Crystal structure of the wild-type and D30A mutant thioredoxin *h* of *Chlamydomonas reinhardtii* and implications for the catalytic mechanism

Valeria MENCHISE*†, Catherine CORBIER*¹, Claude DIDIERJEAN*, Michele SAVIANO†, Ettore BENEDETTI†, Jean-Pierre JACQUOT‡ and André AUBRY*

*Laboratoire de Cristallographie et Modélisation des Matériaux Minéraux et Biologiques, Groupe Biocristallographie, ESA 7036, Université Henri Poincaré-Nancy I, BP 239, 54506 Vandoeuvre-lès-Nancy Cedex, France, †Centro di Studio di Biocristallografia del CNR, Dipartimento di Chimica, Via Mezzocannone 4, 80134 Napoli, Italy, and ‡Interactions Arbres Microorganismes, UMR 1136 INRA, Université Henri Poincaré-Nancy I, BP 239, 54506 Vandoeuvre-lès-Nancy Cedex, France

Thioredoxins are ubiquitous proteins which catalyse the reduction of disulphide bridges on target proteins. The catalytic mechanism proceeds via a mixed disulphide intermediate whose breakdown should be enhanced by the involvement of a conserved buried residue, Asp-30, as a base catalyst towards residue Cys-39. We report here the crystal structure of wild-type and D30A mutant thioredoxin *h* from *Chlamydomonas reinhardtii*, which constitutes the first crystal structure of a cytosolic thioredoxin isolated from a eukaryotic plant organism. The role of residue Asp-30 in catalysis has been revisited since the distance between the carboxylate OD1 of Asp-30 and the sulphur SG of Cys-39 is too great to support the hypothesis of direct proton transfer. A careful analysis of all available crystal structures

reveals that the relative positioning of residues Asp-30 and Cys-39 as well as hydrophobic contacts in the vicinity of residue Asp-30 do not allow a conformational change sufficient to bring the two residues close enough for a direct proton transfer. This suggests that protonation/deprotonation of Cys-39 should be mediated by a water molecule. Molecular-dynamics simulations, carried out either *in vacuo* or in water, as well as proton-inventory experiments, support this hypothesis. The results are discussed with respect to biochemical and structural data.

Key words: molecular dynamics, proton inventory, X-ray structure.

INTRODUCTION

Thioredoxins are ubiquitous small globular proteins (100–120 residues) containing an extremely reactive disulphide bridge with a highly conserved sequence WC(G/P)PC. In bacteria and animal cells, thioredoxins participate in multiple reactions which require reduction of disulphide bonds on selected target proteins [1]. In plants, thioredoxins constitute a large multigenic family with different subcellular locations. The chloroplasts of higher plants contain at least two types of thioredoxin: *f*, which efficiently activates fructose-1,6-bisphosphatase and phosphoribulokinase, and *m*, which activates the NADP-malate dehydrogenase and deactivates glucose-6-phosphate dehydrogenase. Photosynthetic and non photosynthetic plants also contain a cytosolic thioredoxin, called *h* for heterotrophic [2–4]. Thioredoxins belong to a wider group of disulphide oxidoreductases that either reduce or oxidize disulphide bonds. The redox potentials of these catalysts depend on both the nature of the amino acid present between the two cysteine residues and the surrounding amino acid residues [5]. Protein disulphide isomerases, or DsBs, exhibit a CGHC sequence, while glutaredoxin possesses a CPYC active site [6,7]. It has been shown that changing the CGPC site of thioredoxin to CGHC results in the protein behaving more as an oxidant, with an alteration of its redox potential to more positive values [5].

X-ray structures have been reported for both reduced and oxidized forms of human thioredoxin [8]. Oxidized forms were

also reported for *Escherichia coli* thioredoxin [9,10] and for an unusual thioredoxin (trx2) isolated from the photosynthetic cyanobacterium *Anabaena* [11], which closely resembles the prokaryotic *E. coli* structure despite being only distantly related and having some functional differences. In addition, X-ray structures for two chloroplastic thioredoxins, the *m* and *f* isoforms, have been published recently [12]. NMR structures are also available for human [13], *Escherichia coli* [14] and *Bacillus acidocaldarius* [15] thioredoxins as well as for the *h*-type thioredoxin from the green alga *Chlamydomonas reinhardtii* [16]. Very recently, the NMR structure of the chloroplastic *C. reinhardtii* thioredoxin *m* has also been published [17].

Whatever the group or organism they belong to, the catalytic efficiency of thioredoxins is based on the two redox-active Cys residues that undergo a two-electron oxidation to form a mixed disulphide bond. Each Cys of the active site plays a specific role: a nucleophilic attack by the thiolate of Cys-32 (*E. coli* numbering) breaks the disulphide bridge of the target protein, forming a disulphide bridge with the more electrophilic sulphur leaving the other as a thiolate. This transitory intermolecular disulphide bridge is then broken by Cys-35, leaving the target protein reduced and releasing thioredoxin in the oxidized form [18–21]. However, the chemical basis of the catalytic mechanism is not fully understood. Several invariant amino acids such as Asp-26, Trp-31 or Lys-57 (*E. coli* numbering) are of particular interest and could be directly involved in catalysis [19,21,22].

Abbreviations used: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); r.m.s., root mean square; WT, wild-type; Fo, observed structure factor amplitude; Fc, calculated structure factor amplitude.

¹ To whom correspondence should be addressed (e-mail corbier@lcm3b.u-nancy.fr).

² The atomic co-ordinates for wild-type and D30A mutant thioredoxin have been deposited in the RCSB Protein Data Bank under the accession codes 1EP7 and 1EP8, respectively.

