Cadmium and Arsenite Binding by *N*-Dihydrolipoyl-aminoethoxydextran: A Model Study of Enzyme Dithiol Criteria

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ABSTRACT

We have observed the interaction of Cd^{2+}, Zn^{2+}, and AsO_2^- with a dithiol-substituted polymer (*N*-dihydrolipoyl aminoethoxy dextran). Cadmium binding results in an intense uv difference spectrum (λ_{max} = 240 nm). Spectrophotometric titrations with $CdCl_2$ reveal formation of two Cd-dithiol complexes characterized as 2—SH/Cd^{2+} and 3—SH/Cd^{2+}. Stability constants were determined by titration of cadmium-saturated polymer with excess EDTA. For the two complexes, $K_1 = 2.7 \times 10^{14}$ *M* and $K_2 = 7.7 \times 10^{13}$ *M*. NTA is not effective in displacing Cd^{2+}. In competition of Cd^{2+} and Zn^{2+} for dithiol sites, Cd^{2+} is bound about 500 times more firmly than Zn^{2+}. Arsenite binding is sluggish ($K \sim 85$ M^{-1} sec^{-1}) and yields a single complex (2—SH/AsO_2^-). Competition and kinetic data suggest that 10^6 *M* < $K_{AsO_2^-}$ < 10^8 *M*. We have defined basic criteria for evaluation of enzyme active-site dithiols: (1) the binding order "cadium stronger than zinc"; (2) relief of Cd^{2+} inhibition by 10-fold excess EDTA and no relief by 10-fold excess of NTA; (3) inhibition by arsenite.

Key Words: Cadmium, zinc, arsenite, dithiol, lipoic acid, thiol-substituted edxtran, enzyme dithiol criteria, EDTA, NTA, dithiothrietol.

Traditionally inhibition of enzyme action by cadmium (Cd^{2+}) and arsenite (AsO_2^-, $H_2AsO_3^-$) has been used as a test for a functional dithiol at enzyme active centers [1]. Relief of inhibition by dithiol but not by

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monothiol compounds [2, 3] and inhibition by zinc (Zn^{2+}) at concentrations 10 to 100 times higher than those required for cadmium [4] have been invoked as additional requisites. These criteria have evolved from studies on established dithiol enzymes, such as dihydrolipoyl dehydrogenase [5], but have never been subjected to careful chemical evaluation. A direct chemical approach for testing such criteria would compare the binding constants of cadmium, zinc, and arsenite for various dithiols with those for other potential enzyme ligands. However, most attempts at establishing the actual thermodynamic binding affinities of these agents with dithiols have been thwarted by insolubility at pH's relevant for enzyme studies [6]. In order to obtain soluble dithiol complexes of arsenite, cadmium, and zinc at physiological pH's, we have prepared dihydrolipoate covalently linked to a high molecular weight dextran. The dextran derivative is prepared by attaching DL-lipoic acid to an amino-ethoxy-substituted dextran by use of lipoyl-ethyl carbonic anhydride followed by reduction of the cyclic disulfide. The interaction of metals with dithiol polymers is studied by differential spectrophotometry under anaerobic conditions.

Cadmium is bound much more avidly by the dithiol polymer than by analogous monothiol substituted polymers [7]. Under conditions employed for studies of the monothiol polymers, the dithiol compound completely removes cadmium from solution and the binding affinity could only be evaluated by competition with EDTA.[1] a 1:1 complex between metal and dithiol is achieved when the metal is in excess. At lower metal concentrations spectral data suggest a more complex situation in which at least two dithiol sites cooperate in cadmium binding. Zinc is bound between two and three orders of magnitude less strongly than cadmium.

Arsenite is also strongly bound by the dithiol polymer, but the rate of complex formation is slow. Similar kinetic behavior is also observed for the reaction between dithiothreitol and arsenite. While direct binding-constant measurements have not been possible, limits for the dithiol arsenite bindnig affinity are defined by (1) its inability to displace cadmium from the ligand and (2) the kinetics of formation of the complex and the rate of arsenite displacement by cadmium.

