Contribution from the Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XL, U.K., and University Department of Medicine and Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow University, Glasgow G4 0SF, U.K.

Action of Sodium Gold(I) Thiomalate on Erythrocyte Membrane

W. Ewen Smith,*,† John Reglinski,† Shona Hoey,† Donald H. Brown,† and Roger D. Sturrock‡

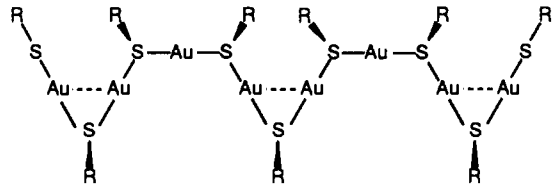
Received October 30, 1989

Reaction of sodium gold(I) thiomalate (myocrisin) with exofacial thiol groups on the membrane of intact erythrocytes was studied by resonance Raman spectroscopy using as a probe molecule 5,5'-dithiobis(2-nitrobenzoic acid) (ESSE). The method has the advantage that separate signals are observed for unreacted ESSE, for hemoglobin, and for the product of the reaction of ESSE with membrane thiols (ES^-). This enables both an internal calibration of the extent of reaction and a continuous monitor of the degree of lysis. The method gives an estimate of thiol concentration and can be used to follow reactions involving thiol site regeneration by the cell. With untreated erythrocytes, ESSE is gradually reduced to ES^- over a period of 4–6 h. Myocrisin-treated cells follow a similar pattern, with the exception that, between 1 and $1^3/_4$ h after treatment with ESSE, these cells take up previously released ES^-, temporarily reversing the apparent reduction process. The magnitude of the reversal is increased if glucose is added. ^1H NMR spectroscopy including ^1H spin-echo NMR experiments on lysate and whole cells indicates that the response of intracellular glutathione to myocrisin attack on the erythrocyte is to form the disulfide rather than a gold complex. A model to explain these results postulates both exchange with accessible surface thiols and the formation of gold–sulfur moieties deeply seated within the membrane-bound proteins of which the hexose transport protein is a dominant species.

Introduction

Gold compounds and sodium gold(I) thiomalate (myocrisin) in particular, have been used as therapeutic agents for the treatment of rheumatoid arthritis for many years.[1-3] They are among the few compounds that can arrest the progress of a chronic inflammatory process. Since these agents are toxic and are effective only with about 70% of patients treated, there is a need for a more specific and effective gold-based therapy. Consequently, the mechanism of action of compounds used currently is a question of practical and theoretical interest.

Myocrisin exists in solution in different forms depending on the method of preparation.[4] Elder[5,6] has examined one form using solution X-ray methods and concluded that it is a linear polymer with a centrosymmetric structure. It has a basic "A frame" unit with a short gold–gold distance and a thiomalate bridge. We have used an NMR titration similar to that of Sadler[7] in conjunction with gold analysis to show that our material has a formula of $Au_8Tm_9^{-8}$ (Tm = thiomalate), and we conclude that in solution the polymer can be diagramatically represented as



$Au_8(SR)_8$: RS = thiomalate

with charge compensation for the gold(I) provided by the thiomalate and sodium ions.

This molecule is readily substituted by other thiols such as cysteine[9] and reacts through exchange with disulfides.[8] Initial reactions are quite rapid, but complete breakdown of the polymer requires some time. For example, reaction of cysteine with myocrisin begins immediately, but the formation of the final products of thiomalate and insoluble gold(I) cysteinate polymer takes about 48 h at neutral pH.[9] Myocrisin reacts with albumin both by absorption of the whole molecule[10] and by reaction at the one active thiol site.[11] However, there are many other possible in vivo reactions with thiols as the gold distribution in plasma proteins illustrates[12] and it may be that there are also exchange reactions in vivo with available disulfide bonds.[8]

Although many enzymes are altered in activity by gold compounds[9] and there is some evidence that the selenium-containing enzyme glutathione peroxidase is involved,[13] no one site of action has been identified positively. It has been suggested that the gold(I) sulfur compound accumulations in vesicles in cells (aurosomes) may play a part[14] or that the gold deposits around the lysosomes (vesicle containing enzymes) may prevent enzyme release and so reduce pain and prevent tissue damage.[15] The latter hypothesis is attractive and may have a part to play in the action of gold, but current in situ in a cell electron microscope techniques only detect gold accumulations and are not sufficiently sensitive to identify chemically active gold moieties dispersed at a molecular level. Consequently, electron microscopy concentrates the theory on the less active deposit of polymeric gold sulfur compounds. Other theories of action of gold are predicted at a cellular level where the action of the compound on the immune system can be studied, but it has not been possible so far to link this immunological approach with the chemistry of the cell.

Table I. Sulfydryl Content of Exofacial Proteins in Erythrocyte Membrane[a]

band	mol wt	no. of thiols/cell	%	function
3	89 000	1.3×10^5	13	anion transport
4.5	40 000–70 000	6.2×10^5	63	hexose transport
6	36 000	1.1×10^5	11	unknown
7	32 000/34 000	1.3×10^5	13	antigens (two proteins)

[a] The exofacial surface is defined by the degree of penetration of the *N*-ethylmalamide radio label used to obtain this data. Depending on the relative permeability of myocrisin, the number of thiols available for binding, especially in the transport proteins, may be larger than predicted by this table.[19-23]

† University of Strathclyde.
‡ Glasgow University.

