Anti-metatype Antibody Stabilization of Fv 4-4-20 Variable Domain Dynamics*

(Received for publication, January 19, 1996, and in revised form, March 4, 1996)

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Anti-metatype (anti-Met) antibodies are immunoglobulins that specifically recognize and stabilize antibodies in their liganded or metatypic state, but lack specificity for either the hapten or the unliganded antibody. Autologous anti-Met antibodies were previously observed in vivo, suggesting that a metatypic autoantibody response could play a physiological role in the immune network, e.g. controlling the clearance of immune complexes from circulation. The first elicited anti-Met antibodies were against the fluorescein-ligated high affinity murine anti-fluorescein monoclonal antibody 4-4-20. The fluorescein-hapten system has proved to be an invaluable tool for both the recognition and characterization of the metatypic response by utilization of its spectral properties. In this investigation, hydrostatic pressure measurements, in conjunction with fluorescence spectroscopy, were performed on the recombinant Fv derivative (Fv 4-4-20) of the high affinity anti-fluorescein monoclonal antibody 4-4-20 complexed to anti-Met antibodies to study the influence of anti-Met antibodies on Fv 4-4-20 intervariable domain interactions. Anti-Met antibodies bound to liganded Fv 4-4-20 were observed to cause a change in the fluorescence properties of fluorescein that was not observed when anti-Met antibodies were bound to the liganded parent immunoglobulin. The variation of these spectral properties upon addition of anti-Met antibodies was shown to be correlated with dissociation of the variable domains in Fv 4-4-20 in response to its interaction with the anti-Met antibody. The ability to cause variable domain dissociation was dependent on whether monoclonal or polyclonal anti-Met antibodies were bound to the metatype. A model was proposed that elucidated the interaction of anti-Met antibodies, polyclonal and monoclonal, with variable domains of the primary anti-antigen antibody.

Anti-metatype (anti-Met)1 antibodies are immunoglobulins that specifically recognize and stabilize antibodies in their liganded state (or metatypic antibodies), but lack specificity for the hapten (1). Autologous anti-Met antibodies were observed in vivo, suggesting that a metatypic autoantibody response could play a physiological role in the immune network, e.g. controlling the clearance of immune complexes from circulation (2). The first anti-Met antibodies were elicited against the liganded high affinity murine anti-fluorescein monoclonal antibody 4-4-20 (1, 3). Since then, investigators have elicited anti-Met antibodies against immunoglobulins specific for other chemical compounds or peptides (4–6). These anti-Met reagents have demonstrated utility in the development of improved analytical assays for small molecules and verified that metatype-anti-Met complexes are not solely a property of anti-fluorescein antibodies. However, detailed information about the effect of anti-Met antibodies on the metatype, which would provide a better understanding of the metatypic response, remains sparse.

The fluorescein-hapten system has proved to be an invaluable tool for both the recognition and characterization of the metatypic response by utilization of its spectral properties (7). Comparison of solution- and solid-phase assays of mAb 4-4-20 and mAb 4-4-20-anti-Met complexes (8–17) has yielded important information about the effect of the anti-Met antibodies on the metatype. For example, dissociation rate analyses demonstrated that the off-rate of the hapten from the metatype is decreased in the metatype-anti-Met complex (18). D2O/fluorescein fluorescence enhancement studies showed that delay in ligand dissociation involves a decrease in rate of conformational fluctuations in the metatype antibody variable domains when part of the metatype-anti-Met complex (19). Solid- and solution-phase studies of anti-Met antibody binding to the single chain derivative scFv 4-4-20 (20, 21), a genetic construct that links the variable domains of a corresponding monoclonal antibody by a polypeptide linker (22, 23), yielded similar results. This indicated that metatopes (or anti-Met-binding site) on the metatype that affected hapten dissociation are specific to the variable domains (18). In summary, it is clear that anti-Met antibodies bound to a metatype variable domain have a significant damping effect on metatypic dynamics, which prolongs the residence time of the hapten in the metatype active site.

