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Three-Dimensional Structures of Influenza Virus Neuraminidase-Antibody Complexes

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Three-dimensional structures of influenza virus neuraminidase–antibody complexes

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[Plate 1]

X-ray diffraction analysis of crystals of a monoclonal Fab fragment NC41 bound to a viral antigen, influenza virus neuraminidase, shows an epitope involving five surface loops of the antigen. In addition it reveals an unusual pairing pattern between the domains of light and heavy chains in the variable module of the antibody. We interpret this result to imply that association with antigen can induce changes in the quaternary structure of the Fab, through a sliding of domains at the variable light/variable heavy chains (V_L–V_H) interface. In addition, Fab binding has altered the conformation of some of the surface loops of the antigen.

The structure of the NC10 Fab–neuraminidase complex has now also been solved. It binds an epitope that overlaps the NC41 epitope. In this structure, there is no electron density for the C-module of the Fab fragment, implying it is disordered in the crystal lattice. The implications of these, and other antibody–antigen structures, for immune recognition are discussed.

1. Introduction

Data on the three-dimensional structure of antigen–antibody complexes are now emerging from studies of two different systems, one using the influenza virus antigen neuraminidase (Colman *et al.* 1987*a*), and the other using hen egg-white lysozyme as antigen (Amit *et al.* 1986; Sheriff *et al.* 1987). A number of reviews have already addressed the central findings of these studies (Mariuzza *et al.* 1987; Colman 1988; Davies *et al.* 1988) although the database of structures remains small and may not be truly representative of the vast number of possible antigen–antibody interactions.

Influenza virus neuraminidase is a tetramer of 60 kDa glycosylated polypeptide chains on the surface of the virus (see review in Colman & Ward (1985)). Antigenic variation in influenza viruses is characterized by amino acid substitutions within both the neuraminidase and haemagglutinin viral-membrane glycoproteins. Liberation of neuraminidase from the virus by pronase results in the loss of approximately 80 residues from the *N*-terminus. Particles so solubilized have been used in all X-ray diffraction studies of this antigen, because neither antigenic nor enzymic properties appear affected by this procedure. The three-dimensional structure of N2 subtype neuraminidase has been determined (Varghese *et al.* 1983) and the antigenic and enzymatic sites located (Colman *et al.* 1983).

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We have recently described the structure of neuraminidase complexed with the Fab fragment of antibody NC41 (Colman *et al.* 1987*a*). Some of those results are repeated and/or extended here. The structure of a second complex with the Fab fragment of antibody NC10 has now also been determined, and preliminary findings from that analysis are given here for the first time.

In both of these studies, the neuraminidase is that of influenza virus subtype N9 (Baker *et al.* 1987), the structure of which is very similar to that of subtype N2 (Varghese *et al.* 1983). The N9 structure refinement is in progress, with an *R*-factor of 0.24 at 2.3 Å† resolution. Some surface regions remain ambiguous, frustrating a high-resolution comparison of the complexed and uncomplexed neuraminidase structures.

2. Neuraminidase–NC41 Fab structure

Crystallographic refinement of the N9–NC41 complex has not yet converged. The current *R*-factor for data in the range of 6–2.9 Å is 0.23, and data to 2.6 Å resolution are available but not yet included in the refinement.

Epitope

A C_α trace of antibody and antigen are shown in figure 1*a*, plate 1, indicating that the epitope is discontinuous and comprised of five separate peptide segments including about 17 amino acid residues. In addition it now appears that complementarity-determining region (CDR) H1 of the NC41 Fab may interact with carbohydrate covalently attached to Asn 200 of a neighbouring subunit of the tetrameric antigen. The chemical composition of this sugar in N9 is not yet known, and it is therefore not clear whether certain features of the density in this region must be ascribed to antibody or antigen. Because the carbohydrate moieties of influenza virus antigens are derived from host cell processes, they are usually considered not to be antigenic, at least in the host in which the virus is grown. However, they could clearly become antigenic in the context of a foreign protein structure, and their involvement in antibody binding would not be surprising. Some data have demonstrated that carbohydrate might mask proteinaceous antigenic sites on influenza virus haemagglutinin (Daniels *et al.* 1983).

