Biochem. J. (1985) **228**, 545–550
Printed in Great Britain

Cysteamine depletes cystinotic leucocyte granular fractions of cystine by the mechanism of disulphide interchange

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(*Received 4 September 1984/11 February 1985; accepted 5 March 1985*)

Cystinotic lysosome-rich leucocyte granular fractions, loaded with [^{35}S]cystine, were exposed to different cystine-depleting agents. During a 30min incubation at 37°C, untreated cystinotic granular fractions lost negligible [^{35}S]cystine when corrected for lysosome rupture. Granular fractions exposed to 0.1mM-cysteamine lost 64% of their initial cystine, and hexosaminidase activity was decreased by 10%. This was accompanied by the formation of high concentrations of [^{35}S]cysteine–cysteamine mixed disulphide within the granular-fraction pellet, and, in the presence of *N*-ethylmaleimide, increasing amounts of [^{35}S]cysteine–*N*-ethylmaleimide adduct outside the granular fraction. In separate experiments, [^{35}S]cystine exited cystinotic leucocyte lysosomes at a negligible rate (half-times 199 and 293min), but [^{35}S]cysteine–cysteamine mixed disulphide exhibited substantial egress (half-times 66 and 88min) and was recovered intact outside the granular-fraction pellet. We conclude that cysteamine depletes lysosomes of cystine by participating in a thiol–disulphide interchange reaction to produce cysteine and cysteine–cysteamine mixed disulphide, both of which traverse the cystinotic leucocyte lysosomal membrane.

Normal polymorphonuclear leucocytes are able to transport non-protein cystine out of their lysosomes by a saturable process exhibiting counter-transport (Gahl *et al.*, 1982*a*,*b*, 1983). Cystinotic leucocytes lack this carrier-mediated transport system (Gahl *et al.*, 1983), and store approx. 50–100 times the normal amount of cystine within their lysosomes (Schneider & Schulman, 1983). The compartmentalized cystine can be depleted by exposure of the intact cells to certain aminothiols both *in vitro* and *in vivo* (Thoene *et al.*, 1976), but the mechanism of cystine depletion by these agents has not been demonstrated. Cysteamine may deplete cystinotic leucocyte granular fractions by damaging lysosomal membranes or may chemically modify a defective membrane carrier in cystinosis in such a manner as to render it functionally normal. Alternatively, Theone *et al.* (1976) proposed that cysteamine participates in a disulphide interchange reaction with cystine to form cysteine and cysteine–cysteamine mixed disulphide, both of which exit cystinotic leucocyte lysosomes by a process not involving the cystine carrier. We present direct evidence supporting this hypothesis.

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Experimental

Expt. 1 examines the cystine-depleting effects of various thiols, and Expt. 2 the egress of [^{35}S]cystine and [^{35}S]cysteine–cysteamine mixed disulphide from cystinotic leucocyte lysosomes.

Cystine loading

Polymorphonuclear-leucocyte-rich cells (0.1–0.2ml packed volume) were prepared from 15–50ml of cystinotic blood (Expt. 1, five subjects; Expt. 2, three additional subjects). The leucocytes were loaded by exposure to 0.25mM-[^{35}S]cystine dimethyl ester prepared from radioactive cystine (specific radioactivity 50–330Ci/mol; Amersham/Searle) (Gahl *et al.*, 1982*b*). Intralysosomal hydrolases cleave the methyl esters to give high concentrations of free [^{35}S]cystine within lysosome-rich granular fractions, which are isolated by limited sonication of washed whole leucocytes

followed by centrifugation (Gahl *et al.*, 1982*b*). This method, with cystinotic leucocytes, consistently yielded approx. 50% of total hexosaminidase activity within the granular fraction, which was purified 2–3-fold with respect to cystine and hexosaminidase per mg of protein. Electron microscopy showed a homogeneous collection of granules with cell debris; slight contamination by intact granules from cells other than polymorphonuclear leucocytes is likely.

In the three studies designated Expt. 1, a 150 μl granular-fraction suspension (0.6–0.8 unit of hexosaminidase activity) contained a total of between 8×10^3 and 73×10^3 c.p.m. in all ^{35}S-containing species identifiable by high-voltage electrophoresis. In Expt. 2 (Table 3), a 300 μl granular-fraction suspension (1.6 units of hexosaminidase activity) contained a total of 118×10^3 c.p.m., equivalent to 0.23 nmol of cystine. This represents a minimal estimate of lysosomal cystine content, since it does not account for endogenous, non-radioactive, cystine.

