Classification of α2-Macroglobulin-Cytokine Interactions Based on Affinity of Noncovalent Association in Solution under Apparent Equilibrium Conditions*

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Kendall P. Crookston†, Donna J. Webb, Beni B. Wolf‡, and Steven L. Gonia§
From the Departments of Pathology and Biochemistry, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

α2-Macroglobulin (α2M) binds numerous cytokines; however, since binding affinities have not been determined, it is difficult to compare various α2M-cytokine interactions or predict whether α2M-cytokine complexes will form in the presence of other cytokine-binding macromolecules. In this investigation, we used a novel method to demonstrate that transforming growth factor-β1 (TGF-β1), TGF-β2, nerve growth factor-β (NGF-β), platelet derived growth factor-BB (PDGF-BB), tumor necrosis factor-α (TNF-α), and basic fibroblast growth factor (bFGF) reversibly associate with α2M-methylamine to form noncovalent complexes. Apparent equilibrium was achieved in less than 15 min. Noncovalent α2M-cytokine complexes were converted into covalent complexes; however, this occurred slowly. Therefore, a rapid equilibrium assumption was determined using a single binding site model. $K_D$ values for the binding of cytokines to α2M-methylamine varied by 2 orders of magnitude. The rank order of affinity was TGF-β2 (13 ± 2 nM) > TGF-β1, NGF-β > PDGF-BB ≥ bFGF > TNF-α. Native α2M bound TGF-β1, TGF-β2, NGF-β, PDGF-BB, and TNF-α. Interferon-γ did not bind to native α2M or α2M-methylamine. Each cytokine bound native α2M with lower affinity than α2M-methylamine except for TGF-β2 which bound both forms with equal affinity. In non-equilibrium systems, α2M-methylamine appeared to bind more TGF-β2 due to the more rapid dissociation of TGF-β2-native α2M complex. The classification of α2M-cytokine complexes according to binding affinity should predict which complexes are most likely to form in cell culture and under various conditions in vivo.

Human α2-macroglobulin (α2M) is a tetrameric glycoprotein ($M_r$ ~ 718,000) and an inhibitor of proteinases from all of the major classes (1–3). The four identical subunits in α2M form two proteinase binding sites. Reaction of α2M with proteinase is initiated when the proteinase cleaves 1 of approximately 12 sensitive peptide bonds in the α2M bait region, which is located near the center of each subunit (1, 4). Bait region cleavage initiates a major conformational change in α2M, which efficiently and irreversibly traps the reacting proteinase without requiring covalent bond formation (5–9). Each α2M subunit has a single β-cysteylnyl-γ-glutamyl thiol ester bond (10, 11). This reaction serves to covalently link α2M to the trapped proteinase (9–13). Thiol esters may also be cleaved directly (without proteinase) by small primary amines, such as methylamine (10, 11). This reaction induces a conformational change in α2M, which is very similar or equivalent to that caused by proteinases (6, 7).

After reaction with proteinase or amine, α2M is recognized by the cellular receptor, α2-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP) (14, 15). LRP is present on the surfaces of many different cell types (16) and responsible for the rapid plasma clearance of conformationally transformed α2M (17, 18). The native (unreacted) conformation of α2M demonstrates no affinity for LRP (17).

Recent studies have demonstrated binding of numerous growth factors, cytokines and hormones to α2M, including transforming growth factor-β1 (TGF-β1) (19–24), TGF-β2 (25, 26), platelet-derived growth factor (PDGF) (27–31), nerve growth factor-β (NGF-β) (32, 33), interleukin-1β (IL-1β) (34–37), interleukin-6 (38), basic fibroblast growth factor (bFGF) (39), tumor necrosis factor-α (TNF-α) (40, 41), interferon-γ (IFN-γ) (41), insulin (42), vascular endothelial growth factor (43), and the inhibin/activin family (44, 45). Cytokine binding to α2M is distinct from the proteinase trapping mechanism (46, 47). Cytokine binding does not induce the α2M conformational change (24, 31). No α2M peptide bonds are cleaved and the thiol esters are not directly disrupted. The extent of cytokine binding depends on the α2M conformation; in most cases, reaction of α2M with proteinase or methylamine

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† Supported in part by the Medical Scientist Training Program GM 07267.
‡ Recipient of Research Career Development Award HL-02272. To whom correspondence should be addressed: University of Virginia Health Sciences Center, Depts. of Pathology and Biochemistry, Box 214, Charlottesville, VA 22908. Tel.: 804-924-9192; Fax: 804-924-5060.

The abbreviations used are: α2M, α2-macroglobulin; LRP, α2-macroglobulin receptor/low density lipoprotein receptor-related protein; TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; NGF-β, nerve growth factor-β; IL-1β, interleukin-1β; bFGF, basic fibroblast growth factor; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; BS6, bis(sulfosuccinimidyl) suberate; BSA, bovine serum albumin; PES, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.
enhances cytokine binding (22, 31, 46). In cell culture, α2M may inhibit (22, 25) or promote cytokine activity (29, 48). For example, the mitogenic effect of TGF-β1 in smooth muscle cell cultures is enhanced up to 20-fold by the simultaneous addition of α3M-methylamine (48). Cytokines that are bound to conformationally transformed α2M are targeted to cells expressing LRP (21); this process may mediate cytokine clearance or regulate cytokine activity.

