Iodination of Proteins by IPy 2 BF 4 , a New Tool in Protein Chemistry †

ARTICLE *in* BIOCHEMISTRY · JUNE 2006

Impact Factor: 3.02 · DOI: 10.1021/bi060103f · Source: PubMed

Articles

Iodination of Proteins by IPy_2BF_4, a New Tool in Protein Chemistry†

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Received January 18, 2006; Revised Manuscript Received March 16, 2006

ABSTRACT: Iodination is a very useful method for protein characterization and labeling. However, derivatization chemistries used in most conventional iodination procedures may cause substantial alterations in protein structure and function. The IPy_2BF_4 reagent [bis(pyridine)iodonium (I) tetrafluoroborate] has been shown to be an effective iodinating reagent for peptides. Herein we report the first application of IPy_2BF_4 in protein iodination in an aqueous medium using three representative substrates: insulin, lysozyme, and the enzyme 1,3-1,4-β-D-4-glucanohydrolase. Our results show that IPy_2BF_4 has clear advantages over existing methods in that the reaction is quantitative, fast, and selective for the most accessible Tyr residues of a protein, and it preserves the functional integrity of the protein when moderate Tyr labeling levels are pursued.

Current techniques for proteome analysis are bringing to light increasing numbers of unexplored proteins, each requiring detailed structural and functional studies to understand its biological role and potential applications. Technologies such as recombinant expression, site-directed mutagenesis, or residue-specific derivatization are useful in this discovery process. In devising chemical derivatization methods for protein chemistry, an important consideration is the fact that proteins dissolve and maintain their native structure only in aqueous media. This requirement constitutes a limitation because many useful derivatizing agents are insoluble in water. The problem is further compounded by the fact that derivatization chemistries often require temperature or pH conditions that may cause substantial protein degradation.

Among the protein modification reactions developed to date (*1*), iodination has been extensively investigated. Because of its high electronic density, iodine is a useful probe in crystallographic studies (*2*), providing information on both the conformation (*3*) and accessibility of Tyr residues (*4*) in the crystal state and on the selective modification of enzyme activity (*5*). Because iodine has several radioisotopes, iodine radiolabels have become very valuable, for example, in the monitoring of metabolic reactions both in vitro (*6*) and in vivo (*7*).

Although a general iodination method is yet to emerge, protein iodination and radioiodination are usually performed on the aromatic side chains of Tyr and His by direct methods based on sodium iodide in the presence of an oxidizing agent (*8*). However, these procedures cause detrimental oxidations on sensitive amino acid residues such as Met, Trp, and Cys. However, indirect methods such as the Bolton-Hunter method (*9*) avoid these unwanted reactions but introduce an additional organic moiety that may affect the biological activity. In addition, they tend to be less specific, by reacting primarily with *N*-terminal, Lys, and Cys instead of Tyr and His functionalities.

MATERIALS AND METHODS

Iodination Reaction. Bovine insulin (1.1 mM in water/HOAc, 2:1 v/v) was reacted with varying amounts (1.1 to 8.8 equiv) of IPy_2BF_4, previously deposited as thin, dry films in plastic vials by the spin-drying of different volumes of a stock acetonitrile solution. The reaction was allowed to proceed for 10 min.

Chicken egg white lysozyme (1.1 mM in PBS, pH 5.9) was reacted with IPy_2BF_4 at different stoichiometries (1.1 to 18.7 equiv) for 10 min as described above. An aliquot (1 μL) of the reaction mixture was subjected to tryptic digestion and MS analysis by either MALDI-TOF or nano LC-ES-Q-TOF techniques (see below).

1,3-1,4-β-D-glucanohydrolase (258 μM in deionized water) was reacted with IPy_2BF_4 (1 to 49.5 equiv) for 10 min, as above. Enzymatic activity of the reaction product was

† This research was supported by the Spanish Ministry of Science and Technology Grants BIO2002-04091-C03-01 to D.A., BQU2001−3853 and CTQ2004-08077-C02-02-01BQU to J.B., BFU2004-06377-C02-02 and BIO2000-0647-C02-02 to A.P., and EET2002-05157-C05 to G.V., J.B., and A.P. and the Generalitat de Catalunya Grant 2001SGR 00327 to A.P.

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10.1021/bi060103f CCC: $33.50
Published on Web 04/22/2006

determined spectrophotometrically (*10*). A 1-μL aliquot of the crude was subjected to tryptic digestion and MALDI-TOF-MS (see below).

