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Biophysical Chemistry 83 (1999) 171–177

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Letter

Aromatic residues mediate the pressure-induced association of digoxigenin and antibody 26-10

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Received 14 October 1999; received in revised form 11 November 1999; accepted 11 November 1999

Abstract

We have previously found that the complex between fluorescently labeled digoxigenin and the monoclonal antibody 26-10 forms with a decrease in volume of approximately 30 ml/mol, leading to increased association of these species under applied hydrostatic pressure. In the present study, we have utilized a panel of mutant antibodies and Fab fragments, previously characterized for their importance in the binding affinity of digoxin:26-10, to probe the molecular basis of pressure sensitivity in this complex, as measured by fluorescence polarization spectroscopy. Several mutations that result in marked decreases in affinity exerted little or no significant effect on the association volume. Mutation at any of several key aromatic residues of the 26-10 Fab heavy chain led to a decrease in the pressure-induced association, and two mutants with Trp → Arg mutations at heavy chain residue 100 exhibited pressure-induced dissociation. The effect of charged groups was found to depend on their proximity to contacting aromatic groups. The ability to understand and control the pressure sensitivity of antigen–antibody complexes has numerous potential applications in immunoseparations and immunosensors.

Keywords: Hydrostatic pressure; Association volume; Antigen–antibody complex; Mutant Fab fragments; Aromatic amino acid residues

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0301-4622/99/$ - see front matter
PII: S0301-4622(99)00139-8

1. Introduction

The study of pressure-sensitive immune complexes is motivated by their potential applications in biotechnology and because they add information regarding volume changes to our understanding of non-covalent interactions in molecular recognition. The effect of pressures up to 4000 bar on binding equilibria has been investigated for a number of antigen–antibody systems, and it has been found that many complexes dissociate when subjected to high pressures; however, others are unaffected and some even associate at high pressure [1–3]. These previous studies have, so far, provided relatively little information regarding the molecular determinants of pressure sensitivity. An improved understanding of the mechanistic basis for pressure sensitivity would provide guidelines for molecular engineering strategies to design selectively pressure-sensitive antibodies for use in applications such as immunoaffinity purification [4] and immunosensors [5].

Various non-covalent interactions in the binding site may regulate the pressure sensitivity of antigen–antibody pairs. The qualitative effect of different chemical groups is believed to be understood — the formation of interfacial voids, salt bridges, or aliphatic contacts is expected to proceed with a positive change in volume, whereas hydrogen bonds and aromatic stacking, due to their efficient packing, are expected to lead to a negative change in volume [6,7]. These conclusions are based largely on small molecule studies; extrapolation to macromolecular binding interfaces is complicated by the heterogeneous nature of the latter. Macromolecular binding pairs for which mutants are available can be useful in extracting information regarding mechanisms in the context of a heterogeneous binding interface. One such system is the complex between the cardiac glycoside digoxin, and the monoclonal antibody 26-10 [8]. The structure of the complex between digoxin and the 26-10 Fab has been solved by crystallography [9], and a number of mutant antibodies to 26-10 have been generated by natural variation and mutagenesis [10,11]. The three-dimensional crystal structure of the Fab 26-10 digoxin: complex has revealed an absence of hydrogen bonds, salt bridges or conformational changes at the interface; as a result, binding of digoxin:26-10 is strongly dominated by non-polar interactions [9]. Notably, the C3 attached tridigitoxose sugar is exposed to solvent and does not participate in binding, and digoxigenin, which is a closely related analog of digoxin lacking the sugar moiety, exhibits similar affinity for 26-10 [9,10]. We have used fluorescently labeled digoxigenin and a selection of 26-10 mutants as a model system for exploring the molecular determinants of pressure-induced association.

2. Materials and methods

2.1. Fluorescein-digoxigenin conjugation

Fluorescein was conjugated to digoxigenin as described elsewhere [12]. The identity and purity ($>98\%$) of fluorescein labeled digoxigenin was determined using mass spectrometry.