Equipment and Materials

Optical spectra were recorded on a Cary Model 15 spectrophotometer. All reagents were analytical grade and were used without further purifica-

tion. Lipoic acid (DL-thioctic acid), dithiothreitol, and *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) were obtained from Calbiochem; ethylchloroformate from Matheson-Coleman-Bell. Aminoethoxy dextran was synthesized by the method of Gaber and Fluharty [7].

Stock solutions of cadmium were prepared by dissolving cadmium metal (99.999% Alpha Inorganic) in concentrated hydrochloric acid. The stock solution was diluted to 2 m*M* with 50-m*M* HEPES,[1] containing 0.1-*M* NaCl and adjusted to pH 7 immediately before use.

Synthesis

One gram of aminoethoxy dextran dissolved in 100 ml of 0.2-*M* potassium bicarbonate buffer, pH 8.4, was purged of oxygen by three cycles of evacuation and gasing with nitrogen. The polymer solution was stirred vigorously while 5 m*M* of freshly prepared DL-lipoic-ethyl carbonic anhydride in about 15 ml of tetrahydrofuran was slowly added over a period of one hour at room temperature. The reaction mixture was stirred for another hour, acidified, extracted three times with diethyl ether, and the pH readjusted to neutrality with dilute sodium hydroxide. Atmospheric oxygen was excluded at all stages by purging with nitrogen and direct illumination was minimized to avoid the formation of disulfide polymers from the lipoate residues. The solution was filtered, EDTA added to 1 m*M* and 200 mg of dithiothreitol was added to reduce the cyclic disulfide. The *N*-dihydrolipoyl aminoethoxy dextran was dialyzed immediately. Deionized water for dialysis was freed of oxygen by prolonged purging with high-purity nitrogen. To avoid exposing the solution to the atmosphere, the water was changed continuously by addition from a large reservoir to a closed dialyzing vessel. At the conclusion of the dialysis the *N*-dihydrolipoyl aminoethoxy dextran was transferred with a gas-tight syringe to 10-ml serum vials which had been purged with nitrogen. The vials were immediately sealed and refrigerated. Solutions of *N*-dihydrolipoyl aminoethoxy dextran were stable for several months if the vials remained unopened. Storage for periods over 6 months resulted in some loss of assayable thiol accompanied by changes in spectra and binding properties.

The extent of thiolation of *N*-dihydrolipoyl aminoethoxy dextran was determined by *p*-chloromercuribenzoate (PCMB) titration [9] and dextran concentration by the Guidici-Fluharty modification of the phenol-sulfuric acid assay for sugars [10]. The sulfur content by commercial analysis was consistent with these titrations. In addition all solutions were assayed with 5,5'-dithiobis(2-nitrobenzoic acid) [11] immediately before use.

As confirmation that dithiols were in fact coupled to the polymer, *N*-dihydrolipoyl aminoethoxy dextran was reacted with excess PCMB and passed through a Sephadex G-25 column. The PCMB-thiolate emerged with the void volume while unreacted reagent was retarded.

The ultraviolet spectrum of *N*-dihydrolipoyl aminoethoxy dextran exhibited a maximum at 240 nm, similar to the monothiol-substituted polymers [7].

Titration

Stock solutions of *N*-dihydrolipoyl aminoethoxy dextran were diluted with oxygen-free buffer, assayed for thiol, and adjusted to about 0.2-m*M* thiol (0.1-m*M* dihydrolipoate). Spectra were recorded as the difference between polymer plus cadmium and polymer plus an equivalent volume of buffer. Additions did not exceed 10% of sample volume. All spectra were corrected to 1 ml, the initial volume. *N*-Dihydrolipoyl aminoethoxy dextran

