IgG Fab Fragments Forming Bivalent Complexes by a Conformational Mechanism That Is Reversible by Osmolytes*§

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Background: Isolated Fab fragments can spontaneously multimerize over time. Results: Distinct conformations are associated with monovalent Fabs versus those in bivalent complexes. Conclusion: Monovalency can be preserved upon conformational stabilization with osmolytes. Significance: A conformational mechanism-informed means for preserving monovalent Fabs increases their potential for use as blocking reagents in clinical applications.

Generated by proteolytic cleavage of immunoglobulin, Fab fragments possess great promise as blocking reagents, able to bind receptors or other targets without inducing cross-linking. However, aggregation of Fab preparations is a common occurrence, which generates intrinsic stimulatory capacity and thwarts signal blockade strategies. Using a panel of biochemical approaches, including size exclusion chromatography, SDS-PAGE, mass spectrometry, and cell stimulation followed by flow cytometry, we have measured the oligomerization and acquisition of stimulatory capacity that occurs in four monoclonal IgG Fab fragments specific for TCR/CD3. Unexpectedly, we observed that all Fabs spontaneously formed complexes that were precisely bivalent, and these bivalent complexes possessed most of the stimulatory activity of each Fab preparation. Fabs composing bivalent complexes were more susceptible to proteolysis than monovalent Fabs, indicating a difference in conformation between the Fabs involved in these two different states of valency. Because osmolytes represent a class of compounds that stabilize protein folding and conformation, we sought to determine the extent to which the amino acid osmolyte L-proline might impact bivalent Fab complexation. We found that L-proline (i) inhibited the adoption of the conformation associated with bivalent complexation, (ii) preserved Fab monovalency, (iii) reversed the conformation of preformed bivalent Fabs to that of monovalent Fabs, and (iv) separated a significant percentage of preformed bivalent complexes into monovalent species. Thus, Fab fragments can adopt a conformation that is compatible with folding or packing of a bivalent complex in a process that can be inhibited by osmolytes.

Fab fragments are classically generated by treating immunoglobulin (Ig) with the protease papain and isolating a ∼50-kDa disulfide-linked intact cleavage product composed of a heavy + light chain fragment that contains only one antigen binding site (1–3). Unlike their bivalent Ig precursor, monovalent Fab fragments can bind their targets without cross-linking and thus possess significant potential as reagents that can block receptors and signaling pathways (4–10). However, although common in a research setting, few Fab fragments are used in clinical applications (11–14), mostly due to two technical limitations. First, Fabs display a relatively short serum half-life compared with intact therapeutic antibodies (Abs)2 (15, 16); however, strategies exist to address this issue, such as conjugating Fabs to polyethylene glycol (17, 18), or engineering Fabs as fusion proteins, together with re-expression as recombinant single-chain products with increased serum half-life (15, 19, 20). The second limitation involves the common observation that Fab preparations may aggregate as a function of time, concentration, temperature, or salt or in correlation with other apparently idiosyncratic observations (21–29). If a Fab preparation is composed mostly of monovalent species but contains a small contaminant of protein aggregate, the few multivalent complexes can display disproportionate activity because they intrinsically possess higher potential for antigen binding avidity and cross-linking and thus may out-compete the potential blocking effects of the more numerous monovalent Fabs. An increased understanding of the molecular mechanisms that mediate Fab aggregation could lead to strategies that would preserve the monovalent state and thus improve the potential use of Fab fragments as blocking reagents in clinical applications.

Beyond Fab fragments, aggregation represents a major problem in the preparation of many therapeutic protein-based

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‡‡1 The abbreviations used are: Ab, antibody; APC, antigen-presenting cell; B6, C57BL/6 mouse strain; Ham, hamster; Ms, mouse; K<sup>B</sup>-OVA, peptide-MHC tetramer of H-2K<sup>B</sup> + OVA; FARL, peptide sequence SSIEFARL; OVA, peptide sequence SIINFEKL; SEC, size exclusion chromatography; MSD, mass-selective detector; PE, phycoerythrin.

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bioreagents (30). In vivo, protein aggregation itself is a mechanism of disease (31), with examples such as systemic light chain amyloidosis (32) and the neurodegenerative disorders of Alzheimer, Huntington, and Parkinson diseases (33). Therefore, a significant aim in biomedicine remains to elucidate the rules that govern protein aggregation, learn how to prevent it, and, where possible, reverse the process after it has happened.

Osmolytes were first described as a class of natural, biologic compounds involved in controlling osmosis and mediating cellular responses to hypo- or hypertonicity (34, 35); however, osmolytes have also been shown to play major roles in protein stabilization, folding, and packing, functions that display significant evolutionary conservation in species as distant as microbes and mammals (36–39). Due to these functions and their general lack of cellular toxicity, osmolytes have been used to stabilize proteins being prepared for biochemical experimentation (40, 41), crystallography (42), and therapeutic application (43, 44). The extent to which osmolytes might impact the preservation of Fab preparations as monovalent species has not previously been addressed.

In the current work, we measure the valency of Fab preparations as they acquire undesirable stimulatory activity, and we observe a protective function for the osmolyte l-proline in preserving monovalency. Our experimental system focuses on four monoclonal Abs (mAbs) with specificity for subunits of the T cell antigen receptor (TCR)/CD3 complex. Because primary T cells are highly dependent on TCR/CD3 triggering for cellular activation, the system provides a sensitive biological readout for monovalent Fab-mediated blockade of the receptor versus multivalent Fab-induced receptor stimulation. Unexpectedly, we observe that all Fabs tested spontaneously form complexes that are precisely bivalent instead of an unpredictable Fab oligomerization that is typically bivalent and that may arise from the dimerization of Fab pairs (45). All mice were used between 6 and 16 weeks of age. Mouse procedures were approved by the Mayo Institutional Animal Care and Use Committee and are consistent with National Institutes of Health guidelines for the care and use of animals.

**EXPERIMENTAL PROCEDURES**

**Mice**—C57BL/6 (B6) mice were purchased from the Jackson Laboratory. OT-I TCR transgenic mice on B6 background were bred from progenitor mice that were kindly provided by Larry Pease (Mayo Clinic, Rochester, MN). T cells from OT-I TCR transgenic mice express a Vα2 + Vβ5 + TCR specific for an octapeptide derived from ovalbumin, SIINFEKL (OVA), presented in the major histocompatibility complex (MHC) H2-Kb (45). All mice were used between 6 and 16 weeks of age. Mouse procedures were approved by the Mayo Institutional Animal Care and Use Committee and are consistent with National Institutes of Health guidelines for the care and use of animals.

