Replacement of Trp^{28} in Escherichia coli Thioredoxin by Site-directed Mutagenesis Affects Thermodynamic Stability but Not Function*

(Received for publication, August 25, 1995, and in revised form, November 21, 1995)

Ivan Slaby†, Vaclava Cerna, Mei-Fen Jeng§, H. J.ane Dyson§, and Arne Holmgren¶

From the Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-17177, Stockholm, Sweden and the Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

Escherichia coli thioredoxin contains two tryptophan residues (Trp^{28} and Trp^{31}) situated close to the active site disulfide/dithiol. In order to probe the structural and functional roles of tryptophan in the mechanism of E. coli thioredoxin (Trx), we have replaced Trp^{28} with alanine using site-directed mutagenesis and expressed the mutant protein W28A in E. coli. Changes in the behavior of the mutant protein compared with the wild-type protein have been monitored by a number of physical and spectroscopic techniques and enzyme assays.

As expected, removal of a tryptophan residue causes profound changes in the fluorescence spectrum of thioredoxin, particularly for the reduced protein (Trx-(SH)\textsubscript{2}), and to a lesser extent for the oxidized protein (Trx-S\textsubscript{2}). These results show that the major contribution to the strongly quenched fluorescence of Trx-S\textsubscript{2} in both wild-type and mutant proteins is from Trp^{28}, whereas the higher fluorescence quantum yield of Trx-(SH)\textsubscript{2} in the wild-type protein is dominated by the emission from Trp^{28}. The fluorescence, CD, and \textsuperscript{1}H NMR spectra are all indicative that the mutant protein is fully folded at pH 7 and room temperature, and, despite the significance of the change, from a tryptophan in close proximity to the active site to an alanine, the functions of the protein appear to be largely intact. W28A Trx-S\textsubscript{2} is a good substrate for thioredoxin reductase, and W28A Trx-(SH)\textsubscript{2} is as efficient as wild-type protein in reduction of insulin disulfides. DNA polymerase activity exhibited by the complex of phage T7 gene 5 protein and Trx-(SH)\textsubscript{2} is affected only marginally by the W28A substitution, consistent with the buried position of Trp^{28} in the protein. However, the thermodynamic stability of the molecule appears to have been greatly reduced by the mutation: guanidine hydrochloride unfolds the protein at a significantly lower concentration for the mutant than for wild type, and the thermal stability is reduced by about 10 °C in each case. The stability of each form of the protein appears to be reduced by the same amount, an indication that the effect of the mutation is identical in both forms of the protein. Thus, despite its close proximity to the active site, the Trp^{28} residue of thioredoxin is not apparently essential to the electron transfer mechanism, but rather contributes to the stability of the protein fold in the active site region.
crease in fluorescence is due to Trp\textsuperscript{28}. Oxidized Trp\textsuperscript{31} mutant proteins have a very low tryptophan fluorescence emission from the remaining Trp\textsuperscript{28}, but reduction results in a large (up to 11-fold) increase of fluorescence intensity (Krause and Holmgren, 1991). The Trp\textsuperscript{28} aromatic ring is in close proximity to the disulfide in wt Trx-S\textsubscript{2} (Katti et al., 1990; Jørgensen et al., 1994), and changes in NMR chemical shifts upon reduction indicate that its position differs slightly in Trx-S\textsubscript{2} (Dyson et al., 1989). Characterization of mutant thioredoxin with Trp\textsuperscript{28} replaced by a nonaromatic residue can be used to determine the role of this residue in the changes of fluorescence emission intensity, as well as to elucidate any involvement in the oxidoreduction mechanism of thioredoxin. Also, since Trp\textsuperscript{28} is a conserved residue in prokaryotes, it might be expected to be of structural importance. In this study, we have chosen to examine the mutant where Trp\textsuperscript{28} is substituted by the smallest hydrophobic residue, alanine. While other substitutions, such as the insertion of serine, would be of some interest, since the mammalian thioredoxins have serine at this position, we considered that the disruption of structure caused by the insertion of a hydrophilic side chain deep into the hydrophobic pocket behind the active site might have a number of effects that could not be analyzed with any certainty. The W28A mutation is the simplest change, with fewest competing effects.

