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METAL-BINDING PROPERTIES OF A CALCIUM-DEPENDENT MONOCLONAL ANTIBODY

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Abstract—The calcium-dependent mAb, M1 (also called anti-Flag or 4E11) was studied using a newly developed metal-sensitive enzyme-linked immunosorbent assay (ELISA). This antibody, specific for a calcium complex of the peptide antigen, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, has found widespread use as a mild purification reagent for Flag-epitope tagged recombinant proteins. Although M1 affinity columns release monovalent Flagged proteins in the absence of calcium, the antibody retains substantial affinity for the Flag sequence even in metal-free conditions, so that it has been impossible to use it to develop a metal-sensitive ELISA assay. This is due to the ability of the antibody to remain bound to polyvalent surface-coated antigen, for instance, when Flagged proteins are bound to ELISA plates or blotting filters. The resultant antigen polyvalence raises the avidity of the Flag antibody to a point where the reaction is essentially calcium-independent. However, when the antibody itself was made monovalent, by proteolytic cleavage to the Fab, this situation was reversed and the ELISA reaction became calcium-dependent. This new metal-dependent ELISA assay was used to explore the metal requirements of the antibody in detail. Among divalent metals, binding tapered off with increasing radius above that of calcium, or with decreasing radius below that of calcium. Several smaller metals, such as nickel, acted as inhibitors of the binding reaction. Substantial binding was demonstrated for heavy metals such as cadmium, lanthanum and samarium. Because it is of interest to use this antibody for the co-crystallization of recombinant Flag-fusion proteins, the ability to bind heavy metals was a significant finding. Copyright © 1996 Elsevier Science Ltd

Key words: monoclonal antibody (mAb), calcium, metal, binding, epitope tag, Flag.

INTRODUCTION

Antibodies that bind to metals or metal complexes are rare, but often interesting, examples of the range of possibilities in an immune response. They have been observed for both natural and artificial immunogens. For example, industrial workers exposed to metal dusts or powders can develop metal-specific allergies which have in many cases been shown to result from the presence of IgE antibodies that bind to metal–protein complexes. Where the reaction specificity has been explored, plasma proteins such as serum albumin are usually found to participate in the complex (Dolovich *et al.*, 1984; Veien *et al.*, 1979). Furthermore, it has been noted that these complexes can be highly specific, in that the metals must be complexed to the N-terminal tripeptide of serum albumin, Asp-Ala-His, in order to be recognized (Nieboer *et al.*, 1984). This tripeptide is known as the Cu^{+2}/Ni^{+2} transport site of albumin, having a physiological role in the chelation and movement of metals in the circulatory system. Antibodies from allergic patients are usually able to discriminate one metal from another in the context of serum albumin, presumably because the metals exhibit distinguishable variations in the geometry of their complexes with Asp-Ala-His (Shirakawa *et al.*, 1992). Whereas Ni^{+2} has frequently been identified as the allergen, other metals such as Co^{+2}, Cr^{+2} and Pt^{+2} have also been implicated (Murdoch and Pepys, 1985).

Artificial systems have also been used to raise and study metal-dependent antibodies. Early work concentrated on synthetic polyamino acid immunogens. Researchers were able to produce mixtures of calcium-dependent antibodies from sheep immunized with the random polymer, $(Glu, Ala, Tyr)_n$ (Liberti *et al.*, 1971). Because of the heterogeneity of both the immunogen and the antibodies obtained, it was not possible to define a precise specificity in this case. In another example, a cyclic-AMP-bovine albumin conjugate was used to raise an anti-cAMP mAb that was shown to require calcium for binding (Luttrell and Henniker, 1991). Chelators such as benzyl-EDTA, conjugated to keyhole limpet hemocyanin, have also been used to produce metal-dependent antibodies (Rearden *et al.*, 1985). In this latter case, highly specific antibodies could be obtained, for example, a mAb which is capable of discriminating indium complexes from other metal complexes with the immunizing chelator (Love *et al.*,

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; mAb, monoclonal antibody; Fab, immunoglobulin antigen-binding fragment.