At least three different mechanisms are recognized whereby α2M binds cytokines (46, 47). First, cytokines may bind non-covalently and reversibly to α2M. This type of binding predominates in the extensively studied TGF-β1/α2M interaction. Second, cytokines may bind covalently to α2M by thiol-disulfide exchange. This reaction principally involves the transformed conformation of α2M, since free Cys residues are generated by thiol ester cleavage. The third mechanism is somewhat more complex, since it requires a temporal relationship between α2M binding of proteinase and cytokine. When α2M is cleaved by an attacking proteinase, the α2M thiol esters become available for reaction with nucleophilic groups in other proteins (in addition to the proteinase) for a short period of time (13). Cytokine Lys residues can function as nucleophiles and cleave the thiol esters, forming covalent linkages with the attacking glutamyl residues (42). The cytokine must be present at the instant of thiol ester cleavage reaction with α2M, since the thiol esters are rapidly hydrolyzed by water in the absence of alternate nucleophiles (13).

Due to the diverse assortment of techniques used previously to study cytokine binding to α2M, it is difficult to assess the significance of each reported interaction. Many previous investigations have included experiments which only detected certain low-affinity (>0.3 μM) disulfide exchange. This reaction principally involves the cytokine binding to α2M, since free Cys residues are generated by thiol ester cleavage. The third mechanism is somewhat more complex, since it requires a temporal relationship between α2M binding of proteinase and cytokine. When α2M is cleaved by an attacking proteinase, the α2M thiol esters become available for reaction with nucleophilic groups in other proteins (in addition to the proteinase) for a short period of time (13). Cytokine Lys residues can function as nucleophiles and cleave the thiol esters, forming covalent linkages with the attacking glutamyl residues (42). The cytokine must be present at the instant of thiol ester cleavage reaction with α2M, since the thiol esters are rapidly hydrolyzed by water in the absence of alternate nucleophiles (13).

Without knowledge of binding affinities, different α2M-cytokine interactions cannot be compared. In addition, it is not possible to predict whether α2M might function as a significant cytokine binder in the presence of other macromolecules which demonstrate affinity for the same cytokine, such as cytokine-specific cellular receptors, proteoglycans, and extracellular matrix proteins. To address this problem, we recently developed an immobilization system to study cytokine binding to α2M under equilibrium conditions (23); however, this system cannot be used to detect certain low-affinity (>0.3 μM) interactions (31). In addition, limitations were identified in the use of immobilized α2M to determine equilibrium dissociation constants (23).

In the present investigation, we used a new method to analyze noncovalent binding of seven cytokines to native α2M and α2M-methylamine under apparent equilibrium conditions. Solutions containing α2M and cytokine were pulse-exposed to the cross-linking agent, bis(sulfosuccinimidyl) suberate (BS3) to stabilize a fraction of the noncovalent α2M-cytokine complex before performing SDS-PAGE. The efficiency of BS3 cross-linking and the extent of covalent binding were accounted for. Then, using a one-site binding model, equilibrium dissociation constants were determined. In previous studies, it has been suggested that each of the seven cytokines studied here form complexes with α2M, which may be physiologically significant. Our results show that the seven cytokines vary in affinity for α2M by at least 2 orders of magnitude.

**MATERIALS AND METHODS**

**Reagents**—Methyamine hydrochloride, chloramine T, 1,4-dithiothreitol, and bovine serum albumin (BSA) were purchased from Sigma. BS3 was purchased from Pierce Chemical Co. Na2HPO4 was purchased from Amersham Corp. ENZYMOMBEADS were purchased from Bio-Rad. Immulon 2 microtiter plates were from Dynatech Laboratories (Chantilly, VA). Tween (enzyme grade) was from Fisher.

α2M—α2M was purified from fresh human plasma by the method of Imber and Pizzo (49) and stored at −20 °C in 40% glycerol. The concentration of α2M was determined by absorbance at 280 nm, using an A280 of 8.93 (5). α2M-methylamine was prepared by dialyzing α2M against 200 mM methylamine HCl in 50 mM Tris-HCl, pH 8.2, for 12 h at 22 °C and then extensively against 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS) at 4 °C. Reaction of native α2M with methylamine was confirmed by loss of trypsin binding activity (greater than 96%).

**Cytokines**—Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). TGF-β2 was also purified from human platelets by the method of Assoian (50). TGF-β1 was radiolabeled as described previously (21) (specific activity, 100–200 μCi/μg) and stored at −20 °C in 4 mM HCl, 100 mM NaCl, 15 μM BSA. Recombinant and platelet-purified TGF-β1 preparations yielded equivalent results in α2M binding experiments, as demonstrated previously (22, 23). Recombinant human TGF-β2 was purchased from Genzyme (Cambridge, MA) and radiolabeled as described for TGF-β1 (100–250 μCi/μg). NGF-β was purified from male mouse submaxillary glands by the method of Darling and Shooter (51) or purchased from Sigma. The two preparations bound α2M equivalently. NGF-β was radiolabeled (5–15 μCi/μg) using ENZYMOMBEADS (lactoperoxidase/glucose oxidase). Recombinant human 125I-βFGF (56 μCi/μg) was purchased from Amersham Corp. Recombinant human 125I-PDGF-BB (45 μCi/μg), human 125I-TNF-α (42 μCi/μg), and human IFN-γ (105 μCi/μg) were from Du Pont NEN.

