Specific Binding Activity of Isolated Light Chains of Antibodies

Abstract. *Free light chains isolated from specifically purified antibody have been shown to bind specific hapten. This proves that part of the binding site does exist on the light chain. The light chains were obtained from antibody directed against the 4-azonaphthalene-1-sulfonate group, and the binding of the simple hapten 4-anilinonaphthalene-1-sulfonate was determined by the fluorescence-enhancement technique. Since this hapten undergoes a striking increase in fluorescence on binding to light chains (and also on binding to specific antibody), the presence of small amounts of bound hapten could be determined, even in the presence of the high concentrations of unbound hapten required because of the low binding constant.*

Antibody molecules can be split after reduction of a few critical disulfide bonds into the component heavy (H) and light (L) chains. Workers in several laboratories have found that the heavy chains show binding activity toward the specific antigen or hapten (*1–3*), proving that the isolated heavy chains still have at least part of the original binding region. The equilibrium constant for binding of heavy chain to hapten is less than that observed with the intact antibody (*2*). Until now there was no direct evidence that there was binding of antigen or hapten by free L-chains.

The light chain from antibody does combine with H-chain, and the product has a stronger binding site than exhibited by H-chain alone (*3*). The increased strength could be due to the possibility that part of the binding site resides on the L-chain so that the L-chain and H-chain together give a more complete and stronger binding site. The possibility remains, however, that L-chain combines with H-chain and does not contribute contact amino acids to the site, but it does affect the conformation of the H-chain to give a stronger site (*4*). There has been chemical evidence that the L-chain contributes to the structure of the site, as shown by (i) paired-labeling experiments in which a tyrosyl residue of the L-chain was found to be primarily affected (*5*) or (ii) by affinity-labeling experiments in which both chains are labeled by the affinity-labeling reagent (*6*). Bacteriophage neutralization by specific L-chain has also been reported (*7*).

We now have direct evidence for the presence of part of the binding site on the L-chain, namely, that L-chain contributes contact amino acids to the site. This can be demonstrated by binding measurements with isolated L-chains. In the system described here, antibodies to the 4-azonaphthalene-1-sulfonate group were used. These antibodies bind 4-anilinonaphthalene-1-sulfonate (4,1-ANS) to give an enhanced fluorescence. Fluorescent enhancement was also observed with 4,1-ANS and L-chains, giving evidence for binding. The fluorescence-enhancement effect permits the measurement of a small amount of binding in the presence of a large amount of free ANS as is required here because of the low binding constant of the L-chains for the hapten. The low binding constant apparently explains the difficulty in determining binding by L-chains in other systems.

The preparations were analyzed to insure that binding was not due to contamination by H-chains which would combine with L-chains to form intact antibody.

The fluorescence-enhancement effect depends on the fact that the fluorescent properties of molecules are affected by their molecular environment (*8*).

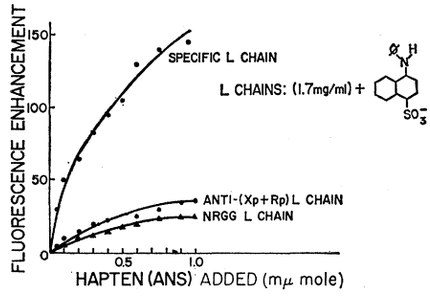

Fig. 1. Specific L-chain binding of 4,1-ANS hapten as shown by fluorescence enhancement. Plot of fluorescence enhancement obtained by adding 4,1-ANS ($5 \times 10^{-6} M$) to 1.7 mg of light chains in an original volume of 1 ml. The final volume after titration was 1.2 ml. The fluorescence values of the original protein solutions were 80 to 90 units on the scale used. The fluorescence for the stock ligand solution ($5 \times 10^{-6} M$) was 8 to 10. Specific L-chains, L-chains prepared from a mixture of anti-Xp antibody and anti-Rp antibody, and also L-chains from normal γ-globulin (NRGG) were used.

Some organic molecules are virtually nonfluorescent in aqueous solution but are highly fluorescent in a nonpolar solvent or when they are bound to certain proteins (*9*). These have been called "hydrophobic fluorescent probes." In 1962 Winkler (*10*) reported that antibody to 4-azonaphthalene-1-sulfonate enhances the fluorescence of 4-(*p*-toludino)naphthalene-1-sulfonates. Yoo *et al.* (*11*) also reported that the above antibody, as well as antibody against 1-azonaphthalene-8-sulfonate, markedly increased the fluorescence of 1-anilinonaphthalene-8-sulfonate and that the emission peak was shifted toward the blue. There was also a highly efficient transfer of excitation energy from the antibody molecule to the bound ligand.

