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In situ FTIR ATR spectroscopic study of the interaction of immobilized human tumor necrosis factor-α with a monoclonal antibody in aqueous environment

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Abstract

By in situ FTIR ATR measurements, the antibody (AB) recognition of human tumor necrosis factor-α (TNFα) immobilized on the Ge surface of a multiple internal reflection element (MIRE) was investigated. The experiments were performed in aqueous environment in a flow-through cell. After immobilization of TNFα on the Ge-MIRE by direct adsorption from aqueous solution, the immobilisate reached stability after about 1 h under flow-through conditions. The remaining sites of the Ge surface were saturated by bovine serum albumin (BSA) in order to prevent unspecific binding of anti-TNFα AB which was then added. The obtained FTIR ATR spectra were shown to result exclusively from AB specifically interacting with TNFα, since the absence of immunoglobulin binding to BSA adsorbed to the Ge MIRE was verified by a reference experiment. Finally, the stability of all adsorbed protein immobilisates was monitored under flow-through conditions for 10.5 h. The TNFα–AB complex showed a decrease of 7.4%, whereas the BSA adsorbate remained stable. IR measurements were performed with polarized light in order to study orientational effects of the immobilized proteins. The dichroic ratios and surface concentrations of all used proteins are available after quantitative analysis of the amide II bands.

Keywords: In situ FTIR ATR spectroscopy; Immobilization; Human tumor necrosis factor-α; Antibody recognition; Quantitative protein analysis

1. Introduction

Among the many cytokines responsible for the regulation of cellular physiology, human tumor necrosis factor-α (TNFα) plays a prominent role as mediator of cellular immune response and inflammation [1]. Inhibition of the effect of TNFα represents the basis for treatment of rheumatoid arthritis and Crohn's disease [2]. Furthermore, this cytokine has been shown to be involved in the pathology of diseases such as septic shock, cancer, AIDS and malaria [3,4]. Mature TNFα is secreted after a posttranslational processing to produce a 17-kDa polypeptide associating into a trimeric form in solution [5]. The carboxyl and amino termini of the molecule are located on the base of the trimer and are considered to be important for receptor binding [6].

The monoclonal anti-TNFα antibody (AB) used in these experiments is also known as Infliximab or Remicade® and consists of the variable regions of a mouse anti-TNFα monoclonal antibody linked to human IgG1 with κ light chains [7]. It is currently approved for treatment of Crohn's disease [8] and clinically applied for therapy in rheumatoid arthritis [9–11].

Changes in the secondary structure of two monoclonal IgGs which differ in their isoelectric point and their corresponding $F(ab')_2$ fragments have been investigated by an FTIR study during adsorption on hydrophilic silica and on hydrophobic methylated silica surfaces showing a decrease of the β-sheet content [12].

By in situ Fourier Transform Infrared Attenuated Total Reflection (FTIR ATR) measurements, we investigated the antibody recognition of TNFα immobilized on the Ge

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surface of a multiple internal reflection element (MIRE). The main targets of these experiments were to find a method to produce a stable and functional TNFα-immobilisate on a Ge MIRE and to unambiguously verify the interaction of this immobilisate with AB by in situ FTIR ATR spectroscopy. For this reason, unspecific AB binding to the Ge MIRE had to be prevented by saturating free MIRE adsorption sites with bovine serum albumin (BSA). The final aim is to replace the immunoglobulin by synthetic peptides binding to TNFα or its receptors and impeding its biological activity [13], as clear disadvantages of large macromolecules such as antibodies exist, e.g., poor bioavailability and stability, expense, and risk of severe side effects in clinical application.

2. Materials and methods

2.1. Chemicals and biochemicals

Water was ultrapure (Elga). All proteins used were dissolved in phosphate buffered saline (PBS): 10 mM Na_2HPO_4/NaH_2PO_4 pH 7.6, 100 mM NaCl, 0.02% (w/v) sodium azide. BSA was purchased from Sigma (A-7906). Human TNFα was a recombinant form from yeast and was purchased from Strathmann Biotec AG. The chimerized monoclonal anti-TNFα antibody (AB) Infliximab or Remicade® was provided by Dieter Falkenhagen, Christian Doppler Laboratory for Specific Adsorption Technologies in Medicine and Center of Biomedical Technology, Danube University Krems, Krems, Austria.

2.2. Spectra acquisition

2.2.1. FTIR ATR equipment

The MIRE was a $51 \times 20 \times 1.5$-mm^3 Ge trapezoid with an angle of incidence of $\Theta = 45°$. All FTIR spectra were recorded at 25 °C with a Bruker IFS 25 spectrometer equipped with a lift-model single-beam-sample-reference (SBSR) ATR mirror attachment, with a hydrodynamically optimized and water-thermostatted SBSR cell (flow-through cuvette) made of Delrin® [14] and with parallel (∥) and perpendicular (⊥) polarized IR light produced by an aluminium grid polarizer on a KRS-5 substrate. A mercury-cadmium telluride (MCT) detector was used. All spectra were scanned at 4-cm^{-1} resolution.