2.2. Production and purification of Fab 26-10 and phage display mutants

Details of the initial construction and expression of these libraries are presented elsewhere [11]. Each colony of *E. coli* was grown in 1 l of super broth (30 g tryptone, 20 g yeast extract and 10 g MOPS per l, pH 7.0) with 20 mM magnesium chloride and 50 μg/ml carbenicillin at 37°C. When absorbance at 600 nm exceeded 1.0 O.D., the cultures were induced with 1 mM IPTG overnight at 30°C. After overnight growth, the cells were separated by centrifugation and resuspended in 20 ml of PBS with 200 μM phenylmethylsulfonyl fluoride. The suspended cells were disrupted by sonication and the cell debris was subjected to centrifugation. The supernatant was filtered through a 0.22-μm filter, applied to a ouabain-BSA-Sepharose affinity column, eluted with 0.2 N ammonium hydroxide (pH 11), and immediately neutralized with 1/10 volumes each of 10× PBSA and 1 M Tris (pH 4.8). The Fabs were concentrated by ultrafiltration and dialyzed into phosphate buffer (0.15 M NaCl and 0.05% azide, pH 7.2) for storage. The

final yields of Fab mutants were 100–400 μg. Size exclusion HPLC (G2000 SWxl column, TosoHaas, Montogomeryville, PA, USA) of each Fab mutant indicated the presence of 5–30% high molecular weight proteins. Since the measured affinities and association volumes of Fab interactions with digoxigenin were close to previously published values, the mutants were used without further purification. The whole IgG mutants LL2 and LB4 were prepared as described previously [10] and deemed to be free of impurities by HPLC using a G3000SW$_{XL}$ column (TosoHaas).

2.3. High pressure fluorescence polarization spectroscopy

The binding of antibodies to fluorescein-labeled digoxigenin was monitored by measuring the change in fluorescence polarization, or anisotropy, corresponding to a decrease in the rotational diffusion coefficient for bound hapten as compared to unbound [13]. Correction for window birefringence and determination of bound fraction, affinity constant and free energy were as described previously [3,14]. The only exception was that the ratio of fluorescence intensities in the bound-to-unbound states was 0.45, in contrast to 1.0 for labeled protein antigens that we had used in previous studies [3]. The experimental protocol was essentially as described [12], allowing for slow ramping of pressurization and sufficient time for sample equilibration at each step.

3. Results

We have previously investigated the interaction of fluorescently-labeled digoxigenin and the 26-10 antibody using high pressure fluorescence polarization spectroscopy [12]. Over the range of 1–2000 bar, pressure-induced association was observed. The finding of a negative association volume was somewhat unusual for a macromolecular system but could be mediated by the efficient packing of aromatic groups present in both digoxigenin and 26-10. We utilized the same experimental approach and a selection of mutant antibodies to investigate the biochemical basis for this behavior in more detail. In each case, the fluorescence anisotropy of a complex was measured at 200 bar intervals from 1 to 2000 bar; greater anisotropy corresponds to an increasing fraction of bound hapten. From the fluorescence anisotropy, the bound fraction and free energy of association (ΔG) were calculated as described [12]. Representative examples of fluorescence anisotropy and free energy vs. pressure (P) are