The present study also examines the role of Trp\textsuperscript{28} in bacte riophage systems. The reduced form of E. coli thioredoxin is essential for an assembly of filamentous phages f1 and M13 (Rusell and Model, 1985) and for phage T7 DNA replication (Mark and Richardson, 1976). T7 DNA polymerase consists of the T7 phage-encoded gene 5 protein (g5p) and reduced E. coli thioredoxin. Isolated g5p has a high single-stranded exonuclease activity, but only a very low 5'-3' polymerase and 3'-5' double-stranded exonuclease activities. Addition of Trx-(SH)\textsubscript{2} to g5p in vitro reconstitutes highly processive DNA polymerase and double-stranded exonuclease activities. To date, little is known about how thioredoxin acts to give T7 DNA polymerase high processivity. The role of Trx in T7 DNA polymerase is apparently different from its well known redox functions and is probably linked to a structural interaction which stabilizes the binding of g5p to a primer-template (Tabor et al., 1987; Slaby and Holmgren, 1989). Site-directed mutagenesis of E. coli thioredoxin with subsequent DNA polymerase assay has been successfully used for probing the participation of specific amino acid residues in the binding interaction with the gene 5 protein (Huber et al., 1986; Krause and Holmgren, 1991; Krause et al., 1991).

**EXPERIMENTAL PROCEDURES**

Materials—Wild-type (wt) Trx was expressed in overproducing E. coli strain SK 3981 containing the plasmid pBR325 (Lunn et al., 1984) and purified as described elsewhere (Dyson et al., 1989). Thioredoxin reductase was prepared from E. coli strain A237-pM13 (Rusell and Model, 1986). Phage T7 gene 5 protein was purified from E. coli BH215-pRS101 (Reutimann et al., 1988) by the Affi-Gel-thioredoxin affinity chromatography method (Slaby and Holmgren, 1989).

The mutagenesis host strain E. coli CUI9276 (hsdR17, mcrAB, recA1, Δ(lac-proAB), F'traD36, proA\textsuperscript{B}, lacΔZAM15) was supplied by Dr. E. Holmgren, Pharma BioScience Center, Stockholm. E. coli strain J F521 (Δlac-proAB), thi, supE, metE46, trn1000: Tn10trxB A2 (7004), recA, F' traD36, proA\textsuperscript{B}, lacΔZAM15) and plasmids pUC18 and pUC138-2 were generous gifts from Dr. J. Fuchs, Department of Biochemistry, Gortner Laboratory, St. Paul, MN.

M13-vector DNA, α-\textsuperscript{32}P-DATP, and other molecular biology materials were purchased from Boehringer Mannheim, Pharmacia Biotech Inc., and Amersham Corp. DNA sequencing was done by the dideoxy method using T7 DNA polymerase kit and equipment from Pharmacia Biotech Inc.

DNA Cloning Techniques—Mutagenesis primer was purchased from Scandinavian Gene Synthesis AB and phosphorylated at the 5' end by T4 polynucleotide kinase. The procedure of Vandevec et al. (1991) for generation of mutants was used. Phage DNA of the subclone in M13mp19, containing antisense orientation of the trxA gene, served as the template. Synthesis of complementary mutated DNA strand, removal of parental strand, transfection of competent E. coli CU9276 strain, and the subcloning into the expression plasmid pUC118 were performed as described previously (Krause and Holmgren, 1991).

Most of the DNA work was done according to standard procedures (Sambrook et al., 1989). Screening for mutants was performed directly by sequencing M13 DNA purified by the method of Vieira and Messing (1987).

Preparation of Mutant Thioredoxin—A 2-liter culture of the J F521/ptraD-wt28 E. coli strain was grown in a fortified batch with 50 μg/ml ampicillin to about A\textsubscript{opt} = 1.0. The cells were then induced with 0.1 mm final concentration of isopropyl-β-D-thiogalactopyranoside and the incubation continued another 8 h with vigorous shaking before harvesting. The purification of W28A thioredoxin was carried out by DEAE-cellulose and Sephadex G-50 chromatography as described (Krause and Holmgren, 1991).

Enzyme Assays of Thioredoxin—The activity of thioredoxin with thioredoxin reductase was assayed with DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) as a substrate (Moore et al., 1964). The reactions, containing 50–200 μM thioredoxin, were started by addition of 100 μM thioredoxin reductase and followed at 412 nm on a Shimadzu UV-2100 recording spectrophotometer (ε\textsubscript{412} DTNB = 13,600 M\textsuperscript{-1} cm\textsuperscript{-1}). The assay mixture contained 100 μM Tris-EDTA, 0.1 mM mgl bovine serum albumin, 0.5 mM DTNB and 0.24 mM NADPH.

