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Conformational Consequences of Coupling Bullous Pemphigoid Antigenic Peptides to Glutathione-*S*-Transferase and their Diagnostic Significance

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Abstract: Recombinant epitopic peptides BP1 and BP2 representing the Bullous pemphigoid autoantigens of BP230 and BP180 bound to the fusion partner glutathione-S-transferase (pGEX-4T-2, Pharmacia) have been previously shown to increase the efficacy of diagnosis of the disease. Using glutathione-*S*-transferase-bound monomer peptides, the sensitivity of the immunological reaction exceeded that of the free synthetic epitopes and was further increased with the number of epitopic blocks in the multimer fusion products. This has been explained by the avidity effect of the fusion partner dimer formation and the high ligand affinity due to the tandem repetitions of epitopic sequences. However, a beneficial conformation of the bound epitopic peptides might also contribute to the above phenomenon. Circular dichroism (CD) and Fourier transform infrared (FTIR) absorption spectroscopic studies revealed the importance of glutathione-*S*-transferase to induce and stabilize ordered secondary structures of the epitopic peptides. The free monomer and multimer peptides in aqueous buffer were present as a mixture of unordered and β-sheet conformation, while binding them to the fusion partner the proportion of ordered secondary structures increased in parallel with the number of antigenic epitopes. The most prominent changes in the conformational state of the monomers in the fusion form were the increase of α-helical and β-sheet and the decrease of unordered conformation, while in the case of oligomeric peptides the adoption of a helical conformation was accompanied by the decrease of β-sheet structure. An outstanding α-helix content (46%) was detected in the case of the trimeric BP1 in its recombinant fusion form. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide conformation; Bullous pemphigoid antigens; recombinant bacterial proteins; glutathione-*S*-transferase; circular dichroism; Fourier-transform infrared

INTRODUCTION

Immune responses against proteins and viruses can be achieved using synthetic antigenic peptides. However, free peptides, by themselves, are poorly immunogenic [1,2]. The interaction of the peptide and antibody is complex and is governed by many factors, one of them is the presence of specific

Abbreviations: GST, glutathione-S-transferase; BP, Bullous pemphigoid; FTIR, Fourier transform infrared; OPD, *ortho*-phenylenediamine; OD, optical density; FSD, Fourier self-deconvolution; Aib, α-aminoisobutyric acid.

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conformation of the antigenic peptide necessary for antibody recognition. The behaviour of peptide antigens is quite variable. B-cell epitopes have conformational preferences for folded forms in solution which are often similar to those found in the antibody-bound peptide and in the intact protein derived from, while T-cell epitopes are mostly present in helical conformation in solution and adopt extended structure in bound state [3]. An approach to increase and diversify the immunogenicity and to achieve a specific conformation in solution is the covalent coupling of small epitopic peptide(s) to an immunogenic carrier protein (reviewed in Reference [4]). One of the alternatives to the use of peptides as immunogens is bacterial expression. Because of its useful properties glutathione-S-transferase (GST) has gained widespread application for bacterial production of polypeptides that are fused to its *C*-terminus [5]. The main advantage of this system is the high level of soluble protein that can be obtained in the cytoplasm of *Escherichia coli* [6].

Bullous pemphigoid (BP) is one member of subepidermal autoimmune blistering disease which is characterized immunologically by circulating antibodies to autoantigens of hemidesmosomes, BP230 (BPAG1) and BP180 (BPAG2) [7,8]. The detection of circulating antibodies against the BP antigens was achieved by means of an ELISA test with recombinant antigens containing only short antigenic epitopes of the two BP antigens bound to a GST partner [9]. The detailed analyses using different immunological techniques revealed that the immunoreactivity of the fusion products containing monomers of antigenic peptides is higher than that of the free peptides. The use of homo- or heterooligomers as substrates further increased the sensitivity of the ELISA assay [9].

The purpose of this work was to show that the high efficiency of the GST/mono- and multimer fusion products is not merely due to the avidity effect of GST dimer formation [6], but that a beneficial conformation of the antigenic peptides in the GST-bound form also can play a role. The conformational consequences of coupling the BP epitopic peptides to the *C*-terminus of GST were studied on the BP1 and BP2 monomers and on their tandem repetitions, BP22 dimer and BP111 trimer antigens. Circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopic studies were performed in solution and the spectroscopic data were evaluated in the context of the immunological results.

