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Article

Robust Approach for Quantifying Glucocorticoid Binding to the Anti-Cortisol Fab Fragment via Native Mass Spectrometry

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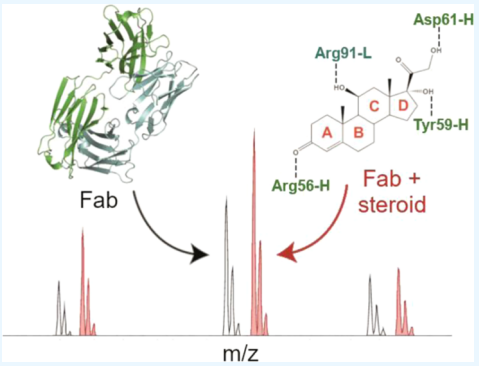
ABSTRACT: In the development of proteins, aptamers, and molecular imprints for diagnostic purposes, a major goal is to obtain a molecule with both a high binding affinity and specificity for the target ligand. Cushing syndrome or Addison's disease can be diagnosed by cortisol level tests. We have previously characterized and solved the crystal structure of an anti-cortisol (17) Fab fragment having a high affinity to cortisol but also significant cross-reactivity to other glucocorticoids, especially the glucocorticoid drug prednisolone. We used native mass spectrometry (MS) to determine the binding affinities of nine steroid hormones to anti-cortisol (17) Fab, including steroidogenic precursors of cortisol. Based on the results, the number of hydroxyl groups in the structure of a steroid ligand plays a key role in the antigen recognition by the Fab fragment as the ligands with three hydroxyl groups, cortisol and prednisolone, had the highest affinities. The antibody affinity toward steroid hormones often decreases with a decrease in the number of hydroxyl groups in the structure. The presence of the hydroxyl group at position C11 increased the affinity more than did the other hydroxyl groups at positions C17 or C21. The binding affinities obtained by native MS were compared to the values determined by surface plasmon resonance (SPR), and the affinities were found to correlate well between these two techniques. Our study demonstrates that native MS with a large dynamic range and high sensitivity is a versatile tool for ligand binding studies of proteins.



INTRODUCTION

Detection of small molecules, such as drugs, toxins, and steroid hormones, in tissues and body fluids is important for many clinical diagnostic and therapeutic applications, such as the discovery and development of drugs and monitoring of human health. Based on molecular recognition, numerous proteins, aptamers, and molecular imprints have been developed for various analytical methods, such as assays or sensors, and have provided powerful tools for diagnostics.[1,2] In the development of usable biomolecular recognition elements, the goal is usually to obtain a molecule with both a high affinity and specificity to the target ligand. Steroid hormones characterize a wide range of physiological and pathological states.[3] For instance, cortisol is a biomarker for numerous diseases, such as Cushing's disease, chronic fatigue, and Addison's disease.[4] However, due to the shared cortisol structure originating from steroidogenesis (Figure 1), high specificity is hard to obtain with biomolecular recognition elements such as antisteroid antibodies.[5−8] Recently, we have reported the crystal structure of an anti-cortisol (17) Fab with and without cortisol as well as in complex with three other glucocorticoids, prednisolone, cortisone, and corticosterone.[9] Of these ligands, the anti-cortisol (17) Fab showed the highest affinity toward cortisol, measured by surface plasmon resonance (SPR). For diagnostic usage, a major disadvantage of anti-cortisol (17) Fab is its high cross-reactivity to prednisolone, which is a commonly used glucocorticoid drug. To better understand the affinity and specificity of glucocorticoid binding, it is important that binding affinities can be accurately measured, preferably with multiple methods independent of each other.

To study protein−ligand interactions and especially to determine their binding affinities, the most common label-free method is SPR, which is based on the interaction of light with a thin metallic surface at the interface of the medium with different refractive indices. In SPR, one molecule (typically a ligand) is covalently immobilized onto the surface of a sensor chip, and the interaction between the ligand and another molecule is then measured. The method enables determination of the kinetics of the ligand binding process, yielding association (k_{on}) and dissociation (k_{off}) rate constants at any given protein concentration. From these rate constants, the overall binding affinity (i.e., the association (K_A) or dissociation (K_D) constant for the binding) can be obtained.