Insulin Characterization, Reduction and Edman Sequencing. After 10 min, the insulin reaction mixture was centrifuged, and the supernatant was analyzed by HPLC (Phenomenex C8 column, 50 × 4.60 mm, 3 μm of particle size) using a 20 to 52% gradient of acetonitrile (+0.036% trifluoroacetic acid) into water (0.045% trifluoroacetic acid) at 40 °C over 8 min. The peak corresponding to the octaiodinated derivative was isolated, lyophilized, redissolved in NH_4HCO_3 (20 μL, 0.1 M, pH 8), treated with DTT (2 μL, 45 mM solution in 0.1 M NH_4HCO_3) to reduce disulfide bridges and alkylated with iodoacetamide (2 μL, 100 mM solution in 0.1 M NH_4HCO_3). The product was analyzed by HPLC using a 10−40% gradient of acetonitrile (+0.036% trifluoroacetic acid) into water (0.045% trifluoroacetic acid) at 40 °C over 30 min, and the two peaks corresponding to fully iodinated A and B chains (mass confirmed by MALDI-TOF-MS) were collected and further analyzed by Edman sequencing in a Procise LC instrument (Applied Biosystems).

Tryptic Digestion (Lysozyme and 1,3-1,4-β-D-Glucanohydrolase). For tryptic digestions of lysozyme and 1,3-1,4-β-D-glucanohydrolase, an aliquot (1 μL) of the reaction mixture was diluted with NH_4HCO_3 (10 μL, 0.1 M), treated with DTT (1 μL, 45 mM) for 15 min at 55 °C to reduce disulfide bonds, and alkylated with iodoacetamide (1 μL, 100 mM solution in 0.1 M NH_4HCO_3) in the dark for 15 min at 25 °C. Then, sequencing-grade trypsin (Promega) (0.5 μg) was added, and the digestion was allowed to proceed for 1 h at 37 °C.

MS Analysis (Lysozyme and 1,3-1,4-β-D-Glucanohydrolase). Prior to MALDI-TOF analysis, the protein digests were desalted on C_{18} StageTips (Proxeon, Odense, Denmark) eluted with MeOH (50%) and formic acid (1%). The desalted sample (0.5 μL) was deposited on the plate, followed by a matrix solution (α-cyano-4-hydroxycinnamic acid, 0.5 μL, 20 mg mL^{-1}) in acetonitrile (70%) in water containing trifluoroacetic acid (0.1%). Spectra were acquired in a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA).

For MS/MS analysis, the protein digests were desalted on a Poros 20 R2 minicolumn eluted with MeOH (50%) in water containing formic acid (1%) into the nanospray needle of a API Q-Star Pulsar mass spectrometer (Applied Biosystems).

For the interpretation of the mass spectra of lysozyme and 1,3-1,4-β-D-glucanohydrolase, the protein sequences were retrieved from the SwissProt database (http://www.expasy.ch), and a list of theoretical tryptic peptide masses was obtained by the PeptideMass (http://us.expasy.org/tools/peptide-mass.html) tool.

RESULTS AND DISCUSSION

We have recently described the use of IPy_2BF_4 [bis-(pyridine)iodonium (I) tetrafluoroborate] (*11*) to iodinate small synthetic peptides in organic (*12*) or aqueous (*13*) media. IPy_2BF_4, a crystalline, air stable solid at room temperature, and one of the most comprehensively studied iodonium (I) complexes, acts as a mild iodinating agent for unsaturated and aromatic compounds. For Tyr-containing peptides, we found that the reaction proceeds almost instantly, selectively, and quantitatively to the diiodinated species. In peptides lacking Tyr, acid catalysis is required for the reagent to attack the nonactivated aromatic amino acid side chains. Thus, under acid catalysis, Phe residues can be regioselectively (ortho) monoiodinated (*14*). Acid conditions are, however, ineffective for the iodination of the indolyl groups of Trp (unpublished results). This is in accordance with the fact that the preferred site for the IPy_2BF_4 iodination of indol is C-3 (*15*) and the fact that Trp can be regarded as a 3-substituted indol derivative.

In any of these cases, oxidation-prone residues such as Met were unaffected (*12a*, *14*). Regarding Trp residues, despite the fact that the oxidative degradation of Trp by acids and oxidants (i.e., oxygen and *N*-bromosuccinimide) are well documented (*16*), oxyindolyl derivatives or other open ring degradation products of Trp have not been detected in acid-free IPy_2BF_4 iodinations of model peptides (unpublished results).

