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Mechanism of Inhibition of Protein-Tyrosine Phosphatases by Disodium Aurothiomalate

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ABSTRACT. Disodium aurothiomalate (AuTM) has been used successfully in the treatment of various autoimmune and inflammatory disorders; however, the molecular target(s) for this agent remains unknown. The aim of this study was to investigate whether the activity of CD45, a protein-tyrosine phosphatase (PTP, EC 3.1.3.48) essential for antigen-receptor-mediated lymphocyte signaling, was modified by AuTM exposure. The effects of AuTM on the activities of CD45 and other PTPs were monitored *in vitro* by a continuous assay using the substrate fluorescein diphosphate. In addition, the inhibition of PTP1B by AuTM was determined using a novel binding assay that employed an optical biosensor (BIAcore). The experimental results are summarized here: AuTM inhibited CD45 activity with an IC_{50} of 1.2 ± 0.1 μM, and inhibition was competitive with substrate. The effect of AuTM, however, was not restricted to CD45, as the cytoplasmic PTP (PTP1B) was also inhibited, with an IC_{50} of 3.6 ± 0.2 μM. AuTM also blocked the binding of GST-PTP1B to an immobilized active site inhibitor: a non-hydrolyzable difluorophosphonomethyl phenylalanine-containing biotinylated hexapeptide. AuTM-inhibited CD45 could be reactivated by the addition of excess dithiothreitol. These findings indicate that AuTM may interact with the essential active site cysteine residue involved in the catalytic mechanism of PTPs. Thus, it is possible that some of the cellular effects of gold result from the inhibition of these important cell signaling molecules. BIOCHEM PHARMACOL 54;6:703–711, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. gold; rheumatoid arthritis; protein-tyrosine phosphatase; enzyme kinetics; BIAcore analysis; drug inhibition

Gold compounds have been used for many years in the treatment of diseases having an autoimmune or inflammatory component to their pathogenesis, including rheumatoid and psoriatic arthritis [1], pemphigus vulgaris [2], and bronchial asthma [3, 4]. Gold compounds have been employed most frequently in the therapy of rheumatoid arthritis, and efforts have thus focused on examining the effects of gold compounds on T-lymphocytes and macrophages, cells that appear to have a primary role in the pathogenesis of this disease [1]. A wide variety of effects has been observed using gold preparations, such as inhibition of interferon-induced C2 production by monocytes [5], partial inhibition of neutrophil protein kinase C activity [6], and inhibition of lymphocyte membrane adenyl cyclase activity [7]. However, the precise mechanism(s) of action of the gold compounds commonly employed in clinical practice (aurothiomalate, aurothioglucose, and auranofin) remains nebulous.

Gold has three common oxidation states (0, I, and III), of which Au(I) has been implicated in its therapeutic usefulness [8]. Au(I) preferentially forms a complex with "soft" ligands such as thiolates and thioethers, with linear two-coordinates. Gold(I) complex can be expected to bind to a large number of proteins, particularly those having nucleophilic sulfhydryl groups in accessible sites.

Recently, two novel mechanisms to explain the effects of gold have been put forward. The first suggested that the gold(I) complex might act at the level of peptide antigen presentation to T-cells [9]. T-cell activation by peptides containing several cysteine residues was found to be inhibited by gold, an effect attributed to a direct interaction between gold ions and the thiol groups within the stimulatory peptides [9]. This effect, however, was limited to peptides having thiol groups within their sequence. The second mechanism suggests that gold compounds are capable of regulating the activity of certain transcription factors, likely via interactions between gold and specific cysteine thiol groups regulated by intracellular redox potential. Gold, for example, was shown to inhibit the function of both the progesterone [10] and glucocorticoid [11] receptors, as well as fos-fos or fos-jun dimer binding to an AP-1

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consensus sequence [12]. Employing cell lines transfected with the appropriate promoter linked to a reporter gene system, Handel *et al.* [12] provided evidence that gold-mediated inhibition of reporter gene transcription might also be occurring *in vivo*. Furthermore, gold thioglucose was shown to inhibit binding of nuclear factor kappa B to DNA [13], possibly via reaction with cysteines normally associated with zinc, and AuTM was also found to inhibit the activity of transcription factor OB2-1 [14].

Thus, if protein cysteine–gold adducts were inhibiting binding of specific transcription factors to their response elements *in vivo*, it would provide an explanation for the diverse effects of gold compounds, which include, for example, inhibition of pokeweed mitogen-induced immunoglobulin secretion from human peripheral blood [15], inhibition of T-lymphocyte and endothelial cell proliferation [16, 17], and reduced T-cell response to interleukin-2 [18].

In addition to transcription factors such as fos and jun, there is another important class of ubiquitous molecules, the PTPs§ (EC 3.1.3.48), with functionally important cysteine groups. Indeed, as with transcription factor inhibition, inhibition of PTPs by gold would have the potential to regulate diverse intracellular signal transduction pathways involving protein-tyrosine phosphorylation. If gold were able to regulate PTP activity under the conditions of gold therapy, it would add another potential mode of action to explain the cellular effects of this metal. For example, the PTP CD45 is indispensable for T-lymphocyte activation and development [19], for B-lymphocyte antigen receptor response to antigen [20], and for degranulation of mast cells in response to cross-linking of cell surface immunoglobulin E receptors [21]. Thus, even a partial inhibition of this PTP could result in the suppression of immune responses. Predicting that AuTM might be able to react with the cysteine thiol anion postulated to exist within the active site of PTPs [22], we tested the ability of AuTM to inhibit these enzymes.