Considerable interest has been focused on the titrating groups of the active site, including the side chain of Asp-26 and its influence on the pK_a values of the two active-site cysteinyl residues. Although it is usually admitted that the pK_a of Cys-32 is close to physiological pH [21], the pK_a value of Cys-35 as well as the pK_a value of Asp-26 in reduced thioredoxin are still a matter of debate. In oxidized thioredoxins, the two active-site Cys residues form a disulphide bridge and Asp-26 titrates unambiguously with a pK_a value of 7.5 and 7.0 for *E. coli* [23] and *C. reinhardtii* [16,17] thioredoxins, respectively. However, in reduced thioredoxins, difficulties due to the proximity of three titrating groups in the active site arise and several pK_a values have been proposed for Cys-35 and Asp-26. In particular, pK_a values of around 7.5 have been reported for the Asp residue by Jeng and Dyson [24] and Vohnik et al. [25], whereas pK_a assignments equal to or higher than 9 have been proposed by Wilson et al. [26] and Jeng et al. [27]. Nonetheless, in all X-ray or NMR structures available, the Asp side chain is buried, which could explain its high pK_a value. It was proposed that the unusually weak acidity of Asp-26 could account for the lower stability of the reduced protein at high pH [28] and that the redox potential of *E. coli* thioredoxin might be modulated by Asp-26 since reduction potentials in thioredoxin-related enzymes are linked to the relative stability of the reduced and oxidized forms. Furthermore, recent kinetic studies of Chivers and Raines [29] and LeMaster et al. [30] carried out on Asp-26 mutants led to the conclusion that Asp-26 acts as a general acid/base catalyst in the thiol–disulphide interchange reaction. However, it cannot be ruled out that the amino acid substitution produces structural perturbations beyond the active site which would be the true cause of the reduced biological activity found for these mutants. Indeed, Dyson et al. [21] and Vohnik et al. [25] concluded that the replacement of this Asp by Ala in *E. coli* thioredoxin (D26A, *E. coli* numbering) leads to conformational disorders near the active site. In order to examine this possibility and with the aim to better understand the implication of these residues in catalysis, we have also solved the X-ray structure of the wild-type (WT) thioredoxin *h* of *C. reinhardtii* and the structure of a mutant in which the essential Asp-30 (*C. reinhardtii* numbering) has been replaced by an alanine. The comparison of the structural data between the WT and the mutant proteins, in conjunction with molecular-dynamics simulations and proton-inventory experiments allows us to conclude that Asp-30 should not interact directly with the side chain of Cys-39 and to propose the involvement of a water molecule for the proton transfer between these two residues.

EXPERIMENTAL

Overexpression, purification and site-directed mutagenesis

Cloning, overexpression and purification of recombinant thioredoxin *h* has already been reported in Stein et al. [31]. The D30A mutagenesis was carried out by PCR essentially as described in Krimm et al. [22].

Crystallization and data collection

Crystals of WT thioredoxin and the D30A mutant (18 mg/ml) were obtained at room temperature using the hanging-drop vapour-diffusion technique. They were grown from a solution composed of 10 % (w/v) 8 kDa poly(ethylene glycol) and 10 % (w/v) 10 kDa poly(ethylene glycol) in 0.1 M sodium cacodylate buffered at pH 6.5. Crystals of the WT and D30A mutant appeared after a few weeks and grew as trigonal sticks in space

Table 1 Crystal and data-collection parameters and refinement statistics

r.m.s.d., root mean square deviation; *a* and *c* are the unit cell parameters in the space group P3₁21; *Z* is the number of molecules in the unit cell. $R_{\text{merge}} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$, where *I* is the intensity of reflection. $\langle I \rangle$ is the ratio of the mean observed intensity to the mean standard uncertainty of the observed intensity. For completeness, R_{merge} and $1/\sigma(I)$ values the outer resolution shells are given in parentheses.

Crystal	WT thioredoxin	D30A mutant thioredoxin
Data collection		
Space group	P3 ₁ 21	P3 ₁ 21
<i>a</i> = <i>b</i> (Å)	49.645	49.692
<i>c</i> (Å)	145.312	145.551
<i>Z</i>	12	12
Resolution (Å)	2.1	2.2
Temperature (K)	298	298
Total reflections	42095	53351
Unique reflections	12186	10823
Completeness (%)		
Overall	95.1	96.4
Outermost data shell	92.6 (2.17–2.1 Å)	91.6 (2.28–2.2 Å)
R_{merge}		
Overall	0.062	0.047
Outermost data shell	0.301 (2.17–2.1 Å)	0.17 (2.28–2.2 Å)
$1/\sigma(I)$		
Overall	15.9	26.8
Outermost data shell	3.5 (2.17–2.1 Å)	6.2 (2.28–2.2 Å)
Refinement		
Resolution range (Å)	20.0–2.1	20.0–2.2
<i>R</i> -factor (%)	20.4	19.6
<i>R</i> -free (%)	25.2	22.0
r.m.s.d. from ideal geometry		
Bond lengths (Å)	0.008	0.018
Bond angles (°)	1.24	1.68
Dihedral angles (°)	24.6	24.9
Number of protein atoms	824	821
Number of water molecules	57	44
Average <i>B</i> -factor (Å ²)		
All atoms	27.57	27.75
Molecule A	26.74	26.44
Molecule B	27.99	28.66
Water molecule	33.55	35.0

group P3₁21 with two independent molecules per asymmetric unit (Table 1).

All data sets were collected at room temperature on our home area detector (DIP2030) with a Phi goniometer using CuK α radiation from a rotating anode generator (Nonius B. V. FR591 model). Crystals of WT thioredoxin and the D30A mutant diffracted beyond 2.1 and 2.2 Å respectively. The two data sets were processed and scaled with the HKL suite of programs [32]. The data-collection results are summarized in Table 1.

Structure determination and refinement

The crystal structure of WT thioredoxin was determined through molecular replacement with the program suite AMoRe [33]. Attempts to determine the crystal structure using either the average NMR structure or all 23 NMR structures of *C. reinhardtii* (PDB code 1TOF) as a model were not successful. Hence the X-ray structure of human monomeric oxidized thioredoxin (PDB code 1ERU), solved to 2.1 Å, was used as the molecular-replacement search model. This protein displays 42 % of sequence identity with the thioredoxin *h* from *C. reinhardtii*, hence all non-conserved amino acids were replaced by alanine. The rotation and translation functions were calculated using data between 10 and 3.5 Å resolution. The one-body translation search, using the centred-overlap function, on the first 20 rotation solutions led

Figure 1 Stereoview of the molecular interface formed between the two independent molecules (A, dark grey; B, light grey) of the asymmetric unit of WT thioredoxin *h* crystal

The residues involved in either the hydrophobic core or the interfacial β -sheet are labelled. Residues Trp-35, Val-64, Ala-71, Ile-76, Ala-78 and Met-79 of both molecules are shown with their lateral chains. The figure was drawn with MolMol [41].

to a single solution with a correlation coefficient of 0.26, but the phased-translation function (AMoRe) failed to locate the second molecule. Finally, the solution was obtained with a correlation coefficient of 0.49 by including a Patterson correlation refinement before the *n*-body translation search. It is interesting to note that the Patterson correlation refinement rotated the second position by 12° with respect to that resulting from the rotation search.

