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Direct Detection of Homocysteine

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At elevated levels in plasma, homocysteine (Hcy) is a risk factor for Alzheimer's[1] and cardiovascular diseases.[2] Methods for the direct detection of Hcy are hampered due to interference from common, structurally related molecules such as cysteine (Cys) and glutathione (GSH). Hcy analyses are thus performed in conjunction with chromatographic separations or immunoassays.[3] As part of our program aimed at developing convenient methods for the selective detection of colorless biomolecules,[4] we herein report the determination of Hcy using inexpensive, commercially available materials. We apply one of our new techniques toward assaying Hcy in human blood plasma.

Hcy has been reported to inhibit the oxidation of luminol and dihydrorhodamine by strong oxidants.[5] Hcy also rapidly reduced ferrylmyoglobin to metmyoglobin.[5] In a comparative study of the ability of GSH, Cys, and Hcy to reduce dehydroascorbic acid (DHA), Hcy promoted much more significant reduction of DHA than Cys and GSH. Furthermore, the reduction occurred at Hcy concentrations that were over an order of magnitude smaller than those of GSH and Cys.[6] Hcy can thus function as a relatively potent reducing agent, although it is also believed to be a causative agent of oxidative stress.[7] The understanding of hyperhomocysteinemia and its associated pathogenicity continues to be of significant current interest.[7]

A key feature of the chemistry of biological thiols is the delicate balance between their oxidizing and reducing functions. Oxidizing thiyl radicals can rapidly equilibrate to reducing, captodative α-amino carbon-centered radicals under physiological, aerobic conditions.[8] Additionally, reducing disulfide radical anions rapidly decay to the reducing α-aminoalkyl radicals.[8] The equilibria involved in the free radical chemistry of biological thiols are pH-dependent and include several radical and recombinant species.

The dication methyl viologen (MV^{2+}) has been previously used as an oxidant during a detailed investigation of the equilibrium kinetics of both the reducing disulfide and the α-amino carbon-centered radicals derived from Hcy, Cys, and GSH.[8] Reducing radical formation was monitored via changes in the UV−vis spectra of solutions containing the methyl viologen radical cation which formed in the presence of the biological thiols.[8]

Importantly, it was surmised that the formation of the reducing α-aminoalkyl radical derived from Hcy should be particularly favorable.[8a] This was attributed to an intramolecular hydrogen abstraction mechanism which involves a five-membered ring transition state (Figure 1).[8a] In contrast, in the cases of Cys and GSH, H-atom abstraction to afford a reducing carbon-centered radical would proceed via less-favored four- and nine-membered ring transition-state geometries, respectively.[8a]

It should thus be possible to develop conditions for the selective detection of Hcy.[9] We find that upon heating colorless solutions of MV^{2+} (4.0 mM) at a very gentle reflux (5 min, pH 7.5, 0.1 M tris buffer, 100% H_2O, 17 mM aminothiol), visual signaling selective for Hcy is observed (Figure 2). The color formation can be monitored via the appearance of absorptions at 398 and 605 nm.[10]

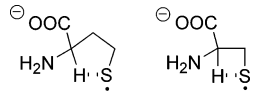


Figure 1. Proton abstraction leading to formation of the α-aminoalkyl radical from the thiyl radical of Hcy (left) and Cys (right).

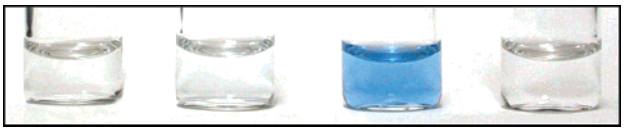
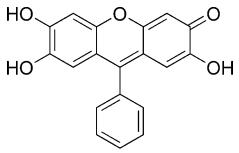


Figure 2. Selective color change in response to Hcy in solutions of MV^{2+}. Left to right: no added analyte, cysteine, homocysteine, and glutathione.

This facile thermal method may be applicable to our recently reported postcolumn HPLC detection system in which analytes are heated in a reactor containing a selective indicator.[11] We also can detect Hcy at room temperature in a fashion more amenable to direct bioassays. The alternative technique employs fluorone black (**1**).