MATERIALS AND METHODS

Materials

AuTM (96%) was from Aldrich (Milwaukee, WI, U.S.A.). FDP and the hexapeptide DADE(F_2PMP)L-NH_2 were synthesized by Dr. Robert Zamboni (Department of Medicinal Chemistry, Merck-Frosst Center for Therapeutic Research). The substrate for papain (Phe-Arg-MCA) was purchased from Bachem Bioscience Inc. (Prussia, PA, U.S.A.). Papain was purchased from Boehringer Mannheim (Laval, Quebec). Tyrosine phosphorylated peptide Lck_{505} (TEGQpYQPQP) was obtained from California Peptide Research Inc. (Napa, CA, U.S.A.). All other chemicals, of reagent grade, were purchased from Sigma (St. Louis, MO, U.S.A.).

Preparation of GST-Fusion Proteins

The catalytic domain of PTP1B (amino acid residues 1–321) was subcloned into the pGEX-2T vector (Pharmacia, Quebec) to generate GST-fusion proteins. *Escherichia coli* cells transformed with the vector were grown in Luria broth containing 100 μg/mL ampicillin at 37° to $O.D._{600}$ ~0.6. The temperature of the culture was reduced to 27°, the expression of the protein was induced by the addition of 0.1 mM IPTG, and the culture was grown overnight at 27°. Cells were harvested, and GST-PTP1B fusion protein was isolated on glutathione Sepharose beads (Pharmacia) and eluted with glutathione as described earlier [23].

The intracellular domain of CD45 (amino acid residues 564–1268) was subcloned into pGEX-2T. Bacterial cells transformed with pGEX GST-CD45 were grown in Terrific Broth [24] containing 100 μg/mL ampicillin and 0.4% (w/v) glucose at 37°; induction was at 27° with 0.1 mM IPTG. GST-CD45 was isolated as described above for GST-PTP1B. The fusion protein was cleaved with thrombin on the beads (GST-CD45:thrombin = 60–80:1) as described earlier [25] and used for analysis. The CD45 obtained in this way was ~70% pure, as determined by Coomassie blue staining.

Assay of PTP Activity

A continuous assay for PTPs using the substrate FDP [26] was utilized. The assay solution contained 20 mM imidazole buffer, pH 6.5, 0.2 mM DTT, and various concentrations of the substrate FDP. The reaction was initiated by the addition of the appropriate amount of enzyme diluted in the above buffer containing 10 μg/mL of BSA and monitored continuously by following the increase in absorbance or fluorescence. Assays were carried out in both 96-well plates and cuvettes. In 96-well plates, the total assay volume was 200 μL, and activity was measured using a CytoFluor II fluorescent plate reader (PerSeptive Biosystems) with excitation at 440 nm (slit width 20) and emission at 530 nm (slit width 30). In cuvettes, the total assay volume was 1.0 mL, and activity was measured by monitoring the increase in either absorption using a Cary-UV spectrometer at 430 nm or fluorescence using a Perkin–Elmer (Buckinghamshire, England) luminescence spectrometer (LS 50 B) with excitation at 475 nm (slit width 10) and emission at 515 nm (slit width 5). The phosphorylated peptide TEGQpYQPQP was used as an alternative substrate for assaying PTP activity. The rates of reactions were obtained by continuously monitoring the increase in fluorescent intensity due to the formation of free tyrosyl peptide at an excitation of 282 nm and an emission of 305 nm [27].

§ *Abbreviations*: AuTM, disodium aurothiomalate; DTT, dithiothreitol; FDP, fluorescein diphosphate; FMP, fluoroscein monophosphate; F_2PMP, difluorophosphonomethyl phenylalanine; GST, glutathione *S*-transferase; IPTG, isopropylthio-β-D-galactoside; and PTP, protein-tyrosine phosphatase.

Preparation of Biotinylated Difluorophosphonomethyl Phenylalanine Peptide

About 0.6 mg of DADE(F_2PMP)L-NH_2 peptide was biotinylated at its N-terminus and purified as described earlier [23]. There was one mole of biotin per mole of peptide, as determined by mass spectrometry.

Real-Time Binding Measurements Using the BIAcore Optical Biosensor

All BIAcore (Biomolecular Interaction Analysis) experiments were carried out with a BIAcore 1000 instrument using a buffer containing 25 mM imidazole (pH 7.2), 150 mM NaCl, 0.2 mM DTT, and 0.005% (v/v) Tween 20 at a flow rate of 5 μL/min, except during dissociation and regeneration where the flow rate was increased to 40 μL/min. The biotinylated DADE(F_2PMP)L-NH_2 peptide was bound to a streptavidin sensor chip by injecting 3 μL of the hexapeptide (0.1 μg/mL in BIAcore buffer supplemented with 10 μg/mL BSA). The binding of protein to the immobilized peptide was monitored by injecting 20 μL of the protein in BIAcore buffer over 4 min. This was followed by buffer flow for another 4 min during which dissociation of bound proteins occurs. The sensor chip surface was regenerated by the injection of two 20-μL pulses of 50 mM HCl. This resulted in completed removal of the bound proteins without disruption of the streptavidin-biotinylated peptide interaction (as monitored by binding of a monoclonal phosphotyrosine antibody to the immobilized peptide).