**Abs and Other Reagents**—The following panel of anti-TCR/CD3 mAbs was purified from hybridoma supernatants: anti-CD3ε, 7D6 (mouse (Ms) IgG2a); anti-CD3ε, 17A2 (rat IgG2b); anti-CD3ε, 145-2C11 (hamster (Ham) IgG1); and anti-TCRβ, H57-597 (Ham IgG2). The 7D6 hybridoma was kindly provided by Balbino Alarcón (Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid). The 17A2 hybridoma was kindly provided by David Wiest (Fox Chase Cancer Center, Philadelphia, PA). The 2C11 and H57 hybridomas were kindly provided by Ed Palmer (University Hospital Basel, Switzerland). Abs from eBiosciences included anti-CD3ε (17A2), anti-Vβ5 (MR9-4), anti-Thy1.2 (53-2.1), anti-CD4 (RM4-5), and anti-CD8α (53.6.7), and anti-CD69 (H12F3). Abs from Jackson ImmunoResearch included nonspecific Ms, rat, and hamster IgG controls and donkey anti-Ms IgG, goat anti-rat IgG, and goat anti-Ham IgG secondary Abs (raised against heavy + light chain immunogens) coupled to horseradish peroxidase (HRP) for Western blots or coupled to FITC for flow cytometry. The PE-labeled H2-Kb/SIINFEKL tetramer (Kb/OVA) represents a tetravalent form of the MHC H-2Kb loaded with OVA peptide (tetramer purchased from Beckman Coulter).

**Preparation of Fab and F(ab′)2 Fragments**—7D6, 17A2, 2C11, and H57 mAbs were purified from hybridoma supernatant by affinity chromatography using a Protein G-Sepharose column (GE Healthcare) equilibrated in PBS or other buffers as noted. After filtration through 0.2-μm filters, mAbs were stored under sterile conditions at 2 mg/ml at 4 °C. Two mg of each IgG were digested with 0.05 mg of the endopeptidase papain (Sigma-Aldrich) at 37 °C following the protocol described by Andrew and Titus (1). After 24 h, digestions were placed on ice, and the papain was quenched by the addition of 30 mCi iodoacetamide (Sigma-Aldrich). Next, digestions were dialyzed with frequent buffer exchanges in PBS over 6 h in a cold room at 4 °C. Fc fragment-containing species were removed by incubating the samples with protein A-Sepharose beads (GE Healthcare) at 4 °C overnight, resulting in Fab preparations that contained at least 2 μg of Fab per undetectable mAb (<1 ng; data not shown). Next, digestions were sterile filtered, and total protein was quantified using a Nanodrop spectrophotometer (Thermo Scientific). All Fab preparations shown in this work were stored in sterile conditions at 4 °C, at 0.2 mg/ml in PBS or in PBS + 1-proline (2 M). For Western blots of Fab preparations, 2 μg of total protein from the digestion samples were subjected to non-reducing SDSE PAGE (8% acrylamide/bisacrylamide (Bio-Rad)) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blotted with probe Abs (anti-Ms, anti-rat, or anti-Ham IgG-HRP). All Western blots were scanned and digitally processed using Adobe Elements software, but no image alteration effects were applied.

**Preparation of F(ab′)2 Fragments**—7D6, 17A2, 2C11, and H57 mAbs were purified from hybridoma supernatant by affinity chromatography using a Protein G-Sepharose column (GE Healthcare) equilibrated in PBS or other buffers as noted. After filtration through 0.2-μm filters, mAbs were stored under sterile conditions at 2 mg/ml at 4 °C. Two mg of each IgG were digested with 0.05 mg of the endopeptidase papain (Sigma-Aldrich) at 37 °C following the protocol described by Andrew and Titus (1). After 24 h, digestions were placed on ice, and the papain was quenched by the addition of 30 mCi iodoacetamide (Sigma-Aldrich). Next, digestions were dialyzed with frequent buffer exchanges in PBS over 6 h in a cold room at 4 °C. Fc fragment-containing species were removed by incubating the samples with protein A-Sepharose beads (GE Healthcare) at 4 °C overnight, resulting in Fab preparations that contained at least 2 μg of Fab per undetectable mAb (<1 ng; data not shown). Next, digestions were sterile filtered, and total protein was quantified using a Nanodrop spectrophotometer (Thermo Scientific). All Fab preparations shown in this work were stored in sterile conditions at 4 °C, at 0.2 mg/ml in PBS or in PBS + 1-proline (2 M). For Western blots of Fab preparations, 2 μg of total protein from the digestion samples were subjected to non-reducing SDS-PAGE (8% acrylamide/bisacrylamide (Bio-Rad)) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blotted with probe Abs (anti-Ms, anti-rat, or anti-Ham IgG-HRP). All Western blots were scanned and digitally processed using Adobe Elements software, but no image alteration effects were applied. 7D6 F(ab′)2 fragments were prepared by pepsin digestion (90 min, 37 °C) of 1 mg of 7D6 mAb using a F(ab′)2 preparation kit (Thermo Scientific Pierce) according to the manufacturer’s instructions.

**Size Exclusion Chromatography (SEC)**—Two Superdex 200 10/300 GL columns (GE Healthcare) were aligned in tandem on an AKTA FPLC system (GE Healthcare), which we refer to as “S-400” column chromatography. The S-400 system was equilibrated either in PBS alone or in PBS + 1-proline (2 M), as indicated, and operated in a chromatography refrigerator, 10–16 °C. Purified mAb, Fab, or F(ab′)2 fragments were injected over the S-400 system in a 1–2-ml sample volume, and...
elution of proteins was monitored by UV absorbance at 280 nm. Absorbance and other elution data were analyzed using Unicorn software (GE Healthcare) to identify the peak volume of elution ($V_e$) and to generate graphics of elution profiles. To calibrate the S-400 system for the molecular weight of IgG species, we used the $V_e$ of 50 μg each of 7D6 mAb (~150 kDa), 7D6 Fab (~100 kDa), and 7D6 Fab (~50 kDa). When fractionating Fab preparations, 28 fractions of 1 ml each were collected, beginning with the void volume at 12–12.99 ml labeled as the 12-ml fraction, and continuing through 39–40 ml (labeled as the 39-ml fraction), a range spanning all fractions that could possibly contain protein. For Western blots of proteins in SEC fractions, 5-μl subsamples of each fraction were used to test for IgG content (except where other gel-loading conditions are specified in the figure legends). S-400 fractions were also tested for their functional activity in T cell stimulation assays described below.

**CD69 Up-regulation Assay of T Cell Stimulation**—Whole cell suspensions from mouse spleen and lymph nodes were plated in triplicate wells, each containing 0.25 × 10⁶ viable cells/ml in RPMI (Invitrogen) supplemented with 10% Cosmic Calf serum (Hyclone) for tissue culture at 5% CO₂, 37 °C. IgG control treatments, as well as Fab preparations and Fab fractions, were added to tissue culture wells at 5 μg/ml. In some experiments, instead of a fixed protein concentration, a fixed volume of each Fab fraction was added to tissue culture, as indicated in the respective figure legends. Storage of Ig species in 2 M l-proline PBS for stock solutions was followed by dilution to a final concentration of 50 mM l-proline additive when added to tissue culture. Following 24-h incubation, plates were placed on ice, washed and subjected to flow cytometry using a C6 flow cytometer (Accuri Cytometers, BD Biosciences). Data were analyzed using CFlow Plus software to observe up-regulation of the activation marker CD69 on Thy1.2⁺ CD8⁺ Vβ5⁺ thymocytes.

