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Homocysteine and cysteine – albumin binding in homocystinuria: assessment of cysteine status and implications for glutathione synthesis?

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Summary. Measurement of plasma total cysteine rather than free dimeric cystine gives a better indication of cysteine status in homocystinuric patients. This is the result of displacement of cysteine from albumin by homocysteine and is related to the plasma homocysteine concentration. In control subjects the free/bound cyst(e)ine ratio was independent of albumin and total cysteine concentrations. In homocystinuric (HCU) patients both free and total cyst(e)ine values differed significantly from control values ($P < 0.001$) but whilst free cystine considerably overlapped control values the total cysteine concentrations were almost invariably lower. The possible consequences of this on glutathione synthesis was explored by assay of plasma total glutathione but no evidence for glutathione deficiency was found. Measurement of total cysteine, rather than free cystine, provides a better indication of cysteine status in HCU.

Keywords: Aminothiols – Homocysteine – Cysteine – Homocystinuria – Glutathione – Albumin

Nomenclature: Cysteine; homocysteine – reduced monomeric form: cystine; homocystine – free oxidised dimeric form: cyst(e)ine; homocyst(e)ine – relating to either, or a mixture of, reduced and oxidised forms: total cysteine;homocysteine – sum of all moieties, both free and protein bound, expressed as sulphydryl equivalents.

Introduction

Treatment of homocystinuric (HCU) patients is traditionally monitored by measuring the free, non-protein bound, dimeric aminothiols, homocystine and cystine. Free aminothiols quickly become protein bound and reported concentrations may be artificially low if specimens are not processed without delay or treated appropriately (Fikerstrand et al., 1993). There are, therefore,

analytical advantages in measuring total aminothiols (Briddon, 1998; Moat et al., 1999). Whilst it is possible that control of free homocystine concentrations is the more important in terms of reducing the risk of vascular disease in HCU (Wilcken and Wilcken, 1997) the relationship between free and total homocyst(e)ine is well established (Moat et al., 1999). However, there is, as yet, insufficient long term clinical outcome data correlated with total homocysteine concentrations to enable clinicians to use this parameter with confidence. Similarly, the measurement of total cysteine is not yet routinely used for assessment of cysteine status in HCU. Since homocysteine is more avidly bound to protein than cysteine (Ueland et al., 1996) increased concentrations of protein bound homocysteine may displace cysteine from albumin and alter the ratio of free to bound (F/B) cyst(e)ine. Thus, for practical and physiological reasons, measurement of free cystine alone may not represent the true cysteine status in HCU patients. This has been investigated in both controls and HCU subjects by measuring free and total plasma cyst(e)ine and exploring the effects of albumin, total cysteine and total homocysteine concentrations on the F/B cyst(e)ine ratio.

In HCU, due to cystathionine-β-synthase deficiency (McKusick 236200), the synthesis of cysteine is not only impaired but is also required for the synthesis of glutathione (Fig. 1). Plasma total glutathione was therefore also measured and assessed relative to cyst(e)ine status.

Materials and methods

Analysis

Non-haemolysed heparinised plasma was separated within 15 minutes and analysis performed within 24 hours. Plasma was immediately frozen at $-20°C$ to minimise thiol redistribution which occurs when stored at 4°C or above (Fikerstrand et al., 1993). Free aminothiols and other amino acids were measured by automated ion-exchange chromatography using a Biochrom 20 analyser (Amersham Pharmacia Biotech, Amersham, UK). Total aminothiols were measured similarly after dithiothreitol (DTT) reduction (Briddon, 1998). Total glutathione, (sum of reduced, oxidised and protein bound fractions), was measured by HPLC after DTT reduction and protein precipitation with perchloric acid (Paroni et al., 1995; Riederer et al., 1989). The CV for this assay was 5.03% at a mean value of 9.5μmol/L, n = 15.

Controls

Aminothiols were measured in 80 patients attending for investigation of neurological symptoms, including ataxia, dystonia and peripheral neuropathy and in whom no biochemical evidence of metabolic dysfunction was detected. 40 samples were pre-treated with DTT to give values for total aminothiols and 40 analysed without DTT to provide values for free aminothiols. In 17 additional control patients both total and free aminothiols together with albumin were assayed.