Cycles of rigid-body refinement using CNS software [34] were performed on the model using data between 10 and 3.5 Å resolution. At this stage the resulting *R*-factor was 0.46 and a 3Fo-2Fc (where Fo and Fc are the observed and calculated structure factor amplitudes, respectively) electron-density map calculated on this model revealed a clear electron density for most of the main-chain atoms of the structure and the intramolecular disulphide bond involving Cys-36 and Cys-39.

Simulated annealing, positional grouped *B*-factor and individual *B*-factor were the procedures used during refinement with CNS programs [34]. Cycles of refinement were alternated with manual rebuilding (TURBO-FRODO [35]) of the protein model and incremental inclusion of higher-resolution data resulted in improved electron-density maps and allowed the introduction of the correct sequence in the model. Finally, the water molecules were added to build the solvent network: each peak contoured at 3 σ on the Fo-Fc maps was identified as a water molecule, provided that favourable interactions would be allowed between this site and the protein. The model was then adjusted and refined, using data between 20 and 2.1 Å resolution, until a final convergence, with *R* and *R*-free values of 20.4% and 25.2%, respectively.

Crystals of the D30A mutant protein were isomorphous to those of WT protein, which allowed the use of the WT structure as a starting model for refinement. Cycles of refinement were performed until crystallographic *R* values of 19.6% and an *R*-free value of 22.0% were reached.

The model's geometry was checked with PROCHECK [36]. There were no outliers on the Ramachandran plot [37] and both models possessed at least 93% of their amino acids in the most

favourable regions. Statistics concerning the geometry of the final models are given in Table 1.

Sequence alignments based on the superposition of the C α coordinates (see Figure 5 below) were done with Modeller [38] and ALSRIPT [39]. Root mean square (r.m.s.) deviation calculations and general crystallographic calculations were performed with the CCP4 suite of programs [40]. The accessibility of protein atoms was calculated using CNS [34]. Figures 1–4 were drawn with MolMol [41].

Molecular dynamics

All calculations and graphical analyses were run on a Silicon Graphics Indigo2 workstation. The INSIGHT/DISCOVER program (Biosym Technologies) [42] was used to perform energy minimizations and molecular-dynamics simulations *in vacuo* and in water solution, pH 7.0, using the AMBER force field [43]. In all simulations, the side chains of Arg and Lys residues were positively charged, whereas the side chains of Glu and Asp were negatively charged. Energy minimizations were carried out using the conjugate gradient algorithm. These procedures were stopped when the maximum derivative was ≤ 0.001 Kcal/mol.

The energy-minimized structures of WT thioredoxin and the D30A mutant, in which the disulphide bridge had been broken, were used as the initial structures for the molecular-dynamics simulations *in vacuo* at 300 and 500 K. These energy-minimized structures were also used for the water simulation at 300 K. The computational conditions were chosen to avoid boundary effects [44]. For the molecular-dynamics simulation of WT thioredoxin in water, the trajectories were run with complete solvation shells of 5 Å thickness (715 and 732 water molecules). All simulations were performed with a time step of 1.0 fs. For the *in vacuo* simulations, the proteins were equilibrated for 80 ps and, after this initial step, additional simulations of 80 ps were carried out without rescaling since the energy was conserved and the average temperature remained essentially constant around the target

values. For the solution simulations, longer runs were performed: the proteins were equilibrated for 80 ps, a sufficient time period for our simulations and, after this initial step, additional simulations of 120 ps were carried out without rescaling since the energy was conserved and the average temperature remained essentially constant around the target values. Co-ordinates and velocities were dumped every 10 steps during the last 80 ps (for molecular-dynamics simulations *in vacuo*) or every 10 steps during the last 120 ps (for simulations in solution). These dumped data were used for statistical analyses and the calculation of the occurrence of hydrogen bonds (the ratio of the number of the simulation steps where hydrogen bonds were present to the total number of simulation steps).

Proton-inventory experiments

All kinetic assays were carried out on a Cary 50 spectrophotometer (Varian) at a constant temperature of 25 ± 1 °C. The reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; 0.1 mM) by thioredoxin (20 nM) was followed at 412 nm. In order to allow measurements under steady-state conditions, a coupled system composed of an excess of thioredoxin reductase (9 μ M) and NADPH (145 μ M) was used, to allow thioredoxin recycling. Proton inventory was performed by measuring the solvent isotope effects at different $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ ratios in 100 mM Tris/HCl buffer, pH 8.5. To determine the solvent isotope effect, aliquots of the solutions were lyophilized and redissolved in adequate $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ mixtures. The reaction was initiated by the addition of DTNB. Five individual measurements were carried out for each $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ ratio. Plots of the measured kinetic solvent isotope effect ($k_x/k_{x_{\text{H}_2\text{O}}}$) versus the $^2\text{H}_2\text{O}$ mole fraction (x) were fitted according to the Gross–Butler equation [45]:

$$\frac{k_x}{k_{x_{\text{H}_2\text{O}}}} = \frac{k_{\text{H}_2\text{O}} (1 - x + \phi_{T_1} x) \times \dots \times (1 - x + \phi_{T_n} x)}{k_{x_{\text{H}_2\text{O}}} (1 - x + \phi_{R_1} x) \times \dots \times (1 - x + \phi_{R_n} x)} \quad (1)$$

Since the SH group of Cys is known to possess a reactant-state fractionation factor that is different from unity and is thus able to contribute markedly to the isotope effect, the corresponding ϕ_R term, usually ignored with other side chains, has been kept in eqn (1). Experimental data were fitted to eqn (1), allowing us to obtain the number of protons (n) as well as the fractionation factors, ϕ_{T_n} in the transition state and ϕ_{R_n} in the reactant state.

RESULTS AND DISCUSSION

C. reinhardtii WT thioredoxin *h* structure

WT thioredoxin crystallizes in the trigonal system and belongs to space group P3₁21 with two independent molecules per asymmetric unit, designated A and B. There were no outliers in the Ramachandran plot [37] and almost all residues of both independent molecules were well defined by the electron density map, except for the N-terminal region (residues 1–3) of molecule A and the C-terminal regions (residues 111 and 112) of both molecules. These two regions account for the main structural differences between the two independent molecules of the asymmetric unit. Indeed, the least-squares superimposition involving all α -carbon atoms of each independent molecule led to an r.m.s. value of 0.64 Å, whereas the superimposition carried out on residues 4–110 led to a r.m.s. value of only 0.26 Å.