MATERIALS AND METHODS

Prediction and Synthesis of Peptide Epitopes

The sequences of the BP antigens were from Swiss-Prot and PIR data banks and were analysed with WISCONSIN Package, Version 8 (Genetics computer Group, Madison, USA) using Peptidestructure and Plotstructure programs. The chosen epitopic segments were as follows:

BP1: WTQEPQPQPLEEKWQHRVVEQIP (BPAG1, AC Q03001, 1814–1834)
BP2: RSILPYGDSMDRIEKDRLQMAP (BPAG2, AC Q02802, 507–528)

Peptide sequences were synthesized by the solid-phase technique utilizing tBoc chemistry [10]. Side-chain protecting groups were as follows: Arg(Tos), Asp(OcHex), Glu(OcHex), His(Z), Ser(Bzl), Thr(Bzl), Tyr(2BrZ) and Lys(2ClZ). The peptide chains were elongated on *p*-methylbenzhydrylamine resin (0.48 mmol/g). Couplings were performed with *N*,*N*′-dicyclohexylcarbodiimide, with the exceptions of Asn and Gln, which were incorporated as their 1-hydroxybenzotriazole esters. The completed peptide resins were treated with liquid HF/dimethyl sulphide/*p*-cresol/*p*-thiocresol (86:6:4:2, v/v), at 0°C, 1 h. HF was removed and the resulted free peptides were solubilized in 10% aqueous acetic acid, filtered and lyophilized. The crude peptides were purified by reverse-phase HPLC. Peptide purities were above 97%. The purified peptides were characterized by mass spectrometry using a Finnigan TSQ 7000 tandem quadrupol mass spectrometer equipped with an electrospray ion source. The measured molecular weights were in good agreement with the calculated values and were as follows: BP1, 2656.97 (calc.) and 2656.8 (measured); BP2, 2648.08 (calc.) and 2648.6 (measured).

DNA syntheses were according to Molnar *et al.* [9]. Each of the four recombinant fusion products is composed of GST (pGEX-4T-2, Pharmacia) coupled with the antigenic epitope(s) at the *C*-terminus. A dipeptide spacer (Gly-Ser) was inserted between GST and the particular epitope sequence. In the multimer constructions the epitopes were separated from one another by a (Pro-Pro-Arg-Ser) tetrapeptide sequence in order to minimize the possible interaction between the epitopic peptides. The recombinant fusion products were as follows:

[GST-BP1]: [GST]–Gly-Ser–[BP1]
[GST-BP2]: [GST]–Gly-Ser–[BP2]

[GST-BP22]: [GST]–Gly-Ser–[BP2]–Pro-Pro-Arg-Ser–[BP2]
[GST-BP111]: [GST]–Gly-Ser–[BP1]–Pro-Pro-Arg-Ser–[BP1]–Pro-Pro-Arg-Ser–[BP1]

Expression and Purification of the Recombinant Fusion Product

The basic protocol was according to [11] and is described here briefly. *Escherichia coli* DH5α cells harbouring the different recombinant fusion expression plasmids were treated with lysozyme and disrupted by sonication. Isolation of the recombinant products was performed directly from the cell lysate with the help of glutathione Sepharose 4B affinity gel (Pharmacia) in the presence of 1% Triton X 100. The recombinant fusion products were eluted by reduced glutathione and were analysed by electrophoresis in 12.5% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) under reducing conditions and by immunological methods.

Immunological Techniques

Immunological reactivity of synthetic peptides and the respective recombinant fusion variants with diseased patients and healthy control sera were tested in ELISA as described earlier [12]. Briefly, antigens were incubated with sera (diluted 1:200). Binding of autoantibodies to antigen was visualized by *ortho*-phenylenediamine (OPD) and H_2O_2, after incubation with a peroxidase labelled anti-human IgG (diluted 1:5000). Optical density (OD) was measured at 492 nm. The cut-off of the ELISA was set at an OD value that was equal to the mean OD value of the negative control sera plus 2 S.D. (S.D. = standard deviation of control OD).

Spectroscopy

CD spectra were recorded on a Jobin–Yvon Mark VI dichrograph at 25°C with a 0.02 cm pathlength cell. Four spectra were accumulated for each sample. The peptide concentration was 0.2–0.5 m*M*. Mean residue ellipticity $[\Theta]_{MR}$ was expressed in degrees $\cdot$ cm^2/dmol using a mean residue weigth of 110. Percentages of secondary structures were calculated by the Provencher and Glöckner curve analysing algorithm [13].