HCU patients

31 adult patients with confirmed cystathionine synthase deficiency attending the adult metabolic clinic for monitoring of treatment were studied. These comprised 10 females,

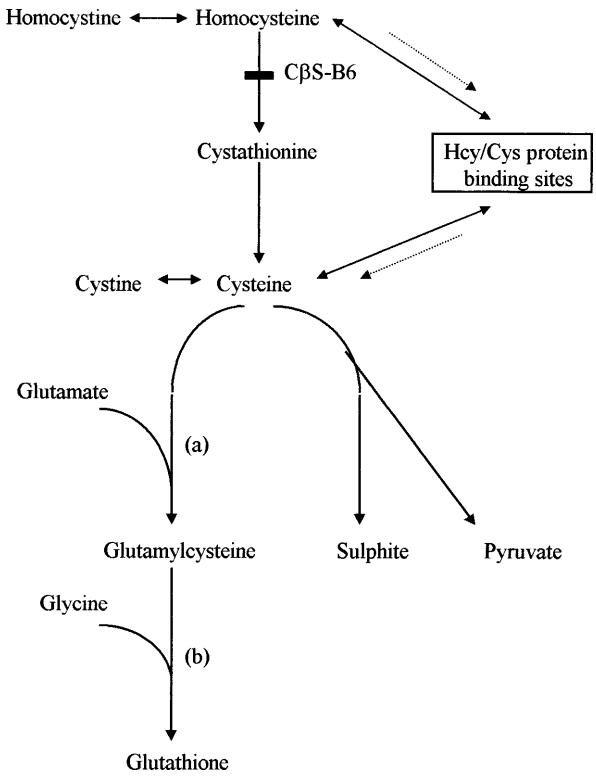

Fig. 1. Simplified metabolic pathway showing enzyme defect in HCU (CβS-B6: cystathione synthase with B6 co-factor) and further metabolism of cysteine to glutathione catalysed by (**a**): γ-glutamyl cysteine synthase and (**b**): glutathione synthase. Dotted line shows effect of increased protein binding of homocysteine with consequent displacement of bound cysteine leading to increased free cysteine and cystine concentrations

mean age 28 years, range 16–46 and 21 males of mean age 29 years, range 13–47. 11 patients were vitamin B6 responsive, as judged by plasma aminothiol response to B6 trial, and the remainder treated with betaine. All patients had free and total aminothiols measured and F/B ratios were calculated allowing for the contribution of mixed disulphides.

Protein binding

The effect of increasing amounts of protein bound homocysteine on free cystine and the F/B cysteine ratio was examined *ex vivo* by adding 1 volume of aqueous oxidised DL-homocystine, adjusted to pH 7.4, to 10 volumes of human albumin solution (Sigma Chemical Co. Poole, UK) and allowing time for protein binding to occur. Two preparations with nominal final concentrations of 200 and 400 μmol/L of homocysteine were used. These were incubated at 37°C and aliquots removed for analysis at 0.5, 1, 2, 3, 4, 6, and 8 hours. No free homocystine, cystine nor other disulphides were detected in the untreated albumin solution prior to homocystine addition; therefore the values obtained for total thiols represented the protein bound concentrations and were used as a baseline for deriving subsequent values. At each time point, concentrations of bound aminothiols were calculated as the difference between the baseline concentration and the actual concentration, including the contribution from mixed disulphide which was also formed in

increasing amounts (Table 1). Cysteine, displaced from albumin, auto-oxidised in solution and was calculated from the free cystine plus mixed disulphide concentration. Values were calculated as sulphydryl equivalents and expressed as μmol/L.

Albumin effect

In 17 controls selected to give a wide range of albumin concentrations (15–44 g/L) both albumin and total cysteine were correlated against the F/B cyst(e)ine ratio.

Statistical treatment

Simple comparisons were made by Students unpaired, 2-tailed, t-test and for regression, significance is quoted for Pearson's correlation coefficient, r. Significance levels were set at 0.05.

Results

Protein binding

Endogenous concentrations of total cysteine and homocysteine in the Sigma albumin solution were 303 and 15 μmol/L respectively with no free aminothiols detected. There was displacement of cysteine from albumin as increasing amounts of added homocystine became protein bound, approximately 50% of the added homocystine being protein bound after 8 hours of incubation, after allowing for MDS formation (Table 1). Figure 2 shows the correlation between protein bound homocysteine and the F/B cyst(e)ine ratio in this *ex vivo* model.

Control data

In the 17 control patients in whom both total and free aminothiols were measured the F/B cyst(e)ine ratio fell within the range 0.22–0.71; mean (SD) = 0.47 (0.139). This ratio was independent of both the albumin and total cysteine concentrations. The correlation of the F/B ratio against albumin, (over the range 15–44 g/L), gave $r = 0.304$, $P = 0.23$, and against total cysteine, $r = 0.303$, $P = 0.27$. In these samples total homocysteine varied between 4 and 15 μmol/L and total glutathione between 3.0–8.3 μmol/L, mean (SD) = 5.7 (1.85). In the groups in which free and total aminothiols were measured separately ($n = 40$) total cysteine was 208–371 μmol/L, mean (SD) = 271 (36.00); free dimeric cystine was 12–61, mean (SD) = 33 (11.74).