Analysis of the contact area between the A and B molecules shows that the largest contact area (1123 Å²) is located at the interface between the two independent molecules and involves the loop Thr-77–Met-79 as well as Trp-35, Val-64, Ala-71 and Ile-76 of both molecules (Figure 1). It is noteworthy that the

arrangement found between monomers A and B is equivalent to that observed in the natural covalent dimeric form of human thioredoxin [3], which results from an intermolecular disulphide bond via the non-conserved residue Cys-73 (human numbering) of each monomer. Since this residue is replaced by an alanine in the *C. reinhardtii* thioredoxin, the resulting dimer is non-covalent. The two monomers are related by a crystallographic 2-fold axis in the human thioredoxin structure, whereas a non-crystallographic 2-fold axis is found in the *C. reinhardtii* structure.

The general fold of thioredoxin *h* from *C. reinhardtii*, as with thioredoxins from other sources [8,10,11,15,17], consists of a five-stranded β -sheet surrounded by four α -helices (Figure 2). Comparison of the NMR average model [16] with the X-ray model of molecule A (residues 4–110) leads to r.m.s. deviations of 1.7 Å. Displacements higher than twice the r.m.s. value are found for residues 21–24 (C-terminal end of the α 1 helix) and residues 36 and 37 (N-terminal end of the α 2-helix). The α 1 helix extends from residue 9 to residue 24 in our model but is supposed to end at residue 19 in the NMR model on the basis of amide–proton-exchange experiments, although distances ($i, i+4$; where i is the number of the residue) characteristic of α -helices were observed up to residue 23 in the latter (J. M. Lancelin, personal communication). Hence, the formation of an additional C-terminal helix turn is probably a transient event in solution and could be stabilized by packing in the crystal state as the α 1-helix is directly involved in intermolecular contacts (Table 2). Another interesting difference concerns the α 2-helix, which contains the active site: this helix begins at residue Gly-37 in the crystal structure, but only at residue Cys-39 in the NMR model. In the crystal structure, a loop immediately preceding the active site (residues Ala-33–Cys-36) is stabilized by intermolecular contacts with molecules from other asymmetric units. As a consequence, the active site could be frozen by the first turn of the α 2-helix in the crystal state whereas it would be more floppy in solution. Interestingly, similar observations have been made when comparing the X-ray and NMR models of human thioredoxin [8].

In the present structure, the two active-site cysteines located at the N-terminal end of the α 2-helix on the surface of the protein are in their oxidized form, with a disulphide bridge forming a 14-membered ring, well defined in the 3Fo-2Fc electron density map. The environment of both Cys residues is similar to that described for *E. coli* [10] or human oxidized thioredoxins [8]. Cys-36 is located on the surface of the protein and is involved in the same intramolecular interactions. Its main-chain carbonyl forms the first hydrogen bond of the α 2-helix with the amide NH of Lys-40 and is also involved with two other hydrogen bonds: the first one between its NH and the carbonyl of Ala-33 and the second one between its sulphur atom and the NH of Cys-39. All these features have been already discussed elsewhere to account for the lower pK_a value of Cys-36 [8,10]. Residue Cys-39 was found to be completely buried and surrounded by hydrophobic residues, with the exception of residue Asp-30 (the distance between Cys-39-SG and the closest oxygen atom of Asp-30-OD1 was 5.9 Å). Such an environment contributes to the lower reactivity of this cysteine when compared with Cys-36. As emphasized in the Introduction, all data available up to now lead to the conclusion that residue Asp-30, which is conserved in most sequences, plays a crucial role in catalysis [21,29,39]. This residue is buried in a hydrophobic core formed by residues Ile-42, Phe-46, Pro-80 and Phe-82, but is nevertheless partially accessible to solvent. The solvent molecule that is hydrogen-bonded to the carboxylate OD2 of Asp-26 and also to the carbonyl CO of Cys-35 in the structures of the oxidized form of *E. coli* thioredoxin [10] and of the chloroplastic *m* and *f* isoforms from spinach [12]

Figure 2 Ribbon diagram of thioredoxin *h* from *C. reinhardtii* in the oxidized form

The side chains of residues Asp-30, Cys-36 and Cys-39 are shown in the ball-and-stick representation. The figure was drawn with MolMol [41].

Table 2 Hydrogen bonds and salt bridges involved in intermolecular contacts between molecules A and B

A and B in bold refer to the two independent molecules. Superscript letters denote symmetry transformations used to generate equivalent atoms: a, x, y, z ; b, $1 - y, x - y, z + 1/3$; c, $x - y, 1 - y, -z + 2/3$; d, $1 - x, 1 - x + y, -z + 1/3$. Atom notations are in agreement with PDB nomenclature.

Atom 1	Atom 2	Distance (Å)
OD1-Asp- A -65	NE1-Trp ^a - B -35	3.22
O-Thr- A -77	N-Met ^a - B -35	2.81
N-Met- A -79	O-Thr ^a - B -79	2.76
NZ-Lys- A -21	O-SER ^b - B -3	3.13
O-Asp- A -87	NE2-Gln ^b - B -16	3.06
OD1-Asp- A -93	NE2-Gln ^c - A -16	2.82
OD2-Asp- A -93	NE-Lys ^c - A -19	2.66
NZ-Lys- A -108	OD2-Asp ^c - A -8	3.37
NZ-Lys- B -108	OD2-Asp ^d - B -63	3.06
N-Lys- B -90	OE1-Glu ^d - B -47	2.73
OD1-Asp- B -92	NZ-Lys ^d - B -61	3.55
OD2-Asp- B -93	N-Gly ^d - B -1	2.82

is also present in our structure between Asp-30-OD2 and Cys 39-CO (Figure 3A).

Crystal structure of the D30A mutant: functional implications for the D30 residue

Crystals of the D30A mutant are isomorphous to those of the WT protein. The superimposition of the WT and D30A structures carried out on C α positions of residues 4–110 leads to a r.m.s.

value of only 0.14 Å. In the crystal structure, the substitution of Asp-30 by Ala does not lead to any distortion of the active-site conformation, as shown in Figure 3. The geometry of the disulphide bridge is conserved and the distance is the same as that between Cys-39-SG and Ala-30-CA in the WT structure.