(1) Brown, D. H.; Smith, W. E. *Chem. Soc. Rev.* **1979**, *9*, 217.
(2) Shaw, C. F. *Inorg. Perspect. Biol. Med.* **1979**, *2*, 287.
(3) Sadler, P. J. *Struct. Bonding (Berlin)* **1976**, *29*, 171.
(4) Kean, W. F.; Lock, C. J. L.; Kassam, Y. B.; Rooney, P. J.; Rischke, J.; Nablo, L.; Buchanan, W. W. *Clin. Exp. Rheumatol.* **1984**, *2*, 321.
(5) Elder, R. C.; Eidsness, K. M. *Chem. Rev.* **1987**, *87*, 1027.
(6) Elder, R. C.; Ludwig, K.; Cooper, J. N.; Eidsness, K. M. *J. Am. Chem. Soc.* **1987**, *107*, 5024.
(7) Isab, A. A.; Sadler, P. J. *J. Chem. Soc., Dalton Trans.* **1981**, 1657.
(8) Reglinski, J.; Hoey, S.; Smith, W. E. *Inorg. Chim. Acta* **1988**, *152*, 261.
(9) Lewis, A. J.; Cottney, J.; White, D. D.; Fox, P. K.; McNeillie, A.; Dunlop, J.; Smith, W. E.; Brown, D. H. *Agents Action* **1980**, *10*, 63.
(10) Kamal, H.; Brown, D. H.; Ottaway, J. M.; Smith, W. E. *Analyst* **1977**, *101*, 645.
(11) Malik, N. A.; Sadler, P. J. *Biochem. Soc. Trans.* **1979**, *7*, 731.
(12) Brown, D. H.; Capel, H. A.; Smith, W. E. *Inorg. Chim. Acta* **1985**, *106*, L23.
(13) Baker, M. A.; Dillart, C. J.; Tappel, Al. A. *Drug-Nutr. Interact.* **1985**, *3*, 141–152.
(14) Elder, R. C.; Eidsness, M. K.; Heeg, M. J.; Tepperman, K. G.; Shaw, C. F.; Shaeffer, N. In *Platinum, Gold and other Chemotherapeutic agents*; Lippard, S. J., Ed.; ACS Symposium Series 209; American Chemical Society: Washington, DC, 1983; Chapter 20, pp 385–400.
(15) Persellin, R. H.; Ziff, M. *Arthritis Rheum.* **1966**, *9*, 57.

0020-1669/90/1329-5190$02.50/0

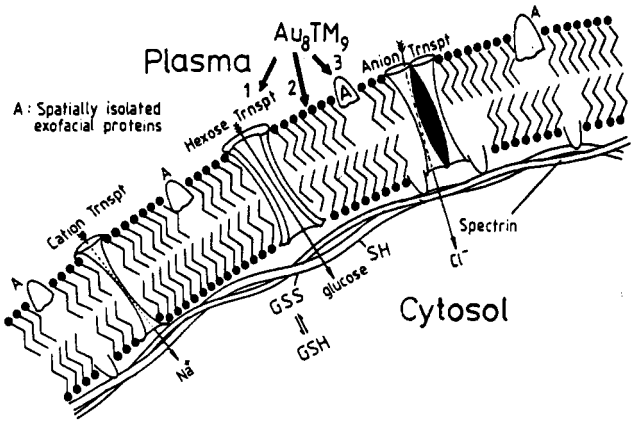


Figure 1. Simplified view of erythrocyte membrane indicating three of the possible targets for the gold drug myocrisin. Target 1 is the thiol on the hexose transport protein and is the largest source of free thiol. Target 2 indicates the possibility of adsorption of the molecule into the membrane and target 3 is one of the antigenic proteins. The anion transport protein is also a likely site for reaction. The conformation of the membrane surface is not reflected by the diagram.

With the use of modern NMR methods, the activity of myocrisin on cytosolic glutathione in the erythrocyte[16] has been studied and a direct interaction postulated. However, in a recent study of 12 patients treated with myocrisin for 12 weeks, the average whole cell gold concentration was 0.2 ± 0.1 μg/mL and the average cytosolic gold concentration was 0.004 ± 0.005 μg/mL.[17] Thus, a considerable percentage of gold is retained in the membrane of cells, and there is insufficient gold transferred to the cytosol for a stoichiometeric reaction with glutathione (0–8 μM Au to 2 mM glutathione). Cigarette smoking can increase cytosolic gold levels substantially,[18] but there is no evidence of any increased therapeutic advantage from smoking. The transport is believed to involve cyanide and thiocyanate. We have reported recently[19] that there is a link between thiophylic receptor sites on the exofacial surface of the erythrocyte membrane and the intracellular glutathione redox balance. This suggests that intracellular biochemistry can be controlled by stimulus of exofacial thiol groups.

Very little has been done to discover the nature or reactivity of the membrane-bound fraction of the gold despite the fact that the exofacial thiol groups are essential to cellular function and are almost inevitably targets for gold compounds (Table I). Figure 1 shows the possible major targets on the membrane for labile gold compounds from the plasma. Two of the sites indicated in the simplified diagram are thiol rich and are representative of groups of proteins that are either transmembrane, such as the hexose and anion transport proteins, or proteins on the exofacial surface at the antigenic sites.[20-23] The diagram also indicates that the adsorption of myocrisin or its metabolites into the membrane may be a feature of its chemistry.

In this study, we use resonance Raman spectroscopy to examine the effect of myocrisin on the active thiol sites present on the exofacial membrane of the intact erythrocytes. The erythrocyte was chosen as a model cell for a number of reasons. It enabled us to study cellular chemistry in the first instance without the need to consider phagocytosis, it is easily obtainable, the biochemistry is relatively well understood, and hemoglobin can be used as an indicator of interference due to lysis. This approach is interfaced with a reanalysis, using ^1H spin-echo NMR spectroscopy, of the cytosolic events that occur on treatment of the intact erythrocyte with myocrisin.[16] The study provides a link between the known chemistry of myocrisin and cellular biochemistry, and it may prove a useful model for further synthetic and metabolic studies with other cell types.

Experimental Section

All chemicals were commercially obtained unless otherwise specified.

The pH of 0.1 M Na_2HPO_4 buffer was adjusted to 7.4 by dropwise addition of concentrated hydrochloric acid. It was used to prepare stock solutions of 1 mM 5,5-dithiobis(2-nitrobenzoic acid), Ellman's reagent (ESSE), and myocrisin.

Heparinized blood samples obtained from healthy adults were centrifuged at 3000 rpm for 10 min. The plasma and white cells were removed by suction, and red cells were washed three times in freshly prepared phosphate buffer.

Resonance Raman Studies. Newly washed red cells were split into 3-mL aliquots, and an equivalent volume of 1 mM myocrisin solution was added. Samples were mixed by gentle inversion and allowed to stand at room temperture for 12 h.

The samples were then centrifuged at 3000 rpm for 10 min, the supernatant was discarded, and the red cells were washed five times with buffer to remove all unreacted or adsorbed material. The samples were then incubated with ESSE as previously outlined[19] and spectra recorded from the supernatant.

Heparinized whole blood, obtained from donors, was split into two aliquots, centrifuged, and separated as before. One aliquot of red cells was washed and incubated overnight in phosphate/glucose buffer, and the second aliquot, in phosphate buffer. Samples were then incubated with ESSE at 25 °C as previously described[19] and spectra recorded from the supernatant.

Each sample of red cells was split into 3-mL aliquots, and an equivalent volume of ESSE solution was added. Samples were mixed by gentle inversion and allowed to stand at room temperature for various lengths of time, ranging from 1 to 6 h. The samples were centrifuged as before, and the supernatant was drawn off with a Pasteur pipet.