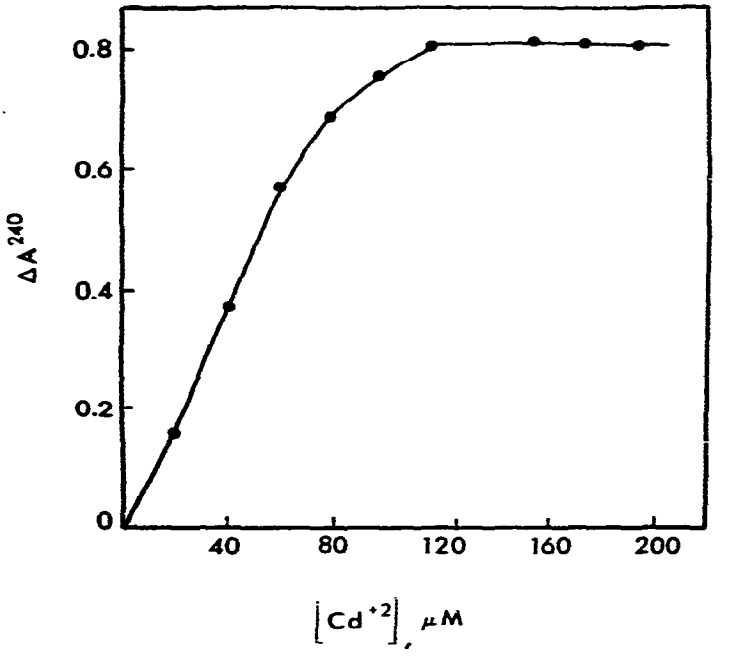


Figure 1. Difference spectrophotometric titration of *N*-dihydrolipoyl aminoethoxy dextran with $CdCl_2$. Dithiol concentration, 115 μM in 25-m*M* HEPES, 50-m*M* NaCl, pH 7. Temperature, 25°. Sample contains polymer plus $CdCl_2$; blank, polymer plus a volume of buffer equal to the added $CdCl_2$ solution.

is rapidly oxidized, particularly in the presence of metal ions; therefore, great care is required to insure that all reagents and reactions vessels are oxygen-free.

Results

On addition of cadmium, the *N*-dihydrolipoyl aminoethoxy dextran absorption at 240 nm is strongly enhanced with no shift in the position of the maximum. No such enhancement is observed with the oxidized polymer (*N*-lipoyl aminoethoxy dextran). Figure 1 shows the change in absorption at 240 nm upon addition of increasing amounts of 2-m*M* cadmium chloride. The absorption change is linear until about 0.5 M of cadmium has been

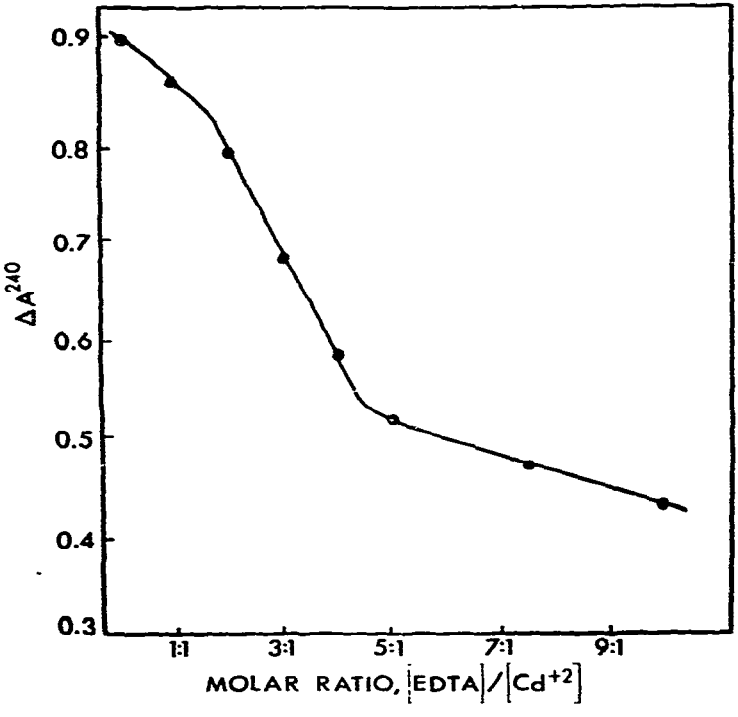


Figure 2(a). Difference spectrophotometric titration of cadmium-*N*-dihydrolipoyl aminoethoxy dextran with EDTA. Dithiol concentration, 115 μM; total Cd^{2+} concentration, 200 μM in 25-m*M* HEPES, 50-m*M* NaCl, pH 7. Blank contains an equivalent amount of cadmium-free *N*-dihydrolipoyl aminoethoxy dextran. Aliquots of EDTA were then added to both reference and experimental cuvettes.

added per mole dithiol. Beyond this point the slope changes until an end point is reached at 1 M cadmium per mole dithiol. Equilibrium dialysis under similar conditions indicates no detectable free cadmium in equilibrium with the polymer until the 1:1 equivalence point is exceeded. More than one cadmium-thiol complex appears to be forming and the binding constants are too large to be measured by equilibrium dialysis or direct spectral titration.