As emphasized in the Introduction, numerous studies have been carried out to elucidate the role of the active-site-buried aspartate residue of thioredoxins. Chivers and Raines [29] and LeMaster et al. [30] established independently from kinetic experiments carried out on *E. coli* WT and Asp-26 (Asp-30 in the *C. reinhardtii* sequence) mutant proteins that this residue serves as a general acid/base catalyst towards Cys-35 (Cys-39 in the *C. reinhardtii* sequence) during the oxidation/reduction reaction. So, if one considers the reduction by thioredoxin of a target protein, Asp-30 would provide a base catalyst, abstracting a proton from Cys-39 in the mixed disulphide intermediate, enhancing the breakdown of this intermediate. Base catalysis is indeed required at this level since the buried Cys-39 exhibits a pK_a value of 11.1 in the mixed intermediate [46].

However, it remains difficult to reconcile the biological results with the available structural data, and LeMaster et al. [30] suggested that this mechanism would require a structural rearrangement because of the great distance observed between Cys-39-SG and Asp-30-OD1.

The distance between Cys-39-SG and Asp-30-OD1 (5.9 Å) in our WT structure is in good agreement with the values found for the X-ray structures of *E. coli* [10] or human [8] thioredoxins. In this context, the involvement of Asp-30 as an acid/base catalyst could only be envisaged (i) if significant conformational changes occur during the reaction or (ii) via a water molecule if the chain flexibility allows access to solvent molecules.

Figure 3 Stereoview showing the active-site region of WT thioredoxin *h* (A) and that of the D30A mutant (B) in the same orientation

The distance between Cys-39-SG and Asp-30-OD1 is 5.9 Å. In the WT structure, a water molecule is hydrogen-bonded with Asp-30-OD2 (2.9 Å) and Cys-39-O (2.8 Å). This water molecule is not found in the D30A mutant structure. The figure was drawn with MolMol [41].

Figure 4 Stereoview showing the hydrophobic environment of residue Asp-30

The Van-der-Waals spheres of Asp-30 (red) and of the neighbouring residues belonging to either the β -sheet (green, Pro-80 and Phe-82) or the α 2-helix (blue, Ile-42 and Phe-46) are represented. The figure was drawn with MolMol [41].

Figure 5 Alignment of the sequences for human, *E. coli* and *C. reinhardtii* thioredoxins based on the superposition of the respective C α co-ordinates

Sequence numbering refers to the *C. reinhardtii* thioredoxin *h* sequence. Positions of deletions are indicated by a point (.). The hydrophobic residues surrounding Asp-30 are shown against a black background.

To examine these two possibilities and to better elucidate the role of Asp-30 in catalysis, we undertook an investigation of the dynamic aspects of these residues *in vacuo* and in relation to solvent accessibility.

Molecular-simulations analysis *in vacuo*

Molecular-dynamics simulations *in vacuo* were carried out on the minimized crystal structures of WT and mutated D30A thioredoxin from *C. reinhardtii* to analyse the behaviour of the protein. Analysis of the mean square displacements of the residues in the two molecules gives an indication of the mutual flexibility of the regions containing the residues of interest, Asp/Ala-30 and Cys-39. Although some flexibility is observed in the active-site region, the relative mobility of residues Asp-30 and Cys-39 does not allow their side chains to come close enough to support the hypothesis of a direct proton transfer between the two residues. Indeed, the distance between the sulphur atom of Cys-39 and the closest oxygen of Asp-30-OD1 during the dynamics simulations *in vacuo* at 300 or 500 K was always greater than 6.5 Å, in good agreement with the distances measured on the 23 *C. reinhardtii* thioredoxin-independent NMR models (6.4–9.9 Å, PDB code 1TOF). This finding supports the conclusions of Vohnik et al. [25] that the aspartate residue does not interact directly with the active-site-buried cysteine. Interestingly, Qin et al. [47] published the NMR structure of a mixed disulphide intermediate (the stage at which Asp-30 is postulated to act as a base catalyst) of a C39S mutant (C35S in the human sequence) of human thioredoxin in complex with a nuclear factor κ B (NF κ B) peptide. This structure, together with the structures of reduced (first stage of the reduction reaction) and oxidized (last stage of the reduction reaction) thioredoxin [8], provides a snapshot of the three-dimensional conformation of the protein at the three stages of the reaction and allows identification of the main conformational events that occur during catalysis. The comparison between the three structures shows that the protein does not undergo any large conformational change upon mixed disulphide formation and that the distance between Cys-39-SG and Asp-30-OD in the mixed disulphide intermediate is too great to support direct proton transfer. Taken together with the results of our molecular-dynamics simulations, this supports the idea that, even if conformational changes occur during catalysis, they do not allow a direct interaction between the side chains of Cys-39 and Asp-30. From a structural point of view, residue Asp-30 is located in the middle of the β -sheet, which constitutes the rigid core of the molecule, and residue Cys-39 is situated at the end of

α 2-helix. Bringing the two residues close together would require a translation movement of the α -helix towards the β -sheet. However, residue Asp-30 is surrounded by hydrophobic, bulky residues belonging to either the β -sheet (Pro-80 and Phe-82) or the α 2-helix (Ile-42 and Phe-46; Figure 4) and a translation of the whole α 2-helix would result in a clash (interpenetration) of the Van-der-Waals surfaces between atoms of the two secondary structures. It is noteworthy that these hydrophobic residues are conserved or replaced by equivalent residues in the structures of other thioredoxins (Figure 5). Alternatively, the destabilization of the N-terminal part of α 2-helix would be sufficient to bring the two residues closer to one another. In order to examine this possibility, we have analysed the situation encountered in the 23 individual NMR models from *C. reinhardtii* thioredoxin, since this helix was significantly shorter in some of them. These models show that the presence of a long, well-structured, α 2-helix correlates with the shortest distance between residues 30 and 39, whereas the models displaying the shortest α 2-helices correlate with the longest distances. It follows that even if the N-terminal turn of the α 2-helix is destabilized in solution, it would always result in an increased distance between the two catalytic residues. Finally, LeMaster et al. [30] suggested that the internal cavity lying between Cys-35-S and Asp-26-OD (*E. coli* numbering) should allow for a χ 1 dihedral angle rotation of Cys-35 to bring these side chains into contact with minimal structural rearrangement. However, such a conformational change to the Cys side chain, which would bring the two atoms to a distance of 4.2 Å in our structure, is neither supported by the molecular-dynamics results (the distance is always above 6.3 Å) nor found in any NMR or crystal structures of thioredoxins.