**Preparation of Incubation Tubes**—Siliconized polypropylene microcentrifuge tubes (National Scientific, San Rafael, CA) were blocked with 0.4 mM BSA in PBS with 3.0 mM sodium azide at 22 °C for 2 h and then with 0.1% (v/v) Tween 20, in the same buffer for an additional 24 h. The tubes were rinsed twice with H2O immediately before use. Under the conditions described for the α2M binding experiments, less than 3% of each 125I-cytokine bound nonspecifically to the prepared test tube walls.

A Model of Cytokine Binding to α2M—The binding of cytokines to α2M in a system which contains no free proteinase (α2M not actively undergoing conformational change) can be described as follows.

$$A + C \rightleftharpoons AC \rightleftharpoons AC^*$$

(Eq 1)

$$k_1 \quad k_2 \quad k_{-1}$$

A is unbound α2M; C is free (unbound) cytokine; AC is reversibly associated (noncovalent) α2M-cytokine complex; and AC* is irreversibly associated (covalent) α2M-cytokine complex. If the rate of conversion of AC to AC* (k2) is slow compared with k1 (rapid equilibrium assumption), then the dissociation constant, Kd, for noncovalent binding of 125I-cytokine to α2M may be expressed as:

$$\frac{C}{AC} = K_d \frac{1}{A}$$

(Eq 2)

Assumptions in this model include the following. 1) Each α2M tetramer has one cytokine binding site. 2) Noncovalent association of 125I-cytokine with the α2M binding site is adequately described by a single Kd. If in fact there are two or four independent and equivalent cytokine binding sites per α2M tetramer instead of one (which is plausible due to the symmetry in the α2M structure) then the actual Kd for the interaction of cytokine with each individual binding site is 2- or 4-fold higher than the values reported here.

125I-Cytokine Binding to α2M as Determined by BS3 Cross-linking—Various concentrations of α2M were incubated with 125I-TGF-β1 (0.5–1.0 nM), 125I-TGF-β2 (0.5–1.0 nM), 125I-NFG-β (1.0–2.0 nM), 125I-PDGF-BB (1.5–2.0 nM), 125I-TNF-α (0.9–1.9 nM), 125I-βFGF (1.2–1.5 nM), or 125I-IFN-γ (1.1–1.6 nM) in PBS with 150 μM BSA, pH 7.4, at 37 °C. At the indicated times, BS3 (dissolved immediately before use}
in H$_2$O) was added to a final concentration of 5 mM. H$_2$O alone was added to identical control incubations. Each tube was incubated for 60 s at 22°C, unless otherwise specified (see below). To stop the cross-linking reaction, solutions were acidified to pH 2.5-3.0 with HCl (0.2). Samples were then denatured in 20% SDS for 30 min at 37°C. Finally, 1% Tris-HCl and 10% glycerol (final concentrations) were added to each sample, and SDS-PAGE was performed (no reducant). Dried gels were subjected to autoradiography. The amount of $^{125}$I-cytokine in each band was determined by cutting the gel into sections and determining the radioactivity in each section using a $\gamma$-counter. Recovery of radioactivity in the gels was typically between 75 and 90%. Free and $\alpha$M-associated $^{125}$I-cytokine were recovered in the gels equivalently.

In the absence of BS3, $^{125}$I-cytokine which was covalently associated with $\alpha$M-comigrated with the high molecular mass $\alpha$M bands by SDS-PAGE. When BS3 was added, additional $^{125}$I-cytokine-$\alpha$M complex was detected. The increase in binding was presumed to represent a fraction of the noncovalent $\alpha$M-cytokine complex. The fraction is determined by the cross-linking efficiency of BS3 ($z$), which is a distinct value for each cytokine, $\alpha$M, and BSA conformation, and BS3 cross-linking time ($0 < z < 1$). When SDS-PAGE was performed using a $\gamma$-counter, recovery of radioactivity in the gels was typically between 75 and 90%. Free and $\alpha$M-associated $^{125}$I-cytokine were recovered in the gels equivalently.

The effectiveness of the procedure for terminating the cross-linking reaction (acidification) was tested by adding 15 mM HCl and BS3 to solutions of $\alpha$M and $^{125}$I-TGF-β1 that had reached apparent equilibrium (see below). Cross-linking was not observed by SDS-PAGE. By contrast, solutions which have been used in other investigations to stop BS3 reactions (0.3 M Tris-HCl, pH 7.4 or pH 8.5; 0.3 M lysine, 60 mM Tris-HCl, pH 7.4 or 8.5; 0.3 M glycine, 60 mM Tris-HCl, pH 7.4 or 8.5) were not instantaneous inhibitors of the cross-linking reaction.

**Analysis of the Results of BS3 Cross-linking Experiments—**Since BS3 was added at high concentration, $\alpha$M-cytokine complex was cross-linked according to pseudo-first order kinetics; the fraction of noncovalent $\alpha$M-cytokine complex cross-linked ($z$) was independent of the concentration of $\alpha$M-cytokine complex. Under these conditions, experimentally detected BS3-stabilized noncovalent $\alpha$M-cytokine complex ($AC_\approx zAC$) is related to $AC$ by the following relationship.

$$AC_\approx zAC$$

(Eq. 3)

$AC$, was determined from the radioactivity in gel slices and corrected for the presence of $AC_\approx$ by subtracting covalent binding detected in the absence of BS3. Experimentally observed BS3-free cytokine and cytokine that was bound to $\alpha$M but not cross-linked by BS3.

$$C_\approx = C_\approx + (1 - z)AC$$

(Eq. 4)

By substituting the expressions for $AC$, and $C_\approx$ into Equation 2, a linear relationship in the form $y = m + b$ was derived.

$$\frac{C_\approx}{AC} = \frac{K_0}{z} \left[ \frac{1}{A} \right] + \frac{1}{z} - \frac{1}{z}$$

(Eq. 5)

where

$$y = \frac{C_\approx}{AC}, \quad m = \frac{K_0}{z}, \quad b = \frac{1}{z} - \frac{1}{z}$$

(Eq. 6)

All results were plotted according to Equation 5. The BS3 cross-linking efficiency ($z$) was determined from the $y$ intercept. This value was used to calculate the $K_0$ from the slope derived by linear regression using GraphPad Inplot (GraphPad Software, San Diego, CA).