Assay of Papain Activity

Rates of papain-catalyzed hydrolysis were determined by the addition of 10 μL of papain (0.1 mg/mL) in 1 mL of incubation mixture containing 25 μM substrate Phe-Arg-MCA in 20 mM imidazole, pH 6.5, 0.2 M NaCl, 5 mM EDTA, and 0.2 mM DTT at room temperature and monitored using a Perkin–Elmer luminescence spectrometer (LS 50 B) with excitation at 380 nm (slit width 10) and emission at 440 nm (slit width 5).

Data Presentation

All the experiments were done in duplicate or triplicate. In some cases, experiments similar to those presented here were also performed under slightly different conditions to confirm the observation. Data presented in the text and graphs are the averages of the individual measurements.

RESULTS

Inhibition of CD45 and GST-PTP1B by AuTM

We investigated the effect of AuTM on the PTP activity of CD45, employing a continuous assay using the substrate

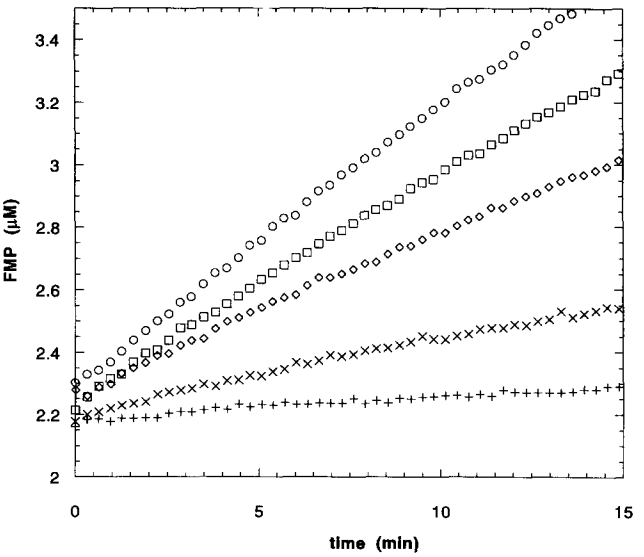

FIG. 1. Inhibition of CD45 by AuTM. Assays were carried out, in duplicate, in 1-mL cuvettes containing 20 μM FDP (K_m = 20 μM for FDP with CD45) in buffer and AuTM at the following final concentrations: 0 (○); 0.5 μM (□); 1.0 μM (◇); 2.0 μM (×); and 5.0 μM (+). The reaction was initiated by the addition of 10 μL of CD45 (final concentration of 0.27 μg/mL), and the time–courses of FMP formation were followed by absorbance at 430 nm (coefficient 34 mM^{-1} cm^{-1}). The IC_{50} was obtained by fitting the percentage of remaining activity versus concentration of AuTM into the IC_{50} equation $(y = (a - b)/[1 + (x/IC_{50})^d] + b)$ using the KaleidaGraph (version 3.05) program (Synergy Software PCS Inc., Reading, PA, U.S.A.).

FDP [26]. Figure 1 shows the time–course of dephosphorylation of FDP by CD45 in the presence of various concentrations of AuTM. The control experiment using 40 mM sodium thiomalate was performed under the same conditions, and no inhibition was observed. AuTM inhibited the activity of CD45 in a concentration-dependent manner, with an IC_{50} of 1.2 ± 0.1 μM. The inhibition was not time dependent in this assay, indicating a rapid rate of inhibition. Gold monochloride (AuCl) also inhibited CD45 with a higher IC_{50} of 15 ± 10 μM under the same buffer conditions. In this case, the quoted concentration of AuCl might not be accurate because of the low solubility of AuCl. The inhibition of CD45 by AuTM does not appear to be substrate dependent as similar results were obtained using the phosphotyrosyl peptide Lck_{505} (TEGQpYQPQP), derived from the C-terminus of the Lck sequence, as an alternative substrate (data not shown).

To determine whether the inhibition of CD45 was specific, AuTM was tested on a cytosolic PTP (PTP1B). AuTM also inhibited the activity of GST-PTP1B in a concentration-dependent manner with an IC_{50} of 3.6 ± 0.2 μM. AuTM was found to be similarly capable of inhibiting the activity of the cytosolic phosphatase SHP-2 as well as a receptor-like phosphatase, PTPε (data not shown). These results suggested that AuTM is likely a pan-specific PTP inhibitor.

