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FOR THE RECORD

Is human thioredoxin monomeric or dimeric?

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Abstract: We have examined the molecular weight and rotational correlation time of human thioredoxin by analytical ultracentrifugation and NMR spectroscopy, respectively. Two variants of human thioredoxin were studied, namely human thioredoxin identical in amino acid sequence to the one whose NMR structure we previously determined (C62A, C69A, C73A, M74T) and human thioredoxin (C62A, C69A, C73A, M74) containing the wild-type amino acid methionine at position 74. In both cases, the experimental data indicate that the predominant species is monomeric and we find no evidence for the existence of a well-defined dimeric form as was observed in the recently reported crystal structure (Weichsel et al., 1996) of human thioredoxin and the C73S mutant.

Keywords: analytical ultracentrifugation; correlation times; heteronuclear relaxation rates; human thioredoxin

In the recently determined crystal structure of human thioredoxin (hTRX), the protein is a dimer, covalently linked via an intermolecular disulfide bond involving Cys73 from each monomer (Weichsel et al., 1996). The active site loop, comprising Trp_{31}-Cys-Gly-Pro-Cys_{35}, forms part of the dimer interface and is blocked. Surprisingly, even a mutant protein in which Cys73 is replaced by Ser, is dimeric in the crystal, which led to the suggestion that the dimer of hTRX may be of physiological importance (Weichsel et al., 1996). In addition, the role of Asp60 in dimer formation was probed both crystallographically and with a dimerization assay using diamide as the oxidant (Andersen et al., 1997). Since no evidence for the presence of a dimer was found in the solution NMR structure of hTRX (Qin et al., 1994), it was suggested that the threonine for methionine substitution at position 74 in the hTRX used for the NMR studies might be responsible for this difference between crystal and solution states (Weichsel et al., 1996). To resolve this issue, we have examined the molecular weight and rotational correlation time of hTRX by analytical ultracentrifugation and NMR spectroscopy, respectively. Two variants were investigated, namely hTRX identical in amino acid sequence to the one whose NMR structure we previously determined (C62A, C69A, C73A, M74T) and hTRX (C62A, C69A, C73A, M74) containing the wild-type amino acid methionine at position 74.

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Results and discussion: Comparison of the two-dimensional ^{1}H-^{15}N correlation spectra of the Met74 and Thr74 hTRX variants revealed only minor chemical shift changes confined to areas adjacent to residue 74 in both the linear sequence and three-dimensional structure, confirming that there are no significant structural differences between the two variants (Fig. 1). Analytical ultracentrifugation was carried out on ^{15}N labeled proteins using a series of buffer conditions. In 50 mM Tris-HCl, 200 mM NaCl, pH 7.5 the Met74 protein behaved ideally, there was no evidence for any intermolecular association, and the molecular weight determined (M_r = 11,720) was almost identical to that predicted (M_r = 11,755) from the DNA coding sequence assuming >95% uniform labeling with ^{15}N (Fig. 2). This is in contrast to the observation of a dimer reported by Andersen et al. (1997) who used a gel filtration assay employing very similar buffer conditions (50 mM Tris-HCl, 200 mM NaCl, pH 8.0). The molecular weight of the Thr74 variant appeared slightly higher (M_r = 13,000), probably due to slight nonspecific aggregation. Since our previous structure determination by NMR was carried out in phosphate buffer, pH 5.5 (Qin et al., 1994), we also investigated the behavior of the Met74 protein in three different concentrations of phosphate buffer. The values obtained for the apparent average mass in 25 mM phosphate buffer, 50 mM phosphate buffer, and 100 mM phosphate buffer, pH 5.5 were 12,195, 12,500, and 13,400, respectively. These data demonstrate that no dimer formation occurs, although a small increase in apparent molecular weight is observed with increasing phosphate buffer concentration. To unambiguously rule out the possibility of dimer formation being responsible for this increase, we calculated the expected weight average masses for equilibrium

