THIOREDOXIN AND GLUTATHIONE IN CULTURED FIBROBLASTS FROM HUMAN CASES WITH 5-OXOPROLINURIA AND CYSTINOSIS

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1. Introduction

A number of metabolic functions are dependent on the interchange between sulfhydryl and disulfide groups [1]. Two important mediators of such interconversions are thioredoxin and glutathione.

Thioredoxins isolated from *E. coli*, yeast, liver and tumor cells [2–6] have approx. mol. wt 12 000. In the reduced form, these proteins have two cysteines at the active site. In the presence of disulfide compounds, the active site sulfhydryls of the reduced form of thioredoxin are readily oxidized to a disulfide bond. The oxidized form of thioredoxin is reduced by NADPH and a specific enzyme, thioredoxin reductase [7]. In vitro thioredoxin acts as a potent reducing agent for both protein and low molecular weight disulfides [5,8,9]. Whether it has the same function in vivo is not yet clear.

Glutathione also participates as a reducing agent in vitro. It is then converted to a disulfide dimer. The oxidized form of glutathione is reduced by NADPH and glutathione reductase. The metabolic function of glutathione in vivo remains to be established (cf. [10]).

In this study we have used cells from two different human mutants in order to study the interplay between the glutathione and the thioredoxin systems, namely 5-oxoprolinuria [11] and cystinosis [12]. In 5-oxoprolinuria, the primary metabolic defect is a generalized deficiency of glutathione synthetase [13]. This leads to a marked decrease in glutathione concentrations. In cystinosis there is a generalized impairment of intracellular cystine reduction yielding massive lyzosomal storage of cystine [14]. However, the primary metabolic defect has so far not been identified.

Cells from 5-oxoprolinuria cases contained markedly decreased levels of glutathione synthetase and also decreased levels of glutathione. The activity of thioredoxin and its reductase were normal indicating that the level of glutathione and thioredoxin are regulated independently. Also fibroblasts from cystinotic patients were found to have normal content of glutathione and the thioredoxin system indicating that the derangement in the conversion of cystine to cysteine in cystinosis is not due to decreased levels of either glutathione or thioredoxin in whole cells.

2. Materials and methods

2.1. *Materials*

Homogeneous thioredoxin was prepared from calf liver [5]. Thioredoxin reductase from calf liver of better than 50% purity [15] was prepared by DEAE-cellulose, TEAE-cellulose and Sephadex G-100 chromatography and affinity chromatography on 2′,5′-ADP–Sepharose. Absorbance and enzymatic activities were measured in a Zeiss PMQIII spectrophotometer with multiple sample changer and a

Servogor 541 recorder. The sources of other materials are in [5,8,15]; the chemicals used were of analytical grade and obtained from commercial sources.

2.2. *Preparation of extracts*

Human skin fibroblasts were obtained from 2 patients with 5-oxoprolinuria [11] and 3 patients with nephropathic cystinosis. The authors are grateful to J. D. Schulman for supplying cells from 2 of the cystinotic patients. Control fibroblasts were also obtained from healthy volunteers.

The fibroblasts were cultivated in 150 mm Falcon petri dishes in Earle's minimum essential medium containing fetal calf serum (10%), penicillin (125 units/ml) and streptomycin (125 μg/ml). The cells were harvested in the late log phase of growth. After rinsing with cold phosphate-buffered saline solution they were detached by scraping with a rubber policeman. The cells from 5–10 petri dishes were pooled, washed twice with cold phosphate buffered saline solution and then immediately frozen in liquid nitrogen until analyzed. The frozen cell pellets were thawed, suspended in 1–2 ml 50 mM Tris-acetate, pH 7.5, disrupted by sonication and centrifuged at 30 000 × *g* for 30 min. The supernatant solution referred to as crude extract was assayed for glutathione and glutathione synthetase.

For analysis of thioredoxin and thioredoxin reductase, the crude cell-free extracts were treated with streptomycin sulfate (200 μl 5% solution/ml) and centrifuged at 20 000 × *g* for 20 min. The precipitate was discarded and to the residual supernatant solution was added ammonium sulfate to give 60% saturation. The precipitate was collected and dissolved in a small vol. 50 mM Tris–Cl, pH 7.5, 1 mM EDTA and incubated with 2 mM dithiothreitol for 30 min at +4°C followed by extensive dialysis (2 × 2 h) against 50 mM Tris–Cl, pH 7.5, 2 mM EDTA.

Protein was measured as in [16].