These results, in addition to the fact that the reagent is water-soluble, encouraged us to explore its use for protein iodination in aqueous solution. For our study, we chose three model proteins of increasing complexity and focused our attention, in particular, on such issues as selectivity, accessibility, and structural−functional integrity.

Bovine insulin (Swiss Prot accession number P01317) was first studied as a model of a small (5734 Da) yet fairly complex protein. It has two peptide chains, each with two Tyr residues and one with two His residues, bridged by two disulfide bonds. To facilitate quick reagent and protein mixing, suitable quantities of IPy_2BF_4 (1.1 to 8.8 equiv) were deposited as thin, dry films in plastic vials by spin-drying different volumes of a stock solution in acetonitrile. The protein in water/HOAc (2:1 v/v) solution was added next, and the evolution of the different partially iodinated species (*17*) was monitored by both HPLC and MALDI-TOF-MS (Figure 1). No traces of partially or totally oxidized insulin chains could be detected, thus confirming the stability of the disulfide bonds. The fully iodinated insulin fraction was isolated by HPLC, reduced, and alkylated with iodoacetamide, and the two fully iodinated A and B chains were separated by HPLC. Sequence analysis of each chain by Edman degradation confirmed that the four diiodinated Tyr residues were at the expected positions. No trace of His iodination was detected.

Our second target was hen egg white lysozyme (P00698), with a view to investigate the selectivity of the reagent toward the different Tyr and His residues within the native protein. A globular protein (129 amino acids, single chain, 14.3 kDa) with a well-defined 3D structure, lysozyme has four potential iodination sites (Tyr20, Tyr23, Tyr53, and His15) and has been the object of early studies in protein iodination (*18*). A series of reactions following the same protocol as that for insulin was performed with the protein dissolved in PBS (pH 5.9) and added to solid IPy_2BF_4 at different stoichiometries (1.1 to 18.7 equiv) (Figure 2). In this instance, the incremental addition of individual iodine atoms to the native protein was not easy to monitor by MALDI-TOF MS, in view of which reduction, iodoacetamide alkylation, and tryptic digestion were performed, followed by MALDI-TOF MS and, as required, tandem MS sequence analysis in a nano-electrospray hybrid quadrupole-TOF instrument. The

