THERMODYNAMIC AND SPECTROSCOPIC COMPARISON OF THE BINDING SITES OF THE MOUSE MYELOMA PROTEIN 315 AND OF ITS LIGHT CHAIN DIMER

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1. Introduction

The homogeneous IgA secreted by MOPC 315 tumor was shown to bind specifically some nitroaromatic derivatives and other electron acceptor aromatic compounds [1]. A detailed investigation of the combining site of the intact molecule (HL) by kinetic mapping resolved several subsites of binding, each contributing elementary interactions with defined portions of the hapten [2,3]. These subsites are: the nitroaromatic binding subsite (S_1), the first and second hydrophobic subsites (S_2 and S_3), and electrostatic positively charged subsite (S_4). Model building of the variable region of this protein, enabled a three dimensional analysis of the binding site which agreed with the available thermodynamic and kinetic data [4]. Recently it was found that the light chain of protein 315 also binds ϵ-*N*(2,4-dinitrophenyl) lysine (DNPL) specifically, though with significantly lower affinity [5]. The light chain was shown to exist in neutral aqueous solutions as a dimer (L_2) which binds homogeneously two moles of DNPL ($K = 6.3 \times 10^3$ M^{-1} at 4°C). This is the first case where the light chain of a homogeneous immunoglobulin is shown to have a significant and specific binding capacity for the same ligand as its parent molecule, providing an experimental approach to examine the role of the light chain in the architecture and specificity of the antibody combining site. Here we report the study of the fine specificity of the L_2 site and the spectroscopic properties of its hapten complexes as compared with those of the parent HL site.

The interactions between several nitrophenyl derivatives and L_2 were studied spectroscopically and by equilibrium dialysis. Both approaches resolved characteristics of the complexes formed between a given hapten and the L_2 site and allowed a comparison with the respective complex formed with the HL site. The affinity of the L_2 site for nitrophenyl derivatives is lower by two to three orders of magnitude than that of the HL site. It is however noteworthy that the fine specificity of the L_2 site with respect to the position of the nitro substituents on the aromatic nucleus remains identical to that of the HL site. These features suggest that the light chain has a dominant role in the construction of the nitroaromatic subsite (S_1) in the parent protein 315. As deduced from kinetic mapping [2,3] and model building [4] the elementary interactions operative in S_1 are a charge transfer complex between trp-93L and the nitroaromatic nucleus, and hydrogen bonding of the nitro group oxygens to Asn-36L and Asn-36H. Two of these interactions may also prevail in the L_2 site. On the other hand, L_2 seems to lack the three additional subsites (S_2–S_4), which interact with hydrophobic and charged portions of side chain attached to the nitroaromatic nucleus. This is so since L_2 shows essentially the same affinity for nitroaromatic compounds irrespective of the presence or absence of an attached side chain (e.g., DNPL and 2,4 dinitrotoluene).

The absorption spectra of L_2–hapten complexes differ in shape and intensity from those formed with HL. Thus in spite of the preservation of the steric

Abbreviations: PBS, phosphate buffered (0.05 M) saline (0.1 M NaCl) pH 7.4; DNPL, ϵ-*N*(2,4-dinitrophenyl)-L-lysine; L-(H-) chain, immunoglobulin light (heavy) chain; HL, intact immunoglobulin or Fab fragment; L_2, light chain dimer

specificity towards the nitro groups, the spectroscopically probed environment of the hapten in L_2 is different from that in the HL site.

The spatial relation between the binding sites for the two ligands on L_2 was examined using haptens containing two DNP moieties. The complexes formed exhibited a markedly enhanced affinity as compared to the mono-DNP derivatives. Sedimentation velocity analysis of complexes with di-DNP derivatives showed a single species of the size of L_2, thus excluding aggregate formation. These findings suggest that the two sites of L_2 are closely localized. Assuming that the binding occurs at the V-region these results are consistent with X-ray data of other L-chain dimers, where the V-regions are closely aligned [6–8].

2. Materials and methods

MOPC 315 bearing mice were kindly provided by Dr M. Potter and were maintained in BALB/c mice. Protein 315 and its light chain were prepared and characterized as described [1,5,9]. [^{3}H]DNPL (1.1 Ci/mmol) was purchased from New England Nuclear. All nitroaromatic derivatives were of analytical grade and were used without further purification.