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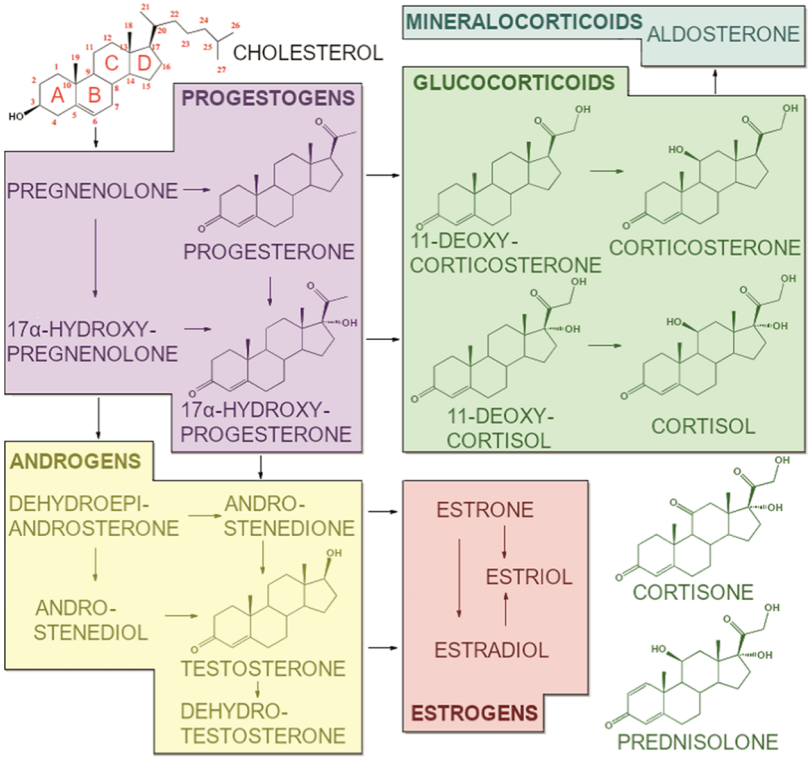

Figure 1. Steroidogenesis with structural formulas of the studied steroid hormones: different steroid classes are represented by different colors; glucocorticoids are shown in green, while progestogens, androgens, estrogens, and mineralocorticoids are shown in purple, yellow, red, and cyan, respectively. Cortisone is a naturally occurring glucocorticoid metabolite, whereas prednisolone is a synthetic analogue.

SPR offers advantages, such as the capability for real-time monitoring, small sample size, reusability of sensor surfaces, and superior sensitivity.[10] However, the immobilization of one component can adversely affect protein−ligand interaction studies.[11]

Another commonly used method for ligand binding studies is isothermal titration calorimetry (ITC), which is based on the quantification of a binding-associated enthalpy change.[12] Its major advantages are its high precision, sensitivity, and capability for simultaneous determination of all thermodynamic parameters, enthalpy, entropy, and binding stoichiometry. ITC measurements are also performed directly in solution and do not require the immobilization of any of the binding components. However, the major limitations of ITC are its high sample consumption/low sensitivity, long analysis times, and the inability to screen multiple ligands at the same time.[11]

Alternatively, protein−ligand interactions can be studied with native mass spectrometry (MS).[13−16] In native MS, the sample molecules are ionized from aqueous buffer solutions, and their mass-to-charge ratios (m/z) are measured. In native MS, proteins remain in their folded, native-like state when "soft" ionization methods, such as electrospray ionization (ESI), are used.[17] The conditions in the ion source are usually adjusted so that undesired protein complex dissociation is prevented upon ion transfer to the gas phase. Since native MS provides separate signals for the free and ligand-complexed forms of the protein, the binding affinity (K_A or K_D) can be readily determined from the respective peak intensities.[11,18] Additionally, detection of separate signals enables simultaneous screening of multiple ligands at once, and it also provides direct information on the binding stoichiometry.[15] Furthermore, native MS does not require the immobilization of any of the binding components, and measurements can be carried out with very small amounts of purified protein materials. To further minimize protein consumption, the utilization of nanoelectrospray ionization (nESI) instead of the conventional ESI effectively reduces the required sample volumes to a few microliters only.[19]

In this work, we used native MS to quantify the binding affinities of a wide array of steroid hormones (i.e., cortisol, prednisolone, cortisone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17α-hydroxyprogesterone, progesterone, and testosterone) to the anti-cortisol (17) Fab fragment. The obtained results were compared to those observed earlier with SPR.[9] To gain further insights into the cross-reactivity of anti-cortisol (17) Fab, the binding affinities were evaluated in light of the previously determined crystal structures.[9] By utilization of nESI as the ionization technique, the protein consumption could be reduced to a nanogram level in a single experiment.