Raman spectra were recorded from the supernatant on a Cary 81 Raman spectrometer modified by Anaspec Ltd. The system is equipped with a cooled photomultiplier and photon counter. A total of 100 mW of 457.9-nm radiation from a Spectra Physica 171-19 argon ion laser was used. The time taken to record a spectrum (1200–1500 cm^{-1}) is 60 s.

Nuclear Magnetic Resonance Studies. The isolated red cell pellet obtained as described above was washed once in a ^2H_2O, Na_2HPO_4 (0.125 M), and NaCl (0.154 M) (pH adjusted to 7.4) solution to facilitate oxygen uptake, thus reducing cell paramagnetism, and twice more in a ^2H_2O/NaCl (0.154 M) solution. A 0.4-mL aliquot of packed erythrocytes was placed in a 5-mm NMR tube with 0.1 mL of (^2H_2O) isotonic saline.

Spin-echo NMR spectra were recorded by using a Hahn spin-echo pulse sequence (90°–t–180°–t) with t = 60 ms. A Bruker (250 MHz) spectrometer was used to record all spectra. Samples were maintained at 20 °C during data collection, and the data from 2000 complete pulse sequences were accumulated for each whole cell spectrum. The free induction decay was collected in 2K of memory, zero filled to 16K. A 1 Hz line broadening function was applied during Fourier transformation. The 90° pulse was generated by using a 7.5 μs pulse width: acquisition time, 0.34 s; spectral width, 3000 Hz. A small presaturation pulse was applied to the water resonance prior to accumulation.

A complete description of the pulse sequence and spectral assignments can be found elsewhere.[16,19,24-26] Lysate was prepared by adding 1 mL of packed erythrocytes prepared for whole cell studies to 2 mL of deuterium oxide.

Spin-echo NMR studies of the lysate and in vitro glutathione studies were conducted in an manner analogous to studies of the whole cell spectra. However, these systems exhibit an increase in sensitivity. As such the free induction decay was collected in 8K of memory: acquisition time, 1.36 s. A total of 200 accumulations were obtained for each spectrum. The FID was not manipulated during Fourier transformation.

The conditions for the addition of gold to these solutions is described in the relevant figure captions.

(16) Otiko, G.; Razi, M. T.; Sadler, P. J.; Isab, A. A.; Rabenstein, D. L. *J. Inorg. Biochem.* **1983**, *19*, 227.
(17) Egilia, J. Ph.D. Thesis, University of Strathclyde, 1987.
(18) Graham, G. G.; Champion, G. D.; Haavisto, T. M.; McNaught, P. J. *Ann. Rheum. Dis.* **1981**, *40*, 210.
(19) Reglinski, J.; Hoey, S.; Smith, W. E.; Sturrock, R. D. *J. Biol. Chem.* **1988**, *263*, 12360.
(20) Haest, C. W. M.; Plasa, G.; Kamp, D.; Deuticke, B. *Biochim. Biophys. Acta* **1978**, *509*, 21.
(21) Haest, C. W. M.; Kamp, D.; Deuticke, B. *Biochim. Biophys. Acta* **1981**, *643*, 319.
(22) Haest, C. W. M.; Kamp, D.; Plasa, G.; Deuticke, B. *Biochim. Biophys. Acta* **1977**, *469*, 226.
(23) Abbott, R. E.; Schachter, D. *J. Biol. Chem.* **1976**, *251*, 7176.
(24) Brown, F. F.; Campbell, I. D. *Philos. Trans. R. Soc. London, B* **1980**, *289*, 395.
(25) Rabenstein, D. L. *Anal. Chem.* **1978**, *50*, 1265A.
(26) Rabenstein, D. L.; Nakashima, T. T. *Anal. Chem.* **1979**, *51*, 1465A.

Results and Discussions

In order to employ the resonance Raman method, a resonant chromophore is required and this was provided by incubation of the cells with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent—ESSE). This compound reacts with thiol to give a mixed disulfide and ESH in the first instance. ESH at neutral pH dissociates to give ES^-, a colored species that can be identified spectrophotometrically.[27]

$$RSH + ESSE \rightarrow RSSE + ESH \quad (1)$$

$$ESH \rightarrow ES^- + H^+ \quad (2)$$

RSH = glutathione or protein thiol

The advantage of detection by Raman spectroscopy is that ES^-, ESSE, and $(ESAu)_x$ species all give identifiable peaks in the same spectrum[8,19] and so can be addressed in the same experiment. ESSE, although impermeant,[28] is liable to cause cell lysis at high concentrations. Consequently, hemoglobin released can interfere with the standard UV–visible determination, and in addition, lysis may release unacceptably high levels of glutathione from the cytosol of the erythrocyte. With the resonance Raman method, a discrete peak due to hemoglobin (ν_4) is observed and estimates of lysis are routinely <1%. From a previous study[29] on the estimation of the thiol content of lysate, the relative intensity of the hemoglobin to thiol signal in lysate is known. Consequently, the degree of lysis can be continuously monitored, and a correction to the surface thiol concentration can be applied. Thus, by centrifugation of the cell suspension at appropriate times, resonance Raman spectra of the supernatant free of cells and therefore of hemoglobin from the intact cell, can be measured and used to estimate the ES^-/ESSE ratio and, through the ν_4 band of the hemoglobin, the extent of lysis. By this means the extent of reaction of ESSE with the cell surface can be estimated.

The thiol concentration on the exofacial membrane of erythrocytes arises mainly from proteins that are present in the membrane and that are involved in transport and immune functions[20-23] (Table I). The magnitude of the changes found in this study indicate that gold affects most of the available thiols, and since it is possible with the methods used to determine only the total thiol concentration, the changes observed to a large extent reflect the activity of the hexose transport protein, which accounts for 60% of the available thiol and is a proven receptor site for thiophillic activity.[19,30] In addition to the surface sites, the two transmembrane proteins are rich in sulfur and have a number of thiols and disulfide links throughout their length.[31,32] On incubation of the cell with a disulfide such as Ellmans reagent, an exchange reaction occurs (eqs 1 and 2). However, because the cell remains viable, the surface thiol can be regenerated by the cell (eq 3).[19] This latter process requires energy, and consequently,

$$\text{membrane-SSE} + 2H^+ + 2e^- \rightarrow \text{membrane-SH} + ESH \quad (3)$$

the rate and extent of reaction depends on the nutritional state of the cell among other factors. It would seem that in general the rate of this regeneration (eq 3) is slower than disulfide formation (eq 1) between membrane thiol and Ellman's reagent.[33]

Ellman's anion (ES^-) has an absorption band closer to the 457-nm emission from the laser than has ESSE, and consequently, it is closer to resonance and gives a larger signal per molecule.