Altogether, these structural considerations constitute a further argument to rule out the hypothesis of a direct proton transfer between the two residues and led us to investigate a possible role of water molecules in catalysis. For this purpose, two molecular-dynamics simulations in water solution at 300 K were performed on the structures of the WT and mutant proteins.

Molecular-dynamics simulations in solution

The simulation carried out on the WT protein reveals that during the last 120 ps, one water molecule from the water bulk gets into the active site, occupying a position that is at hydrogen-bonding distance from both the Cys-39 sulphur atom and the Asp-30-OD1 atom (Figure 6). Note that although this water molecule is not present in our crystal structure, another one occupies a close position between Asp-30-OD1 and Cys-39-CO.

Figure 6 Molecular-dynamics simulations in water at 300 K for WT thioredoxin *h*

Shown are distance-versus-time-course plots between: **(A)** the water molecule (oxygen atom) and Cys-39-SG; **(B)** the water molecule (oxygen atom) and Asp-30-OD1; and **(C)** the water molecule (oxygen atom) and Asp-30-OD2.

During the simulation, the water molecule (Ow) acts as a bridge between the two residues, with Ow-Cys-39-SG and Ow-Asp-30-OD1 average distances of 3.86 and 3.06 Å, respectively (Figures 6A and 6B), with the occurrence of a hydrogen-bond network (ratio of the number of the simulation steps where hydrogen-bonds are present between the three atoms to the total number of simulation steps) of 56 % between the three atoms. In addition, the average distance between the Ow-Asp-30-OD2 is 4.26 Å (Figure 6C) with a hydrogen-bond occurrence between the two atoms of 26 %. The results concerning the occurrence of the hydrogen-bond network between the Ow, the Cys-39-SG and the Asp-30-OD1 are significant since well-established crystallographic structures of small peptides that contain an hydrogen-bond network through water molecules do give similar occurrences [48]. Therefore, in the reduction reaction, the water molecule could act as a hydrogen-bond donor towards the Asp-30 side chain and as a hydrogen-bond acceptor from the Cys-39 sulphur atom. The results of the statistical analysis of the hydrogen-bonds for the WT enzyme have to be compared with those concerning the D30A mutant. In the case of the D30A mutant, the simulation suggests that water molecules might be involved in an interaction with the Cys-39-SG atom, but with low occurrence of hydrogen-bonds (14 %). These water molecules

are linked by hydrogen-bonds, giving rise to a water-molecule network near the Cys-39 residue.

Solvent isotope effect and proton inventory

The reduction of DTNB by reduced thioredoxin proceeds in two chemical steps via a mixed disulphide intermediate, and gives rise to two molecules of 2-nitro-5-thiobenzoate (NTB⁻). Stopped-flow analysis of this reaction established that the release of the first NTB⁻ molecule following the nucleophilic attack of the first catalytic Cys residue occurs at a very high rate, whereas the release of the second one from the mixed disulphide occurs more slowly [29]. Since the breakdown of the mixed disulphide intermediate constitutes the rate-determining step of the reduction reaction, it is possible to follow this second step under steady-state conditions by the use of a coupled system containing an excess of thioredoxin reductase and NADPH, which allows thioredoxin recycling.

To give experimental support for the involvement of a water molecule, the solvent isotope effect was examined and a proton inventory was carried out. Substituting ²H₂O for H₂O results in a 1.6-fold decrease in the steady-state constant of the WT enzyme. In order to establish how many protons contribute to

Figure 7 Proton inventory for the breakdown of the mixed disulphide intermediate at pH 8

S. E. values were less than 2%. The solid line represents the best fit of experimental data to eqn. (1). The dotted line represents the theoretical curve that would have been obtained for a single proton transfer and a ϕ_R value of 0.56 for Cys-39.

the solvent isotope effect, a proton inventory was performed for the WT enzyme by measuring the dependence of k_x (the differential isotope effect on the steady-state rate constant) upon the $^2\text{H}_2\text{O}$ mole fraction (x ; Figure 7). The best fit of the data against eqn (1) was obtained for $n = 2$, with values of 0.59 ± 0.05

and 0.58 ± 0.05 for the fractionation factors of the two protons in the transition state (ϕ_{T_1}, ϕ_{T_2}) and a value of 0.56 ± 0.1 for the fractionation factor of Cys-39 in the reactant state (ϕ_R). This latter value is in close agreement with those reported for the catalytic cysteines of proline racemase (ϕ_R , 0.55 ± 0.1) [49] or for small thiol compounds (ϕ_R , 0.61 ± 0.17) [45]. Fitting the data to the Gross–Butler equation adapted for one proton contribution to the transition state ($n = 1$) with a fixed $<\phi_R$ value of 0.56 gives rise to a dome-shaped curve, represented Figure 7 (dotted-line), that does not fit our experimental data. On the other hand, keeping ϕ_R unconstrained and setting the number of protons to one allows us to fit the data quite well but the resulting ϕ_R value obtained from the adjustment becomes equal to 1.0, an unprecedented value for a thiol group [45]. Consequently, the most probable conclusion of this experiment is that the solvent isotope effect originates from the transfer of two protons with equivalent contributions to the rate-limiting transition state. Therefore, at pH 8, the deprotonation of residue Cys-39 required for the breakdown of the mixed disulphide intermediate would involve a water molecule (Scheme 1), itself activated by Asp-30 acting as a base catalyst.