1993). Immunizations with naturally occurring proteins have also led to the production of metal-dependent antibodies. Among the most common are antibodies against the family of vitamin K-dependent plasma proteins. These proteins contain regions bearing multiple gamma-carboxy glutamic acid residues (Gla domains) that readily form complexes with metals. As a result, antibodies against these regions are frequently metal-dependent (Wakabayashi *et al.*, 1986). Several studies have investigated the fine specificities of such antibodies, and a common epitope has been identified within the Gla domains of several proteins (Church *et al.*, 1988). Other metal-dependent antibodies have been obtained against the calcium binding sites of the EGF domains of thrombomodulin (Kimura *et al.*, 1989) and protein C (Ohlin and Stenflo, 1987), as well as the trypsin-like calcium site found in factor IX (Bajaj *et al.*, 1992), a calcium-binding domain in troponin C (Strang and Potter, 1992) and the calcium-binding site of the platelet-aggregating protein GPIIb (Gulino *et al.*, 1990). Among the variety of metal-dependent antibodies, some seem to have narrow metal specificities whereas others react with a broad range of different metals. Most are still poorly characterized at the level of molecular structure.

The calcium-dependent mAb M1, also referred to as anti-Flag, has been characterized in more detail than most other metal-binding antibodies. This antibody was raised by immunizations with the Flag epitope sequence, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (Hopp *et al.*, 1988) and has found wide use in the identification and purification of a number of recombinant proteins which bear the Flag epitope tag (Chiang and Roeder, 1993; Gerard and Gerard, 1994; Gerszten *et al.*, 1994; Guan *et al.*, 1992; Knappik and Plueckthun, 1994; Park *et al.*, 1989; Prickett *et al.*, 1989). A preliminary amino acid scan established that the antibody binds primarily to the first four amino acids and has a strong requirement for a free N-terminal amino group (Prickett *et al.*, 1989). Studies with peptide combinatorial libraries further clarified the importance of the N-terminal region, and established that the Asp at position 7 is also required for complex formation (Pinilla *et al.*, 1995). However, until now, the extent of the metal specificity has been unknown. This paper describes the development of a metal-sensitive ELISA assay and a detailed exploration of the metal specificity of the Flag-anti-Flag reaction.

MATERIALS AND METHODS

Materials

The anti-Flag mAb M1 was obtained from Kodak Imaging Systems (formerly IBI), New Haven, CT. Metals were the highest quality available, and were obtained as chloride salts, or occasionally, acetate or sulfate salts. Flagged interleukin 3 was prepared as described (Park *et al.*, 1989) by expression in yeast cells. Culture supernatants containing the product were clarified by passing through a 0.45 mm filter, and used for anti-Flag affinity chromatography without further treatment. Flag peptide-conjugated ovalbumin was prepared as described previously (Prickett *et al.*, 1989).

Preparation of affinity columns

The Flag peptide affinity column consisted of 5 ml of Sulfolink resin (Pierce, Rockford, IL) conjugated via a thioether bond to a version of the Flag peptide which has a cysteine residue near its C-terminus (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Gly-Cys-Gly). Approximately 8.7 mg of this peptide were attached to the support, using the manufacturer's recommended procedures. To prepare the anti-Flag antibody affinity column, whole M1 antibody was coupled to AffiGel-Hz (BioRad, Hercules, CA) according to the manufacturer's instructions. Sugars on the antibody were oxidized with sodium periodate, and it was then transferred into "Hz" coupling buffer (BioRad) by gel filtration on a Sephadex G-25 fine column (Pharmacia, Piscataway, NJ). Then 64 mg (12 ml) of this material was reacted with 6 ml of AffiGel-Hz, for 14 hr at room temperature. Coupling was 64% complete, yielding a gel that contained 6.8 mg of antibody per ml.

Fab production and purification

The Fab fragment of M1 was produced according to published procedures (Demignot *et al.*, 1989; Harlow and Lane, 1988). Papain (Sigma P-9886, St Louis, MO) was activated for 15 min after diluting it to a concentration of 95 mg/ml in a buffer containing 20 mM cysteine and 2 mM Na_2EDTA adjusted to pH 7.5 with NaOH. The enzyme (65 ml) and M1 (65 ml of a solution of antibody at 9.5 mg/ml in 20 mM HEPES, 150 mM NaCl, pH 7.5 buffer) were combined to yield an enzyme-to-substrate ratio of 1/100. The reaction was incubated at 37°C for 3 hr, then was stopped by adding iodoacetamide to a concentration of 25 mM. After dialysis against 10 mM Tris–HCl pH 7.5, the products were applied to a 0.7×20 cm column of DEAE Sepharose CL-6B (Sigma) equilibrated with 10 mM Tris–HCl pH 7.5, and eluted with a gradient from 0 to 1 M NaCl in the same buffer. The first peak, containing Fab, was pooled then dialysed against 5 mM Tris–HCl pH 7.5. The second peak containing Fc was discarded.