An assessment of the contributions of various metatype structural components to the metatype/anti-Met interaction remains to be addressed: 1) the role of constant domains of the metatype antibody and 2) the role of intervariable domain interactions. mAb 4-4-20-anti-Met complexes were previously noted to have an identical fluorescence intensity quenching maximum (Qmax) and steady-state polarization (18) as the native liganded mAb 4-4-20. In contrast, scFv 4-4-20-anti-Met complexes showed decreased fluorescein fluorescence Qmax and polarization values (24) with regard to the parent immunoglobulin. This indicated that anti-Met antibodies caused an additional disturbance at the active-site level of the anti-hapten antibody in the scFv 4-4-20-anti-Met complex that did not occur in the mAb 4-4-20 anti-Met complex. A process that would have resulted in the decrease of fluorescein Qmax and polarization in

* Work performed in the Laboratory for Fluorescence Dynamics was supported jointly by National Institutes of Health Division of Research Resources Grant RR03155-01 and the University of Illinois at Urbana-Champaign. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: anti-Met, anti-metatype; mAb, monoclonal antibody; scFv, single chain Fv.
liganded scFv 4-4-20 was interdomain dissociation. Another process was the dissociation of fluorescein from the metatype active site. In total, these observations inferred that constant domains influenced the steady-state metatype anti-Met complex equilibrium by stabilizing the metatype against the effects of binding an external reagent such as the anti-Met antibodies.

To investigate this further, hydrostatic pressure measurements, in conjunction with fluorescence spectroscopy, were performed with the recombinant Fv derivative (Fv 4-4-20) (25) of the high affinity mAb 4-4-20 complexed to anti-Met antibodies (Fv 4-4-20-anti-Met complex) to study the effect of the absence of metatype constant domain and intervariable domain interactions on the stabilizing influence of anti-Met antibodies. Fv 4-4-20 was an ideal system for this study. Fv fragments are composed of heavy (VH) and light chain (VL) variable domains bound by noncovalent interactions, thus allowing investigation of variable domain interactions in the absence of the constant domains of mAb or the polypeptide linker of scFv. The recombinant Fv 4-4-20 derivative was recently constructed, expressed, and characterized using solid- and solution-phase assays and was found to be identical to scFv 4-4-20 in all respects (25). In this investigation, similar to scFv 4-4-20 (24), Fv 4-4-20-anti-Met complexes showed decreased fluorescein fluorescence Qmax and steady-state polarization compared with mAb 4-4-20. These spectral properties were shown to be correlated with dissociation of the variable domains of the metatype in response to interaction with the anti-Met antibody. Although anti-Met antibodies caused a decrease in Fv 4-4-20 variable domain association, hydrostatic pressure measurements demonstrated that when the hapten was locked into the Fv 4-4-20-anti-Met complex, it was stabilized against dissociation relative to the native liganded Fv 4-4-20. Dissociation of the variable domains observed with Fv 4-4-20 when bound by anti-Met antibodies did not occur with immunoglobulins or Fab fragments because the constant domains play a significant role in stabilizing intervariable domain interactions. In vivo, dissociation of Fv domains by autologous anti-Met antibodies may explain the enhanced degradation of Fv compared with other immunoglobulin proteins stabilized by covalent bonds (26, 27).

RESULTS

Sample Preparation—Aliquots of the Fv 4-4-20 active site using the isothiocyanate derivative (isomer I) of fluorescein were serially diluted (1:2) over the concentration range of 23.5 μM to 4 nM. The domain dissociation was calculated using Equation 1 and was plotted as a function of Fv 4-4-20 protein concentration. Fv 4-4-20 concentrations used in Fig. 2 were corrected for the R value or the amount of affinity-labeled protein of Fv affinity labeling. The concentration of fluorescein was determined on a Beckman DU-64 spectrophotometer using absorbance at 492 nm and an extinction coefficient (ε 492) of 72.000 cm−1 μM−1. The R value or the amount of affinity-labeled protein was calculated as the ratio of fluorescein to Fv 4-4-20 protein concentration. The concentrations for the polyclonal and monoclonal 4A6 anti-Met antibodies were constant at 200 and 118 μM, respectively. Fluorescein was covalently coupled in the Fv 4-4-20 active site using the isothiocyanate derivative (isomer I) of fluorescein (Sigma). A 1.1 molar excess of fluorescein isothiocyanate was incubated with Fv 4-4-20 for 4–5 h with agitation at 37°C. Protein samples were extensively dialyzed against phosphate-buffered saline to remove free fluorescein isothiocyanate.