An advantage of this derivation is the separation of BS3 cross-linking efficiency and $\alpha$M-cytokine binding affinity. Affinely, a tight interaction between $\alpha$M and cytokine may be detected as limited binding by SDS-PAGE if $z$ is small. Unless otherwise indicated, $K_0$ values reflect the mean of four separate determinations ± standard error (S.E.). Despite the large excess of BSA which was present in our incubations with $\alpha$M, significant BS3 cross-linking of $^{125}$I-cytokines to BSA was not observed.

**Variation in BS3 Incubation Time—**If $z$ is high and the reversible interaction between $AC$ and $C_\approx$ is rapid compared with the BS3 pulse time (1.0 min), then BS3 might shift the equilibrium toward the apparent concentration of $^{125}$I-cytokine by preventing $AC$ dissociation. In order to assess the importance of this effect, the time of BS3 pulse exposure was varied between 1 and 7 min in experiments with $^{125}$I-TGF-β1 and $^{125}$I-Methylamine. The $K_0$ and $z$ values were determined for each BS3 exposure time.

**TGFB Binding to Immobilized αM—**$^{125}$I-TGF-β2 binding to immobilized $\alpha$M was examined according to the method of Webb et al. (23). Briefly, $\alpha$M-methylamine was incubated in each well of a 96-well microtiter plate so that 90 fmol of the $\alpha$M-cytokine complex became immobilized. The wells were then blocked with Tween 20 (0.1% v/v). $^{125}$I-TGF-β2 (0.2 nM) in PBS with 15 mM BSA was equilibrated in the microtiter wells with immobilized $\alpha$M-methylamine at 22°C. Different concentrations of native $\alpha$M or $\alpha$M-methylamine (0.1 nM to 0.25 μM) were included in solution with the $^{125}$I-TGF-β2. After 1 h, the wells were washed. Surface-associated $^{125}$I-TGF-β2 was recovered by incubation with 0.1 M NaOH, 2% SDS and quantified in a $\gamma$ counter. When $^{125}$I-TGF-β2 was incubated in microtiter wells that did not have $\alpha$M-methylamine, binding was decreased by greater than 95%. Reported results represent the average of three separate experiments with duplicate determinations.

**Dissociation of $^{125}$I-TGF-β2 from Immobilized $\alpha$M—**The apparent rate of dissociation of $^{125}$I-TGF-β2 from immobilized $\alpha$M-methylamine and immobilized native $\alpha$M was determined according to the method used previously with TGF-β1 (23). $\alpha$M-methylamine and native $\alpha$M were incubated in separate microtiter wells so that 90 fmol of either species was immobilized. $^{125}$I-TGF-β2 was then incubated in the wells as described above. The wells were washed and equilibrated in fresh buffer (PBS with 15 mM BSA) containing 0.1 μM $\alpha$M-methylamine (to inhibit reassociation of dissociated $^{125}$I-TGF-β2) at 22°C. At various times, the wells were washed and treated with NaOH/SDS; free $^{125}$I-TGF-β2 and $^{125}$I-TGF-β2 remaining surface-associated were determined in a $\gamma$ counter.

Other Methods for Analyzing Binding of TGF-β2 and TNF-α to $\alpha$M—$^{125}$I-TGF-β2 (0.5 nM) was incubated with native $\alpha$M or $\alpha$M-methylamine (2.8 μM) in PBS containing 9 μM BSA for 50 min at 37°C. $^{125}$I-TNF-α (1.5 nM) was incubated with $\alpha$M-methylamine (2.8 μM) in PBS with 3 μM BSA for 2 h at 37°C. The reaction mixtures were subjected to FPLC gel filtration on Superox-6 equilibrated in PBS (0.4 ml/min). Binding of $^{125}$I-TGF-β2 or $^{125}$I-TNF-α to $\alpha$M was determined by the radioactivity coeluting with $\alpha$M (percent of total radioactivity loaded) as described previously (22, 31). Similar incubations were also analyzed by nondenaturing PAGE (21, 22) and by SDS-PAGE.