Experiment 1

The [^{35}S]cystine-loaded granular fractions were suspended in 750 μl of 0.25 M-sucrose/10 mM-Hepes/NaOH buffer, pH 7.0, containing either no addition (control), 0.1 mM-cysteamine (Sigma Chemical Co.), 0.1 mM-cystamine (Sigma Chemical Co.), 1 mM-glutathione (Sigma Chemical Co.), 0.1 mM-cystamine + 1 mM-glutathione or 0.1 mM-dithiothreitol (Bethesda Research Laboratories). After 0, 3, 6 and 30 min at 37°C, a 10 μl sample of the suspension was assayed for total hexosaminidase activity, and 150 μl was placed on ice and centrifuged at 17000 *g* for 10 min at 4°C. A 10 μl portion of the supernatant (10 μl) was assayed for hexosaminidase activity, and another portion (90 μl) was added to 10 μl of *N*-ethylmaleimide (Pierce Chemical Co.) (final concn. 10 mM) before high-voltage electrophoresis. The pellet was resuspended in 200 μl of 10 mM-sodium phosphate buffer, pH 7.0, containing 10 mM-*N*-ethylmaleimide, and, after 20 min, the protein was precipitated with 20 μl of sulphosalicylic acid (final concn. 4% w/v). Both the protein-free pellet extract and the original supernatant were analysed for radioactivity corresponding to [^{35}S]cystine, cysteine and cysteine–cysteamine mixed disulphide by high-voltage electrophoresis (Steinherz *et al.*, 1982).

Mean total recovery (supernatant and pellet) of ^{35}S radioactivity was constant during the incubation period. Unhydrolysed mono- and di-methyl esters of cystine accounted for less than 14% of the total pellet ^{35}S radioactivity at zero time.

Ascorbic acid (Calbiochem) (5 mM) and 0.1 mM-pantetheine, prepared from pantethine (Calbiochem), were similarly tested for cystine-depleting activity.

Experiment 2

[^{35}S]Cystine-loaded cystinotic granular fractions were exposed to 0.1 mM-cysteamine in sucrose/Hepes buffer for 15 min at 37°C. *N*-Ethylmaleimide was added to the reaction mixture (final concn. 2 mM), and the granular fraction was collected by centrifugation and washed with 2 ml of sucrose/Hepes buffer containing 2 mM-*N*-ethylmaleimide. It was then suspended in the same buffer at 37°C, and 300 μl portions were removed at 0, 10, 25, 40, 50 and 60 min, separated into pellet and supernatant by centrifugation, and analysed for ^{35}S-containing compounds as described above. A second experiment was performed with the use of 0, 10, 30 and 60 min time points.

Assay of hexosaminidase activity

The extent of lysosomal rupture was assessed by dividing supernatant hexosaminidase activity by total hexosaminidase activity in the granular-fraction suspension. Hexosaminidase activity was assayed by 3 min incubation as previously described (Gahl *et al.*, 1982*a*). One unit of hexosaminidase hydrolysed 1 nmol of substrate/min at 37°C.

Determination of protein

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

In Expt. 1, the effects of various thiols on the cystine content of cystinotic leucocyte lysosomes were examined. [^{35}S]Cystine-loaded granular fractions in control incubation medium or in medium containing 1 mM-glutathione lost cystine at a rate commensurate with the loss of hexosaminidase (Table 1). Loaded granular fractions suspended in either 0.1 mM-cystamine or 0.1 mM-dithiothreitol lost [^{35}S]cystine only slightly more rapidly than hexosaminidase. In contrast, exposure of granular fractions to 0.1 mM-cysteamine resulted in a mean 64% depletion of cystine with only a 10% decrease in hexosaminidase. Exposure to cystamine + glutathione was accompanied by a similar, though less dramatic, effect (Table 1). Pantetheine (0.1 mM) or 5 mM-ascorbic acid failed to deplete cystinotic leucocyte granular fractions of [^{35}S]cystine (results not shown).

In media containing either cysteamine or cystamine + glutathione the amounts of cysteine–cysteamine mixed disulphide were similar inside and outside the granular-fraction pellet, averaging 9.0 and 11.1% of total recoverable radioactivity re-

Table 1. *Effects of various thiol-containing compounds on* [^{35}S]*cystine inside cystinotic leucocyte granular fractions (Expt. 1)*

Cystinotic leucocyte granular fractions loaded with [^{35}S]cystine were suspended at 37°C in medium containing the indicated compound. Samples of the suspension were removed at 0 to 30 min, centrifuged, and the pellets suspended in 10 mM-*N*-ethylmaleimide. The protein-free acid extracts were analysed for [^{35}S]cystine by high-voltage electrophoresis. Cystine contents are expressed as percentages (means ± S.D.) of the amount of cystine present in the granular-fraction pellet at zero time. For three experiments, the amounts of radioactivity identified in cystine at zero time were 60.0×10^3, 18.5×10^{-3} and 5.6×10^3 c.p.m. per sample. Hexosaminidase activities are expressed as percentages (means ± S.D.) of total hexosaminidase activity recovered in the 17000*g* pellet of each granular-fraction sample.