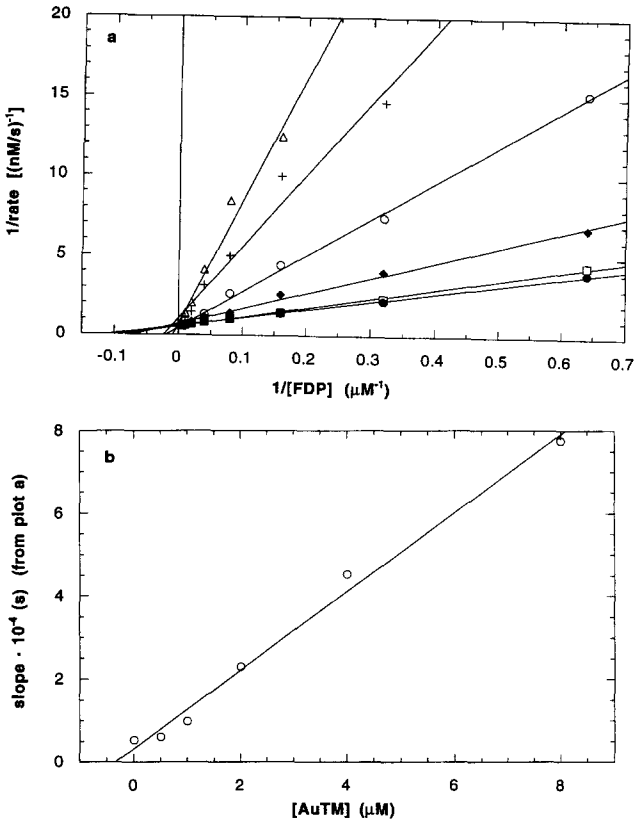

FIG. 2. Lineweaver–Burk analysis of AuTM inhibition. (a) To a 96-well plate, 170 μL of FDP (1–200 μM) in buffer and 10 μL of AuTM (0–160 μM) were added so that the final AuTM concentrations were 0 (●); 0.5 μM (□); 1.0 μM (◆); 2.0 μM (○); 4.0 μM (+); and 8.0 μM (△). The reaction was initiated by the addition of 20 μL of CD45 (final concentration of 0.27 μg/mL). The initial rate of dephosphorylation was determined, and 1/v versus 1/[S] was plotted. (b) Replot of slopes from Fig. 2a versus concentration of AuTM. The value reported in the text for K_i was calculated from a linear fit.

Competition between AuTM and Substrate

To determine if the inhibition of PTP by AuTM was competitive, the rates of CD45-catalyzed dephosphorylation were measured at various FDP concentrations (ranging between 1/7 and 7 · K_m) and at various AuTM concentrations (ranging between 0 and 10 μM). The Lineweaver–Burk plot of 1/v versus 1/[S] is shown in Fig. 2a. According to the kinetic equation for competitive inhibition as shown in equation 1,

$$1/v = \{(K_m/V_{max})(1 + [I]/K_i)\}1/[S] + 1/V_{max} \quad (1)$$

in our case, the V_{max} (reciprocal of intercept on the Y axis) remained constant as the AuTM concentration increased, while the K_m^{app} ($K_m[I]/K_i + K_m$) increased as the AuTM concentration increased. Therefore, this result is consistent with the competitive inhibition of CD45 by AuTM. Replotting the slope of Fig. 2a versus the concentration of AuTM yielded a binding constant K_i of 0.3 ± 0.1 μM (Fig. 2b).

Inhibition of Binding of GST-PTP1B to Biotinyl-DADE(F_2PMP)L-NH_2

The hexapeptide DADE(F_2PMP)L-NH_2 containing the non-hydrolyzable phosphotyrosyl mimetic F_2PMP was shown previously to be a potent inhibitor of PTP1B [28]. We have synthesized this hexapeptide and have confirmed the observations. The peptide inhibited PTP1B with an IC_{50} of 80 ± 2 nM, and this inhibition was reversible and competitive with substrate (data not shown). The recently published crystal structure of the PTP1B $Cys_{215}Ser$ mutant revealed that a phosphotyrosyl peptide having the above sequence bound to the active site of the phosphatase [29]. Therefore, to determine whether the inhibition of PTP1B by AuTM also took place at the same site as the inhibition by the hexapeptide, a binding experiment using BIAcore was developed. BIAcore measures the change of surface plasmon resonance (SPR), which is an optical phenomenon arising in the metal films under conditions of total internal reflection [30]. One interactant is immobilized on the sensor surface. Solution containing the other interactant(s) flows continuously over the sensor surface. As molecules from solution bind to the immobilized interactant, the resonance angle changes, and this response is recorded continuously. BIAcore has many applications [31]; here we used it to determine whether AuTM blocks the binding between PTP1B and the hexapeptide inhibitor. The uppermost sensogram in Fig. 3a, where 10 μg/mL GST-PTP1B was injected over the sensor surface containing biotinylated hexapeptide inhibitor, revealed rapid binding of the protein to the immobilized peptide, as indicated by a progressive increase in resonance signal, which reached an apparent equilibrium. At the end of the injection, during the free buffer flow "wash off" phase, the bound protein dissociated rapidly from the peptide. The observed binding was specific for PTP1B, as GST alone (bottom sensogram in Fig. 3a) failed to bind to the sensor chip surface. In the absence of immobilized biotinylated peptide, there was no significant binding of GST-PTP1B to the streptavidin sensor surface (data not shown). As shown in Fig. 3a, the rate of binding and the apparent steady-state depended on the concentration of GST-PTP1B flowing over the surface. The binding of GST-PTP1B to the biotinylated peptide was inhibited by incubation of the enzyme with non-biotinylated hexapeptide inhibitor (middle two sensograms in Fig. 3b), or by vanadate (bottom sensogram in Fig. 3b), a well known inhibitor of PTPs [32–34], prior to injection. These experiments suggested that the observed inhibition of PTP1B binding was brought about by competition of vanadate, or hexapeptide inhibitor, for the active site of PTP1B. Similarly, incubation of GST-PTP1B with 11, 33, and 100 μM aurothiomalate (lower three sensograms in Fig. 3c),

