Crystallization and preliminary X-ray analysis of anti-digoxin antibodies.

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Abstract

The Fab fragments of several monoclonal antibodies that bind digoxin and other cardiac glycosides have been screened for crystallization conditions. We have crystallized two of these in forms suitable for X-ray analysis. The anti-digoxin antibody 40-50 in complex with ouabain crystallizes with symmetry consistent with space group $C2$, with $a = 92.6$, $b = 85.0$, $c = 73.0$ Å and $\beta = 131.7°$. This crystal form shows considerable non-isomorphism between crystals. A second anti-digoxin antibody, 49-10, crystallizes with symmetry consistent with space group $P2_12_12$, with $a = 95.3$, $b = 147.1$ and $c = 76.2$ Å.

The development of somatic cell fusion has made it possible to examine the specificity of homogeneous (monoclonal) antibodies. In particular, it has been possible to determine the three-dimensional structures of antibody–antigen complexes. Crystal structures have been determined for antibody fragments complexed with proteins, peptides, nucleotides, carbohydrates and small molecules (Fischmann *et al.*, 1991; Sheriff *et al.*, 1987; Padlan *et al.*, 1989; Tulip, Varghese, Laver, Webster & Colman, 1992; Tulip, Varghese, Webster, Laver & Colman, 1992; Tulip *et al.*, 1989; Bentley, Boulot, Riottot & Poljak, 1990; Prasad *et al.*, 1993; Jacobo-Molina *et al.*, 1993; Chitarra *et al.*, 1993; Stanfield, Fieser, Lerner & Wilson, 1990; Rini, Schulze-Gahmen & Wilson, 1992; Garcia, Ronco, Verroust, Brünger & Amzel, 1992; Rini *et al.*, 1993; Shoham, 1993; Herron *et al.*, 1991; Cygler, Rose & Bundle, 1991; Padlan, Cohen & Davies, 1985; Herron, He, Mason, Voss & Edmunson, 1989; Alzari *et al.*, 1990; Brünger, Leahy, Hynes & Fox, 1991; Vix, Rees, Thierry & Altschuh, 1993; Arevalo, Stura, Taussig & Wilson, 1993; Jeffrey *et al.*, 1993).

Digoxin (digoxigenin tridigitoxoside, Fig. 1) is a cardiac glycoside derived from the common foxglove (*Digitalis purpurea*) that has long been prescribed for patients with congestive heart failure and certain cardiac rhythm disturbances. Digoxigenin consists of a 5β,14β steroid with an α,β unsaturated five-membered lactone ring attached at position 17 of the steroid nucleus. Digoxin consists of digoxigenin with three β(1→4)-D-glycoside linked digitoxoses attached at position 3 of the steroid nucleus (Fig. 1a). Digoxigenin is a rather rigid hapten with rotation about the steroid–lactone bond being the only degree of freedom. In digoxin free rotation may also occur about the glycosidic linkages. A large number of structurally defined digoxin congeners are known and have been useful in studying affinity and specificity of anti-digoxin antibodies (Mudgett-Hunter, Margolies, Ju &

Haber, 1982; Mudgett-Hunter, Anderson, Haber & Margolies, 1985; Schildbach *et al.*, 1991; Schildbach *et al.*, 1993). The anti-digoxin antibody 26-10 has been the subject of previous structural study (Rose *et al.*, 1983; Brünger, 1991; Jeffrey *et al.*, 1993).

Crystallization of ten anti-digoxin antibodies was attempted in a broad-based effort to understand the structural basis of antibody specificity and affinity. Crystals of 26-10 hapten complexes (Rose *et al.*, 1983) are difficult to reproduce and display a high degree of twinning by hemi-merohedry (Strong, 1990), limiting the usefulness of 26-10 as the only model system for digoxin-specific antibodies. Therefore, a more tractable crystal form was sought. Reported here are the crystallizations of two different anti-digoxin antibodies: 40-50 and 49-10. These two antibodies differ in sequence, affinity and fine specificity from 26-10 (Table 1; Mudgett-Hunter *et al.*, 1985).

The monoclonal anti-digoxin antibodies 40-50 and 49-10 were produced in A/J mice by somatic cell fusion as described in Mudgett-Hunter *et al.* (1985). Antibodies 40-50 and 49-10
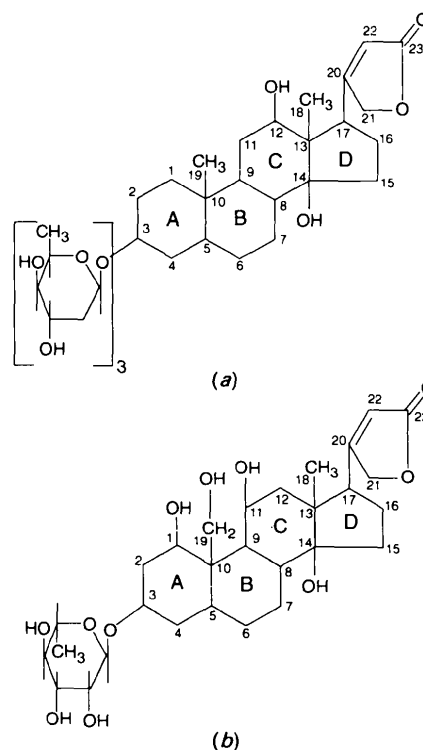

Fig. 1. (*a*) Structure of digoxin (digoxigenin tridigitoxoside). (*b*) Structure of ouabain (ouabagenin rhamnoside).