**RESULTS**

**Demonstration of Apparent Equilibrium—**Since noncovalent AC complex may be slowly converted into AC*, methods that measure total $\alpha$M-cytokine complex may fail to detect apparent equilibrium for the reversible association step ($k_1/k_\approx$) in the reaction. Using BS3, we measured AC, as a function of time for each cytokine and $\alpha$M (1.0 μM). Representative plots with $^{125}$I-TGF-β1 and $^{125}$I-TGF-β2 are shown in Fig. 1. AC, (and therefore AC) reached maximum values within about 10 min in experiments with $\alpha$M-methylamine and $^{125}$I-TGF-β2.
\( \beta_1 \) or \( \beta_2 \)-TGF-\( \beta_2 \). Comparable results were obtained with the other cytokines. In experiments with native \( \alpha_2M \) and \( ^{125I}- \) TGF-\( \beta_1 \) or \( ^{125I}- \) TGF-\( \beta_2 \) and other cytokines not shown, maximum AC levels were lower (compared with \( \alpha_2M \)-methylamine); however, the times required to achieve apparent equilibrium were essentially unchanged. Based on these experiments, all apparent equilibrium measurements were made at 1 h, except when TGF-\( \beta_1 \) and bFGF were studied. The later two growth factors formed AC with \( \alpha_2M \)-methylamine more rapidly (see below) and therefore were incubated with \( \alpha_2M \)-methylamine or native \( \alpha_2M \) for 15 min to limit this conversion.

\( \alpha_2M \)-Cytokine Binding Isotherms—Different concentrations of \( \alpha_2M \)-methylamine were incubated with each \( ^{125I} \)-cytokine. Binding was detected by SDS-PAGE after exposing the solutions to BS\(^3\). Fig. 2 shows representative autoradiographs from experiments in which \( \alpha_2M \)-methylamine was incubated with \( ^{125I}- \) TGF-\( \beta_2 \) (panel A) and \( ^{125I}- \) NGF-\( \beta \) (panel B). The low-mobility bands included BS\(^3\)-stabilized AC complex (AC\(_i\)) and covalent \( \alpha_2M \)-methylamine-cytokine complex (AC\(_e\)). Comparable low-mobility bands were observed in experiments with each of the other cytokines, except \( ^{125I}- \) IFN-\( \gamma \). We were not able to detect BS\(^3\)-stabilized \( \alpha_2M \)-methylamine-\( ^{125I}- \) IFN-\( \gamma \) complex or native \( \alpha_2M \)-\( ^{125I}- \) IFN-\( \gamma \) complex, even when the \( \alpha_2M \) concentration was 4.0 \( \mu M \) or when the time of incubation with BS\(^3\) was extended to 7.0 min.

In each cytokine binding experiment, AC\(_e\) was determined by correcting for AC\(_i\) (detected in the absence of BS\(^3\)). AC\(_e\)/(AC\(_i\) + C\(_i\)) varied as a hyperbolic function of \( \alpha_2M \)-methylamine concentration. A composite of results from five separate experiments with TGF-\( \beta_2 \) is shown in Fig. 3. Similar plots were generated for the other cytokines that bound \( \alpha_2M \)-methylamine or native \( \alpha_2M \).

Analysis of Cytokine Binding Experiments—In Fig. 4, results obtained in representative experiments with TGF-\( \beta_2 \) (panel A), TGF-\( \beta_1 \) (panel B), and NGF-\( \beta \) (panel C) were plotted according to Equation 5. Similar graphs were constructed to analyze the results of experiments with PDGF-BB, TNF-\( \alpha \), and bFGF. Apparent equilibrium dissociation constants were determined from the slopes in each graph. These values were then averaged to generate the results presented in Table I. All of the cytokines, with the exception of TGF-\( \beta_2 \), bound \( \alpha_2M \)-methylamine with greater affinity than native \( \alpha_2M \). Our native \( \alpha_2M \) preparations did not contain detectable levels of \( \alpha_2M \) that had undergone conformational

\[
\frac{1}{[\alpha_2M]} \begin{array}{c}
\text{[CM]} \\
[\mu M^{-1}]
\end{array}
\]



change, as determined by nondenaturing PAGE and electron microscopy (53). If such species were present, they would be expected to decrease the apparent \( K_D \) values for interaction of native \( \alpha_2M \) with TGF-\( \beta_1 \), NGF-\( \beta \), PDGF-BB, TNF-\( \alpha \), and bFGF.

Great variability was observed in the \( K_D \) values for the
**Table I**

Equilibrium dissociation constants for $\alpha_2$M binding to 125I-cytokines

| Cytokine          | $K_D$ with $\alpha_2$M-methylamine (nM) | $K_D$ with native $\alpha_2$M (nM) |
|-------------------|----------------------------------------|-----------------------------------|
| TGF-β2            | 80 ± 11                                 | 0.33 ± 0.13                       |
| TGF-82            | 13 ± 2                                  | 11 ± 3                            |
| NGF-β             | 0.11 ± 0.01                             | 0.34 ± 0.06                       |
| PDGF-BB           | 0.37 ± 0.20                             | ND ($^*$)                         |
| TNF-α             | $>0.75$ ± $0.10$ μM                     | $>1.27$ ± $0.17$ μM              |
| bFGF              | 0.50 ± 0.04 μM                          | 0$^*$                             |
| IFN-γ             | $^*$                                    | 0$^*$                             |

$^*$ Not determined. Binding was evident but insufficient for the determination of $K_D$.

$^*$ Binding was sufficient for analysis only after extending the BS3 exposure time to 7 min. Therefore, the $K_D$ values represent minimum estimates.

$^*$ Binding was not detected using a 1-min BS3 exposure time.

$^*$ Binding was not detected even when BS3 exposure time was extended to 7 min.