To determine binding constants for these strong complexes, it has been necessary to add a ligand capable of competing with the polymer for cadmium. Nitrilotriacetic acid (NTA) in tenfold molar excess is not effective in removing cadmium from the polymer. A similar concentration of EDTA removes more than 50% of the metal, indicating that the affinity of the thiol sites on *N*-dihydrolipoyl aminoethoxy dextran for cadmium is about an order of magnitude larger than that of EDTA. Figure 2(a) shows the spectrophotometric titration of cadmium-saturated *N*-dihydrolipoyl aminoethoxy dextran with increasing amounts of EDTA. The stability constant, K'_{PS}, for the cadmium-dithiol complex at neutral pH is defined by

$$K'_{PS} = \frac{[\text{PS-Cd}] \times [\text{EDTA}]}{[\text{PS}] \times [\text{EDTA-Cd}]} \times K'_{EDTA}.$$

Here K'_{EDTA} is the stability constant of the cadmium-EDTA complex at pH 7 and [PS] is the concentration of polymer binding sites. The avidity of the polymer for cadmium assures that the amount of cadmium bound to EDTA, [EDTA-Cd], can be determined from the total metal added and a comparison of the ΔA^{240} with the titration of Fig. 1. The concentration of free EDTA, [EDTA], is $[\text{EDTA}]_{total} - [\text{EDTA-Cd}]$. We assume that at saturation the amount of cadmium added equals the concentration of cadmium binding sites regardless of complex stiochiometry at lower metal concentrations. Thus [PS-Cd] can also be determined by reference to the standard titration (Fig. 1) and $[\text{PS}] = [\text{PS-Cd}]_{sat} - [\text{PS-Cd}]$. The data in Fig. 2(a) is used to determine these values and plotted in Fig. 2(b) as [PS]/[PS-Cd] vs [EDTA]/[EDTA-Cd]; the slope is K'_{EDTA}/K'_{PS}. The failure of the plot to intersect the origin is due to the presence of a slight excess of cadmium over polymer sites at the start of the titration. The change in slope upon the removal of cadmium from the polymer corroborates the suggestion from the direct titration that two types of complexes exist. From this analysis, values for the equilibrium constants for the two types of complex are: $K'_{PS(I)}$ (that at metal saturation) $= 7.7 \times 10^{13}\ M$; and $K'_{PS(II)}$ (that at lower metal to dithiol ratios) $= 2.7 \times 10^{14}\ M$.

Zinc-*N*-dihydrolipoyl aminoethoxy dextran has a different absorption maximum below 225 nm, but reliable spectrophotometric titrations are prevented by intense end absorptions of both polymer and buffer. In competi-

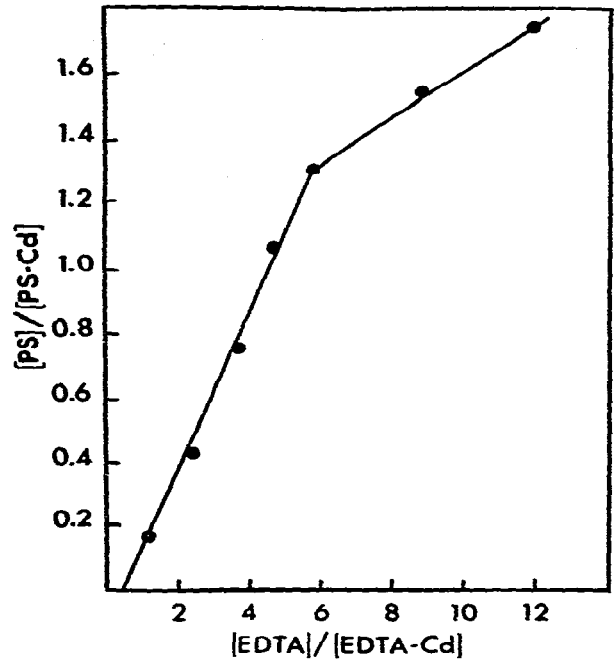


Figure 2(b). Data in Fig. 2(a) replotted as ratio of dithiol sites free, [PS], to those occupied, [PS—Cd], versus ratio of EDTA free, [EDTA], to EDTA-Cd complex, EDTA-Cd].