RESULTS

Binding and Titration Curves—Fig. 1 shows the effect of polyclonal and monoclonal anti-Met antibodies on the fluorescein anisotropy of a constant concentration of liganded Fv 4-4-20. The concentration of liganded Fv 4-4-20 with an initial anisotropy value of 0.355 ensured that all the fluorescein was bound. Increasing concentrations of monoclonal anti-Met antibody decreased the anisotropy of fluorescein to a value known to be indicative of free fluorescein. Polyclonal anti-Met antibodies also decreased the anisotropy of fluorescein from its initial value to a value that leveled off significantly above that obtained with equal monoclonal antibody.

In Fig. 2, affinity-labeled Fv 4-4-20 was used to monitor the effect of increasing anti-Met antibody concentrations on the...
intervariable domains by observing its effect on the Fv dimer dissociation concentration. Both monoclonal and polyclonal anti-Met antibodies caused a decrease in fluorescein anisotropy in affinity-labeled Fv 4-4-20 compared with that in the absence of anti-Met antibodies, indicating that domain-domain dissociation had occurred. As in Fig. 1, the monoclonal antibody had a greater effect on the fluorescein fluorescence anisotropy compared with the polyclonal antibody.

Hydrostatic Pressure Fluorescence Measurements—The effects of the anti-Met antibody on liganded Fv 4-4-20 variable domains were also monitored using steady-state fluorescence spectroscopy in conjunction with hydrostatic pressure. To ensure that the change in intensity of the fluorescein fluorescence was not due to a first-order effect for liganded Fv 4-4-20, high pressure steady-state measurements were performed as a function of liganded Fv 4-4-20 concentration. Fig. 3 shows the comparison of fluorescein fluorescence recovery for two ratios of Fv 4-4-20 to a constant concentration of fluorescein. The protein concentration dependence of hydrostatic pressure-induced fluorescein intensity recovery indicated that the intensity increase was at least a bimolecular event (34).

Pressure-induced dissociation of fluorescein from the active site of liganded scFv 4-4-20 had previously been confirmed by Coelho-Sampaio and Voss (14). In that study, it was shown that when hydrostatic pressures were applied to liganded scFv 4-4-20, the increase in fluorescein fluorescence intensity below 1.6 kilobars was solely due to fluorescein dissociation. Fig. 4 shows comparative pressure-induced dissociation curves for liganded Fv 4-4-20 and liganded scFv 4-4-20 at the same concentrations of active site and fluorescein. The identity of these two curves indicated that dissociation of fluorescein from the active site of Fv 4-4-20 was not due to the variable domain dissociation that would have been expected to occur in liganded Fv 4-4-20 because it was devoid of linker.

On the other hand, pressure-induced dissociation experiments performed on affinity-labeled Fv 4-4-20 as a function of protein concentration confirmed that the fluorescein fluorescence increase was due to a first-order reaction (data not shown). This was an expected result since the fluorescein covalently binds to the active site and cannot dissociate. Pressure was applied to affinity-labeled scFv 4-4-20 at an equal protein concentration to compare the role of the linker in pressure-induced fluorescein fluorescence recovery on affinity-labeled derivative antibodies. The intensity recovery for equal concentrations of affinity-labeled Fv 4-4-20 and affinity-labeled scFv 4-4-20 active sites demonstrated more intensity recovery for affinity-labeled Fv 4-4-20 (Fig. 5). The smaller fluorescence recovery for affinity-labeled scFv 4-4-20 compared with affinity-labeled Fv 4-4-20 indicated that the first-order reaction may be restricted by the linker. It was therefore concluded that the first-order reaction involved rearrangement of the fluorescein microenvironment, which was more restricted in affinity-labeled scFv 4-4-20.

Anti-Met antibodies were added to liganded Fv 4-4-20 to observe the effect on fluorescein dissociation as a function of pressure (Fig. 6). Comparison of the fluorescein dissociation of liganded Fv 4-4-20 with and without polyclonal anti-Met antibodies demonstrated that anti-Met antibodies hindered dissociation of fluorescein from the active site, as previously observed with liganded scFv 4-4-20 by Coelho-Sampaio and Voss (24). Fig. 7 shows the effect of pressure on fluorescein fluorescence enhancement of affinity-labeled Fv 4-4-20 and affinity-labeled scFv 4-4-20 with polyclonal anti-Met antibodies. Anti-Met antibodies stabilized affinity-labeled Fv 4-4-20 and affinity-labeled scFv 4-4-20 against fluorescein fluorescence enhancement in the two samples equally.