Amino acid sequence changes at positions 367 (Ser to Asn), 369 (Ala to Asp), 370 (Ser to Leu), 400 (Asn to Lys) and 432 (Lys to Asn) abolish binding of NC41 to neuraminidase, whereas changes at positions 329 (Asn to Asp) and 368 (Ile to Arg) reduce binding (Webster *et al.* 1987). All these amino acids contribute to the surface area of the antigen that is buried when NC41 antibody binds. The only mutant for which no effect on binding is observed is at position 220, well outside the binding site for this antibody.

Several experiments have now shown directly that single amino acid sequence changes on antigens that are sufficient to abolish antibody binding result only in local perturbation of the antigen structure (Knossow *et al.* 1984; Varghese *et al.* 1988; see also below). We have discussed elsewhere an explanation for the observation that a change of only one amino acid in an interface of 17 or more can prejudice the formation of the complex through that interface (Colman *et al.* 1987*b*). The argument rests on the strength of the binding constant, but for values of around 0.1 μM, the loss of a single hydrogen bond could reduce the affinity to around 0.1 mM, although this does not imply that the other 16 or so amino acids in the interface play

† 1 Å = 10^{-10} m = 10^{-1} nm.

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no role. Rather, their contributions are essential in offsetting the various negative contributions to the stabilization of the complex that derive from factors such as rotational entropy and hydrophobic free energy.

Changes in antigen structure

A comparison of the complexed and uncomplexed N9 structures indicated shifts of the order of 1 Å in the 370 loop of neuraminidase (Colman *et al.* 1987*a*). Elsewhere, local structural changes in the epitope of the antigen, involving mostly sidechain rotations, are becoming apparent. Further analysis of these and other possible structural alterations in the antigen resulting from antibody binding awaits completion of the refinement.

Changes in antibody structure

In the absence of a structure of the uncomplexed NC41 Fab fragment, no hard statements can be made on this subject. Comparison with other Fab fragment structures can, at best, suggest possibilities.

Many of the CDR loops of the antibody conform to the canonical conformations described by Chothia & Lesk (1987). For example, L3 has a *cis*-proline at position 95, and the mainchain conformation and the positions of the CDR sidechains are similar to that seen in McPC603 (Satow *et al.* 1986). Some uncertainties remain over the H1 loop structure, in consequence of ambiguities in the neuraminidase carbohydrate at Asn 200, referred to above. We cannot yet eliminate the possibility of structural changes in the CDRs of the antibody as an important aspect of the interaction with antigen.

Another level of antibody structure that might be influenced by binding to antigen is the quaternary structure of the V module, i.e. the interaction between the V_L and V_H domains. The Interface Adaptor Hypothesis (Colman *et al.* 1987*a*; Colman 1988) states that the interface between V_L and V_H domains has been designed as a flexible adaptor, allowing antigen to mould slightly different CDR surfaces by repositioning the V_L CDRs with respect to the V_H CDRs. The basis for the hypothesis is the observation that interactions between the variable domains are structurally unique (Novotny & Haber 1985; Chothia *et al.* 1986) and are influenced by contributions from the CDR loops at the distal (antigen-binding) end of the V_L–V_H interface (Davies *et al.* 1975). This implies a 'softness' in the interface, which may well be exploited when the CDR surface binds a macromolecular antigen.

The V_L–V_H domain pairing in the NC41 antibody has been compared with that in other Fab fragments, and reported to be an outlier within that family of interactions (Colman *et al.* 1987*a*). Refinement of the structure has required a softening of that view, because differences in the domain pairing between NC41 and the Fabs Kol (Marquart *et al.* 1980), McPC603 (Satow *et al.* 1986) and J539 (Suh *et al.* 1986) are currently 8.4°, 5.5° and 11.6° respectively. However, small differences in the pairing can result in displacements of 3 or 4 Å in the relative position of the heavy- and light-chain CDRs, and in this way the precise pattern of interactions between the two variable domains directly influences the antigen specificity of the antibody. Thus the hypothesis that the V_L–V_H pairing can be modulated by engaging antigen at the CDR surface (Colman *et al.* 1987*a*; Colman 1988) is not sensitively assayed by measuring the pairing through framework residues alone, as was done previously (Colman *et al.* 1987*a*).