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Upon addition of thiols (Hcy, Cys, GSH, 100 μM) to a solution of **1** (10.0 μM) in 70% MeOH/H_2O (phosphate buffer, H_2O, pH = 7.3), an increase in absorbance occurs at 510 nm in the UV−vis spectra at room temperature (analysis after 5.0 min).[10] The absorbance increase is greatest for Hcy compared to that for equimolar amounts of other biothiol analytes.[10] Amino acids such as L-alanine, L-arginine, L-glutamine, L-glycine, L-lysine, L-methionine, L-serine, and L-threonine do not produce detectable spectral changes compared to solutions of **1** without analyte.[10]

Our findings suggest a process whereby **1** is involved in the redox chemistry of the thiols. ^1H NMR studies show that Hcy conversion to homocystine (disulfide of Hcy) is enhanced in the presence of **1**.[10] Additionally, the MALDI mass spectrum of products formed in a solution containing **1** and Hcy exhibits prominent peaks for glycine sodium salt and the disodium and dipotassium salts of a glycine-derived dimer.[10,12] Glycine and its dimerization products are termination products of α-amino acid carbon-centered radicals.[8b,13]

Thiol analysis in biological fluids typically requires disulfide reduction. This is often accomplished by using phosphines. Since disulfide radicals (vide supra) are involved in the reduction of MV^{2+},[8] we propose that PPh_3 may inhibit biothiol chemistry, leading to greater colorimetric selectivity.[14]

When PPh_3 (5 equiv to thiol) is present in a 70% MeOH/H_2O (phosphate buffer pH = 7.3), solution of **1** (10 μM), absorbance

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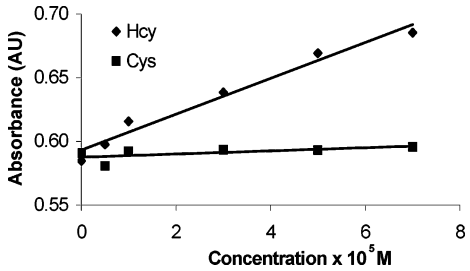

Figure 3. Absorbance (510 nm) vs varying concentrations of Cys and Hcy in solutions of **1** (1.0×10^{-5} M) and PPh_3 (4.5×10^{-4} M).

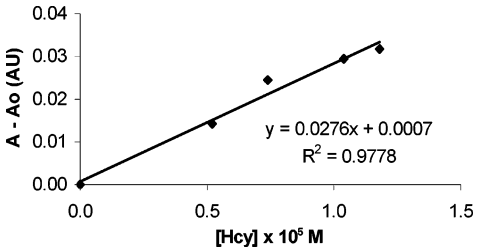

Figure 4. Calibration curve (510 nm) for the determination of Hcy in human plasma in the presence of PPh_3 and **1** after reduction and deproteinization, using an indirect standard addition approach. **A** is the absorbance of plasma sample with added Hcy, and **Ao** is the absorbance of plasma sample without added standards.

changes only occur in response to Hcy. Insignificant changes are observed for Cys (Figure 3) and related thiols.[10] If a 30-fold molar excess of Cys (to Hcy) is added to a solution of Hcy (the naturally occurring proportion of Cys to Hcy[15]) and **1**, no absorbance change is observed corresponding to excess Cys.[10]

The studies involving MV^{2+} and **1** show that the properties of biothiols can be controlled to afford selective detection methods. Furthermore, compound **1** shows great potential as a colorimetric agent in the determination of total Hcy in human plasma by a standard addition method.[16] A calibration curve derived from the solutions containing added Hcy standards is shown in Figure 4. It exhibits linearity in the working range from 0 to 15 μM, which is inclusive of the upper limit of healthy Hcy concentration.[15] The percent recovery of Hcy is 102.9 $\pm$ 7.3%. The relative standard deviation (RSD) is 7.1% ($n = 3$)**.** We are currently investigating monitoring Hcy concentrations well beyond its healthy level in plasma.[1,2] We are also studying further details of the biochemical and sensing mechanisms. These results will be reported in due course.

