Iodophenylarsine Oxide and Arsenical Affinity Chromatography: New Probes for Dithiol Proteins

APPLICATION TO TUBULINS AND TO COMPONENTS OF THE INSULIN RECEPTOR-GLUCOSE TRANSPORTER SIGNAL TRANSDUCTION PATHWAY*

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In our studies of the effects of the trivalent arsenical phenylarsine oxide on insulin-dependent hexose uptake in 3T3-L1 adipocytes, we needed direct methods to study arsenical-protein interactions. In this report, we describe two such new tools. The first is the radiolabeled covalent affinity reagent 4-[^125]Iiodophenylarsine oxide. This compound has effects on 3T3-L1 adipocytes similar to those of phenylarsine oxide both with respect to effects of hexose uptake and the accumulation of pp15, a phosphotyrosine-containing putative mediator of insulin action. Iodophenylarsine oxide labels numerous proteins in intact cells in a concentration-dependent, but apparently insulin-independent fashion. The second tool is trivalent arsenical affinity chromatography, which we use to show novel direct interactions between trivalent arsenicals and several proteins from 3T3-L1 adipocytes including the insulin-responsive glucose transporter GLUT4, the insulin proreceptor, and both the α and β subunits of tubulin. The non-insulin-dependent glucose transporter GLUT1, the mature insulin receptor, and the fatty acid-binding protein 422(aP2) do not show strong interactions with arsenical resin. These results provide a new chemical approach to the study of both insulin-dependent hexose transport and tubulin function.

Arylarsine oxides are oligomeric anhydrides of arylarsonous acids, compounds of trivalent arsenic that have been studied for over 90 years for their wide range of biological effects (1, 2). The basis for the action of these compounds is believed to be the formation of heterogeneous covalent adducts between closely spaced sulfhydryl groups on proteins. Among the most sensitive and best studied arsenical-sensitive enzymes is the lipoic acid-containing enzyme complex pyruvate dehydrogenase (2-4). During the catalytic cycle with lipoic acid in its reduced form (dihydrolipoic acid), organoarsine oxide can react to form a six-membered 1,3,2-dithiarsolane heterocycle. Treatment of the inhibited enzyme with an excess of the dithiol antidote 2,3-dimercaptopropanol restores enzyme activity by abstracting the inhibitory arsenical in the form of a five-membered 1,3,2-dithiarsolane cyclic adduct. Other arsenical-sensitive enzymes are believed to be affected by an analogous interaction of arsenicals with sulfhydryl groups of closely spaced protein cysteinyl residues, although these interactions are generally less well understood (2). Conceivably, a large variety of potential arsenical-binding sites may exist, differing from one another with respect to the formation of reactive groups (i.e., closely spaced in primary amino acid sequence versus brought together only in secondary, tertiary, or even quaternary structure), the presence of activating groups (e.g., general bases that cause deprotonation of thiol to form thiolate anion) that might facilitate the chemical interaction, or the existence of reactions that compete with arsenical binding (e.g., closely spaced thiols that may become unreactive by oxidation to a disulfide bond), among other possibilities.

Our laboratory has studied the complex effects of phenylarsine oxide (PAO) on insulin-stimulated glucose uptake in 3T3-L1 adipocytes (5-11). In the absence of insulin, low concentrations of PAO (4 μM) were found to cause a 2-3-fold enhancement of basal hexose uptake rate, which was abrogated by higher concentrations of arsenical. Of more interest was the observation that higher concentrations of PAO (35 μM) completely inhibited the insulin-stimulated component of hexose uptake (5). The simultaneous addition of 35 μM PAO and insulin was furthermore associated with the accumulation of a 15-kDa phosphotyrosine-containing protein (pp15) that we have subsequently identified as phospho-O-Tyr19,422(aP2) protein, a product and potential transducer of the insulin receptor protein-tyrosine kinase that accumulates because of PAO-mediated inhibition of specific membrane-associated protein-tyrosine-phosphatases or phosphotransferases (7-11).

