Redox status and protein binding of plasma homocysteine and other aminothiols in patients with hyperhomocysteinemia due to cobalamin deficiency[1-3]

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ABSTRACT We determined reduced, oxidized, and protein-bound homocysteine, cysteine, and cysteinylglycine in plasma from 13 patients with hyperhomocysteinemia (total homocysteine in the range 30.6–159.8 μmol/L) due to cobalamin deficiency. Reduced homocysteine ($\bar{x}$ ± SD: 1.87 ± 2.06 μmol/L) was markedly above normal (0.24 ± 0.12 μmol/L) in most patients, and the reduced fraction increased as an exponential function of the total homocysteine concentration. The ratio of reduced homocysteine to total homocysteine was positively correlated with the reduced-total ratio for cysteine and cysteinylglycine, suggesting redox equilibrium between different aminothiol species. The free oxidized and the protein-bound forms of homocysteine account for most of the homocysteine in plasma of these patients. The amount of protein-bound homocysteine was negatively correlated with the concentrations of both protein-bound cysteine and cysteinylglycine, indicating displacement of these aminothiols by homocysteine. *Am J Clin Nutr* 1994;59:631–5.

KEY WORDS Homocysteine, cysteine, cysteinylglycine, cobalamin, redox status

Introduction

Cobalamin deficiency (1) is among the clinical conditions (2) that cause a marked elevation in the plasma concentration of the sulphur amino acid homocysteine, ie, hyperhomocysteinemia. This is explained by impaired function of the cobalamin-dependent enzyme, methionine synthase (5-methyltetrahydrofolate homocysteine methyltransferase (EC 2.1.1.13.), which catalyzes remethylation of homocysteine to methionine (3).

The hyperhomocysteinemia in cobalamin deficiency has been exploited as a tool for the diagnosis and follow up of this deficiency state (1, 4, 5). In addition, elevated homocysteine may itself be a pathogenic factor. Results from clinical studies including > 1800 patients suggest that plasma homocysteine level is an independent risk factor for premature cardiovascular disease (6, 7). However, sparse data connect cobalamin deficiency to cardiovascular disease (8). Such a relationship may be confounded by thrombocytopenia, defects of platelet function, and hypocholesterolemia, which are signs often encountered in cobalamin-deficient patients (6, 9). These alterations may protect against cardiovascular disease (6). In addition, nutritional cobalamin deficiency usually develops at advanced age (9).

Clinical studies on hyperhomocysteinemia are based on the measurement of either the acid-soluble homocysteine-cysteine mixed disulfide or total plasma homocysteine, which comprises all homocysteine species in the plasma (2, 6). However, a more differentiated picture may be obtained by determination of reduced, oxidized, and protein-bound forms. We recently measured these species in healthy subjects during fasting (10), after methionine (10) and homocysteine loading (11), and in patients with homocystinuria (12), using a procedure for trapping thiols by collecting blood directly into evacuated tubes containing thiol-reactive agents (13). These investigations show the presence of small amounts of reduced homocysteine in fasting healthy subjects, and this fraction increases markedly after methionine and homocysteine intake. Notably, increased concentrations of reduced homocysteine had marked effects on the redox status and protein binding of other aminothiols in plasma. Similar findings were made in homocystinurics where reduced homocysteine was the species showing the largest increase compared with healthy subjects (10–13).

The studies on redox status and protein-binding of aminothiols cited above were conducted under conditions of transient hyperhomocysteinemia caused by increased homocysteine production or entry into the vascular compartment, or in homocystinurics with steady-state increases in plasma homocysteine caused by impaired homocysteine catabolism. Conceivably, the redox status of and dynamic relation between plasma aminothiols may be related to their turnover rates, which in turn may be influenced by the activity of homocysteine metabolizing enzymes. We therefore investigated these variables in 13 patients with long-term hyperhomocysteinemia caused by cobalamin deficiency, which is a chronic elevation of plasma homocysteine under conditions of impaired remethylation.