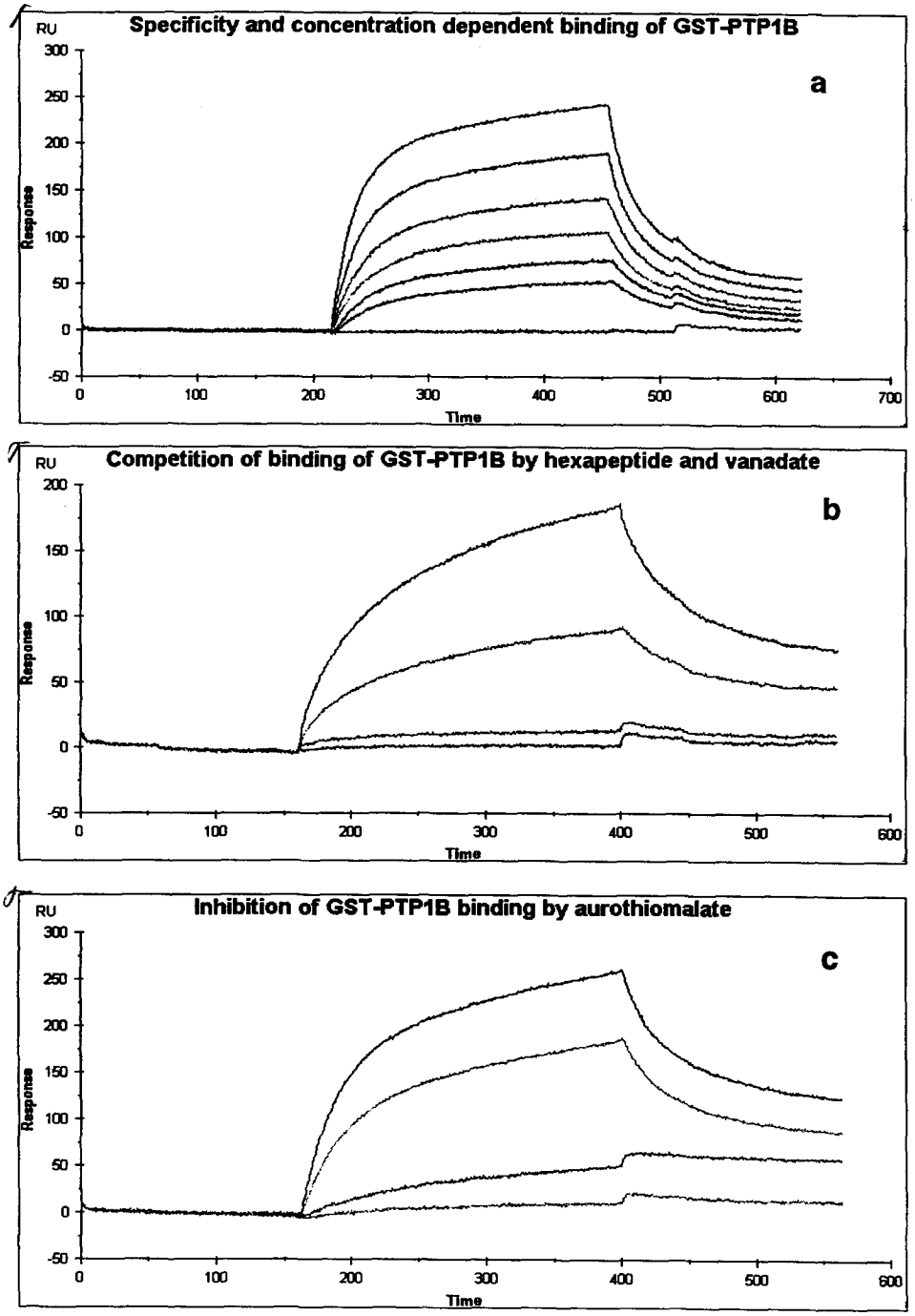

FIG. 3. Binding and competition of GST-PTP1B to the immobilized hexapeptide. (a) Specificity and concentration-dependent binding of GST-PTP1B to immobilized hexapeptide. The overlay sensograms indicate the injection of 10, 7.5, 5, 3.75, 2.5, 1.8 μg/mL GST-PTP1B, or 10 μg/mL GST from top to bottom, respectively. (b) Inhibition of binding of GST-PTP1B by hexapeptide inhibitor or vanadate. The overlap sensograms from top to bottom represent 10 μg/mL GST-PTP1B in BIAcore buffer preincubated with 0, 0.2, or 2 μM hexapeptide inhibitor, or with 0.5 μM vanadate, respectively, before injection over the streptavidin-biotinyl DADE(F_2PMP)L-NH_2 sensor chip surface. (c) Concentration-dependent inhibition of binding of GST-PTP1B to DADE(F_2PMP)L-NH_2 by aurothiomalate. GST-PTP1B (10 μg/mL) in BIAcore buffer was preincubated with 0, 11, 33, and 100 μM (top to bottom sensograms) AuTM before injection over a streptavidin-biotinyl DADE(F_2PMP)L-NH_2 sensor chip surface. RU = resonance units. On the X-axis of each panel, time is expressed in seconds.