RESULTS AND DISCUSSION

Initial Ligand Screening Experiments. Prior to the ligand binding experiments, the desalted anti-cortisol (17) Fab fragment was analyzed in both native and reducing solution conditions. In 200 mM ammonium acetate (pH 6.8), the mass spectrum of the Fab fragment displayed a narrow charge state distribution (15+ to 13+ at m/z 3200−3800), which indicated that the protein remained in its near-native form in these conditions (Figure 2a).[20] Thus, 200 mM ammonium acetate was used as the solvent for further ligand binding experiments. In contrast, when the Fab fragment was measured in the presence of acetonitrile, acetic acid, and 10 mM dithiothreitol (DTT) used as a reducing agent, separated light (L) and heavy (H) antibody chains were observed and the charge state

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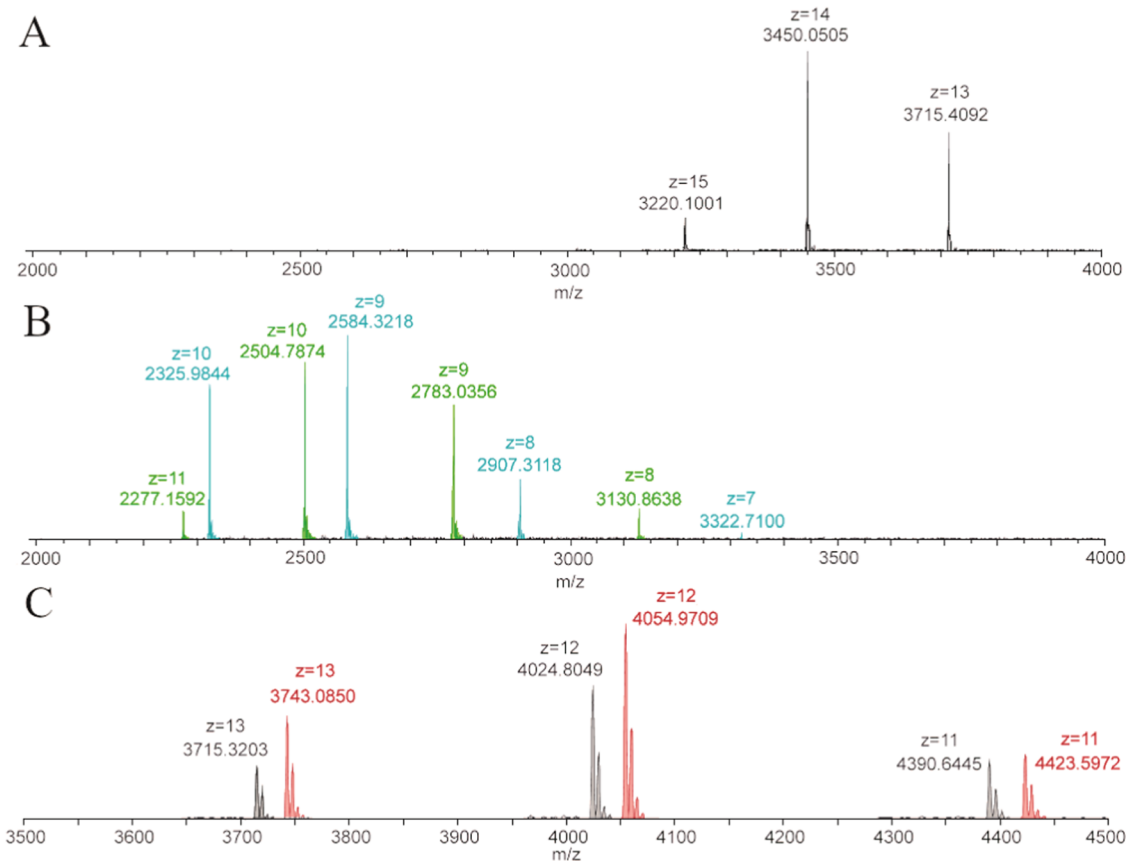