Insulin was used to determine the protein disulfide reductase activity of thioredoxin coupled to NADPH oxidation via thioredoxin reductase (Holmgren, 1979). The reactions, containing 0.5–1.5 μM thioredoxin, 0.4 mM NADPH, 160 μM insulin, 2 mM EDTA in 0.1 M phosphate buffer, pH 7.0, were started by addition of 200 μM thioredoxin reductase. The rate of NADPH oxidation was calculated from the decrease in absorbance at 340 nm, using a molar extinction coefficient for NADPH (6,200 M\textsuperscript{-1} cm\textsuperscript{-1}).

DNA Polymerase Assay—Stimulation of DNA polymerase activity of gene 5 protein (3 mM final concentration) by wild-type (wt) or mutant thioredoxins of varying concentrations was measured by a standard assay of polymerase activity on denatured salmon sperm DNA at 37 °C (Slaby et al., 1984). Thioredoxin was added directly to the assay mixtures containing gene 5 protein followed by at least 10-min preincubation. The reactions were started by a transfer to 37 °C water bath. One unit of DNA polymerase catalyzes the incorporation of 10 nmol of total nucleotide into an acid-insoluble product in 30 min at 37 °C (Huber et al., 1986).

Fluorescence Measurements—Protein fluorescence was measured with a thermostatted Shimadzu RF-510C spectrofluorimeter at 18 °C. Excitation of fluorescence was at 283 nm and emission spectra from 300 to 400 nm were recorded. The samples contained 1 μM concentrations of thioredoxin in potassium phosphate buffer, pH 7.0. Oxidized thioredoxin was reduced by dithiothreitol (DTT) in a concentration of 1 mM. The denaturation experiments were carried out by addition of stock 8 mM guanidine hydrochloride to the samples.

NMR Measurements—All spectra were acquired on a Bruker AMX500 spectrometer operating at 500 MHz for protons as described previously (Dyson et al., 1986, 1989). 2QF COSY (Rance et al., 1983) and 2D (Braunschweiger et al., 1983) spectra were acquired at 298 K for both the oxidized and reduced forms of the W28A mutant. Samples were approximately 4 mM in 100 mM potassium phosphate buffer, pH 6.0. Spectral widths were commonly 7042 Hz with 4,000 complex points in ω\textsubscript{2} and 14085 Hz with 512 complex points in ω\textsubscript{1}. Spectra were Fourier transformed on a Sun workstation using the FTNMR software of Dennis and Cavanagh to give total matrix size of 2,000 × 1,000 real points. Spectra were referenced to an internal standard of dioxane at 3.75 ppm.

Circular Dichroism Measurements—Circular dichroism spectra were recorded on J ASCO J 700 or Aviv 61DS spectropolarimeters using a 1-mm path length at 24 °C. Protein samples in 50 mM phosphate buffer, pH 7.0, and varying concentrations of guanidine HCl were prepared by gradual dialysis of thioredoxin to the respective concentrations. The samples were reduced with DTT at a final concentration of 1 mM and equilibrated for 15 min before CD spectra recorded from 250 to 215 nm were recorded. The fraction of unfolded thioredoxin at each concentration of guanidine HCl was determined from the percent ellipticity change at 222 nm relative to the values at 0 and 5 mM guanidine HCl.

Analytical Methods—Protein concentrations were determined from the absorbance at 280 nm using molar extinction coefficients of 13,700 M\textsuperscript{-1} cm\textsuperscript{-1} for wt and 8,200 M\textsuperscript{-1} cm\textsuperscript{-1} for W28A thioredoxin and 134,000 M\textsuperscript{-1} cm\textsuperscript{-1} for gene 5 protein. ε\textsubscript{280} for thioredoxin reductase was 67,500.
Protein absorbance and ultraviolet spectra were determined with a Zeiss PMQ3 spectrophotometer. To determine the amino acid composition, protein samples were lyophilized and hydrolyzed with 6 M HCl, 0.5% phenol for 24 h at 110 °C in vacuo and analysis was performed with Beckman 121M amino acid analyzer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was run on 8–12% gradient gels using the Phast System from Pharmacia.