Spectrophotometric measurements were carried out on a Cary 118 double beam spectrophotometer equipped with a thermostated cell holder. Unless otherwise stated, measurements were at 4 ± 0.2°C. Differential absorption spectra were made using a matched pair of tandem cells (Hellma). One compartment of each cell contained the ligand solution and the other an exactly equal volume of the protein solution. Difference spectra were recorded after mixing the solutions in one of the cells. The reference baseline was recorded before mixing and also after mixing both cells. Difference spectrophotometric titrations were made by gradual addition of ligand to both the mixed cell and the ligand compartment of the unmixed cell, making the proper adjustement to maintain the equality of volume and concentration. The extinction coefficient differences were evaluated from the slope of a plot of the absorption changes versus the concentration of the bound hapten using the reported association constants. Equilibrium dialysis was performed in 200 μl lucite cells, as described [5]. Association constants of different haptens to L_2 were obtained in inhibition measurements in which DNPL was displaced by the hapten in question. These were performed as follows: the inhibiting hapten (designated 2) was added to a mixture of the reference hapten DNPL (designated 1). The bound (X_1) and free (C_1) DNPL concentrations were determined, monitoring radioactive counting of [^{3}H]DNPL in equilibrium dialysis, or changes of the DNPL difference spectrum in spectrophotometric measurements. Using the previously determined [5] values of the association constant for DNPL (K_1) and the number of binding sites per L_2 molecule (n), the bound inhibitor concentration (X_2) was calculated from the equation

$$X_2 = nA_0 - X_1\,(1 + 1/K_1C_1)$$

with A_0 being the known total L_2 molar concentration. K_2, the association constant of the inhibiting ligand, was then obtained from the equation

$$K_2 = X_2/[nA_0 - X_1 - X_2)\,(H_2 - X_2)]$$

with H_2 being the known total inhibitor concentration. The tabulated values of association constants represent averages of several such measurements performed at different inhibitor concentrations. Both equations above are based on simple mass-action-law considerations and involve no approximations. In cases of homogeneous binding their use may be advantageous compared to that of the Karush equation [10]. This is because: (a) a control measurement at exactly the same free reference hapten concentration is not required here. (b) All variables in our formuli are directly measurable quantities (D. Lancet, manuscript in preparation).

3. Results

Figure 1 shows the difference spectrum of the L_2–DNPL complex versus free DNPL in the range 250–500 nm. For comparison we remeasured the difference spectrum of the HL–DNPL complex from 300–550 nm (insert). The difference spectra of the

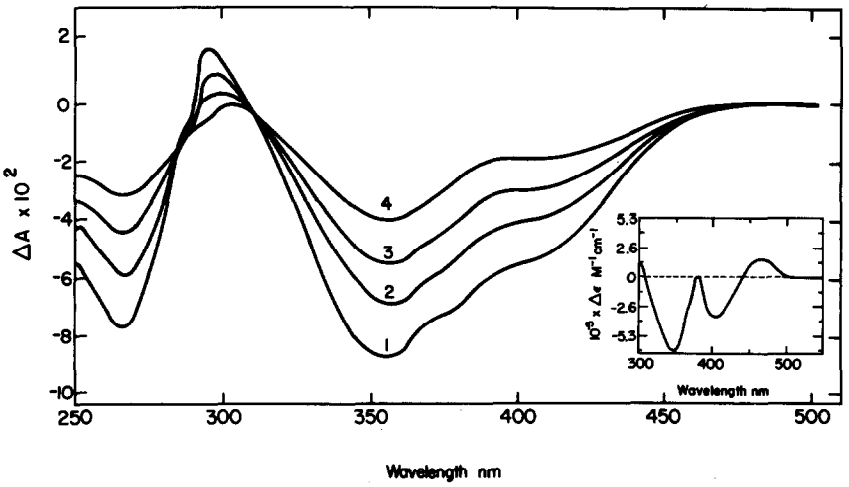

Fig.1. Difference spectrum of DNPL bound to L_2 versus free DNPL in PBS, at different temperatures corresponding to: (1) 4°C. (2) 15°C. (3) 25°C. (4) 35°C. Concentration of L_2 3×10^{-5} M and of DNPL 1.15×10^{-4} M. The spectrum at 4°C is the result of a complete spectrophotometric titration. For further details see Materials and methods. Insert: Difference spectrum of DNPL bound to HL versus free DNPL at 4°C in PBS. Concentrations: 9×10^{-6} M 315-HL and 2.3×10^{-5} M DNPL.

two complexes are different. The pronounced positive band at around 470 nm typical for the complexes of DNPL with 315 HL and with other anti-DNP antibodies [1,11,12] is totally missing in the L_2 complex. Also, the trough at 380 nm of the HL complex is absent in the L_2 complex. The extinction coefficient differences are $\Delta\epsilon_{356} = -4600\ M^{-1}\ cm^{-1}$ for the L_2 complex and $\Delta\epsilon_{345} = -6600\ M^{-1}\ cm^{-1}$ for the HL complex. The difference spectrum of the L_2 complex with ϵ,α-(2,4-dinitrophenyl)lysine (fig.2) is distinctly different from that of the complex with the mono-DNP derivative (DNPL). For the former two positive maxima are apparent at around 390 nm and 470 nm while the 350 nm band remains negative. The extinction coefficient difference per DNP group of the di-DNP hapten is $\Delta\epsilon_{352} = -5260\ M^{-1}\ cm^{-1}$, somewhat larger than that of L_2–DNPL.