The complexity of the effects of PAO on hexose uptake, presumably caused by covalent interactions with proteins, inspired our interest in studying such interactions more directly. Two independent approaches seemed feasible. The first of these was to study hexose uptake effects and differential labeling of proteins in cells by a radiolabeled analog of phenylarsine oxide. To this end, we synthesized 4-[^125]Iiodophenylarsine oxide by a radiochemical microscale adaption of a method reported by Mameli and Patta in 1909 (12). The second approach was to study the interaction of proteins in cell lysates with a PAO affinity resin by studying the differential elution of proteins by increasing concentrations of thiol.

The abbreviations used are: PAO, phenylarsine oxide; SDS, sodium dodecyl sulfate; DMP, 2,3-dimercaptoethanol; DETT, 2-threo-1,4-dithio-2,3-butanediol; MeSO, dimethyl sulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; FMSW, phenylmethylsulfonyl-fluoride.

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Arsenical-Protein Interactions

PREPARATION OF PA0 AFFINITY RESIN

To fractionate native proteins from cell extracts on the basis of their interaction with aromatic trivalent arsenical compounds like PA0, we prepared an affinity matrix from a commercially available agarose derivative bearing activated spacer arms by reaction with a solubilized commercial preparation of 4-aminophenylarsine oxide. Because this arylarsine oxide preparation was apparently in highly polymeric insoluble form, it was first solubilized by brief exposure to a stoichiometric amount of HCl and rapid neutralization in the presence of 2 eq of mercaptoethanol to inhibit reoligomerization. Based on colorimetric analysis of incorporated arsenical, ~1 μmol of arsenical was incorporated per g of wet resin. To demonstrate the specificity of the column, the resin was treated with either excess 2,3-dimercaptopropanol or an equivalent amount of thiol in the form of mercaptoethanol before washing the column and loading cell extract. As shown in Figs. 11 and 12, treatment with 2,3-dimercaptopropanol caused total inactivation of the resin; essentially all of the applied protein was recovered in the flow-through fraction. In contrast, the mercaptoethanol-pretreated column bound considerable protein. A small amount of this protein could be eluted with SDS, but the bulk required up to 100 mM mercaptoethanol for elution. Fig. 11 shows that the retained fractions were particularly enriched in proteins capable of binding 4-[125I]iodoPA0, and the flow-through fractions were reciprocally depleted.

Identification of Specific Proteins in PA0 Affinity Column Fractions

The interaction of trivalent arsensials with specific proteins was analyzed by Western blot analysis of column fractions eluted from a PA0 affinity column. Specific antibodies to synthetic peptides of the mouse insulin receptor, mouse 422(aP2) protein, and both mouse and rat glucose transporters GLUT1 and GLUT4 were used to probe replica blots. By this means, we hoped to assess differential interactions between the immobilized arsenical and detergent-solubilized proteins mediating different steps of a proposed insulin signal transduction pathway. In addition, mouse monoclonal antibodies specific for the α and β subunits of tubulin were used to demonstrate the interactions of abundant proteins that we found to show high avidity for the resin. For these experiments, each Triton/octyl glucoside extract of cells (treated or not with 1 μM insulin) was divided among four affinity columns, two containing active PA0 resin and two containing inactive PA0 resin. After loading, the columns were eluted pairwise (one active, one inactive) with buffers containing incremental concentrations of thiol eluants in buffer containing either 1% Triton X-100 or 1% SDS to compare elution of native and denatured proteins, respectively. As shown in Fig. 13A, most of the insulin receptor (as detected by the processed β subunit of 95 kDa) and nearly all of the detectable 422(aP2) protein passed through the column and were recovered in the flow-through fraction and the first eluted fraction. Because the columns were loaded and washed in the absence of exogenous reducing agents, we presumed that both the disulfide-