**TCR/Antigen Binding Assay**—Triplicate samples of OT-I splenocytes (0.25 × 10⁶ viable cells/ml) were preincubated on ice for 60 min with one anti-TCR/CD3 IgG Fab fragment preparation from 7D6, 17A2, 2C11, or H57 (or a matching IgG species control) at 5 μg/ml. Six 2-fold serial dilutions of K⁺/OVAP-PE reagent (Beckman Coulter) were prepared with the first concentration being 1:50 of the purchased stock tetramer. The K⁺/OVAP-PE dilutions were mixed with anti-Thy1.2-APC and incubated with the Fab-pretreated OT-I splenocytes for 60 min on ice. In parallel, some Fab-pretreated cells were washed and then stained with anti-Thy1.2-APC mAb together with relevant anti-Fab secondary Ab (anti-Ms, anti-Rat, or anti-Ham IgG-FITC) to detect binding of the different anti-TCR/CD3 Fabs. All samples were washed following staining and fixed in 0.25% formaldehyde for analysis by flow cytometry.

**RESULTS**

**Several Anti-TCR/CD3 Fab Fragments Acquire the Capacity to Stimulate T Cells**—We used papain to generate Fab fragments from several mAbs specific for mouse TCR/CD3, with subsequent Fab purification in PBS: 7D6 (mouse IgG2a, anti-CD3εγ), 17A2 (rat IgG2b, anti-CD3εγ), 2C11 (hamster IgG1, anti-CD3ε), and H57 (hamster IgG2, anti-TCRβ). A monovalent Fab would be predicted to fail to induce TCR/CD3 cross-linking and T cell stimulation. To test this, we subjected the Fab preparations to a sensitive in vitro CD69 up-regulation assay. In one example, peripheral T cells from B6 mice were cultured in the presence of either nonspecific Ms IgG, 7D6 Fab (fresh preparation), or 7D6 mAb, each at 5 μg/ml. Under these conditions, we observed that only 7D6 mAb was stimulatory for T cells (Fig. 1, A and B, left), and we concluded that freshly generated 7D6 Fab was not capable of T cell stimulation. However, routine testing soon revealed that Fabs were able to acquire stimulatory capacity with time. This was exemplified when the 7D6 species above were compared with an aged 7D6 Fab preparation, which proved capable of T cell stimulation (Fig. 1, A and B, right). The amount of time that passes prior to acquisition of stimulatory capacity by these Fab preparations stored in our standard conditions (sterile PBS, 4 °C, 0.2 mg/ml) varies apparently idiosyncratically from weeks to months (data not shown). We have observed this pattern of acquisition of stimulatory capacity not only with 7D6, but also with the other Fab specificities: 17A2, 2C11, and H57 (Fig. 1C).

**7D6 Fab Spontaneously Forms a Bivalent Complex (Bi-7D6-Fab) That Stimulates T Cells**—We wished to determine why the Fab preparations acquired stimulatory activity with time. To do so, we chose to focus on 7D6 as a model Fab due to the best yield of its parent mAb upon purification from hybridoma supernatant. Two types of Fab preparations were categorized: “inactive” (negative for CD69 up-regulation) and ‘active’ (positive for CD69 up-regulation). We hypothesized that aggrega-
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A

| T cell Counts | CD69 | CD69 |
|---------------|------|------|
| 7D6 Fab Fresh | 50   | 100  |
| 7D6 Fab Aged  | 100  | 50   |

B

| CD69 (% Max. Stim.) | 7D6 Fab Fresh | 7D6 Fab Aged |
|----------------------|---------------|--------------|
| 7D6 Fab Fresh        | 50            | 100          |
| 7D6 Fab Aged         | 100           | 50           |

C

| CD69 (% Max. Stim.) | IgG non-specific | IgG Fab Aged | IgG mAb |
|---------------------|-----------------|--------------|---------|
| 7D6 Fab Aged        | 50              | 100          | 100     |
| 2C11 Fab Aged       | 100             | 50           | 50      |
| H57 Fab Aged        | 100             | 50           | 50      |

FIGURE 1. Several anti-TCR/CD3 Fab fragments acquire the capacity to stimulate T cells. A and B, 7D6 Fab fragments were tested for stimulatory capacity either right after completion of papain digestion of the corresponding mAbs (7D6 Fab fresh; left panels), or after 4 months of sterile storage in PBS at 4 °C (7D6 Fab aged; right panels). A, overlays of the CD69 profiles of CD8⁺ T cells treated with 5 μg/ml nonspecific Ms IgG control, 7D6 Fab (fresh versus aged), or 7D6 mAb. B, replicate data for CD69 expression is represented on the y axis as a percentage of the maximum, which was observed on Thy1.2⁺ CD8⁺ T cells treated with 2C11 mAb. Error bars, S.E.

tained protein species with masses of 47.87 and 96 kDa (supplemental Table 1), although in the inactive 7D6 Fab preparation, the larger species must have been present at relatively low quantities because it was not detectable by UV absorbance (Fig. 2A). Under denaturing, non-reducing conditions, the 96-kDa species was decomposed into a single mass of 47.87 kDa, indicating that the larger species was a non-covalent dimer of the 47.87-kDa species (supplemental Table 1). Meanwhile, when either inactive or active 7D6 Fab preparations were analyzed under reducing conditions, the 47.87 kDa mass decomposed into smaller masses of 23.84 and 24.09 kDa, representing the heavy and light chain fragments that compose the Fab (supplemental Table 1). We conclude that preparations of 7D6 Fab contained both monovalent species (Mono-7D6-Fab, ~50 kDa by SEC and 47.87 kDa by MSD/TOF) and putatively bivalent species (Bi-7D6-Fab, ~100 kDa by SEC and 96 kDa by MSD/TOF). MS was sensitive enough to find both species in both inactive and active 7D6 Fab preparations, whereas SEC detected Bi-7D6-Fab in the active preparation only.

It was possible that Bi-7D6-Fab possessed stimulatory capacity in the active 7D6 Fab preparation, whereas Mono-7D6-Fab was expected to be inert for T cells. To test this hypothesis, two 7D6 Fab preparations, categorized respectively as “inactive” and “active” according to their capacity to up-regulate CD69, were subjected to S-400 SEC, and 28 fractions (1 ml each) were isolated that together spanned all sizes that could contain protein. Each fraction was tested for stimulatory capacity in the CD69 up-regulation assay. For the inactive 7D6 Fab preparation, we observed that fractions corresponding to Bi-7D6-Fab were positive for CD69 induction in T cells, whereas fractions corresponding to Mono-7D6-Fab were negative (Fig. 2C). These data confirmed the MS data indicating that the “inactive” 7D6 Fab preparation indeed contained some bivalent Fab complexes that proved to be functional when purified away from the rest of the preparation. Likewise, the “active” 7D6 Fab preparation also showed that most stimulatory activity originated from fractions corresponding to Bi-7D6-Fab (Fig. 2D). Whereas Mono-7D6-Fab fractions produced little if any T cell stimulation, there was some activity from fractions that would correspond to larger Fab aggregates (Poly-7D6-Fab), most notably the system void volume in the 12-ml elution fraction (Fig. 2D). Interestingly, fractions corresponding to the size of intact mAb (fractions 24–27; Fig. 2A) did not display peak activity (Fig. 2D), reinforcing the notion that these “active” 7D6 Fab preparations were devoid of undigested mAb, and higher-order valency and stimulatory activity were due to Fab oligomerization. We conclude that two-copy complexes of 7D6 Fab can spontaneously form and accumulate over time, which by molecular weight and stimulatory capacity are functionally bivalent, possessing most of the stimulatory capacity of the “active” 7D6 Fab in these preparations.

