The structure of a reduced mutant T4 glutaredoxin

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Abstract The mutant T4 glutaredoxin where the active site residues Val[15] and Tyr[16] have been substituted by Gly and Pro, respectively, crystallizes in a form where the active site disulfide is accessible to reagents. Treatment of the crystals with dithiotreitol causes very subtle changes in the overall glutaredoxin structure. The main differences are seen around the active site where the sulfurs of Cys[14] and Cys[17] move apart slightly.

Key words: Glutaredoxin; Redox-active thiols; Disulfide reduction; Crystallography; Mutant protein

1. Introduction

Ribonucleotide reductase, a key enzyme in DNA synthesis, is responsible for the de novo synthesis of the necessary deoxyribonucleotides. In this reaction, thiol groups of the enzyme are oxidized to form a disulfide bridge which has to be reduced for the next catalytic cycle. There are at least two systems in cells which are able to reduce ribonucleotide reductase: the thioredoxin and glutaredoxin systems [1]. Thioredoxins and glutaredoxins are small α/β proteins with redox active thiols. Both systems use NADPH-dependent flavoproteins for the regeneration of reduced thioredoxin and glutaredoxin. Thioredoxin is reduced by thioredoxin reductase while glutaredoxin is reduced by glutathione, which has previously been reduced by glutathione reductase.

When bacteriophage T4 infects *Escherichia coli*, a bacteriophage encoded ribonucleotide reductase and a glutaredoxin are synthesized for the production of bacteriophage DNA. This T4 glutaredoxin can use both reducing systems for its regeneration. It was originally called T4 thioredoxin since it is reduced by thioredoxin reductase, but sequence comparisons clearly show that the T4 protein belongs to the glutaredoxin family [2].

The three-dimensional structure of T4 glutaredoxin has a central pleated sheet with both parallel and antiparallel strands surrounded by helices [3,4]. *E. coli* glutaredoxin [5] and thioredoxin [6,7] as well as human thioredoxin [8], have structures similar to T4 glutaredoxin except that the thioredoxins have an additional β-strand and α-helix at their N-terminus. The active site disulfide is located at equivalent positions at the amino-end of the longest helix in all these proteins.

All crystallographic investigations of thioredoxins and glutaredoxins have so far been performed on oxidized disulfide forms of the proteins. Direct reduction of the crystals has not been possible since both *E. coli* thioredoxin and T4 glutaredoxin were crystallized with help of metal ions which link molecules together in layers. All attempts to reduce these crystallized proteins resulted in destruction of the crystals since the metals are reduced and/or removed.

NMR investigations have recently given a detailed picture of what happens when the human and *E. coli* thioredoxins are reduced [8,9]. The overall structures of the reduced thioredoxins are similar to those of oxidized thioredoxins, but differ in the local conformation of the active site cysteines. In the *E. coli* thioredoxin, the side chain sulfur atom of Cys[32] is tilted away from that of Cys[35] in the reduced form [9]. In human thioredoxin, Cys[35] is turned away in the reduced form, while Cys[32] remains in a similar position in the two forms [8]. Residues 9–12 in the active site of oxidized *E. coli* glutaredoxin is part of a locally disordered region which makes the detailed comparison of the residues at the active site difficult [10].

In order to investigate the role of the active site residues of T4 glutaredoxin, a series of mutant proteins were produced [2,11,12]. The double mutant Val[15]→Gly and Tyr[16]→Pro crystallized in a new crystal form. These crystals diffract much better than the wild-type crystals which made possible a structure determination to higher resolution (1.45 Å) [4]. The mutant was crystallized in the absence of metals, in contrast to wild-type crystals which were obtained in presence of cadmium. This has now given us a possibility to obtain reduced crystalline glutaredoxin. In these orthorhombic crystals, the main crystal interaction is formed between the amino end of the last helix and the carboxyl end of the same helix in another molecule [4]. The crystal packing leaves the active site accessible for reagents. We here report the differences between the reduced and oxidized mutant glutaredoxin determined from crystallographic investigations of this crystal form.