FTIR measurements were performed on a Bruker IFS-55 FTIR spectrometer at a resolution of 2 cm^{-1} using a 0.05 cm cell with CaF_2 windows. Peptides were dissolved in D_2O at a concentration of 0.2 m*M* and maintained at room temperature for 1 h to ensure that the isotopic H-D exchange reached equilibrium, as judged by a constant minimal absorbance at the residual amide II band. The amide I region of the spectra was decomposed to individual bands by the Levenberg–Marquardt nonlinear curve-fitting method using weighted sums of Lorentz and Gauss functions. The choice of the starting parameters was assisted by Fourier self-deconvolution (FSD) [14].

RESULTS AND DISCUSSION

CD Studies

Monomer BP1 and BP2. The CD spectra of the monomer antigenic peptides, BP1 and BP2 in phosphate buffered saline (PBS), pH 7.4 and in 2,2,2-trifluoroethanol (TFE) are shown in Figure 1. Although the spectra in aqueous buffer of both peptides are marked by a strong negative band at $\sim$200 nm (a sign of the predominance of unordered conformer populations) the Provencher–Glöckner curve analysing algorithm [13] indicated a substantial contribution of β-sheet structure. The red shift of the negative maximum and the higher intensity of the negative $n \rightarrow \pi^*$ transition at around 222 nm in the case of BP2 peptide reflect also the presence of α-helical conformation (4% α-helix, Table 1).

TFE is well-known to promote α-helical and β-turn conformations, that is, structures which are stabilized by intramolecular H-bonds [15]. The two peptides show typical helical CD spectra in this solvent differing only in band intensities. Applying the curve

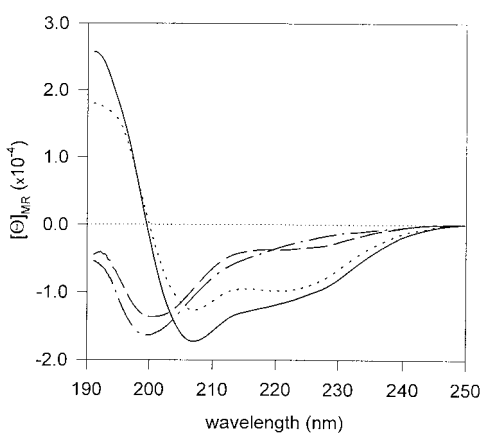

Figure 1 CD spectra of the free monomer epitopic peptides. BP1 in TFE (—) and in PBS buffer, pH 7.4 (— - —). BP2 in TFE (···) and in PBS buffer, pH 7.4 (---).

Table 1 Percentages of Secondary Structural Elements in BP1 and BP2 Antigenic Peptides in Free and in GST-bound Form Obtained from the Deconvolution of CD Spectra by the Provencher and Glöckner Method [13]

Peptide	α-Helix	β-Sheet	Unordered
BP1 free monomer	0 (48)[a]	52 (32)	48 (20)
BP1 fusion form	9	65	26
BP2 free monomer	4 (30)	51 (25)	45 (45)
BP2 fusion form	9	69	22
BP22 free dimer	6	62	36
BP22 fusion form	11	53	36
BP111 free trimer	0	76	24
BP111 fusion form	46	36	18

[a] In parenthesis the secondary structure percentages in TFE solution.

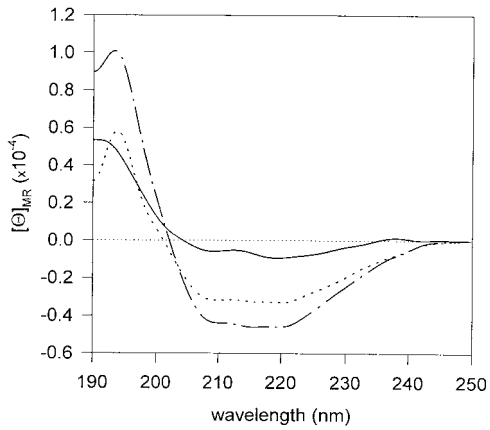

Figure 2 CD spectra in PBS buffer, pH 7.4 of the GST fusion partner (—), the [GST-BP1] fusion construction (— - —) and the BP1 monomer epitopic peptide in GST-bound form (···).