Figure 2. Native mass spectra of the anti-cortisol (17) Fab fragment in (A) 200 mM ammonium acetate (pH 6.8), (B) 200 mM ammonium acetate/acetonitrile/acetic acid (50:40:10, v/v) with 10 mM DTT, and (C) 200 mM ammonium acetate/1.5 vol % DMSO/0.5 vol % methanol with 0.1 μM cortisol. The protein concentrations were 0.1, 2.5, and 0.1 μM, respectively. In (B), the signals representing free H- and L-chains are shown in green and cyan, respectively. In (C), the signals of the free protein are shown in gray, while the signals of the protein−ligand complex are shown in red (charge states 11+, 12+, and 13+).

distributions shifted to lower m/z. In these conditions, the narrow charge state distributions (11+ to 8+ for H-chain and 10+ to 7+ for L- chain at m/z 2200−3400) similar to native MS were seen (Figure 2b). The most abundant isotopic mass determined for the L-chain (23249.876 Da) has an excellent fit with the calculated value (23249.655 Da), and the mass determined for the H-chain (25037.778 Da) also matched perfectly with the calculated value (25037.780 Da), when considering four extra alanine residues and the C-terminal 6xHis-tag in the protein construct due to the expression vector pKKTac was used.[21]

The initial ligand screening experiments were then performed to determine the approximate binding affinities of the ligands toward the anti-cortisol (17) Fab fragment. To maintain ligand solubility, dimethyl sulfoxide (DMSO) and methanol (MeOH) were used as the cosolvents (1.5 and 0.5 vol %, respectively). While the use of these cosolvents did not affect the binding affinity (Figure S1), the charge state distribution clearly shifted to lower charge states (13+ to 11+ at m/z 3800−4500) (Figure 2c) due to the presence of DMSO, which is reported to shift the charge state distribution to lower charge states. This type of "subcharging" at low DMSO concentrations has been previously reported and discussed in detail.[22] It has been argued that this phenomenon is attributable to the gas-phase or solution-phase compaction of protein ions in the presence of DMSO but was not observed to affect the overall stability of any of the studied oligomeric proteins in the previous work. As we did not observe any marked differences in the binding affinities of our Fab fragment, in the absence or presence of a small amount of DMSO/methanol, we conclude that the solution conditions can be considered native-like, at least with the studied protein−ligand system. Based on the results of the previous SPR analyses,[9] the binding affinities were expected to be in the nanomolar range for ligands with the highest affinity. Thus, the protein concentration was kept as low as possible, while having a sufficiently high signal-to-noise ratio to obtain accurate results. Based on the initial screening experiments, the Fab concentration of 100 nM was chosen for further affinity measurements. In the ligand screening experiments, the ligand concentration varied between 100 nM and 10 μM, depending on the ligand. At ligand concentrations of <5 μM, native MS showed only 1:1 binding, implying high binding specificity. In contrast, at higher ligand concentrations, the binding of the second ligand was observed, which likely represents non-specific binding to sites other than the primary binding site. Based on the initial ligand screening experiments, cortisol, prednisolone, corticosterone, and cortisone were recognized as high-affinity ligands with K_D values in the nanomolar range. In addition, 11-deoxycortisol showed submicromolar affinity, while the rest of the ligands showed lower binding affinities, with K_D values in the micromolar range (Figure S2).

Affinity Measurements of Ligands with Anti-Cortisol (17) Fab. Based on the initial ligand screening experiments,