	Control		0.1 mM-Cysteamine		0.1 mM-Cystamine		1 mM-Glutathione		Cystamine + glutathione		0.1 mM-Dithiothreitol	
Time (min)	[^{35}S]-Cystine (%)	Hexos-aminidase (%)	[^{35}S]-Cystine (%)	Hexos-aminidase (%)	[^{35}S]-Cystine (%)	Hexos-aminidase (%)	[^{35}S]-Cystine (%)	Hexos-aminidase (%)	[^{35}S]-Cystine (%)	Hexos-aminidase (%)	[^{35}S]-Cystine (%)	Hexos-aminidase (%)
0	100	95 ± 2	100	94 ± 2	100	94 ± 0	100	93	100	96	100	94
3	97 ± 7	90 ± 3	69 ± 9	91 ± 0	94 ± 8	88 ± 0	93	—	84	—	91	—
6	94 ± 5	87 ± 4	56 ± 22	88 ± 2	89 ± 16	89 ± 1	98	85	78	85	84	90
30	84 ± 6	83 ± 4	36 ± 21	84 ± 3	77 ± 20	85 ± 1	93	80	58	76	72	79

spectively. Throughout the course of the experiment, the amount of mixed disulphide inside the granular fraction pellet increased slightly (results not shown).

At the same time, the expected product of a reaction between [^{35}S]cystine and cysteamine, i.e. [^{35}S]cysteine, increased substantially outside the cysteamine-treated granular fractions, measured as [^{35}S]cysteine–*N*-ethylmaleimide adduct (Table 2). This finding, not observed for granules exposed to cystamine, reflects the relatively rapid escape of cysteine from cystinotic leucocyte lysosomes (Gahl *et al.*, 1982*b*).

Next, cystinotic leucocyte lysosomes were loaded with [^{35}S]cystine and exposed to cysteamine; washing and centrifugation yielded a granular fraction containing both [^{35}S]cystine and [^{35}S]cysteine–cysteamine mixed disulphide. The simultaneous egress of these two disulphides was assessed in the absence of exogenous cysteamine and in the presence of *N*-ethylmaleimide (Expt. 2). After 1 h at 37°C, 28% of granular-fraction [^{35}S]cystine was lost from the sedimentable fraction, presumably as a consequence of lysosomal rupture (Table 3). During the same time, 55% of the original pellet [^{35}S]cysteine–cysteamine mixed disulphide was lost from the granular fraction and largely recovered in the supernatant. After 1 h the amount of mixed disulphide in the supernatant was more than 83% of the amount remaining in the pellet. At the same time, only 33% of the pellet [^{35}S]cystine was found in the supernatant.

Table 2. [^{35}S]*Cysteine outside cystinotic leucocyte granular fractions exposed to cysteamine or cystamine (Expt. 1)*

Supernatants, removed from the respective pellets described in Table 1, were assayed for [^{35}S]cysteine–*N*-ethylmaleimide adduct by high-voltage electrophoresis. [^{35}S]Cysteine–*N*-ethylmaleimide adduct appearing in control supernatants originated from the small amount of [^{35}S]cysteine found together with [^{35}S]cystine in granular fractions loaded with [^{35}S]cystine dimethyl ester (Gahl *et al.*, 1982*b*). Values for [^{35}S]cysteine–*N*-ethylmaleimide adduct are expressed as percentages (means ± S.D.) of total recoverable radioactivity that was identified as [^{35}S]cysteine–*N*-ethylmaleimide adduct and appeared in the supernatant of a 17000*g* centrifugation.