The only convincing test of this aspect of induced fit of antibody to antigen will come from determination of the structure of complexed and uncomplexed Fab fragments. In this regard, neither of the Fab fragments that crystallize as complexes with neuraminidase has yet yielded crystals suitable for X-ray diffraction analysis in the absence of antigen.

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Isosteric binding to a mutant neuraminidase

The NC41 Fab fragment crystallizes when complexed with the monoclonal variant of N9 known as OX2, Asn 329 to Asp (Laver *et al.* 1986). Residue 329 is at the edge of the epitope for NC41. These crystals are isomorphous with crystals of wild-type complex and diffraction data from them have been interpreted as implying that the binding of NC41 to wild-type and variant neuraminidase is isosteric, although the binding affinity for the variant is less than for wild-type (Laver *et al.* 1986; Colman *et al.* 1987*a*). A Fourier map at 3 Å resolution of the difference (variant complex minus wild-type complex) amplitudes with current model phases shows the largest negative peak (6 σ) coincident with the position of the Asn 329 sidechain in the wild-type complex. An adjacent positive peak (4 σ) might be interpreted as the position of the Asp 329 sidechain in the variant complex, rotated approximately 180° from the position occupied by Asn 329 in wild type, but it is not greater than other unexplained features of the same peak electron density. (The difference map in this case was calculated by using only one half of the theoretically observable data between 10 and 3 Å.)

A difference electron-density map of the two neuraminidase variants, OX1 minus OX2 (where OX1 is Ser 370 to Leu) shows significant electron density only at and near the position of the sidechain of residue 370 (9.4 σ) and no features above 3.0 σ around residue 329. This result shows that the substitution of Asn to Asp at position 329 results in no detectable redistribution of electron density in neuraminidase in the absence of NC41 antibody. Thus the altered position of Asp 329 in complex with NC41 may be interpreted as resulting from combination with the antibody.

The present result is consistent with the earlier interpretation, namely that local changes in the interface accommodate the substitution of Asn for Asp at position 329, and, in a manner which remains to be determined, reduce the binding affinity. No gross rearrangement of antibody on antigen occurs. This observation provides a starting model for visualizing the structural effects of somatic mutation during secondary immune responses where point mutations in the antibody are selected for higher binding affinity to the antigen. These mutations may exert their influence on binding in a purely local fashion, as observed here.

3. Neuraminidase–NC10 Fab structure

The N9 neuraminidase used here was isolated from an influenza virus found in whales in Maine, U.S.A. in 1984. It differs from the neuraminidase of virus from tern (isolated in Australia in 1975 and used in the NC41 complex study) by 14 amino acids (Air *et al.* 1987), mostly on the underside of the globular neuraminidase head (Varghese *et al.* 1983). The NC10 antibody was raised against the tern virus neuraminidase (Webster *et al.* 1987), but is seen to bind an epitope where the tern and whale virus neuraminidases are identical in sequence. Crystals of tern N9–NC10 Fab have also been grown, but are disordered and unsuitable for structure analysis.

Structure determination

Crystals of complex are tetragonal prisms belonging to the space group I422 with unit cell dimensions $a = b = 171.5$ Å, $c = 160.2$ Å. The structure was determined by a combination of Patterson search and isomorphous replacement procedures, with potassium platinum tetrachloride and diamino-dinitro-platinum as heavy atom reagents. Initially, the orientation