2.2.2. SBSR method

Sample (S) and reference (R) compartments were placed above each other on either side of the MIRE. Each compartment (area: 310 mm^2, thickness: 0.26 mm) was sealed by a viton O-ring (diameter: 1.0 mm) and had a volume of about 80 μl. In order to get IR spectra from both, a parallel beam was passed alternatively by means of a lift through S at the lower half and R at the upper half of the MIRE. As S and R are accessible at any time by the single beam of the instrument, this method is referred to as the single-beam-sample-reference (SBSR) technique [14–18].

2.3. Sample preparation

2.3.1. Clean Ge surface

Before a new experiment was begun, each side of the Ge ATR plate was polished by machine (Logitech PM5) with a pellon cloth by means of a 0.1-μm diamond particle suspension (AB Technics, Tribuswinkel, Austria) rotating at 30 rpm for 10 min. Subsequently, the plate was subjected to various cleaning procedures, using consecutively acetone, ultrapure water and ethanol, until there were no visible impurities left. In order to remove small traces of organic compounds, all glassware and ATR plates were finally cleaned for 3 min by plasma (Harrick Sci. Corp.) before use. The Ge plate was considered to be clean if the $\nu(CH_2)$ bands at ~ 2920 and ~ 2850 cm^{-1} disappeared completely in the FTIR ATR spectrum (single beam mode). Finally, the plate was mounted in the SBSR cell for flow-through experiments.

2.3.2. Immobilization of TNFα, saturation with BSA and addition of anti-TNFα antibody

All solutions used in these experiments were transported to the flow-through cuvette with the help of peristaltic pumps (Ismatec SA, Switzerland) using viton tubings (inside diameter I.D. = 1mm). The flow rates used were 1 ml/20 min, 1 ml/10 min and 1 ml/5 min and corresponded nominally to one exchange of the compartment volume (80 μl) per 1.6, 0.8 and 0.4 min, respectively. A schematic representation of the sequence of experimental steps in the R and S compartments is given in Fig. 1.

At first, all compartments were filled and rinsed with PBS at a flow rate of 1 ml/10 min for 50 min. Every few minutes spectra were recorded to check the stability of the system. Then the pump was stopped and reference spectra were recorded. Afterwards, 1.2 ml of a 1.5-ml TNFα solution (66.7 μg/ml PBS) was pumped into the S compartments at a flow rate of 1 ml/5 min; then the pumps were stopped. The R compartments were treated in the same way with PBS instead of TNFα solution. After the TNFα adsorption to the Ge MIRE had taken place (1 h), R and S compartments were rinsed with PBS for 76 min (1 ml/10 min). After acquiring spectra of immobilized TNFα, remaining binding sites of the S compartments and all binding sites of the R compartments were saturated by a concentrated solution of BSA (25 mg/ml PBS) which was pumped through the cuvette for 38 min (15 min at a flow rate of 1 ml/5 min and for additional 23 min at a flow rate of 1 ml/20 min). Then, in the same way, all compartments were rinsed with lowly concentrated BSA solution (100 μg/ml in PBS) for 38 min, and after recording spectra of the S side (S compartments with immobilized TNFα and BSA) and of the R side (R compartments with immobilized BSA), a solution of anti-TNFα antibody (AB) and BSA (both 100 μg/ml in