HCU patients

In HCU patients ($n = 31$) free dimeric cystine was 3–43 μmol/L, mean (SD) = 22 (10.95) and total cysteine 43–338 μmol/L, mean (SD) = 168 (69.34). There was a statistically significant difference, ($P < 0.001$) for both free cystine and

Table 1. Incubation of albumin solution with added homocystine: two experiments with nominal starting concentrations of 200 and 400 μmol/L homocysteine. Values in μmol/L expressed as sulphydryl equivalents

Time (hours)	1. Nominal 200 μmol/L Hcy added					2. Nominal 400 μmol/L Hcy added				
	Free Hcy	Free Cys	MDS	Unbound Cys	F/B Cys ratio	Free Hcy	Free Cys	MDS	Unbound Cys	F/B Cys ratio
0	198	0	0	0	0	380	0	0	0	0
30 min	168	4	31	35	0.13	320	4	39	43	0.17
1	154	10	39	49	0.15	288	16	59	75	0.34
2	114	26	52	78	0.35	219	38	70	108	0.55
3	94	36	56	92	0.44	200	48	76	124	0.70
4	76	44	51	95	0.46	168	50	70	120	0.66
6	50	48	40	88	0.41	134	60	70	130	0.75
8	40	54	41	95	0.46	128	64	70	134	0.79

MDS: Hcy-Cys mixed disulphide

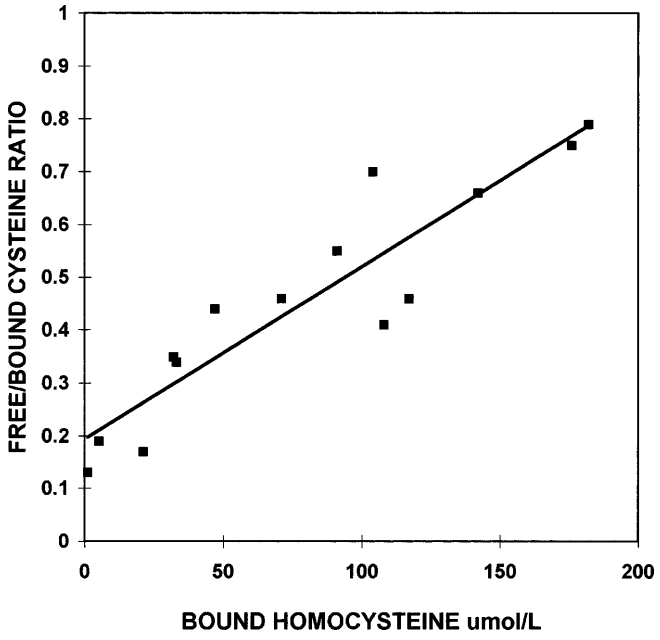

Fig. 2. Relationship between bound homocysteine and the free/bound cysteine ratio *ex vivo* in albumin solution. Combined data from two experiments. For the trend line $y = 0.003x + 0.212$, $r = 0.907$, $P < 0.001$

Table 2. Comparison of results for B6 responsive and non-responsive HCU patients under treatment. Values given as mean ± SD (range). * = significant at 0.05 level

Analyte	B6 responsive	B6 non-responsive	P (t-test)
Free hcy	3.2 ± 3.48 (0–9)	17.7 ± 26.37 (0–96)	0.024*
Total hcy	119 ± 45.2 (37–179)	175 ± 107.0 (49–445)	0.051
Free cys	25 ± 10.5 (15–52)	19 ± 10.7 (3–43)	0.158
Total cys	207 ± 61.8 (124–338)	145 ± 62.9 (43–245)	0.017*
Bound cys	137 ± 52.0 (68–222)	86 ± 48.2 (13–180)	0.018*
Total glutathione	5.4 ± 2.26 (2.3–9.3)	5.7 ± 2.71 (1.1–11.0)	0.762
Free/bound cys	0.59 ± 0.22 (0.26–1.07)	0.90 ± 0.48 (0.40–1.71)	0.022*

total cysteine compared to the control group. However, there was a considerable overlap with poor discrimination from the control group for free cystine whereas total cysteine was almost invariably lower than the control range (Fig. 3). The F/B cyst(e)ine ratio, mean (SD) = 0.76 (0.343), range 0.32–0.75, was significantly different from controls ($P = 0.0005$). This ratio was directly related to the total homocysteine concentration (Fig. 4A) and indirectly related to the total cysteine concentration (Fig. 4B). Plasma total glutathione was 1.1–11.0μmol/L; mean (SD) = 5.6 (2.56) which was not significantly different from the control group, $P = 0.65$. Two patients with particularly low free cystine and total cysteine values also had low total plasma glutathi-