Further biochemical analysis was performed to determine if biophysical differences could be identified between monovalent Fabs and those Fabs participating in the bivalent complex, Bi-7D6-Fab. To segregate 7D6 Fab species by native molecular weight, S-400 SEC fractions were obtained as above. These fractions were further analyzed by SDS-PAGE under either non-reducing or reducing conditions, followed by Western blotting...
with anti-Ms IgG. For both inactive and active 7D6 Fab preparations (Fig. 3, A and B), we observed that fractions corresponding with Mono-7D6-Fab produced in non-reducing SDS-PAGE a single band of the expected molecular size for 7D6 Fab fragment (band X, ~48 kDa; Fig. 3A). No other major bands were detected by Western blot for the inactive 7D6 Fab preparation. In contrast, for the active 7D6 Fab preparation, a different band pattern with faster mobility Ig-containing species was obtained from fractions corresponding to Bi-7D6-Fab (bands Y and Z; Fig. 3B). Thus, a biophysical difference existed between lone monovalent Fabs and those that composed bivalent complexes.

We wished to determine whether this difference in non-reducing SDS-PAGE migration might be due to a difference in molecular weight of the electrophoretically migrating proteins. First, we observed that if SDS-PAGE were changed to reducing conditions, then IgG-containing species from Mono-7D6-Fab (SEC fraction 32) and Bi-7D6-Fab (SEC fraction 28) migrated identically to each other (Fig. 3C). This indicated that bands Y and Z were only apparent in non-reducing conditions (Fig. 3B). To determine the extent to which non-reducing SDS-PAGE-specific conditions were involved in generating bands Y and Z, we studied the molecular size of 7D6 Fabs using an alternative procedure, reverse phase HPLC-MS. Fab samples were added to denaturation buffer (5 μL of Fab in PBS, added to 95 μL of 0.1% formic acid), followed by column binding and elution in mobile phase gradient buffers (Buffer A: 97.9% H2O, 1% acetonitrile, 1% isopropyl alcohol, 0.1% formic acid; Buffer B: 9.9% H2O, 80% acetonitrile, 10% isopropyl alcohol, 0.1% formic acid). MS analysis showed that both Mono- and Bi-Fabs displayed the expected masses for 7D6 Fab whether in non-reducing (48 kDa) or reducing conditions (24 kDa) (data not shown; molecular weight data similar to the data reported in supplemental Table 1). Thus, because both reducing SDS-PAGE and denaturing reverse phase HPLC-MS data showed apparently full-length
Fab heavy and light chain fragments for Bi-7D6-Fab, this implied that native Bi-7D6-Fab from which those analyses originated must have also been full-length.

Further MS analysis was undertaken to examine molecular weight through amino acid sequence analysis. First, we sought sequence confirmation that the uniform bands resulting from reducing SDS-PAGE were full-length and equivalent between Mono- and Bi-7D6-Fabs. Samples of each Fab type were subjected to reducing SDS-PAGE, and the 24 kDa bands generated were isolated from Coomassie-stained gels and digested with endoproteinases trypsin and Asp-N for mass/charge ratio (m/z) and sequence analysis by nano-HPLC-electrospray tandem mass spectrometry (supplemental Table 2). We found the same set of peptides originating from the Mono- and Bi-7D6-Fab samples. Peptides were identified as derived from the heavy or light chain constant domains after sequence comparison using the Swissprot database (supplemental Table 2). Reported sequences of the constant domains of the IgG2a heavy chain were confirmed as expected for 7D6 Fab. Additionally, several peptides aligned with sequences from the variable domains of the heavy and light chains from other mouse antibodies reported in the NCBI RefSeq database (supplemental Table 3). We conclude that under conditions of reducing SDS-PAGE, no amino acid sequence differences were observed comparing Mono- and Bi-7D6-Fabs.

In contrast, a different result was obtained when performing MS-based sequence analysis comparing bands X and Z (see Fig. 3) that had been isolated from non-reducing SDS-PAGE. Coomassie-stained gels of Mono- and Bi-7D6-Fab samples. Band Y could not be confidently distinguished from band X in SDS-PAGE alone; nor could band Y be generated in sufficient quantity in SEC-x-SDS-PAGE to be included in this analysis. We observed that although Mono-Fab band X was composed of the full-length Fab sequences, Bi-Fab band Z had lost 74 amino acids (8 kDa) from the C terminus of the Fab heavy chain constant domain (supplemental Table 2). Other sequences in constant and variable domains of heavy and light chains were equivalent between bands X and Z (not shown; sequence data similar to that reported in supplemental Tables 2 and 3). Therefore, band Z was composed of a full-length light chain in disulfide-linked association with a truncated heavy chain, together having a molecular mass of ~40 kDa (SDS-PAGE apparent molecular mass <37 kDa). Because native Bi-7D6-Fab does not have this truncation, we conclude that band Z resulted from the breaking of covalent bonds, proteolysis, of 7D6 Fab, which specifically occurred during non-reducing SDS-PAGE analysis. In sum, these experiments yield the following conclusions (Fig. 4): (i) there is no evidence for differences in native mass between 7D6 Fabs that compose a monovalent versus a bivalent complex, whether analyzed by MS or reducing SDS-PAGE; and (ii)
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**FIGURE 4.** Schematic representation of 7D6 Fab species found in different experimental conditions. In native conditions, 7D6 Fab can be found in both monovalent and bivalent species of 48 and 96 kDa, respectively (observed by SEC and MSD/TOF/MS). We propose that the bivalent species (round-edged) adopts an alternative conformation from that of the monovalent species (straight-edged). In non-reducing SDS-PAGE, monovalent Fabs contain variable and constant domains of full-length Fab heavy and light chains. In contrast, bivalent Fabs contain two bands that migrate faster, bands Y and Z. Band Z contains intact light chain, but the heavy chain is truncated, having lost a segment of ~8 kDa from the C terminus, a reproducible proteolytic cleavage occurring under non-reducing SDS-PAGE conditions. Band Y, migrating slightly faster (~47 kDa) than band X (48 kDa), is not fully characterized because its proximity to band X currently prevents its isolation with high confidence. However, band Y might represent a less truncated product of non-reducing SDS-PAGE than band Z, a speculation that is represented in the schematic as a likely possibility. When monovalent and bivalent Fab species are subjected to reducing SDS-PAGE, they both generate a single band of mouse immunoglobulin of identical mobility, which contains both full-length Fab heavy and light chains.

non-reducing SDS-PAGE conditions reveal an increased susceptibility to proteolysis in Fabs composing a bivalent complex but not in lone monovalent Fabs. Differential susceptibility to proteolysis is a property known to be linked to protein folding and conformation (21, 49, 50). Therefore, we hypothesize that Fabs composing a bivalent complex display a conformation distinct from that of lone monovalent Fabs, and this distinct conformation imparts a unique susceptibility to proteolysis in the presence of conditions associated with non-reducing SDS-PAGE (Fig. 4). This hypothesis predicts that osmolytes, known to stabilize protein folding/packing/conformation, might affect formation of bivalent Fab complexes.