The affinity of several nitroaromatic compounds for L_2 was studied using equilibrium dialysis and spectrophotometric titrations. The selection of haptens was guided by the objective of comparing the fine specificity of the L_2 site with that of the HL sites, mainly with regards to the nitroaromatic subsite (S_1). Table 1 summarizes the binding constants of several haptens to L_2 and HL. A remarkable difference between the two sites is the comparable affinity of 2,4-dinitrotoluene and DNPL to L_2, as contrasted by a four-fold difference in HL. The common specificity of the two sites is manifested in that:

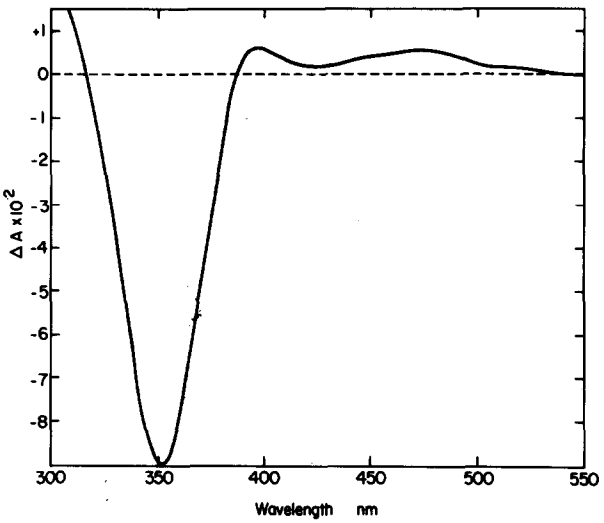

Fig.2. The difference spectrum of the L_2 complex with α,ϵ-di-DNP lysine versus the free hapten. Concentrations: L-chain = 2.9×10^{-5} M, di-DNP = 2×10^{-6} M (4°C in PBS). This spectrum was obtained in a complete spectrophotometric titration.

Table 1
Binding constants of nitrophenyl derivatives to protein 315 (HL) and its light chain dimer (L_2)

Hapten	K_{HL} M^{-1}	K_{L_2} M^{-1}	
		ED	DS
1. ε-*N*-(2,4-dinitrophenyl)lysine	$1.0 \cdot 10^{7}$[a]	$6.3 \cdot 10^{3}$[d]	–
2. 2,4-dinitrotoluene	$2.5 \cdot 10^{6}$[b]	$5.0 \cdot 10^{3}$	$6.2 \cdot 10^{3}$
3. 2,4,6-trinitrotoluene	$1.2 \cdot 10^{7}$[b]	$1.0 \cdot 10^{4}$	$9.0 \cdot 10^{3}$
4. 1,3-dinitrobenzene	$\sim 1.0 \cdot 10^{4}$[b]	$1.5 \cdot 10^{3}$	$1.4 \cdot 10^{3}$
5. 2,6-dinitrotoluene	$< 1.0 \cdot 10^{4}$[b]	$< 1.0 \cdot 10^{3}$	–
6. α,ε-*N*-di(2,4-dinitrophenyl)lysine	–	–	$2.3 \cdot 10^{5}$
7. Bis[β-*N*-(2,4-dinitrophenyl)]-meso-diamino succinate	$\sim 1.0 \cdot 10^{7}$[c]	$1.7 \cdot 10^{5}$	$3.0 \cdot 10^{5}$

ED: determined by equilibrium dialysis
DS: determined from difference spectrum
All data are at 4°C in PBS
[a] Taken from ref. [1]
[b] Extrapolated from value at 21°C, ref. [2]
[c] From ref. [16] (value for Fab fragment)
[d] From ref. [5]

(a) the introduction of a third nitro group into the toluene ring (2,4,6-trinitrotoluene) causes an increase in the affinity to both L_2 and HL. (b) Both have comparatively low affinity towards 1,3 dinitrobenzene and an even lower binding of 2,6 dinitrotoluene. An interesting observation is that the di-DNP derivatives bind to L_2 with a 30–50-times higher association constants than the mono-DNP derivative, while for HL the affinity of these compounds is the same [16].

