Microinjected glutathione reductase crystals as indicators of the redox status in living cells

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Abstract The flavoenzyme glutathione reductase catalyses electron transfer reactions between two major intracellular redox buffers, namely the NADPH/$NADP^+$ couple and the 2 glutathione/glutathione disulfide couple. On this account, microcrystals of the enzyme were tested as redox probes of intracellular compartments. For introducing protein crystals into human fibroblasts, different methods (microinjection, particle bombardment and optical tweezers) were explored and compared. When glutathione reductase crystals are present in a cytosolic environment, the transition of the yellow E_{ox} form to the orange-red 2-electron reduced charge transfer form, EH_2, is observed. Taking into account the midpoint potential of the E_{ox}/EH_2 couple, the redox potential of the cytosol was found to be < -270 mV at pH 7.4 and 37°C. As a general conclusion, competent proteins in crystalline – that is signal-amplifying – form are promising probes for studying intracellular events.

Key words: Protein crystal; Glutathione reductase; Cytosol; Biolistic crystal transfer; Microinjection; Redox potential

1. Introduction

Numerous biological processes such as glycolysis, coenzyme-A-dependent reactions, and synthesis of deoxyribonucleotides depend on free SH-groups. Consequently, a reducing milieu favoring the presence of thiols must prevail in the cytosol [1,2]. The predominant enzyme maintaining a high thiol/sulfenate and high thiol/disulfide ratio in extant eukaryotic cells is the homodimeric 105 kDa flavoprotein glutathione reductase (GR). This enzyme which catalyzes the reaction $NADPH+GSSG+H^+ \rightarrow NADP^+ +2$ GSH also represents a major protective principle against the effects of reactive oxygen species [3].

As shown by crystallographic [4,5] and spectroscopic studies [6,7] the active site structure and, as a consequence, the color of glutathione reductase depend on the redox potentials of the dominating cytosolic redox buffers, namely NADPH/$NADP^+$ and 2 GSH/GSSG. The catalytic cycle of GR has been studied both in solution [6,7] and in crystals [8,9]. The cycle starts with the so-called E_{ox} form of the enzyme which is characterized by oxidized flavin and a disulfide (Cys-63-Cys-58) at the catalytic site. Reduction with two electrons leads to EH_2, a stable intermediate of the enzyme, in which Cys-58 is present as a free thiol while the thiolate of Cys-63 forms a charge-transfer complex with the flavin [10–12]. This CT complex gives rise to a new absorption band around 540 nm corresponding to a color change of the enzyme from yellow to orange-red. In thiol:disulfide interchange reactions among the active site dithiol and glutathione disulfide, the latter is reduced to give 2 GSH molecules while E_{ox} is reformed. EH_2 can be generated by physiologic concentrations of either NADPH or GSH. When excess NADPH is present, EH_2 forms a complex with NADPH which further increases the absorbance at 540 nm.

The redox sensitivity of the enzyme suggested to us that glutathione reductase crystals might be suitable indicators for the relevant redox potential of the cytosol [13]. The three major steps required to substantiate this hypothesis – production of suitable and stable crystals, transfer of the crystals into living cells, and detection of the expected color change – are described.

2. Materials and methods

2.1. Crystals

Recombinant human glutathione reductase was isolated and assayed as described [13,14]. Since GR crystals grown in the presence of $(NH_4)_2SO_4$ are not stable in the cytosol, that is, under conditions where stabilizing precipitants and mother liquor are absent, a method for crystallizing GR at low ionic strength was developed. Ten mg/ml enzyme was dialysed exhaustively at 4°C against 50 mM ammonium bicarbonate, pH 7.0, and then for 24 h against 30 mM, 10 mM and 5 mM NH_4HCO_3, respectively, at a constant pH of 7.0. To allow crystal growth the dialysis buttons were kept in 5 mM NH_4HCO_3. The resulting crystals were harvested and stored in water; before use, they were filtered through Whatman membranes in order to obtain particles of defined sizes; crystal dimensions were measured under the microscope.