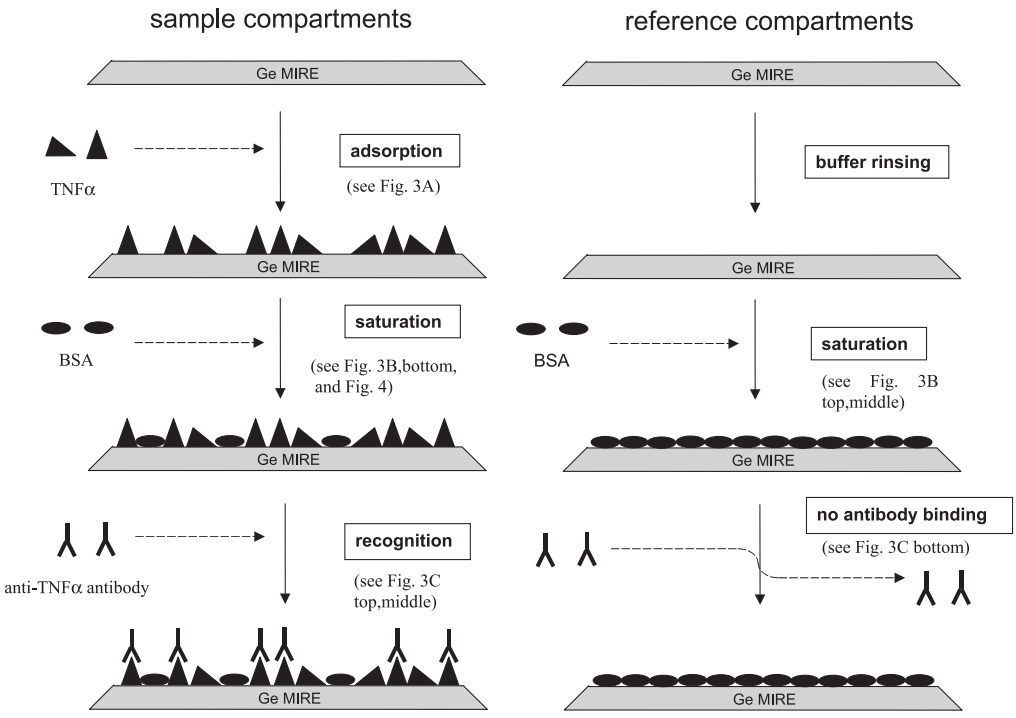

Fig. 1. Schematic description of the pathways of the ATR experiment for the sample and reference compartments. Each step was monitored by IR spectra acquisition. Steps of buffer rinsing after protein adsorption and antibody binding are not shown. Sample: after adsorption of TNFα to the Ge MIRE, remaining free binding sites were saturated with BSA. Finally, antibody for recognition of TNFα was added. Reference: all binding sites of the Ge MIRE were saturated with BSA. Finally, antibody was added to ensure that no recognition of BSA took place.

PBS) was pumped into all compartments for 38 min (in the same way as previously described for the saturation with BSA and the subsequent rinsing process). After washing with BSA solution (100 μg/ml in PBS) for 38 min at a flow rate of 1 ml/5 min and acquiring spectra of AB bound to immobilized TNFα in the S compartments and of immobilized BSA in the R compartments, where no bound AB was detected, the loss of protein in all compartments was checked by extensive washing with BSA solution (100 μg/ml in PBS) at a flow rate of 1 ml/20 min for 10.5 h.

Each of the steps described above was monitored by recording FTIR ATR spectra. During the rinsing and protein adsorption steps, every few minutes short-term spectra were recorded in order to monitor the propagation of the process. After BSA saturation, 100 μg BSA/ml PBS instead of PBS buffer was used for rinsing in order to prevent loss of BSA adsorbed to the Ge MIRE in the R compartments and adsorbed to the inner surfaces of the viton tubings and of the Delrin® flow-through cuvette. Preceding experiments showed that BSA desorbed slowly unless small quantities of this protein were present in the aqueous environment.

2.4. Determination of protein surface concentrations

For the calculation of protein surface concentrations, the thin film approximation was applied [19]. The concept of determination of surface concentration Γ for FTIR ATR spectrosopy is discussed with many examples in Refs. [18,20,21]. Input parameters used in all calculations such as the angle of incidence, refractive indices, integrated molar absorption coefficient of amide II and the number of active internal reflections are listed in Table 1. The uncertainties correspond to approximately a 95% limit of confidence. Parameters specific for each protein such as molecular weights, numbers of amide groups per molecule, integrated absorbances of amide II bands and ranges of

Table 1
Magnitudes and uncertainties of input parameters equal for TNFα, BSA and AB

Parameter	Symbol	Magnitude	Uncertainty[a]
Angle of incidence/deg	Θ	45.00	1.50
Refractive index of germanium MIRE	n_1	4.00	0.00
Refractive index of an adsorbed/bound protein layer	n_2	1.45	0.05
Refractive index of the aqueous environment at 1547 cm^{-1}	n_3	1.33	0.05
Integrated molar absorption coefficient of amide II/(cm/mol). Linear baseline. Range of integration: $1585 \pm 1 - 1500 \pm 1$ cm^{-1}	$\int \varepsilon d\tilde{\nu}$	8.25×10^6	2.90×10^5
Number of active internal reflections	N	26.8	1

[a] The limit of confidence is approximately 95%.

Table 2
Protein-specific input parameters, surface concentrations, dichroic ratios and wavenumbers of selected absorption peaks

	TNFα	BSA	TNFα–antibody
Parameter			
Molecular weight (kDa)	52.05	66.40	146.0
Number of amide groups per molecule	468	582	1316
Integrated absorbance $a_{\parallel}$ of amide II band of parallel polarized spectra (cm^{-1}). Linear baseline.	1.536 ± 0.077	1.439 ± 0.072	0.792 ± 0.040
Integrated absorbance $a_{\perp}$ of amide II band of perpendicular polarized spectra (cm^{-1}). Linear baseline.	0.843 ± 0.042	0.808 ± 0.040	0.468 ± 0.023
Range of integration (cm^{-1})	1482.0–1592.5	1485.1–1600.2	1482.0–1592.5
Results			
Surface conc. Γ calculated from $a_{\parallel}$ and $a_{\perp}$ of amide II band (10^{-12} mol/cm^2)	7.51 ± 0.66	5.70 ± 0.49	1.41 ± 0.12
Experimental dichroic ratio $R_{exp}=a_{\parallel}/a_{\perp}$ of amide II (for comparison: $R_{iso,thin\ film}=1.67\pm0.12$)	1.82 ± 0.13	1.78 ± 0.13	1.69 ± 0.12
Concentration in solution (mg/ml)	0.067	25	0.100
Wavenumbers of selected peaks in the amide I region of parallel polarized spectra (cm^{-1})	1636 (max), 1690 (shoulder)	1655 (max)	1638 (max), 1690 (shoulder)
Wavenumbers of selected peaks in the amide II region of parallel polarized spectra (cm^{-1})	1521 (shoulder), 1546 (max), 1562 (shoulder)	1548 (max)	1517 (shoulder), 1550 (max)
$\tilde{\nu}_{max}$ (amide III) of parallel polarized spectra (cm^{-1})	1259 (sharp)	1246 (broad)	1235 (broad)

integration are listed in Table 2. For the determination of results in Table 2, the amide II band was evaluated because of the interference of the water bending vibration (~ 1645 cm^{-1}) with the amide I band (~ 1650 cm^{-1}). The uncertainties of the results were evaluated by a straightforward error propagation calculation [22]. Analytical expressions for partial derivatives were evaluated by means of the Symbolic Mathematical Toolbox of MATLAB [23], whereas the final numeric calculation of overall uncertainties was performed by means of the standard MATLAB software.