**DISCUSSION**

Fv 4-4-20, previously constructed and characterized by Mallender et al. (25), may be viewed as a protein dimer. It exists as two pleated \( \beta \)-sheet variable domains of \( \sim 12-13 \) kDa lacking...
the interdomain polypeptide linker of scFv 4-4-20. The crystal structure of Fab 4-4-20 (35, 36) revealed a total buried surface area of 1366 Å² using van der Waals (119), hydrogen bond (6), and ion pair (1) interactions between the two VL and VH domains for association. Hydrostatic pressure had previously been shown to be a means of separating dimer subunits without changing solvent parameters (33) or causing substantial alteration of protein tertiary conformations at pressures below 5 kilobars (34). The results of Fig. 4 are in agreement with conclusions made by Mallender et al. (25) that Fv 4-4-20 antigen binding characteristics are identical to those of scFv 4-4-20. At low concentrations, the linker in scFv 4-4-20 minimizes dissociation of the variable domains compared with Fv 4-4-20. However, at antibody concentrations above the K_d, the interdomain interactions composed of the previously mentioned van der Waals, hydrogen bond, and ionic pair interactions appear sufficient to stabilize the domains against interdomain dissociation as evidenced by the lack of discernible difference between the derivative antibodies under application of hydrostatic pressure.

The addition of polyclonal and monoclonal anti-Met antibodies to liganded Fv 4-4-20 caused a decrease in fluorescein fluorescence Q_max and polarization when bound by anti-Met antibodies was examined more fully. Coelho-Sampaio and Voss (24) attributed variation in fluorescence Q_max and polarization to idiotype antibodies in the polyclonal anti-Met reagent. Anti-Met antibodies were elicited against a specific primary ligand-antibody by immunization of a host with the affinity-labeled antibodies (18, 19). Anti-idiotype antibodies were specific for unliganded antibodies and were generated in a similar manner. Anti-idiotype antibodies present in the anti-Met reagent would bind to the unliganded antibody, shifting the equilibrium between the liganded and unliganded anti-fluorescein antibodies. The decrease in Q_max would result from a greater amount of free fluorescein than that present in the absence of anti-Met antibodies. This explanation for the change in fluorescein fluorescence parameters upon addition of anti-Met antibodies was not entirely satisfactory because the same anti-Met reagent caused no appreciable effect on the Q_max and λ_max shift of liganded mAb 4-4-20. The absence of any variation in fluorescein fluorescence Q_max and polarization upon addition of anti-Met antibodies to liganded mAb 4-4-20 showed that the delay on the off-rate by anti-Met antibodies was not a consequence of new bonds formed between antigen and the active site. Conversely, the effect on the Q_max and polarization of liganded scFv 4-4-20 bound by anti-Met antibodies demonstrated that constant domains played an important role.

To examine the mode of the effect of anti-Met on the fluorescence of liganded Fv 4-4-20, increasing concentrations of anti-Met antibodies were added to liganded Fv 4-4-20 (Fig. 1). Monoclonal anti-Met antibodies, when present in large
excess, decreased the fluorescence polarization of liganded Fv 4-4-20 to a value that correlated with free fluorescein. Polyclonal anti-Met antibodies decreased both $Q_{\text{max}}$ and polarization, but not as efficiently as monoclonal antibodies. The decrease in polarization observed with the monoclonal antibody ensured that the changes in $Q_{\text{max}}$ and polarization were not due to anti-idotype antibodies. To probe the effect of anti-Met antibodies on the intervariable domain binding affinity, a constant concentration of anti-Met antibodies was added to increasing amounts of affinity-labeled Fv 4-4-20, and the fluorescein fluorescence anisotropy was plotted in the form of a binding curve (Fig. 2). In affinity-labeled Fv 4-4-20, fluorescein was covalently bound to a lysine in the Fv 4-4-20 active site. A decrease in fluorescein fluorescence $Q_{\text{max}}$ or polarization upon addition of anti-Met was possible only if the domains dissociated. At concentrations of Fv 4-4-20 below the $K_d$, the interdomain dissociation was relatively the same for affinity-labeled Fv 4-4-20, affinity-labeled Fv 4-4-20 with monoclonal antibody 4A6, and affinity-labeled Fv 4-4-20 with polyclonal anti-Met antibodies. At concentrations of affinity-labeled Fv 4-4-20 above the $K_d$ (25), both monoclonal and polyclonal anti-Met antibodies provoked domain dissociation. The affinity-labeled interdomain binding curves (Fig. 2) indicated that polyclonal and monoclonal anti-Met antibodies caused an instability between the two variable domains that resulted in the subsequent decrease in fluorescein polarization, which was in agreement with Fig. 1. In conjunction, the results of Figs. 1 and 2 demonstrate that the effect of the monoclonal antibody on the interdomain affinity was greater than that of the polyclonal antibodies, and both provoked variable domain dissociation.

