The Insulin-like Growth Factor (IGF) Binding Site of Bovine Insulin-like Growth Factor Binding Protein-2 (bIGFBP-2) Probed by Iodination*

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The insulin-like growth factor (IGF) binding site of bovine insulin-like growth factor binding protein 2 (bIGFBP-2) has been probed by chemical iodination. Tyrosyl residues of bIGFBP-2 were reacted by chloramine T-mediated iodination. The modification patterns of free bIGFBP-2 and bIGFBP-2 associated with insulin-like growth factor II (IGF-II) were compared by tryptic mapping using electrospray mass spectrometry and N-terminal sequencing. The presence of bound IGF-II resulted in protection of tyrosine at position 60 from iodination measured by the relative loss of tyrosine specific fluorescence and the incorporation of the radioisotope 125I. In addition, the pattern of iodine incorporation of bIGFBP-2 was not different whether IGF-I or IGF-II was the protective ligand. bIGFBP-2, when iodinated alone sustained a 8-fold loss of binding affinity for IGF-I and a 4-fold loss in binding affinity for IGF-II. In contrast, bIGFBP-2 iodinated while complexed with either IGF-I or IGF-II retained the same binding affinity for IGF-I or IGF-II as non-iodinated bIGFBP-2. We conclude that tyrosine 60 lies either in a region of bIGFBP-2 which directly interacts with both IGF-I and IGF-II or lies in a region of bIGFBP-2 which undergoes a conformational change that is important for IGF binding. Furthermore, iodination of tyrosine residues at positions 71, 98, 213, 226, and 269 has no detectable impact on binding of bIGFBP-2 to the IGFs.

The insulin-like growth factors (IGF-I and -II) are polypeptide mitogens which play diverse roles in development and metabolism across a wide range of vertebrate species (recently reviewed in Ref. 1). While the mitogenic actions of IGFs are mediated via specific type-I IGF receptor interactions, the bio-availability and localization of IGFs are considered to be largely determined by a family of proteins known as the insulin-like growth factor binding proteins (IGFBPs) (1–3). Detailed molecular structures of both IGF-I (4–6) and IGF-II (7, 8) have been determined. Moreover, some of the structural elements which are involved in IGFBP binding have been determined by a number of approaches which include chemical modification (9) and the generation of IGF-I and IGF-II mutants (10–14). In contrast, there are no published structures for the IGFBPs and furthermore, the residues of IGFBPs which are important for IGF binding are largely unknown.

Most of the studies which address structural and functional aspects of the IGFBP family have not sought to directly identify the sites to which IGFs bind. Rather, researchers have focused mainly on issues such as IGFBP phosphorylation (15), IGFBP proteolysis (16–19), and the functional significance of motifs identified in the primary sequences of IGFBPs, for example, the Arg-Gly-Asp sequence of IGFBP-1 (20).

There is evidence to suggest that both the N- and C-terminal regions of IGFBPs are important for complex formation with IGFs (21, 22). Notably, the strongest regions of sequence homology are found in the N- and C-terminal cysteine-rich regions of these molecules. Included in this sequence homology is the conserved alignment of 16 cysteine residues, 10 in the N-terminal and 6 in the C-terminal third of IGFBPs. While all of the cysteine residues of IGFBPs are believed to participate in disulfide bonds (23), it remains to be shown whether all IGFBPs share the same disulfide-bridging pattern. In contrast, the respective middle regions of IGFBP sequences show greater heterogeneity.

We have used chemical iodination to characterize the binding interaction between IGFs and bIGFBP-2. We have achieved this by identifying tyrosine residues of bIGFBP-2 which are iodinated when this molecule is not bound to IGFs, but on the other hand are protected from iodination when either IGF-I or IGF-II is bound. The primary structure of bIGFBP-2 contains six tyrosine residues which are spread throughout the amino acid sequence of this molecule. This study has shown that an approximate 5-fold protection against modification specifically at Tyr60 occurs when bIGFBP-2 is iodinated while in a complex with either IGF-I or IGF-II. Finally, iodination at Tyr60 leads to a significant reduction in the affinity of IGFs for bIGFBP-2, indicating that Tyr60 may be one of a number of residues which play an important role in the bIGFBP-2 binding reaction with the IGFs.