analysing algorithm, 48% and 30% α-helical content were estimated for BP1 and BP2, respectively (Table 1). This result is consistent with the data obtained from the Chou–Fasman–Prevelige secondary structure prediction algorithm [16] (data not shown) which shows a high propensity of α-helical conformation for the *N*-terminal and central part of BP1, while good helix forming ability was found to be restricted only for the central part of the BP2 peptide sequence. In the latter case the adoption of periodic ordered conformation of the *N*-terminal region is prevented by a sequence of amino acids (Pro-Tyr-Gly-Asp-Ser) with high β-turn forming potential. Comparing the percentages of the secondary structures in PBS buffer and in TFE, BP1 shows a higher increase in α-helix content in TFE (48% helix induction) while in the case of BP2 only 26% α-helix induction was detected. The difference between the α-helical content in aqueous buffer and in TFE may reflect the relative ability of peptides to undergo structural transition upon binding to a carrier. A similar correlation was found in the case of antimicrobial peptides upon interaction with membranes [17].

Figure 2 shows the CD spectra in PBS of the GST fusion partner, the [GST-BP1] recombinant fusion construction and the monomer BP1 peptide in the GST-bound form. The CD spectrum of [GST-BP2] is not significantly different from that of [GST-BP1] and hence is not shown. In general, the CD spectra of BP antigenic peptides in GST-bound form were obtained by subtracting the CD spectrum of GST from those of [GST-epitopic peptide] fusion constructions. The low-intensity CD spectrum of GST possibly reflects a mixture of various conformations. As shown in Figure 2, the CD spectrum of BP1 monomer changed considerably in GST-bound form compared to that of the free peptide in PBS in Figure 1: more specifically, the appearance of a positive maximum at 195 nm and the broad negative band between 200 and 240 nm reflect an increased contribution of ordered secondary structures. The low intensity of the positive maximum, however, indicates still a substantial amount of unordered conformers. On the basis of the curve analysing algorithm, the most prominent change in the conformational state of the monomer BP1 in GST-bound form is the increase of the α-helix and β-sheet conformations and the decrease of unordered structure. The percentages of the individual conformations, however, are not significantly different in the two GST-bound BP1 and BP2 peptides (Table 1).

Multimer BP22 and BP111. Figure 3 illustrates the CD spectra in PBS of the dimeric BP22 and trimeric BP111 peptides in free and GST-bound form. The deconvolution of the CD spectra shows that both free multimeric peptides contain a substantial amount of β-sheet structure (Table 1). In the case BP22 dimeric peptide a 6% of helical conformation was also estimated. When coupled to GST, some increase of helicity was found for BP22 (11% in GST-bound form), while an outstanding α-helix content (46%) was detected in the case of BP111 trimeric peptide. In

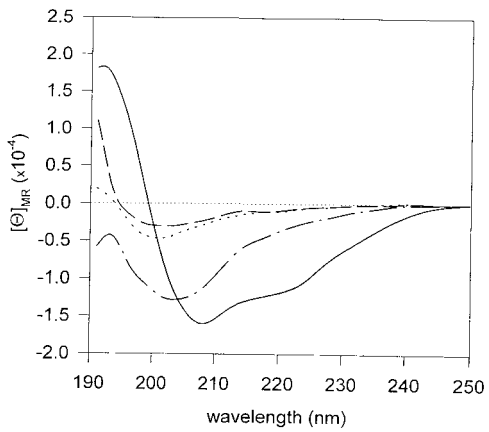

Figure 3 CD spectra of BP22 dimer and BP111 trimer epitopic peptides in PBS. BP22 in free (- - - -) and in GST-bound form (— - —); BP111 in free (···) and in GST-bound form (—).

both peptides the adoption of helical conformation was accompanied by a substantial decrease of β-sheet structure, particularly in the case of BP111. (It is worth noting that the amount of α-helix in monomer BP1 and BP2 peptides upon binding to GST increased at the expense of unordered structures.)

FTIR Studies

Infrared measurements were performed in D_2O solutions of the GST fusion partner, the trimer epitopic peptide BP111 and the [GST-BP111] fusion construction. The FSD-based curve fitted FTIR spectra are presented in Figure 4(a–c). Figure 4(d) shows the spectrum of BP111 in the GST-bound form obtained by subtracting the spectrum of GST protein from that of the [GST-BP111] recombinant fusion form. The component band frequencies and intensities are listed in Table 2.