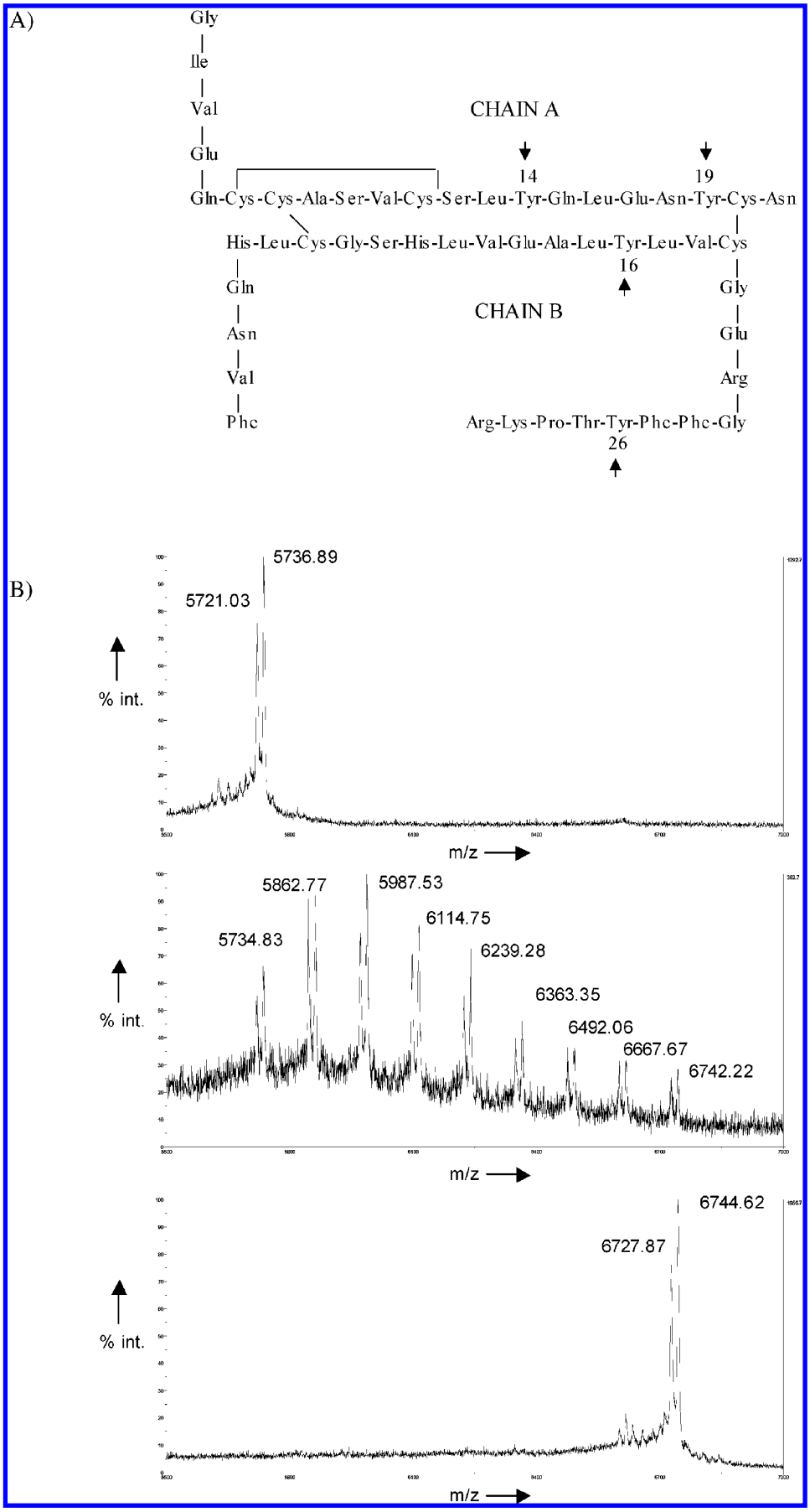

FIGURE 1: (A) Bovine insulin sequence with Tyr residues indicated with arrows. (B) MALDI-TOF mass spectra of bovine insulin iodination reactions. Top, untreated insulin (*m*/*z* 5736.89); middle, treated with 3.3. equiv (mixture of iodinated species); and bottom, treated with 8.8 equiv of IPy_2BF_4 (*m*/*z* = 6744.62 corresponding to the mass of insulin plus 8 iodine atoms).