The temperature dependence of the difference spectrum of the L_2–DNPL complex is also presented in fig.1. From these spectra (after correction for the temperature dependence of the extinction coefficient of the hapten) a van 't Hoff plot was constructed and $\Delta H = -5.6$ Kcal/mol was calculated. Using this value of ΔH and that of K at 4°C, we obtain $\Delta G = -4.8$ kcal/mol and $\Delta S = -2.9$ cal/mol deg. (at 4°C). The respective values for HL are $\Delta H = -16.6$ kcal/mol (van 't Hoff), $\Delta G = -8.7$ kcal/mol and a calculated $\Delta S = -28.5$ cal/mol deg. [13].

4. Discussion

The L_2 site is found to resemble the HL site in its steric requirements with respect to the nitroaromatic nucleus, but differs from it by its insensitivity to the presence of an attached side chain. This suggests that the L_2 site preserves many of the structural features of the nitroaromatic subsite (S_1) of HL, but is apparently devoid of the other subsites (S_2–S_4). Out of the three amino acid residues proposed to interact with DNP in S_1 of HL, two are in the light chain (Trp 93L and Asn 36L [4]. If these are also responsible for binding in the L_2 site, then the preservation of the specificity is not surprising, as both types of binding interactions are expected to show preference for certain nitroaromatic isomers. Thus, charge transfer stacking to the indole of tryptophan is favored with the meta-, di- or trinitrophenyl derivatives, where the nitro groups can remain coplanar with the ring [14] and hydrogen bonding requires optimal distance and angular relations with the proton donors [15]. The markedly lower affinity of L_2 towards nitroaromatic compounds as compared with that of HL may be due to:

(i) The absence of one of the two hydrogen-bonding residues (Asn 36H).

(ii) Less efficient charge-transfer complexation as evidenced by the disappearance of the charge transfer absorption band [14] at 470 nm.

(iii) Other differences that result from replacement of the heavy chain in HL by another light chain in L_2 (see below).

The decrease of binding free energy appears to be purely an enthalpic effect as the entropy of binding in L_2 is even more favourable than in HL.

X-Ray crystallography of light chain dimers [6–8] suggests that they resemble an Fab fragment with one monomer having its original configuration, and the other playing the structural role of a heavy chain [6]. The observation that two nitroaromatic molecules bind at apparently identical sites on 315-L_2 indicates that the two binding sites are related to each other by a two-fold rotation symmetry axis (local diad), though the molecule as a whole is not strictly symmetric. This suggestion is consistent with X-ray data of other light chain dimers showing diad symmetry in the pocket formed by the hypervariable regions [6–8].

In general, two situations may occur in light chain dimers. (a) The set of residues on the L chain which interacts with the hapten is situated close to the local diad. Consequently, the two symmetric sets may accomodate only one hapten between them as in REI-L_2 (R. Huber private communication) or two hapten molecules can be bound but at non-equivalent positions due to steric hindrance as in Mcg-L_2 [6]. This is clearly not the case for 315-L_2 as the two bound haptens do not hinder each other sterically [5]. (b) The hapten-binding residues are relatively distant from the local diad so that the sites are far enough and steric hindrance between the haptens does not occur. This appears to be the case for 315-L_2. It is however possible that in addition to the contact residues contributed by the L chain in HL, the hapten in L_2 interacts with some others L chain residues, positionally homologous to those originally contributed by the H chain. Thus, each hapten bound to 315-L_2 may interact with residues from both light chains. The 315-L_2 binding sites may therefore differ from that of HL not only in that H-chain residues are missing, but also in that 'new' L-chain residues form a contact with the hapten. Hindrance by such residues may account for part of the reduced affinity of L_2 toward the nitroaromatic ligands. The absence of subsite S_2–S_4 in L_2 does not necessarily mean that only the H chain is involved in their construction. In the HL site, L-chain residues possibly participate in S_2–S_4 (e.g., Arg. 95L [4] may be part of S_4), but in L_2 their interaction may be perturbed by the 'new' contact residues.

L_2 is found to bind di-DNP ligands with an association constant about 40-times higher than that of DNPL. This suggests that both DNP moieties of the di-DNP hapten interact simultaneously with the two adjacent sites on L_2. Considering the structure of the di-DNP ligand, an upper limit of about 12 Å is estimated for the distance between the two DNP binding sites on L_2. The increase of free energy of binding is only 40% as compared to the expected increase of 100%, probably due to a somewhat altered mode of binding which is also expressed in absorption difference spectra of the L_2–di-DNP complex. Further characterization of the L_2 sites using a series of di-DNP ligands is underway.

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