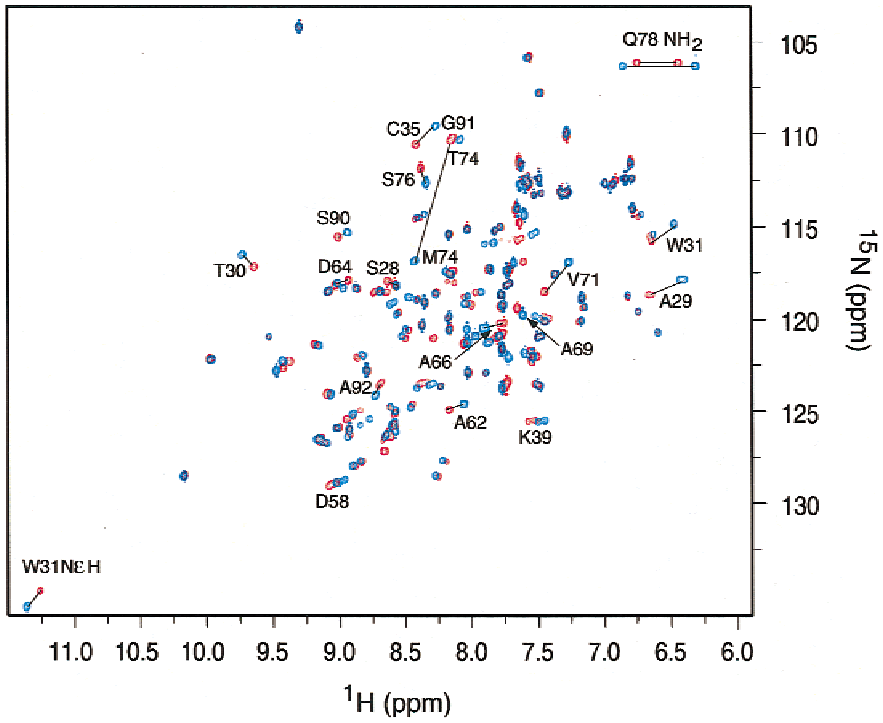

Fig. 1. ^{1}H-^{15}N heteronuclear single quantum coherence correlation spectra of the Met74 (blue contours) and Thr74 (red contours) variants of hTRX. Sample conditions: 0.3 mM hTRX in 50 mM sodium phosphate buffer, pH 5.5 at 35 °C. Note that the doubling of several resonances is due to N-terminal heterogeneity of the proteins with respect to the presence or absence of methionine as the first amino acid as described previously (Forman-Kay et al., 1992).

dissociation constants (K_d) of 6 and 166 μM at protein concentrations of 1 and 10 mg/mL. Equilibrium dissociation constants varying between these two values were reported by Andersen et al. (1997) for a pH range of 3.8–8.0. Table 1 summarizes the data for these K_d values assuming a monomeric mass of 11,755. As can be seen, the predicted apparent masses are far higher than any of those determined by equilibrium analytical centrifugation, ruling out significant dimer formation for the Met74 protein.

As an independent check we also determined the rotational correlation time from ^{15}N T_1 and $T_{1\rho}$ relaxation data (Kay et al., 1989; Clore et al., 1991) which is summarized in Table 2. The oxidized form (which has a disulfide bridge between Cys32 and Cys35 in the active site) of the Thr74 variant has a τ_c value at 35 °C of ~4.7 ns at a protein concentration of ~0.3 mM. The oxidized form of the Met74 variant has a τ_c value at 35 °C of ~6.1 and ~6.8 ns at protein concentrations of 0.3 and 3 mM, respectively. For comparison, interleukin-8 (a 16 kDa dimer), interleukin-1β (a 18 kDa monomer), and staphylococcal nuclease (a 18 kDa monomer) have τ_c values at 35 °C of 7.5 ns (corrected for temperature), 8.3 and 9.1 ns, respectively (Kay et al., 1989; Clore et al., 1991; Grasberger et al., 1993). Thus the measured τ_c values for human thioredoxin are consistent with a monomer, and the slightly larger τ_c values for the Met74 variant relative to the T74 species can be accounted for by a small degree of nonspecific aggregation, under NMR sample conditions.