Concluding remarks

Altogether, our results are in agreement with previous conclusions [29,30] that the conserved Asp residue serves as an acid/base in the oxidation/reduction reactions catalysed by thioredoxins, by protonating (during substrate oxidation) or deprotonating (during substrate reduction) the thiol of the most buried cysteine residue. However, comparison of the structural data for the WT and mutant proteins, in conjunction with

Scheme 1 The role of Asp-30 in the catalytic mechanism of thioredoxins

On the basis of our crystal structures, molecular dynamics and proton inventory, we propose that, in the second step, the proton transfer between Cys-39 and Asp-30 is mediated by a water molecule.

molecular-dynamics simulation and proton-inventory experiments, suggests that this proton transfer is mediated by a water molecule (Scheme 1). Furthermore, the structural details obtained from the crystal structure suggest that the presence of a polar residue, such as aspartic acid, should restrict the access to the active site to one water molecule. This hypothesis is also supported by the molecular-dynamics simulation. In addition, Asp-30 is able potentially to provide hydrogen-bond acceptors to activate and to orient the water molecule. On the contrary, in the D30A mutant, the presence of a smaller side chain could allow the active site to be occupied by two or more water molecules. This different behaviour gives rise to a network of water molecules that could assist the Cys residue and might explain why the mutant protein retains some catalytic efficiency. This hypothesis is also supported by the data of Chivers and Raines [29] showing that, in the case of mutants at position Asp-30, external buffers (such as imidazole) can access the active site and enhance the breakdown of the mixed disulphide intermediate, thus partly restoring the loss of activity caused by the replacement of the Asp residue. Such a water molecule network that mediates the proton transfer in the absence of a catalytic residue has also been reported for an aldehyde dehydrogenase [50] on the basis of proton-inventory experiments.

We are greatly indebted to Dr J. Navaza (Laboratoire de génétique des virus, Gif sur Yvette, France) and to Dr K. Berndt (Department of Bioscience, Center for Structural Biochemistry, Karolinska Institutet, Huddinge, Sweden) for helpful discussions. We would like to thank the Service Commun de Diffraction X sur Monocristaux (Université Henri Poincaré-Nancy 1, Nancy, France) for providing access to crystallographic experimental facilities. This work was supported by the Centre National de la Recherche Scientifique (PCV98-099), the University Henri Poincaré-Nancy I, the Consiglio Nazionale delle Ricerche and the Ministero dell'Università della Ricerca Scientifica e Tecnologica.

REFERENCES

- Holmgren, A. (1985) Thioredoxin. *Annu. Rev. Biochem.* **54**, 237–271
- Schürman, P. and Jacquot, J. P. (2000) Plant thioredoxin systems revisited. *Annu. Plant Physiol. Plant Mol. Biol.* **51**, 371–400
- Buchanan, B. B., Schürmann, P., Decottignies, P. and Lozano, R. M. (1994) Thioredoxin: a multifunctional regulatory protein with a bright future in technology and medicine. *Arch. Biochem. Biophys.* **314**, 257–260
- Buchanan, B. B., Schürmann, P. and Jacquot, J. P. (1994) Thioredoxin and metabolic regulation. *Semin. Cell Biol.* **5**, 285–293
- Krause, G., Lundstrom, J., Barea, J. L., Pueyo de la Cuesta, C. and Holmgren, A. (1991) Mimicking the active site of protein disulfide-isomerase by substitution of proline 34 in *Escherichia coli* thioredoxin. *J. Biol. Chem.* **266**, 9494–9500
- Creighton, T. E. (1997) Protein folding coupled to disulphide bond formation. *J. Biol. Chem.* **378**, 731–744
- Aslund, F., Berndt, K. D. and Holmgren, A. (1997) Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein-protein redox equilibria. *J. Biol. Chem.* **272**, 30780–30786
- Weichsel, A., Gasdaska, J., Powis, G. and Montfort, W. R. (1996) Crystal structures of reduced, oxidized, and mutated human thioredoxins: evidence for a regulatory homodimer. *Structure* **4**, 735–751
- Holmgren, A., Soderberg, B. O., Eklund, H. and Branden, C. I. (1975) Three-dimensional structure of *Escherichia coli* thioredoxin-S2 to 2.8 Å resolution. *Proc. Natl. Acad. Sci. U.S.A.* **6**, 2305–2309
- Katti, S., LeMaster, D. and Eklund, H. (1990) Crystal structure of thioredoxin from *Escherichia coli* at 1.68 Å resolution. *J. Mol. Biol.* **212**, 167–184
- Saarinen, M., Gleason, F. and Eklund, H. (1995) Crystal structure of thioredoxin-2 from *Anabaena*. *Structure* **3**, 1097–1108
- Capitani, G., Markovic-Housley, Z., Delval, G., Morris, M., Jansonius, J. N. and Schürmann, P. (2000) Crystal structures of two functionally different thioredoxins in spinach chloroplasts. *J. Mol. Biol.* **302**, 135–154
- Qin, J., Clore, G. and Gronenborn, A. (1994) The high-resolution three-dimensional solution structures of the oxidized and reduced states of human thioredoxin. *Structure* **2**, 503–522
- Jeng, M., Campbell, A., Begley, T., Holmgren, A., Case, D., Wright, P. and Dyson, H. (1994) High-resolution solution structures of oxidized and reduced *Escherichia coli* thioredoxin. *Structure* **2**, 853–868
- Nicasro, G., De Chiara, C., Pedone, E., Tatò, M., Rossi, M. and Bartolucci, S. (1999) NMR structure of a novel thioredoxin from *Bacillus acidocaldarius*: possible determinants of protein stability. *Eur. J. Biochem.* **267**, 403–413
- Mittard, V., Blackledge, M., Stein, M., Jacquot, J. P., Marion, D. and Lancelin, J. M. (1997) NMR solution structure of an oxidized thioredoxin h from the eukaryotic green alga *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* **243**, 374–381
- Lancelin, J. M., Guilhaudis, L., Krimm, I., Blackledge, M. J., Marion, D. and Jacquot, J. P. (2000) NMR structures of thioredoxin m from the green alga *Chlamydomonas reinhardtii*. *Proteins Struct. Funct. Genet.* **41**, 334–349
- Kallis, G. B. and Holmgren, A. (1980) Differential reactivity of the functional sulphydryl groups of cysteine-32 and cysteine-35 present in the reduced form of thioredoxin from *Escherichia coli*. *J. Biol. Chem.* **255**, 10261–10265
- Krause, G. and Holmgren, A. (1991) Substitution of the conserved tryptophan 31 in *Escherichia coli* thioredoxin by site-directed mutagenesis and structure-function analysis. *J. Biol. Chem.* **266**, 4056–4066
- Gleason, F. K. (1992) Mutation of conserved residues in *Escherichia coli* thioredoxin: effects on stability and function. *Protein Sci.* **5**, 609–616
- Dyson, H. J., Jeng, M. F., Tennant, L. L., Slaby, I., Lindell, M., Cui, D. S., Kuprin, S. and Holmgren, A. (1997) Effects of buried charged groups on cysteine thiol ionization and reactivity in *Escherichia coli* thioredoxin: structural and functional characterization of mutants of Asp 26 and Lys 57. *Biochemistry* **36**, 2622–2626
- Krimm, I., Lemaire, S., Ruelland, E., Miginiac-Maslow, M., Jacquot, J. P., Hirasawa, M., Knaff, D. and Lancelin, J. M. (1998) The single mutation Trp35 → Ala in the 35–40 redox site of *Chlamydomonas reinhardtii* thioredoxin h affects its biochemical activity and the pH dependence of C36-C39 ¹H-¹³C NMR. *Eur. J. Biochem.* **255**, 185–195
- Langsetmo, K., Fuchs, J. and Woodward, C. (1991) The conserved, buried aspartic acid in oxidized *Escherichia coli* thioredoxin has a pKa of 7.5. Its titration produces a related shift in global stability. *Biochemistry* **30**, 7603–7609
- Jeng, M. and Dyson, H. (1996) Direct measurement of the aspartic acid 26 pKa for reduced *Escherichia coli* thioredoxin by ¹³C NMR. *Biochemistry* **35**, 1–6
- Vohnik, S., Hanson, C., Tuma, R., Fuchs, J., Woodward, C. and Thomas, G. J. (1998) Conformation, stability, and active-site cysteine titrations of *Escherichia coli* D26A thioredoxin probed by Raman spectroscopy. *Protein Sci.* **7**, 193–200
- Wilson, N., Barbar, E., Fuchs, J. and Woodward, C. (1995) Aspartic acid 26 in reduced *Escherichia coli* thioredoxin has a pKa > 9. *Biochemistry* **34**, 8931–8939
- Jeng, M., Holmgren, A. and Dyson, H. (1995) Proton sharing between cysteine thiols in *Escherichia coli* thioredoxin: implications for the mechanism of protein disulphide reduction. *Biochemistry* **34**, 10101–10105
- Ladbury, J., Wynn, R., Hellinga, H. and Sturtevant, J. (1993) Stability of oxidized *Escherichia coli* thioredoxin and its dependence on protonation of the aspartic acid residue in the 26 position. *Biochemistry* **32**, 7526–7530
- Chivers, P. T. and Raines, R. T. (1997) General acid/base catalysis in the active site of *Escherichia coli* thioredoxin. *Biochemistry* **36**, 15810–15816
- LeMaster, D., Springer, P. A. and Unkefer, C. J. (1997) The role of the buried aspartate of *Escherichia coli* thioredoxin in the activation of the mixed disulphide intermediate. *J. Biol. Chem.* **272**, 29998–30001
- Stein, M., Jacquot, J. P., Jeannette, E., Decottignies, P., Hodges, M., Lancelin, J. M., Mittard, V., Schmitter, J. M. and Miginiac-Maslow, M. (1995) *Chlamydomonas reinhardtii* thioredoxins: structure of the genes coding for the chloroplastic m and cytosolic h isoforms; expression in *Escherichia coli* of the recombinant proteins, purification and biochemical properties. *Plant Mol. Biol.* **3**, 487–503
- Otwinowski, Z. and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**, 307–326
- Navaza, J. (1994) AMoRe: an automated package for molecular replacement. *Acta Crystallogr. A* **50**, 157–163
- Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S. et al. (1998) Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D* **54**, 905–921
- Roussel, P. A. and Cambillau, C. (1991) TURBO-FRODO, Silicon Graphics applications directory, Silicon Graphics, Mountain View, CA
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. and Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**, 283–291
- Ramakrishnan, C. and Ramachandran, G. N. (1965) Stereochemical criteria for polypeptide and protein chain conformations. II. Allowed conformations for a pair of peptide units. *Biophys. J.* **5**, 909–933
- Sali, A. and Blundell, T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779–815
- Barton, G. J. (1993) ALSCRIPT: a tool to format multiple sequence alignments. *Protein Eng.* **6**, 37–40