The amide I region (1700–1600 cm^{-1}) contains the C=O stretching vibrations of the amide group which are sensitive to changes in the peptide/protein backbone conformation. The strong component band near 1595 cm^{-1} is associated with amino acid side-chain vibrations: the aromatic ring vibrations of Tyr, Phe and Trp and the ν_{as} vibration of deprotonated COOH groups of Glu and Asp residues [18,19].

The bands between 1674 and 1668 cm^{-1} can be assigned to amide carbonyls exposed to the solvent but not involved in strong oriented intramolecular or intermolecular H-bonds of ordered secondary structures and also may be contributed by the satellite band of antiparallel β-sheet [14,20]. This component is most intense in the case of the GST protein, while it is not present in the spectrum of [GST-BP111]. In the [GST-BP111] fusion construct, GST is also expected to undergo a conformational change. This explains the appearance of the negative component in the difference spectrum (Figure 4d).

A group of component bands is found in the 1656–1648 cm^{-1} region. Bands between 1657 and 1650 cm^{-1} in D_2O are generally assigned to α-helix [14]. This band, which is most intense in the case of BP111 in GST-bound form, is shifted to 1648 cm^{-1}. On the basis of the CD spectrum, BP111 in bound form is predominantly in helical conformation. Therefore, we think that the shift to a lower frequency is caused by unusual amide–solvent interactions, already observed in proteins known to be largely α-helical [21,22].

An additional component is appearing at 1663–1662 cm^{-1} in the spectrum of [GST-BP111] recombinant construction and BP111 fusion form. In the spectra of peptides containing repeating α-aminoisobutyric acid (Aib) residues a component band at 1663–1662 cm^{-1} has been associated with the 3_{10} helical conformation [23]. Thus, this high frequency component band may reflect a typical 3_{10} or weakly H-bonded (nascent) α-helical conformation.

The amide I component between 1641 and 1635 cm^{-1} can be correlated with the acceptor C=O group of $1 \leftarrow 4$ H-bonded β-turns. On the basis of comparative NMR and FTIR absorption spectroscopic studies on bridged cyclic and linear peptides with well-characterized turns [24], a band at 1640 ± 3 cm^{-1} can be assigned to the acceptor amide carbonyl of type I and type II β-turns.

The most significant feature in the amide I region of all peptides, except BP111 in bound form, is the band between 1622 and 1616 cm^{-1} which arises from β-sheet and/or strongly H-bonded extended polypeptide chains (β-aggregates) [25]. The GST-bound BP111 contains significantly lower amount of β-sheet conformation than the free peptide.

In general, the results of our FTIR absorption spectroscopic study are in good agreement with those of CD measurements. The bands at 1662, 1648 and 1635 cm^{-1} in the IR absorption spectrum of BP111 in GST-bound form mainly represent helical or folded conformations, that is 3_{10}-helix, α-helix and β-turn. The sum of their relative amounts