The Osmolyte L-Proline Inhibits Formation of Bi-7D6-Fab Complexes—Our strategy was to store Fab preparations in the presence of osmolytes to assess any effects on preservation of the monovalent state. Because the ultimate goal was to use Fab fragments as blocking reagents, we first assessed the compatibility of two osmolytes, L-proline (37, 43) and glycerol (34, 38), with viable cell culture and T cell stimulation. We found that when B6 peripheral T cells were stimulated with either 7D6 mAb or 2C11 mAb, up-regulation of CD69 was unaffected by the presence of 50 mM L-proline in the cultures (supplemental Fig. 1A). By comparison, glycerol also failed to inhibit CD69 up-regulation on responding cells, but glycerol caused a ~50% reduction in T cell viability (data not shown) and is therefore not emphasized in the experiments that follow.

Mono-7D6-Fabs were purified and stored at 4 °C in either PBS or PBS + 2 mM L-proline (PBS-Pro). To achieve this, the S-400 system was equilibrated in either PBS or PBS-Pro, and an inactive 7D6 Fab preparation was subjected to SEC fractionation (Fig. 5A). From each SEC fractionation, subsamples were collected corresponding with Poly-7D6-Fab (elution 12–23 ml), Bi-7D6-Fab (elution 28–30 ml), and Mono-7D6-Fab (elution 32–34 ml) (Fig. 5A). Each Fab subsample was tested for its effect on B6 T cell viability and CD69 up-regulation at a fixed protein concentration of 5 μg/ml. Dilution of reagents in tissue culture resulted in a final concentration of 50 mM L-proline. We found that T cell viability was not affected by these reagents in the presence or absence of L-proline (supplemental Fig. 1B); nor did PBS- or PBS-Pro-containing fractions intrinsically induce significant CD69 up-regulation when compared with the positive (2C11 mAb) and negative (Ms IgG) assay controls (Fig. 5B), suggesting the absence of detectable amounts of the Bi-Fab species at the time of this first stimulation test. After 2 months of sterile storage at 4 °C, all samples were retested for their stimulatory capacity. We observed that PBS-stored Fabs, which originally eluted in Mono- or Bi-Fab fractions, became positive for CD69 up-regulation (Fig. 5C, left). In contrast, Fabs stored in PBS-Pro possessed little if any stimulatory capacity (Fig. 5C, right). The same effect was observed if Fabs were stored in glycerol, although overall T cell viability was decreased in those cultures (data not shown). These data demonstrated that (i) Mono-7D6-Fabs purified by SEC and stored in PBS, and proven to be non-stimulatory/inactive early after purification, later acquired T cell stimulatory capacity, and (ii) storage in PBS-Pro inhibited Fab acquisition of T cell stimulatory capacity.

We wished to determine whether the Mono-7D6-Fab that had been purified and stored in PBS had indeed formed a bivalent complex and whether the Mono-7D6-Fab stored in PBS-Pro had not. Both Fab types were refractionated by S-400 SEC, and the fractions were subjected to non-reducing SDS-PAGE followed by Western blotting with anti-Ms IgG. The refractionation profile of PBS-stored Mono-7D6-Fab showed the presence of Mono-7D6-Fab as well as Bi-7D6-Fab with the characteristic faster migrating band pattern (Fig. 5D, bands Y and Z; band Y is faint here, but clearly visible in longer film exposures that overexpose the rest of the blot (data not shown)). In contrast, the profile of Mono-7D6-Fab stored and refractionated in PBS-Pro showed only the presence of Mono-7D6-Fab (Fig. 5E). We conclude that purified Mono-7D6-Fab stored in PBS forms bivalent complexes over time and that purification and storage in PBS-Pro inhibits the formation of these bivalent complexes.

L-Proline Reverts Some Preformed Bi-7D6-Fab Complexes Back into Mono-7D6-Fab—We sought to determine the extent to which preformed Bi-7D6-Fabs could be separated by L-proline into Mono-7D6-Fabs. Freshly generated 7D6 Fab was purified in PBS and then diluted for storage with an equal volume of either PBS or PBS + 4 mM L-proline, for a final concentration of 2 mM L-proline (PBS-Pro) in the latter case. After 1 week of storage, both preparations were inactive, failing to induce CD69 up-reg-
ulation upon incubation with B6 peripheral T cells (Fig. 6A, top). However, after 1 month of storage, the PBS-stored 7D6 Fab acquired stimulatory capacity, whereas the PBS-Pro-stored 7D6 Fab did not (Fig. 6A, bottom). The difference in storage conditions (with versus without L-proline) did not impact the ability of 7D6 Fabs to bind their epitope on T cells, as shown by flow cytometry (Fig. 6B).

To determine whether L-proline could reduce the stimulatory capacity of the PBS-stored 7D6 Fab, this preparation that had acquired activity was now diluted in an equal volume of either PBS or PBS + 2 M L-proline (PBS-Pro). Immediately following collection of 7D6 Fab fractions from S-400/PBS or S-400/PBS-Pro as depicted in A, B6 peripheral T cells were treated for 24 h with the indicated Ig species and fractions at 5 μg/ml to monitor T cell stimulation. The final concentration of L-proline was adjusted to 50 mM in the cultures related with the PBS-Pro condition. CD69 expression is represented on the y-axis as a percentage of the maximum induction of CD69 on Thy1.2+ CD8+ T cells treated with 2C11 mAb (PBS-Pro). B, immediately following collection of 7D6 Fab fractions from S-400/PBS or S-400/PBS-Pro as depicted in A, B6 peripheral T cells were treated for 24 h with the indicated Ig species and fractions at 5 μg/ml to monitor T cell stimulation. The final concentration of L-proline was adjusted to 50 mM in the cultures related with the PBS-Pro condition. CD69 expression is represented on the y-axis as a percentage of the maximum induction of CD69 on Thy1.2+ CD8+ T cells treated with 2C11 mAb. D and E, Mono-7D6 Fab PBS and PBS-Pro fractions remaining after C were refractionated by S-400 SEC, and 28 fractions of 1 ml each were collected as described for Fig. 2, C and D. In preparation for SDS-PAGE and Western blotting, fractions were concentrated by evaporation-centrifugation, and 20 μl of each concentrated fraction was subjected to non-reducing SDS-PAGE (8% acrylamide/bisacrylamide). Western blot was performed with donkey anti-Ms IgG-HRP. As was seen in Fig. 3, excised lanes 26 and 36–39 were blank (lanes not shown). The arrows indicate the different size Ms IgG bands X, Y, and Z. Error bars, S.E.