Hydrostatic pressure was used to examine the effect of anti-Met antibodies on antibody active-site dynamics by using the affinity-labeled antibody. Application of hydrostatic pressure in affinity-labeled Fv 4-4-20 and affinity-labeled scFv 4-4-20 demonstrated an increase in fluorescein fluorescence intensity that was the result of a first-order effect. The independence of fluorescein fluorescence recovery as a function of protein concentration allowed a comparison between affinity-labeled Fv 4-4-20 and affinity-labeled scFv 4-4-20 at the same protein concentrations. The greater increase in fluorescence intensity observed in affinity-labeled Fv 4-4-20 and affinity-labeled scFv 4-4-20 compared with affinity-labeled scFv 4-4-20 demonstrated that the first-order reaction was restricted by the linker. Fluorophore fluorescence intensity is known to be a sensitive measure of the fluorophore microenvironment (7, 38). The difference in the results between affinity-labeled Fv 4-4-20 and affinity-labeled scFv 4-4-20 implied that the first-order reaction was most likely a molecular rearrangement of the fluorescein active site. Fluorescein is 93% buried in the 4-4-20 active site across the interdomain interface (38). The difference in behavior between affinity-labeled scFv 4-4-20 and affinity-labeled Fv 4-4-20 is probably a reflection of the increased flexibility of the active site in Fv 4-4-20 due to the lack of a linker. Polyclonal anti-Met antibodies, added to each of these affinity-labeled antibodies, were found to stabilize against a pressure-induced fluorescein fluorescence increase. This was interpreted as stabilization of the dynamic fluctuations of the microenvironment of the fluorescein, and therefore the active site, and was in agreement with $D_2O$ enhancement studies (19) that showed anti-Met to stabilize conformational dynamics.

Pressure-induced ligand dissociation was performed on liganded Fv 4-4-20 to examine the efficiency of anti-Met antibody stabilization of fluorescein in an antibody active site devoid of interdomain polypeptide linker or constant domains (Fig. 6). Polyclonal anti-Met antibodies were found to stabilize fluorescein in the active site of liganded Fv 4-4-20 against dissociation with the same efficiency as for liganded scFv 4-4-20. The effect of polyclonal anti-Met antibodies on anti-fluorescein antibody ligand off-rate delay was antibody concentration-dependent, in agreement with results obtained with liganded scFv 4-4-20 (24). The concentration dependence was most likely because higher concentrations of anti-Met antibodies ensured that more Fv 4-4-20 variable domain metatopes were bound. In summary, polyclonal anti-Met antibodies had caused both the fluorescein release and stabilization in the Fv 4-4-20 active site. This apparent paradox is resolved in the following model proposed to explain general metatype/anti-Met interaction (Fig. 8).

In this model, a concentration-dependent five-stage equilibrium exists between the individual variable domains (VH and VL), fluorescein, and anti-Met antibodies. Individual domains are known not to bind fluorescein (25). At variable domain concentrations below the $K_d$, the domains are not associated with each other or with fluorescein (stage 1). At concentrations above the $K_d$, Fv dimer is formed (stage 2). Upon formation of Fv dimer, fluorescein is quickly bound (stage 3). Liganded Fv in the presence of anti-Met antibodies will form a liganded Fv-anti-Met encounter complex (stage 4), which will either go on to stage 5, the stabilized liganded Fv-anti-Met complex, or back to stage 1, the basic components. The course of the reaction from stage 4 to either stage 5 or 1 depends on the variety and concentration of anti-Met antibodies.

Dimer destabilization observed with monoclonal anti-Met antibodies is believed to be similar, but not identical, to a phenomenon with the $\beta$-subunit of tryptophan synthetase observed by Silva et al. (39) and bovine lactate dehydrogenase.