ELISA conditions and inhibition

ELISA assays were carried out in 96-well Pro-Bind plates (VWR, Chester, PA). Plates were coated with a Flag peptide-ovalbumin conjugate, as described (Prickett *et al.*, 1989). Binding of M1 (usually present at 10 μg/ml) or its Fab fragment (also 10 μg/ml) was carried out in TBS–Tween buffer (20 mM Tris–HCl, 150 mM NaCl, pH 7.2 containing 0.02% v/v Tween-20) with or without metals or other additives. After binding for 1 hr at room temperature, plates were washed with TBS–Ca–Tween (TBS–Tween with 1 mM $CaCl_2$ added) then reacted with goat anti-mouse Fab peroxidase conjugate (Sigma A-4416) for 45 min at room temperature. After another wash in TBS–Ca–Tween, the plates were reacted with substrate solution (tetramethylbenzidine; Sigma T-3405.

dissolved in phosphate-citrate buffer with urea-hydrogen peroxide; Sigma P-9305) and read at 450 nm on a Titertek Multi-scan microtiter plate reader after quenching with H_2SO_4 at 30 min. The results shown in this paper represent averaged values for two to four determinations per point. Metals other than calcium were diluted into TBS–Tween lacking calcium for testing. For inhibition experiments, inhibitors (at various concentrations) were combined with Fab in TBS–Ca–Tween and incubated at room temperature for 2 hr, before transfer into the antigen coated wells.

RESULTS

Antibody purification

In order to remove contaminating proteins from the antibody we used the Cys-Flag-Sulfolink affinity column for antigen-specific chromatography. Chromatography on this column removed all detectable contaminating proteins. Up to 181 mg of antibody could be purified on this column in a single run, based on an extinction coefficient of 1.35 at 280 nm. This represents a capacity of at least 36.3 mg of antibody per ml of gel. A low pH buffer is required to elute the antibody from this column, with pH below 4.0 required before substantial material elutes. We chose to use this approach to purify the anti-Flag antibody because of the high purity achieved. The only contaminant remaining after this procedure was a faint band seen on heavily loaded SDS–PAGE gels, which can be accounted for by the presence of a small amount of naturally occurring Fabc fragment that has been reported by others working with mouse IgG2b (Demignot *et al.*, 1989). Because it would be converted to Fab, we did not attempt to remove this material.

Fab production

The Fab fragment of the anti-Flag antibody was produced by papain digestion using published procedures (Harlow and Lane, 1988). The digestion was monitored by SDS–PAGE after aliquots were quenched with iodoacetamide. As expected, whole antibody disappeared within 30 min and fragments began to appear. The Fab appeared quickly and was stable, even during prolonged digestion times (out to at least 4 hr). Yields of Fab were high, typically around 50 mg per 100 mg of antibody digested. The reaction was stopped by adding iodoacetamide, and the preparation was dialysed against 150 mM NaCl, 20 mM Tris/HCl pH 7.2 buffer and stored at 4°C with 0.02% NaN_3 added.

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up to 1 M had no

Results from ELISAs

Figures 1 and 2 contrast the effects of calcium on ELISAs carried out with whole antibody or Fab. The sigmoid curves obtained with whole antibody in the presence or absence of calcium in the binding medium are nearly superimposable. This contrasts with the results for the Fab, where a much lower maximum color reaction was obtained for the Fab in the absence of calcium.

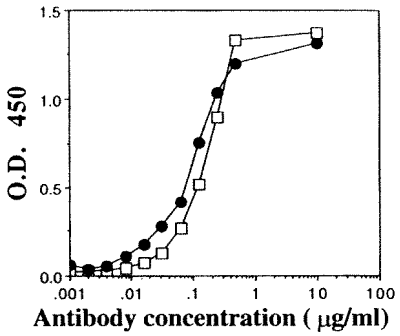

Fig. 1. Calcium-insensitive whole-antibody ELISA. The two data plots indicate ELISA reactions using the whole M1 antibody with (□), or without (●), calcium in the medium (1 mM $CaCl_2$). The two curves obtained are nearly superimposable, indicating that the ELISA is calcium-insensitive when performed with whole antibody. This contrasts with the Fab ELISA results shown in Fig. 2.