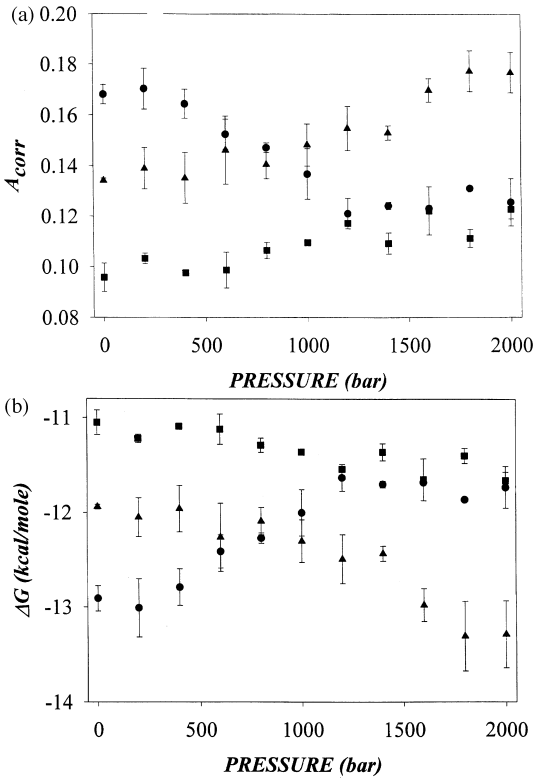

Fig. 1. (a) Anisotropies (A_{corr}) and (b) free energies (ΔG) of the interaction of 26-10 Fab (▲) and two mutants, G20 (■) and D9 (●), with fluorescently labeled digoxigenin. The association of the indicated complexes was monitored by fluorescence polarization spectroscopy over the pressure range 1–2000 bar. Anisotropy values were corrected for birefringence by standard methods [14]. Anisotropies were converted to fractions bound and free energies, and association volumes (ΔV) were determined by regression of ΔG vs. P. Note that the complexes involving 26-10 and G20 each exhibited pressure-induced association, whereas that involving D9 exhibited pressure-induced dissociation.

shown in Fig. 1. The association volume was regressed in each case from the ΔG vs. P plot.

3.1. Single residue variants of 26-10

First, we used previously characterized mutants of the 26-10 monoclonal antibody that were observed to exhibit marked effects on affinity. Single substitutions in the CDR2 of tyrosine with a histidine at H:50 (mutant LL2) and of serine with a phenylalanine at H:52 (mutant LB4) resulted in significantly lower affinities for digoxin [10]. We chose to study LL2 and LB4 to verify whether these key contacting residues also influence the association volume. The observed affinities of 26-10 and the two mutants at atmospheric pressure were somewhat less than reported previously for their interaction with digoxin [10,15]; this may be due to the use of fluorescently labeled digoxigenin as the ligand here. The association volume of LB4 and digoxigenin ($\Delta V = -28 \pm 5$ ml/mol) was close to that of wild-type 26-10 and digoxigenin ($\Delta V = -31 \pm 4$ ml/mol, Table 1). On the other hand, LL2 exhibited a lower pressure sensitivity (ΔV of -18 ± 5 ml/mol) as compared to 26-10, but a substantial pressure-induced association remained. Thus, residues that are important determinants of binding affinity at atmospheric pressure do not necessarily play a major role in the pressure response of the complex.

3.2. Mutant Fabs of 26-10

Two important aromatic residues in 26-10, that make contact with the hapten, are tyrosine at position 33 (H:CDR1) and tryptophan at position 100 (H:CDR3). To investigate the importance of these non-polar contacts, we selected 10 mutants with substitutions in the vicinity of these residues. The mutants were chosen from a phage display library of mutants in the H:30–35 [11] and H:99–101 (M.K. Short and M.N. Margolies, manuscript in preparation) loci. Those selected exhibit generally comparable affinities for digoxi-

Table 1
Association volumes of 26-10 and mutant complexes with fluorescently labeled digoxigenin

Antibody	K_a (10^9 M^{-1})[a]	H:CDR1, residues 30–35	H:CDR2, residues 50–52	H:CDR3, residues 99–101[b]	ΔV (ml/mol digoxigenin)
26-10 and variant IgGs					
26-10 IgG	5.0	–	Y I S	–	-31 ± 4
LL2	0.6	–	H I S	–	-18 ± 5
LB4	0.08	–	Y I F	–	-28 ± 5
Phage display selected Fabs					
26-10 Fab	5.3 ± 1.9	T D F Y M N	–	K W A M D	-29 ± 4
G6-1	4.3 ± 0.6	G E R F F N	–	–	-33 ± 5
D4-5	3.5 ± 0.2	M P S Y I N	–	–	-19 ± 8
D4-1	3.4 ± 0.2	W D L W V N	–	–	-23 ± 5
D4-12	4.0 ± 0.2	A R M Y I N	–	–	-5 ± 7
A4-19	21.7 ± 8.1	P S F Y Y N	–	–	-20 ± 2
D36	22.0	–	–	Y W A L M	0 ± 7
D32	18.6	–	–	A W A L Q	-23 ± 7
D6	1.6	–	–	G R A L N	$+11 \pm 2$
D9	1.6	–	–	R R A L D	$+25 \pm 6$
G20	0.6	–	–	L Q A L S	-13 ± 2