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 by a method adapted from Szabo et al. (24). Receptor grade IGF-I and IGF-II were the kind gift of GroPep Pty. Ltd. (Adelaide, Australia). Radiolabeled 125I-IGF-I and 125I-IGF-II peptides were kindly provided by Spencer Knowles (CRC for Tissue Growth and Repair, Adelaide, Australia). Modified, sequencing grade trypsin was purchased from Boehringer Mannheim (North Ryde,
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New South Wales, Australia). HPLC columns were purchased from Brownlee Laboratories (Santa Clara, CA) or C8 Aquapore (Sydney, Australia). Carrier-free Na125I was purchased from Amersham International (Sydney, Australia). Pre-siliconized tubes (Sorenson BioScience, Inc., Salt Lake City, UT) were used for reaction vessels and for the collection of fractions during chromatography. All HPLC was carried out using Waters 510 solvent pumps, a Waters 490 4-channel absorbance detector (Millipore-Waters, Lane Cove, N.S.W) and a Perkin Elmer LS4 fluorescence spectrometer (Sorensby, Victoria, Australia). The Waters Maxima software package was used to control solvent gradients and for data collection. HPLC-grade acetonitrile was purchased from Merck (Kilsyth, Victoria, Australia) and trifluoroacetic acid from Sigma-Aldridge (Castle Hill, N.S.W, Australia). The Waters Maxima software package was used to control solvent gradients and for data collection. HPLC-grade acetonitrile was purchased from Merck (Kilsyth, Victoria, Australia) and trifluoroacetic acid from Sigma-Aldridge (Castle Hill, N.S.W, Australia). All other reagents were analytical grade.

Optimization of Iodination Conditions—At the scale of 0.1 nmol of bIGFBP-2, a series of iodinations were performed by the chloramine-T method (9). The amount of Na125I (specific radioactivity 50 Ci/nmol) was maintained at 4 nmol/reaction and the molar ratio of chloramine-T/Na125I per reaction was varied between 0.125 and 1.25. Two stock solutions were prepared, one containing bIGFBP-2 and the other containing both IGF-II and IGF-I in the following manner. In two separate tubes, 20 nmol of [125I]lyophilized IGF-I (5 nmol in 1 ml of 0.1 M acetic acid) was added to the bIGFBP-2 stock solution and 3.2 nmol of IGF-II in 40 ml of 10 mM acetic acid was added to the bIGFBP-2+IGF-II stock solution (resulting in a 4-fold molar ratio of IGF-II to bIGFBP-2). The vials were left at room temperature for 2 h to allow the complex to form. Then 36 nmol of NaI was added to each reaction mixture in 10 ml of 5 mM NaOH. This was the equivalent of a 40-fold molar excess of NaI over bIGFBP-2. After mixing, both of the reaction mixtures were divided into 8 × 50-µl (0.1 nmol) aliquots of bIGFBP-2. Iodination was initiated by the addition of 5 µl of the appropriate chloramine-T solution (prepared in a 10-fold serial dilution in 8 steps from 0.5 to 0.05 mM with water) followed by an incubation at room temperature for 45 s. Each reaction was quenched by the addition of 20 nmol of sodium metabisulfite in 10 ml of water. The 16 reaction mixtures were stored at −20°C prior to HPLC gel filtration at acid pH.