To determine whether L-proline could reduce the stimulatory capacity of the PBS-stored 7D6 Fab, this preparation that had acquired activity was now diluted in an equal volume of either PBS or PBS + 2 M L-proline (final concentration, 2 M L-proline). We observed that upon dilution in PBS-Pro, stimulatory capacity was immediately reduced by ~40% (Fig. 6C, left), and this effect remained after 10 days of storage (Fig. 6C, right). Next, we tested the effect of diluting in L-proline a confirmed Bi-7D6-Fab species that had been obtained via S-400 SEC fractionation in PBS from an active 7D6 Fab preparation. Again, dilution in L-proline immediately reduced T cell stimulatory capacity by ~50%, and this reversion remained after 7 and 11 days of storage (Fig. 6D). We conclude that, after forming bivalent complexes, subsequent dilution in PBS-Pro can reduce the stimulatory capacity of active Fab preparations.

Next, we compared by non-reducing SDS-PAGE and Western blot the activity-reduced 7D6 Fab that had been diluted in PBS-Pro (from Fig. 6C) with preparations whose storage was originally and always in PBS or PBS-Pro. As expected, PBS-stored 7D6 Fab displayed the characteristic band pattern associated with bivalent Fab complexes (X, Y, and Z), whereas PBS-Pro-stored Fab displayed only band X (Fig. 6E), as described above (Figs. 3 and 5). The slight mobility difference in band X versus band Y was controlled for by loading PBS-stored 7D6 Fab on both sides of the gel surrounding the other samples, showing that band mobility was uniform on opposite sides of the gel. We observed that activity-reduced 7D6 Fab in PBS-Pro displayed a band pattern with a predominant band X and decreased bands Y and Z, approaching that of the sample that had always been stored in PBS-Pro (Fig. 6E). Relative decrease in bands Y and Z could not have been due to protein loss from the sample, because the compared samples had been equalized for total protein amount, concentration, and volume, and measurements of protein amount and
concentration were taken before and after experimentation, with no differences noted between experimental groups (data not shown). Therefore, we conclude that the presence of l-proline induced some Fabs to migrate as band X, when without l-proline they would have migrated as band Y or Z. Because we showed above (Figs. 3 and 5) that, in non-reducing SDS-PAGE, predominant band X indicates Mono-Fab, and predominant bands Y and Z indicate Bi-Fab, we conclude that dilution in PBS-Pro can revert some Bi-7D6-Fabs back into monovalent Fabs.

Other Anti-TCR/CD3 Fabs Form Bivalent Fab Complexes That Stimulate T Cells—Fabs generated from the other mAbs (17A2, 2C11, and H57) were stored for up to 1 week in PBS and then subjected to S-400 SEC, with purified fractions being assessed for stimulatory capacity using the CD69 up-regulation assay. The UV absorbance profiles at 280 nm from S-400 SEC for the three Fab preparations showed single, major elution peaks for 17A2 Fab (Ve/H1 = 33.06 ml, supplemental Fig. 2A), 2C11 Fab (Ve/H1 = 33.37, supplemental Fig. 2C), and H57 Fab (Ve/H1 = 32.19 ml, supplemental Fig. 2E). Upon comparison with the S-400 SEC elution peaks of Bi-7D6-Fab (Ve/H1 = 28.79 ml, Fig. 2B) and Mono-7D6-Fab (Ve/H1 = 32.69 ml, Fig. 2A and B), we concluded that the 17A2, 2C11, and H57 Fab S-400 major elution peaks represented the monovalent ~50-kDa species of each preparation. Purified SEC fractions subjected to the CD69 up-regulation assay revealed stimulatory activity only in fractions that would correspond with bivalent Fab species (supplemental Fig. 2A, B, D, and F), as we had previously observed with 7D6 Fabs (Fig. 2C).

FIGURE 6. l-Proline reverts some preformed Bi-7D6-Fab complexes back into Mono-7D6-Fab. A, stimulatory capacity of a 7D6 Fab preparation stored in PBS (7D6 Fab PBS) or PBS-Pro (7D6 Fab Pro) for 1 week (top) or 1 month (bottom), as measured by CD69 up-regulation on B6 peripheral T cells. All IgG controls and Fab samples were tested at 5 μg/ml. CD69 expression is represented on the y axes as a percentage of the maximum induction of CD69 on Thy1.2+ CD8+ T cells treated with 7D6 mAb. B, CD3 cell surface staining on B6 peripheral CD8+ T cells by 7D6 Fab PBS and 7D6 Fab Pro shown in A after 2 months of storage. All IgG controls and Fab samples were tested at 5 μg/ml. C, aliquots of the 7D6 Fab PBS shown as active in A (bottom) were diluted in either PBS or PBS-Pro (Pro) and then restested at 5 μg/ml for their T cell stimulatory capacity in a CD69 up-regulation assay on the day of dilution (day 0, left) and after 10 days of storage at 4 °C (day 10, right). CD69 expression is represented for each time point on the y axis as a percentage of the maximum induction of CD69 on B6 Thy1.2+ CD8+ T cells treated with the active 7D6 Fab PBS diluted in PBS. D, two aliquots of a Bi-7D6-Fab obtained by the S-400/PBS SEC fractionation protocol summarized in Fig. 4A were diluted either in PBS or PBS-Pro (Pro). The resulting Fab samples were tested at 5 μg/ml for their capacity to stimulate B6 peripheral T cells immediately upon dilution (day 0) or after 7 and 10 days of storage at 4 °C. CD69 expression is represented on the y axis as a percentage of the maximum induction of CD69 on Thy1.2+ CD8+ T cells treated with the Bi-7D6-Fab diluted in PBS. E, 0.5-μg samples of the 7D6 Fab PBS shown as active in A (bottom), the same 7D6 Fab PBS diluted in Pro as shown in C, and the 7D6 Fab Pro shown as inactive in A were subjected to non-reducing SDS-PAGE in an 8% acrylamide/bisacrylamide gel and transferred to nitrocellulose for Western blotting with donkey anti-Ms IgG-HRP. An additional sample from another batch of active 7D6 Fab stored in PBS (7D6 Fab PBS) was loaded at the right end of the gel to aid in the identification of bands X, Y, and Z. The arrows indicate these bands as described in the legends to Figs. 3 and 5. Error bars, S.E.
L-Proline Decreases the Stimulatory Capacity of Anti-TCR/CD3 Fabs While Stabilizing a Proteolysis-protected Conformation Associated with Monovalency

We wished to determine the extent to which L-proline might decrease the stimulatory capacity of 17A2, 2C11, and H57 Fab preparations. Samples from the stimulatory fractions (identified in supplemental Fig. 2) were diluted in either PBS or PBS-Pro (Pro). Aliquots of the resulting dilutions were tested for their T cell stimulatory activity in a CD69 up-regulation assay with B6 peripheral T cells on days 0 (the day of dilution), 7, and 11. CD69 expression is represented for each time point on the y axis as a percentage of the maximum induction of CD69 on B6 Thy1.2<sup>+</sup> CD8<sup>+</sup> T cells treated with PBS-stored, active Fabs diluted in PBS. D–G, Mono-Fab species were isolated by S-400 SEC fractions in PBS for 7D6 (D), 17A2 (E), 2C11 (F), and H57 (G). Immediately following fractionation, these Mono-Fab species were diluted in either PBS or PBS-Pro (Pro) and stored for 2 months at 4 °C. At this point, subsamples of 0.5 μg were subjected to either non-reducing (8% acrylamide/bisacrylamide; top panels) or reducing SDS-PAGE (13% acrylamide/bisacrylamide; bottom panels), followed by transfer to nitrocellulose membranes for Western blots. The arrows indicate the different size IgG bands X, Y, and Z found in each sample. Error bars, S.E.