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of the neuraminidase tetramer around the fourfold crystallographic symmetry axis was determined by searching the Patterson with the known fourfold symmetric neuraminidase structure. Data between 6 and 4 Å were used with an integration radius of 50 Å, and the highest correlation was at the level of 4.5 σ. An R-factor search for the position of the tetramer along the fourfold axis yielded a single solution, and one which was consistent with packing requirements. The correlation coefficient between the observed and calculated structure factors for data between 6 and 5 Å peaked at 0.24 at the correct translation position, compared with background levels of less than 0.1. This result may be less surprising in the light of the ultimate finding that one half of the Fab fragment (the C-module) is disordered in the crystals, and the search fragment, the neuraminidase tetramer, represents effectively two thirds, and not one half, of the scattering volume. Although the derivatives were of marginal quality, in combination with phasing from the neuraminidase component and from solvent flattening an interpretation of the Fab structure, at least in the V-module, has been possible. No electron density has been observed for the C-module of the Fab fragment, although all solvent flattening procedures assumed protein occupancy by volume of the crystals in excess of 41%, the expected figure for 100 kDa of protein per asymmetric unit. Restrained crystallographic refinement of the structure has so far yielded an R-factor of 0.30 for data between 6 and 3 Å for a model with bond-length root mean square deviations from ideality of 0.017 Å.

Protomer structure

The general shape of the tetrameric N9–NC10 Fab protomer is similar to that of the NC41 complex (and other complexes examined by electron microscopy (Tulloch *et al.* 1986)), appearing as an inverted table where the tetrameric antigen is the table top and the Fab fragments the four legs. Packing of the protomers is as suggested in Tulloch *et al.* (1986) for the N9–NC35 Fab crystals, which share the same space group and similar cell dimensions with the N9–NC10 complex crystal. Along the fourfold axis, two neuraminidase heads make close contact via their undersides (i.e. proximal to the viral membrane) surface, which in the present description constitutes the table top. The other type of contact along the fourfold axis results from two sets of table legs, Fab fragments, interdigitating and interacting through the V-modules of the Fab. Crystal growth does not require the participation of the disordered C-module.

Absence of electron density for the C-module is reminiscent of other studies of antibody structures where flexibility of polypeptide segments between globular modules is the cause (Colman *et al.* 1976; Ely *et al.* 1978). In the lysozyme–HyHEL5 complex (Sheriff *et al.* 1987), two crystal forms grow under the same conditions, each with a different elbow angle between V- and C-modules. The presence of even a limited number (2 or 3) of different elbow angles in the crystals of the N9–NC10 complex would suffice to make visualization of the C-module difficult at this stage.

Epitope

The epitope recognized by the NC10 antibody is, to a large degree, common to that seen by the NC41 antibody (figure 1*b*). The centre of the epitope appears to be a little further from the particle fourfold axis in NC10 than in NC41, but contacts with surface loops around residues 330, 342, 369, 401 and 432 appear possible. These observations are consistent with binding studies showing reduced binding of NC10 to neuraminidase variants with sequence changes at positions 329, 369, 370 and 432 (Webster *et al.* 1987).

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It is also possible that the carbohydrate at Asn 200 on a neighbouring subunit will form part of the NC10 epitope. Although the two antibodies, NC10 and NC41, are binding similar surfaces, the arrangements of the two antibodies on that surface are strikingly different (figure 1*a*, *b*). Thus whereas of all the NC41 CDRs L1 is most remote from the antigen, L2 and H1 of NC10 are most distant from the epitope. These observations on the differential involvement of the six CDRs in engaging the epitope provide one rationalization for the observations of one antibody servicing two different antigens (Srinivisappa *et al.* 1986).

Antibody structure

Analysis of the V_L–V_H pairing awaits completion of the refinement, because the magnitudes of the differences between NC41 and other Fabs have altered by several degrees during refinement of that structure. Modelling of the CDRs of the NC10 antibody is not yet complete, but will eventually allow a detailed comparison of the interactions between the neuraminidase and the two antibodies, in this case indicating how one largely common antigenic surface can accommodate two different antibodies.

4. Summary

The molecular details of antigen–antibody interactions that are emerging appear to be consistent with a larger body of data on protein–protein interactions. The fundamental stereochemical issues include flexible/rigid interactions, complementarity, buried surfaces and adaptibility of subunit interfaces. These factors, and their role in immune recognition, have been discussed in more detail elsewhere (Colman 1988).