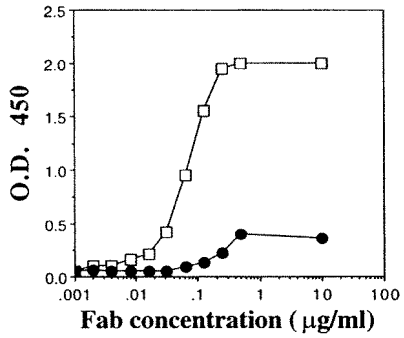

Fig. 2. Calcium-sensitive Fab ELISA. As in Fig. 1, the data plots represent binding in the presence (□), or absence (●), of calcium. The great decrease in color development when binding is carried out in the absence of calcium reflects a lower avidity for the monovalent Fab fragment compared to the divalent whole antibody (see text). This differential binding and color development were used as the basis for the metal-sensitive ELISA tests done in this study.

Further experiments were carried out to determine the latitude of conditions under which the antibody/antigen complex is stable. We found that the Fab fragment was much more sensitive to changes in the binding medium, compared to the whole anti-Flag antibody, when used in the ELISA format. This provided a sensitive test for the effects of various salts and pHs on Flag-anti-Flag binding. Despite this sensitivity, the Fab remained reactive with the Flag sequence over a wide range of environmental conditions. This is consisten
conclusion that the Flag-anti-Flag
affinity interaction with an assoc
around 10^9 M^{-1}, which shifts to a lo
significant interaction (K_a around
calcium ion is removed.

Examples of media components te
lowing: (a) *pH*: values of pH were
from 3.0 to 10.0 in the ELISA react
color development was seen across a
pH 5.0 to pH 8.5. The reactivity taper
and below these values, and was nea
and above pH 10.0; (b) *salts*: NaCl

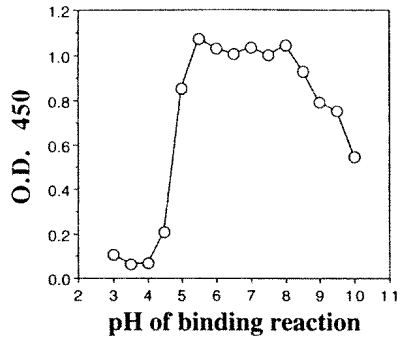

Fig. 3. Effect of pH on the ELISA. The Fab ELISA was developed under normal conditions, with 1 mM $CaCl_2$, 150 mM NaCl and 0.02% Tween-20 present in the primary Flag-anti-Flag binding medium, except that a wide-range buffer was used. This buffer consisted of formic acid, acetic acid, MES, HEPES, Tris and ethanolamine, each at a final concentration of 10 mM, adjusted to various pH values with either HCl or NaOH.

inhibitory effect on the reaction, $MgCl_2$ inhibited only partially at concentrations above 0.5 M, and the same was true for Tris–HCl (Fig. 4); (c) *chelators*: EDTA, EGTA and citrate inhibited the reaction completely in concentrations above 16 mM for the first two, and 120 mM for citrate (Fig. 4, Table 1) in the presence of 1 mM $CaCl_2$; (d) *metals*: nickel inhibited the Ca-dependent reaction (Fig. 4, Table 1). This finding is distinct from its failure to promote binding, described below. As already mentioned, Mg^{2+} in high concentrations also showed a slight inhibitory activity.

Divalent metals

These metals were added to the TBS–Tween ELISA binding medium in varying concentrations and gave sigmoid curves of color development based on metal concentration (Fig. 5). The results obtained correlate well with the ionic radius of each metal tested (Fig. 5 and Table 2), with those metals having radii near that of calcium showing the greatest reactivity. The reactivity of the alkaline earth series correlated closely with radius, with the small radius element Mg^{2+} inactive in Flag binding, Ca^{2+} showing maximal activity, and Sr^{2+} and Ba^{2+} less active in relationship to their larger radii.