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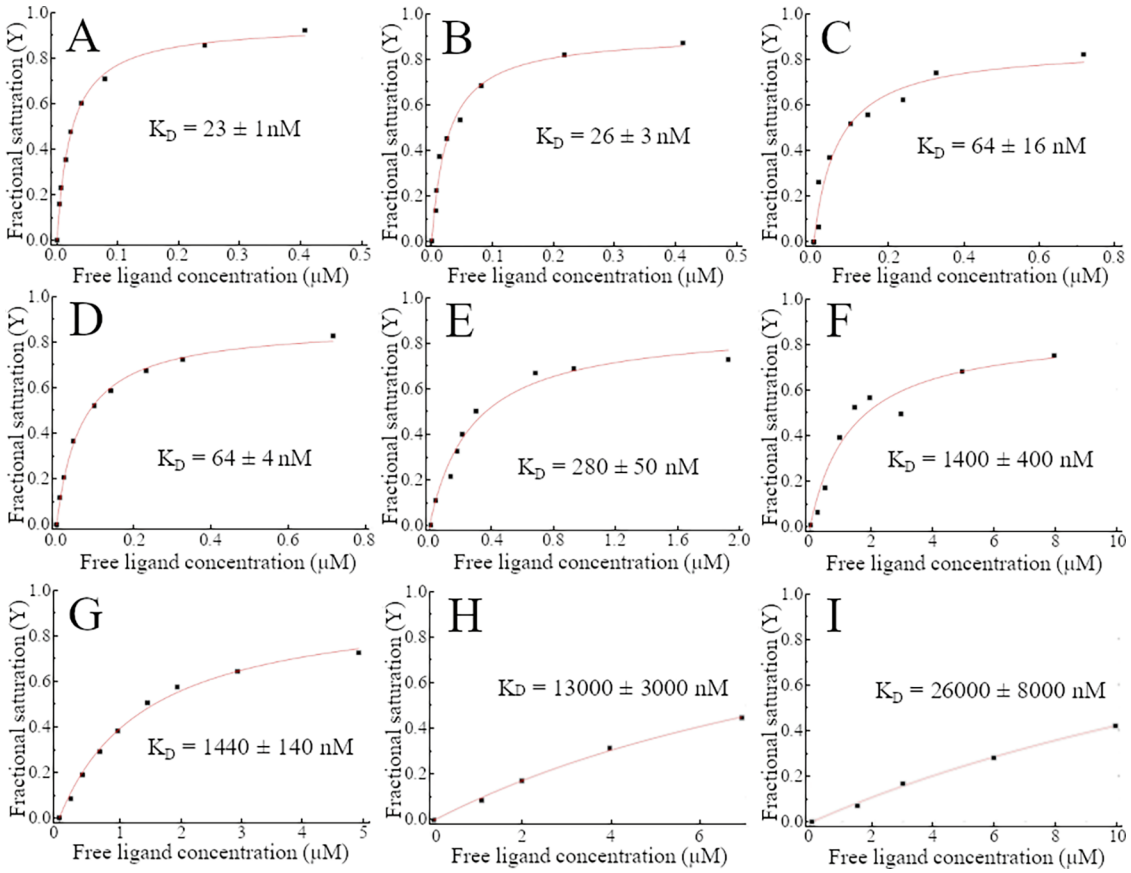

Figure 3. Titration plots (fractional saturation vs free ligand concentration) for the steroid hormone binding to the anti-cortisol (17) Fab fragment: (A) cortisol, (B) prednisolone, (C) corticosterone, (D) cortisone, (E) 11-deoxycortisol, (F) 11-deoxycorticosterone, (G) 17α-hydroxyprogesterone, (H) progesterone, and (I) testosterone. Each data point is an average value from the three replicate samples. The solid red line represents the best fit to the specific one-site binding model.

ligand titration experiments were designed for more accurate affinity determination. For the titration of each ligand, a series of eight different concentrations was designed at a fixed Fab concentration of 100 nM. For the ligands with the highest presumed affinities, cortisol and prednisolone, the concentrations ranged from 20 to 500 nM, whereas for cortisone and corticosterone, they were 20−800 nM. The concentration of 11-deoxycortisol was between 50 nM and 2 μM. For the ligands with micromolar affinities, 17α-hydroxyprogesterone and 11-deoxycorticosterone, the concentration varied between 200 nM and 8 μM. Initially, the ligand concentrations were 1−45 and 1.5−60 μM for progesterone and testosterone, respectively. However, since ligand concentrations greater than 10 μM resulted in halted ligand saturation, probably due to nonspecific binding[23] or limited ligand solubility, only four concentrations (1 to 7 μM with progesterone and 1.5 to 10 μM with testosterone) were used.

From the measured native mass spectra, the equilibrium concentrations of the free and ligand-bound proteins were obtained using the respective peak ratios, which enabled the deduction of the free ligand concentration (Table S1). The dissociation constants (K_D) were then obtained by fitting the fractional saturation (Y) against the free ligand concentration using a specific one-site binding model (Figure 3). For all tested ligands, the determined K_D values are reported in Table 1. As expected, the highest affinities were obtained for cortisol and prednisolone (K_D values of 23 ± 1 and 26 ± 3 nM, respectively). In turn, for ligands with the lowest binding affinities, progesterone and testosterone, the K_D values were 13 ± 3 and 26 ± 8 μM, respectively.