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Table 1. *Comparison of anti-digoxin antibodies*

Antibody name	Class	Heavy chain Sequence group‡	Type	Light chain Sequence group‡	Affinity for digoxin (M^{-1})*	Affinity for ouabain (M^{-1})†
26–10	IgG2a	II(A)	κ	II	9.1×10^9§	3.3×10^8¶
40–50	IgG2b	I(B)	κ	III	1.7×10^9¶	3.6×10^8¶
49–10	IgG1	II(B)	κ	V	1.6×10^9**	1.4×10^8¶

* Affinites for digoxin were measured by saturation equilibrium assays using either filtration through glass fiber (26-10 and 40-50) (Schildbach *et al.*, 1991) or incubation with dextran-coated charcoal (49-10) (Mudgett-Hunter *et al.*, 1985) to separate free from bound [³H]digoxin.

† Affinites for ouabain were measured using solution-phase competition assays using either filtration through glass fiber (26-10 and 40-50) or incubation with dextran-coated charcoal (49-10) to separate free from bound [³H]digoxin. Values were converted from K_i (26-10 and 40-50) or estimated from relative 50% inhibitory concentrations (49-10).

‡ Kabat, Wu, Perry, Gottesman & Foeller (1991).

§ Schildbach *et al.* (1993).

¶ J. F. Schildbach & MNM, unpublished data.

** Mudgett-Hunter *et al.* (1985).

were purified by affinity chromatography on ouabain–amine Sepharose columns (Zurawski, Novotny, Haber & Margolies, 1978). Originally, both antibodies were eluted from the column with 20 mM ouabain (Fig. 1*b*) in phosphate-buffered saline. Following elution, the antibodies were dialyzed against five changes of phosphate-buffered saline and concentrated. Subsequently, antibody 40-50 was eluted from the ouabain affinity column with 200 mM ammonia into 1 M Tris–HCl, pH 8.0 in 10× phosphate-buffered saline to avoid the potential for residual ouabain at the antigen-binding site despite extensive dialysis.

Fabs from 40-50 and 49-10 were prepared from purified antibody by first dialyzing against 50 mM Tris–HCl, pH 7.2, 0.1 M NaCl, 1 mM EDTA and concentrating the antibody to 5 mg ml⁻¹. The appropriate papain concentration and digestion time were determined in preliminary experiments (Cygler, Boodhoo, Lee & Anderson, 1987; Boodhoo, Mol, Lee & Anderson, 1988) which were analyzed by isoelectric focusing gel electrophoresis using the Phastgel system (Pharmacia). Optimal conditions were found to be a papain/antibody (*w/w*) ratio of 1/800 (40-50) or 1/200 (49-10) for 30 min at 310 K in the presence of 25 mM β-mercaptoethanol. The digestions were stopped by the addition of iodoacetamide to a final concentration of 30 mM. The digestion mixture was passed through a column of immobilized protein A (Pierce) to separate Fab fragments from Fc fragments and undigested IgG using Immunopure buffers from Pierce. Using a Centriprep (Amicon) concentrator, the Fabs were concentrated and exchanged into 20 mM Tris–HCl, pH 8.0 (40-50) or distilled water (49-10).

The 40-50 Fab was shown to have several components by DEAE-HPLC (high-pressure liquid chromatography). Using a DEAE-15HR, AP-2 column (Waters/Millipore) two major components were separated by a combined linear gradient of 0–195 mM NaCl and 20 mM Tris–HCl, pH 8.0–7.2. Each component was concentrated and buffer exchanged into 10 mM imidazole–HCl, pH 7.0 using a Centricon 10 (Amicon) so that the final protein concentration was ∼10 mg ml⁻¹. Similarly, at least four components were present in the 49-10 Fab preparation and were separated with a linear gradient from 0 to 260 mM NaCl in 20 mM Tris–HCl, pH 8.2. Each component collected from the DEAE column was concentrated to ∼10 mg ml⁻¹ and buffer exchanged into 10 mM imidazole, pH 7.0, 0.02% NaN₃ with a Centricon 10.