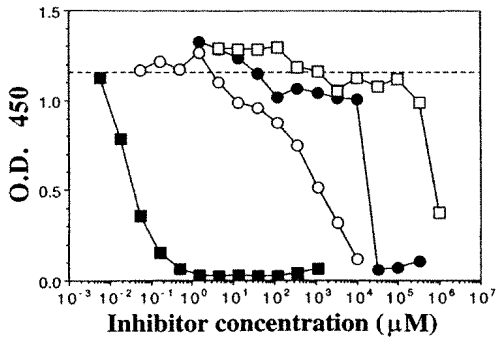

Fig. 4. ELISA inhibition assays. Assays were set up under optimal conditions (TBS–Ca–Tween) and varying concentrations of inhibitors were incubated with the Fab before it was added to the plates. Curves represent: Flag peptide (■), $NiCl_2$ (○), EDTA (●) and Tris–HCl (□). The dashed line is shown at the level of the average result obtained in the normal reaction with 1 mM Ca (positive control).

Table 1. Inhibition of the ELISA reaction

Inhibitor	IC50[b]	Remarks
Flag peptide	22 nM	AspTyrLysAspAspAspAsp-LysGlyCysGly[a]
EDTA	14 mM	–
EGTA	16 mM	–
Citrate	32 mM	–
Ni^{2+}	470 μM	–
Mg^{2+}	> 1 M	Slight inhibition at 1 M
Tris	560 mM	–
Na^+	> 1 M	No effect at 1 M

[a]The acidic peptide was neutralized with NaOH before use.
[b]Concentration that reduced the ELISA color reaction by half.
Substances with significant inhibitory activity in the ELISA are shown, and compared to NaCl, which had no effect. The acid materials EDTA, EGTA and citrate were tested as sodium salts, while the cations Ni^{2+}, Tris, Mg^{2+} and Na^+ were tested as chloride salts.

It appears from the data obtained that the Flag/Fab binding site has more tolerance for ions that are larger than Ca^{2+} than it does for smaller ions (Table 2, columns 3 and 4), with Sr^{2+} being the best alternative to Ca^{2+} when present at 10 mM levels. Among other divalent metals, Cd^{2+} was the next most reactive, consistent with its radius and the fact that it is often used as a calcium substitute. Ba^{2+}, Mn^{2+} and Pd^{2+} had lesser, but significant reactivity. Other divalent ions fell into two groups: the first being those that were tested and found to be non-binding, including all small radius ions in the transition series, namely Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}. The second group included those metals too insoluble to test, namely Sn^{2+}, Hg^{2+} and Pb^{2+}.

Trivalent metals

A variety of trivalent, and several tetravalent metals were examined as well. Many were too insoluble in ELISA buffer to work with, including all of the tetra-

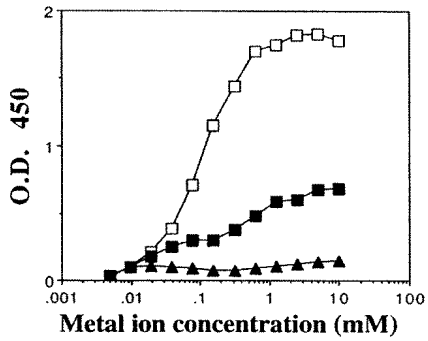

Fig. 5. Divalent metal ELISA data. Effect of increasing metal ion concentrations on binding of the Fab to Flag ovalbumin-coated plates. Data plots represent: Ca^{2+} (□), Mn^{2+} (■) and Mg^{2+} (▲), added as chloride salts. Concentrations of Fab and other salts were held constant, while increasing amounts of the indicated metal salts were added.

Table 2. Ionic radius vs binding

Cation	Ionic radius	EC50mM	ELISA reactivity, % Ca at 10 mM[a]
Ba^{2+}	1.34	>10	36
Sr^{2+}	1.12	2.3	91
Ca^{2+}	0.99	0.11	100
Cd^{2+}	0.97	0.62	70
Mn^{2+}	0.80	>10	35
Pd^{2+}	0.80	>10	14
Mg^{2+}	0.66	–	3
Ni^{2+}	0.69	–	−1
La^{3+}	1.14	0.003	N.D.[b]
Sm^{3+}	1.00	0.033[c]	N.D.[b]

[a]Color value of the ELISA reaction when 10 mM calcium was present represents 100%. Other metals are also compared at 10 mM, expressed as a percentage of the color developed with 10 mM Ca^{2+}.

[b]N.D., not determined.

[c]Estimated from curve in Fig. 6.