	[^{35}S]Cysteine–*N*-ethylmaleimide adduct (%)		
Time (min)	Control	0.1 mM-Cysteamine	0.1 mM-Cystamine
0	3.3 ± 2.3	4.4 ± 1.6	3.4 ± 2.7
3	5.4 ± 3.2	9.3 ± 2.2	4.3 ± 2.2
6	6.2 ± 5.7	17.5 ± 6.6	4.8 ± 2.6
30	11.1 ± 6.0	32.0 ± 14.1	4.2 ± 1.9

Table 3. *Loss of [^{35}S]cystine, [^{35}S]cysteine–cysteamine mixed disulphide and protein from cystinotic lysosome-rich leucocyte granular fractions (Expt. 2)*

Cystinotic leucocyte granular fractions, loaded with [^{35}S]cystine and [^{35}S]cysteine–cysteamine mixed disulphide (Expt. 2), were suspended in medium containing 2mM-*N*-ethylmaleimide. Samples (300μl) were removed at 0 to 60min, separated into pellet and supernatant by centrifugation, and analysed for ^{35}S-containing compounds by high-voltage electrophoresis. Note that each cystine molecule will contain two ^{35}S atoms, whereas the mixed disulphide contains only one. Results are not corrected for loss due to lysosomal rupture as estimated by supernatant hexosaminidase, which increased from 2% at 0 time to 9% at 60min.

	10^{-3} × Radioactivity (c.p.m.)						
	[^{35}S]Cystine			[^{35}S]Cysteine–cysteamine			Total pellet protein (μg)
Time (min)	Pellet	Supernatant	Total	Pellet	Supernatant	Total	
0	69.6	4.1	73.7	14.5	0.9	15.4	244
10	61.5	5.9	67.4	11.4	2.3	14.7	220
25	57.8	8.9	66.7	10.1	4.0	14.1	238
40	56.1	10.9	67.0	8.6	4.1	12.7	216
50	52.8	12.2	65.0	7.9	4.9	12.8	204
60	50.1	17.0	67.1	6.5	5.4	11.9	232

Table 4. *Half-times for egress of [^{35}S]cystine, [^{35}S]cysteine–cysteamine mixed disulphide and [^{35}S]cysteine–N-ethylmaleimide from cystinotic leucocyte granular fractions (Expt. 2)*

Results are for the experiment described in Table 3 (A) and for a separate experiment (B). Half-times and correlation coefficients were determined by linear-regression analyses of plots of ln(c.p.m./mg of protein) against time.

	10^{-3} × Radioactivity in granular-fraction pellet (c.p.m./mg of protein)					
	[^{35}S]Cystine		[^{35}S]Cysteine–cysteamine		[^{35}S]Cysteine–*N*-ethylmaleimide	
Time (min)	A	B	A	B	A	B
0	285	489	59	43	130	92
10	280	491	52	32	109	76
25	243	—	42	—	74	—
30	—	391	—	25	—	54
40	260	—	40	—	75	—
50	259	—	39	—	68	—
60	216	439	28	26	53	54
Half-time (min)	199	293	66	88	51	79
Correlation coefficient	0.80	0.58	0.95	0.81	0.96	0.88

When pellet ^{35}S-containing species were expressed as c.p.m./mg of protein, half-times for exit from the granular fractions could be calculated (Table 4). In two experiments, the half-times for [^{35}S]cystine egress (199 and 293min) were prolonged, consistent with previous data (Gahl *et al.*, 1982*b*). In contrast, significant rates of egress for [^{35}S]cysteine–cysteamine mixed disulphide (half times = 66 and 88min) and [^{35}S]cysteine–*N*-ethylmaleimide adduct (half-times 51 and 79min) were observed (Table 4).

Discussion

Cysteamine caused depletion of cystine (Table 1) in cystinotic leucocyte granular fractions that was initially accompanied by a very high intralysosomal, compared with extralysosomal, concentration of cysteine–cysteamine mixed disulphide; this follows from the fact that the amounts of the mixed disulphide inside and outside the granular-fraction pellet were similar, whereas the estimated volume of the pellet was less than one-tenth the volume of the incubation medium. These findings suggest that the disulphide interchange reaction occurred within the lysosomes, as Thoene *et al.* (1976) proposed. Moreover, [^{35}S]cysteine–cysteamine mixed disulphide left the granular fraction and appeared in the supernatant proportionately more rapidly than did [^{35}S]cystine (Tables 3 and 4). Since there was no cysteamine in the medium, and the presence of 2mM-*N*-ethylmaleimide precluded the possibility that intralysosomal [^{35}S]cysteine–

cysteamine mixed disulphide was reduced to free thiols and reoxidized to the mixed disulphide outside the granular fraction, the mixed disulphide itself apparently traversed the lysosomal membrane, despite the absence of a functional cystine carrier. The other product of a reaction between cystine and cysteamine, cysteine, has previously been shown to exit cystinotic leucocyte granular fractions rapidly (Gahl *et al.*, 1982*b*).