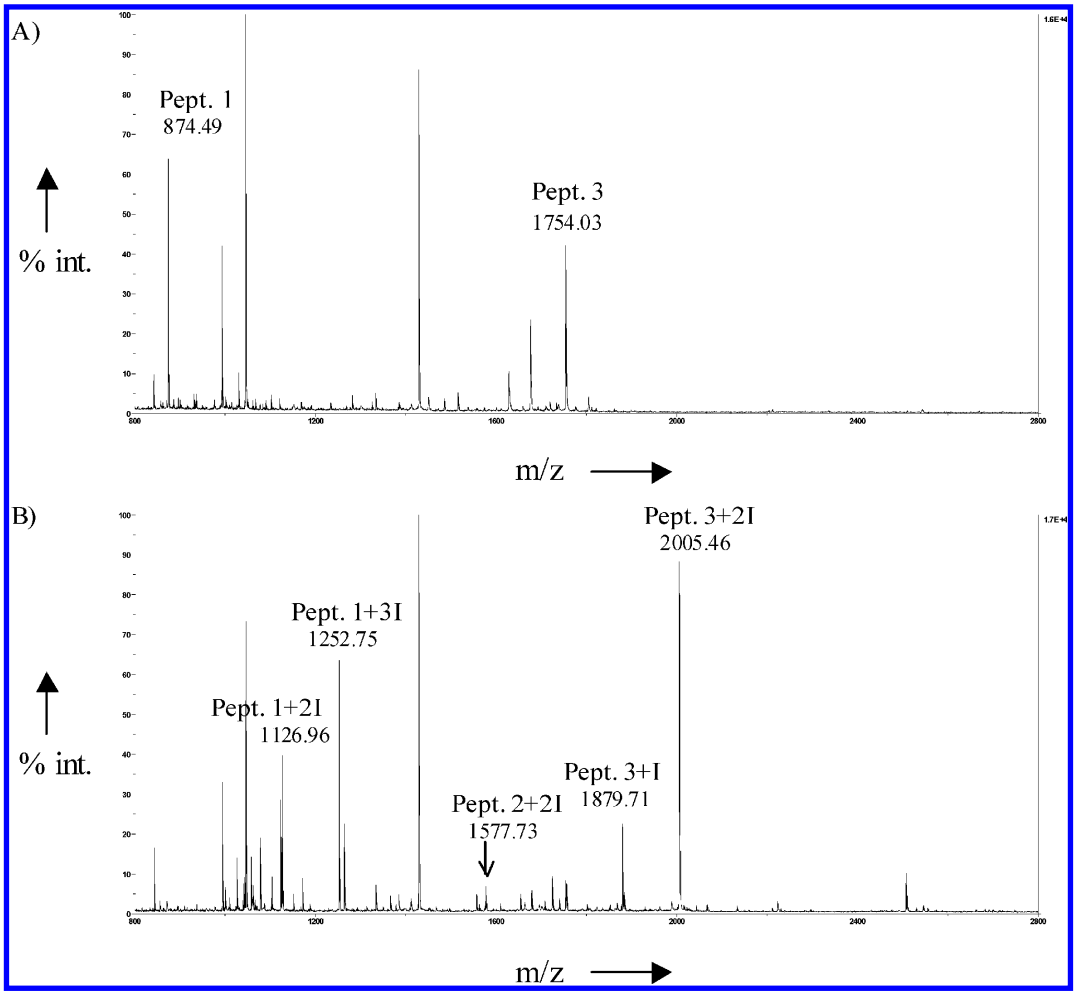

FIGURE 2: MALDI-TOF-MS of tryptic digestion of (A) lysozyme and (B) iodinated lysozyme with 18.7 equiv of IPy_2BF_4. Peptides **1**, **2**, and **3** are represented as Tyr- and His-containing fragments. (See the description in the text and in the Supporting Information.)

MS analysis focused on three tryptic peptides containing Tyr or His residues (in bold): **His15**-Gly-Leu-Asp-Asn-**Tyr20**−Arg21 (peptide **1**); Gly22-**Tyr23**−Ser-Leu-Gly-Asn-Trp-Val-Cys-Ala-Ala-Lys33 (peptide **2**); and Asn46-Thr-Asp-Gly-Ser-Thr-Asp-**Tyr53**-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg61 (peptide **3**). Peptide **2** was not detected by MALDI-TOF MS in thetryptic digestion of the noniodinated lysozyme (Figure 2A) This is consistent with the behavior of certain peptides (i.e., chain A of insulin), which are known to fly poorly under MALDI-TOF conditions. The results show that when 18.7 equivalents of IPy_2BF_4 were used, the peaks corresponding to peptide **1** with both two and three iodine atoms were detected, whereas peptide **2** was diiodinated, and peptide **3** gave peaks consistent with the noniodinated, mono and diiodinated species (Figure 2B). A detailed analysis of the reaction at the highest stoichiometry was provided by MS/MS sequence analysis (Supporting Information), which confirmed that peptide **1** was present in two forms: one with diiodinated Tyr20 and another with both diiodinated Tyr20 and monoiodinated His15; peptide **2** was exclusively diiodinated at Tyr23, and peptide **3** coexisted with the mono and diiodinated versions at Tyr53. In summary, in lysozyme, a clear trend toward the diiodination of Tyr20 and Tyr23 at low reagent-to-protein ratios is initially observed. On increasing the reagent load, nascent iodination of Tyr53 takes place along with further iodine incorporation at His15. Quantitative diiodination of Tyr53 is never achieved even at the highest molar ratio of IPy_2BF_4. These results suggest that the Tyr53 of lysozyme has limited accessibility to the iodonium electrophile as expected for a buried residue in the protein structure (*18*). Interestingly, this iodination of lysozyme at slightly acidic conditions (pH 5.9) is selective for Tyr and His residues. As already noted, in the absence of strong acids, nonactivated aromatic amino acid residues such as Phe and Trp of model peptides are inert against IPy_2BF_4. This was also checked in lysozyme by MS/MS sequencing of two tryptic peptides (**Phe34**-Glu-Ser-Asn-**Phe38**−Asn-Thr-Gln-Ala-Thr-Asn-Arg45 and Cys115-Lys-Gly-Thr-Asp-Val-Gln-Ala-**Trp123**−Ile-Arg125), which showed neither iodination nor oxidation.