The metals that were tested are shown, along with their ionic radii and results of the metal-binding ELISA reaction. Because some metals were not soluble at levels that would allow the determination of an EC50, the value obtained at 10 mM is included as a measure of relative reactivity. Lanthanum and samarium were not soluble at 10 mM.

valent ions as well as Sc^{3+}, Cr^{3+}, Fe^{3+}, Y^{3+} and Bi^{3+}. On the other hand, the lanthanide series yielded several members with sufficient solubility to be tested, and the results are shown in Fig. 6. Although all of the lanthanides exhibited insolubility above 1 mM concentration in ELISA buffer, interesting data were obtained at lower concentrations of lanthanum (La^{3+}), samarium (Sm^{3+}), and terbium (Tb^{3+}).

At concentrations near their solubility maxima, these ions caused an artifactual color development to values above 2.0, even in the absence of specific anti-Flag antibody. However, in the micromolar range and below, this

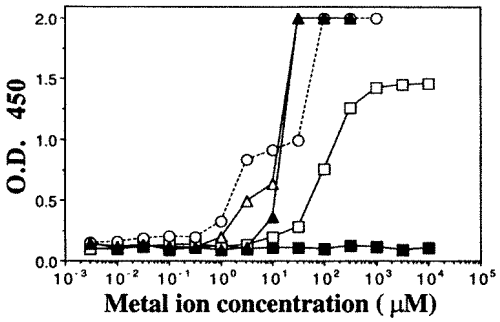

Fig. 6. Trivalent metal ELISA data. Effect of increasing metal ion concentrations on binding of the Fab to Flag ovalbumin coated plates. Data represent: $CaCl_2$ (□), no metal (■), or La^{3+} (○), Sm^{3+} (△) and Tb^{3+} (▲), added as acetate salts. An artifactual color development causes the curves for the lanthanide metals to exceed 2.0 in the higher concentration range, above 20 μM (compare to Ca^{2+} curve). However, the portions of the curves below 2–20 μM were shown in control experiments to be dependent on the presence of specific anti-Flag Fab and the indicated metal.

effect was not seen. Instead, a Fab-dependent reaction developed, giving a biphasic curve for color development vs. ion concentration. The portion of this curve at and below 1 μM was useful for determining relative binding affinities, which are included in Table 2. As was seen with the alkaline earths, the lanthanides show a preference for larger over smaller ionic radii.

Samarium in affinity chromatography

In order to confirm the ELISA-based observation that Sm^{3+} binds in the Flag/Fab complex, we carried out a separate set of experiments using affinity chromatography on the same anti-Flag column that was used to purify Flag IL-3 in the Ca^{2+} dependent process. In this case, however, we substituted Sm^{3+} for the Ca^{2+} normally incorporated in the loading and washing buffers of the column. The presence of 10 μM samarium was sufficient to retain Flag IL-3 on the column while contaminating proteins were washed away, then addition of EDTA to the washing buffer caused elution of purified Flag IL-3. The affinity chromatography behavior was identical to the results reported in the original description of the calcium-mediated Flag affinity purification system (Hopp *et al.*, 1988). This confirms the ability of Sm^{3+} to bind in the Flag-anti-Flag complex, because normally the absence of Ca^{2+} leads to premature elution of Flagged proteins in the wash fractions.

DISCUSSION

The calcium dependence of the M1 mAb was discovered serendipitously while searching for a mAb that would work effectively as a purification tool for Flag-fusion proteins (Hopp *et al.*, 1988). Subsequent studies characterized the specificity of the interactions of M1, demonstrating a requirement for a free N-terminal amino group on the Flag sequence, as well as strict requirements for Asp, Tyr, Lys and Asp at positions 1–4, respectively. A preliminary investigation of metals indicated that calcium strongly promoted binding, whereas several other metals were ineffective (Prickett *et al.*, 1989).