2. Experimental

The isolation of the glutaredoxin gene, mutagenesis and overproduction of the mutant protein have been described by Joelson et al. [11]. Crystals were obtained by the hanging drop method using 50 mM MES buffer, pH 6.5, and 5% PEG 4000 as precipitant. The crystals are orthorhombic $P2_12_12_1$ with cell dimensions a = 30.2 Å, b = 47.8 Å and c = 61.3 Å and with one molecule in the asymmetric unit. The structure of the mutant protein was solved by molecular replacement methods and has been refined to an R-value of 17.5% at 1.45 Å resolution [4].

Crystals were reduced by soaking in 50 mM dithiotreitol in 50 mM MES, pH 6.0, for 2 h. A data-set was collected on one crystal using a Xentronics area detector (Nicolet) mounted on a Rigaku rotating anode. The data was evaluated using the BUDDHA program [13]. The obtained data-set extends to 2.4 Å resolution and contains 80% of all possible reflections to this resolution. The dataset was scaled using the CCP4 programs (Daresbury, UK). The internal R_{sym} for symmetry related reflections was 5.4%. The data set from the reduced crystals scaled to a dataset of the oxidized glutaredoxin mutant with 10.2% differences in Fs.

The structure was refined with the XPLOR program [14] using pos-

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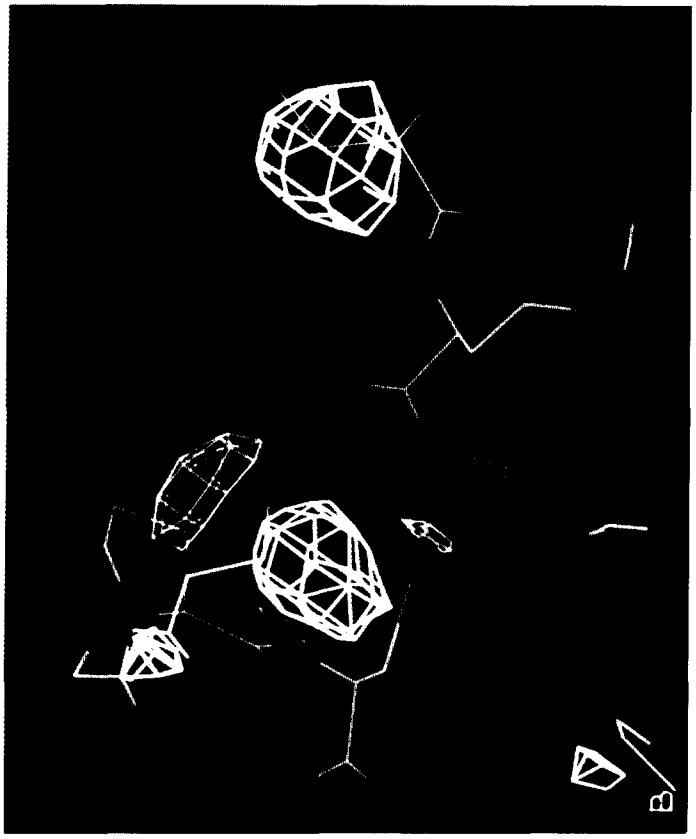
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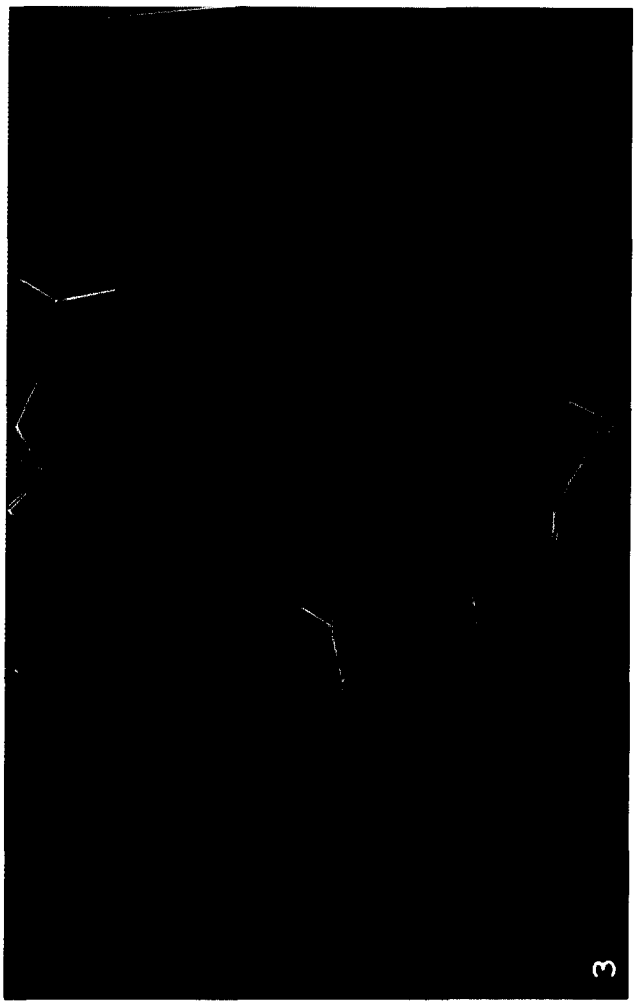
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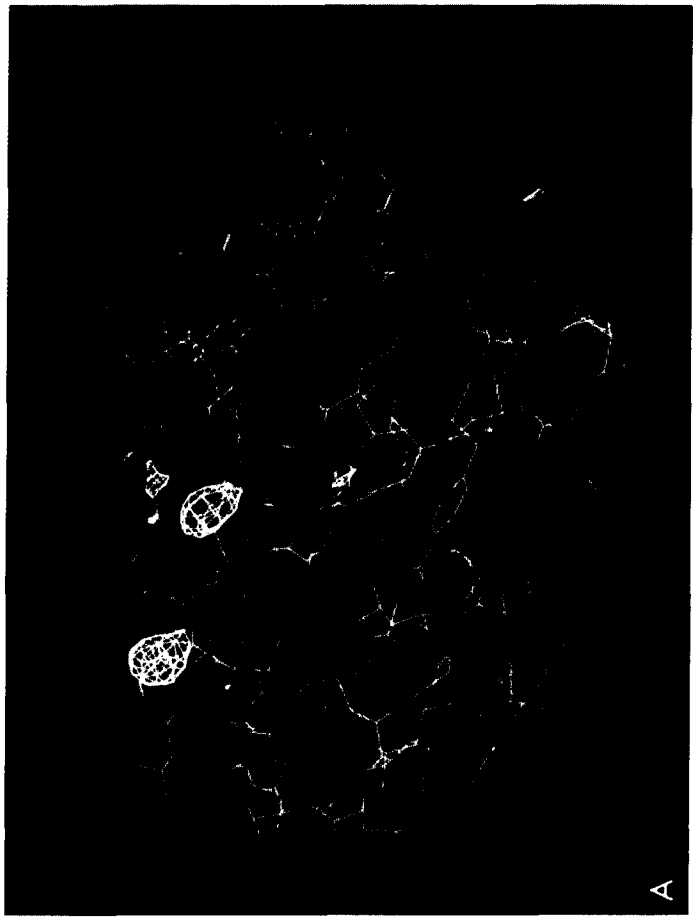
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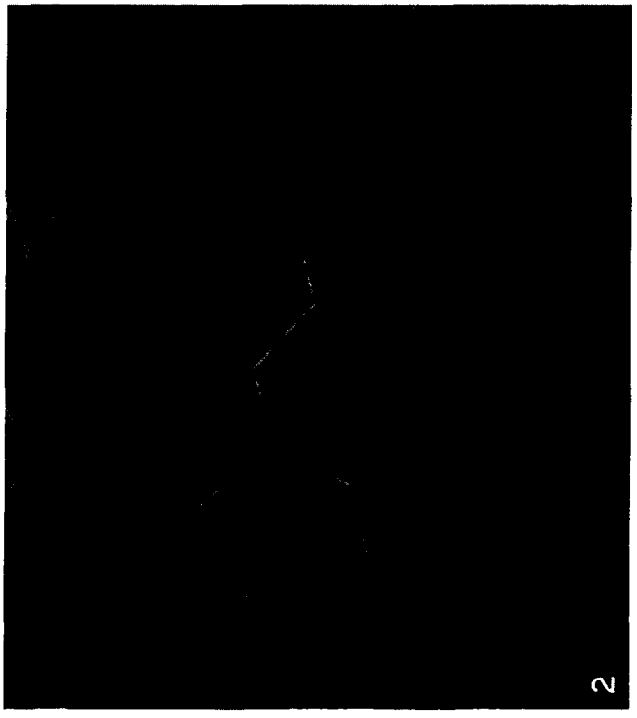
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itional and temperature factor refinement. The starting coordinates were from the refined oxidized glutaredoxin [4]. In this model the active site cysteines were changed according to the $F_{red}-F_{ox}$ difference Fourier map before refinement. The final R-value for the refinement of the reduced glutaredoxin mutant is 14.3% for the reflections between 7.0 and 2.4 Å.