tion binding experiments, a fivefold molar excess of zinc displaces about 10% of the thiol-bound cadmium. Thus, zinc is bound between 2 and 3 orders of magnitude less firmly than cadmium by the polymeric dithiols. Higher concentrations of zinc, which would give more extensive displacement of cadmium and allow a more precise estimate of the relative binding affinity, can not be maintained in solution at pH 7.

To evaluate whether two types of dithiol-cadmium complexes occur with dithiol ligands free in solution, an attempt was made to study the stoichiometery of cadmium complexes with a low molecular weight dithiol, dithiothreitol. Addition of cadmium to dithiothreitol maintained at pH 7 by addition of sodium hydroxide, results in an insoluble complex and the liberation of 2.1 M of proton per M cadmium at 1:1 metal:dithiol ratio. There is no indication of any but a 1:1 adduct. It is also possible to titrate dithiothreitol spectrophotometrically with zinc. The absorbance change is linear to the end point at 1 M zinc per mole dithiothreitol. When titrated at constant pH, 1.78 M proton per mole zinc is liberated. In contrast to the

cadmium complex, zinc-dithiothreitol does not precipitate at pH 7. However, there is no indication that either zinc or cadmium form any complex other than a 1:1 adduct with dithiothreitol.

Arsenite Studies

When sodium arsenite is added to *N*-dihydrolipoyl aminoethoxy dextran, an ultraviolet difference spectrum can be obtained. Although it is devoid of any peak, the absorption increases with decreasing wavelength down to around 230 nm where buffer absorption becomes intense. A spectral titration at 240 nm results in a linear increase in absorption with arsenite up to a sharp end point at an arsenite-dithiol ratio of 1·1. There is no evidence for site cooperativity between dithiol centers as with cadmium. Cadmium and zinc binding occurs rapidly, and is always complete within the mixing time. In contrast, arsenite binding is observed to occur slowly and it is possible to measure the rate of complex formation. Figure 3 shows that the formation of the *N*-dihydrolipoyl aminoethoxy dextran-arsenite complex follows second order kinetics with respect to arsenite and polymeric dithiol sites. The rate constant derived from this data is 85 M^{-1} sec^{-1}.