In conclusion, we have developed colorimetric methods for the selective detection of homocysteine at neutral pH. Methyl viologen solutions turn color selectively in the presence of Hcy upon heating. In solutions containing **1** and PPh_3, we selectively detect Hcy via UV−vis spectroscopy at room temperature. The latter technique shows great potential for directly assaying Hcy levels in human plasma. Current studies in our lab include developing fluorescence and electrochemical methods based on these results.

Acknowledgment. We dedicate this paper to Professor Amos B. Smith, III, on the occasion of his 60th birthday. We also very gratefully acknowledge the generous support of the National Institutes of Health (R01 EB002044).

Supporting Information Available: ^{1}H NMR, mass, and UV−vis spectra related to the interactions of biological thiols with methyl viologen or **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Absorbance changes due to the formation of reduced MV^{2+} were previously[8a] found to be promoted by not only Hcy, but also GSH and Cys. In addition, the rate constants experimentally derived for the proton abstraction step were found to be surprisingly similar for GSH and Hcy. Simulated kinetics of the decay of the disulfide radical anion to the reducing alkyl radical agreed with experimental results only in the case of GSH. These experiments were each performed at pH 10.5. On the basis of the significant pH dependence of the equilibria involving biothiol chemistry, as well as the relatively favored proton abstraction mechanism previously proposed for Hcy, we find appropriate experimental conditions for the highly selective detection of Hcy, as shown conclusively in Figure 2. Selective colorimetric detection of Hcy is attained at neutral pH. This result embodies strong evidence for the geometrically favored proton abstraction mechanism (Figure 1) for Hcy compared to other thiols.
(10) See Supporting Information.
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(12) MALDI TOF MS (anthracene matrix), calcd for glycine sodium salt $C_2H_4NNaO_2$ $(M + Na)^+$ 97.01, found 96.89; calcd for glycine dimer (2,3-diaminosuccinic acid disodium salt) $C_4H_6Na_2N_2O_4$ $(M + 2Na)^+$ 192.01, found 193.05; calcd for glycine dimer (2,3-diaminosuccinic acid dipotassium salt) $C_4H_6K_2N_2O_4$ $(M + 2K)^+$ 223.96, found 223.86.
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(14) Additionally, PPh_3 and congeners can undergo redox chemistry and/or reactions with thiols in the presence of electron acceptors (e.g., MV^{2+}), see (a) Yasui, S.; Shioji, K.; Tsujimoto, M.; Ohno, A. *Chem. Lett.* **1995**, 784. (b) Yasui, S.; Shioji, K.; Tsujimoto, M.; Ohno, A *J. Chem. Soc., Perkins Trans. 2* **1999**, 855.
(15) Healthy plasma total homocysteine concentrations are ca. 12 μM. Cysteine concentrations are typically 20−30 times that of homocysteine (see refs 1 and 2).
(16) Commercial lyophilized human blood plasma (5.0 mL) is reconstituted with distilled H_2O. Bound thiols are liberated from proteins by stirring the plasma solution in a commercial disulfide reducing gel, TCEP (tris-[2-carboxyethyl]phosphine hydroxide). This is followed by deproteinization upon addition of MeOH which also contains PPh_3 (1.5×10^{-3} M). After centrifugation (5.0 min, 3000*g*) the supernatant is filtered through a 0.45-mm filter. Hcy standards in H_2O (0.3 mL, pH = 7.3, phosphate buffer) containing **1** are added to the filtrate. For % recovery determinations, known amounts of Hcy are added to plasma samples before the reduction and deproteinization steps, and the absorbance difference with respect to the original sample is correlated with the Hcy concentration from the calibration curve. The Hcy concentration present in the commercial plasma sample is determined from the calibration curve by subtracting the absorbance of a solution containing **1** and PPh_3 from the absorbance of the plasma sample containing **1** and PPh_3. The total Hcy content in the commercial plasma sample is found to be 3 μM.

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