A–C, fractions identified in supplemental Fig. 2 as containing Bi-Fab species of 17A2, 2C11, and H57 Fabs were diluted in either PBS or PBS-Pro (Pro). Aliquots of the resulting dilutions were tested for their T cell stimulatory activity in a CD69 up-regulation assay with B6 peripheral T cells on days 0 (the day of dilution), 7, and 11. CD69 expression is represented for each time point on the y axis as a percentage of the maximum induction of CD69 on B6 Thy1.2<sup>+</sup> CD8<sup>+</sup> T cells treated with PBS-stored, active Fabs diluted in PBS. D–G, Mono-Fab species were isolated by S-400 SEC fractions in PBS for 7D6 (D), 17A2 (E), 2C11 (F), and H57 (G). Immediately following fractionation, these Mono-Fab species were diluted in either PBS or PBS-Pro (Pro) and stored for 2 months at 4 °C. At this point, subsamples of 0.5 μg were subjected to either non-reducing (8% acrylamide/bisacrylamide; top panels) or reducing SDS-PAGE (13% acrylamide/bisacrylamide; bottom panels), followed by transfer to nitrocellulose membranes for Western blots. The arrows indicate the different size IgG bands X, Y, and Z found in each sample. Error bars, S.E.

storage for 2 months at 4 °C, these Fabs were analyzed by non-reducing/reducing SDS-PAGE and Western blot to assess any changes that might occur in their band profiles. We found that in non-reducing conditions, all PBS-stored Fabs produced pattern that favored faster mobility bands when compared with PBS-Pro-stored Fabs (Fig. 7, D–G, top panels). Bands are labeled as unknowns X, Y, and Z, which are unique for each Fab (Fig. 7, D–G, top panels). In contrast, in reducing conditions, only the band patterns expected for full-length Fab heavy and light chains (~23–25 kDa) were observed in all experimental groups (Fig. 7, D–G, bottom panels). Thus, like Bi-7D6-Fab, other bivalent Fabs were associated with faster mobility bands observed specifically under non-reducing SDS-PAGE conditions. Although Fabs stored in PBS-Pro can display the faster migrating band Y and/or Z to some extent, an observation common to all of the Fabs is that, when stored in PBS alone, the relative amounts of Y and Z increase, along with bivalency and stimulatory capacity. We interpret these data as indicating that...
l-proline functions as an osmolyte for all of these Fabs, stabilizing a proteolysis-protected conformation that favors monovalency while inhibiting adoption of a proteolysis-susceptible conformation associated with formation of a bivalent, stimulatory complex.

Storage in l-Proline Is Compatible with Anti-TCR/CD3 Fab-mediated Inhibition of T Cell Stimulation

We tested the panel of anti-TCR/CD3 Fab preparations stored in PBS-Pro for their capacity to block TCR antigen recognition without causing T cell activation intrinsically. First, OT-I peripheral T cells were incubated on ice together with the different PBS-Pro-stored Fabs, followed by washing and staining with corresponding FITC-labeled secondary anti-IgG Abs and APC-labeled anti-Thy1.2 mAb. Histograms show the staining of the OT-I TCR/CD3 complex on Thy1.2+ T cells (anti-IgG-FITC fluorescence, x axis). E–H, antigen binding to the OT-I TCR in the presence of PBS-Pro-stored anti-TCR/CD3 Fabs. OT-I peripheral T cells incubated with the indicated PBS-Pro-stored Fabs or nonspecific IgG controls were subsequently stained with a serial dilution of a Kb/OVA-PE tetramer and APC-labeled anti-Thy1.2 mAb. Graphs show the Kb/OVA-PE staining dilution tested on x axes and Kb/OVA-PE mean fluorescence intensity (MFI ± S.E.) on Thy1.2+ T cells on y axes. I–L, CD69 up-regulation assay of OT-I thymocytes preincubated with the indicated PBS-Pro-stored Fabs or nonspecific IgG controls at 5 μg/ml and subsequently co-cultured with T2-Kb cells preloaded with 2 μM null versus antigenic peptide (OVA). After 24 h, the co-cultures were stained with anti-Thy1.2-PerCP, anti-CD8a-APC, anti-Vβ5-FITC, and anti-CD69-PE mAbs. Graphs show CD69 expression on OT-I thymocytes (y axis) as a percentage of the maximum stimulation observed on Thy1.2+ CD8+ Vβ5+ thymocytes when co-cultured with T2-Kb APCs loaded with the peptide OVA and treated with the nonspecific IgG control. Error bars, S.E.
Bivalent Fab Complex Formation Reversible by Osmolytes

Previously (46, 47)), Analysis by flow cytometry was used to confirm the binding of the Fabs to the TCR/CD3 complex (data not shown) and show the extent of CD69 up-regulation by the thymocytes in response to antigenic stimulation. Consistent with the tetramer interefere test, we found that 7D6 and 17A2 Fabs did not perturb the response of OT-I thymocytes to OVA stimulation (Fig. 8, I and J), whereas 2C11 and H57 Fabs displayed an inhibitory blocking effect (Fig. 8, K and L). Importantly, all Fabs stored in PBS-Pro failed to induce CD69 intrinsically, indicating that they were in a monovalent state that did not cross-link TCR/CD3. We conclude that the osmolyte principle can be applied to Fab fragment preparations, stabilizing a proteolysis-protected conformation associated with monovalency and improving the potential application of Fabs as non-cross-linking, monovalent, blocking reagents.

DISCUSSION

Fab fragments are commonly used in laboratory investigations as monovalent binding reagents that can occupy their ligands without inducing cross-linking (4–10). However, it is a fairly common experience that Fab preparations that initially display blocking properties can acquire inherent stimulatory capacity. This occurrence is often attributed to aggregation of Fabs, which eliminates the ability of the preparation to be used for signaling blockade. As a result of this phenomenon, common practice often involves discarding old, stimulatory Fab preparations and preparing new, fresh Fab stocks on a routine basis. In addition, this tendency of Fab preparations to acquire inherent stimulatory capacity represents a significant impediment to the more widespread development of Fab fragments for clinical applications.

A preliminary expectation might be that Fab aggregation represents a diverse process that results in protein aggregates of heterogeneous copy number and valency. According to this idea, it might be predicted that this process is probably distinct and irreproducible between different Fab preparations and/or different monoclonal IgG specificities. However, in studying four Fab fragments with specificity for the TCR/CD3 complex, we were surprised to observe that each formed a complex that was precisely bivalent (Figs. 1, 2, and 7). This bivalent species displayed most of the stimulatory capacity of the preparation.