Both the 40-50 Fab–ouabain complex and 49-10 Fab were crystallized by hanging-drop vapor diffusion using Linbro plates (Flow Laboratories) and plastic coverslips. For 40-50

the well contained 1 ml of 50–100 mM Tris–HCl, pH 8.0–8.5, 12–18%(*w/v*) polyethylene glycol 3350 (Sigma). With 40-50 Fab eluted with ouabain from the ouabain affinity column the hanging drop contained 5 μl of the above protein solution, 3 μl of buffer and PEG solution, and 2 μl 5 mM CuCl₂ so that the starting protein concentration in the drop was ∼5 mg ml⁻¹ and the starting CuCl₂ concentration was 1 mM. The best crystals were obtained with a well solution of 50 mM Tris–HCl, pH 8.0, 15%(*w/v*) PEG 3350 at room temperature (∼294 K) and grew in a week. Both major components of 40-50 Fab could be used to grow these crystals, which grew without the formation of precipitate in the drop. Diffraction quality crystals could only be grown with the addition of copper ions; nickel was the only other metal that yielded crystals, but they were small and of irregular morphology. The crystals were blocky rods with axial ratios of approximately 1:1:3. With 40-50 Fab eluted with 200 mM ammonia from the ouabain affinity column crystals could be grown under identical conditions without adding ouabain, but these grew in droplets with a heavy precipitate and could not be grown larger than about 0.025 mm with axial ratios of 1:10. Protein eluted with ammonia required the addition of 4.5 mM ouabain to the buffer and PEG solution (initial ouabain concentration in the drop of 1.4 mM) to obtain diffraction-size crystals. Crystals of the 40-50 Fab eluted with ammonia could not be obtained in the presence of digoxin solubilized with ethanol.

For 49-10, the well contained 1 ml of 0.1 M Tris–maleate buffer, pH 3.0–4.0, and 14–16%(*w/v*) PEG 8000 (Sigma). The hanging drop contained 4 μl of the above protein solution and 4 μl of well solution. The largest crystals were obtained at pH 3.5 at room temperature (∼294 K). The 49-10 Fab crystallizes very quickly, generally within 1–2 d. The crystals are thin plates with axial ratios of approximately 1:5:15. Only the major component yielded usable crystals under these conditions.

Crystals for both 40-50–ouabain complex and 49-10 Fab were mounted in thin-walled glass capillaries (Charles Supper Company) and examined on a Siemens (Xentronics) area-detector system. The data were reduced with the *XENGEN* package of programs (Howard *et al.*, 1987). Two crystals were examined for 40-50. The first data set extended to 3.3 Å resolution and the second to 2.7 Å resolution with reflections observable to 2.2 Å resolution. With the aid of the program *XPREP* (Siemens Analytical X-ray Inc.), the space group was determined to be C2. The first crystal had unit-cell dimensions *a* = 92.5, *b* = 84.9, *c* = 73.0 Å and β = 131.6°. The second crystal had unit-cell dimensions *a* = 93.7, *b* = 84.8, *c* = 70.1 Å and

$\beta = 128.0°$, indicating considerable non-isomorphism between crystals. The unit-cell volume is consistent with one Fab ($M_r \simeq 50\,000$) per asymmetric unit with a V_M of $< 2.2\,\text{Å}^3\,\text{Da}^{-1}$ suggesting a tightly packed cell (Matthews, 1968).

For 49-10, data were observable to 2.8 Å resolution. The unit cell was orthorhombic with $a = 95.3$, $b = 147.1$ and $c = 76.2$ Å. The space group was determined from precession photographs (not shown) to be $P2_12_12$. The size of the unit cell is consistent with two Fabs per asymmetric unit which yields a V_M of 2.7 $\text{Å}^3\,\text{Da}^{-1}$. Interestingly, the $h0l$ zone is pseudo-centered at low (to ~ 7.0 Å) resolution. A native Patterson map with data from 50 to 7 Å resolution showed a peak consistent with the observed pseudo-centering at $u = 0.5$, $v = 0.1$, $w = 0.5$ that was 44% of the origin peak height.

The non-isomorphism observed with the 40-50 crystals could be the result of complexed and uncomplexed antibody, but the protein that was eluted from a ouabain affinity column with ammonia would only grow diffraction-size crystals in the presence of ouabain. Thus, we are left with the conclusion, that despite extensive dialysis, ouabain is likely to be present in the binding site of protein that was eluted with ouabain from the ouabain affinity column.

The 40-50 crystal form appears to be the more promising of these two different Fabs based on the size of the unit cell, the number of molecules per asymmetric unit and the resolution obtainable. We are pursuing structure determination for both crystal forms by the method of molecular replacement. The structures of one or both of these antibodies and their complexes with cardiac glycosides will enable us to compare their mode of binding with that of antibody 26-10 (Jeffrey *et al.*, 1993). From these comparisons we will be able to explore how the different specificities for cardiac glycosides are achieved by different antibodies that utilize different variable regions (Mudgett-Hunter *et al.*, 1985).

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