3. Results

The $F_{red}-F_{ox}$ difference Fourier map shows two large negative peaks, a few smaller negative peaks and a few positive peaks (Fig. 1). As expected, one of the major negative peaks occur at the place of the former disulfide bridge with the largest positive peak outside the sulfur atom of Cys^{14} indicating that the side chain of this residue has turned outward towards the solvent. A small positive density beside the sulfur atom of Cys^{17} indicate that the cysteine residue has turned slightly inward. There was also small negative density on the main chain of Gly^{15} and Pro^{16} suggesting a small displacement of these residues or higher mobility. Small positive-negative peaks around Phe^{33} and Lys^{21} show that these residues close to the active site also experience small positional shifts. Met^{1} and Met^{37} also have small positive densities.

One of the largest negative peaks in the difference Fourier map was located outside the protein. After analysis of the corresponding position in the oxidized T4 glutaredoxin we interpreted it to be the sulfate part of a MES buffer molecule (Fig. 2). In the refined map of the oxidized glutaredoxin the density corresponding to the sulfate is clear and one of the oxygen atoms makes hydrogen bonds to the main chain nitrogen of residue 64, to the OH group of the side chain of Tyr^{7} and a water molecule [4]. This molecule is bound about 6 Å from the disulfide bridge of the active site.

The refined structure of the reduced glutaredoxin confirms the observations made from the difference Fourier map. The overall structure is little affected by the reduction and the root mean square difference for all atoms is only 0.1 Å. The only change larger than 0.5 Å is found for the Sγ atom of Cys^{14} which differs by 0.6 Å in the two structures (Fig. 3).

4. Discussion

Our experiment shows that mutant T4 glutaredoxin can be reduced in the crystalline state. The amount of remaining oxidized glutaredoxin molecules is very small as judged from the height of the difference electron density maps calculated from the reduced protein at the end of the refinement and the oxidized protein at the start.

The T4 glutaredoxin accommodates the breaking of the disulfide bridge by moving the exposed sulfur atom away from the other and keeping the internal cysteine side chain approximately in the same place as in the oxidized form. The limited conformational changes seem to be compatible with reduction of *E. coli* glutaredoxin as investigated by NMR methods [10]. Similar types of conformational changes take place in both human and *E. coli* thioredoxins and in glutathione reductase when their active site disulfide bridge is reduced [8,9,15].

The reason why the MES buffer molecule does not bind to the reduced form of glutaredoxin may be that the negative charge of the reduced Cys^{14} repels the negative charged sulfate of the buffer molecule. The pK_a of the cysteine residues has not been determined but investigations on the *E. coli* glutaredoxin indicate that the pK_a of the corresponding residue is lower than 5 [17,18]. Similarly, the corresponding residue in a mammalian glutaredoxin has been suggested to have a pK_a of 2.5 [19]. For the *E. coli* thioredoxin the corresponding residue has a pK_a value of 6.7 [20].