**Table II**

Variation of apparent $K_D$ with length of incubation with BS3 cross-linker

| BS3 incubation time | Apparent $K_D$ |
|---------------------|----------------|
| min                 | nM             |
| 1                   | 76             |
| 4                   | 47             |
| 7                   | 21             |

**Table III**

BS3 cross-linking efficiencies (2) and covalent binding of cytokines to $\alpha_2$M

| Cytokine     | Native $\alpha_2$M | $\alpha_2$M-methylamine |
|--------------|---------------------|--------------------------|
|              | $x^*$ (AC$^*$/AC + AC) | $x^*$ (AC$^*$/(AC + AC)) |
| TGF-82$^{11}$| 0.13 ± 0.02         | 0.38 ± 0.06               |
| TGF-β2       | 0.18 ± 0.03         | 0.34 ± 0.02               |
| NGF-β        | 0.15 ± 0.03         | 0.33 ± 0.04               |
| PDGF-BB      | 0.14 ± 0.01         | 1.3-3.3                  |
| TNF-α        | 0.12 ± 0.01         | 0.16 ± 0.01               |
| bFGF         | 0.16 ± 0.03         | 6.0-11.4                 |
| IFN-γ        | $^*$                | 0$^*$                     |

$^*$ Mean ± S.E.

$^*$ Range of values from four to five experiments (10 $\alpha_2$M concentrations per experiment).

$^*$ Values determined after incubating cytokine with $\alpha_2$M for 15 min.

All other values determined after incubating cytokine with $\alpha_2$M for 1 h.

Binding of $\alpha_2$M-methylamine to the seven cytokines. The absolute ranking (from highest to lowest affinity) was TGF-β2 > TGF-β1, NGF-β > PDGF-BB ≥ bFGF > TNF-α > IFN-γ. In experiments with TNF-α, binding was insufficient for analysis after a 1.0 min pulse-exposure to BS3. Therefore, we extended the pulse exposure time to 7 min. This longer cross-linking time may have artificially decreased the apparent $K_D$ of the TNF-α-$\alpha_2$M interaction. To examine this possibility, we analyzed the binding of $^{125}$I-TGF-β1 to $\alpha_2$M-methylamine using different BS3 pulse exposure times. As shown in Table II, slightly lower $K_D$ values were determined with longer BS3 incubation times.

The rank order of affinity for cytokine binding to native $\alpha_2$M was similar to that presented for $\alpha_2$M-methylamine. The most significant difference involved the unique ability of TGF-β2 to bind native $\alpha_2$M and $\alpha_2$M-methylamine with equal affinity. As a result, the gap in affinity between TGF-β2 and TGF-β1 was much larger (TGF-β2 > TGF-β1) for native $\alpha_2$M compared with $\alpha_2$M-methylamine.

Values and Covalent Binding of Cytokines to $\alpha_2$M—The BS3 cross-linking efficiencies ($x^*$) were determined from the $y$ intercepts in the plots shown in Fig. 4. Although many factors may influence the efficiency with which BS3 cross-links a complex, it is interesting that the $x$ values were consistently higher in experiments with $\alpha_2$M-methylamine (Table III). Using the presented $x$ values, we estimated the total amount of noncovalent $\alpha_2$M-cytokine complex (AC) for each $\alpha_2$M concentration in all of the experiments, according to Equation 3. The percentage of total binding which was covalent (AC$^*$/ AC + AC$^*$) was then determined (Table III). Under the conditions of our studies (1-h incubation with each cytokine except for TGF-β1 and bFGF which were incubated for 15 min), 88–98% of the $\alpha_2$M-cytokine complexes were noncovalent. Greater fractional covalent binding was observed with

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**Fig. 5. Inhibition of binding of $^{125}$I-TGF-β2 to immobilized $\alpha_2$M-methylamine.** $^{125}$I-TGF-β2 (0.2 nM) was incubated in wells with immobilized $\alpha_2$M-methylamine for 1 h at 22°C. Native $\alpha_2$M (C) or $\alpha_2$M-methylamine (C) was included in the incubation buffer.

$\alpha_2$M-methylamine, as expected due to the free Cys residues in this structure. The extent of covalent binding of $\alpha_2$M-methylamine to TGF-β1 and PDGF-BB (shown in Table III) is consistent with previous studies (22, 31).

**TGF-β2 Binding to Immobilized $\alpha_2$M—** When $^{125}$I-TGF-β2 was incubated with immobilized $\alpha_2$M-methylamine, 2.7 ± 0.2 fmol of $^{125}$I-TGF-β2 bound per well (n = 5). Binding of $^{125}$I-TGF-β2 to immobilized $\alpha_2$M-methylamine was inhibited equivalently by native $\alpha_2$M and $\alpha_2$M-methylamine in solution (Fig. 5). The IC$\alpha_2$ values were 19 ± 3 nM for native $\alpha_2$M and 19 ± 5 nM for $\alpha_2$M-methylamine. These results are markedly different from those obtained previously with TGF-β1 (23). In the earlier studies, $\alpha_2$M-methylamine in solution was seven times more effective than native $\alpha_2$M in inhibiting $^{125}$I-TGF-β1 binding to immobilized $\alpha_2$M-methylamine. The results of the experiments with immobilized $\alpha_2$M confirm the finding that TGF-β2 is unique in its ability to bind to native $\alpha_2$M and $\alpha_2$M-methylamine with comparable affinity.