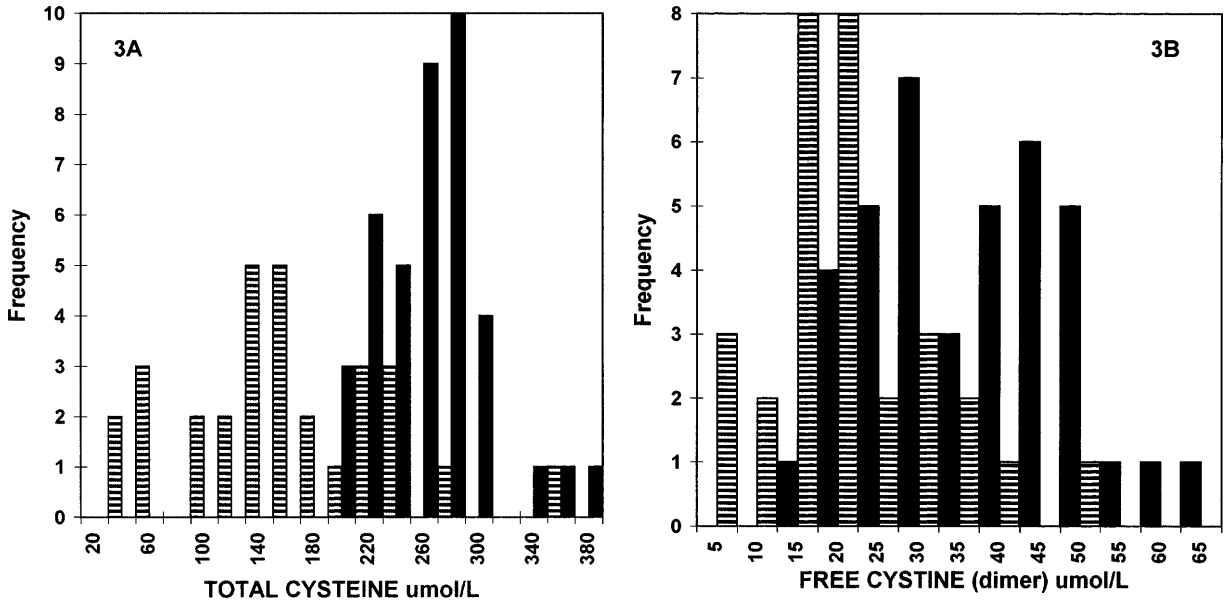

Fig. 3. Relative distributions of total cysteine (**A**) and free (dimeric) cystine (**B**) in controls (solid bars) and HCU patients (hashed bars)

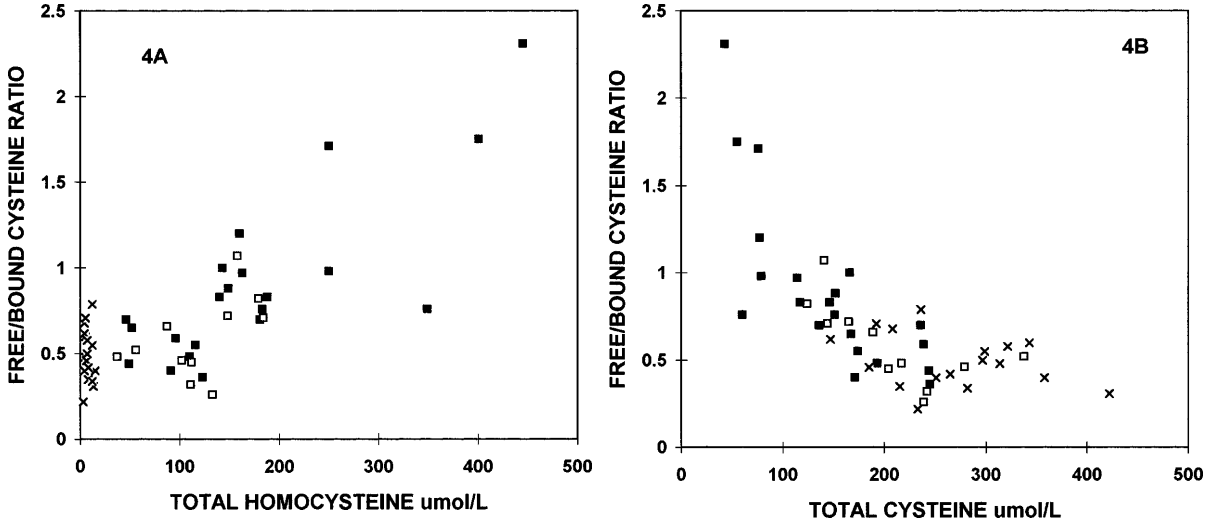

Fig. 4. Relationship between the free/bound cysteine ratio and total homocysteine (**A**) and total cysteine (**B**) showing control data (*) and HCU patients: B6 responsive (open square) and B6 non-responsive (filled square)