[a] Affinities of H:CDR1 mutants for digoxin are from Short et al. (1995) [11]; those of H:CDR2 mutants are from Schildbach et al. (1991) [10]; those of H:CDR3 mutants are from M.K. Short and M.N. Margolies, manuscript in preparation.

[b] The numbering convention of these residues proceeds as 99, 100, 100a, 100b, 101, according to standard convention, Kabat et al. (1991) [21].

genin as 26-10, with a few somewhat higher and a few somewhat lower (Table 1).

The association volumes of the Fab:digoxigenin complexes did not correlate with affinity (see Table 1). Among the H:CDR1 mutants, D4-12 displayed the greatest change in reaction volume, compared to wild-type, with a ΔV of -5 ± 7 ml/mol. The other H:CDR1 mutants exhibited roughly the same ΔV as the 26-10 Fab. Note that the 26-10 Fab exhibited identical (within experimental uncertainty) ΔV as compared to the whole antibody (-29 ± 4 vs. -31 ± 4 ml/mol).

The greatest changes in ΔV were recorded for the H:CDR3 mutants D36, D6 and D9. The association volume of D36, which has several mutations that increase the non-polar character of the region, was approximately zero. For two mutants, D6 and D9, the pressure sensitivity changed directions compared to wild-type, resulting in pressure induced dissociation with ΔV of 11 ± 2 and 25 ± 6 ml/mol, respectively. D6 and D9 contain identical insertions at H:100 and H:100b, although it is the former (W100R) that appears to make the most significant contribution (see Section 4). Not all mutants from the H:CDR3 exhibited abolished or reversed pressure sensitivity. Two additional H:CDR3 mutants, D32 and G20, interacted with digoxigenin with association volumes of 80% (-23 ± 7 ml/mol) and 45% (-13 ± 2 ml/mol) of wild-type 26-10 Fab.

4. Discussion

While some general features of the effects of binding site chemistry (e.g. charged, polar, aliphatic or aromatic) on association volumes have been established, the identification of critical amino acid residues for pressure sensitivity has been accomplished in only a few cases, principally involving salt bridges. We have previously reported pressure-induced association of the digoxigenin:26-10 hapten:antibody complex, with a magnitude ($\Delta V \sim -30$ ml/mol at 25°C) greater than that observed in other pressure-associating systems to date. The negative association volume exhibited by digoxigenin:26-10 appears to be due to the presence of a large number of aromatic groups among the contacting residues of this complex, consistent with the behavior of small aromatic dyes [16].

There are a number of aromatic residues from the 26-10 Fab that contact the hapten; among these, more than 50% of the pairwise atomic contacts involve four aromatic amino acids from the heavy chain of the Fab — H:Tyr33, H:Tyr47, H:Tyr50, and H:Trp100 [9]. We utilized antibodies mutated at three of these residues. We first looked at residues in the H:CDR2 region of the Fab, using mutants that we had previously characterized as having significant effects on affinity [10,15]. Mutation of H:Tyr50 to an aliphatic or polar residue leads to a significant loss in affinity [15]. The pressure sensitivity of Y50H, which has a 10-fold lower affinity for fluorescently labeled digoxigenin than wild-type, was reduced by almost half, from -31 to -18 ml/mol, as compared to wild-type. The reduction in pressure-induced association exhibited by this mutant could arise from a combination of two factors — the loss of an aromatic (Tyr) residue, and the introduction of a charged (His) residue, which favors dissociation under pressure with an estimated contribution of ~ 10 ml/mol charges [7]. A nearby mutation involving a non-aromatic residue, S52F (designated LL2), also exhibits a 10-fold weaker affinity for fluorescently labeled digoxigenin than 26-10, yet we found no significant difference in the association volume (ΔV) between LL2 and wild-type. While the Y50H mutation did have a significant effect on pressure sensitivity, the H:CDR2 mutations did not account for all of the pressure-induced association.

