app)}$ | $K_D$ (nM) | Relative $K_D$ |
|---------------|------------------|-----------------|----------------|----------------|----------|----------------|
| bIGFBP-2      | 1.1              | 22.6            | 0.95           | 2.3            | 3.0      | 5.0           |
| Val-59 $\rightarrow$ Ala | 1.4 | 9.2 | 0.70 | 3.7 | 1.7 | 6.3 | 11.5 |
| Tyr-60 $\rightarrow$ Ala | 1.7 | 10.1 | 0.70 | 4.2 | 1.9 | 7.9 | 30.9 |
| Thr-61 $\rightarrow$ Ala | 1.1 | 1.0 | 1.1 | 0.5 | 12.6 | 3.3 | 0.7 | 1.5 |
| Pro-62 $\rightarrow$ Ala | 0.8 | 30.3 | 0.95 | 2.3 | 1.1 | 7.9 | 1.0 | 0.2 | 0.04 | 0.2 |
| Arg-63 $\rightarrow$ Ala | 0.7 | 21.1 | 0.95 | 1.7 | 0.8 | 7.0 | 3.6 | 0.7 | 0.2 | 1.0 |

$^a$ IGF-I biosensor data: $K_D = k_{off}/k_{on}$.
$^b$ Chi$^2$ values for sensogram curve fits were equal to 1.0 or were less. $R^2$ values generated during the linear regression derivation of binding constants were equal to 0.95, or were greater. Interchip variation was 25% of the value or less for the apparent kinetic constant values $k_{on}$, $k_{off}$, and $K_D$.

The ranking of the bIGFBP-2 mutants relative to bIGFBP-2 with respect to $k_{on}$, $k_{off}$, and $K_D$ was the same on both biosensor chips. The interchip variation in the fold differences of kinetic constants was 12% of the mean value or less.

**TABLE II**

| Summary of kinetic parameters derived from the interaction of bIGFBP-2 peptides on IGF biosensor surfaces |
|---------------------------------------------------|
| IGF-I (60 RU) | $k_{on}$ $\times 10^5$ (1/Ms) | Relative $k_{on}$ | $k_{on(app)}$ $\times 10^{-5}$ (1/s) | Relative $k_{on(app)}$ | $K_D$ (nM) | Relative $K_D$ |
|---------------|------------------|-----------------|----------------|----------------|----------|----------------|
| bIGFBP-2      | 0.6$^b$           | 1.0$^b$         | 7.3            | 32             | 1.0      | 0.3           |
| Val-59 $\rightarrow$ Ala | 3.3 | 5.5 | 6.6 | 300 | 9.4 | 0.9 | 1.8 |
| Tyr-60 $\rightarrow$ Ala | 3.0 | 5.0 | 6.0 | 94 | 2.5 | 4.2 | 8.4 |
| Thr-61 $\rightarrow$ Ala | 3.2 | 2.2 | 3.0 | 45 | 1.4 | 0.4 | 0.8 |
| Pro-62 $\rightarrow$ Ala | 0.5 | 0.8 | 18.0 | 34 | 1.1 | 0.9 | 1.8 |
| Arg-63 $\rightarrow$ Ala | 0.4 | 0.7 | 18.0 | 34 | 1.1 | 0.9 | 1.8 |

| IGF-II (66 RU) | $k_{on}$ $\times 10^5$ (1/Ms) | Relative $k_{on}$ | $k_{on(app)}$ $\times 10^{-5}$ (1/s) | Relative $k_{on(app)}$ | $K_D$ (nM) | Relative $K_D$ |
|---------------|------------------|-----------------|----------------|----------------|----------|----------------|
| bIGFBP-2      | 1.1              | 22.6            | 0.95           | 2.3            | 3.0      | 5.0           |
| Val-59 $\rightarrow$ Ala | 1.4 | 9.2 | 0.70 | 3.7 | 1.7 | 6.3 | 11.5 |
| Tyr-60 $\rightarrow$ Ala | 1.7 | 10.1 | 0.70 | 4.2 | 1.9 | 7.9 | 30.9 |
| Thr-61 $\rightarrow$ Ala | 1.1 | 1.0 | 1.1 | 0.5 | 12.6 | 3.3 | 0.7 | 1.5 |
| Pro-62 $\rightarrow$ Ala | 0.8 | 30.3 | 0.95 | 2.3 | 1.1 | 7.9 | 1.0 | 0.2 | 0.04 | 0.2 |
| Arg-63 $\rightarrow$ Ala | 0.7 | 21.1 | 0.95 | 1.7 | 0.8 | 7.0 | 3.6 | 0.7 | 0.2 | 1.0 |

$^a$ IGF-I biosensor data: $K_D = k_{off}/k_{on}$.
$^b$ Chi$^2$ values for sensogram curve fits were equal to 1.0 or were less. $R^2$ values generated during the linear regression derivation of binding constants were equal to 0.95, or were greater. Interchip variation was 25% of the value or less for the apparent kinetic constant values $k_{on}$, $k_{off}$, and $K_D$.

The ranking of the bIGFBP-2 mutants relative to bIGFBP-2 with respect to $k_{on}$, $k_{off}$, and $K_D$ was the same on both biosensor chips. The interchip variation in the fold differences of kinetic constants was 12% of the mean value or less.