For estimating enzyme activity, a suspension of crystals was assayed by spectroscopically monitoring the GSSG-dependent consumption of NADPH [13,14]. Between individual measurements the suspension was manually shaken. To test for intermolecular cross-linking among GR dimers, crystals were dissolved in 0.1 M Tris-HCl, pH 8.7 containing 0.2% sodium dodecyl sulfate. Subsequent polyacrylamide electrophoresis revealed 70% dimeric enzyme (bridged by the intersubunit disulfide Cys-90-Cys-90′ [5]) and $< 30\%$ higher aggregates.

The crystals were found to be stable – also at 25°C and 37°C – for hours in PBS-based quasi-physiological solutions, in cell culture media, as well as in living cells. In order to test the redox sensitivity of glutathione reductase, particles ranging in size from 10 to 1000 μm^3 were incubated at 37°C in solutions having a quasi-physiologic redox milieu. For instance, addition of 100 μM NADPH, 1.25 μM $NADP^+$, and 2 mM GSH in PBS to yellow oxidized GR crystals of 100 μm^3 led to the characteristic deep orange color of the reduced enzyme within

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Abbreviations: CHO cells, Chinese hamster ovary cells; CT, charge transfer; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide; PBS, phosphate-buffered saline of pH 7.4

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2 min; addition of 3 mM serum albumin representing a physiologic protein concentration of 20% had no influence on rate and extent of this color change (Fig. 1).

2.2. Crystal transfer into human fibroblasts

To manipulate GR crystals into living cells we applied and compared three different methods: microinjection, biolistic particle transfer and optical tweezers.

2.3. Microinjection and localization of intracellular crystals

The AIS system from Zeiss was used [15]. In order to obtain a homogeneous suspension of injectable microcrystals, the stock suspension was filtered through 2 μm Whatman membranes. During injection the cells – human fibroblasts or CHO cells grown on glass coverslips – were kept under 20 mM HEPES, pH 7.4, at 37°C. Crystals of <10 μm^3 were successfully injected into both cell types. (Larger crystals required rather big injection capillaries which often destroyed the cells.) Cells were mounted in Petri dishes on a motorized scanning stage and observed under an inverted microscope (Axiovert 10, Zeiss). Images were acquired with a color CCD video camera (DXC-101P, Sony) and stored on video tape or digitalized with a real time video board (DVS 3000, Hamamatsu) before storage on a PC. Real time observation of the cells was possible by video monitors. Moving of the stage and microinjection were computer-controlled.

The intracellular localization of the crystals was subsequently confirmed by immunostaining. For this purpose cells were fixed with 3% formaldehyde, washed twice in phosphate-buffered saline and permeabilized with 0.1% Triton X-100. Staining was carried out with rabbit (anti-hGR) serum and fluorescein-5-isocyanate-conjugated mouse anti-(rabbit-IgG) antibodies. Pictures of immunostains were taken with a cooled slow scan CCD camera (Photometrics CH250), digitalized and stored on a SUN workstation for further processing and printing. By this procedure GR crystals were clearly marked and homogeneous background staining was obtained (Fig. 2).

2.4. Biolistic particle transfer

The PDS-1000/He device [16,17] was used. The cell culture medium was removed before the biolistic process, and the cells were kept under vacuum (−0.6 bar). Crystals were mounted on a Kapton membrane and accelerated by different pressures towards the cells. Immediately after the bombardment, the cells received fresh medium. Using this method, it was possible to insert larger GR crystals (>10 μm^3) into human fibroblasts (Fig. 3). Best results were achieved with an acceleration pressure of 6 bar, a distance between cell surface and mesh of 5 cm, and a target distance of 2 cm. The cells survived the bombardment procedure; the crystals changed their color to dark orange and remained in that form for hours indicating that the cellular reducing milieu was not drastically disturbed.

2.5. Optical tweezers

To introduce GR crystals into cells with optical tweezers we applied a combined laser-microscope system [18,19] consisting of an Nd:Yag lasertrap, a pulsed UV nitrogen laser, and an inverted microscope equipped with a color CCD video camera. The lasers were focused through objectives with high numerical aperture and could be attenuated continuously. As crystals could be moved more readily in protein-free buffer we replaced the cell culture medium by PBS prior to crystal manipulation. GR microcrystals (25–100 μm^3) were added to the cell culture and caught in the Nd:Yag lasertrap at 100 mV; they were moved easily and steered into the cytosol when the laser energy was increased to 200 mW in the cytosol. Crystal transition through the cell membrane was made possible by punching the membrane with a single shot of the nitrogen laser.