In conclusion, analytical ultracentrifugation and NMR spectroscopy both indicate that the predominant species of both the Met74 and Thr74 variants of hTRX is monomeric, and we find no evidence for the existence of a well-defined dimeric form.

Table 1. *Predicted weight average masses that would be observed by analytical ultracentrifugation for a reversible monomer-dimer equilibrium of hTRX*

Concentration (mg/mL)	K_d = 6.1 μM	K_d = 166 μM
1	21,495	18,300
10	22,800	21,500

Material and methods: Both the Thr74 and Met74 variants of hTRX were produced and purified essentially as described previously (Forman-Kay et al., 1992). Samples for analytical ultracentrifugation were exchanged into the different buffers by gel filtration using a PD-10 column (Pharmacia Biotech, Piscataway, New Jersey). The initial concentrations varied between 0.2–2 mg/mL. One hundred microliter samples were analyzed in a Beckman XL-I analytical ultracentrifuge at 27,500 rpm at 20 °C using standard double sector cells. After the samples approached equilibrium (14–16 h), they were overspeeded at 45,000 rpm for 4 h for the determination of the baseline correction. Data analysis and other determinations were carried out as described previously (Wingfield et al., 1997). Samples for NMR spectroscopy were exchanged into 100 mM sodium phosphate buffer, pH 5.5, by centrifugation