3. Results and discussion

3.1. TNFα adsorption and coverage of S compartments

3.1.1. Adsorption of TNFα and stability of the immobilisate

As described in detail in Section 2.3.2, TNFα was directly adsorbed to the Ge MIRE of the S compartments from aqueous solution. During this process, the increase of the amide II band caused by TNFα adsorption was evaluated. Within 9 min, 92% of the protein were immobilized. From this point of time, maximum coverage was reached after 39 min, and the last 12 min of monitoring (all in all 1 h) exhibited no further increase of protein which indicated a final coverage not exceeding a protein monolayer. After rinsing with PBS to remove loosely adsorbed TNFα (about 8%) and to check the stability of the immobilisate which was reached after about 45 min (see Fig. 2), the spectra of adsorbed TNFα shown in Fig. 3A were recorded. The maximum of the amide I band at 1636 cm^{-1}, the weak shoulder at 1690 cm^{-1} and the shoulders of the amide II band at 1521 and at 1562 cm^{-1} are typical of the existence of antiparallel β-sheet structures [24–27] indicating that this typical feature of TNFα(no α-helices, 45% antiparallel β-sheets) (see pdb-file 1TNF of Ref. [6]) was predominantly conserved after immobilization. It should be noted that TNFα has a very characteristic amide III component at 1259 cm^{-1}, which enables the unambiguous discrimination from the anti-TNFα AB (Fig. 3C, top, middle).

3.1.2. Quantitative evaluation of the TNFα immobilisate

Quantitative evaluation of the amide II band led to a surface concentration of $\Gamma_{TNF\alpha}=(7.51\pm0.66)\cdot10^{-12}$ mol cm^{-2}. X-ray data [6] showed that the outer shape of a TNFα molecule can be approximated by a cone with a basal plane of approximately 2400 $Å^2$ ($d \approx 55Å$) and a height of about 55Å. For the estimation of the upper and lower limits for the average area per molecule after adsorption on the Ge MIRE plate, two boundary states were considered: (1) The molecules adsorb with the basal plane and occupy an area of ~ 2400 $Å^2$ per molecule corresponding to a surface concentration of $\Gamma_{min} \approx 6.9\times10^{-12}$ mol cm^{-2}. (2) The molecules lie on the MIRE plate like thrown cones and occupy an area of a circle sector which results in ~ 1800 $Å^2$ per molecule corresponding to a surface concentration of $\Gamma_{max} \approx 9.2\times10^{-12}$ mol cm^{-2}.

The average value of the experimentally determined surface concentration $\Gamma_{TNF\alpha}=7.51\times10^{-12}$ mol cm^{-2} is located between Γ_{min} and Γ_{max}. As described in Section 3.1.1, the protein coverage should not exceed a monolayer. Assuming 100% coverage corresponding to a tightly packed monofilm of TNFα molecules, this would be in accordance with ~ 73% of the adsorbed protein molecules with the theoretical molecular area of boundary state (1) and ~ 27% with the molecular area of boundary state (2).

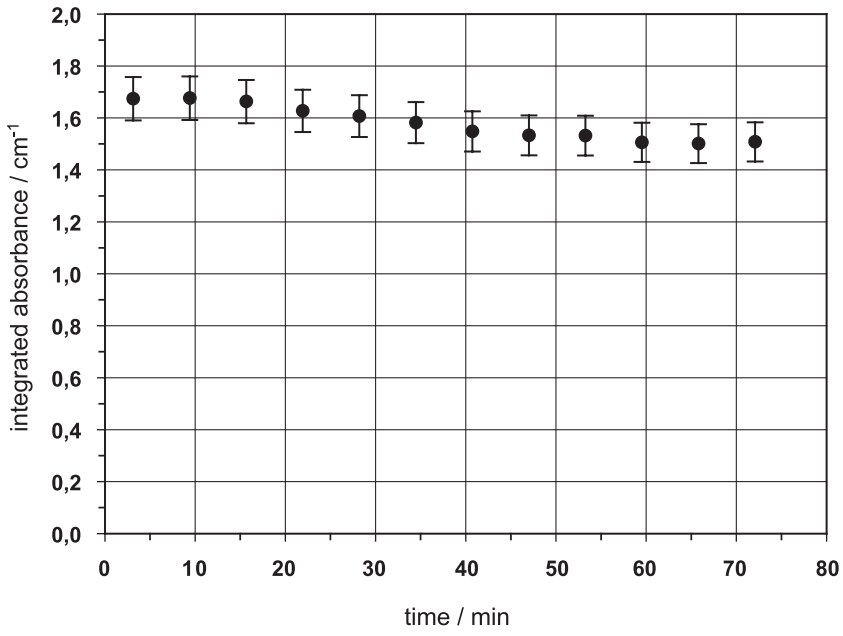

Fig. 2. Time course of ∥ polarized integrated absorbance of the amide II band of adsorbed TNFα in the S compartments during rinsing with PBS (1 ml/10 min). At first, loosely bound TNFα was washed away from the Ge MIRE (about 8%) but after about 45 min, the adsorbate kept stable within the limits of uncertainty.