Bivalent Fab complexes appear to be composed of two monovalent Fabs associated by non-covalent interactions (Figs. 2 and 3 and supplemental Fig. 2 and Table 1). The experiments focusing on 7D6 Fab showed no differences in native mass between the Fabs that compose a monovalent versus a bivalent complex, when non-covalent associations were separated and Fabs were analyzed by MS (supplemental Table 1) or reducing SDS-PAGE (Fig. 3C). This observation was also true for the other Fab specificities (reducing SDS-PAGE analysis; Fig. 7, D–G, bottom panels). Amino acid sequence analysis showed that the same peptide fragments were identified in digests originating from reduced samples of both Mono- and Bi-7D6-Fabs (supplemental Tables 2 and 3), corroborating the presence of both heavy and light chains in both states of valency. It remains formally possible that an unconventional stoichiometry of full-length Fab heavy and light chains composes the bivalent complex, because the present data do not directly exclude a model where, for example, one conventional Fab might associate with either two lone heavy chains, or two lone light chains. However, we consider this unlikely, given the data showing that the Bi-Fabs possess two functional binding sites (T cell activation data; Figs. 1, 2, and 7 and supplemental Fig. 2). It is rare for unassociated heavy or light chains to inherently possess the antibody’s binding specificity, because the binding site is usually formed combinatorially via the complementarity-determining regions of the folded heavy and light heterodimer (51, 52). Therefore, it is most likely that a bivalent Fab represents a complex containing two conventional Fabs.

We propose a model in which Fabs composing a bivalent complex possess a conformation distinct from that of Fabs composing a monovalent complex. Differential susceptibility to proteolysis is a property known to be linked to protein folding and conformation (21, 49, 50), and we observed that bivalent Fabs display a unique, reproducible susceptibility to proteolysis under conditions associated with non-reducing SDS-PAGE (Figs. 3, 4, and 7 and supplemental Table 2). We conclude that the generation of faster mobility protein band patterns in non-reducing SDS-PAGE can be used as a “marker” indicating that the sample contained bivalent Fab complexes. These data cause us to hypothesize that Fabs composing a bivalent complex display a conformation distinct from that of lone monovalent Fabs.

The chemical mechanism by which this non-reducing SDS-PAGE-associated proteolysis occurs specifically in bivalent but not monovalent Fabs is not yet understood. Peptide bond hydrolysis in band Z (Fig. 3 and supplemental Table 2) does not occur until the 7D6 Fab sample is prepared and run in non-reducing SDS-PAGE (schematically represented in Fig. 4). Sample heating is not absolutely required, because guanidine HCl-mediated denaturation at 4 °C followed by SDS-PAGE also generates bands Y and Z (data not shown). We are currently investigating whether the mechanism involves acid hydrolysis together with complete protein denaturation, but we do not yet have conclusions on this point. Extrinsic enzymatic protease activity is unlikely to generate the faster mobility bands from Fab bivalent complexes. There is no papain in our preparations of purified 7D6 Fab fragments, as per intact mass determination and peptide sequencing by MS techniques (data not shown), and neither storage at 4 °C nor heating to 95 °C inhibits generation of bands Y/Z for any of the Fabs in the panel, nor do mixtures of various kinds of protease inhibitors (data not shown). In particular, serine protease inhibitors do not impact this proteolysis (data not shown), arguing that it is unlikely that this proteolytic activity originates from intrinsic protease activity from the Fab itself; an uncommon but reported activity of some antibodies is that they can possess catalytic protease activity toward the antigens they bind (53), and this activity, when observed, is most often via serine protease activity (54, 55). Elucidation of the chemical mechanism behind the specificity of this conditional proteolysis remains a focus of investigation.

Formation of precisely bivalent complexes implies that Fab “aggregation” can involve an ordered process conserved between different IgG specificities. We do not yet know if the
two mono-Fabs that comprise a bivalent complex are arranged in a universally conserved spatial orientation when considering Fabs that originated from different IgGs. However, we know that all of the bivalent Fabs preserve their epitope binding capability and acquire stimulatory capacity. Therefore, this implies that both antigen binding sites in bivalent Fabs are functional, and the resultant TCR/CD3 receptor cross-linking is what causes signaling (56). In practical terms, an ordered, reproducible process of Fab complexation could evoke the generation of targeting strategies to prevent it.

The conformation-based hypothesis generated the prediction that osmolytes, known to stabilize protein folding/packing/conformation, might affect formation of bivalent Fab complexes. We employed a known osmolyte, l-proline (37, 43), and found that it effectively prevented formation of bivalent Fab complexes and also separated a significant portion of preformed bivalent complexes into monovalent species (Figs. 5–7). A second osmolyte, glycerol (34, 38), also displayed the ability to prevent formation of bivalent Fab complexes, but we did not fully pursue experiments using glycerol because l-proline was more compatible with viable T cell culture (data not shown). Osmolytes have been previously shown to play important roles in protein folding, packing, and conformation. The proposed mechanism to explain this property of osmolytes is based on the solvophobic effect (36, 39), which drives polypeptides to exclude osmolyte molecules from the protein surface by stabilizing a folded conformation that minimizes its solute-exposed surface area. In this regard, it seems that bivalent Fab complexation involves adoption by the mono-Fab of a specific folding/packing/conformation that either mediates or accompanies bivalency. Evidence for this alternative conformation involves increased susceptibility to proteolysis and altered electrophoretic migration displayed by Fabs composing a bivalent complex when they are subjected to heat denaturation and non-reducing SDS-PAGE (Figs. 5–7). Treatment of preformed bivalent Fabs with l-proline reverts their SDS-PAGE migration back to the profile observed for non-complexed mono-Fabs while impairing their capacity to stimulate T cells (Fig. 6). As shown in Figs. 5 and 7, l-proline preserves the proteolysis-protected Fab conformation associated with monovalency, consistent with functions that are attributed to this amino acid as an osmolyte that stabilizes native protein folding (37, 43).

The mechanism that drives bivalent complexation of Fabs is not yet clear. Any favored mechanism must take into account the fact that all four Fabs tested in the present study displayed the propensity to form these complexes. It has been reported that 50–70% of proteins display some positive level of homodimerization (57), which brings up the possibility that the observations in the current work may reflect a more universal tendency among proteins. The phenomena of “protein breathing” and “folding funnels” (58, 59) suggest that proteins can vacillate between a number of different conformations that represent entropically favorable free energy states. Perhaps certain of these spontaneous conformations create binding opportunity and entropic favorability for a bivalent Fab complex. Whatever the mechanism may be, l-proline separates some bivalent complexes back into monovalent complexes. This is consistent with the idea that protein conformation and folding are probably important for controlling Fab complexation.

These observations regarding the effect of osmolytes on the Fab complexation allow the proposal of a prescription to minimize Bi-Fab formation and thus minimize the acquisition of stimulatory capacity of Fab preparations. Following Ab digestion with papain and protein A-mediated removal of Fc-containing species, Mono-Fab fragments can be purified by SEC in buffers containing 2 M l-proline. We have examined such Fab fragments following up to 5 months of storage in l-proline, at which time they still retain a lack of stimulatory activity (data not shown). Therefore, we conclude that employment of the osmolyte principle can improve the maintenance of Fab fragments in a non-aggregated, monovalent state. This effect can potentially make a significant contribution to the development of more Fab-based reagents for clinical therapies.

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