RESULTS AND DISCUSSION

Preparation of PA0 Affinity Resin

Columns containing 1 ml each of PA0 affinity resin were pretreated for 1 h with 2 ml of solubilization buffer supplemented with either 20 mM 2-mercaptoethanol (2-ME) (active PA0, 2nd-7th lanes) or 10 mM 2,3-dimercaptopropanol (inactive PA0, 8th-13th lanes) and then were extensively washed with solubilization buffer. The columns were each charged for 1 h with 2 ml of cell lysate (1st lane) prepared by treating washed monolayers of 3T3-L1 adipocytes with solubilization buffer for 1 h, pelleting insoluble material, and filtering off the floating fat droplets. The column flow-through fractions were each cooled with a 3-ml wash with solubilization buffer (2nd and 5th lanes). Both columns were then sequentially eluted with 5-ml aliquots of solubilization buffer with 1% SDS (instead of Triton X-100 supplemented with 0 (3rd and 9th lanes), 1 mM (4th and 10th lanes), 10 mM (5th and 11th lanes), 100 mM (6th and 12th lanes), or 1 M (7th and 13th lanes) 2-mercaptoethanol. The fractions were analyzed on two replica reducing SDS-polyacrylamide gels. The first gel was double-stained with Coomassie Blue (upper) and 4-[125I]iodo-PA0 (middle). The second gel was silver-stained (lower). The indicated molecular masses correspond to standards as described in Fig. 7.

FIG. 11. Interaction of proteins with PA0 affinity resin. Extracts from 3T3-L1 cells treated or not with 1 μM insulin were loaded onto columns containing 1 ml of either active or inactive PA0 resin. The columns were eluted with increasing concentrations of 2-mercaptoethanol (2-ME) and then 10 mM 2,3-dimercaptopropanol (BAL) in buffer containing either 1% Triton X-100 (A) or 1% SDS (B). Eluted proteins were precipitated with trichloroacetic acid, and the pellets were washed with isopropyl alcohol/hexane (2:3) before resolubilization in 250 mM sodium phosphate (pH 7.5), 1% SDS. Protein concentrations were measured using Pierce bicinchoninic acid reagent. F/T, flow-through fraction.
TRITON buffer containing Triton X-100. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis blotting. Detected proteins are insulin receptor proreceptor and 422(aP2) protein present in the flow-through fractions. In contrast, the 210-thiol within a specified radius under "Experimental Procedures." Results are plotted for 1 mM, 10 mM, 100 mM, and 1 M thiol such that the areas under the curves of GLUT1 and GLUT4 are widely separated in primary sequence at residues 223 and 429. Since we have demonstrated that the Cys-Gly-Cys tripeptide could function as a PAO-binding motif (Figs. 6 and 7) and that the binding of GLUT4 was relatively insensitive to denaturation (Fig. 13B), we speculate that arsenical binding is mediated by cysteines 361 and 363 of mouse GLUT4. It will be of interest to determine whether our prediction can be substantiated in the intact molecule by oligonucleotide-directed mutagenesis of the 4 cysteine residues of the GLUT4 sequence.

The functional alteration caused by trivalent arsenicals will be a subject of continued investigation. Earlier studies suggested that toxic interaction may not directly affect the catalytic activity of the glucose transport apparatus (5, 36), but rather may affect another of its functions, for example, its stability to degradation or its translocation to the plasma.

Comparison of the amino acid sequences of mouse GLUT1 and GLUT4 (24) shows a divergent motif in the proposed ninth transmembrane region: GLUT1, 338 LLGLAGMAGC AVLMTIALALL 358; and GLUT4, 354 LLGLAGMCCGAI LMTVALLLL 374. The only other cysteine residues in GLUT4 are widely separated in primary sequence at residues 223 and 429. Since we have demonstrated that the Cys-Gly-Cys tripeptide could function as a PAO-binding motif (Figs. 6 and 7) and that the binding of GLUT4 was relatively insensitive to denaturation (Fig. 13B), we speculate that arsenical binding is mediated by cysteines 361 and 363 of mouse GLUT4. It will be of interest to determine whether our prediction can be substantiated in the intact molecule by oligonucleotide-directed mutagenesis of the 4 cysteine residues of the GLUT4 sequence.

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It was demonstrated previously that the nascent proreceptor monomer undergoes dimerization, giving rise to a disulfide-cross-linked dimer (29). Then after extensive conformational rearrangement involving the formation and isomerization of intrachain disulfide bonds, it undergoes final proteolytic cleavage to produce the mature disulfide-linked $\beta$-$\alpha$-$\alpha$-$\beta$ heterotetrameric receptor (29). Our present results suggest that at least one arsenical-binding dithiol motif persists through the proreceptor dimer stage and is lost either coincidently or nearly contemporaneously with the proteolytic cleavage between the $\alpha$ and $\beta$ subunits. The formation of a final disulfide bond may possibly signal the proteolytic event that gives rise to mature receptor.