one results of 1.1 and 1.2 μmol/L,. One HCU sample had a particularly high total glutathione of 11.0 μmol/L not obviously attributable to cellular contamination of the sample as judged by normal plasma taurine and glutamate concentrations. Results were also analysed according to B6 responsiveness (Table 2 and Fig. 4). No correlation was found between total glutathione and

either the F/B cyst(e)ine ratio ($r = 0.177$, $P = 0.34$) or free cystine ($r = 0.076$, $P = 0.68$).

Discussion

In HCU due to CBS deficiency the transulphuration pathway is impaired and cysteine synthesis compromised (Fig. 1). It might be expected therefore that glutathione synthesis, which is dependent on a supply of non-protein bound cysteine would also be impaired, but normal blood concentrations of free reduced glutathione in HCU patients have been reported (Kozich et al., 2000). This may be a consequence of the increased F/B cyst(e)ine ratio in HCU which resulted in most patients in our group having free cystine concentrations within the control range (Fig. 3). The relationship between free and bound cysteine has been explored previously *in vivo* (Ueland et al., 1996) as a component of the thiol redox system. The mechanisms of plasma total protein binding using reduced homocysteine have been reported (Togawa et al., 2000). Additionally, we have shown here that oxidised dimeric homocystine, as opposed to reduced homocysteine, will also displace cysteine from pure albumin, in a non-physiological environment, in a dose dependent manner supporting the concept of preferential protein binding of homocyst(e)ine (Mansoor, 1992), (Table 1 and Fig. 2) and that albumin is probably the major thiol binding protein in plasma. In our control subjects the F/B cyst(e)ine ratio was independent of the plasma albumin concentration. Albumin has a single cysteine residue, Cys_{34}, available for disulphide bonding (Gilbert et al., 1996). Based on a formula weight for albumin of 69KDaltons the molar fraction of binding sites occupied by cysteine varied between 30 and 76%. In non-HCU controls, therefore, the ratio of free to albumin bound cysteine is probably mediated solely by the thiol redox.

As glutathione protein binding may also be affected by increased homocysteine concentrations with possible alteration of F/B glutathione ratios and glutathione aminothiol dipeptides, total glutathione was measured as a potentially more reliable indicator of glutathione synthesis. Results confirmed that HCU patients do not appear to be glutathione deficient, even when the metabolic defect is severe as judged by free and total aminothiol concentrations and B6 responsiveness. Our findings suggest one reason for this is displacement of cysteine from protein due to increased protein binding of homocyst(e)ine. This increases the supply of free reduced cysteine, which is an intermediary between protein bound cysteine and oxidised dimeric cystine, for glutathione synthesis (Fig. 1). In our patient series, although most had low concentrations of total cysteine, free concentrations were generally within the reference interval. (Fig. 3B). Although two patients had low concentrations of total glutathione of 1.1 and 1.2 μmol/L and very low free cystine concentrations of 5 and 4 μmol/L respectively, they did not exhibit signs associated with inherited disorders of glutathione synthesis, e.g. haemolytic anaemia. Other patients with similarly low cystine results had normal plasma total glutathione levels. This suggests that plasma concentrations of

glutathione and, possibly, of aminothiols do not adequately reflect intracellular metabolism.

It is interesting to note that, even in HCU subjects, the F/B cyst(e)ine ratio is normal at total homocysteine concentrations below about 140 μmol/L (Fig. 4A). Since this approximately corresponds to the total homocysteine concentration above which free homocystine rapidly increases in plasma (Moat et al., 1999) this may define the correspondingly low concentration of total cysteine of about 150 μmol/L (Fig. 4B) below which the plasma thiol redox can no longer be maintained by cysteine moieties alone.

Our data show that the measurement of total cysteine is necessary in HCU subjects to obtain a reliable assessment of cysteine status and it may provide an additional indication of disease severity (Fig. 4 and Table 2). In addition, examination of the F/B cyst(e)ine ratio may be of clinical use in evaluating metabolic control.

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