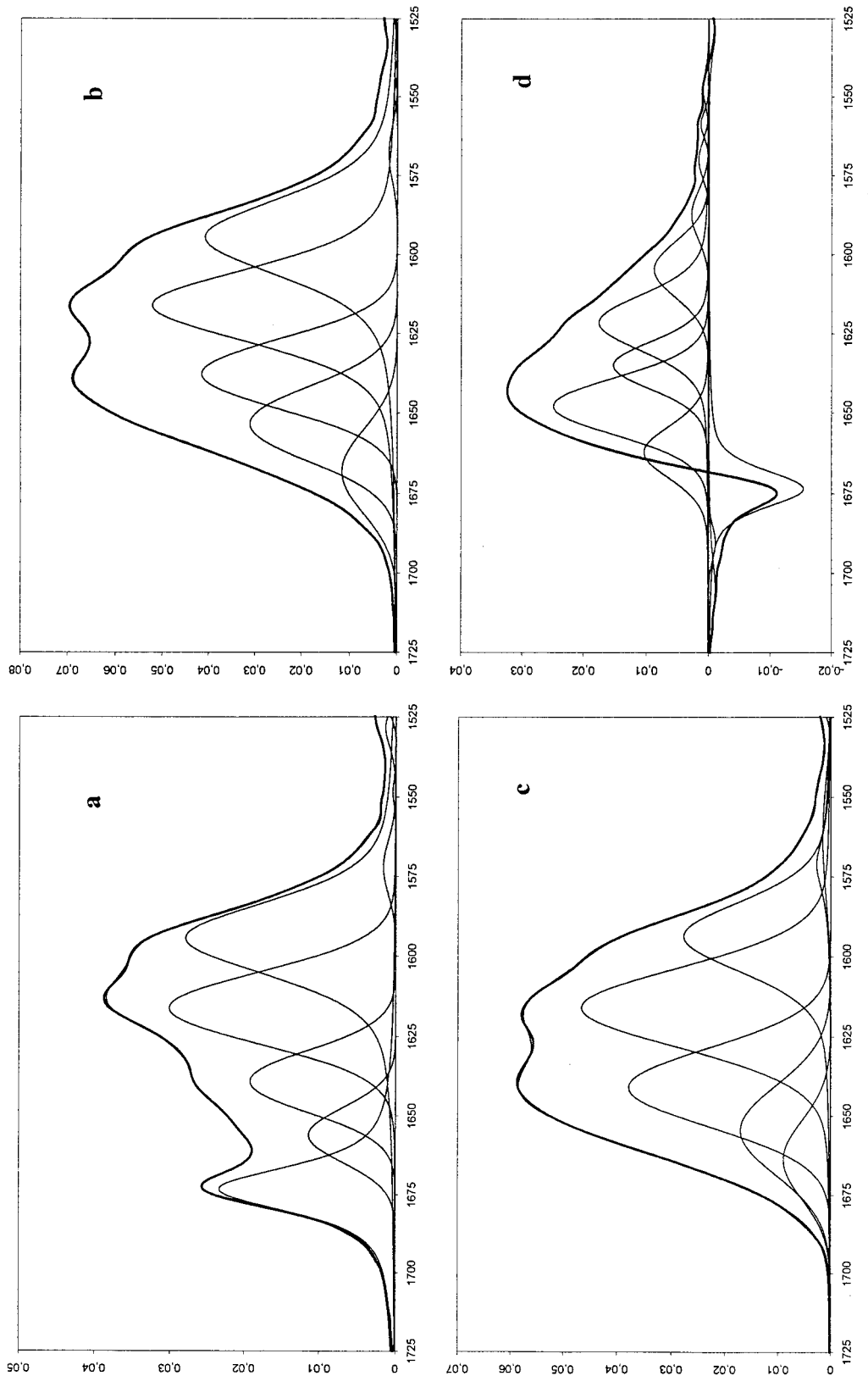

Figure 4 FTIR spectra in D_2O of GST protein (a), free trimer BP111 epitopic peptide (b), [GST-BP111] recombinant fusion construction (c), BP111 in GST-bound form (d). The spectrum of BP111 in GST-bound form was obtained by subtracting the spectrum of GST from that of the [GST-BP111] recombinant fusion construction. Concentration of samples: 0.2 m*M*.

Table 2 Characteristic Frequencies (in cm^{-1}) in D_2O in the Amide I Region of the GST Fusion Protein, the Free Trimeric BP111 Epitopic Peptide, the [GST-BP111] Fusion Construction and the BP111 Peptide in its Fusion Form

Assignment	BP111 free trimer	[GST-BP111]	GST	BP111 fusion form
β-Aggregation/β-sheet	1616 (43)[a]	1616 (43)	1616 (40)	1622 (28)
β-Turn[b]	1638 (27)	1641 (31)	1639 (21)	1635 (20)
α-Helix[b]	1654 (21)	1654 (18)	1656 (12)	1648 (37)
3_{10} or nascent helix		1663 (8)		1662 (15)
Solvent exposed amide carbonyls	1668 (9)		1673 (27)[c]	

[a] In parenthesis the relative intensities (%). The strong component band near 1595 cm^{-1} in Figure 4 arises from amino acid side-chain vibrations; hence it is not involved in the calculation of the relative amounts of the different secondary structures.
[b] Both bands may get contribution from the unordered conformation [14,19].
[c] This component may also be contributed by the satellite band of antiparallel β-sheet [14,19].

is much higher (72%) compared to that of the free peptide (48%, Table 2). In agreement with this finding, a high proportion of α-helix was calculated from the deconvolved CD spectrum of the GST-bound BP111 peptide (46%, Table 1). It should be noted that the relative amount of helical and folded structures cannot be rigorously determined by means of CD and FTIR absorption spectroscopies. Both the low frequency β-turn and α-helix bands may get a contribution from that of the unordered structure [14,19]. On the other hand, the subtraction of the spectrum of GST from that of the recombinant construct [GST-BP111] is also a source of uncertainties.