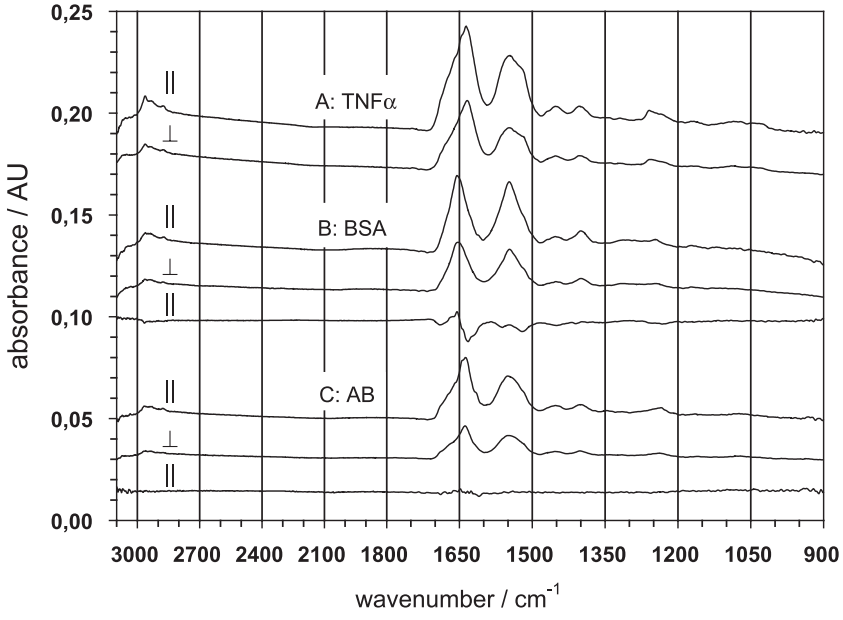

Fig. 3. Polarized IR ATR absorbance spectra of immobilized TNFα, immobilized BSA and anti-TNFα AB bound to TNFα. (A) TNFα adsorbed to Ge MIRE of the S compartments, with the Ge MIRE in contact with PBS as reference; surface concentration $\Gamma_{TNF\alpha}=(7.51\pm0.66)\times10^{-12}$ mol cm^{-2}. (B) Top, Middle: BSA adsorbed to Ge MIRE of the R compartments in contact with 100 μg BSA/ml PBS, with the Ge MIRE in contact with PBS as reference; surface concentration $\Gamma_{BSA}=(5.70\pm0.49)\times10^{-12}$ mol cm^{-2}. Bottom: Difference spectrum between immobilized TNFα rinsed with 100 μg BSA/ml PBS after treatment with concentrated BSA solution and immobilized TNFα in contact with PBS before BSA addition. During the BSA saturation process, a small amount of adsorbed TNFα was displaced by BSA molecules as revealed by negative bands of TNFα and positive bands of BSA, see Fig. 4. (C) Top, Middle: Anti-TNFα AB bound to an adsorbed TNFα monolayer on the Ge MIRE of the S compartments obtained by subtracting the spectrum of TNFα adsorbed to Ge MIRE after saturation with BSA corresponding to the sum of the spectra in A (top) and B (bottom) from the protein sum spectrum of TNFα after saturation with BSA and bound AB in Fig. 5A; surface concentration $\Gamma_{AB}=(1.41\pm0.12)\times10^{-12}$ mol cm^{-2}. Bottom: Difference spectrum between immobilized BSA of the R compartments after rinsing with AB solution and immobilized BSA in contact with 100 μg BSA/ml PBS before rinsing with AB solution corresponding to the spectrum in B (top). No detectable binding of the anti-TNFα antibody to BSA adsorbed to Ge MIRE was found.

3.2. Saturation with BSA and quantitative evaluation of the BSA immobilisate adsorbed to the Ge MIRE in the R compartments

As shown in preceding experiments, the saturation of remaining binding sites of the S compartments is necessary because of adsorption of the anti-TNFα AB on a clean Ge MIRE surface and probably also on the inner surfaces of the viton tubings and of the Delrin® flow-through cuvette. As described in detail in Section 2.3.2, BSA was directly adsorbed to the Ge MIRE of the R (clean Ge surface) and S (Ge surface with immobilized TNFα) compartments from aqueous solution.

In the R compartments, the increase of the amide II band caused by BSA adsorption was evaluated. After 9 min, 93% of the protein were immobilized. From this point of time, maximum coverage was reached after 13 min, and the last 16 min of monitoring (all in all 38 min) exhibited no further increase of protein which indicated—as in the case of the previously described TNFα adsorption—a final monolayer coverage. The absorbance spectra shown in Fig. 3B (top, middle) were recorded after saturation with BSA. Quantitative evaluation of the amide II band resulted in a BSA surface concentration of $\Gamma_{BSA}=(5.70 \pm 0.49) \times 10^{-12}$ mol cm^{-2}.