The studies reported here are addressing aspects of both the strengths and weaknesses of the immune system. On the one hand, two different antibodies are capable of binding a common (or at least substantially overlapping) epitope, and in addition at least one amino acid sequence substitution (Asn 329 to Asp) within that epitope does not greatly affect binding. On the other hand, there are now many examples of single amino acid sequence changes that effectively abolish antibody binding to antigen.

Conformational changes in antigen are a possible (Colman *et al.* 1987*a*; Sheriff *et al.* 1987) but not essential (Amit *et al.* 1986) element of binding by antibody. Whether the small changes in antigen structure observed to date are representative of the upper limit of structural alteration that an antibody can cause in an antigen remains to be seen.

No direct evidence yet exists for conformational change in an antibody on binding an antigen, but the very symmetry of the interaction, involving as it does in these cases simply a protein–protein interaction, suggests that such structural changes are likely, at least at the level of CDR loop structures, and possibly even at the level of V_L–V_H interactions. For these reasons we have suggested that antigen–antibody interactions have some of the character of a handshake.

Description of plate 1

Figure 1. (*a*) C_α drawing of one neuraminidase subunit–NC41 Fab interaction viewed normal to the molecular fourfold axis (at left rear). The C-module of the Fab fragment is observed in this structure but has been omitted from the figure. (*b*) Similar drawing of the interaction of neuraminidase with the NC10 Fab fragment. In this case, the C-module of the Fab fragment appears to be disordered within the crystal. (Neuraminidase yellow, epitope white, VH domain purple, VL domain blue, CDRs orange.)

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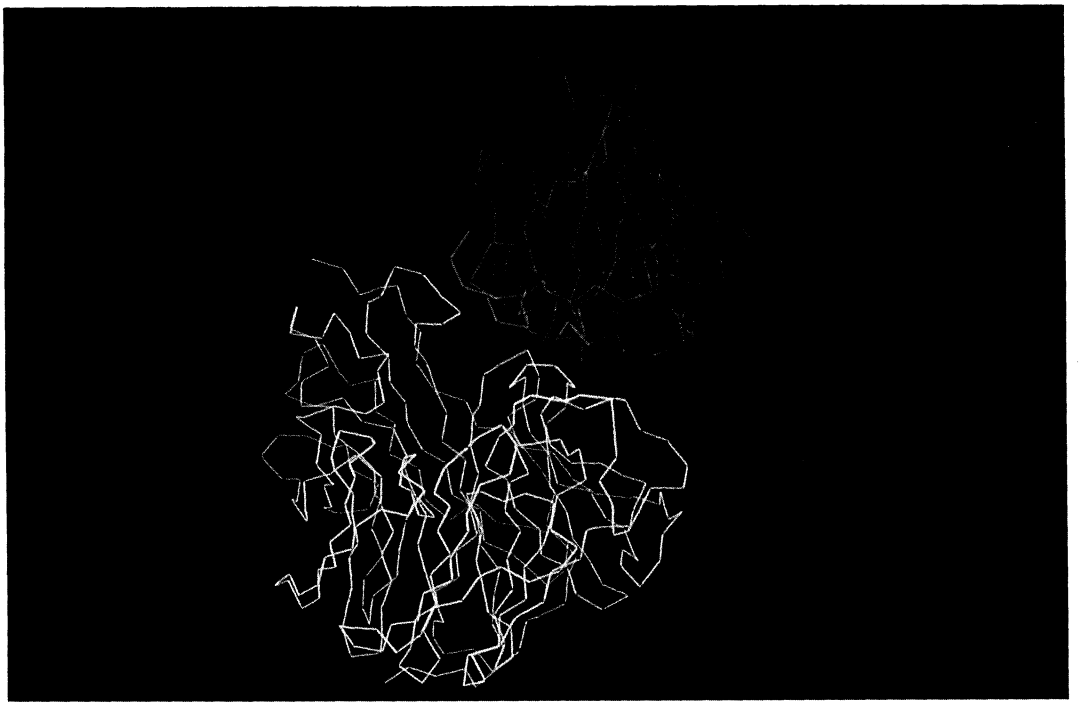