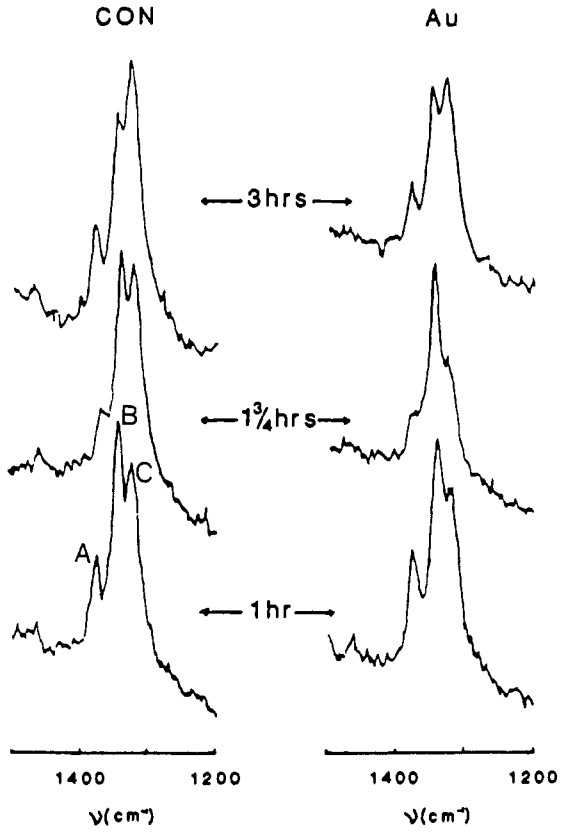


Figure 2. Raman spectra of erythrocytes following 12 h of incubation with myocrisin and subsequent treatment with ESSE (Au). Controls (Con) were obtained from a second aliquot of the same red cell sample treated in an identical manner except that no myocrisin was added. The effect is more pronounced at $1^3/_4$ h.

Signals of equal intensity mean a ratio of ES^- to ESSE of about 1:5.[29] The lower intensity of the ESSE signal compared to ES^- means that an excess of Ellman's reagent can be used, and the signal from it will vary more slowly with thiol concentration changes than does that from ES^-. Thus, a calibration graph that uses ESSE as internal calibrant has only a slight curvature.

The resonance Raman spectra from the supernatant of a suspension of erythrocytes incubated with ESSE illustrates these points (Figure 2, control). Peak C arises from ES^-, peak B from ESSE, and peak A from hemoglobin (ν_4). The hemoglobin peak is about 20% larger that the ES^- in a lysate, and so an estimation of the degree of lysis can be made and the ES^- peak height corrected before measurement of the ES^- to ESSE ratio. The initial spectrum, which is similar to that found at shorter incubation times, represents the number of thiols per cell. A longer period of incubation causes an increase in thiol concentration as sites are regenerated and become available for further reaction. The magnitude of the hemoglobin peak indicates that the ES^- peak is not due to lysis releasing intracellular glutathione but arises from interaction with the surface thiol.

To study the effect of myocrisin on the sulfydryl status of the membrane, the cells were incubated with compound for 12 h and then thoroughly washed to remove any free myocrisin or metabolites such as free thiomalate (Figure 2). They were then incubated with Ellman's reagent as before. A complex series of reactions is likely to occur, involving thiol exchange with possible release of thiomalate. Consequently a second type of control experiment incubating with thiomalate instead of myocrisin was used to distinguish the thiomalate control from the buffer only control (Figure 3). The thiol content in this control experiment, even after copious washing to remove excess thiomalate, was higher with a more rapid production of Ellman's anion than that obtained from the untreated cells. This suggests that thiomalate either exchanges with disulfide sites or absorbs into the membrane and is released slowly to provide the additional thiol.

(27) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.
(28) Famey, J. P.; Whitehouse, M. W. *Biochem. Pharmacol.* **1975**, *24*, 1609.
(29) Banford, J. C.; Brown, D. H.; McConnell, A. A.; McNeil, C. J.; Smith, W. E.; Hazelton, R. A.; Sturrock, R. D. *Analyst* **1982**, *107*, 195.
(30) Reglinski, J.; Smith, W. E.; Sturrock, R. D. *Magn. Reson. Med.* **1988**, *6*, 217.
(31) Mueckler, M.; Caruso, C.; Baldwin, S. A.; Panico, M.; Blench, I.; Morris, H. R.; Allard, W. J.; Leinhard, G. E.; Lodish, H. F. *Science* **1985**, *229*, 941.
(32) Jay, D.; Cantley, L. *Annu. Rev. Biochem.* **1986**, *55*, 511.
(33) Chilles, C.; Mulheron, M.; McCrae, F.; Reglinski, J.; Smith, W. E.; Brezski, M.; Sturrock, R. D. *Ann. Rheum. Dis.* **1990**, *49*, 668.

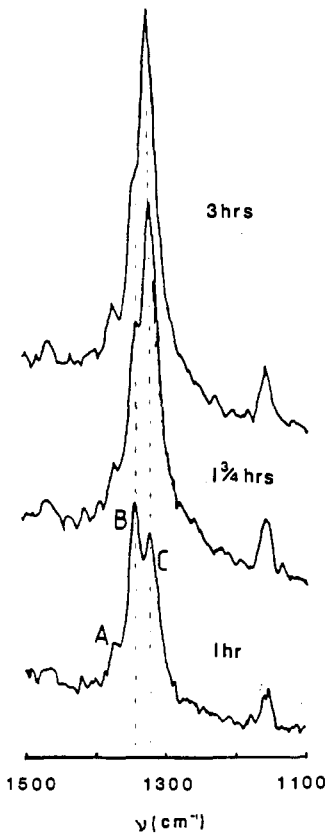


Figure 3. Raman spectrum of erythrocytes following 12 h of incubation with thiomalic acid and subsequent treatment with ESSE.

With myocrisin, an initial thiol level similar to untreated cells is observed, but after $1^3/_4$ h the signal from ES^- is significantly reduced (Figure 2). No similar reduction is found at 1 or 4 h. In the absence of myocrisin, erythrocytes incubated with glucose prior to treatment with Ellman's reagent produce a higher and more active sulfydryl population.[34-37] This experiment has been repeated a number of times and the reproducibility of an estimation of this type is better than 5%,[29] and consequently, the difference between the controls and the treated samples is significant.