RESULTS AND DISCUSSION

Site-directed Mutagenesis and Expression of W28A Thioredoxin—To obtain a template for mutagenesis, the trxA gene from pUC118-trxA (Lim et al., 1985) was inserted into M13mp19 via the EcoRI and HindIII sites. A primer with the sequence 5'-CTCTGCCGCGAAATCGACG-3' containing a 2-base mismatch in its central region was hybridized to the single-stranded M13mp19-trxA DNA. Mutant thioredoxin genes were identified by sequencing at a frequency of about 10%. The replicative form of M13mp19 carrying the trxA mutation was cleaved and recloned to the pUC118 expression vector utilizing the EcoRI and HindIII sites. The host E. coli strain JF521 lacking the chromosomal wild-type trxA gene was used for expression of the pUC118-trxA-encoded W28A thioredoxin. The expression was inducible by isopropyl-β-D-thiogalactopyranoside, since pUC118 contains the lac promoter just in front of the trxA gene. The insertion of the fragment into the pUC118 polylinker region introduces a TAA stop codon between lacZ and trxA, thus excluding a protein fusion (Krause and Holmgren, 1991).

Purification and Physical Properties—A yield of about 30–50 mg of mutant thioredoxin/liter of culture was obtained from the JF521/pUC118-trxA-W28A cells. The W28A protein has the same chromatographic properties during purification as wild-type thioredoxin, and electrophoretic properties are also similar: W28A thioredoxin appears as a single band in the same position as wt thioredoxin in sodium dodecyl sulfate gels (data not shown). The amino acid composition indicates that W28A thioredoxin contains only one tryptophan residue (data not shown), and this is confirmed by analysis of the ultraviolet and fluorescence spectra. The ultraviolet spectra of the mutant protein show the expected decrease of tryptophan absorbance at 280 nm (data not shown), and the corresponding lower molar extinction coefficient is in good agreement with the disulfide content of W28 thioredoxin as determined by NADPH oxidation catalyzed by thioredoxin reductase.

NMR Spectra of W28A—Diagnostic 2Q and 2QF COSY NMR spectra of the mutant thioredoxin confirm that the protein is folded in solution and that its structure is very similar to that of the wt protein. A portion of the 1H 2Q spectrum of Trx-(SH)2 is shown in Fig. 1, and several of the resonance assignments, obtained by analogy with those of wt (Dyson et al., 1989) are indicated.

Activity of W28A Thioredoxin in Oxidoreduction Catalysis—The activity of wt and mutant Trx with thioredoxin reductase was assayed with DTNB as a substrate (Moore et al., 1964). At pH 8.0, DTNB reduction rates with relatively high concentrations of thioredoxin reductase (100 nM) are 63 μM·min⁻¹ for wt and 73 μM·min⁻¹ for W28A. The rates of reduction of insulin
disulfides at pH 7.0 are 26 μM Trx-S2 min⁻¹ for wt and 30 μM Trx-S2 min⁻¹ for the mutant. No decrease in the rates of reaction is evident; indeed W28A thioredoxin appears to be a marginally better substrate for thioredoxin reductase and slightly more efficient in the reduction of insulin disulfides than the wild-type protein.

Interaction with the Bacteriophage T7 Gene 5 Protein—The interactions of mutant and wt thioredoxins with g5p were tested in a reconstitution assay in vitro with pure proteins. The results obtained under identical conditions with wt thioredoxin and W28A mutant are shown in Fig. 2, where polymerase activity is plotted against the logarithm of increasing thioredoxin concentration at a constant concentration of g5p of 3 nM. A quantitative evaluation of the reconstitution assays was achieved by using Scatchard plot analysis to determine an observed equilibrium dissociation constant, K_obs (Huber et al., 1986). This constant depends on the initial concentration of g5p and corresponds to the thioredoxin concentration yielding half-maximum enzyme activity, assuming that the polymerase activity is proportional to the concentration of the g5p-thioredoxin complex. In our experiment, K_obs of 4 nM for wt and 6 nM for W28A thioredoxin were calculated. The maximal polymerase activities of the reconstituted complexes were similar with both thioredoxins (1,800 units/nmol).