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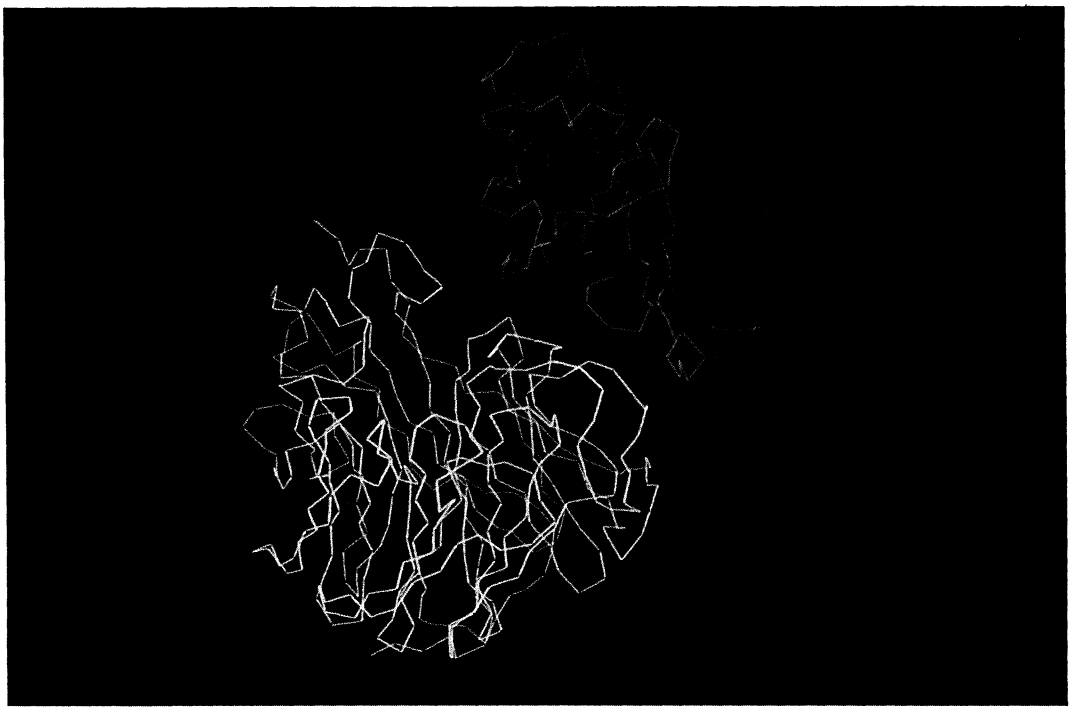

FIGURE 1. For description see opposite.

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The extent to which antibody and antigen might induce structural changes in each other is likely to be limited. The immune system is clearly very, though not singularly, specific, and that specificity should not be unduly compromised by the economy that enables one antibody to bind more than one antigen.

In cellular immune responses, as distinct from humoral responses mediated by antibody, there are two levels of interaction where the induced fit of interactants may be important. The first concerns the binding of processed antigen to histocompatibility antigen (HLA). The structure of HLA-A2 is being determined in two different crystal forms (Bjorkman *et al.* 1987) which may in itself provide some measure of the structural heterogeneity of the processed-antigen binding site, although the preferred comparison would be of the structures of the same HLA molecule complexed to two significantly different bound processed antigens. It would not be inconsistent with other observations on protein–protein interactions (see, for example, Colman 1988) if the helix/β-sheet contacts in the HLA structure were subject to small modifications during the course of binding processed antigen in the groove between the two helices (Bjorkman *et al.* 1987). Such modifications could be somewhat different for each different processed-antigen–HLA interaction, and may be one mechanism by which a small number of different HLA molecules are able to bind a large number of very different peptides.