However, when the experiment was repeated in a glucose solution, the rate of SH production was greater and the degree of reduction of the ES^- signal by myocrysin at $1^3/_4$ h was smaller (Figure 4). These results indicate that the gold moiety present in the membrane as a specific effect on the biochemistry of the intact cell. Furthermore, a broad link is established between myocrisin activity, glucose metabolism, and the hexose transport protein. This is not surprising in that the hexose transport protein is the major (60%) resevoir of thiol on the erythrocyte surface (Table I), the thiol is essential for transport, and there is an increase in the exposed thiol concentration by two per protein on binding sugars.[34-37] The ribbon structure of myocrisin may allow the hexose transport protein to accommodate either myocrisin or its polymeric metabolites. Indeed, the Raman experiments indicate a direct chemical reaction with the thiols and disulfides in the protein.

The resonance Raman results discussed above clearly target the membrane sulfydryl population as a major site of action for both Ellman's reagent[19] and myocrisin. This observation for myocrisin is at variance with the ^1H spin-echo NMR study reported by Otiko et al.,[16] where the data is interpreted in terms of membrane transport and a subsequent reaction between glutathione and the gold compound. Furthermore, a ^1H spin-echo NMR study by our group on the action of Ellman's reagent on the erythrocyte clearly indicates that the membrane thiol is the target for the aromatic disulfide.[19] As a result, it was decided to reinvestigate the action of myocrisin on the intact erythrocyte by using ^1H spin-echo NMR spectroscopy.

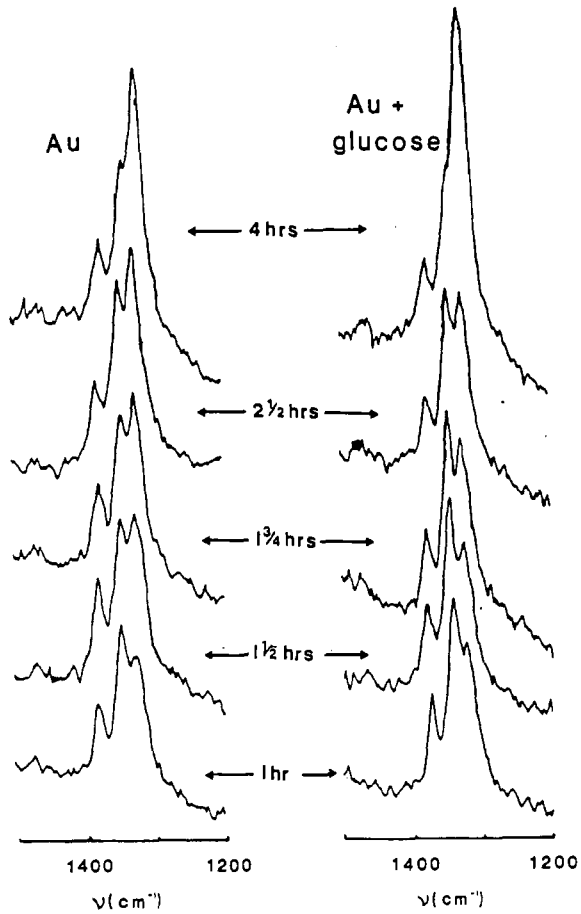


Figure 4. Effect of glucose on the sulfydryl group activity. There is a greater degree of lysis in this sample, resulting in a higher thiol level at $1^3/_4$ h in both samples.

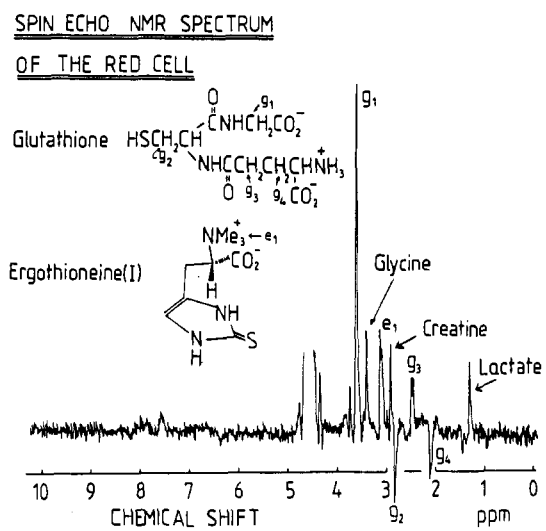


Figure 5. 250-MHz ^1H spin-echo NMR spectrum of the intact erythrocyte.

Glutathione is the dominant non-protein thiol in the cytosol of the human erythrocyte and is clearly identifiable by ^1H spin-echo NMR spectroscopy (Figure 5).[16,19,24-26,30] Model studies on the reaction of glutathione and myocrisin in aqueous solution show that exchange reactions occur whereby glutathione replaces thiomalate in the polymer and that the polymer subsequently

(34) Pinkofsky, H. B.; Jung, C. Y. *Arch. Biochem. Biophys.* **1985**, *240*, 94.
(35) Abbot, R. E.; Schachter, D.; Batt, E. R.; Flamm, M. *Am. J. Physiol.* **1986**, *250*, C853.
(36) Batt, E. R.; Abbott, R. E.; Schachter, D. *J. Biol. Chem.* **1976**, *251*, 7184.
(37) Jung, C. Y. *J. Biol. Chem.* **1974**, *249*, 3568.

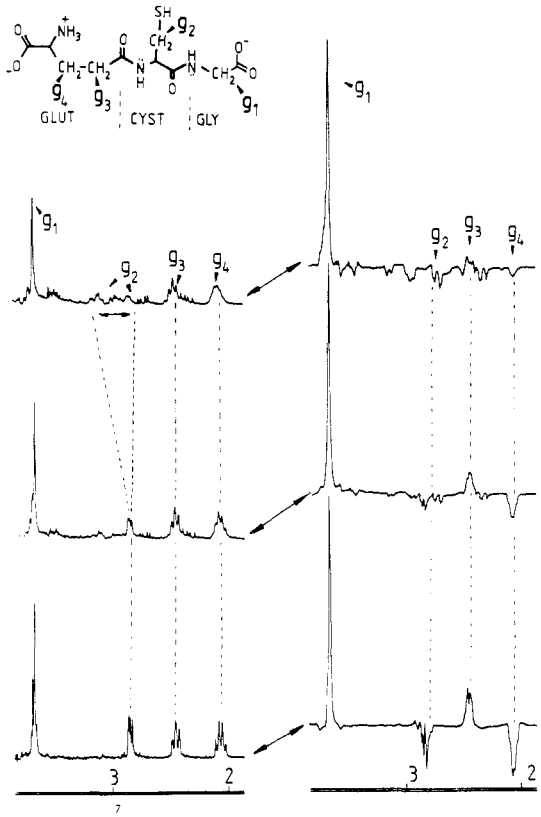


Figure 6. Titration of glutathione (0.033 M) with myocrisin solution (0.025 M) (a) in the standard FT NMR made and (b) in the spin-echo mode.