Flag technology

When covalently attached to a solid support, the anti-Flag antibody can be used for the rapid purification of Flag-fusion proteins in a mild, calcium-dependent affinity chromatography procedure (Hopp *et al.*, 1988; Park *et al.*, 1989; Prickett *et al.*, 1989). Flag-fusion proteins are typically purified to homogeneity in a single step, starting from a crude cell homogenate or supernatant, without ever exposing the protein to conditions other than physiological saline at pH 7.2 (with calcium or EDTA). The entire purification process can be carried out in several hours. This process has been applied to several important classes of molecules, including gene-regulatory proteins (Chiang *et al.*, 1993), members of the seven-transmembrane helix receptor family (Gerszten *et al.*, 1994; Guan *et al.*, 1992) and immunoglobulins (Knappik and Plueckthun, 1994) in addition to the cytokines purified

in our laboratories (Hopp *et al.*, 1988; Prickett *et al.*, 1989). Further exploration of the metal specificity of M1 may lead to improved performance in affinity chromatography. For example, nickel-mediated elution seems possible, given the results presented in this paper.

ELISA behavior of the M1 antibody

The performance of the antibody and its Fab fragment in the ELISA assay is unusual. This can be observed in Figs 1 and 2, which demonstrate the calcium insensitivity of the reaction when using whole antibody and the calcium-dependence of the reaction with the Fab. First, considering the whole antibody, the near superimposability of the curves obtained with and without calcium indicates that a complex is indeed formed by the antibody and antigen in the absence of calcium, and that this binding is strong enough to withstand the washing and subsequent incubations without dissociation. This lack of a detectable calcium effect contrasts with the known behavior of this antibody in affinity chromatography, where calcium is critical to successful binding.

Because this study required a rapid, sensitive way to measure metal reactivity in this system, it was necessary to overcome this lack of metal-sensitivity by trying alternative approaches. Fortunately, when the Fab was tested in the same ELISA format, the results were dramatically different. As can be seen in Fig. 2, the Fab ELISA shows a powerful calcium requirement. It should be noted that whereas color development is greatly reduced when calcium is omitted from the binding medium, both curves have a sigmoid character, indicating a saturation of the plate-bound antigen in both cases. This in turn indicates that sufficient binding takes place to bind all of the Flag peptide that is available even when calcium is absent, but that some subsequent step in the series of reactions fails to occur to the same extent as when calcium is present. A simple explanation that we favor is that, whereas binding may proceed to saturation either in the presence or absence of calcium, the dissociation of the antibody takes place much more rapidly when calcium is absent from the complex. This leads to the observed results because the Fab, when complexed to both antigen and calcium, remains bound to the plate for a sufficient time to allow the washes and secondary antibody–enzyme conjugate reactions to take place, whereas the Fab dissociates more rapidly when calcium is not present in the complex. Because the excess of Fab has been removed by the first wash after the Fab incubation, this dissociation is no longer in equilibrium with a counteracting association, which leads to a net loss of Fab-antigen complex throughout the subsequent washing and binding reactions.

In the light of the above rationale, the most unexpected result may be the strong color reaction seen for the whole antibody, even in the absence of calcium. This apparent inconsistency can be explained in the following way: because the antigen-antibody complex formed in the absence of calcium is the same for whole antibody as it is for Fab, it follows that this complex will also dissociate rapidly, as is the case for the Fab. However, the divalent nature of the whole antibody makes a critical difference. Because dissociation of the first of the two Fab arms of the antibody leaves the second arm still attached, there exists the possibility of a re-association of the first arm before the second arm is released. Because calcium is present in all washes and subsequent reactions, any reformed complexes will contain calcium and therefore will resist further dissociation. This effect is not seen for the Fab, because, once it has dissociated, the Fab diffuses away from the surface and becomes diluted in the washing media. This dilution effect is the key difference between Fab and whole antibody in terms of the ability to maintain binding long enough to produce a color reaction. Valency-dependent affinity effects of this type have been characterized before, especially for polymeric antibodies such as IgA and IgM, where the effect is most dramatic (Crothers and Metzger, 1972). To our knowledge, this is the first time that such an effect has also had the property of being metal-dependent.

Inhibitors

Because Flag-anti-Flag binding requires calcium ions, and depends on several ionic amino acids in the Flag sequence, we tested various ionic and chelating materials for their ability to interfere in the reaction. Table 1 lists and compares the inhibitors identified in this study. The chelators EDTA, EGTA and citrate all had substantial inhibitory ability. However, none had inhibitory activity near that of the Flag peptide itself, which was approximately six orders of magnitude more effective. Although the peptide is expected to block the antibody binding site, and the chelators are presumed to inhibit by removal of calcium from the complex, it is unclear what mechanism might explain the effectiveness of nickel as an inhibitor. Whereas Ni^{2+} was found to be incapable of participating in stable complex formation in the ELISA, possibly due to its small radius, its inhibitory ability is difficult to explain. To block binding of the peptide to the antibody, Ni^{2+} must react with one or the other of these components in a complex that cannot go on to bind the last component. Therefore it seems that Ni^{2+} is capable of complexing with either the antibody binding pocket, or with the Flag sequence to yield a complex that has an inappropriate steric fit to the other component. Whether such an inhibitory Ni^{2+} complex is formed with the antibody or the peptide must await further testing.