Hydrodynamic measurements showed that the outer shape of a BSA molecule can be approximated as oblate ellipsoid (40 × 40 × 140 Å) [28,29]. For the estimation of the upper and lower boundaries for the average area per molecule after adsorption on the Ge MIRE plate, the ellipsoid is approximated as a cuboid with a base area of 40 × 40Å = 1600 $Å^2$ and a height of 140 Å. As in the case of TNFα (see Section 3.1.2) two boundary states were considered: (1) The molecules adsorb with the lateral surface which results in 40 × 140Å = 5600 $Å^2$ per molecule corresponding to a surface concentration of $\Gamma_{min} \approx 3.0 \times 10^{-12}$ mol cm^{-2}. (2) The molecules adsorb with the base plane and occupy an area of about 1600 $Å^2$ per molecule corresponding to a surface concentration of $\Gamma_{max} \approx 10.4 \times 10^{-12}$ mol cm^{-2}.

The average value of the experimentally determined surface concentration $\Gamma_{BSA} = 5.70 \times 10^{-12}$ mol cm^{-2} is located between Γ_{min} and Γ_{max}. As previously described, the protein coverage should not exceed a monolayer. Indeed, a quite tightly packed monofilm was produced in this case. It should be noted that in preceding experiments about the same final BSA surface concentration could be reached even with lower concentrated BSA solutions. Furthermore, as described in Section 2.3.2, there was no significant adsorption of AB to the Ge MIRE in the R compartments after BSA saturation, indicating that all free adsorption sites had been occupied by BSA molecules. Assuming 100% coverage corresponding to a tightly packed BSA monofilm, this would be in accordance with ~ 64% of the adsorbed protein molecules with the theoretical molecular area of boundary state (1) and ~ 36% with the molecular area of boundary state (2).

The main secondary structure of BSA is the α-helix (~ 60%); the remainder of the backbone is random-coil with no evidence for β-pleated sheet conformation [30]. The maximum of the amide I band at 1655 cm^{-1} and of the amide II band at 1548 cm^{-1}, respectively, of adsorbed BSA in the R compartments (Fig. 3B, top, middle) are in the ranges of absorption expected for α-helical structure [24,31].

However, in the equally treated S compartments, where immobilized TNFα has been rinsed with 100 μg BSA/ml PBS after treatment with concentrated BSA solution, the spectra in Fig. 3B (bottom) and Fig. 4 show a small replacement of already adsorbed TNFα molecules by BSA molecules as documented by the increase of α-helical structures [positive peaks at 1655 cm^{-1} (amide I) and 1548 cm^{-1} (amide II)] and the decrease of β-sheet structures [negative peaks at 1690 and 1633 cm^{-1} (amide I) and at 1562 and 1521 cm^{-1} (amide II)].

3.3. Binding of anti-TNFα antibody to TNFα

After recording spectra of the S compartments with immobilized TNFα and BSA and of the R compartments with immobilized BSA, a solution of anti-TNFα antibody (AB) and BSA (both 100 μg/ml in PBS) was pumped into all compartments as described in detail in Section 2.3.2. The spectra in Fig. 3C (top, middle) of the S compartments were acquired immediately after washing with BSA solution (100 μg/ml in PBS) for 38 min and show that AB has bound to immobilized TNFα with a surface concentration of $\Gamma_{AB}=(1.41 \pm 0.12) \times 10^{-12}$ mol cm^{-2}. The corresponding spectrum of the R compartments in Fig. 3C (bottom) shows that no detectable amount of AB was adsorbed to the Ge MIRE with immobilized BSA, indicating that the tightly bound BSA saturated all MIRE adsorption sites (see also Section 3.2). It should be noted that preceding experiments resulted in that without BSA saturation AB molecules unspecifically adsorb to a clean Ge MIRE surface.

Thus, BSA could be used in this case for saturating remaining MIRE binding sites of the S compartments, leading to exclusively specific binding of the AB due to recognition of its antigen. It should be noted that the amide I and amide II bands of TNFα and AB are quite similar but the amide III bands of these two proteins are different (see Table 2 and Fig. 3A and C, top, middle). The spectra in Fig. 5A show a combination of these different amide III features, indicating again the formation of TNFα/AB complex in the S compartments. Comparison of surface concentrations resulted in a 5 to 1 molecular ratio between TNFα and AB. A summary of concentrations of all used proteins is given in Table 2. Finally, the stability of the adsorbed proteins was checked by washing with lowly concentrated BSA solution (100 μg/ml in PBS) for 10.5 h. The overall absorbance spectra of the complex of TNFα and its AB in the S compartments before rinsing are shown