resulted in a concentration-dependent decrease in the binding of GST-PTP1B to the immobilized hexapeptide. The relatively higher concentration of AuTM required for the same amount of inhibition of GST-PTP1B in this case than in the IC_{50} determination in enzyme activity assays might be due to the higher enzyme concentration and different assay conditions employed in the BIAcore study. Taken together, these studies suggested that aurothiomalate was interacting with the peptide binding site of PTP1B, results that were consistent with the observed kinetics.

Dependence of IC_{50} on DTT Concentration and Reversibility of AuTM Inhibition

PTPs are enzymes having a cysteine as an active site nucleophile [35, 36]. Since Au(I) preferentially binds to thiol groups, such as cysteine-SH in proteins [8], it was likely that AuTM was inhibiting PTPs via an interaction with the active site cysteine residue. To investigate this possibility, we used DTT, a thiol reagent, to interfere with the inhibitory effect of AuTM on CD45. As shown in Fig. 4, the IC_{50} for CD45 was shifted from 1.2 ± 0.1 μM at

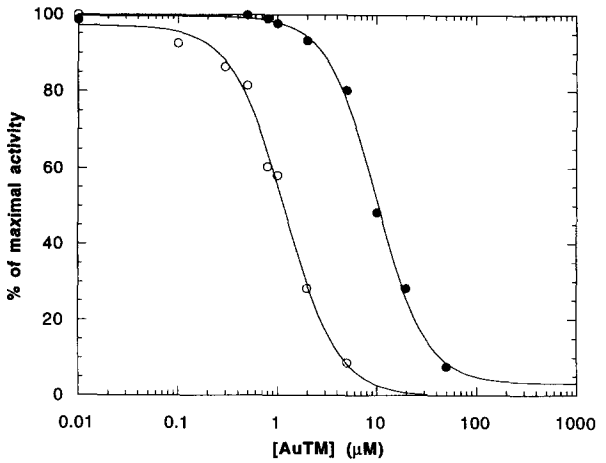

FIG. 4. Dependence of the IC_{50} of CD45 inhibition by AuTM on the concentration of DTT. CD45 (0.27 μg/mL, final assay concentration) was assayed with 20 μM FDP in buffer containing 0.2 mM DTT (○) or 2 mM DTT (●) in the presence of various concentrations of AuTM. The initial rate of dephosphorylation was determined as a percent of the uninhibited rate and plotted against the concentration of AuTM. The data were fitted into an IC_{50} equation ($y = (a - b)/[1 + (x/IC_{50})^d] + b$) using the KaleidaGraph (version 3.05) program.

0.2 mM DTT to 10.3 ± 0.8 μM at 2 mM DTT. The decreased potency could have been due to the reduced effective concentration of Au(I) because of the formation of Au(I)–DTT complexes by addition of DTT in the buffer solution.

To test whether the Au(I)-inhibited CD45 complex could be reactivated by thiol agents such as DTT, CD45 was first incubated in assay buffer with 20 μM FDP and 50 μM AuTM in the presence of 0.2 mM DTT. Under these conditions, approximately 99% of CD45 phosphatase activity was inhibited (Fig. 5). At 22 min (indicated by the arrow in Fig. 5), an aliquot of DTT was added to the reaction mixture, bringing the final DTT concentration to 10 mM. As seen in Fig. 5, CD45 phosphatase activity was restored by the DTT addition. The recovery of phosphatase activity, however, was relatively slow, with 85–100% being regained within ~15 min after the addition of DTT. This slow recovery may be due to the low concentration of "active thiol anion" under the assay condition (pH 6.5) since the pK_a of DTT is ~9.2 [37]. We observed that the reactivation of CD45–Cys–Au(I) by the addition of 2 mM DMH [*N*, *N'*-dimethyl-*N*, *N'*-bis(mercaptoacetyl)hydrazine], which has a pK_a of 7.6 [37], was instantaneous. In the absence of AuTM, addition of 10 mM DTT did not alter the activity of CD45 significantly (Fig. 5).