When compared to the previous SPR analysis,[9] the binding affinities measured with native MS were systematically lower for the high-affinity ligands, with the determined K_D values being roughly twice as high as compared to the SPR results (Table 1, Figure 4). This is in agreement with the studies of Jacklin et al., in which lower K_D values have been observed using SPR, compared to the other techniques.[11] In contrast, for the ligands with the lowest affinities, progesterone and testosterone, we observed K_D values similar to those observed by SPR analysis. While accurate determination of K_D values for low-affinity ligands is challenging with either method, both methods work well from low nanomolar to high micromolar binding affinities. This is well in line with our previous work with the thyroid hormone binding to the antibody Fab fragment, whose affinity spanned from a low nanomolar to a high micromolar range (i.e., 5 orders of magnitude).[24]

Structural Analysis. Based on the previously determined crystal structures, the hapten is almost entirely embedded in the paratope of the anti-cortisol (17) Fab fragment.[9] Cortisol has been observed to have extensive hydrophobic interactions together with four hydrogen bonds with the paratope. The carbonyl oxygen O3 of cortisol forms a hydrogen bond with the side chain of Arg56-H, while the oxygen atoms of hydroxyl groups OH11, OH17, and OH21 are hydrogen-bonded to the carbonyl oxygen of Arg91-L, to the carbonyl oxygen of Tyr-59-H, and to the side chain of Asp61-H, respectively (Figure 5).

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Table 1. Dissociation Constants and Major Structural Features for Steroid Binding to the Anti-Cortisol (17) Fab fragment

ligand	K_D (nM)		structural features						H-bond formation			
	native MS[1]	SPR	1–2	3	11b	17a	17b	structure (PDB ID)	Arg56-H	Arg91-L	Tyr59-H	Asp61-H
cortisol	23 ± 1[R1]	7 ± 1[R2]	C–C	=O	OH	OH	Ac(OH)	8cby	O3	OH11b	OH17a	OH21
prednisolone	26 ± 3[R1]	13 ± 1[R2]	C=C	=O	OH	OH	Ac(OH)	8cc1	O3	OH11b	OH17a	OH21
corticosterone	64 ± 16[R1]	31 ± 2[R2]	C–C	=O	OH	H	Ac(OH)	8cbz	O3	OH11b		OH21
cortisone	64 ± 4[R1]	32 ± 2[R2]	C–C	=O	=O	OH	Ac(OH)	8cc0	O3		OH17a	OH21
11-deoxycortisol	280 ± 50[R1]	120[R1]	C–C	=O	H	H	Ac(OH)	modeled	O3			OH21
11-deoxycorticosterone	1400 ± 400[R1]	N.D.	C–C	=O	H	H	Ac(OH)	modeled	O3			OH21
17α-hydroxyprogesterone	1440 ± 140[R1]	80[R1]	C–C	=O	H	OH	Ac(OH)	modeled	O3		OH17a	
progesterone	13000 ± 3000[R1]	15000 ± 3000[R2]	C–C	=O	H	H	Ac(OH)	modeled	O3			
testosterone	26000 ± 8000[R1]	23000 ± 4000[R2]	C–C	=O	H	H	OH	modeled	O3			

[1]Average value obtained from three technical replicates. [R1]Determined in this work. [R2]Determined in our previous work[9] N.D., *not determined*

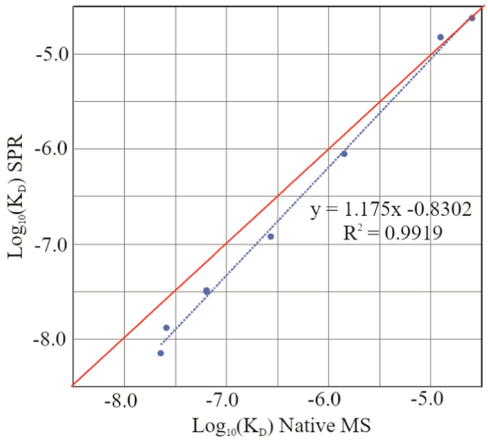

Figure 4. Correlation diagram (double-log plot) of the K_D values determined for the steroid hormone binding of the anti-cortisol (17) Fab fragment by native MS and SPR. The correlation observed between native MS and SPR is shown with a blue dashed line, while the red solid line represents the equal affinity between the two methods.