Finally, because a number of charged amino acids of the peptide are involved in the specificity of the reaction, it is clear that this antigen–antibody complex is highly ionic in nature. Such an interaction should be interfered with by salts, as has been used in affinity chromatography elution methods for years. Indeed, slight inhibition was seen for all salts near 1 M concentration, suggesting that salt-bridges between the Flag peptide and M1 probably do exist. However, given that high concentrations of salts such as Tris–HCl, $MgCl_2$ and NaCl were only weak inhibitors of the ELISA, it seems likely that hydrophobic effects must also be relatively important in the binding

reaction, because these effects are typically resistant to salt interference.

Relationship to other metal-binding antibodies

Some of the findings presented here demonstrate that this antibody is unique in its metal specificity. The ability of some metals to promote binding and others to inhibit binding is unusual. The rather tight specificity based on ionic radius contrasts with several other mAbs that can bind to complexes with metals as widely separated in radius as calcium and magnesium (0.99 and 0.66 Å). On the other hand, inhibition by nickel suggests that the antigen or antibody may be able to take on different and distinguishable conformations while binding either Mg^{2+} or Ni^{2+}, because these metals have greatly different inhibitory activities but very similar radii (0.66 and 0.69 Å). Because Ni^{2+} is known to bind avidly to amino groups, it may be that the N-terminus of the Flag is bound by Ni^{2+} in a way that is similar to its binding of the N-terminus of the albumin Cu^{2+}/Ni^{2+} site, whereas the Ca^{2+} and Mg^{2+} complexes do not use this amino group (these metals are known to favor chelation exclusively with oxygen ligands). Alternatively, if a histidine residue exists in the binding site of the antibody, this might result in the type of behavior seen, due to the strong affinity of histidine for nickel.

The ability to substitute trivalent metals into a calcium binding site has been reported before, both for antibodies and also for other proteins. Therefore the ability to substitute them in the Flag-anti-Flag reaction complex is not entirely unexpected. However, the discovery that Sm^{3+} and other heavy metals bind to Fab/Flag complexes is a significant finding in this study, because of the planned use of these antibodies in crystallographic studies.

Antibodies and metals in crystallization

A large number of crystallized Fab fragment structures have now been reported in the scientific literature (Boulot *et al.*, 1988; Laver, 1990). Furthermore, a substantial number of Fabs have been shown to co-crystallize in complexes with the protein antigens to which they normally bind (Chitarra-Guillon *et al.*, 1988; Griest *et al.*, 1992) and it has been proposed that Fab complexation actually enhances the crystallizability of most proteins (Air *et al.*, 1987; Davis *et al.*, 1990; Jacobo-Molina *et al.*, 1991; Laver, 1990). One aspect of protein crystallography that is particularly troublesome is the need to obtain a second set of crystals that differ from the first by the substitution of a heavy atom at one or only a few sites. Unfortunately, most proteins do not contain a pre-existing heavy metal binding site. However, given the present finding that heavy atoms can be incorporated in the Flag-anti-Flag complex, it would appear that co-crystallization with anti-Flag Fab may simultaneously facilitate the crystallization of Flagged proteins, while introducing a single, specific heavy metal binding site using the calcium site in the Flag-anti-Flag complex. Because heavy metal substitution can be critical to the success of crystallographic studies, this approach may substantially reduce the time needed for the crystallization and structure analysis of many proteins.

Flag technology has already proved to be a widely applicable means to produce and purify recombinant proteins, so that its extension to crystallization and metal substitution will enable researchers to generate protein crystals by a more systematic process than has been possible before. Although the addition of the Fab adds to the complexity of X-ray structure determination, the net result nonetheless should be an increase in the rate at which crystallographic data can be obtained for proteins of interest. In preliminary experiments, we have found that the M1 Fab does indeed crystallize. Progress with this antibody-mediated crystallization system will be the subject of future reports.

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