Finally, we have investigated the effect of iodination on the enzymatic activity of a large protein, 1,3-1,4-β-D-glucanohydrolase (EC 3.2.1.73) from *Bacillus licheniformis* (pdb 1GBG). As recombinantly expressed in *Escherichia coli*, this protein is a monomeric endoglycosidase with a molecular mass of 24 513 Da (*19*), well characterized from both functional and structural points of view (*20*). The enzyme cleaves β-(1→4) glycosidic bonds of mixed β-(1→3,1→4)-linked glucans. A total of 21 potentially iodinatable residues (17 Tyr and 4 His) are present in its sequence, of which three Tyr (Tyr26, Tyr96, and Tyr125) residues appear in the 3D structure (*21*) as solvent-accessible and located at the binding site cleft. These three residues play a crucial role in substrate binding, and accordingly, the

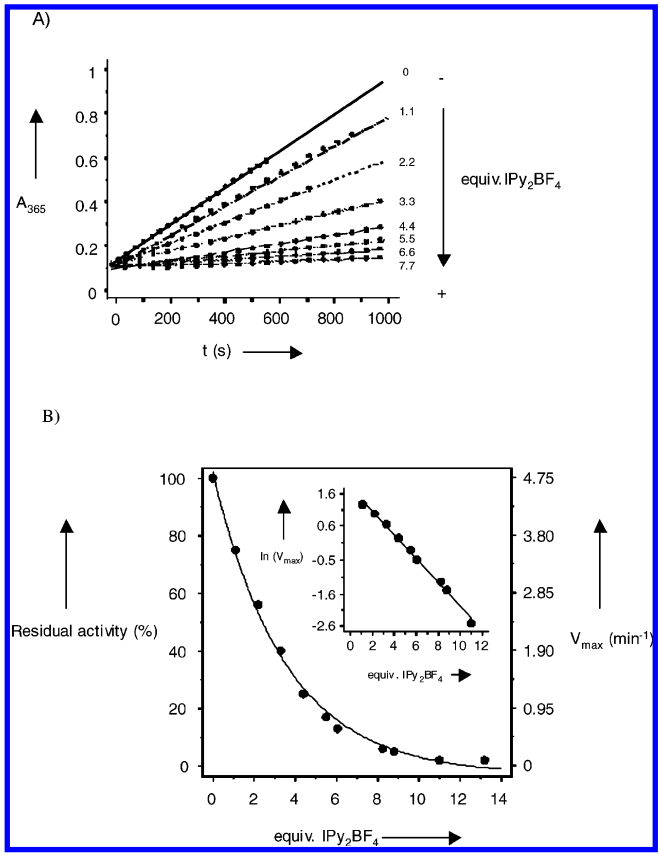

FIGURE 3: Activity of 1,3-1,4-β-D-4-glucanohydrolase after treatment with different concentrations of IPy_2BF_4. Enzymatic activity was measured at 365 nm by incubating the chemically modified enzyme with a constant concentration of substrate, Glcβ1,4Glcβ1,3Glcβ-MU (2.4 mM) at 30 °C in a citrate/phosphate buffer (6.5 mM citric acid, 87 mM Na_2HPO_4, pH 7.2) and $CaCl_2$ (0.1 mM). (A) Time-course monitoring of the release of 4-methylumbelliferone (MU) from the substrate at different IPy_2BF_4 concentrations. (B) Residual activity vs IPy_2BF_4 concentration.

modifications affecting them may result in a loss of affinity and enzyme activity. The substrate binding site of this enzyme is a particularly large hydrophilic protein surface that faces the solvent because it is designed to accommodate up to six glucopyranosyl units of the linear, high-molecular-weight substrate beta-glucan (average MW 215 000, 1200 Glc units). Varying amounts of IPy_2BF_4 that were calculated to provide reagent-to-enzyme ratios in the range of 1 to 49.5 equivalents were used. After 10 min, aliquots of the reaction mixture were assayed for enzymatic activity with the specific substrate 4-methylumbelliferyl 3-*O*-β-cellobiosyl-β-D-glucopyranoside (Glcβ4Glcβ3Glcβ-MU) (*22*). The native enzyme follows Michaelis−Menten kinetics with substrate inhibition ($k_{cat} = 0.75\ s^{-1}$, $K_m = 0.93$ mM, $K_i = 15.2$ mM) (*10*). Time courses of substrate hydrolysis for a series of iodinated enzyme probes (Figure 3A) clearly show a progressive reduction in activity, already detectable at reagent ratios as low as 2.2 equivalents of IPy_2BF_4, where activity is reduced to 55% relative to that of the wild-type enzyme. At 7.7 equivalents, complete inactivation is observed. Inactivation follows a first-order kinetics ($A_x = A_0e^{-kx}$) as shown by the plot of the residual enzyme activity versus the amount of iodinating reagent added ($k = 0.308 \pm 0.001\ mol^{-1}$) (Figure 3B).