On the second level of cellular immune responses, T-cell receptor molecules may be subject to conformational changes on binding HLA–processed-antigen complex. The structural character of such induced changes could be expected to be similar to those anticipated in antibody–antigen complexes, i.e. in the CDR-equivalent loop structures or in V-module-equivalent quaternary structure, as discussed above for antibodies.

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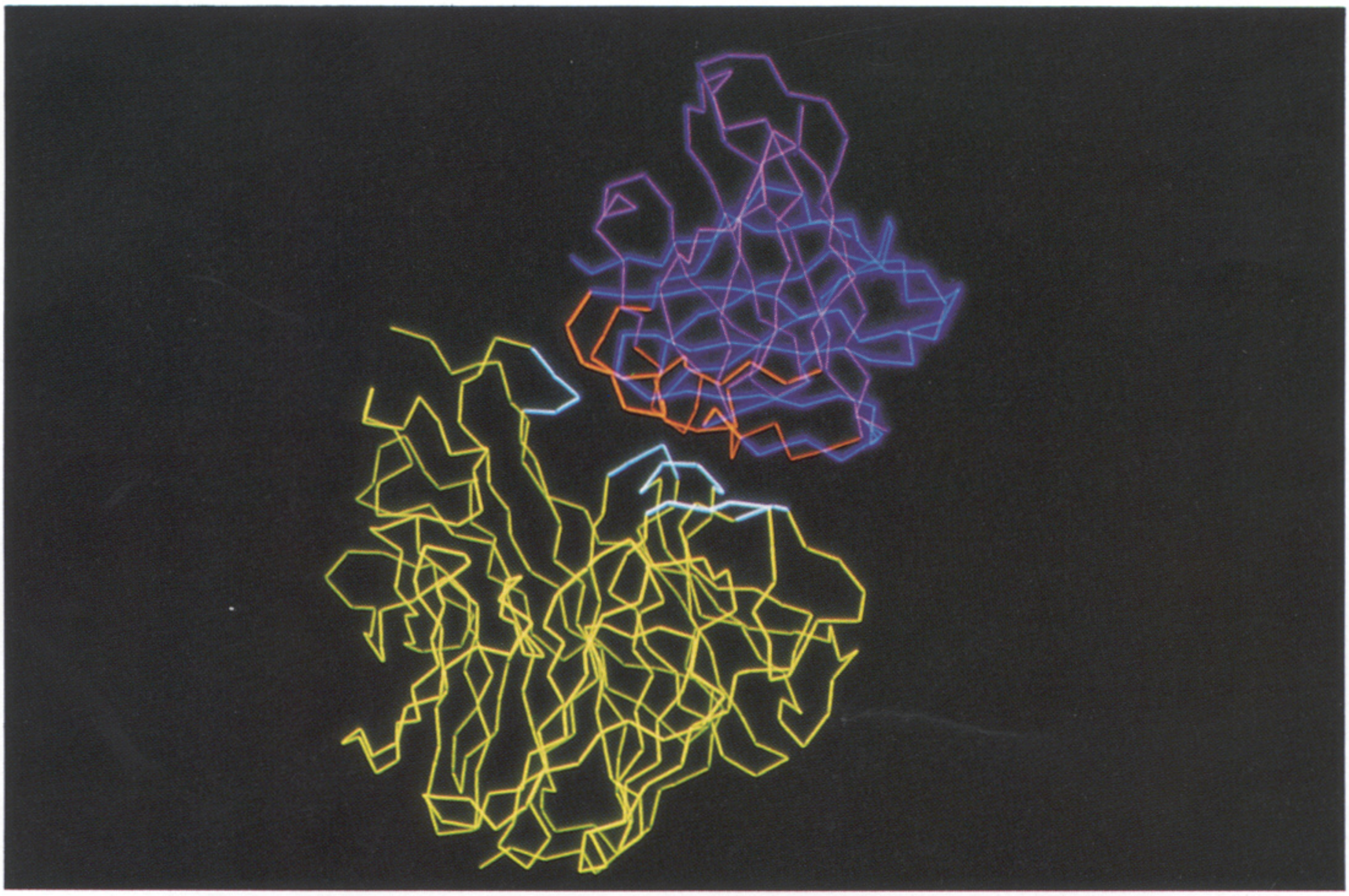