degrades,[7] forming smaller polynuclear gold clusters and perhaps some mononuclear gold species.

$$Au_8Tm_9 + \text{excess GSH} + H^+ \rightarrow 8Au(SG)_x + 9TmH \quad (4)$$

GSH = glutathione

$1 \ll x < 2$: excess charges omitted for clarity

This reaction can be studied by NMR methods (Figure 6). As myocrisin is titrated into a glutathione solution, ligation occurs via the thiol function on the cysteinyl residue. The standard FT NMR resonance of the g_2 ($-CH_2-$) β-cysteinyl residue changes from an asymmetric quartet into a much larger group of lines that are dispersed (δ 2.8–3.2) to a greater extent by the proximity of the metal center. The two resonances derived from the glutamyl residue of the tripeptide are also affected, but to a smaller extent.

The interpretation of the whole cell and lysate spectra is facilitated by repeating the above experiment in the spin echo mode (Figure 6). The changes observed in the resonance from glutathione manifest themselves as changes in the signal intensities of the respective g_2 ($-CH_2-$, β-cysteinyl), g_3, and g_4 (glutamyl residue) resonances, where binding of the gold center to the thiol would seem to shorten the T_2 value of the g_3 and g_4 resonances by a measurable extent. In contrast however, the g_1–glycyl residue remains relatively unchanged. This may result from the folding of the tripeptide in the vicinity of the metal. Red cell lysate, although a complex mixture, still contains glutathione as a major component. Also present in this complex mixture are hemoglobin and ergothioneine, both of which have reactive sulfur centers and would normally be expected to react with myocrisin. However, it is known that, in terms of reactivity, glutathione is dominant among these components,[38] with ergothioneine being the least reactive. Reactions of gold with hemoglobin are unlikely to be observed in these experiments.

Titrating gold into red cell lysate (0.5 mL; 1:2 v/v cells; ^2H_2O lysis) produces a series of spectra (Figure 7), which are in general agreement with the model study discussed above (Figure 6). As myocrisin is introduced into the mixture, the glutathione resonances g_2, g_3, and g_4 respond in characteristic fashion. The g_3 and g_4 (glutamyl residues) diminish in signal intensity whereas the g_2 signal is dispersed. As such the reactivity of myocrisin in

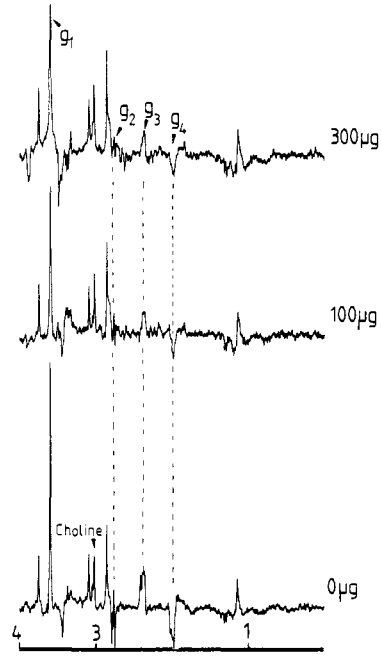


Figure 7. Titration of red cell lysate (0.5 mL; 1:2 v/v erythrocyte/^2H_2O) with myocrisin solution (0.025 M). Prior to the addition of myocrisin the choline/g_4 intensity = 1.26; post myocrisin addition choline/g_4 intensity = 2.75.

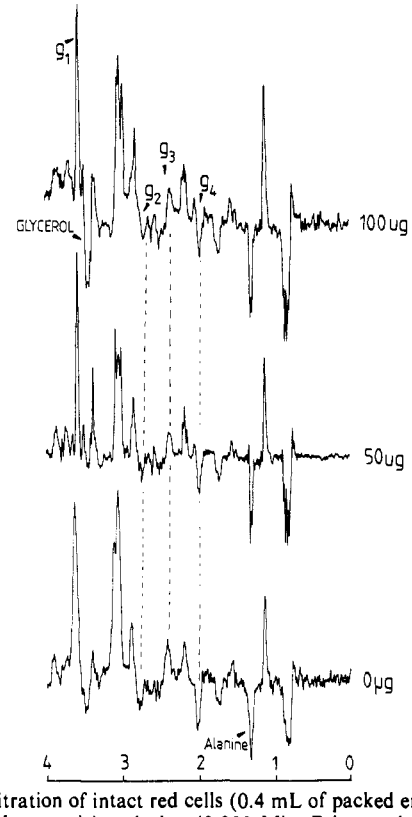


Figure 8. Titration of intact red cells (0.4 mL of packed erythrocyte in 0.5 mL) with myocrisin solution (0.033 M). Prior to the addition of myocrisin the alanine/g_4 intensity is 1.84; post myocrisin addition alanine/g_4 – 1.64. Cell lysis would seem to affect the relative concentrations of the cytosolic components. To this effect, major differences are observed in the *relative* cytosolic concentrations of the amino acids alanine and valine in the intact cell (above), as compared to the cell lysate (Figure 7).

(38) Reid, R. S.; Rabenstein, D. L. *J. Am. Chem. Soc.* **1982**, *104*, 6733.

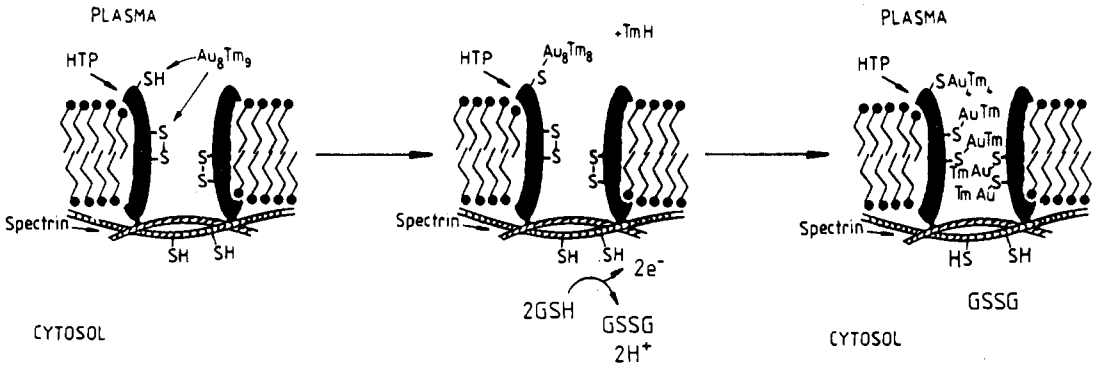


Figure 9. Illustration of one mode of action of myocrisin on the membrane. The protein illustrated is a simplified model of the hexose transport protein, showing only one exofacial thiol to illustrate likely sites for gold addition. Such changes would effect protein function; for example, in the second stage of the reaction, some of the internal protein disulfides are reduced to thiolates (as ligands).

lysate is identical with that of the model discussed above and suggests that one product is a gold glutathione complex. The end point of the titration lies somewhere between 200 and 300 μg of myocrisin (1.0–1.5 mM) for an initial intact cell volume of 0.15–0.20 mL (equivalent to 0.6–0.8 mM glutathione).