TGF-β2 Binding to $\alpha_2$M as Determined by FPLC and Non-denaturing PAGE—Danielpour and Sporn (26) performed non-denaturing PAGE experiments and demonstrated that TGF-β2 binds almost exclusively to conformationally transformed $\alpha_2$M. We hypothesized that their results differ from those presented here due to the use of a nonequilibrium system (nondenaturing PAGE) to analyze TGF-β2 binding. To address this hypothesis, we studied $^{125}$I-TGF-β2 binding to native $\alpha_2$M and $\alpha_2$M-methylamine by FPLC and nondenaturing PAGE. In FPLC experiments, $^{125}$I-TGF-β2 binding to $\alpha_2$M-methylamine and native $\alpha_2$M was 65 ± 2% and 20 ± 0% (of total cytokine), respectively. In native PAGE experi-
ments, $^{125}$I-TGF-β2 binding to α2M varied depending on the time of electrophoresis. After 1.5 h of electrophoresis, $^{125}$I-TGF-β2 binding to α2M-methylamine and native α2M was 85 and 51%, respectively (determined by radioactivity in gel slices). After electrophoresis for 3.5 h, $^{125}$I-TGF-β2 binding to α2M-methylamine and native α2M was 72 and 32%, respectively (autoradiograph shown in Fig. 6). These results demonstrate that TGF-β2-α2M complexes dissociate during electrophoresis. The extent of dissociation depends on the time of electrophoresis and possibly the α2M conformation.

$^{125}$I-TGF-β2 dissociation from immobilized native α2M and immobilized α2M-methylamine was studied at 22 °C. The dissociation curves were nonlinear (Fig. 7), as has been shown previously with TGF-β1 (23). Nonlinearity might represent heterogeneity in the structure of the α2M which is immobilized. $^{125}$I-TGF-β2 dissociated more rapidly from immobilized native α2M compared with α2M-methylamine. The time required for 50% dissociation was 57 min with native α2M and 128 min with α2M-methylamine. Although cytokine dissociation from immobilized α2M may differ from the process as it occurs in solution, these results support the hypothesis that different rates of dissociation influence recovery of native α2M-cytokine and α2M-methylamine-cytokine complexes by FPLC or non-denaturating PAGE.

TNF-α Binding to α2M as Determined by FPLC and Non-denaturating PAGE—Since the $K_\text{D}$ values determined for the interaction of α2M with TNF-α were unexpectedly low, we performed non-denaturating PAGE and FPLC experiments similar to those performed by Wollenberg et al. (40). As shown in Fig. 6, $^{125}$I-TNF-α bound preferentially to α2M-methylamine, as determined by non-denaturating PAGE. In FPLC experiments, 44% of the $^{125}$I-TNF-α bound to α2M-methylamine. Therefore, the low affinity of the TNF-α/α2M interaction does not preclude recovery in nonequilibrium systems.

**DISCUSSION**

The function of α2M as a cytokine carrier is complicated due to the different conformational states of α2M. Only conformationally transformed α2M (α2M-proteinase, α2M-methylamine) is recognized by the α2M receptor (LRP) and binding of cytokines, including TGF-β1, PDGF-BB, and TNF-α, does not inhibit the α2M/LRP interaction (21, 24, 40). Therefore, cytokines which are bound to transformed α2M may be targeted to cells expressing LRP, either for catabolism or potentially to induce cellular responses. By contrast, cytokines which are bound to native α2M are stabilized. The plasma clearance of these cytokines is retarded (24, 31), and the susceptibility to proteinase cleavage may be decreased (38). Due to LRP, native α2M is present in large molar excess to transformed α2M in the blood and most likely in many extravascular spaces. The form of α2M to which a cytokine binds will depend not only on the affinity of the interaction but also on the concentration of each α2M species.

We demonstrated previously that PDGF-BB and TGF-β1 bind predominantly to native α2M in the blood in vivo (31). This result was not entirely unexpected, since the plasma concentration of α2M is 2–4 μM (17). For the seven cytokines studied here, plasma represents a relatively simple system since there are few plasma proteins which compete with α2M for cytokine binding, including cellular receptors, solubilized forms of cellular receptors, proteoglycans, and extracellular matrix proteins. In order to model the function of α2M in complex systems, we must have some understanding of the strength of α2M-cytokine interactions. We hypothesized and our results confirmed that the major component of many α2M-cytokine interactions is non-covalent complex formation. Information regarding the affinity of non-covalent α2M-cytokine binding was only available in our previous study of TGF-β1 and immobilized α2M (23). Therefore, we focused the present investigation on the non-covalent association step in the α2M/cytokine binding mechanism. We examined cytokines which had previously been shown to associate with α2M. In our studies, conversion of non-covalent α2M-cytokine complex into disulfide-stabilized complex occurred slowly but progressively with time. This conversion may occur more rapidly and therefore be more important in the α2M regulatory mechanism of some cytokines such as IL-1β (24–37).

In experiments with α2M-methylamine, the equilibrium dissociation constants for the seven cytokines varied from 13 nM (TGF-β2) to undetectable (greater than 5 μM in the BS² cross-linking system). The $K_\text{D}$ determined for the binding of TGF-β1 to α2M-methylamine in solution (80 nM) was equiv-
alent to that determined previously (79 nm) in the immobilized α2M system (23). The relatively weak interaction of α2M-methylamine with bFGF, TNF-α, and PDGF-BB suggests that relatively high concentrations of conformationally transformed α2M would be necessary to compete with other macromolecules which bind these cytokines (including cytokine-specific cellular receptors). Using the BS² method, we were not able to demonstrate complex formation between IFN-γ and α2M-methylamine or native α2M.