3. Results and discussion

Vis-à-vis an oxidizing environment, the cytosol of aerobic organisms requires a reducing milieu for its basic metabolic reactions. As alterations of intracellular redox homeostasis are known to occur in stressed or apoptotic cells [1,2,13] and in various pathological conditions such as infection or cancer there is a need for methods which allow to monitor the redox status of intracellular compartments. Here we report on crystals of the flavoenzyme glutathione reductase as indicators of the redox milieu in living cells. Crystals grown in the presence of 5 mM ammonium bicarbonate of pH 7.0 were found to be suitable as they do not disintegrate in the absence of mother liquor.

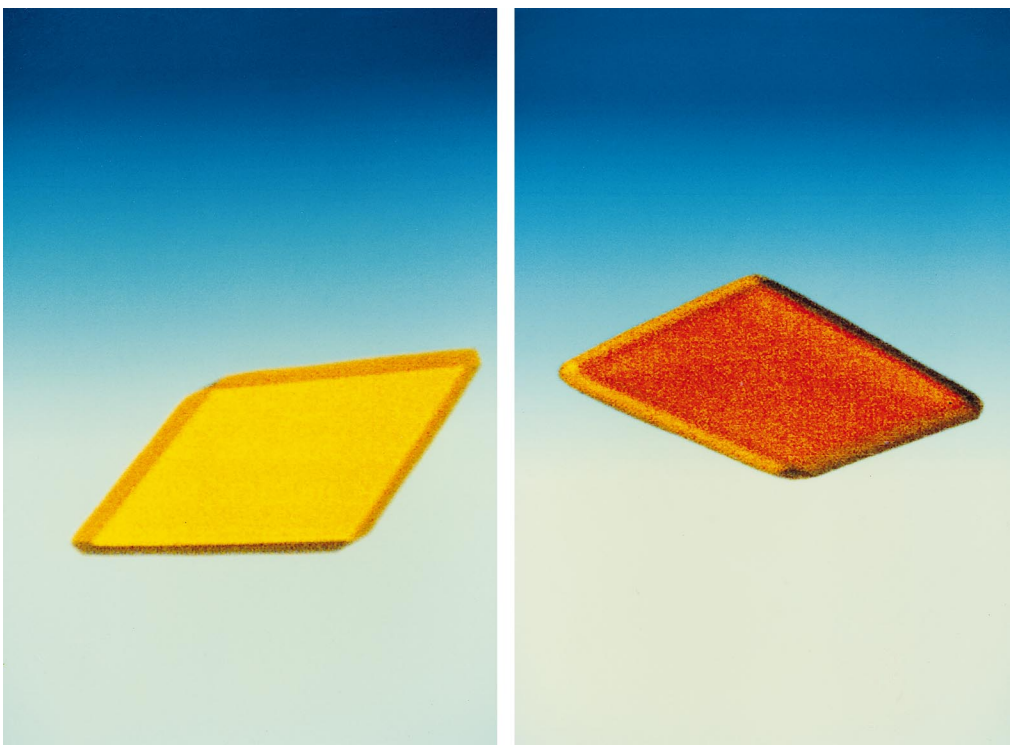

Fig. 1. A glutathione reductase crystal (300×) in oxidized (left) and reduced (right) form. For reduction, the oxidized crystal was transferred from water to PBS containing 5 mM GSH, 100 μM NADPH and 3 mM bovine serum albumin.