**Fig. 8.** Five-stage model outlining the dynamics of anti-Met antibody interaction with Fv variable domains.
by King and Weber (40). Both groups found that pressure-induced dissociation of the dimer caused the reassociated species to have decreased subunit affinity. Bovine lactate dehydrogenase showed reduced enzymatic activity upon removal of pressure, although subunit reassociation occurred immediately. It was concluded that upon dissociation of the aggregate, the subunits had undergone a change in conformation that resulted in a loss in affinity for each other (34). Polyclonal anti-Met antibodies were shown previously (31) and in this study with the pressure experiments to have an effect on the antibody active-site dynamics. The affinity-labeled anti-Met titration curve utilizing the monoclonal anti-Met antibody, which by definition bound to one metatype on the $V_{\gamma}$ domain, showed the monoclonal antibody to have the effect of destabilizing the dimer. Although only bound to one domain, monoclonal anti-Met antibodies were previously shown to be capable of stabilizing scFv 4-4-20 variable domain dynamics (30). Stabilization of one domain in the dimer by a monoclonal anti-Met antibody would make the two domains dynamically incompatible, causing dissociation to occur readily if no additional stabilizing element such as constant domains or a linker is present. Therefore, for anti-Met antibodies to stabilize ligand in an anti-antigen antibody-antigen complex as observed in this and previous studies (24, 30, 31), the binding of an anti-Met antibody to one domain on the metatype has to be balanced by binding to the other domain. Indeed, this was observed when polyclonal anti-Met antibodies were added to affinity-labeled and liganded Fv 4-4-20, where the decrease in $Q_{\text{max}}$ and polarization was much less than for monoclonal antibodies (Figs. 1 and 2). Once the complex was formed, the metatype-anti-Met complex was more resistant to hapten dissociation, as observed in Figs. 6 and 7. In addition, this would explain the lack of cooperativity observed by Coelho-Sampaio and Voss (24) when trying to stabilize liganded scFv 4-4-20 by using different combinations of monoclonal anti-Met antibodies. All the monoclonal anti-Met antibodies used in that investigation were specific for the $V_{\gamma}$ domain (30). To observe cooperativity by combining different monoclonal antibodies, an important requirement is that monoclonal antibodies specific for both domains be combined. The combination of monoclonal antibodies specific for both domains may create the balance of the domain dynamics needed for metatype/anti-Met stabilization. The small decrease in $Q_{\text{max}}$ and polarization observed with liganded Fv 4-4-20 when bound by polyclonal antibodies in Fig. 1 was probably due to a population of Fv dimers that were not bound on both domains.

CONCLUSION

Anti-Met antibodies were shown to affect the interdomain interactions. The decrease in fluorescence $Q_{\text{max}}$ and polarization observed with affinity-labeled Fv 4-4-20 upon addition of both polyclonal and monoclonal anti-Met antibodies indicated that some incompatibility was initiated between the Fv 4-4-20 domains. When only one domain was bound, as in the case of a monoclonal anti-Met antibody (30), the Fv interdomain associative interaction was particularly weakened. Polyclonal anti-Met antibodies caused fluorescence to dissociate from a fraction of the Fv 4-4-20 population while further stabilizing fluorescence in the other fraction. The degree of stabilization of the variable domains was believed to depend on whether both variable domains were bound by anti-Met antibodies.

Monoclonal derivative antibodies have the potential to be used as agents for diagnosis and therapy of cancer (37). Functional Fv fragments, which are smaller in mass and have reduced in vivo half-lives compared with immunoglobulins, have been found to be unsuitable for these purposes because of the in vivo dynamic dissociation of the dimer (26). In accordance with the results in this study, the in vivo dynamic dissociation of the Fv dimer may be due to an analogous anti-Met antibody response elicited against Fv fragments that significantly contributes to Fv instability in vivo, favoring dimer dissociation.

Acknowledgments—We thank the Laboratory for Fluorescence Dynamics for instrumentation and technical support. We are grateful to Mark Mumma and Donald J. Weaver, Jr. for stimulating discussion in reference to this work. We thank Steve D. Miklasz and Christopher Martin for assistance with ascites preparation.
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J. Biol. Chem. 1996, 271:11247-11252.
doi: 10.1074/jbc.271.19.11247

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