Utilization of the Recombinant Fusion Peptides in Test System

A representative analysis of the efficiency of recombinant fusion products compared to synthetic peptides are summarized with a scattered blot in Figure 5. The use of recombinant fusion oligomers as substrates increased the sensitivity of the diagnosis. Sixty percent of patients proved to be positive to a mixture of BP1 and BP2 synthetic peptides (Figure 5a), whereas this value increased to 90% in the case of a mixture of multimer [GST-BP22] + [GST-BP111] fusion products (Figure 5b).

CONCLUSION

In the ELISA system the sensitivity of the immunological reaction of BP1 and BP2 epitopic peptides representing Bullous pemphigoid autoantigens in the GST recombinant fusion form exceeded that of the chemically synthesized peptides. The sensitivity of the system increased to 90% for multimer fusion products. The high efficiency of the GST/multimer fusion

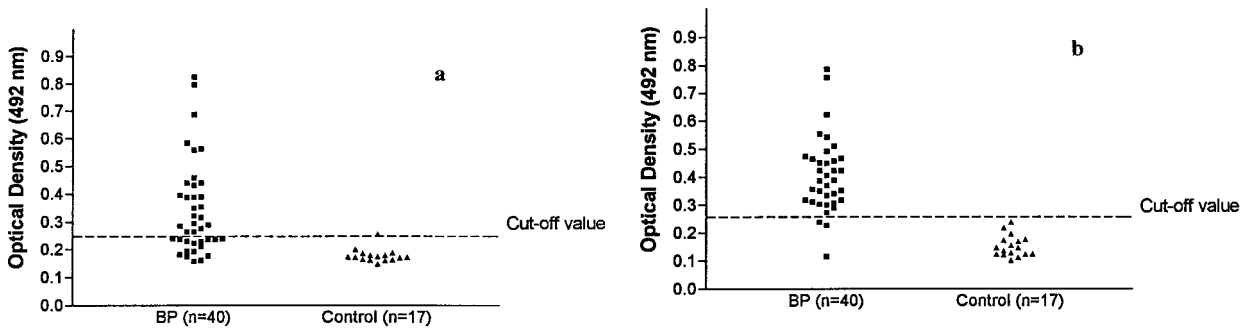

Figure 5 Scatter blot of reactivities of sera from patients with BP and healthy controls using synthetic peptides (a) and their recombinant constructs in fusion form (b) as antigens in ELISAs. The cut-off of the ELISA was set at an OD value that was equal to the mean OD of the negative control sera plus 2 S.D. The cut-off is indicated by a dashed line. (a) BP1 and BP2 synthetic peptides as antigens in equimolar quantities (1 μg/well); (b) [GST-BP22] and [GST-BP111] fusion constructs as antigens in equimolar quantities (1 μg/well).

products has been explained by the avidity effect of the GST dimer formation and the high ligand affinity due to the tandem repetitions of antigenic epitopes [6,12,26]. However, spectroscopic measurements revealed that GST also induces a measurable beneficial conformation of the fused peptides.

CD and FTIR absorption analyses indicate that in PBS buffer (pH 7.4) the free monomeric BP1 and BP2 and the free multimeric BP22 and BP111 epitopic peptides are present mainly in unordered and β-sheet conformation. In the case of multimeric forms an increase of β-sheet structure at the expense of unordered structures was seen. The observed high α-helix induction in TFE (48%) in the free monomeric peptides, especially in the case of BP1, are in accordance with the high helix forming ability of this peptide upon binding to the GST fusion partner. Upon binding of the monomer and multimer peptides to GST the proportion of ordered secondary structures increased in parallel with the number of antigenic epitopes. The most prominent changes in the conformational state of the monomers in the fusion form were the increase of α-helical and β-sheet confirmation and the decrease of the unordered conformation, while in the case of oligomeric peptides the adoption of a helical conformation was accompanied by the decrease of β-sheet structure. An outstanding 46% α-helix content was detected in trimeric BP1 in the recombinant fusion construction. As a result of the structural analysis, the predominance of helical and folded (α-helix, 3_{10}-helix and β-turn) conformations might also be responsible for the increased sensitivity of immunological recognition in the case of a mixture of GST/multimer fusion products.

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