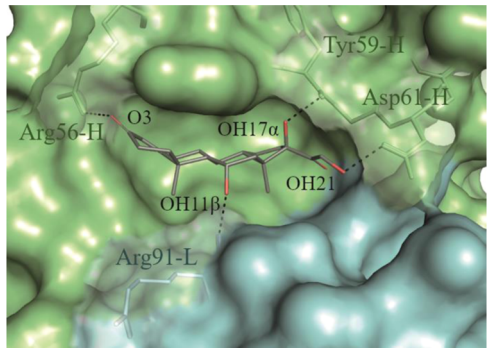

Figure 5. Paratope of the anti-cortisol (17) Fab fragment complexed with cortisol (PDB: 8CBY). H- and L-chains are represented with green and cyan colors, respectively. Cortisol is shown as a gray stick model, and hydrogen bonds are shown with black dashed lines.

The crystal structures of anti-cortisol (17) Fab in complex with cortisol, prednisolone, cortisone, and corticosterone show similar binding modes.[9] Based on the solved crystal structures, other steroids can be modeled into the binding site. The main structural differences between steroids are the number and location of the hydroxyl groups. Prednisolone has a double bond between C1 and C2 in the A-ring, but this has only a minor impact on its three-dimensional structure. In testosterone, a side chain at C17 in the D-ring is substituted with a hydroxyl group with a β-conformation. However, based on the modeled complex structure, there is no sterical reason why testosterone would not be able to bind similarly to cortisol in the binding site of the anti-cortisol (17) Fab.

Due to the ability to form hydrogen bonds with anti-cortisol (17) Fab, three hydroxyl groups attached to cortisol have a vital role in antigen recognition. Hydroxyl groups provide electrostatic and shape complementarity, and they may also significantly affect the solubility and entropic cost of the binding. Based on our affinity measurements, the total number of hydroxyl groups in the steroid structure is indeed significant, since the absence of one hydroxyl group reduces the affinity of the ligand roughly 5- to 10-fold. It is clear that structural complementarity is a major factor in antigen recognition by the

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anti-cortisol (17) Fab fragment. Because the K_D value for 11-deoxycortisol was more than 4-fold higher compared to corticosterone and 11-deoxycorticosterone and 17α-hydroxyprogesterone had almost equal affinities, the hydroxyl group OH11 had the most significant influence on the binding. However, corticosterone and cortisone were found to have equal affinities and both possess an equal number of hydrogen bonds, but cortisone has carbonyl oxygen, oxygen, O11, which is incapable of forming a hydrogen bond to Arg91-L. Instead, cortisone is hydrogen-bonded via OH17 to the Fab fragment. Presumably, the additional augmentation of the affinity is due to the hydrophobic effect caused by the orientation of the ligand in the binding site, where a hydrophobic steroid core structure is buried in the hydrophobic binding pocket, while the polar hydroxyl group OH11 is pointing away from the pocket.

CONCLUSIONS

The binding affinities of nine steroids to the anti-cortisol (17) antibody Fab fragment were successfully determined using native mass spectrometry, and the results were compared with the ones measured with surface plasmon resonance. For most ligands, native MS resulted in slightly lower binding affinities compared to SPR, which is in agreement with earlier studies.[11] For the ligands with low binding affinities ($K_D \sim 10^{-5}$ M), the obtained affinities were very similar compared to SPR. From a structural point of view, three hydroxyl groups in the structure of cortisol play a key role in antigen recognition by anti-cortisol (17) antibodies, as cortisol and prednisolone with three hydroxyl groups had the highest affinities. The affinity of a steroid to anti-cortisol (17) Fab reduces as the number of hydroxyl groups decreases. The hydroxyl group at position C11 had the most significant contribution to affinity. This increase in binding affinity results from the hydrophobic effect rather than solely from the chemical complement.

Increased concentration of cortisol can be used as a biomarker for stress and stress-related disorders, and there is a clear need for the development of a cortisol immunoassay method. Abnormal levels of cortisol can also indicate Cushing syndrome or Addison's disease. For better understanding of the cross-reactivity, the binding specificity and affinities of cortisol and related glucocorticoid ligands to the anti-cortisol Fab fragment were systematically studied. In general, our study shows that native MS serves as an excellent method for accurately determining dissociation constants for protein–ligand complexes within a large dynamic range. Furthermore, nanoelectrospray ionization allows measurements from minute amounts of protein materials. In addition to the affinity determination of closely related ligands, native MS could also serve as a promising tool for the characterization of specificity to a wide range of small molecules with different receptors and carrier proteins.