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References

[1] Holmgren, A. (1989) J. Biol. Chem. 264, 13463–13460.
[2] Nikkola, M., Gleason, F., Saarinen, M., Joelson, T., Björnberg, O. and Eklund, H. (1991) J. Biol. Chem. 266, 16105–16112.
[3] Söderberg, B.-O., Sjöberg, B.-M., Sonnerstam, U. and Brändén, C.-I. (1978) Proc. Natl. Acad. Sci. USA 75, 5827–5830.
[4] Eklund, H., Ingelman, M., Söderberg, B.-O., Uhlin, T., Nordlund, P., Nikkola, M., Joelson, T. and Petratos, K. (1992) J. Mol. Biol. 228, 596–618.
[5] Sodano, P., Xia, T.-H., Bushweller, J.H., Björnberg, O., Holmgren, A., Billeter, M. and Wüthrich, K. (1991) J. Mol. Biol. 221, 1311–1324.
[6] Holmgren, A., Söderberg, B., Eklund, H. and Brändén, C.-I. (1975) Proc. Natl. Acad, Sci. USA 72, 2305–2309.
[7] Katti, S., LeMaster, D.M. and Eklund, H. (1990) J. Mol. Biol. 212, 167–184.
[8] Qin, J., Clore, G.M. and Gronenborn, A.M. (1994) Structure 2, 503–522.
[9] Jeng, M.-F., Campbell, A.P., Begley, T., Holmgren, A., Case, D.A., Wright, P.E., and Dyson, H.J. (1994) Structure 2, 853–868.
[10] Xia, T.-H., Bushweller, J.H., Sodano, P., Billeter, M., Björnberg, O., Holmgren, A. and Wüthrich, K. (1992) Protein Sci. 1, 310–321.
[11] Joelson, T., Sjöberg, B.-M. and Eklund, H. (1990) J. Biol. Chem. 265, 3183–3188.
[12] Nikkola, M., Gleason, F. and Eklund, H. (1993) J. Biol. Chem. 268, 3845–3849.
[13] Blum, M., Metcalf, P., Harrison, S.C. and Wiley, D.C. (1987) J. Appl. Crystallogr. 20, 235–242.
[14] Brünger, T.A., Kuriyan, J. and Karplus, M. (1987) Science 235, 458–46.
[15] Karplus P.A., Pai, E.F. and Schulz, G.E. (1989) Eur. J. Biochem. 178, 693–703.
[16] Eklund, H., Cambillau, C., Sjöberg, B.-M., Holmgren, A., Jörnvall, H., Höög, J.-O. and Brändén, C.-I. (1984) EMBO J. 3, 1443–1449.
[17] Björnberg, O. (1990) Thesis, Karolinska Insitutet, Stockholm, Sweden.
[18] Sandberg, V., Kren, B., Fuchs, J.A. and Woodward, C. (1991) Biochemistry 30, 5475–5484.
[19] Gan, Z.-R. and Wells, W.W. (1987) J. Biol. Chem. 262, 6704–6797.
[20] Kallis, G.-B. and Holmgren, A. (1980) J. Biol. Chem. 255, 10261–10265.

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Fig. 1. (A, top left) Difference Fourier ($|F_{red}|-|F_{ox}|$) contoured at a $\pm 3\sigma$ level for the part corresponding to one glutaredoxin molecule. Positive densities are magenta and negative densities are white. The cysteines are located in the middle of the bottom of the picture with the MES molecule at the bottom right. (B, top right) The part corresponding to the active site area with the cysteines to the left and the MES molecule to the right.
Fig. 2. The position where a MES buffer molecule was interpreted in the electron density map of oxidized crystals. (Positively contoured map at the 1σ level).
Fig. 3. The final model of the active site of reduced glutaredoxin after refinement with the corresponding part of the oxidized glutaredoxin. The main difference is that the cysteine sulfur atoms move apart upon reduction.