Mutation of the aromatic residues in the H:CDR1 region from residues 30 to 35 can also lead to altered affinity and/or specificity [11]. The five H:CDR mutants studied all exhibited negative association volumes, although the value for one (D4-12) was close to zero (Table 1). The extent of pressure-induced association in this region correlated with the number of aromatic (Phe, Tyr, Trp) residues in positions H:30–35.

The H:CDR3 region makes the largest contribution to the complementarity of digoxin:26-10 binding in terms of both the number of pairwise contacts (26/59 total) and buried surface area

(126/383 Å^2 total) [9]. This region was found to have the greatest effect on pressure sensitivity, with several mutations resulting in decreased pressure-induced association; in fact, two (D6 and D9) even displayed pressure-induced dissociation (Table 1). The two mutants displaying pressure-induced dissociation share a WAM to RAL mutation in bases 100, 100a and 100b. Since the leucine at 100b is present in mutants of both positive and negative association volumes, we conclude that the W100R mutation is responsible for the reversal in sign of the association volume. The greater ΔV of D9 is likely due to its three charged groups in the 99–101 region, compared to only one for D6. Another clone with the Trp100 mutated, G20, possesses no charged groups and exhibited reduced pressure-induced association. Thus, the ΔV of Trp100 mutants correlates with the number of charged groups, with increasing charge leading to increased ΔV.

Although charged groups appear to drive positive association volumes (pressure-induced dissociation) in the cases where aromatic groups are deleted, our results suggest that, when aromatic groups are present, proximal charge groups may act synergistically to drive negative association volumes. In the wild-type 26-10 antibody, which exhibits a large negative association volume (~ -30 ml/mol), charged groups are proximal to the key aromatic residues in the H:CDR1 and H:CDR3 regions (i.e. H:Asp31, H:Phe32; H:Lys99, H:Trp100; see Table 1), albeit that the charged side chains point away from the ligand in the digoxin:26-10 complex [9]. Among the H:CDR1 mutants, the ones with the most negative association volumes (G6-1 and D4-1) possess multiple aromatic groups with at least one neighboring charge, the next most negative (A4-19) possesses multiple aromatics but no neighboring charges, and the two least negative (D4-5 and D4-12) possess only one aromatic residue and no neighboring charges. Synergy of charged and aromatic groups could also be responsible for the pressure-induced association observed in complexes involving nucleic acids [17,18]

In summary, we have used a selection of mutants to the anti-digoxin antibody 26-10 to probe the molecular basis for the high degree of pressure-induced association observed in its complex with fluorescently labeled digoxigenin. We found no apparent correlation between affinity and association volume, in contrast to previous observations of a correlation between ΔG and ΔV for the mutation of salt bridges in the complex of cytochrome b_5–cytochrome c [19,20]. It appears that the effect of aromatic groups on pressure sensitivity is more dependent on context, particularly on the nature of neighboring residues. For the pressure-induced association of digoxigenin:26-10, we conclude that aromatic residues within the antibody Fab, particularly H100-Trp, are key determinants of ΔV. Furthermore, our results suggest that the effect of charged groups on ΔV may be different depending on whether they are proximal to aromatics. Clearly, further studies on other well-defined systems are required to determine whether these conclusions are general to all molecular complexes.

Acknowledgements

The authors acknowledge the technical assistance of Robert Bieganski and Rou-Fun Kwong. This work was partially supported by NSF Grant BES-9696066 (to MLY) and NIH Grant R01 HL47415 (to MNM).

References

[1] T. Coelho-Sampaio, E.W. Voss, Jr, Pressure-induced dissociation of fluorescein from the anti-fluorescein single-chain antibody 4-4-20, Biochemistry 32 (1993) 10929–10935.