In contrast, the interaction of the two glucose transporter species (GLUT1 and GLUT4) with the arsenical resin is contrasted. Although nearly all of GLUT1 was recovered in the flow-through fraction, over half of GLUT4 was bound by the active resin. Treatment with SDS alone eluted some of the bound GLUT4, but complete elution required up to 1 M 2-mercaptoethanol. Insulin pretreatment of cells did not affect the binding of GLUT4. The effects of trivalent arsenicals on insulin-stimulated glucose uptake in adipocytes and 3T3-L1 cells have been extensively studied by our laboratory (5–11) and others (30–36). Recent reports have been extended to the study of inhibition by arsenicals of hexose transport in skeletal muscle (37–39). Direct methods to study arsenical-protein interactions have only recently been applied.

Frost and Schwalbe (35) recently demonstrated the use of N-[H]acetyl-4-aminophenoxyarsine oxide to study arsenic-protein interaction in 3T3-L1 adipocytes. Our results support their finding that many proteins are labeled in intact cells and further demonstrate the dependence of the protein labeling pattern on the concentration of labeled arsenical. These findings suggest a hierarchy of avidities of cellular proteins for organic arsenicals.

The selective binding of GLUT4 to arsenical resin has obvious bearing on the effects of arsenicals on insulin-stimulated glucose uptake. The direct interaction of glucose transporters with arsenicals has previously been suggested by Douen et al. (30) on the basis of apparent competition between PAO and cytochalasin B for binding to plasma membranes from insulin-stimulated rat adipocytes. Such competition would not be able to distinguish between the two classes of glucose transport proteins.

The linked $\alpha$ and $\beta$ subunits of the mature insulin receptor were present in the flow-through fractions. In contrast, the 210-kDa insulin proreceptor was retained by the columns and was eluted with 100 mM 2-mercaptoethanol. All protein interactions with the resin were prevented by preinactivation of the resin with 2,3-dimercaptopropanol.

These results both support and augment our understanding of the post-translational processing of the insulin proreceptor.

FIG. 13. Western blot detection of specific proteins in affinity column fractions. Cells treated or not with 1 mM insulin were extracted and applied to active or inactive PAO resin for elution with buffer containing Triton X-100 or SDS as described for Fig. 12. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis on replica 12.5% polyacrylamide gels before transfer and immunoblotting. Detected proteins are insulin receptor (INS R) $\beta$ subunit/prereceptor and 422(aP2) protein (A), GLUT1 and GLUT4 (B), and $\alpha$- and $\beta$-tubulins (C). BAL, 2,3-dimercaptopropanol.

FIG. 14. Interthiol distances in monothiol buffers used for column elution. Probabilities of finding at least one molecule of thiol within a specified radius of another were calculated as described under "Experimental Procedures." Results are plotted for 1 mM, 10 mM, 100 mM, and 1 M thiol such that the areas under the curves represent the probability distributions.
membrane in response to insulin. Direct demonstration of the effects of arsenicals on glucose transporters will require much additional effort.

To demonstrate that the partial absorption of GLUT4 by the resin was not caused by column overloading, Fig. 13C shows the elution of the α and β subunits of tubulin, abundant proteins that appear to show high avidity for the arsenical resin. Under all conditions, both tubulin subunits were completely absorbed by the active resin and required up to 1 M 2-mercaptoethanol for elution. Interestingly, SDS elution of either α- or β-tubulin, but not GLUT4, required ~10 times less 2-mercaptoethanol than did Triton X-100 elution.