Tryptophan Fluorescence Measurements—The fluorescence emission spectra between 300 and 400 nm with excitation at 283 nm are shown in Fig. 3 for wt thioredoxin and mutant thioredoxins W28A and W31A. The fluorescence behavior of the three proteins differs significantly. The quantum yield of tryptophan fluorescence in wt Trx-S2 is very low (Q = 0.02). Reduction of the disulfide bond by DTT causes a 3.5-fold increase in fluorescence at pH 7.0 (Holmgren, 1972). The oxidized form of the W31A mutant shows a decrease of approximately 50% in tryptophan fluorescence compared to wt Trx-S2 and a large increase in fluorescence (10-fold) upon reduction to give a fluorescence significantly greater than that wild-type Trx-S2 (Krause and Holmgren, 1991). The fluorescence quantum yield of W28A Trx-S2 is about 20% lower than wt Trx-S2 and increases only...
Thioredoxin contains a significant amount of protein. Since thioredoxin contains a significant amount of helical structure, the negative ellipticity at 222 nm can be used as a measure of the extent of unfolding of the molecule, although contributions from the ellipticity of the five-stranded β-sheet (λ<sub>max</sub> = 215 nm) may also be present. Differential stability of the helix and sheet portions of thioredoxin have also been inferred from hydrogen exchange studies (Jeng and Dyson, 1995).

The ellipticity at 222 nm as a function of GdnHCl concentration is shown in Fig. 5 for wt and W28A thioredoxin. For both proteins, the oxidized form is significantly more stable than the reduced form to GdnHCl denaturation, consistent with the greater thermal stability of Trx-S<sub>2</sub> (see below). Interestingly, the concentration of GdnHCl at which half of the protein is denatured differs by exactly the same amount between the two forms of W28A Trx compared with wt, indicating that changing Trp<sup>28</sup> does not affect the relationship between the structures of the two oxidation states. This observation is entirely consistent with the results of the enzymatic assays, which indicate no significant difference in activity for the mutant.

However, it is clear from Fig. 5 that the stability of W28A in either oxidation state is significantly decreased relative to the wild-type protein. The GdnHCl concentration for half of the protein to be denatured is decreased from 2.38 M to 1.73 M for Trx-S<sub>2</sub> and from 1.60 M to 1.00 M for Trx-(SH)<sub>2</sub>. A similar plot (not shown) for the fluorescence intensity results shown in Fig. 3 is much more complex due to the complexity of the fluorescence changes, including changes in emission wavelength, upon reduction of either wt or W28A mutant Trx, but still shows the same overall behavior. These results indicate that although the protein is correctly folded under native conditions, as indicated by the similarity of the mutant and wt NMR spectra (Fig. 1), the stability of the W28A mutant protein has been significantly decreased by the change of tryptophan for alanine at position 28.

The thermal stability of the oxidized and reduced forms of the mutant and wild-type thioredoxins was estimated by following the ellipticity at 222 nm as a function of temperature (data not shown). The transition temperatures of the mutant proteins are significantly lower than those of the wild-type proteins (80 °C compared with 88 °C for Trx-S<sub>2</sub> and 70 °C compared with 80 °C for Trx-(SH)<sub>2</sub>). Once again, the relative difference between the two oxidation states has been preserved, consistent with the functional similarity of the mutant to the wild-type and with the behavior of the proteins toward denaturation in GdnHCl.

**CONCLUSIONS**

The two tryptophan residues are highly conserved in thioredoxins (Eklund et al., 1991). Trp<sup>31</sup> is conserved throughout prokaryotic and eukaryotic proteins and its replacement with alanine causes significant changes in function (Krause and Holmgren, 1991). Consistent with this, Trp<sup>31</sup> is situated in an unusual position on the surface of the molecule (Katti et al., 1990; Jeng et al., 1994). By contrast, Trp<sup>28</sup> is conserved only in prokaryotic thioredoxins (Eklund et al., 1991) and is largely buried in the hydrophobic core of the molecule (Jeng et al., 1994). The residue that replaces Trp<sup>28</sup> in eukaryotes, which is not even remotely homologous to tryptophan; this argues that tryptophan at this position is probably not necessary for the function of thioredoxin. Its conservation among such a large and varied group as the prokaryotes argues that it is necessary for some other reason, possibly for stabilizing local structure in the vicinity of the active site cysteine residues.