HPLC Gel Filtration at Acid pH—Reaction products were thawed and acidified by the addition of 50 µl of 2 M acetic acid to dissociate the IGF-bIGFBP-2 complex. Iodinated bIGFBP-2 was separated from free iodide and iodinated IGF (when present) by gel filtration on a Tosk G3000SW Ultrogel HPLC column (7.5 × 600 mm) equilibrated with a solution containing 150 mM NaCl, 10 mM HCl, and 0.05% (v/v) Tween 20. The column was eluted at a flow rate of 0.5 ml/min and 1 ml fractions were collected for γ-counting. Protein was detected by absorbance at 215 nm, aromatic residues at 275 nm and tryptophan at 295 nm. The position of P1, P2, P3, P4, P5, and P6 and the iodinated derivatives will be referred to as Ps (mono-iodotyrosyl derivative) and Pss (di-iodotyrosyl derivative), where (s) refers to the peptides 1–6. The positions of P1, P2, P3, P4, P5, P6 and the iodinated derivatives of these peptides in the elution profile of the tryptic map were established by N-terminal sequencing. The identities of tyrosine containing peptides were confirmed by electrospray mass spectrometry (EMS) on a Perkin Elmer Pxi-ex trap quadrupole mass spectrometer by Yoji Hayawaka at the Australian Research Council EMS unit, Adelaide.

Iodinations of bIGFBP-2 and IGF Binding Assays—bIGFBP-2 was modified as described in the large-scale iodinations except that the radioactive iodine isotope125I was omitted from the reaction. Again, bIGFBP-2 was iodinated alone and in a complex with both IGF-I and IGF-II. The iodinated bIGFBP-2 species were then purified by HPLC gel filtration as described above and the incorporation of iodine was confirmed by the loss of tyrosine-specific fluorescence during the chromatography. The relative affinities of the respective iodinated bIGFBP-2 species for both 125I-labeled IGF-I and IGF-II were determined by charcoal binding assay, as described previously (24).

RESULTS

We have used IGF-mediated protection against chemical modification of the tyrosine residues of bIGFBP-2 to investigate the association between bIGFBP-2 and IGF-I or IGF-II at the molecular level. The 6 tyrosine residues of bIGFBP-2 are distributed throughout the amino acid sequence and so enable the IGF-binding site to be localized to the N-terminal, middle, or C-terminal regions of this molecule. In this study, we have identified tyrosine residues which are protected from chloramine T-mediated iodination when IGFs are bound as outlined in Fig. 1.

Optimization of Iodination Conditions—When the concentration of NaI was held at 40-fold excess over bIGFBP-2, an increase in the molar ratio of chloramine-T/NaI in the iodination reaction resulted in an increase in the amount of iodine...
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which became incorporated into bIGFBP-2 (Fig. 2a). This figure also shows that when bIGFBP-2 was iodinated in a complex with IGF-II, the molar incorporation of iodine into bIGFBP-2 was reduced. A linear increase in iodine incorporation was observed for both IGF-protected bIGFBP-2 and unprotected bIGFBP-2 over the chloramine-T/NaI molar ratio range of 0.125 and 0.75. However, when this ratio was increased beyond 0.75, the subsequent increase in iodine incorporation into both protected and free bIGFBP-2 was less, on a molar basis. For IGF-protected bIGFBP-2 this change in the reactivity of the bIGFBP-2 tyrosine residue population was observed to occur when approximately 5 mol of iodine had been incorporated per bIGFBP-2 molecule compared with approximately 8 mol of iodine for bIGFBP-2 alone. These results suggest that the number of reactive tyrosine residues of bIGFBP-2 was reduced when IGF-II was bound.

The tyrosine fluorescence of the intact bIGFBP-2 molecule was a useful measure of the degree of iodination of the tyrosine residues. The chloramine T-dependent increase in the iodination of bIGFBP-2 was seen by a corresponding drop in tyrosine-specific fluorescence (Fig. 2b). As the ratio of chloramine-T/NaI per reaction was increased between 0.125 and 0.75, there was a rapid loss of bIGFBP-2-associated tyrosine fluorescence. However, negligible further losses in the tyrosine fluorescence of the iodinated bIGFBP-2 species were observed when this ratio was increased beyond 0.5. Fig. 2b shows that the tyrosine fluorescence ultimately dropped to approximately 10% of the unmodified molecule when bIGFBP-2 was iodinated alone. In contrast, bIGFBP-2 which had been iodinated while protected by IGF-II still retained 40% of the tyrosine fluorescence of the unmodified molecule. Both sets of data outlined above, which are representative examples of four independent experiments, suggest that ordinarily reactive tyrosine residues of bIGFBP-2 were protected from iodination when IGF-II was bound.