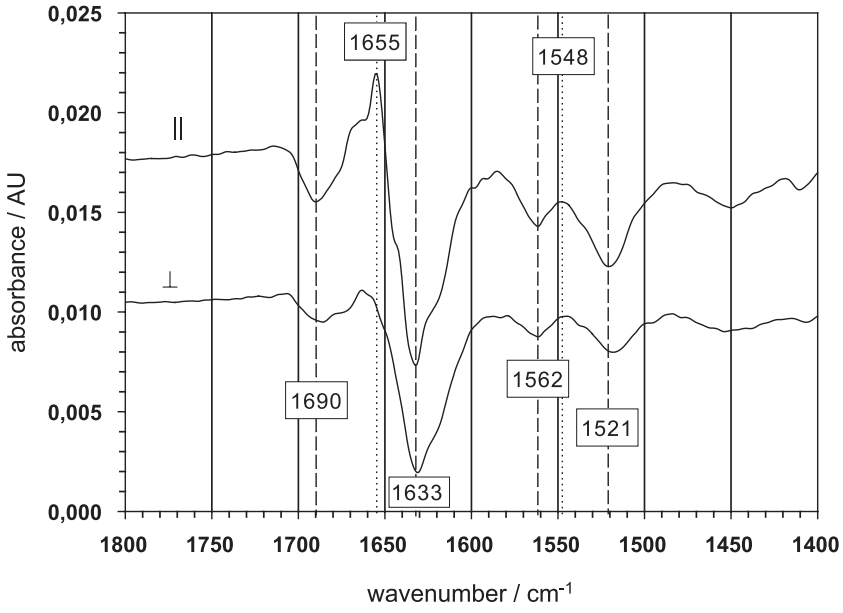

Fig. 4. Magnified presentation of amide regions of Fig. 3B, bottom, with both polarizations. For details, see Fig. 3B (bottom). The spectra demonstrate the slight displacement of adsorbed TNFα (negative bands at 1690, 1633, 1562 and 1521 cm^{-1}) by BSA (positive bands at 1655 and 1548 cm^{-1}) after rinsing with 100 μg BSA/ml PBS.

in Fig. 5A, and the decrease of adsorbed total protein of about 7.4% after rinsing is shown in Fig. 5B. On the other hand, the adsorbed BSA in the R compartments treated with the same washing procedure remained quite stable (Fig. 5C).

3.4. Spatial alignment of protein molecules in the adsorbates

Orientation measurements require independent spectra of the sample by means of parallel (∥) and perpendicular

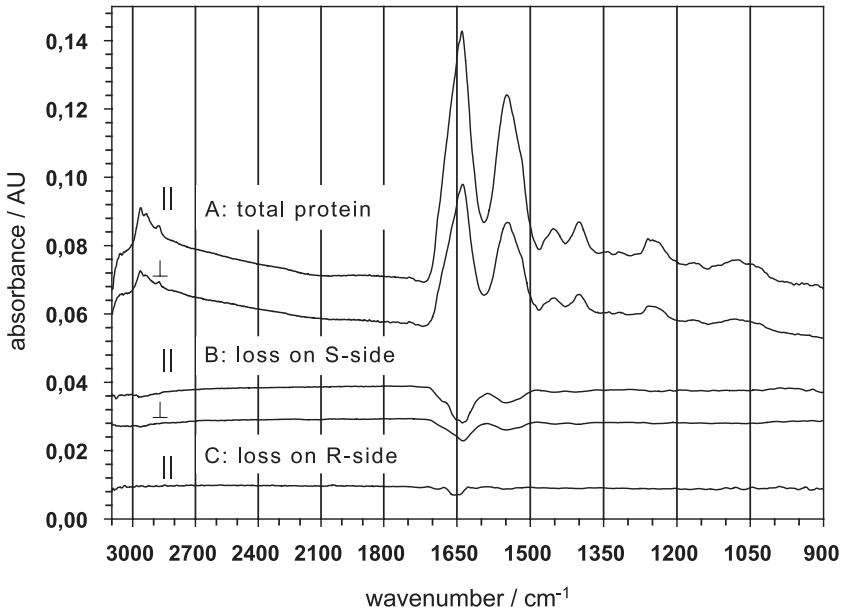

Fig. 5. Polarized IR ATR absorbance spectra of total amount of protein adsorbed to Ge MIRE of the S compartments and spectra of loss of protein of R and S compartments after rinsing with 100 μg BSA/ml PBS. (A) Spectra of the total amount of protein adsorbed to Ge MIRE of the S compartments consisting mostly of TNFα and anti-TNFα AB and of a small amount of BSA in contact with 100 μg BSA/ml PBS, with the Ge MIRE in contact with PBS as reference; dichroic ratio $R = 1.72 \pm 0.12$. (B) Loss of adsorbed protein of the S compartments after rinsing with 100 μg BSA/ml PBS for 10.5 h (1 ml/20 min). The total amount of adsorbed protein of the S compartments at the beginning of the washing process in contact with 100 μg BSA/ml PBS (see A) was taken as reference. Due to the small loss of 7.4%, it is not possible to discriminate between the fractions of loss of individual proteins. (C) Loss of adsorbed BSA of the R compartments after rinsing with 100 μg BSA/ml PBS for 10.5 h (1 ml/20 min). The adsorbed BSA in contact with 100 μg BSA/ml PBS of the R compartments at the beginning of the washing process (see Fig. 3B, top, middle) was taken as reference. There is practically no loss of BSA due to the fact that the washing solution contained 100 μg/ml of BSA.

(⊥) polarized incident light. In order to get rid of physical and molecular constants, such as the magnitude of the transition moment, the so-called dichroic ratio $R = a_{\parallel}/a_{\perp}$ is generally used as basic quantity. $a_{\parallel}$ and $a_{\perp}$ denote the peak absorbances or integrated absorbances of measured spectra with parallel and perpendicular polarized incident light.