Although arsenical affinity chromatography has been used to study small dithiol molecules like dihydroxylic acid (40), arsenical affinity chromatography of proteins, to our knowledge, has never been reported. In designing the resin, we chose a straightforward spacer arm-linked active group to allow maximal access to protein reactive groups. Instead of eluting with dithiol, we chose to use step gradients of mercaptoethanol to allow regeneration of the columns with a buffer wash. Regeneration is not easily accomplished and therefore is not practical after treatment of columns with 2,3-diaminopropanol. Mercaptoethanol elution may also allow an analytical approximation in the secondary or tertiary structure that is apparent only in the folded state of the protein. We would expect to be able to distinguish between these possibilities by testing for a change in the arsenical-binding avidity upon denaturation. Proteins with dithiol motifs that are assayed by primary structure should, by this reasoning, exhibit arsenical--binding avidity that is relatively insensitive to denaturation, whereas proteins with dithiol motifs that are assayed by tertiary structure should become significantly less avid in their arsenical binding upon denaturation. This latter case may, in fact, apply to β-tubulin. Using a bifunctional reagent that allows no more than 9 Å between reactive sulphydryl groups, Little and Luduena (41, 42) and Luduena and Roach (43) have identified two pairs of cysteinyl residues that are distant in the primary structure of β-tubulin (residues 239 and 354 and residues 12 and either 201 or 211) and that are brought together in the folded state. A similar dithiol in α-tubulin is less well characterized. Accordingly, the thiol concentration required for complete elution of tubulins from arsenical affinity resin was decreased from 1 M (8.7-Å average mobile phase interthiol distance) to 100 mM (~20-Å average mobile phase interthiol distance) upon denaturation with SDS (Fig. 13C). The current belief that the vicinal dithiols of tubulin have important roles in tubulin function raises the possibility of developing rationally designed arsenical-based antimotic agents (43). The interactions of tubulin with arsenicals have, to our knowledge, never been reported.

In a broader sense, arsenical affinity chromatography may have applications in other systems. The finding that so many different proteins appear to interact with arsenical resin implies that this technique may prove to be valuable not only as an important adjunct to study the toxic and therapeutic effects of arsenical compounds, but also as a unique method of protein purification.

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Note Added in Proof—Since submission of the manuscript, we learned of a recent report of the use of arsenical affinity chromatography for the purification of the plasma enzyme lecithin cholesterol acyltransferase (44). We also have learned of an early reference to the colchicine-like effect of trivalent arsenic (45).

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EXPERIMENTAL PROCEDURES

Materials: Arsenic acid, p-arsenobetaine and 3,7,11-tetrabromo-1,0,13-trimethoxyarsine were obtained from Aldrich, p-arsanilic acid and from Kodak. Affigel 10 and 5% agarose were obtained from BioRad. Carrier-free sulfur (35S) dioxide was from American. Pressure injection cocking (10) consisted of a stainless steel and unsintered alumina (460 ml) (PIA) chromatographic. Prior to injection cocking (10) contained fresh chromatographic and provided A (Sulfate) in MegS04.

Sodium 4-iodo-p-arsenobetaine (PIAB): The general scheme for the synthesis of 3-4 iodobenzyl arsenic acid from arsinite, iodination, and reduction (Fig. 1) was described recently by Melander and Parks (12). The oxidation allows the iodination and reduction to be performed conveniently in the vial used for stopping the reaction.

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As shown in Figure 8, the effects of IPAO on glucose uptake paralleled closely those of PAO, although IPAO appeared to be slightly less potent in its effects than PAO. Specifically, both arsenicals caused a two-fold increase in 2-deoxy-glucose uptake in the absence of insulin reaching maxima at approximately 10 mU/ml for PAO and 30 mU/ml for IPAO. Glucose uptake in the absence of insulin was significantly reduced in the presence of PAO or IPAO (at 10 mU/ml) with 30 mU/ml of IPAO being quite nearly the nadir level of cells not exposed to insulin, although about twice as much IPAO (40 mU/ml) in PAO (10 mU/ml) was required to maximize such reductions in uptake.

A second insulin-dependent process affected by arsenicals ar sosulation is the accumulation of p31, mainly characterized as phospho-Ser-473(Thr-32) insulin (9). This positive insulin mediator accumulates in 3T3-L1 adipocytes treated with both PAO and IPAO because of concern insulin-dependent activities of the insulin receptor protein tyrosine kinase and PAO-mediated ribosomal protein S6 kinase, which mediates the synthesis of phospholipids (7, 11). We have recently described the correlation labeling with 125I-IPAO of two such purified phosphoproteins with molecular masses of 30 and 36kDa (11). Phosphatase labeling to maximal cellular accumulation of p31 was performed as previously described (9). Differentiated 3T3-L1 adipocytes in 25 mm dishes were serum-cultured overnight in culture in DMEM. The monolayers were then washed with phosphate buffer (PBS) and left for 3 h with insulin (10 mU/ml) to allow p31 to accumulate into the cytoplasm. The cells were then washed and incubated with 125I-IPAO for 30 min.