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Subjects and methods

Materials

N-Ethylmaleimide (NEM), *N*-ethylmorpholine, dithioerythritol, homocysteine, and cysteine were obtained from Sigma Chemical Co (St Louis) and cysteinylglycine was from Serva Chemicals (Heidelberg, Germany). Sodium tetrahydroborate was from Fluka Chemie AG (Buchs, Switzerland). Dimethyl sulfoxide, hydrogen bromide, 5-sulfosalicylic acid (dihydrate), perchloric acid, acetic acid, phosphoric acid, and acetonitrile (for chromatography) were purchased from Merck AG (Darmstadt, Germany), and monobromobimane (mBrB) was from Molecular Probes, Inc (Eugene, OR). Tetrabutylammonium hydroxide was obtained from Aldrich-Chemie (Steinheim, Germany). ODS Hypersil, octadecylsilane, (3-μm) was obtained from Shandon Southern Ltd (Chesire, UK). Columns for reversed-phase liquid chromatography (3-μm Hypersil, 150 $\times$ 4.6 cm) were slurry packed at 63 MPa by using a Shandon column packer.

Patients

We studied 13 patients (7 males and 6 females) with cobalamin deficiency. Their mean ($\pm$SD) age was 69.9 $\pm$ 12.2 y. The diagnosis was confirmed by measurement of cobalamin and methylmalonic acid in serum and total homocysteine in plasma.

Analysis

Blood was routinely collected into three evacuated tubes containing either mBrB or NEM as thiol-derivatizing reagent, or no additions. The blood was immediately centrifuged at 10 000 $\times$ *g* for 1 min at room temperature to remove blood cells.

From the analysis of blood collected in the solution containing mBrB we obtained reduced thiols, analysis of blood collected into NEM gave the oxidized forms, and the total amount of thiol components was assayed in nontreated plasma. The protein-bound fraction is calculated by subtracting reduced and free oxidized species from the total amount. Details on the construction and performance of these assays were described in a separate publication (13). Methylmalonic acid in plasma was assayed by a method based on HPLC and fluorescence detection (14, 15). Plasma methionine was determined in deproteinized plasma with an assay based on derivatization with *o*-phthaldialdehyde and fluorescence detection (16). Serum cobalamin was determined with a microparticle enzyme-intrinsic factor assay run on an IMx instrument from Abbott (Abbott Park, IL).

Statistical analysis

The relation between total homocysteine and reduced homocysteine was evaluated by fitting data to an exponential equation. The relation between the reduced-total ratios for various plasma thiols was assessed by using linear correlation. The curve fitting was done by using the software program *Cricket Graph* (Cricket, Malvern, PA). *P* values are given for a test for zero correlation and are given as two-tailed.

Results

Patient characteristics

The patients (n = 11) had a mean hemoglobin concentration of 109 $\pm$ 22 g/L, and four had values below normal (normal range 116–166 g/L). The folate (18.3 $\pm$ 8.9 nmol/L), methionine (20.6 $\pm$ 7.4 μmol/L), cysteine (265.7 $\pm$ 64.6 μmol/L) and cysteinylglycine (31.2 $\pm$ 7.2 μmol/L) concentrations were normal in all patients, except in one who had elevated plasma cysteine (412.2 μmol/L). The normal values for these variables were > 5.7 nmol/L (serum folate), 30 $\pm$ 5 μmol/L (plasma methionine), 250 $\pm$ 30 μmol/L (plasma cysteine), and 30 $\pm$ 5 μmol/L (plasma cysteinylglycine) (13, 17).

Ten patients had serum cobalamin concentrations (range 31–97 pmol/L) below normal, two patients below the detection limit (< 20 pmol/L), and only one had serum cobalamin (180.0 pmol/L) within the normal range, which is 150–840 pmol/L. All patients (n = 13) has serum methylmalonic acid (range 0.69–77.4 μmol/L) that was markedly above normal (< 0.34 μmol/L) (15).