A 0.75 molar ratio of chloramine-T/NaI was chosen to generate iodinated bIGFBP-2 at a scale which enabled the modification pattern of iodinated IGF-associated and free bIGFBP-2 to be determined by tryptic mapping. These reaction conditions are indicated by arrows in Figs. 2a and b.

Identification of IGF/IGFBP Binding Sites—Complete tryptic digestion of bIGFBP-2 would theoretically generate 32 fragments ranging in size from single amino acids up to a peptide of 45 residues (Fig. 3). Trypsin was chosen as the proteolytic mapping enzyme because in theory it could liberate each tyrosine residue in a discrete peptide fragment. Following tryptic digestion, peptides of bIGFBP-2 were separated by reverse-phase HPLC (Fig. 4). Autodigestion of trypsin (Fig. 4d) generated a background of 7 fluorescent peaks. Digestion of non-iodinated bIGFBP-2 (Fig. 4a) resulted in the generation of 9 tyrosine containing peptides in addition to those generated by trypsin autodigestion as detected by tyrosine fluorescence. The pattern of digestion shown in Fig. 4 is representative of the many experiments performed (data not shown). N-terminal sequence analysis and EMS identified the fluorescent tryptic peptides of bIGFBP-2 to be in (order of elution) P1, P6, P2, and P3, + P6, P4 + P5, +P4 + P5, where + indicates an intact trypsin-sensitive bond, as defined in Fig. 3. The tyrosyl peptides P4, P5, and P6 were all identified as partial digestion fragments while the peptides P2 and P3 were not resolved by this chromatography. The mass of the peptide which was identified as P3 by N-terminal sequencing was between 15 and 16 mass units greater than the predicted mass. One possible explanation for this observation is the previously documented (28, 29) oxidation of methionine by chloramine-T. The EMS and N-terminal sequencing results are summarized in Table Ia.