We have shown that the replacement of Trp<sup>28</sup> with alanine results in no significant change in the functional properties of thioredoxin. The NMR, fluorescence, and CD spectra of the mutant protein under native conditions all indicate that the
protein is correctly folded, consistent with its correct function both in assays that require reduction or oxidation of the cysteines and in assays such as the T7 DNA polymerase reaction that measure tightness and specificity of binding. However, both fluorescence and CD measurements as a function of added guanidine hydrochloride and temperature clearly show that the stability of the W28A protein is significantly decreased relative to wild type. Furthermore, the effects of the mutation on the stability of the protein are the same for both Trx-S2 and Trx-(SH)2, consistent with the integrity of function in this mutant. It is perhaps somewhat surprising that such a significant mutation as the replacement of a tryptophan by an alanine in close proximity to the active site should have as little effect on the function of the protein as it does in this case. These results are an indication of the robust nature of thioredoxin and of its stability of function toward a wide variety of mutational challenges.

Acknowledgments—We thank Dr. Bjo¨rn Nilsson (Pharmacia, Stockholm) for help with the CD experiments. The technical assistance of Monica Lindell is gratefully acknowledged.

REFERENCES

Braunschweiler, L., Bodenhausen, G., and Ernst, R. R. (1983) Mol. Physiol. 48, 535–560

Dyson, H. J., Holmgren, A., and Wright, P. E. (1988) FEBS Lett. 228, 254–258

Dyson, H. J., Holmgren, A., and Wright, P. E. (1989) Biochemistry 28, 7074–7087

Dyson, H. J., Gippert, G. P., Case, D. A., Holmgren, A., and Wright, P. E. (1990) Biochemistry 29, 4129–4136

Eklund, H., Gleason, F. K., and Holmgren, A. (1991) Proteins Struct. Funct. Genet. 11, 13–28

Gleason, F. K., and Holmgren, A. (1988) FEMS Microbiol. Rev. 54, 271–298

Holmgren, A. (1972) J. Biol. Chem. 247, 1992–1998

Holmgren, A. (1979) J. Biol. Chem. 254, 9113–9119

Holmgren, A. (1981) Biochemistry 20, 3204–3207

Holmgren, A. (1985) Annu. Rev. Biochem. 54, 237–271

Holmgren, A. (1989) J. Biol. Chem. 264, 13963–13966

Huber, H. E., Russel, M., Model, P., and Richardson, C. C. (1986) J. Biol. Chem. 261, 15006–15012

Jeng, M.-F., and Dyson, H. J. (1995) Biochemistry 34, 611–619

Jeng, M.-F., Campbel, A. P., Begley, T., Holmgren, A., Case, D. A., Wright, P. E., and Dyson, H. J. (1994) Structure 2, 853–868

Katti, S. K., Lemaître, D. M., and Ekland, H. (1990) J. Mol. Biol. 212, 167–184

Krause, G., and Holmgren, A. (1991) J. Biol. Chem. 266, 4056–4066

Krause, G., Lundström, J., Barea, J. L., Pueyo de la Cuesta, C., and Holmgren, A. (1991) J. Biol. Chem. 266, 9494–9500

Lim, C.-J., Geraghty, D., and Fuchs, J. A. (1985) J. Bacteriol. 163, 311–316

Lunn, C. A., Kajhu, S., Wallace, B. J., Kushner, S. R., and Piguet, V. (1984) J. Biol. Chem. 259, 10469–10474

Mark, D. F., and Richardson, C. C. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 780–784

Moore, E. C., Reichard, P., and Thelander, L. (1964) J. Biol. Chem. 239, 3445–3452

Mérida, F., Rigler, R., Holmgren, A., and Brochon, J.-C. (1989) Biochemistry 28, 3383–3398

Rance, M., Sörensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., and Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. 117, 479–485

Reutimann, H., Sjöberg, B.-M., and Holmgren, A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6783–6787

Russel, M., and Model, P. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 29–33

Russel, M., and Model, P. (1986) J. Biol. Chem. 261, 14997–15005

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Slaby, I., and Holmgren, A. (1989) J. Biol. Chem. 264, 16502–16506

Slaby, I., Lind, B., and Holmgren, A. (1984) Biochem. Biophys. Res. Commun. 122, 1410–1417

Tabor, S., Huber, H. E., and Richardson, C. C. (1987) J. Biol. Chem. 262, 16212–16223

Vandeyar, M. A., Weiner, M. P., Hutton, C. J., and Batt, C. A. (1988) Gene (Amst.) 65, 129–133

Vieira, J., and Messing, J. (1987) Methods Enzymol. 153, 3–11