The addition of myocrisin to a suspension of intact erythrocytes produces a different pattern of reactivity (Figure 8). The myocrysin acting at the membrane proteins (Figure 9) causes an indirect and multiplicitive effect on the redox chemistry of the cytosol. Consistent with data reported by Otiko et al.,[16] the g_2 (β-cysteinyl) residue responds to the presence of the gold compound but the g_3 and g_4 (glutamyl) resonances do not. This suggests that a gold–glutathione complex is not forming in the cytosol in an analogous manner to the lysate and model system discussed above. A diminition of the g_2 resonance in the ^1H spin-echo NMR spectrum independent of g_3 and g_4 is normally regarded as characteristic of glutathione oxidation in the cytosol.[24,30,39-43] This process is normally accompanied by a reduction in the signal intensity of the g_1–glycyl residue. The signal broadening associated with this process cannot easily be discerned in this system due to the inhomogeneity of the mixture. Thus, it would seem that the best interpretation of these observations is that myocrisin stimulates oxidation of intracellular glutathione as does penicillamine[41] and ESSE.[19]

The oxidation of the cytosolic glutathione pool does not require that the two redox-active species, myocrisin and glutathione, be in the same chemical environment. Previous studies[19,30,41,43] have shown that the membrane can mediate in redox processes. This mechanism will be elaborated on below. Two other factors support the interpretation that the membrane is mediating these reactions. The rate of transport of myocrisin across the membrane is slow.[44] Consequently, the kinetics of the reaction with glutathione, being rate limited by transport, would also be expected to be slow. This is not the observed pattern. The response of glutathione to myocrisin in the extra cellular fluid is rapid ($t_{1/2}$ < 15 min), suggesting that transport is not required. However, this does not rule out that slower processes associated with transport may occur in vivo. The response observed in the whole cell spectra is inconsistent with the lysate experiment insofar as appreciable diminuition of the g_2 (β-cysteinyl) resonance is observed in the whole cell system at levels of myocrisin (50 μg of myocrysin (0.25 mM)/0.4 mL of packed erythrocytes) much too low, even assuming complete transport and no other reaction, to completely exchange with the entire cytosolic pool of glutathione. The cytosolic response observed is roughly equivalent to a 10-fold magnification of the applied chemical stress. This magnification of response is observed in other systems where the membrane is the target of the chemical agent.[19] As such, we suggest that consistent with the resonance Raman data, myocrisin is reacting at the membrane.

Proposed Mechanistic Interpretation

Myocrisin is observed by resonance Raman spectroscopy to react at the thiol and disulfide sites on the exofacial surface of the membrane proteins. From in vitro experiments, two main types of reaction should occur on the protein. With available thiols, an exchange reaction will occur.

$$\text{membrane–SH} + Au_8Tm_9 \rightarrow \text{membrane–S–}Au_8Tm_8 + TmH \quad (5)$$

This reaction can proceed further if further thiol is available until ultimately the gold polymer is used up. However, with small thiol molecules, the rate of reaction with the terminal groups is fast, occurring within a few minutes whereas the reaction to completely break up the myocrisin molecule takes some time, (for example 48 h to form an insoluble gold cysteinate complex). With proteins, where there will be steric restrictions and problems with availability of additional thiol, these reactions would be expected to be slower.

Myocrisin can also react with disulfides in proteins[8] primarily by exchange at the terminal Au–Tm moieties.

(6)

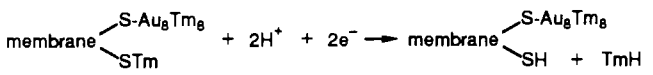
In model experiments with ESSE and lipoic acid, this type of reaction occurs within a few minutes. Subsequent reactions then take place in which the polymeric myocrisin molecule is further broken down, but the time taken for complete breakdown varies from a few hours with ESSE to about 2 weeks with lipoic acid.[8] Most NMR titrations[7,8] are carried out on a short time scale and show bound and unbound thiomalate even with excess thiol. The latter produces dithiomalate as an end product in contrast to ESSE, which produces a mixed disulfide, the former being a better model for protein reactivity.

$$2Au_8Tm_9 + 17\text{ESSE} \rightarrow (16/x)(\text{Au SE})_x + 18\text{ESTm}$$

$$2Au_8Tm_9 + 16n(\text{lipoic acid}) \rightarrow (16/n)(\text{AuLipoic})_n + 9Tm_2 \quad (7)$$

The exchange process (eq 5) does not entail any redox behavior on the part of the sulfydryl functions in the membrane proteins, and subsequently, a stimulus to the glutathione system in the cytosol would not be expected (in contrast to Ellman's reagent[19]). However, the insertion mechanism (eq 6) is effectively a reductive process where the sulfur in the membrane disulfide changes oxidation state in becoming a thiolate ligand to gold (eq 6). Thus in vivo new disulfides and gold thiolate species are generated. However, it appears that the former is more likely to be reduced by reactions analogous to that of eq 3. For example

(39) Brown, F. F.; Campbell, I. D.; Kuchel, P. W.; Rabenstein, D. L. *FEBS Lett.* **1977**, *82*, 12–16.
(40) Rabenstein, D. L.; Brown, D. W.; McNeil, C. J. *Anal. Chem.* **1985**, *57*, 2294.
(41) McKay, C. N. N.; Brown, D. H.; Reglinski, J.; Smith, W. E.; Capell, H. A.; Sturrock, R. D. *Biochim. Biophys. Acta* **1986**, *888*, 30.
(42) Butler, A. R.; Calsy-Harrison, A. M.; Glidewell, C.; Johnson, I. L.; Reglinski, J.; Smith, W. E. *Inorg. Chim. Acta* **1988**, *151*, 281.
(43) Reglinski, J.; Smith, W. E.; Belch, J. J. F. *J. Chem. Res. Miniprint* **1989**, 316.
(44) Lewis, D.; Capell, H. A.; Smith, W. E. *J. Rheumatol.* **1984**, *11*, 111.