Reduced homocysteine and other thiol components

The concentration of reduced homocysteine in plasma from the patients with cobalamin deficiency (1.87 $\pm$ 2.06 μmol/L) was markedly elevated compared with the normal concentration [0.24 $\pm$ 0.12 μmol/L (13)] **Fig 1**. The fraction of homocysteine that existed in the reduced form increased exponentially as a function of the total homocysteine concentration (**Fig 2**).

We also investigated the relation between the ratios of reduced to total homocysteine, cysteine, and cysteinylglycine (**Fig 3**). There was a linear relationship between the reduced-total ratio for homocysteine vs cysteine and cysteinylglycine, and cysteine vs cysteinylglycine (Fig 3).

Free oxidized species

Free oxidized homocysteine was significantly elevated in all patients with cobalamin deficiency and accounted for 10–20% of total homocysteine. The concentrations of free oxidized cysteine (91.2 $\pm$ 21.5 μmol/L) and free oxidized cysteinylglycine (7.5 $\pm$ 1.6 μmol/L) were close to the values found in a healthy population (13) (Fig 1).

Protein-bound species

Protein-bound homocysteine was above normal and accounted for most homocysteine in plasma from the cobalamin-deficient patients (Fig 1). Protein-bound cysteine (and cysteinylglycine) were close to normal for most patients, except patients 1, 9, and 10, who also had low concentrations of protein-bound cysteine. Notably, these patients were among those with the highest concentration of plasma homocysteine (Fig 1). The relation between the protein-bound species is further illustrated in **Fig 4**, showing that protein-bound cysteine was negatively correlated with the amount of protein-bound homocysteine. Notably, the linear regression line showed a slope of ≈ -1, suggesting that increased protein-bound homocysteine is associated with an equivalent reduction in protein-bound cysteine.

Discussion

We previously measured the amount of reduced homocysteine, the associated changes in the redox status and protein binding of other aminothiols in plasma from subjects with impaired homocysteine catabolism (12) and in healthy subjects given methionine (10) or homocysteine (11) by mouth.

The present work extends these studies by investigating these variables in hyperhomocysteinemic patients with cobalamin deficiency. The number of observations and the large range of total