This rapid inactivation of the enzyme with increasing amounts of iodinating reagent correlates with increasing

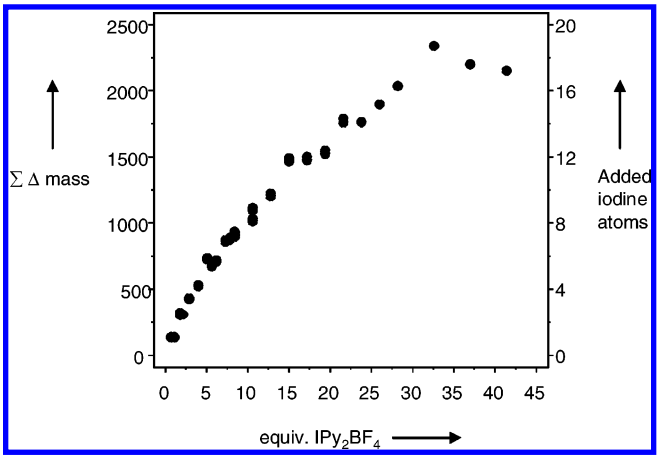

FIGURE 4: Changes in the mass and number of incorporated iodine atoms of1,3-1,4-β-D-4-glucanohydrolase at different stoichiometries of IPy_2BF_4. Experimental conditions are described in the text.

numbers of iodine atoms incorporated on the enzyme molecule. As shown by MALDI-TOF MS, (Figure 4), the average mass of the main peak increases up to a maximum of about 17 iodine atoms for ca. 40 equivalents of IPy_2BF_4. These data suggest that the first incoming iodine atoms target readily accessible, key residues at the catalytic pocket, causing rather fast enzyme inactivation and that further addition affects noncatalytic, possibly, remote residues.

For a more detailed picture of which amino acids are modified early but still allow the enzyme to retain most of its functionality, we analyzed reaction mixtures corresponding to 1.1, 2.2, 3.3, and 7.7 equivalents of IPy_2BF_4 by trypsin digestion and MALDI-TOF-MS as described above. The MS results together with in silico data on the accessibility of Tyr residues (Table 1 and Figure 5) showed that the residues that were altered early were the most accessible ones, as follows: (a) with 2.2 equivalents of IPy_2BF_4, Tyr58, Tyr26, and either Tyr9 or Tyr15 (the last two Tyr residues found in the same tryptic peptide, residues 1−22) are partially monoiodinated; (b) with 3.3 equivalents of IPy_2BF_4, the modifications observed in (a) were accompanied by the diiodination of the peptide 1−22 (two iodines at either Tyr9 or Tyr15 or one at each position), monoiodination of Tyr161 and Tyr96, monoiodination of the peptide 181−212, involving either Tyr198, Tyr205, Tyr208, or His207, with Tyr205 being the most likely one because of its high accessibility and monoiodination of the peptide 68−85, involving either Tyr72, Tyr74, or Tyr77, with Tyr74 being the most likely one because of its high accessibility; and (c) with 7.7 equivalents of IPy_2BF_4, substantial (vs partial in (a) above) monoiodination and diiodination of Tyr58 were observed. In addition, the extent of monoiodination at Tyr26 increased, and one new monoiodination peak at peptide 120−134 (Tyr124, Tyr125, or His132 as possible targets) was found. Tyr26 is located at the edge of the binding cleft, and it is involved in substrate binding with long oligomeric substrates (*23*). Its modification is not fully detrimental for enzyme activity when the trisaccharide substrate in the activity assay is used because it does not completely reach the edge of the binding cleft where Tyr26 is located. With 7.7 equivalents, the enzymatic activity was completely lost, consistent with the iodination of an active site residue such as Tyr125 located in the center of the binding cleft. Additional information on these experiments is enclosed as Supporting Information.