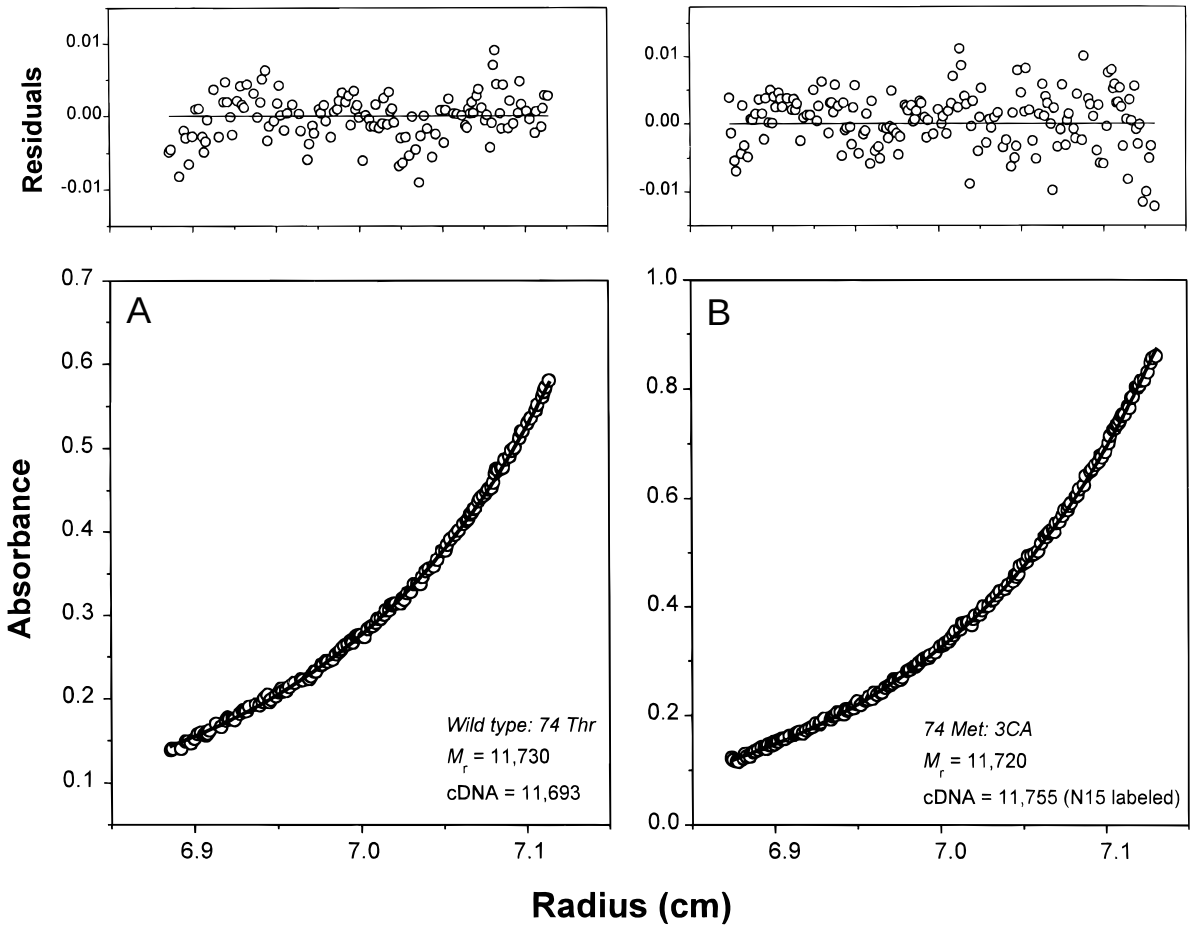

Fig. 2. Analytical ultracentrifugation data for human thioredoxin. **A:** Wild-type variant Thr74. **B:** Mutant (C62A, C69A, C73A, M74). Samples were in 50 mM Tris-HCl, 200 mM NaCl, pH 7.5 and the measurements were made using an Optima XL-I analytical centrifuge. The absorbance gradient measured at 280 nm in the centrifuge cell after attaining sedimentation equilibrium at 27,500 rpm is shown in the bottom panel. The solid line is the result of fitting to a single ideal species and the open circles are the experimental values. The corresponding top panels show the difference in the fitted and experimental values (residuals) as a function of radial position. Also indicated are the experimentally determined molecular weights versus those predicted from the gene coding sequence.

in a centricon concentrator (Amicon, Beverly, Massachusetts). NMR spectra were recorded on a Bruker AMX600 spectrometer at 35 °C. The ^{15}N T_1 and $T_{1\rho}$ relaxation data for the amide envelope were obtained by recording a series of one-dimensional ^{15}N inverse detected NMR spectra for a series of T_1 delays (20, 68, 260, 516, 1,028 and 2,052 ms) and 2.5 kHz spin-lock durations (10.16, 17.84, 32.16, 50.0, 64.4, 78.8, 98.0, and 102.8 ms) as described by Tjandra et al. (1996). Average ^{15}N T_1 and $T_{1\rho}$ values were obtained by fitting the decay of the amide envelope to a single exponential. The decay of the amide envelope was uniform, and no evidence for any significant variation in ^{15}N T_1 or $T_{1\rho}$ values for individual resonances was detected.

Table 2. *Summary of ^{15}N relaxation data on the Met74 and Thr74 variants of hTRX*

Sample	^{15}N $\langle T_1 \rangle$ (ms)	^{15}N $\langle T_{1\rho} \rangle$ (ms)	τ_c[b] (ns)	τ_c[c] (ns)	$\langle S^2 \rangle$[c]	^1H_N $\langle T_2 \rangle$ (ms)
0.3 mM Thr74 hTRX	498 ± 14	160 ± 5	4.7 ± 0.2	4.7	0.83	~46
0.3 mM Met74 hTRX	562 ± 20	125 ± 5	6.1 ± 0.2	6.0	0.87	~38
3.0 mM Met74 hTRX	629 ± 16	115 ± 2	6.8 ± 0.2	6.8	0.84	~30–33

[a] Data were recorded at 600 MHz and 35°C.
[b] Obtained by fitting the ^{15}N $T_1/T_{1\rho}$ ratio by optimization of the value of τ_c (Clore et al., 1991).
[c] Obtained by fitting the ^{15}N T_1 and $T_{1\rho}$ values by optimization of the values of τ_c and $\langle S^2 \rangle$ (Clore et al., 1991).

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