α2M-methylamine has frequently been used as a model of the transformed α2M conformation and therefore α2M-proteinase complexes in general (17, 46, 47). The structure of α2M-methylamine is identical or nearly identical to that of most α2M-proteinase complexes (6, 7, 46). α2M-methylamine and α2M-proteinase complexes are recognized equivalently by LRP (17) and by antibodies which do not recognize native α2M (54). By contrast, in cytokine binding studies, α2M-methylamine has not consistently behaved equivalently to α2M-proteinase complexes. For example, Huang et al. (20) and LaMarre et al. (26) reported that the TGF-β1 binding activity of α2M-methylamine is increased compared with native α2M, whereas the TGF-β1 binding activity of α2M-trypsin is decreased. Hall et al. (22) subsequently demonstrated that this apparent discrepancy depends upon α2M-proteinase binding stoichiometry. Although α2M is capable of binding up to 2 mol of proteinase/mole, under most physiologic conditions (α2M present in excess to proteinase), almost exclusively 1:1 (binary) α2M-proteinase complexes form (8). These binary α2M-proteinase complexes uniformly demonstrate increased TGF-β1 binding activity unlike the ternary (2:1) α2M-proteinase complexes which demonstrate decreased TGF-β1 binding activity (22). Equivalent results have now been obtained in studies with PDGF-BB (31) and NGF-β². Therefore, we consider α2M-methylamine an appropriate model of physiologically significant α2M-proteinase complexes in cytokine binding experiments.

The interaction of TGF-β2 with α2M was unique in a number of ways. First, the affinity of the interaction of TGF-β2 with α2M-methylamine was the highest measured in this series. Second, TGF-β2 was the only cytokine which bound with comparable affinity to α2M-methylamine and native α2M. This result was confirmed in two systems that measure apparent equilibrium binding (BS² cross-linking and immobilized α2M). In experimental systems that are affected by α2M-cytokine dissociation (FPLC, nondenaturing PAGE), greater TGF-β2 binding to α2M-methylamine was observed. This result probably reflected different rates of dissociation of TGF-β2-α2M-methylamine complex and TGF-β2-native α2M complex. A second factor which may have been important in earlier studies is covalent binding. In our experiments, only a small percentage of the α2M-methylamine-TGF-β2 complex was stabilized by disulfide bonds. If higher levels were achieved in other studies (perhaps due to longer incubation times), then this subpopulation of α2M-methylamine-TGF-β2 complex would be resistant to dissociation during nondenaturing PAGE. Since native α2M forms far less covalent complex with cytokines, stabilization would not be factor for this α2M conformation.

Our results with TGF-β2 add insight into previous studies comparing the regulation of TGF-β1 and TGF-β2 by α2M. Danielpour and Sporn (25) reported that α2M significantly inhibits the receptor binding and biological activity of TGF-β2. In the same experimental systems, α2M was only 5% as effective in inhibiting receptor binding of TGF-β1 and had no effect on TGF-β1 biological activity. LaMarre et al. (55) reported a similar finding based on studies of primary hepatocyte cultures. α2M counteracted the mitoinhibitory activity of TGF-β2 but did not affect the activity of TGF-β1. The α2M used in these earlier studies (25, 55) was a commercially available bovine preparation which consists primarily of the native form. The large difference in results obtained with TGF-β2 versus TGF-β1 (25, 55) probably reflected not only the generally increased TGF-β2 binding activity of all α2M species, but also the unique capacity of TGF-β2 to bind with equal affinity to native α2M and conformationally transformed α2M.

The KD values reported in this investigation assume one cytokine binding site per α2M. If instead there are two or four independent, noninteracting cytokine binding sites, then the reported KD values are 2- or 4-fold lower than the actual values, respectively. In our experiments, α2M was always present at large molar excess to ¹²⁵I-cytokine. Therefore, our results would not permit an assessment of cooperativity between multiple cytokine binding sites if they exist. Finally, it is possible that radioiodination affected the structure of some cytokine molecules so that a subpopulation of ¹²⁵I-cytokine was incapable of binding to α2M. If this is true, the determined KD values are still accurate for the fraction of ¹²⁵I-cytokine which retains α2M binding activity. Variability in the fraction of ¹²⁵I-cytokine which can bind to α2M would be reflected in the z values.

In a recent study, Liebl and Koo (56) compared the binding of nine cytokines to α2M. Some of the studied cytokines (TGF-β, NGF-β, PDGF, TNF-α) were the same as those surveyed here; however, the other investigators performed only electrophoresis experiments. Their results and the conclusions drawn from these results are highly disparate from the results and conclusions presented here. We attribute this discrepancy, at least in part, to the exclusive use (in the other study) of a nonequilibrium system (PAGE) to analyze cytokine binding to α2M. As mentioned above, this approach is problematic due to unpredictable levels of α2M-cytokine dissociation and the absence of a clear correlation with actual α2M/cytokine binding affinity.

We hypothesize that the KD values determined here can be used to predict which cytokines will be most readily affected by α2M in cell culture and in vivo. Our hypothesis is supported by the relation of our results to previous biological studies of TGF-β1 and TGF-β2 (25, 55). Based on the presented KD values, we propose that TGF-β2 is unique, since this cytokine may be regulated by even low concentrations of native α2M. TGF-β1 and NGF-β demonstrate fairly high affinity for α2M-methylamine and therefore may be regulated by this α2M conformational variant. PDGF-BB, bFGF, and TNF-α are low-affinity α2M binders which may still interact with α2M in the blood and in other solutions containing high concentrations of α2M. Finally, IFN-γ is considered a noninteracting cytokine.

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