In the experiments described here, the integrated absorbances of the amide II bands were used to calculate the dichroic ratio for each protein as presented in Table 2. For an isotropic arrangement of molecules in a thin film on a Ge MIRE with the optical parameters $\Theta = 45°$, $n_1 = 4.0$, $n_2 = 1.45$ and $n_3 = 1.33$ (see also Table 1), a calculated dichroic ratio of $R_{\mathrm{iso,thin\ film}} = 1.67 \pm 0.12$ is obtained [18,21]. If the experimental dichroic ratio R is lower, the mean angle between the average direction of the transition dipole moments of the molecules and the normal to the Ge MIRE lies between the magic angle $\alpha_M \approx 54.7°$ and 90° ($\alpha_M < \alpha_{exp} < 90°$), if it is higher, the mean angle lies between 0° and the magic angle ($0° < \alpha_{exp} < \alpha_M$).

TNFα exhibited a dichroic ratio of $R_{exp} = 1.82 \pm 0.13$ and BSA resulted in $R_{exp} = 1.78 \pm 0.13$. Both give evidence for a certain alignment of amide groups along the normal to the Ge MIRE. In both cases, such an alignment should be expected from the different physico-chemical behaviour of the adsorbed part of the protein molecule in contact with the Ge MIRE surface and of the other part of the molecule being oriented towards buffer solution.

The dichroic ratio of anti-TNFα AB ($R_{exp} = 1.69 \pm 0.12$) gives no evidence for a certain alignment, because the dichroic ratio is within the limits of uncertainty for an isotropic arrangement. However, a spatial anisotropy of the AB molecules adsorbed to TNFα cannot be excluded unambiguously, because if ordered secondary structural elements are isotropically distributed within a single molecule, the overall dichroic ratio always equals R_{iso}. This case should especially hold for large molecules, and the size of the AB is much larger than the size of the other two proteins (see molecular weights in Table 2).

4. Conclusions

In this paper we described a possible approach to study specific interaction between an immobilized receptor and a dissolved substrate quantitatively on a molecular level by means of SBSR ATR FTIR spectroscopy. TNFα acted as receptor and an anti-TNFα antibody as substrate. The most important criteria for such investigations are on the one hand that the receptor is not denatured upon immobilization, and on the other hand that unspecific binding of the substrate can be excluded unambiguously.

Unspecific adsorption of anti-TNFα antibody could be avoided by a procedure well known from ELISA, i.e. treatment of the solid support by a serum albumin solution. BSA turned out to adsorb tightly to a clean germanium surface, preventing any detectable adsorption of anti-TNFα antibody. Posttreatment of the immobilized TNFα layer by BSA was therefore applied to avoid adsorption of anti-TNFα antibody into possible gaps of the TNFα monolayer. The proof that immobilized TNFα was still native followed from the fact that no anti-TNFα antibody adsorbed in the reference channel, consisting of a BSA coated germanium surface, while in the sample channel the antibody was captured by the molar ratio of one antibody per five TNFα molecules.

Quantitative analysis of the FTIR ATR spectra gave strong evidence that TNFα adsorbed in a nearly compact monolayer to the germanium surface of the MIRE, where about three quarters of the cone-like shaped molecules are expected to be attached by the basal planes and the rest of them are lying attached by the outer coverages as schematically shown by Fig. 1. After interaction with the antibody, about 40% of the TNFα monolayer is expected to be coated by an antibody monolayer as may be estimated from data summarized in Table 2. The molar ratio TNFα/anti-TNFα antibody resulted in 5.33 with a corresponding mass ratio of 0.357. From the latter, a rough estimation of the area ratio between TNFα and its antibody based on spherical molecular shapes resulted in $(0.357)^{2/3} \approx 0.50$, meaning that one antibody molecule covers about two TNFα molecules, thus resulting in a binding efficiency of ~ 38%.

This layer assembly turned out to be very stable even under flow-through conditions. However, it should be noted that the presence of 100 μg BSA/ml PBS was essential for the stabilization of the BSA protection layer. This low concentrated BSA solution did not exchange molecules from the TNFα assembly as to be concluded from the stable shapes of the amide I, II and III bands.

The application of SBSR ATR technique turned out to be most valuable, especially for long-term experiments. The main advantages are significantly improved background compensation and permanent availability of a reference with the same age as the sample. On the other hand, independent evaluation of the single channel spectra measured in the sample and reference path, respectively, is also possible.

The TNFα/antibody assembly will now be used to study the interaction with fragments of anti-TNFα antibodies as well as with synthetic peptides mimicking an anti-TNFα antibody.

The more general meaning of these experiments is that a germanium MIRE surface can be protected against unwanted protein adsorption by a physisorbed BSA or HSA monolayer. The latter was found in a more recent experiment. Moreover, tight adsorption of a receptor to the germanium surface is the simplest way of immobilization. Whether biological activity is maintained has to be proven in any case. Obviously with TNFα there was no spacer between the solid support and the protein required in order to maintain biological activity due to the enhancement of the orientational flexibility of the molecule.

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