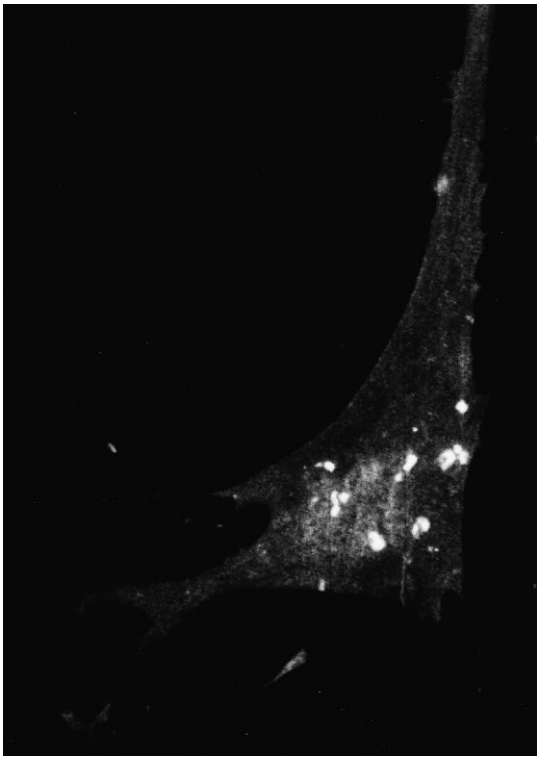

Fig. 2. Human fibroblast after microinjection of GR crystals (600×). The crystals were located by fluorescent immunostaining.

When the crystals were dissolved in a denaturing solution, subsequent polyacrylamide electrophoresis revealed less than 30% protein aggregates. Thus it is unlikely that the stability of the crystals is due to spontaneous cross-linking among the constituent GR molecules. It should be pointed out, however, that the introduction of chemical cross-links could serve as a general procedure for stabilizing protein crystals which are to be used as in vivo probes.

In the GR crystals, color and absorption spectrum of oxidized (E_{ox}) and reduced (EH_2) glutathione reductase are clearly distinguishable; both forms of the protein represent stable enzyme species (Fig. 1). Absorption changes of the protein caused by substrate turnover can be neglected since only the outer molecular layer of the crystal appears to be effective in catalysis. Alternatively, one could consider enzyme mutants which bind substrates but do not turn them over – a case in point being the GR mutant H467F [9].

Using different techniques, crystals of oxidized GR were manipulated into human fibroblasts. Particle bombardment was most efficient for crystal transfer into a large number of cells at the same time. When studying selected cells, microinjection (for crystals of less than 10 μm^3), or optical tweezers (for crystals of 25 to 100 μm^3) were found to be appropriate methods [20]. Using the latter technique, the protein crystal could be kept in the focus of the Nd:Yag lasertrap at a remarkably low energy (100 mW). For moving the crystal towards the target cell, the laser energy was doubled.