The loss of tyrosine fluorescence due to iodination can clearly be seen when the tryptic map of non-iodinated bIGFBP-2 (Fig. 4a) is compared with the tryptic tyrosine fluorescence maps of bIGFBP-2 iodinated ether alone (Fig. 4b) or complexed with IGF-II (Fig. 4c). Table Ib summarizes the differences in resid-
ual tyrosine fluorescence of bIGFBP-2 which was iodinated in a complex with IGF-II or alone. An additional fluorescent peak (A) was present in the iodinated bIGFBP-2 maps but was absent in the non-iodinated bIGFBP-2 map. However, this peak was most probably an artifact of the iodination reaction as both N-terminal sequence and EMS analysis showed conclusively that this peak was not a peptide. The order of tyrosine reactivity when calculated in terms of the residual tyrosine fluorescence (Table Ib) changed from P6 \( \rightarrow \) P1 \( \rightarrow \) P2 and P3 \( \rightarrow \) P4 \( \rightarrow \) P5 when bIGFBP-2 was modified alone to P2 and P3 \( \rightarrow \) P6 \( \rightarrow \) P4 \( \rightarrow \) P5 \( \rightarrow \) P1 when bIGFBP-2 was modified in a complex with IGF-II. However, the major difference in the iodination pattern of both free bIGFBP-2 and IGF-II associated bIGFBP-2 was the large residual tyrosine fluorescence of the peptide P1 in the map of IGF-II associated bIGFBP-2 (Fig. 4c). When quantified, the residual fluorescence of this peptide was 94% of the tyrosine specific fluorescence yielded by the same peptide isolated from non-iodinated bIGFBP-2 (Fig. 4a) and was 5.7-fold more intense than for the same peptide from bIGFBP-2 which had been iodinated free of IGF-II (Fig. 4b). The amount of non-iodinated P6 peptide was very low in all of the iodinated bIGFBP-2 tryptic maps which indicated that this residue was freely available for iodination, although complex formation with IGF-II afforded a 2.5-fold protection against modification at this site (Fig. 4c) in comparison to bIGFBP-2 modified alone (Fig. 4b). The non-iodinated tyrosyl peptides P2 and P3 were 2.8 times more abundant in the peptide map of bIGFBP-2 which had been modified alone to P2 and P3 \( \rightarrow \) P6 \( \rightarrow \) P4 \( \rightarrow \) P5 \( \rightarrow \) P1 when bIGFBP-2 was modified in a complex with IGF-II. However, the major difference in the iodination pattern of both free bIGFBP-2 and IGF-II associated bIGFBP-2 was the large residual tyrosine fluorescence of the peptide P1 in the map of IGF-II associated bIGFBP-2 (Fig. 4c). When quantified, the residual fluorescence of this peptide was 94% of the tyrosine specific fluorescence yielded by the same peptide isolated from non-iodinated bIGFBP-2 (Fig. 4a) and was 5.7-fold more intense than for the same peptide from bIGFBP-2 which had been iodinated free of IGF-II (Fig. 4b). The amount of non-iodinated P6 peptide was very low in all of the iodinated bIGFBP-2 tryptic maps which indicated that this residue was freely available for iodination, although complex formation with IGF-II afforded a 2.5-fold protection against modification at this site (Fig. 4c) in comparison to bIGFBP-2 modified alone (Fig. 4b). The non-iodinated tyrosyl peptides P2 and P3 were 2.8 times more abundant in the peptide map of bIGFBP-2 which had been modified alone (Fig. 4b) compared with bIGFBP-2 which was iodinated with IGF-II bound (Fig. 4c). The additive residual tyrosine specific fluorescence of partially digested peptides which contained P4 and P5 were similar (Fig. 4, b and c) regardless of IGF association prior to the iodination reaction. The tyrosine fluorescence associated with P4+P5 peptides was significant, accounting for 36% of the total remaining fluorescence of the non-iodinated tyrosyl peptides.

The tryptic peptides of bIGFBP-2 which had incorporated iodine were also directly identified by their \(^{125}\text{I} \) radioactivity. Fig. 5 shows an alignment of the tryptic map fractions of bIGFBP-2 iodinated free (a) and bIGFBP-2 iodinated as a complex with IGF-II (b) which contain \(^{125}\text{I} \) radioactivity. Fractions which contained iodinated peptides were characterized by both N-terminal sequencing and EMS as summarized in Table IIa.

### FIG. 3. Tryptic peptides of bIGFBP-2. The amino acid sequence of bIGFBP-2 is in the single letter code. Trypsin-sensitive peptide bonds are indicated ( ) and tyrosine containing tryptic peptides of bIGFBP-2 are boxed and labeled P1 to P6 with respect to their order in the sequence. The tyrosine residues of bIGFBP-2 are shown in bold. Partial trypsin digestion products from the C-terminal region of bIGFBP-2 are shown under the main sequence.
A summary of bIGFBP-2 tryptic peptides identified by tyrosine specific fluorescence

Tyrosine containing peptides were the only peptides of bIGFBP-2 which were found to incorporate iodine under our reaction conditions. As shown in Fig. 5a, the order of elution of iodinated tyrosyl peptides of bIGFBP-2 was determined to be P1*, P1**, P6*, P6**, and P3*; and P3**, P2*, P2**, +P6**, (+P4+P5)***; and (P4+P5)***, (P4+P5)***, and (P4+P5)****, where parentheses indicate that the locations of the iodine atoms in these peptides were not characterized. The incorporation of iodine was observed to increase the hydrophobicity of tyrosine containing peptides as can be seen in the longer retention times of modified peptides (Table Ia). The exceptions to this were the mono-iodotyrosyl (*) and di-iodotyrosyl (**) derivatives of P3, which did not exhibit significantly increased retention times, presumably due to the larger size of this tyrosyl peptide (45 residues). Edman degradation of the iodinated, partially digested derivatives of the peptides P4 and P5 indicated that the tyrosine residue in P4 (Tyr213) and not P5 (Tyr226) was the major site of iodine incorporation in these peptides.