**DISCUSSION**

In this study, the role of the Tyr-60 region of bIGFBP-2 in IGF binding has been investigated by alanine screening mutagenesis. In our chemical modification study (32), Tyr-60, which lies in the N-terminal cysteine-rich domain of bIGFBP-2, was shown to be protected from iodination when either IGF-I or IGF-II was bound. The high degree of sequence homology in the Tyr-60 region of the whole IGFBP family (Fig. 1) suggested that other residues in this vicinity might also play a role in IGF binding. To investigate this possibility, Val-59 $\rightarrow$ Ala, Tyr-60 $\rightarrow$ Phe, Thr-61 $\rightarrow$ Ala bIGFBP-2 were recombinantly expressed, purified, and characterized for changes in IGF affinity. The only bIGFBP-2 mutants that were observed to exhibit significantly reduced affinity for IGF-I and IGF-II using a variety of complementary analyses were Tyr-60 $\rightarrow$ Ala and Tyr-60 $\rightarrow$ Phe bIGFBP-2. All of the Tyr-60 region bIGFBP-2 mutants produced essentially the same CD spectra (Fig. 4) and all ran as single bands at the same molecular weight as bIGFBP-2 in SDS-PAGE analysis (Fig. 3). Therefore, any changes in the IGF binding characteristics of the bIGFBP-2 mutants were considered to be due to the loss of side chain interactions at the mutagenic site in question and not to gross changes in protein structure.

There was good agreement between the three functional analyses that were used to investigate the IGF binding abilities of the bIGFBP-2 mutants. Western ligand blot (Fig. 3b), solution binding assays (Fig. 5, Table I), and the BIACore analyses (Fig. 6, Table II) all indicated that both Ala and Phe substitution for Tyr-60 resulted in a bIGFBP-2 molecule with reduced affinity for IGFs. However, some differences were noted. For example, Val-59 $\rightarrow$ Ala bIGFBP-2 bound 125I-IGF-II at a visibly reduced level compared with bIGFBP-2 in the Western ligand blot (Fig. 2), yet bound 125I-IGFs as well as bIGFBP-2 in the solution binding assay (Fig. 4, Table I). Similarly, the decrease in 125I-IGF-II binding of Tyr-60 $\rightarrow$ Ala bIGFBP-2 was far more apparent in the Western ligand blot (Fig. 3b) than in the
solution binding assay (Fig. 4, Table I).

Direct measurement of the association and dissociation kinetics of the IGFBP-2/IGF interaction in the BIAcore experiments could explain the observed differences between the Western ligand blot and the solution binding assay results. Whereas Val-59 → Ala and Tyr-60 → Ala bIGFBP-2 dissociated 2.3- and 6.2-fold more rapidly from the IGF-II biosensor surface than bIGFBP-2, respectively, both also associated with this surface approximately 2-fold more rapidly than bIGFBP-2. Under the equilibrium conditions of the solution binding assay, the increased dissociation rates of Val-59 → Ala and Tyr-60 → Ala bIGFBP-2 were offset by the increases in the association rate. However, in the Western ligand blot, nonspecifically bound 125I-IGF-II was washed from the filter by buffer replacement, and so the possibility of IGF and IGFBP-2 reaching a binding equilibrium was prevented. Therefore, we propose that the Western ligand blot ranked the bIGFBP-2 mutants with respect to their relative dissociation rates, whereas the solution binding assay ranked the bIGFBP-2 mutants according to their overall affinities.

Insight into the reduced IGF binding affinity of Tyr-60 → Ala and Tyr-60 → Phe bIGFBP-2 was provided by BIAcore analysis. Substitution of Tyr-60 of bIGFBP-2 with Ala and Phe produced very different changes in the kinetics of IGF interactions. Tyr-60 → Ala bIGFBP-2 associated with and dissociated from IGF biosensor surfaces more rapidly than bIGFBP-2. In contrast, Tyr-60 → Phe bIGFBP-2 exhibited a reduced rate of association with, and an increased rate of dissociation from, IGF biosensor surfaces compared with bIGFBP-2. Interestingly, Tyr-60 → Ala and Val-59 → Ala bIGFBP-2 exhibited very similar kinetics on the IGF-I and IGF-II biosensor surfaces. The enhanced association and dissociation kinetics of Val-59 → Ala bIGFBP-2 with immobilized IGF suggests that this mutation produced subtle changes to the structure of the IGF-binding site of bIGFBP-2, without detriment to the net energy of IGF binding. In contrast, Tyr-60 → Ala bIGFBP-2 exhibited a net reduction in the energy of IGF binding due to large increases in the dissociation rate from IGF surfaces. Therefore, the shape and volume of the side chains of Val-59 and Tyr-60 may help to define the IGF-binding site of bIGFBP-2. However, the aromatic function and the hydrogen bonding potential of Tyr-60 are clearly the most significant contributors to the stability of the bIGFBP-2/IGF complex. The replacement of Tyr-60 with Phe was anticipated to be the most subtle mutation, with the aromatic side chain packing maintained and the loss of the tyrosyl hydroxyl group the only change. Surprisingly, Tyr-60 → Phe bIGFBP-2 exhibited the lowest affinities for IGFs, thus providing further evidence to suggest that Tyr-60 participates in hydrogen bond(s) that stabilize IGF interactions.

In circulation, IGFBPs bind IGFs with a 1:1 stoichiometry (reviewed in Ref. 7), and therefore a single site kinetic model should provide a valid approximation of the IGFBP/IGF interaction. Indeed, the apparent affinity constants that were calculated with the derived association and dissociation constants of bIGFBP-2 and the IGF-I and IGF-II surfaces (0.5 nM for IGF-I and 0.2 nM for IGF-II) correspond very well with published constants generated by competition solution binding assays (44, 45). Yet, the interactions of bIGFBP-2 and the bIGFBP-2 mutants with immobilized IGFs deviated from pseudo-first order kinetics in the BIAcore (Fig. 6). Multiple phase kinetics for single site interactions can be due to artifacts of the BIAcore assay conditions (46, 47). In this study, steps have been deliberately used to minimize mass transfer limita-

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