[2] C.Y. Cheung, D.J. Green, G.J. Litt, J.A. Laugharn, Jr, High-pressure-mediated dissociation of immune complexes demonstrated in model systems, Clin. Chem. 44 (1998) 299–303.

[3] S. Sundaram, C.M. Roth, M.L. Yarmush, Pressure-induced dissociation of antigen–antibody complexes, Biotechnol. Prog. 14 (1998) 773–781.

[4] M.L. Yarmush, A.M. Weiss, K.P. Antonsen, D.J. Odde, D.M. Yarmush, Immunoaffinity purification: basic principles and operational considerations, Biotechnol. Adv. 10 (1992) 413–446.

[5] H.A. Fishman, D.R. Greenwald, R.N. Zare, Biosensors in chemical separations, Annu. Rev. Biophys. Biomol. Struct. 27 (1998) 165–198.

[6] K. Heremans, High pressure effects on proteins and

other biomolecules, Annu. Rev. Biophys. Bioeng. 11 (1982) 1–21.
[7] V.V. Mozhaev, K. Heremans, J. Frank, P. Masson, C. Balny, High pressure effects on protein structure and function, Proteins 24 (1996) 81–91.
[8] M.M. Hunter, M.N. Margolies, A. Ju, E. Haber, High-affinity monoclonal antibodies to the cardiac glycoside, digoxin, J. Immunol. 129 (1982) 1165–1172.
[9] P.D. Jeffrey, R.K. Strong, L.C. Sieker et al., 26-10 Fab–digoxin complex: Affinity and specificity due to surface complementarity, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 10310–10314.
[10] J.F. Schildbach, D.J. Panka, D.R. Parks et al., Altered hapten recognition by two anti-digoxin hybridoma variants due to variable region point mutations, J. Biol. Chem. 266 (1991) 4640–4647.
[11] M.K. Short, P.D. Jeffrey, R.F. Kwong, M.N. Margolies, Contribution of antibody heavy chain CDR1 to digoxin binding analyzed by random mutagenesis of phage-displayed Fab 26-10, J. Biol. Chem. 270 (1995) 28541–28550.
[12] P. Roy, C.M. Roth, M.N. Margolies, M.L. Yarmush. Effect of pressure on antigen–antibody complexes: modulation by temperature and ionic strength. Mol. Immunol. (in press).
[13] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Press, New York, 1983.
[14] A.A. Paladini, G. Weber, Absolute measurements of fluorescence polarization at high pressures, Rev. Sci. Instrum. 52 (1981) 419–427.
[15] J.F. Schildbach, R.I. Near, R.E. Bruccoleri et al., Heavy chain position 50 is a determinant of affinity and specificity for the anti-digoxin antibody 26-10, J. Biol. Chem. 268 (1993) 21739–21747.
[16] G. Weber, F. Tanaka, B.Y. Okamoto, H.G. Drickamer, The effect of pressure on the molecular complex of isoalloxazine and adenine, Proc. Natl. Acad. Sci. U.S.A. 71 (1974) 1264–1266.
[17] D. Rentzeperis, D.W. Kupke, L.A. Marky, Volume changes correlate with entropies and enthalpies in the formation of nucleic acid homoduplexes: differential hydration of A and B conformations, Biopolymers 33 (1993) 117–125.
[18] P.M. Torgerson, H.G. Drickamer, G. Weber, Effect of hydrostatic pressure upon ethidium bromide association with transfer ribonucleic acid, Biochemistry 19 (1980) 3957–3960.
[19] K.K. Rodgers, T.C. Pochapsky, S.G. Sligar, Probing the mechanisms of macromolecular recognition: the cytochrome b_5–cytochrome *c* complex, Science 240 (1988) 1657–1659.
[20] K.K. Rodgers, S.G. Sligar, Mapping electrostatic interactions in macromolecular associations, J. Mol. Biol. 221 (1991) 1453–1460.
[21] E.A. Kabat, T.T. Wu, H.-M. Perry, K.S. Gottesman, C. Foeller, Sequences of Proteins of Immunological Interest vol. 5th ed., (U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD, 1991).