It is immediately evident on comparison of panels a and b in Fig. 5 that the peptide P1 was shielded from iodination when IGF-II was bound. Quantification of the iodinated peptides helped to decipher the impact of IGF-association on the modification of other bIGFBP-2 tyrosyl peptides. The extent of iodine incorporation at each tyrosine residue could be calculated as a fraction of the total radioactivity incorporated into bIGFBP-2 thereby allowing the relative reactivity of each tyrosine residue to be compared, as summarized in Table Ib. These data also show that the association of IGF-II with bIGFBP-2 did not significantly reduce the incorporation of iodine into peptides P2 and P3, or P6. In fact, there was a 1.5-fold increase in the modification of the peptides P2, P3, and P6 when IGF was associated with bIGFBP-2. The tyrosine-specific fluorescence data described above also showed that an increase in the degree of modification of the peptides P2 and P3 occurred when bIGFBP-2 was iodinated in a complex with IGF. A slight protection against modification in either P4 or P5 was observed when bIGFBP-2 was iodinated in a complex with IGF-II (Table Ib). The peptide P1 accounted for 15% of the total iodine incorporation when bIGFBP-2 was iodinated alone. In contrast, the labeling of this peptide was reduced by a factor of 4.3 to only 3.5% of the total iodine incorporation when bIGFBP-2 was iodinated in a complex with IGF. This result is also in strong agreement with the tyrosine fluorescence data reported above.

Presented in Fig. 5b is the profile of 125I radioactivity in tryptic peptides of bIGFBP-2 iodinated while in a complex with IGF-II. The same modification pattern was observed when IGF was associated with bIGFBP-2. The tyrosine-specific fluorescence data described above also showed that an increase in the degree of modification of the peptides P2 and P3 occurred when bIGFBP-2 was iodinated in a complex with IGF. A slight protection against modification in either P4 or P5 was observed when bIGFBP-2 was iodinated in a complex with IGF-II (Table Ib). The peptide P1 accounted for 15% of the total iodine incorporation when bIGFBP-2 was iodinated alone. In contrast, the labeling of this peptide was reduced by a factor of 4.3 to only 3.5% of the total iodine incorporation when bIGFBP-2 was iodinated in a complex with IGF. This result is also in strong agreement with the tyrosine fluorescence data reported above.

Determination of the Biological Activity of Iodinated Forms of bIGFBP-2—The abilities of non-radioactive iodinated bIGFBP-2 species to bind radiolabeled IGF-I or IGF-II were compared with each other and non-iodinated bIGFBP-2. Fig. 6 shows the binding of radiolabeled IGF-I (a) and IGF-II (b), respectively, by increasing amounts of non-iodinated and iodinated bIGFBP-2 species. All of the iodinated bIGFBP-2 species