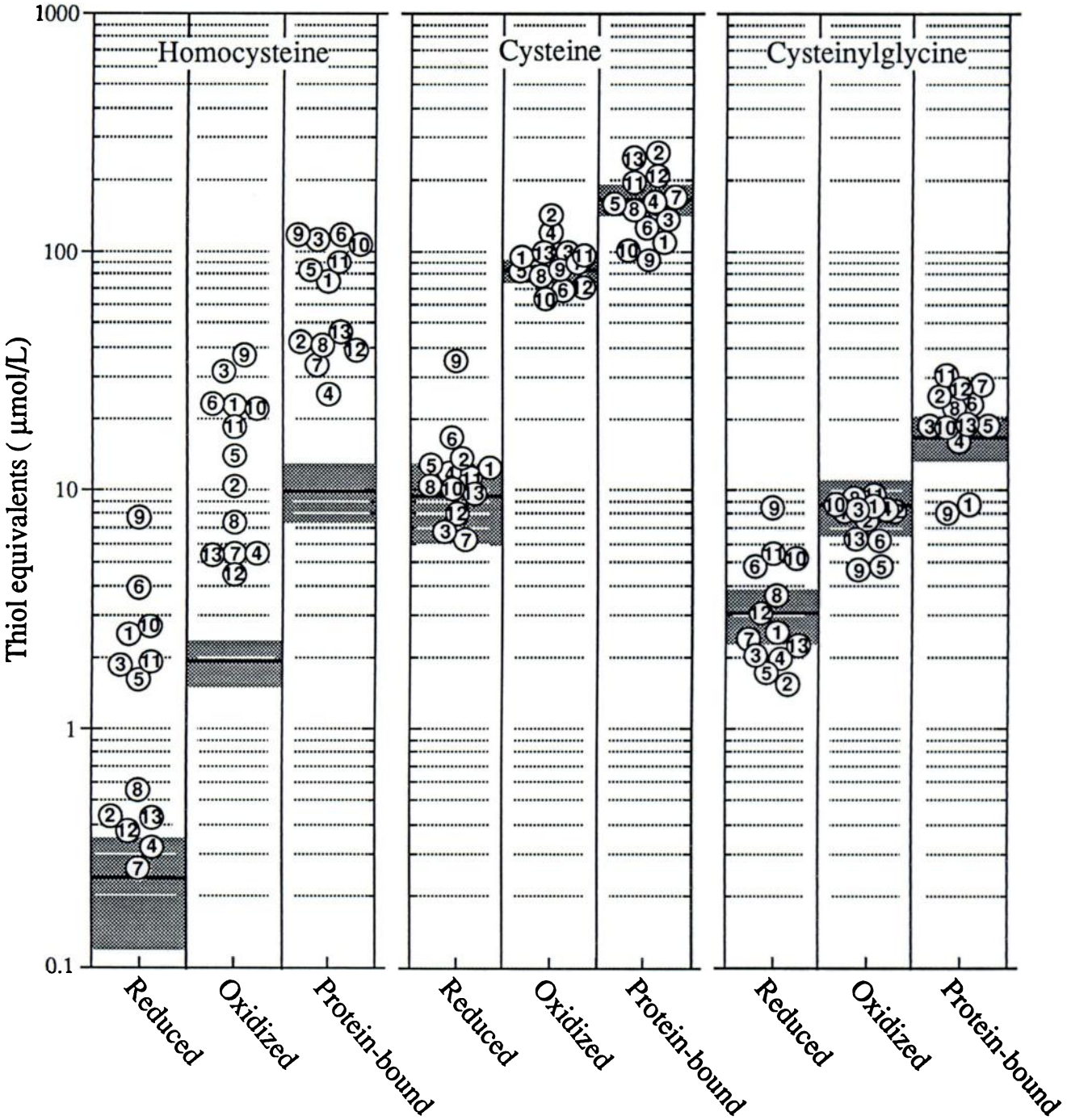

FIG 1. Thiol components in plasma of 13 patients with cobalamin deficiency. Patients are identified by the numbers placed on the data points. The means for normal nonfasting subjects are given by the bold horizontal lines and shaded areas indicate the corresponding SDs. Data are from reference 13.

homocysteine values (30.6–159.8 μmol/L) demonstrate that the reduced fraction increases exponentially as a function of the total homocysteine concentration (Fig 2). When total homocysteine exceeded 100 μmol/L, the reduced-total ratio was 2–5% (Fig 2), which approaches the value found in two patients with homocystinuria (12). In most of the patients with homocystinuria, who have total homocysteine between 150 and 400 μmol/L, the reduced total ratios were in the range 10–25% (12). Thus, the redox status of homocysteine may be a continuous function of the total homocysteine concentration, independent of the metabolic defect, and reduced homocysteine may become a significant species when total homocysteine amounts are > 150 μmol/L. This relationship should be taken into account when evaluating the deleterious effects from elevated plasma homocysteine.