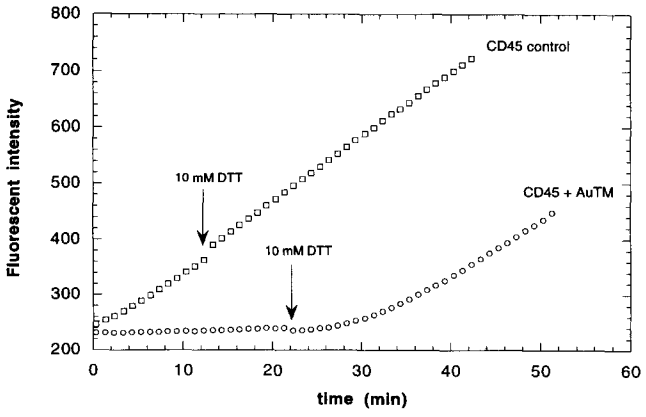

FIG. 5. Reversibility of AuTM-inhibited CD45 with DTT. CD45 (0.27 μg/mL final assay concentration) was assayed with 20 μM FDP in cuvettes as described in Materials and Methods. The time–course experiment shows the activity of CD45 assayed in the absence (□) or presence (○) of 50 μM AuTM. At 22 min, as indicated by the arrow, 10 μL of 1 M DTT was added into the cuvettes so that the final concentration of DTT was 10 mM.

Inhibition of the Thiol Protease Papain by AuTM

To determine if AuTM would also inhibit the activity of another class of enzymes having an active site cysteine residue as a nucleophile, we investigated the effect of gold on the thiol protease papain. The fluorogenic substrate Phe-Arg-MCA was used to monitor the activity of papain. AuTM inhibited the activity of papain when assayed with 25 μM substrate (K_m 25 μM for this substrate), yielding an IC_{50} of 8 ± 5 μM. The higher error level in these data was due to the low solubility of substrate in the assay conditions employed. As with CD45, inhibition of papain by AuTM was reversible by the addition of DTT (data not shown). These results suggested that the inhibitory mechanism was similar in both cases.

DISCUSSION

We have demonstrated that AuTM in the micromolar range is capable of inhibiting the activity of PTPs, a class of enzymes employing a nucleophilic cysteine at the active site to catalyze dephosphorylation of phosphotyrosine residues within proteins [22, 35, 36, 38–41]. As AuTM preferentially binds to thiol groups to form a Cys–Au(I) complex [8, 42], inhibition of this class of phosphatases by AuTM is likely due to complex formation between AuTM and the active site cysteine residue of these enzymes, as shown in the mechanism scheme in Fig. 6. We cannot exclude, however, the possibility that AuTM forms complexes with other thiol groups in these proteins. There were two pieces of evidence suggesting that the active site cysteine was the favored target of AuTM. One was that the active site cysteine has an unusually low pK_a ~4.7 [43], so in the assay conditions (pH 6.5), this cysteine exists as a thiol anion, a form that has greater activity toward AuTM; the second was that other conserved cysteine groups may not be essential for the activity of PTPs as suggested by mutagenesis studies with the receptor-linked PTP LCA [44]. In that case, 4 conserved cysteines in positions 737, 828, 1047, and 1144 were changed to serine and then the PTPase activity of the resultants was tested. Only cysteine at position 828, equivalent to the active site cysteine in

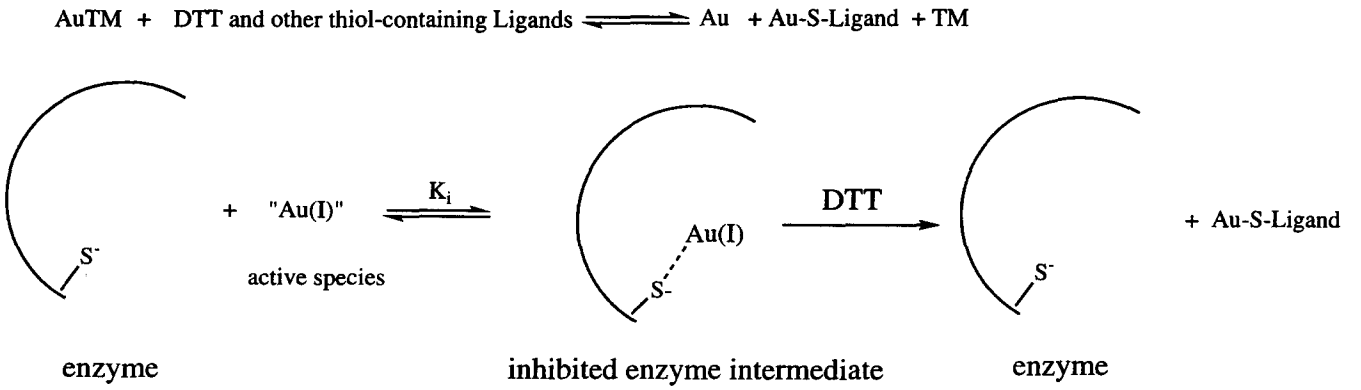

FIG. 6. Kinetic mechanism scheme for the inhibition of PTPs by AuTM.

PTP1B, was essential for the PTPase activity. Also, all of the following results are consistent with the hypothesis that AuTM interacts with the active site cysteine.