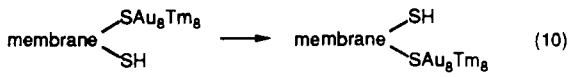
(8)

The electrons for the process are ultimately provided by the glutathione in the cytosol, where, as has been shown above by using ^1H spin-echo NMR, the thiol is oxidized (Figure 9) as a result of myocrisin addition

$$2GSH \rightarrow GSSG + 2H^+ + 2e^- \quad (9)$$

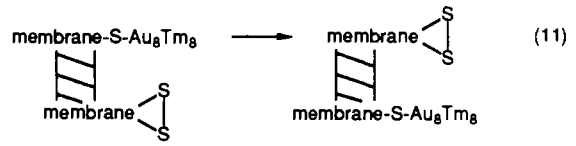
The gold moiety on the other hand cannot be removed unless another suitable ligand is available to exchange with the membrane thiolate and coordinate with the metal. Thus the likely explanation of the reduction in Ellman's anion signal (Figure 2) is that the ES^- is adsorbed by the cells through ligation with the gold species on the membrane.

The 12-h incubation with myocrisin is sufficiently long for exchange to occur down into the protein through the large numbers of thiols and disulfides present, and consequently, subsequent reactions of a type alluded to above may occur during the incubation process. For example, by simple exchange with a thiol



(10)

or by further reaction with a disulfide

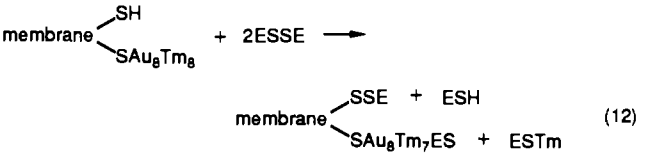


(11)

We have restricted our argument to exchange with the terminal sites on the myocrisin polymer, as it is clear[8] that this is the most rapid process. However, polymer breakdown could also occur.[7,8] The extent, depth, and detail of such reactions require further study, but it seems likely that some deep-seated gold sites will be created and that the polymer will not always be completely destroyed. Given that the overall rates of reaction are on a time scale similar to those for untreated cells, it seems likely that the reactive surface sites dominate the processes observed. Further, exchange reactions between disulfides and myocrisin release no free ES^-. Thus the observation that the initial ES^- concentration is similar to that in untreated cells suggest, regeneration of the active surface thiols possibly during gold incubation and certainly prior to the first measurements.

A simplified explanation for the dominant processes occurring at the cell surface would be that the protein in its initial form has both thiols and disulfides in its structure. Myocrisin can react with the exposed sulfydryl functions (eq 5) and migrate via exchange reactions to the more deep-seated sites, especially if polymer breakdown occurs. This would remove a number of thiols from the available pool capable of reacting with Ellman's reagent. However, interaction of myocrisin with disulfides (eq 6) and subsequent reduction of the thiomalate mixed disulfide formed (eq 8) will generate what is effectively a new thiol site on the membrane.

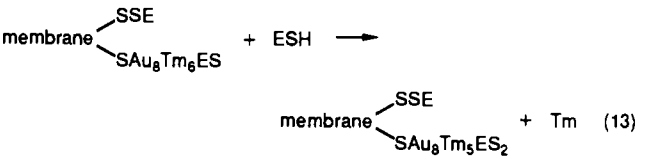
This explains why approximately the same thiol concentration is initially found in treated and untreated cells. On the addition of Ellman's reagent, the initial fast reactions would be thiol disulfide exchange and an exchange of the terminal thiomalate at the myocrisin site; it should be remembered that although complete exchange of thiols with myocrisin is slow, exchange at the terminal ends of the polymer is rapid. The initial reaction would be of the type



(12)

From in vitro experiments, both reactions would be expected to be complete in less than 30 min, but clearly kinetics associated with membrane structure could vary the rate.

The ESH produced initially is released into solution, and will be detected as ES^- but can be effective in further breaking up the polymer. At no time is $(ESAu)_x$ or a related ESAu species detected, so that no such species is released into solution at any stage. The likelihood is that the attack of ES^- on the polymer results in complex exchange and redox reactions. On the basis that thiol exchange is fast compared to regeneration, it is likely that after $1^3/_4$ h one common form of the hexose protein is that with a thiol exchanged to a disulfide and the terminal thiomalate of a complexed myocrisin moiety. Such a reaction with ESH could involve insertion of ES^- into bridging positions.



(13)

There is some evidence that the ESAu species are polymeric. If the ES^- species splits the polymer, it need not be released since it may bond to other thiols or disulfides or may be adsorbed in the protein or membrane. This reaction removes ES^- from solution, reducing the signal in the cytosol. It creates a thiol in a more deep-seated site that can undergo further exchange or eventually generate more ES^-, but it would account for the temporary reduction in ES^-.

Thus, initially Ellman's reagent reacts with the available thiol over a period of 15–30 min. A slower reaction taking about 1–2 h involves ES^- attack on complexed gold, and consequently, a loss of ES^- from the supernatant then takes place. Further, over a period of several hours, the slower cell regeneration process takes over. These time scales are consistent with in vitro experiments.

Thus the largest fraction of cellularly bound gold is in the membrane, but little to no attention has been paid to the action of this fraction in therapeutic studies despite the belief that gold compounds act at a cellular level to mediate the immune response. The action of gold compounds on thiol sites in different cell types remains a problem for the future, but this study shows that gold compounds do act on the functional thiols on the membrane surface and mediate cellular metabolism (Figure 9). This type of behavior may help to explain the fact that many cellular enzymes are affected by gold drugs but as yet no one target for specific action has been identified. It is perhaps significant that in a study of gold and platinum compounds of therapeutic interest, the cytosolic concentrations of small molecules in HeLa[45] cells were affected by the gold compounds and not by the platinum ones. This suggests a role for gold in the leukocyte membrane that is not shared with platinum and that mediates cellular activity. Thus, this paper opens up the possibility of a new target for drug research, namely compounds modeled to react with specific active thiols in the functional proteins of cell membranes.

Registry No. Myocrisin, 12244-57-4; glutathione, 70-18-8.

(45) Reglinski, J.; Smith, W. E. Unpublished results.