The plasma concentration of reduced homocysteine is low and shows great variability in normal males and females (10, 13). In

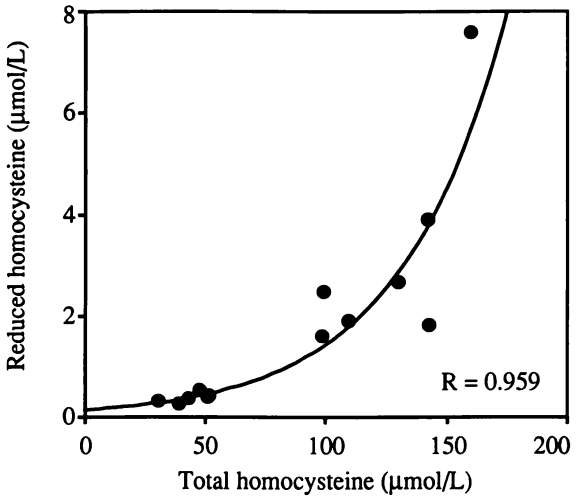

FIG 2. The relation between total homocysteine and reduced homocysteine in plasma from 13 patients with cobalamin deficiency. The data points were fitted to the equation $y = 0.146 \cdot 10^{0.0099}x$.

cobalamin-deficient patients, this species showed the greatest increase compared with the concentrations in healthy subjects (Fig 1). We also observed a linear relationship between the reduced-total ratios for homocysteine, cysteine, and cysteinylglycine (Fig 3). Because inhibition of homocysteine remethylation primarily affects the concentration and thereby the redox status of homocysteine, altered redox status of other aminothiols like cysteine and cysteinylglycine is probably due to secondary events. These secondary changes may develop as a results of disulfide interchange reactions, and the linear relationship may indicate that an equilibrium state exists. This conclusion is supported by previous observations that homocysteine loading has pronounced effects on redox status of cysteine and cysteinylglycine (11).

Protein-bound homocysteine was markedly above normal and accounts for the major portion of plasma homocysteine in the cobalamin-deficient patients (Fig 1). Protein-bound cysteine was close to normal in most patients, except the three or four patients with the highest concentration of homocysteine (Fig 1). There was a negative correlation between protein-bound homocysteine and protein-bound cysteine (Fig 4). Low concentrations of protein-bound cysteine in patients with homocystinuria has been demonstrated by others (18, 19) and could be explained by decreased formation of cysteine due to impaired function of cystathionine β-synthase. However, the negative correlation and the apparent stoichiometry of the relation (slope of the regression line ≈ -1) demonstrated in the present study adds to the data suggesting that homocysteine displaces cysteine from its binding sites in plasma (11). Decreased protein binding of these aminothiols may be a general consequence of elevated homocysteine, and may reflect the presence in plasma of saturable binding sites for aminothiols, which preferentially interact with homocysteine (20–22).

In conclusion, the hyperhomocysteinemia caused by cobalamin deficiency is associated with an increased fraction of reduced homocysteine, which in turn affects the redox status of other aminothiols in plasma. In addition, elevated concentrations of protein-bound homocysteine cause an equivalent decrease in protein-bound cysteine and cysteinylglycine, and this effect is probably due to displacement. The secondary changes in plasma aminothiols in cobalamin-deficient patients seem to be general effects from hyperhomocysteinemia. Conceivably, altered redox status and protein-binding may involve various sulfhydryl residues in blood or tissues, and such changes may contribute to the pathogenesis of cobalamin deficiency. Elevation of different species of homocysteine may cause vascular damage. This possibility is strengthened by the recent finding that reduced, oxidized, and protein-bound homocysteine are elevated in 60 patients with premature peripheral arterioscleroses, as compared with healthy