MATERIALS AND METHODS

Materials. Anti-cortisol (17) Fab is a murine antibody isolated from a VTT steroid-specific antibody library of 1.8×10^8 unique clones. The discovery, isolation, production, and purification of the anti-cortisol (17) Fab fragment are described elsewhere.[9] Prior to the native MS measurements, the protein was desalted by Bio-Rad Micro Bio-Spin 6 columns using 200 mM ammonium acetate as an eluent. For protein concentration determination, both the Bio-Rad DC (detergent compatible) protein assay (with bovine serum albumin as the standard) and UV absorbance measurements at 280 nm were utilized. All the studied ligands, cortisol (hydrocortisone; Sigma–Aldrich, product no. H4001), prednisolone (Sigma–Aldrich, P6004), cortisone (Sigma–Aldrich, C2755), corticosterone (Sigma–Aldrich, C2505), 11-deoxycortisol (Sigma–Aldrich, R0500), 17α-hydroxyprogesterone (Sigma–Aldrich, H5752), 11-deoxycorticosterone (BioNordika, 22916), progesterone (MilliporeSigma, P0130), and testosterone (Sigma–Aldrich, 86500), were carefully weighed and dissolved in MeOH. Additionally, UV absorbance at 242 nm was used as a control for the concentration determination of cortisol, using a specific extinction coefficient, $\varepsilon_{1\%,1\ cm} = 445$.[25]

Mass Spectrometry. All mass spectrometric experiments were performed using a Q Exactive UHMR hybrid quadrupole Orbitrap instrument (Thermo Scientific, Bremen, Germany) equipped with a static nanoelectrospray ion source (Nanospray Flex). Calibration of the instrument was performed using cesium iodide clusters obtained from a 2-propanol/water (1:1 v/v) solution of cesium iodide at 2 mg/mL. Thermo Scientific ES380 borosilicate glass capillaries were used in the experiments. The most important instrument parameters used are listed in Table S2.

For all of the native MS experiments, 200 mM ammonium acetate was used as a solvent. Since the binding affinities were expected to be in the nanomolar range for the high-affinity ligands, the protein concentration was screened to find the lowest possible concentration that still provided a sufficiently high signal-to-noise (S/N) ratio for accurate measurements. The Fab concentration was finally fixed at 0.1 μM for affinity determination. For the reducing solution conditions, 200 mM ammonium acetate/acetonitrile/acetic acid (50:40:10, v/v) with 10 mM DTT was used as a solvent at 2.5 μM protein concentration.

Due to the low water solubility of the tested steroids, all samples were prepared in 200 mM ammonium acetate with 1.5 vol % DMSO and 0.5 vol % MeOH. Prior to the measurements, all samples containing ligands were incubated at 4 °C overnight.

Ligand Affinity Determination. For all ligands, direct ligand titration experiments were performed to determine the dissociation constants (K_D) for the binding. While the concentration of the anti-cortisol (17) Fab fragment was kept constant, the concentrations of the ligands varied depending on the binding affinities. The degree of protein saturation (Y) was calculated from the ratio of the protein–ligand complex and the free protein peak integrals, summed over all observed charge states, and the data were fitted into the specific, single-site binding model[26]

$$Y = \frac{B_{max}[L]}{K_D + [L]}$$

where B_{max} is the number of binding sites (maximum occupancy) and $[L]$ is the free ligand concentration, which is deduced from the calculated equilibrium concentrations. For each ligand concentration, three replicate samples were prepared and measured.

For spectral deconvolution and determination of the degree of protein saturation, UniDec software was used.[27] For designing the ligand binding experiments, protein thermodynamics simulation applets developed by Pääkkönen et al. were further utilized.[28] The final curve fittings were performed using

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OriginPro 2023 software (Originlab Corporation, Northhampton, MA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09027.

Obtained saturation degrees and the calculated free ligand concentrations, instrument parameters, comparison of native MS with and without DMSO and methanol, and native MS spectra of initial ligand screening experiments (PDF)

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Author Contributions

T.N. supervised the antibody development work, and K.I. determined the binding affinities for steroids using SPR. J.P. counseled about the native MS measurements, and V.E. designed and carried out all MS experiments and data analysis under the supervision of J.J., J.R., and N.H. All the authors contributed to the article writing and revision.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

K_A, association constant; k_{on}, association rate constant; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; K_D, dissociation constant; k_{off}, dissociation rate constant; ESI, electrospray ionization; Fab, fragment antigen-binding region; H, heavy chain; ITC, isothermal titration calorimetry; L, light chain; MeOH, methanol; MS, mass spectrometry; nESI, nanoelectrospray ionization; SPR, surface plasmon resonance

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