Cell monolayers were then washed with ice-cold PBS and cytosolic contents including 125I-p31 were released by the addition of 1000 mU/ml glycylglycine in 10 mM HEPES, 1 mM NaN3, pH 7.4. A 45-min aliquot of released protein from each dish mixed with 10 μl of ESI H2O and 185 CHAPS and of 10 μl of the 185 CHAPS. The cytosolic proteins were then analyzed by two-dimensional electrophoresis and autoradiography. The spot or area corresponding to p31 was excised for measurement of incorporated radiolabel.

Figure 9. Induction of p31 accumulation by PAO and insulin. Serum-starved monolayers of differentiated 3T3-L cell were cultured in medium in the presence of various concentrations of PAO and insulin for 3 h. The accumulation of p31 was then measured by the in vivo labeling technique as described above. The results were normalized and presented in the form of a graph. As shown in Figure 9, (PAO) causes concentration-dependent accumulation of p31 in insulin-treated 3T3-L1 adipocytes over the concentration range where it elicits effects on glucose uptake. A slight insensitivity of the effects of IPAO on glucose uptake paralleled closely those of PAO, although IPAO appeared to be slightly less potent in its effects than PAO. Specifically, both arsenicals caused a two-fold increase in 2-deoxy-glucose uptake in the absence of insulin reaching maxima at approximately 10 mU/ml for PAO and 30 mU/ml for IPAO. Glucose uptake in the absence of insulin was significantly reduced in the presence of PAO or IPAO (at 10 mU/ml) with 30 mU/ml of IPAO being quite nearly the nadir level of cells not exposed to insulin, although about twice as much IPAO (40 mU/ml) in PAO (10 mU/ml) was required to maximize such reductions in uptake.

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Arsenical-Protein Interactions

HCl and was quickly resuspended with 400 μl of 1M potassium HCO₃. The mixture was rapidly ejected into a sealed test-tube containing 250 μl of 10 mg/ml each of bovine serum albumin. 24 hr at room temperature on a rotating shaker. Unbound was blocked by adding 300 μl of ethanolamine base and mixing for one more hour at room temperature. The mixture was then subjected to three washes with 1 ml of 200 μl portions of HCl and 1 ml of 200 μl portions of saline. The washed mixture was stored at -20°C for 5 min. For quantitation of unbound arsenicals, advantage was taken of the similarity between arsenic and phosphorus in chromatographic assays based on reduction of their respective complexes with molybdate [17, 20]. Briefly, a weighed sample of each standard was added to a test tube containing 500 μl of an ethanolamine buffer solution at 500 μM. 250 μl of 10 mg/ml of bovine serum albumin. After 1 hr, the solution was filtered through a 0.2 μM filter and then diluted with 1 ml of 500 μM ethanolamine buffer solution at 500 μM. The filter solution was then analyzed by flameless spectrophotometry.

To test the protein binding capacity, 0.5 ml of 10 mg/ml of bovine serum albumin was added to 0.5 ml of 200 μM arsenic (III) solutions. The mixture was then incubated at 37°C for 1 hr, centrifuged at 10,000 rpm for 10 min, and the supernatant was analyzed by flameless spectrophotometry.

For the purpose of the experiment, the mixture was divided into two equal parts of 250 μl each. To one part, 1 μM of molybdate was added, and to the other, 20 μM of molybdate was added. After 1 hr, the solutions were analyzed by flameless spectrophotometry.

Determination of protein binding capacity: In order to measure the percentage of unbound arsenic, 250 μl of 10 mg/ml of bovine serum albumin was added to 250 μl of 0.1 M HCO₃ buffer solution. After 1 hr, the mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was analyzed by flameless spectrophotometry.