-
- 40 Collaborative Computational Project No. 4 (1994) The CCP4 Suite: programs for protein crystallography. *Acta Crystallogr. D* **50**, 760–763
- 41 Koradi, R., Billeter, M. and Wuthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graphics* **14**, 51–55
- 42 Weiner, S. J., Kollman, P. A., Nguyen, D. T. and Case, D. A. (1986) An all atom force field for simulations of proteins and nucleic acids. *J. Comp. Chem.* **7**, 230–252
- 43 Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) The Protein Data Bank: a computer-based archival file for macromolecular structures. *J. Mol. Biol.* **112**, 535–542
- 44 Saviano, M., Aida, M. and Corongiu, G. (1991) Molecular dynamics simulation *in vacuo* and in solution of cyclolinopeptide A: a conformational study. *Biopolymers* **31**, 1017–1024
- 45 Cook, P. F. (1994) Enzyme mechanism from isotope solvent effect, CRC Press, Boca Raton, FL
- 46 LeMaster, D. (1996) Structural determinants of the catalytic reactivity of the buried cysteine of *Escherichia coli* thioredoxin. *Biochemistry* **35**, 14876–14881
- 47 Qin, J., Clore, G. M., Kennedy, W. M., Huth, J. R. and Gronenborn, A. M. (1995) Solution structure of human thioredoxin in a mixed disulfide intermediate complex with its target peptide from the transcription factor NF kappa B. *Structure* **3**, 289–297
- 48 Saviano, M., Rossi, F., Pavone, V., Di Blasio, B. and Pedone, C. (1992) Molecular dynamics simulation *in vacuo* and in solution of [Aib^{5,6}-D-Ala⁸]cyclolinopeptide A: a conformational and comparative study. *J. Biomol. Struct. Dyn.* **9**, 1045–1060
- 49 Belasco, J. G., Bruice, T. W., Alberly, W. J. and Knowles, J. R. (1986) Energetics of proline racemase: fractionation factors for the essential catalytic groups in the enzyme-substrate complex. *Biochemistry* **25**, 2558–2564
- 50 Marchal, S., Rahuel-Clermont, S. and Branlant, G. (2000) Role of glutamate 268 in the catalytic mechanism of non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase from *Streptococcus mutans*. *Biochemistry* **39**, 3327–3335
-

Received 23 October 2000/13 June 2001; accepted 30 July 2001