Table 1: Accessibility of Tyrosine Residues of 1,3-1,4-β-D-glucanohydrolase from *Bacillus licheniformis* and Its Relationship with Position and Extent of Iodination[a]

accessibility	residue	IPy_2BF_4 (equiv)[b]	comments
≥45%	58	2.2/7.7	on the protein surface; far from the active site
≥40%	9[c]	2.2	on the protein surface; far from the active site
≥27%	205[c]	3.3	on the protein surface; far from the active site
≥25%	26	2.2	in the loop covering the active site; interacts with the substrate in subsites −3 and −4
≥22%	146		far from the active site
≥20%	74,[c] 161	3.3	far from the active site
≥19%	96	3.3	in the substrate binding site on the surface loop
≥15%	125	7.7	in the active site; it interacts with the substrate in subsites −1 and +1
≥14%	15		buried residue
≥12%	198		buried residue
≥2%	124		buried residue
≥0%	72, 77, 149, 208, 213		buried residue

[a] The accessibilities were calculated using the accessible aa function of the Swiss-PDBViewer v3.7 program available at http://www.expasy.org/spdbv/. [b] The amount of iodinating reagent required for the modification to be observed. [c] The Tyr residue most likely to be modified in a tryptic peptide containing several Tyr residues is indicated.

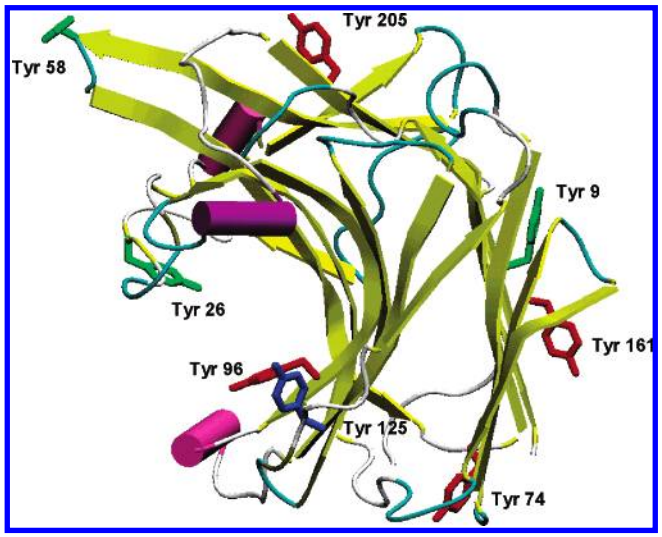

FIGURE 5: Glucanohydrolase model showing some of the most accessible Tyr residues.

An interesting finding in this series of experiments was that monoiodination at Tyr58 was observed with the exclusion of diiodination (see (a) in the previous paragraph) in contrast with the general trend toward fast diiodination observed for small peptides (*13*), suggesting an important role for the protein microenvironment in the iodination reaction.

Taken together, our data indicate that this rather large protein is progressively iodinated, starting at the most accessible residues, while still retaining significant enzymatic activity up to 3.3 equivalents of IPy_2BF_4. This fact provides further insight on the potential of IPy_2BF_4 as a tool in protein chemistry.

Our results show that proteins react with IPy_2BF_4 to yield iodinated species. The reaction has clear advantages because (i) extensive and fast iodination occurs in aqueous media; (ii) iodination preferentially proceeds with the most accessible Tyr residues of a protein; and (iii) it is expected that the moderate labeling levels of Tyr may preserve the functional integrity of a given protein provided that Tyr residues that are crucial for activity are relatively shielded from solvent exposure.

ACKNOWLEDGMENT

We thank Dr. C. Chiva and Dr. M.L. Valero of the Proteomics Unit, Universitat Pompeu Fabra for expert technical help in MS/MS and Edman sequencing. We also thank Dr. F. Canals for preliminary MALDI-TOF MS results and Mrs. M. Abel for collaboration in the enzymatic experiments.

SUPPORTING INFORMATION AVAILABLE

Selected data are presented for three proteins in one file. Insulin HPLC results, MALDI-TOF-MS spectra, and Edman sequencing for iodinated insulin chain A and B, respectively, are included. After calculating values for the tryptic digestion of lysozyme, MS/MS sequence analyses are shown, and the calculated values of recombinant 1,3-1,4-β-D-glucanohydrolase tryptic peptides are presented with the MALDI-TOF-MS spectrum of tryptically digested recombinant glucanohydrolase (after treatment with IPy_2BF_4). This material is available free of charge via the Internet at http://pubs.acs.org.

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BI060103F