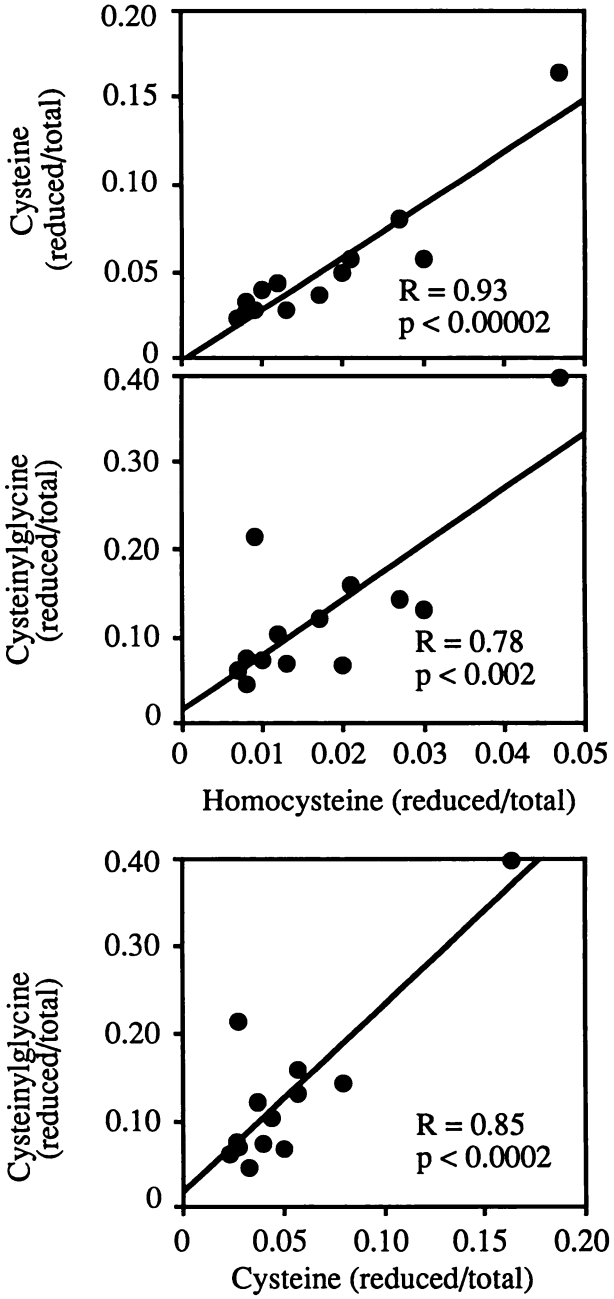

FIG 3. The relation between the redox status of homocysteine, cysteine, and cysteinylglycine in plasma. The fraction existing in reduced form is calculated as the amount of reduced thiol divided by the total amount of the particular thiol, ie, the reduced-total ratio. The straight lines are obtained by linear regression.

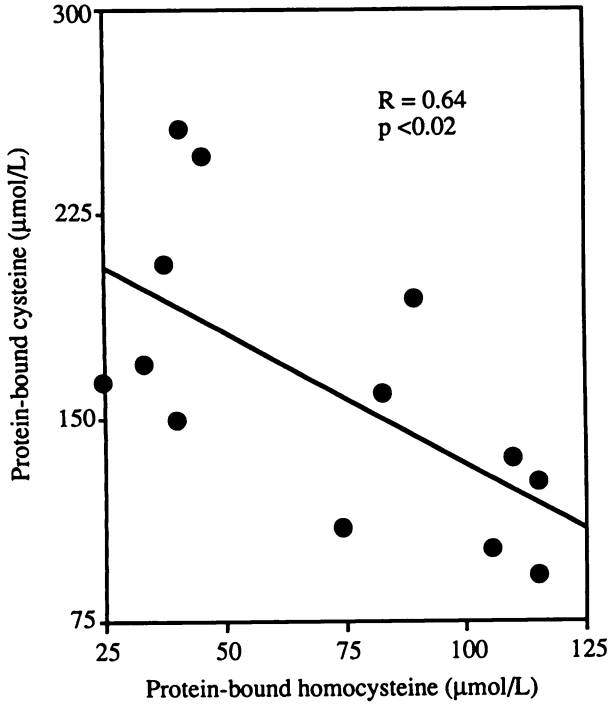

FIG 4. The relation between protein-bound homocysteine and protein-bound cysteine. Linear-regression analysis of the relation between these two variables is given by the equation $y = -0.965x + 230$.

control subjects (MA Mansoor, C Bergmark, AM Svardal, PE Lønning, and PM Ueland, unpublished data, 1993).

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