Antibody was induced in rabbits by injecting them in the footpad with bovine γG-4-azonaphthalene-1-sulfonate in complete Freund adjuvant. Several weeks after the first injection, the rabbits were bled. They were then given alternate booster injections and bled frequently. The γ-globulin was prepared by the method of Kekwick (*12*). Antibody was specifically purified by adsorbing it on an immunoadsorbant [prepared essentially by the method of Onoue *et al.* (*13*)] and eluting with $1M$ propionic acid. This purified antibody was reduced and alkylated (*14*), but $0.2M$ mercaptoethanol was used for the reduction and an equivalent amount of iodoacetamide for alkylation. The chains were separated by chromatography on Sephadex G-100 equilibrated with $1M$ propionic acid. The tubes containing the L-chains were pooled. The pooled material was dialyzed against cold distilled water and pervaporated with intermittent dialysis against $0.01M$ propionic acid. The L-chains were then dialyzed against borate buffer at *p*H 8.

The ligand (4,1-ANS) was prepared by the method of Houben and Weyl (*15*) with some modification. It was considered pure as judged by thin-layer chromatography and in that the emission spectrum was independent of excitation wavelength within the range examined. Also the chemical analysis agreed with the molecular formula.

Fluorescence was measured with the Aminco-Bowman spectrofluorometer equipped with a high-pressure xenon arc and a water jacket for temperature control. For measurement, a 1.0-ml portion of the protein solution was placed in a 1-cm square quartz cuvette, and portions of the ligand solution were added from micropipettes. The fluores-

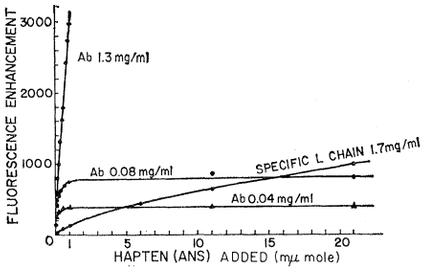

Fig. 2. Comparison of fluorescence enhancement by specific antibody and by specific L-chain. Antibody (Ab) at concentrations of 1.3 mg/ml, 0.08 mg/ml, and 0.04 mg/ml and L-chain with concentration of 1.7 mg/ml were titrated with 4,1-ANS as in Fig. 1, but after the addition of 1 mμ mole of 4,1-ANS as $5 \times 10^{-6} M$ 4,1-ANS, $5 \times 10^{-4} M$ 4,1-ANS was used for further additions. The final volume was 1.24 ml.

cence was measured after each addition. The excitation wavelength used was 365 mμ, and the emission was measured at 435 mμ where the antibody-ligand complex shows a maximum.

The binding of ligand to L-chain is shown in Fig. 1 where the fluorescence enhancement (the increment of fluorescence intensity above the sum of the intensities for the ligand and the protein alone) is plotted against the amount of ligand added to a given amount of specific L-chain. Comparisons were made with L-chains from normal rabbit γ-globulin and with L-chains from a mixture of specifically purified antibody to *p*-azobenzoate (anti-Xp) and antibody to *p*-azobenzenearsonate (anti-Rp). There was a much stronger fluorescence enhancement with the specific L-chains.

The observed binding due to the L-chain was not due to the presence of a small amount of H-chain in the L-chain preparation. This was shown by a comparison of the fluorescence enhancement observed for specific light chain with that observed for small amounts of antibody as well as with small amounts of heavy chain added to the light chain. Furthermore, the concentration of heavy chain in the light chain preparation was shown to be less than 2.5 μg per milligram of light chain by radioimmunoelectrophoresis (*16*).