Reported half-times for cystine loss from cystinotic leucocyte granular fractions range from 142min to infinity; normal half-times are 34–60min (cf. Table III in Gahl *et al.*, 1982*b*). Since normal lysosomes do not accumulate cystine *in vivo*, we would not expect the cystinotic leucocyte granules used in the present study, whose half-times for cysteine–cysteamine mixed disulphide egress were 66–88min, to accumulate the mixed disulphide *in vivo*. They would be expected, however, to retain cystine, whose half-times for egress (199 and 293min; Table 4) were within the range reported for granules that store 50–100 times normal amounts of cystine (Gahl *et al.*, 1982*b*).

It was theoretically possible that cysteamine might directly alter the lysosomal cystine carrier, e.g. by modification of a mutant free thiol group, as demonstrated for apolipoprotein E (Weisgraber *et al.*, 1982). However, if this were the mechanism, cystine, for which the carrier has approx. 3 times the affinity compared with the mixed disulphide cysteine–cysteamine mixed disulphide (Gahl *et al.*, 1983), should leave the cystinotic leucocyte lysosomes more rapidly than does the mixed disulphide. In fact, the opposite was true (Table 4), arguing against normalization of a mutant transport protein as the basis for cystine depletion by cysteamine. (However, the possibility that cysteamine has some rapidly reversible activating effect on the carrier *in vivo* is not rigorously excluded by this experiment.)

Reduced glutathione did not deplete cystine from cystinotic leucocyte granular fractions (Table 1; Thoene *et al.*, 1976), consistent with the relatively large size of glutathione. Glutathione may be unable to enter lysosomes freely (Schulman & Bradley, 1970) to participate in a disulphide interchange reaction.

Cystamine depleted granular fractions of cystine only moderately, probably because it is not itself a reducing agent, although it may readily enter lysosomes. In the presence of excess glutathione, however, 0.1mM-cystamine (equivalent to 0.2mM-cysteamine) effected substantial intralysosomal cystine depletion (Table 1). Given the high cytoplasmic glutathione concentration, cystamine would be expected to prove an excellent cystine-depleting agent in intact cells, and Thoene *et al.* (1976) have shown this to be the case.

Dithiothreitol, which depletes cystinotic fibroblasts of cystine (Goldman *et al.*, 1970) and has been used therapeutically in cystinosis (Goldman *et al.*, 1974), may deplete cystine by reduction to cysteine within lysosomes. It is moderately effective in isolated granular fractions (Table 1).

Although the thiol pantetheine did not deplete lysosomes of cystine, it has been reported (Butler & Zatz, 1984) that pantethine's depletion of cystine from cystinotic fibroblasts in culture occurs because pantethine is reduced to pantetheine in the cytoplasm of intact cells and then degraded to cysteamine by pantetheinase, an enzyme known to be present in human fibroblasts and leucocytes (Orloff *et al.*, 1981).

Ascorbic acid, which did not deplete cystinotic leucocyte granular fractions of cystine, would appear incapable of reducing cystine directly. Although it does modestly decrease fibroblast cystine content in cystinosis (Kroll & Schneider, 1974), it has been used without clinical success in cystinosis (Schneider *et al.*, 1979).

All the cystine-depleting studies (Table 1) used supernatant hexosaminidase as a measure of lysosome breakage or leakage. This may underestimate disruption, since the enzyme may re-adsorb on sedimentable material, and since damaged vesicles may leak small molecules, such as cystine, without leaking hexosaminidase. Nevertheless, it is encouraging that cystinotic granules in control medium, which might be expected to lose cystine only by vesicle rupture, lost 16% of initial cystine, which corresponds closely to the 12% of initial hexosaminidase lost during a 30min incubation (Table 1).

The correlation between cystine depletion in isolated leucocyte granular fractions and in intact leucocytes *in vivo* suggests that potential cystine-depleting agents can be tested with the system described in the present paper as an alternative or adjunct to studies with whole cells in culture. From the present study we conclude that cysteamine can react with cystine inside human leucocyte lysosomes to produce cysteine and cysteine–cysteamine mixed disulphide, both of which can leave cystinotic lysosomes to effect cystine depletion.

Note added in proof (received 25 March 1985)

A transport system for lysine has now been described in human fibroblast lysosomal membranes. The system is intact in cystinotic cells and recognizes cysteine–cysteamine mixed disulphide, suggesting that this compound may exit from cysteamine-treated lysosomes by a carrier-mediated process rather than by diffusion (Pisoni *et al.*, 1985*a*,*b*).

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