A direct estimation of the binding affinity from spectral titration data is

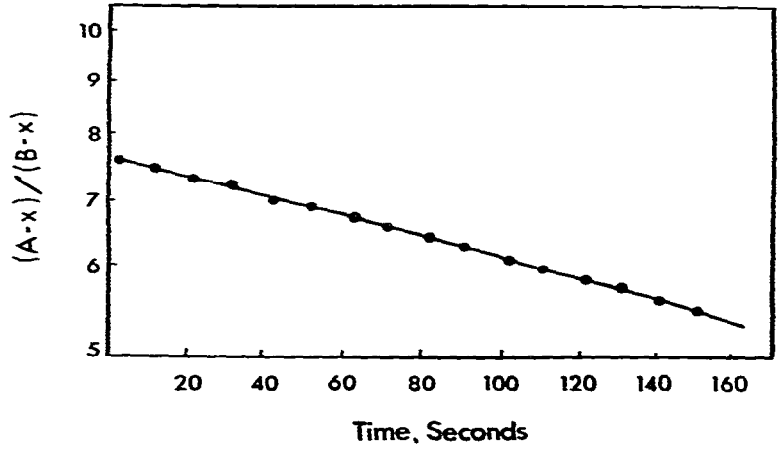


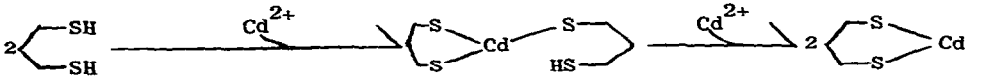
Figure 3. Rate of formation of *N*-dihydrolipoyl aminoethoxy dextran-arsenite complex. Initial dithiol concentration, A, was 1.52×10^{-4} M; initial arsenite concentration, B, was 1.97×10^{-4} M; the concentration of product, x, at time, t, is $x = \alpha_t E$, where α_t is absorbance (240 nm) at t and $E = 2.58 \times 10^3$ M^{-1} cm^{-1}, the extinction coefficient of the complex. Solutions were in 0.025-M HEPES, 0.05-M NaCl, pH 7. The reaction was run at 25°.

precluded by the sharp saturation of the titration curve implying a binding constant in the range of 10^6 to 10^7 M or greater. Experiments on arsenite and cadmium competition for the same sites establish that a thousand fold excess of arsenite will not displace cadmium from the polymer, while cadmium can slowly replace arsenite. If one assumes that the rate limiting step in the displacement of arsenite by cadmium is the dissociation of the arsenite complex, and that cadmium does not effect this rate, such data can be used to obtain the rate constant for the breakdown of the arsenite dithiol complex. This in turn permits a calculation of a rough binding constant since an estimate of the rate constant for complex formation is available. The first order rate constant estimated from the half time of displacement is 1×10^{-4} sec^{-1}. The dissociation reaction is not strictly first order or entirely independent of cadmium concentration, and may therefore reflect a contribution from ligand exchange processes. Nevertheless from these rough kinetic constants we can estimate an affinity constant of about 8.5×10^5 M. A similar dissociation experiment on dithiothreitol-arsenite complex yields a formation rate constant of 30 M^{-1} sec^{-1}, a dissociation rate constant of 7.7×10^{-4} sec^{-1}, and an estimated binding constant of 3.9×10^4 M. Although the kinetically determined binding constants are not completely independent of cadmium concentration they are useful in that they provide lower limits for the actual constants.

Since the experiments on cadmium displacement by arsenite will easily show a 5% replacement, the failure of a thousandfold excess of arsenite to displace any discernible amount of cadmium means that the binding constant for the two ions must differ by 5 to 6 orders of magnitude. Thus an upper limit on the arsenite-dithiol complex binding constant is between 10^8 and 10^9 M.

Discussion

The preparation of *N*-dihydrolipoyl aminoethoxy dextran has permitted the first direct determination of the stability of a cadmium-dithiol complex. At saturation the complex is a 1:1 adduct of cadmium and dithiol. At cadmium concentrations below about 50% saturation, the spectral data indicate a more complex situation with more than 2 thiols per cadmium involved in the complex. Assuming that the spectral increment per thiol coordination is equal to that observed at saturation, the stoichiometry at low metal ion concentrations approaches 3 thiols per cadmium. The following model most simply explains the titration data:



An equally satisfactory model would link all four sulfurs to the cadmium but with a reduced spectral increment for the intermediate complex. In view of the established propensity of cadmium to induce the formation of 3:1 binding sites [7], we favor the former model. The difficulty of explaining why the fourth sulfur appears not to be bound, may actually suggest that the binding is considerably more complex than the model proposed. Regardless of the exact formulation, cadmium- and polymer-bound dithiol systems can form at least two different complex species of similar stability.

When cadmium is added to dithiothreitol, proton titrations give no evidence for anything but a 2-thiol-per-metal complex. The formation of complexes characterized by 3 (or more) thiols per cadmium appears, at least for the present, to be a property peculiar to polymer-bound thiols. The synthesis of *N*-dihydrolipoyl glucosaminitol [8] was originally undertaken to obtain a soluble, low-molecular-weight analog of the polymer system; but the cadmium complex of this compound is insoluble, precluding meaningful studies at high dithiol to metal ratios.

Thiol complexes of cadmium and zinc are more stable than those with oxygen and nitrogen ligands, and the usual order of affinity, "zinc stronger than cadmium," is reversed for thiol complexes [12–14]. We have previously confirmed these facts for polymer-bound monothiols [7]. In such systems a polythiol macrocyclic chelate binds cadmium about 2 orders of magnitude more firmly than zinc. The same binding order and high selectivity are also seen with a dithiol-substituted polymer in which binding affinities (cadmium vs. zinc) differ by 10^2–10^3.