The expected color change of glutathione reductase crystals

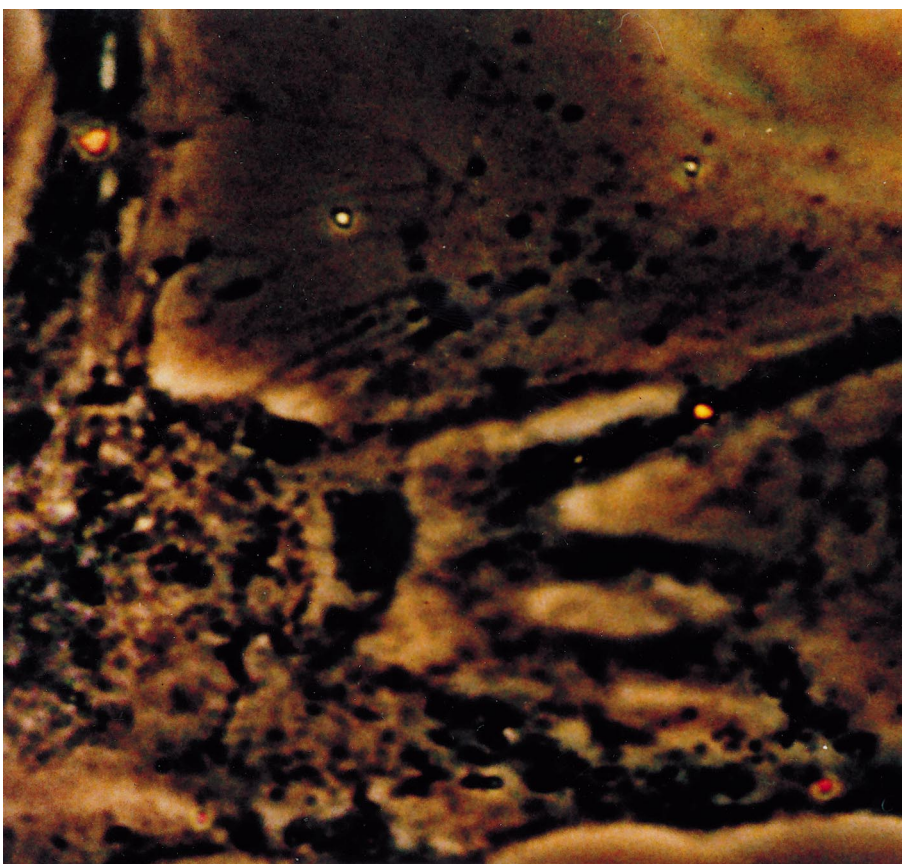

Fig. 3. Human fibroblast (600×) 3 min after successful introduction of GR crystals by particle bombardment. Focus is on the upper membrane. A small fragment is already fully reduced (lower right-hand corner) whereas it took longer for a larger intracellular crystal (upper left-hand corner). Two small crystals were caught on the cell membrane (upper third) and therefore remained in the yellow E_{ox} form.

from yellow (E_{ox}) to dark orange (EH_2 and EH_2-NADPH complex) was detectable when the crystals reached the reducing cytosolic environment. The colors of intracellular crystals (Fig. 3) were compared visually with crystals of equal size incubated in N_2-flushed PBS solutions containing either 5 mM GSH and varying concentrations of GSSG or 20 μM $NADP^+$ and varying concentrations of NADPH as redox buffers. According to these comparisons the redox potential at 37°C and pH 7.4 in the cytosol is less negative than −270 mV. When accounting for temperature (−1.3 mV/°C) and pH (−40 mV/pH unit) [21], this estimation is consistent with the midpoint potential of −229 mV determined for the E_{ox}/EH_2 redox couple at 20°C and pH 7.0 [7,12].

As shown in Fig. 2, the intracellular localization of microinjected crystals was confirmed by immunostaining. In order to assess the possible biochemical perturbations an injected GR crystal could cause in a cell, the following estimations (see [3] for a review) might be useful. A GR microcrystal of 1 μm^3 (corresponding to 1 fl) contains approximately 10 amol FAD corresponding to 5 amol dimeric enzyme. If such a crystal is injected into a cell with a cytosolic space of 30 pl, the GR concentration would be increased by 5 amol/30 pl which is by approximation 200 nmol/l. Although this value corresponds to typical intracellular GR concentrations (50 to 500 nM), it is unlikely that the enzyme activity of the crystals represents a perturbation since it just adds to the enormous spare capacity of GR activity observed in cytosolic spaces [3]. The amount of NADPH or glutathione sequestered by a 1 μm^3 GR crystal would alter the cytosolic concentrations of these compounds (100 μM and 2–5 mM, respectively) by less than 1%.

In conclusion, glutathione reductase crystals might become a useful tool for studying the redox status of the cytosol and of compartments such as the endoplasmic reticulum [22]. Mechanisms of drug action as well as (patho)physiologic alterations of antioxidant status, occurring e.g. in apoptotic or parasitized cells [23,24], could be monitored. As a case in point, when reduced glutathione is exposed to nitrosative stress represented by *S*-nitrosoglutathione and other physiologic NO-carriers, EH_2 is oxidized at Cys-63; this results in a yellow enzyme species with an absorption spectrum resembling E_{ox} [25]. Work is in progress to quantitate the speed and extent of the color change of intracellular GR crystals by absorption spectroscopy.

One may also consider to employ microinjected crystals of other proteins, such as catalase or myoglobin, as dynamic indicators of intracellular functions. The protein represents the authentic sensor, and the crystalline state guarantees the amplification needed for signal detection. A prerequisite is, of course, that the signal-yielding compounds have access to the solvent channels of the crystal.

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