AuTM inhibited CD45 and GST-PTP1B with IC_{50} values of 1.2 ± 0.1 and 3.6 ± 0.2 μM, respectively (Fig. 1 and data not shown), in the presence of 0.2 mM DTT as a required component of the assay buffer. That the presence of DTT likely reduced the effective concentration of AuTM suggested that the IC_{50} values actually represent an over-estimate. These inhibitory concentrations of AuTM were similar to those (~5 μM) required for inhibition of AP-1 transcription factor binding to oligonucleotides [12]. Handel and coworkers [12] found that the inhibitory effects of gold were abolished by 1 mM DTT, and also presented evidence that gold at ~10 μM was capable of inhibiting AP-1 activity within breast cancer cell lines. Such levels are below those concentrations of gold that can be achieved in serum (10–25 μM) after approximately 2 months of weekly 50 mg doses of intramuscular aurothiomalate [45, 46] during treatment of rheumatoid arthritis. While gold compounds may not be particularly potent inhibitors of tyrosine phosphatases *in vitro*, it should be pointed out that the intracellular concentrations of gold compounds are unknown. Indeed, therapeutically administered gold accumulates within cells of the monocyte-macrophage lineage, leading to the generation of "aurosomes" within these cells [45]. This finding raises the possibility that the intracellular gold concentrations within such cells might be considerably higher than those observed in the extracellular fluid compartment. As macrophages play important roles in antigen presentation to T-lymphocytes, and are responsible for the release of a variety of mediators and enzymes that contribute to synovial inflammation and cartilage destruction [1], it is tempting to speculate that tyrosine phosphatases within this population may be particularly vulnerable to gold administration.

Disodium thiomalate alone failed to inhibit PTPs; in addition, gold monochloride inhibited PTPs, suggesting that gold or gold complex were the active components. While the IC_{50} value obtained with AuTM was higher than the expected ratio of 1:1 between the PTP and AuTM, nonspecific interactions between gold and other thiol groups in the protein and in the reaction mixture, especially DTT, probably accounted for this difference. In Fig. 6, we have not specified the gold form, which could have been either free Au(I) or Au–ligand. As shown by Takahashi *et al.* [47], gold in the oxidation state +1 will form a complex with ligands, such as thiomalate and thiosulfate. However, *in vivo*, Au(I) dissociates from carrier molecules, such as thiomalate, binding to thiol groups present within proteins [8, 42].

Further evidence to support the proposed reaction scheme that AuTM might be interacting with the active site cysteine was obtained from two additional studies. First, on a kinetic basis, the inhibition of PTPase activity was competitive with respect to substrate. As indicated by the Lineweaver–Burk plot (Fig. 2a), V_{max} did not change while K_m^{app} increased as the concentration of AuTM increased. A second approach made use of an optical biosensor instrument (BIAcore). In this experiment, a hexapeptide inhibitor, DADE(F_2PMP)L-NH_2, a potent, competitive, and reversible inhibitor for PTP1B [28], was immobilized to the biosensor chip surface and the binding of GST-PTP1B to the chip surface was monitored. Binding was specific for GST-PTP1B, as no binding was observed with GST alone (Fig. 3a). Binding of GST-PTP1B was dependent on the concentration of the injected protein (Fig. 3a) and could be reduced by incubation with either the hexapeptide inhibitor or vanadate, both of which are active-site directed inhibitors [29, 32] (Fig. 3b). Incubation of GST-PTP1B with various concentrations of AuTM inhibited the binding of GST-PTP1B to the hexapeptide inhibitor (shown in Fig. 3c), suggesting that AuTM was able to compete for the hexapeptide inhibitor binding site on GST-PTP1B. However, gold-mediated disruption of other critical non-active site thiol groups on the enzyme, leading to denaturation of the protein, was an alternative, although a less likely explanation for the reduced binding of PTP1B brought about by AuTM. Indeed, the finding that AuTM-inhibited CD45 could be reactivated by the addition of excess thiol reagent such as DTT (shown in Fig. 5) suggested that the active site cysteine was modified by AuTM and that protein unfolding was not occurring. The recovery of almost all of the enzyme activity with relatively small amounts of added

DTT suggested that this molecule was able to effectively react with the Au(I)–cysteine–CD45 intermediate, with the ensuing release of free enzyme.

Additional support for the mechanism shown in the scheme in Fig. 6 was obtained by studying the inhibitory effect of AuTM on the activity of the thiol protease papain. The potency of AuTM against PTPs and this thiol protease was similar, and both were also reversible by excess DTT addition. Inhibition of papain by gold salts was described previously [48] in agreement with the prediction that any thiol reagent should bind to gold [8]. The affinity of AuTM for a thiol reagent within a protein is dependent on the nucleophilicity of the thiol, which in turn is dependent on the protein environment around the thiol group. The thiol group must also be accessible and not buried within the protein. Since thiol proteases, like PTPs, have a cysteine as the active site nucleophile, modifications of this cysteine would be predicted to result in enzyme inhibition. Thus, the active site cysteine-containing enzyme papain, having no structural similarity to PTPs, was inhibited by AuTM, likely by a mechanism similar to that responsible for PTP inhibition.

In conclusion, our results demonstrated that AuTM, in the low micromolar range, inhibited PTPs *in vitro*, and also that the inhibition was competitive with substrate. The mechanism likely involved the interaction of gold with the active site cysteine nucleophile within these molecules. It remains to be determined whether the activities of key regulatory PTPs, such as CD45 [19], are altered within intact cells exposed to AuTM treatment. Indeed, inhibition of lymphocyte CD45 would constitute a novel manner by which AuTM administration might bring about suppression of diseases where lymphocytes have important pathogenic roles. These issues will be the subject of future studies.

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