Figure 2 shows the fluorescence enhancement as a function of ligand added for 1.3, 0.08, and 0.04 mg of antibody in 1 ml. These curves were compared with the fluorescence-enhancement curve for the specific L-chains, 1.7 mg/ml. The binding to the L-chains is much weaker than the binding to antibody, and the curve is therefore quite different in shape. Since the binding to antibody is very strong, the fluorescence enhancement increases rapidly with added ligand until the antibody is almost saturated and until there is no further increase in the fluorescence increment with additional ligand. The initial portion of the curve is very steep. Antibody at 1.3 mg/ml gives a very large fluorescence enhancement, and, at the lowest amount of antibody used (0.04 mg/ml), there is also a similar very steep initial rise in fluorescence enhancement followed by a plateau. The fluorescence enhancement shown by the specific L-chain is quite different. It follows a much less steep slope even though the L-chain is present at the relatively high concentration of 1.7 mg/ml. Thus the fluorescence enhancement of the L-chain preparation was not due to the presence of intact antibody.

We have also found that with added heavy chain, the curves show the steep initial slope observed with intact antibody rather than the more gradual slope shown by our L-chain preparations. This was demonstrated by adding to the samples of L-chain small amounts (0.02, 0.2, and 0.4 mg each) of H-chain from the same pool of antibody. The mixtures were then dialyzed against $1M$ propionic acid for 8 hours in the cold room and then against cold water and cold borate buffer at *p*H 8.0. Light chains alone were similarly treated. No precipitates formed during the treatment. The samples were titrated in the same way as in Fig. 2, and the curves are shown in Fig. 3. The addition of small amounts of heavy chain to the L-chains did change the shape of the titration curves. First, there was a rapid increase of fluorescence enhancement as with intact antibody. This was followed by a more gradual increase that resembles the enhancement by L-chain to which no H-chain had been added. If the fluorescence increment of specific L-chains shown in Figs. 1, 2, and 3 were due to contamination by a small amount of heavy chain, the shape of the titration curves would resemble that of the L-chain sample to which the H-chain had been added. Thus there is no detectable amount of heavy chain in the preparations of light chain by this criterion.

To demonstrate in another way that the binding by the light chain preparation did not result from residual antibody activity due to the presence of intact antibody formed by recombination of a small amount of H-chain contaminant in the light chain preparation, radioimmunoelectrophoresis was carried out (*16*). In this procedure a solution containing 1.6 mg of normal rabbit γ-globulin per milliliter and 1.2 mg of L-chain per milliliter was subjected to electrophoresis on one side of a slide, and a solution of the normal rabbit γ-globulin at the same concentration but containing 3 μg of intact antibody per milliliter on the other side. The patterns were then developed by placing in the trough a mixture of goat antiserum to rabbit heavy chains, goat antiserum to rabbit light chains, and ^{125}I-labeled insulin bearing 4-azonaphthalene-1-sulfonate groups. The stained slide showed the presence of L-chains and globulin on one side of

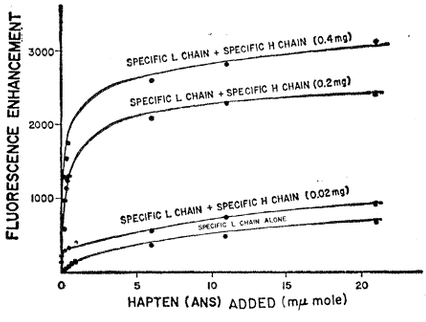

Fig. 3. Effect on fluorescence enhancement of L-chain by the addition of small amounts of H-chain. Specific L-chain (0.9 mg) and H-chain (0.02, 0.2, and 0.4 mg) from the same antibody pool, were mixed individually, and each sample in an original volume of 1 ml was titrated by adding ANS as in Fig. 2.

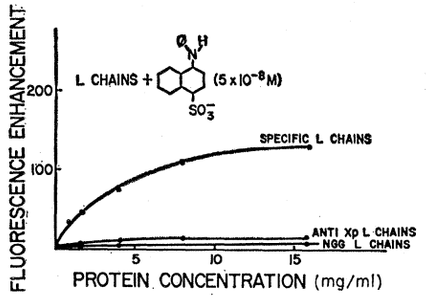

Fig. 4. Effect of L-chain concentration on fluoresence enhancement. The fluorescence enhancement is shown for solutions $5 \times 10^{-8} M$ in 4,1-ANS containing L-chains from specific antibody, L-chains from anti-Xp antibody, and L-chains from normal γ-globulin at the indicated concentrations.

the slide and the presence of only globulin on the other. After radioautography there was no line of exposure corresponding to the position of the normal rabbit γ-globulin in the side where L-chain had been applied, whereas in the other part of the slide a line could be seen which was due to the antibody (3 μg/ml) which had been added to the normal rabbit γ-globulin on that part of the slide. This means that there was less than 3 μg of intact antibody per milliliter in the preparation containing 1.2 mg of L-chain per milliliter.