![Fig. 5. The tryptic map of iodinated bIGFBP-2. 125I radioactivity. Shown are the radioactivity chromatograms of bIGFBP-2 iodinated free (a) and associated with IGF-II (b). Peaks which were shown by N-terminal sequencing and mass spectroscopy to correspond to modified tyrosyl peptides of bIGFBP-2 are identified in chromatogram a. Peptides with both one (*) and two (**) iodine atoms were identified. Iodinated derivatives of tyrosyl peptides P4, P5, and P6 were also identified as partial digestion products as indicated (+).](image)
were observed to bind the same maximal percentage of radio-
labeled IGF-I or IGF-II tracer as non-iodinated bIGFBP-2.
However, when bIGFBP-2 was iodinated in the absence of IGF,
the half-maximal binding concentration of this molecule was
increased from 2.3 to 17.9 ng for IGF-I and 0.8 to 3.2 ng for
IGF-II, when compared with the half-maximal binding concen-
tration of non-iodinated bIGFBP-2. In contrast, bIGFBP-2, io-
dinated in complex with IGF-II showed a further loss of the tyrosine
fluorescence associated with this molecule (Fig. 2b). Whereas bIGFBP-2 had lost 90% of tyrosine
fluorescence before a stable fluorescence minimum was
reached, IGF-II associated bIGFBP-2 had only lost a maximum
of 60% of its original tyrosine specific fluorescence. Therefore
these results cannot be explained by the presence of additional
tyrosine residues of IGF-II titrating the available iodous ion
to an apparent drop in bIGFBP-2 labeling.

DISCUSSION

Iodination is a very sensitive chemical modification tech-
nique which, under mildly acidic conditions, specifically labels
tyrosine residues (30). Iodination has been used in this study
as a structural and functional probe to investigate the interaction
between bIGFBP-2 and its ligands IGF-I and IGF-II, at a
molecular level. Previously, this approach has been success-
fully used to identify tyrosine residues of IGF-I which are
important for interaction with the type-1 IGF receptor (31, 32)
and tyrosine residues of both IGF-I and IGF-II which are
important for association with bIGFBP-2 (9). The primary struc-
ture of bIGFBP-2 contains six tyrosine residues. The tyrosine residues Tyr60 and Tyr71 are
located in the cysteine-rich N-terminal region, Tyr98 is located in the middle region, and the three remaining tyrosine residues Tyr213, Tyr226, and Tyr269 lie in the cysteine-rich C-terminal region of the molecule (33).
Iodine incorporation into the bIGFBP-2 molecule was measured both directly by 125I associated γ radiation counting and
by monitoring the loss of tyrosine specific fluorescence (26).

In this study, a range of iodination conditions of both free and
IGF-associated bIGFBP-2 were investigated, all of which
clearly showed that the presence of IGF-I or IGF-II bound to
bIGFBP-2 directly modified iodination of bIGFBP-2 tyrosine
residues. It could be argued that the additional tyrosine resi-
dues of IGF-II which are present in the “complex” iodination
reaction simply titrate the reactive iodous ion and therefore
nonspecifically reduce bIGFBP-2 modification. However, iodina-
tions were performed at reaction conditions in which the
concentration of the reactive iodous ion was sufficiently high to
label all of the available tyrosine residues of bIGFBP-2, irrespec-
tive of the presence of IGF-II. This was evident when
further incorporation of iodine into bIGFBP-2 did not result in
a further loss of the tyrosine fluorescence associated with this
molecule (Fig. 2b). Whereas bIGFBP-2 had lost 90% of tyrosine
fluorescence before a stable fluorescence minimum was
reached, IGF-II associated bIGFBP-2 had only lost a maximum
of 60% of its original tyrosine specific fluorescence. Therefore
these results cannot be explained by the presence of additional
 tyrosine residues of IGF-II titrating the available iodous ion
thus leading to an apparent drop in bIGFBP-2 labeling.

Tryptic mapping established that all of the tyrosine residues of bIGFBP-2 (Tyr60, Tyr71, Tyr98, Tyr213, Tyr226, and Tyr269 in
tryptic peptides P1, P2, P3, P4, P5, and P6, respectively) were readily
identified when iodinated free. Furthermore, these trends in tyrosine reactivity
 remained consistent when the iodinations were repeated at