While the relative metal preferences are indistinguishable for mono- and dithiol-substituted dextrans, the actual binding affinities are considerably different. The close juxtaposition of two thiols in a preformed site enhances the complex stability by approximately 10^8 over the randomly substituted monothiol polymers where formation of a binding site must be induced by the metal. Therefore, from the actual stability of the cadmium complex it should be possible to discriminate a preexistent polythiol site from one generated by the addition of metal.

The ready reversal of cadmium inhibition by dithiols, but not by monothiols, is a commonly accepted criterion for an enzymatic dithiol [2]. Our studies provide chemical evidence for what, to now, has been only an intuitive understanding of this phenomenon. Dithiols bind cadmium *much* more strongly than do monothiols and would be expected to compete far more effectively for an enzyme-bound inhibitor. An understanding of the basic chemistry does not obviate the fundamental disadvantages of the "reversal by dithiol but not monothiol" criterion. As we have emphasized, there are no applicable data on the cadmium-complex stability of simple low molecular weight monothiols, such as mercapoethanol, traditionally em-

ployed by enzymologists. When polymer-bound and able to form polythiol sites, monothiol adducts with cadmium exhibit stability constants around 10^6 M; simple monothiols would form even less stable complexes. Therefore, at moderate concentrations, they could not complete effectively for even oxygen/nitrogen-bound cadmium in which case reversal by a dithiol is moot.

As the experiments with dithiothreitol and N-dihydrolipoyl glucosaminitol [8] vividly demonstrate, low molecular weight thiol-cadmium complexes are highly insoluble—even when the organic moiety is quite hydrophilic. Another, and perhaps more severe hazard, accompanies the use of thiols as inhibition-reversing reagents—thiols readily reduce protein disulfides and can exert independent effects on the enzyme.

We suggest an alternative reversal criterion which is not compromised by the problems attendent with thiols. From our measurements, we would expect cadmium inhibition of a preformed polythiol to be relieved by about a tenfold excess of EDTA, but not by an equivalent amount of NTA. The absolute affinity within the enzyme could differ from that in our models, but probably not by more than a factor of 10^2 in either direction. Thus reversal of cadmium inhibition by EDTA, but not by NTA, would suggest an enzymatic dithiol. Reversal by both EDTA and NTA would indicate either a weaker induced polythiol site, a sulfur-nitrogen, or an oxygen-nitrogen system. If EDTA failed to reverse the inhibition, a more complex preformed site such as the apparent trithiol site of metallonthionein [15] might be indicated.

Direct spectral titration of dihydrolipoyl aminoethoxy dextran with zinc indicates a 1:1 (metal:dithiol) complex is formed at metal saturation and this is confirmed by precipitation and elemental analysis of the complex. It is difficult to ascertain if higher order complexes are present at dithiol excess because of high solvent blanks at the absorption maximum of zinc thiolate. The binding affinities of the dithiol polymer system for zinc and cadmium can be compared by measuring the effect of zinc on the cadmium complex. Cadmium is bound approximately 500 times more firmly than zinc and the criterion that cadmium should form stronger complexes than zinc with dithiols is confirmed within the model.

Arsenite also forms a strong 1:1 complex with the polymer-bound dithiol and this reagent would also be expected to titrate a preformed enzymatic dithiol under the reaction conditions employed in the model system. No spectral evidence for arsenite binding to monothiol polymers can be observed and no interference by arsenite on the cadmium binding by these polymers can be detected. Arsenite does not appear to bind monothiols at levels usually employed in testing for enzyme dithiols. It is, therefore, a more specific reagent than cadmium for preformed dithiol functions. Be-

cause the arsenite dithiol binding is too strong to be evaluated by direct spectrophotometric techniques and no arsenite-sequestering agents of known affinity are available for competition studies, it has not been possible to determine the actual binding constant. However, several lines of evidence allow us to limit this value to within two orders of magnitude. The inability of a thousandfold excess of arsenite to displace even 5% of the cadmium from its complex with the polymer-bound dithiol sets an upper limit between 10^8 and 10^9 M. Under similar conditions cadmium completely displaces arsenite. These experiments were continued for several days, minimizing the possibility that displacement is kinetically rather than thermodynamically limited. On the other hand, the arsenite-dithiol binding constant must be greater than 10^6 since a lower value would result in curvature of the spectral titration and allow direct evaluation.