Although there was a line on the radioautograph corresponding to the L-chain region, this may not be due to specific binding of the ^{125}I-labeled insulin-4-azonaphthalene-1-sulfonate antigen, but may be due to nonspecific binding of this antigen by L-chain in general.

The L-chain preparation was also free of serum albumin because the addition of small amounts of 9-chloro-2-methoxy-acridinylbenzenesulfonate, a fluorescent probe analytical reagent for albumin (*17*), showed no fluorescence enhancement.

The equilibrium constant (K_0) for the binding of L-chain to ligand or ligand to L-chain was calculated to be about 10^{-2} liter/mole. This was done by using the data of Fig. 1 and the value for the fluorescence enhancement of the ligand by binding with L-chains. The latter value was obtained by measuring the fluorescence enhancement of the ligand at a low concentration upon the addition of increasing amounts of L-chain (Fig. 4). From this fluorescence-enhancement value, the free and bound concentrations of ligand on the point in Fig. 1 were calculated, and, by use of the Sips (*18*) equation, an average value for K_0 was found.

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Cytoplasmic and Chloroplast Ribosomes of Chlamydomonas: Ultracentrifugal Characterization

Abstract. *Ribosomes isolated from the cytoplasmic and chloroplast fractions of* Chlamydomonas *were characterized in the ultracentrifuge. The cytoplasmic ribosomes belong to the 80S class of ribosomes, and, like animal ribosomes, dissociate to 60, 50, and 40S subunits. However, like the ribosomes of microorganisms, they contain smaller RNA's, 24 and 16S, and require 0.01 mole of magnesium ions per liter for stability. Chloroplast ribosomes are 70S like those of higher plants but are very unstable. A stable 50S subunit has been observed.*

The comparative biochemistry of ribosomes is relevant both in the study of evolution and in the analysis of ribosomal function. By 1963, bacterial ribosomes were generally accepted (*1, 2*) to have sedimentation coefficients of 70*S* with RNA components of 23 and 16*S*, while the ribosomes of higher plants and animals are 80*S* with correspondingly larger RNA components (for example, 28 and 18*S* in mammals). Since that time, several investigators have reported further distinctions among ribosomes from different classes of organisms.

Taylor and Storck (*3*) showed that the sedimentation coefficients of ribosomes of yeast and fungi are about 80*S*, and they proposed that 70*S* ribosomes are characteristic of procaryotic organisms and 80*S* ribosomes of eucaryotes, from yeast to man. More recently, distinctions among ribosomes have been proposed on the basis of the size of the RNA (*4, 5*) and on ribosomal sensitivity to low concentrations of Mg^{2+} (*6*). Furthermore, higher plants have been shown to contain two classes of ribosomes: 80*S* in the cytoplasm and 70*S* in the chloroplasts (*5, 7, 8*).

Electron microscopic studies (*9*) of *Chlamydomonas* show that the chloroplasts of normal green cells are packed with ribosome-like particles lying free between the lamellae. If these particles are ribosomes, their number may be estimated as at least 25 percent that of the cytoplasmic ones, since the chloroplast represents 50 percent of the cell volume. Thus they should be seen readily in the ultracentrifugal analysis of crude lysates.

We now report ultracentrifugal studies of ribosomes extracted from the cytoplasm and chloroplast of the green alga *Chlamydomonas*, a phytoflagellate considered to be on the main evolutionary line of both the higher plants and the animals (*10*). We have found that the cytoplasmic ribosomes of *Chlamydomonas*, like those of animals and higher plants, are approximately 80*S*, but that, like bacterial ribosomes, they require high concentrations of Mg^{2+} (0.01*M*) for monosome stability. The larger RNA component has a sedimentaion coefficient of 24*S*, closer to that of higher plants (*5*), fungi (*4*), and bacteria (*2*) than to the 28*S* typical of animal ribsomes (*2*). *Chlamydomonas* ribosomes contain about 40 percent RNA (*11*); in this respect they are more like plant and animal ribosomes than they are like those of bacteria.

The chloroplast ribosomes apparently are 70*S*, but they are extremely unstable. They have only been observed as such in homogenates of whole cells. After fractionation only a stable 50*S* subunit has been observed.

Sonic oscillation was used to prepare homogenates of *Chlamydomonas* for immediate examination in the ultra-