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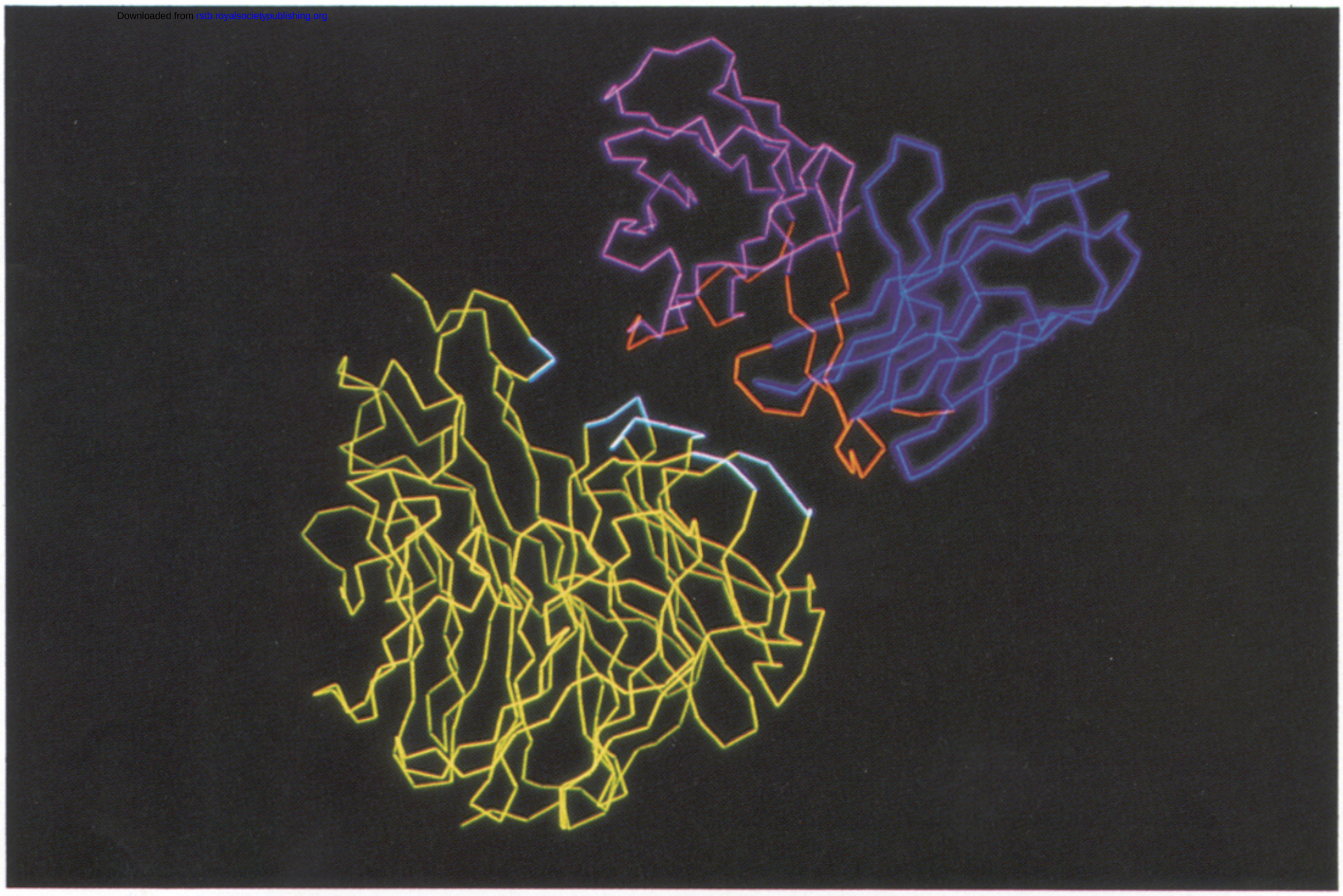

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FIGURE 1. For description see opposite.