An indirect approximation of the arsenite binding constant for the polymer-bound dihydrolipoate residue can be made from the kinetics of complex formation and breakdown. This gives a value near 10^6 M. This is actually only a lower limit, as the rate constant for dissociation is not corrected for the small rate enhancement by cadmium. This "kinetic" binding constant should at least be within an order of magnitude of the true value.

Analogous kinetic experiments with arsenite and dithiothreitol give a rate constant for complex formation similar to that for the polymer-bound dihydrolipoate, a larger dissociation rate constant with more pronounced cadmium dependence and an indirect binding affinity between 10^4 and 10^5 M. The formation of the arsenite-dithiothreitol complex has also been studied by Zahler and Cleland [16]. Their indirect estimate of the formation-rate constant is about an order of magnitude larger than the value we have observed directly. This probably reflects differences in pH, reaction medium, and/or an overcorrection for monothiol in their calculations. However, their estimate of the binding constant is close to the lower limit estimated from the sharpness of the spectral titrations. We thus expect binding constants for arsenite-dithiol complexes to be in the vicinity of 10^6–10^7 M, and no greater than 10^9 M.

The most important aspect of arsenite-dithiol complex formation, relative to enzyme inhibition studies, is that it is quite slow. The sluggish reactivity of arsenite must be considered in any attempt to evaluate arsenite-inhibition as a dithiol criterion. Our results suggest that in static inhibitor studies, an arsenite preincubation period of less than 5–10 minutes would produce misleading results. A review of the literature of dithiol enzymes [1] reveals several ambiguous arsenite-inhibition studies; results which may be due to inadequate preincubation.

From this work and our earlier study [7], we can define what we consider valid enzyme dithiol criteria. The binding order "cadmium stronger than

zinc" has been established for both induced polythiols and preformed dithiols. The binding constant for the two metals differs by about two orders of magnitude in both systems. This binding order is not typical of other ligand systems. The use of cadmium and zinc is complicated by the ability of both metals to organize random monothiols into stable polythiol binding sites. Although the binding order is maintained—making this a diagnostic clue to polythiol ligands of all types—the relative affinity is many orders of magnitude below that for a preformed dithiol site. In the absence of other complexing agents cadmium and zinc should titrate the enzyme. The choice of assay conditions is critical since phosphate, citrate, and several other common buffer ions bind metal ions. Chelates of graded affinity for cadmium and zinc can provide information on the stability of an enzyme-metal complex and be of value in discriminating between induced and preformed polythiols. NTA should reverse inhibitions due to binding at an induced site; an excess of EDTA would be required to free a cadmium-blocked dithiol site; and EDTA would not be expected to easily remove the cadmium from a preformed trithiol center.

Arsenite appears to be relatively selective for preformed dithiols under conditions commonly used for enzyme inhibition studies and does not tend to induce polythiol centers. Thus arsenite can be used to titrate a dithiol enzyme with considerably less interference from other thiol proteins that would be possible with cadmium. However, experimental conditions must take into account the slow rate of arsenite-dithiol interaction. Further, enzyme-bound arsenite should be displaced by cadmium. It should be also noted that arsenite at high concentration has been reported [17] to inhibit xanthine oxidase, an enzyme unaffected by cadmium. This observation, attributed to formation of an arsenite-molybdenum complex, should be an adequate reminder that arsenite is not absolutely specific for dithiols. Cadmium and arsenite can be valuable reagents for detecting enzyme polythiol centers, but the use of multiple criteria and careful attention to details in both inhibition and reversal studies will be required.

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FOOTNOTE

[1] The following abbreviations are used: HEPES, *N*-2-hydroxethyl piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; NTA, nitrilotriacetic acid; PCMB, *p*-chloromercuribenzoic acid and its products in aqueous solution.

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