| Peptide identity | Retention time (min) | partial N-terminal sequence | Predicted mass (Da) | Identified mass (Da) |
|------------------|----------------------|-----------------------------|---------------------|---------------------|
| P1               | 21.79                | CGVYTPR.                    | 979                 | 979                 |
| P1**             | 24.00                | CGVYTPR.                    | 1104                | 1105                |
| P6               | 26.91                | GDFECHLFYNBQGGAR.           | 2048                | 2048                |
| P6**             | 28.57                | GDFECHLFYNEQGGAR.           | 2173                | 2174                |
| P3               | 28.57                | HGDAEYSAQPBGVADNGEEHS       | 4802                |                     |
| P3**             | 28.57                | HGDAEYSAQPBGVADNGEEHS       | 4728                | 4746                |
| P2               | 29.68                | CYNPNGSELPILR.             | 1530                | 1529                |
| P2**             | 31.36                | CYNPNGSELPILR.             | 1654                | 1655                |
| +P6              | 32.36                | LIQGAPT.                    | 3124                | 3124                |
| P4** + P5        | 35.52                | Not done                    | 3389                | 3389                |
| P4** + P5        | 35.82                | CPCLELYLS                  | 2846                | 2846                |
| P4** + P5        | 37.04                | GPLELYLS                   | 2972                | 2972                |
| P4** + P5**      | 37.60                | Not done                    | 3098                | 3098                |

| Species          | Iodine incorporation (% of total) |
|------------------|----------------------------------|
| IGF-protected    |                                  |
| Free             | P1  | P2  | P6 and P3 | P4 + P5 |
| Iodinated bIGFBP-2 | 3.5 | 29.8| 39.2 | 19.5 |
| Iodinated bIGFBP-2 | 15.2 | 19.8| 29.0 | 25.6 |
| Fold protection  | 4.3 | 0.7 | 0.7 | 1.3 |

* The predicted mass takes into account the extra 58 mass units of the S-carboxymethyl group and an extra 128 mass units per iodine atom.

** The iodinated peptides P6**, P5**, and P3** were not resolved by the tryptic map chromatography.

The mass of P3 was 16 mass units larger than the mass based on the amino acid composition (see “Results”).

A summary of bIGFBP-2 tryptic peptides identified by 125I radioactivity
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chloramine-T/NaI molar ratios of 0.125, 0.375, and 1.25 (data not shown).

There are two likely explanations for IGF-II mediated protection of bIGFBP-2 at Tyr60. First, this residue may lie in a region of bIGFBP-2 over which IGF-II binds, thus preventing modification at this site. Second, Tyr60 may become less accessible for iodination following a conformational rearrangement of the bIGFBP-2 tertiary structure on IGF-II binding. These results are the mean of triplicate determinations and error bars show standard deviation values when greater than the size of the symbols.

Despite protection from iodination at Tyr60, the binding capacity of all iodinated bIGFBP-2 species were the same (Fig. 6, a and b). However, this mutant was also observed to aggregate, presumably through non-native disulfide formation. Therefore it is likely that the loss of IGF binding was due to a similar disruption of structure through the loss of native disulfide bonds in the putative N- and C-terminal domains (36, 37).

In conclusion, this study has identified a region located in the putative N-terminal domain of bIGFBP-2 which includes Tyr60 that is important for the association of this molecule with IGF-II and IGF-I. To minimize the structural disruption which
may be introduced by the random substitution of amino acids, bIGFBP-2 was iodinated both free and in complex with IGF-I or IGF-II as a probe for the binding interaction. Iodination at Tyr<sup>60</sup> and not Tyr<sup>71</sup>, Tyr<sup>80</sup>, Tyr<sup>123</sup>, or Tyr<sup>260</sup> lead to an 8- and 4-fold reduction in affinity for IGF-I and IGF-II, respectively. These results are further proof to suggest that major determinants of IGF binding reside in the N-terminal region of IGFBPs and they also raise implications for the generation of iodinated IGFBPs for radioimmunossay and ligand blot analysis. We therefore recommend that IGFBPs should be iodinated while in a complex with IGF to subsequently retain their full affinity for IGFs.

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