2.3. *Enzymatic determinations*

Analysis of glutathione was performed on duplicate 0.1 ml aliquots of the crude extracts which were incubated at 0°C for 30 min in final vol. 0.5 ml containing 50 μmol dithiothreitol and 1 μmol EDTA. The volume was then adjusted to 1 ml and a final concentration of 5% trichloroacetic acid. After 10 min at 0°C, the protein precipitate was removed by centrifugation and the supernatant solution was extracted 4 times at room temperature with 5 ml diethyl ether in order to remove excess dithiothreitol. Glutathione, reduced as well as oxidized, was analyzed according to the enzymatic method in [17].

Glutathione synthetase was analyzed as in [13].

Thioredoxin was determined as in [5,15] by measuring its catalytic activity in the reduction of insulin disulfides by excess NADPH and thioredoxin reductase. Dithiothreitol activated calf liver thioredoxin was used as standard [15].

Thioredoxin reductase was analyzed by Method 2 as described [15] in the presence of 10 μM dithiothreitol-activated calf liver thioredoxin. The activity was also measured by the 5,5′-dithio-bis-(2 nitrobenzoic acid) assay [15] and was expressed as μmol thionitrobenzoate formed/min. The two methods gave results in good agreement.

3. Results and discussion

The concentration of glutathione and the activity of glutathione synthetase, thioredoxin and thioredoxin reductase in extracts from tissue cultured fibroblasts are shown in table 1.

The levels of glutathione and glutathione synthetase activity were significantly lower in 5-oxoprolinuria cells compared to control cells. This confirms the observation [13] that the primary enzymatic defect in 5-oxoprolinuria, i.e., the glutathione synthetase mutation is expressed in cultured fibroblasts. Furthermore, it is apparent that the fibroblast model system also exhibits decreased glutathione concentrations as a consequence of the enzymatic block. This observation indicates that 5-oxoprolinuria fibroblasts in culture can be used as a model system for studies of the metabolism and biological functions of glutathione.

Fibroblasts from 3 cystinotic patients did not differ from control cells with respect to the levels of glutathione and glutathione synthetase.

The results also show that the levels of thioredoxin and thioredoxin reductase were very similar in cultured fibroblasts from control individuals and from cases of 5-oxoprolinuria and cystinosis. Glutathione deficiency did not result in a compensatory increase in either thioredoxin or thioredoxin reductase. The defective cystine reduction in cystinosis did not seem to be due

Table 1
Levels of glutathione, glutathione synthetase, thioredoxin and thioredoxin reductase in extracts of tissue cultured fibroblasts

	Source of fibroblasts		
	Control subjects	5-oxoprolinuria patients	cystinosis patients
No. individuals	5	2	3
Glutathione (nmol/mg protein)	7.6 (5.9–11.1)	1.6 (0.8– 2.2)	7.9 (5.9– 9.8)
Glutathione synthetase (nmol/h/mg protein)	27 (21 –33)	4.0 (3.4– 4.6)	25 (21 –29)
Thioredoxin (units/mg protein)	5.8 (5.3– 6.4)	6.0 (5.9– 6.1)	6.0 (5.6– 6.4)
Thioredoxin reductase (munits/mg protein)	62 (50 –84)	52 (48 –56)	56 (50 –65)

Several separate analyses of each extract and multiple batches of harvested cells were analyzed from each individual. Mean values are given and the range is indicated in parenthesis. Thioredoxin activity is expressed as units/mg protein where 1 unit corresponds to the activity of 1 μg calf liver thioredoxin activated by treatment with dithiothreitol [15]. Thioredoxin reductase activity is expressed as munits/mg protein where 1 unit is equivalent to the formation of 1 μmol thionitrobenzoate/min as defined [15]

to a general deficiency of either thioredoxin or thioredoxin reductase. The present results were obtained with whole cell extracts in vitro and cannot of course exclude a subcellular enzyme defect. However, with the present small amounts of material this was not possible to study.

Thioredoxin does not possess any enzyme activity by itself and the methods for its measurement in mammalian cells depend on coupling to disulfide reduction in a protein-like insulin. A prerequisite for this was the availability of highly purified thioredoxin reductase from calf liver, and the observation [8,15] that the thioredoxin systems of human and bovine organs show a high degree of cross-reactivity. The pretreatment of the fibroblast extracts prior to analysis served to eliminate glutathione and its reductase which will disturb the activity measurements [15].

This study is a first quantitative measurement of the thioredoxin system in human cells. Thioredoxin had been shown to be present in human platelets [8]. The levels of thioredoxin and thioredoxin reductase in the fibroblasts were similar to the corresponding levels in calf thymus [15].

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