Articles

Folding of the Reduced Form of the Thioredoxin from Bacteriophage T4

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ABSTRACT: The folding pattern for bacteriophage T4 thioredoxin is similar to that of the oxidized form [Borden, K. L. B., & Richards, F. M. (1990) *Biochemistry 29*, 3071–3077]. Equilibrium and kinetic studies were carried out by fluorescence and circular dichroism techniques. The same box model proposed for the oxidized form, with four identifiable states, can accommodate most of the data: $N \rightarrow U_c \rightarrow U_t \rightarrow I_t \rightarrow N$, where N is the native state, U_c is the unfolded species with Pro 66 in the cis form, U_t is the unfolded species with Pro 66 in the trans form, and I_t is a *trans*-Pro 66 intermediate with a volume comparable to that of N. However, the relative importance of the different components is shifted between the oxidized and reduced proteins. In spite of the small size of the disulfide loop, the Cys 14–Cys 17 bond appears to be important in stabilizing I_t. The tertiary structure as monitored by near-UV CD and fluorescence indicates that the reduced form is significantly less stable than its oxidized counterpart; however, the two secondary structures, as seen by far-UV CD, are very similar. The intermediate I_t behaves as though it is cold denaturated at 4 °C.

The thioredoxins are a widely distributed group of proteins with a variety of different biological functions, a number of which depend on the redox properties of the single disulfide bond. The 108-residue protein from *Escherichia coli*, EcTrx,[1] and the 87-residue protein from bacteriophage T4, T4Trx, are two members of this group. The latter protein lacks the first 21 N-terminal residues of EcTrx and has only seven sequence identities among the remaining 87 residues. However, the general peptide chain conformation is known to be very similar from comparison of the two crystal structures, EcTrx by Holmgren and Brändén (1975) and Katti et al. (1990) and T4Trx by Söderberg et al. (1978). The active site region with the disulfide loop and a neighboring *cis*-proline residue is located in the same part of both proteins even though none of the other nearby residues are identical. EcTrx has two tryptophan and two tyrosine residues while T4Trx has four tyrosine residues. None of the aromatic rings occupy the same three-dimensional positions in the two proteins. EcTrx has five proline residues; T4Trx has four. Only the *cis*-prolines near the disulfide groups occupy identical positions.

There have been a number of studies on the structure and folding of EcTrx in both its oxidized and its reduced forms (Dyson et al., 1989; Hiroaki et al., 1988; Kelley et al., 1986; Kelley & Stellwagen, 1987; Langsetmo et al., 1989). The cleavage of the single disulfide bond is thought to only cause minor conformational changes (Stryer et al., 1967; Holmgren & Roberts, 1976; Hiraoki et al., 1988). On the basis of CD studies, Brown et al. (1987) suggested that upon reduction there is a 6% increase in the α-helical content and a 3% decrease in β-sheet content. Higher resolution techniques such as NMR confirm that only small changes occur upon reduction of EcTrx (Hiraoki et al., 1988; Dyson et al., 1989). Differences in the structures of the oxidized and reduced EcTrx appear to be restricted to the active site near the disulfide bond. The crystal structure of the reduced protein is not yet known.

Langsetmo et al. (1989) have studied the folding of both forms of EcTrx as well as some mutants via urea gradient electrophoresis. The native reduced form contains two species, the cis and the trans isomers of Pro 76. In the folded oxidized state, they only found evidence for the trans form of Pro 76 at low temperatures. In both oxidized and reduced forms the native species containing the trans isomers are less stable than their cis counterparts. The oxidized form is more stable than the reduced to denaturation by urea, by GuHCl (Kelley et al., 1987), and by pH and temperature (Hiraoki et al., 1988). Both Kelley et al. (1987) and Langsetmo et al. (1989) suggest a four-state box mechanism to describe the folding pathway of EcTrx.

The extensive sequence differences combined with the three-dimensional structural similarities between the *E. coli* and T4 thioredoxins suggested that a comparison of their folding pathways would be useful. Borden and Richards (1990) have studied the folding of the oxidized form of T4Trx and found that a four-state box mechanism would serve as a minimal model in this system also. A study of the reduced form of T4Trx is reported in this paper.

Experimental Procedures

The preparation and purification of T4Trx, the fluorescence and CD procedures, and explanations of the phase amplitude and time constant calculations are provided in the earlier paper (Borden & Richards, 1990).

All buffers contained 50 mM sodium phosphate, pH 6.0, 1 mM DTT, and 0.5 mM EDTA and were degassed for 1 h before use. All test tubes were flushed with argon or dry N_2 before samples were placed into them. The samples were allowed to sit overnight at room temperature to ensure complete reduction.

A separate set of experiments were carried out where the far-UV CD, near-UV CD, and fluorescence were monitored in the oxidized state; the sample was then reduced by addition of a small amount of concentrated DTT, and the same physical

[1] Abbreviations: EcTrx, thioredoxin isolated from *E. coli*; T4Trx, thioredoxin isolated from bacteriophage T4; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); near-UV CD, circular dichroism in the 210–260-nm range; far-UV CD, circular dichroism in the 260–310-nm range; GuHCl, guanidine hydrochloride.

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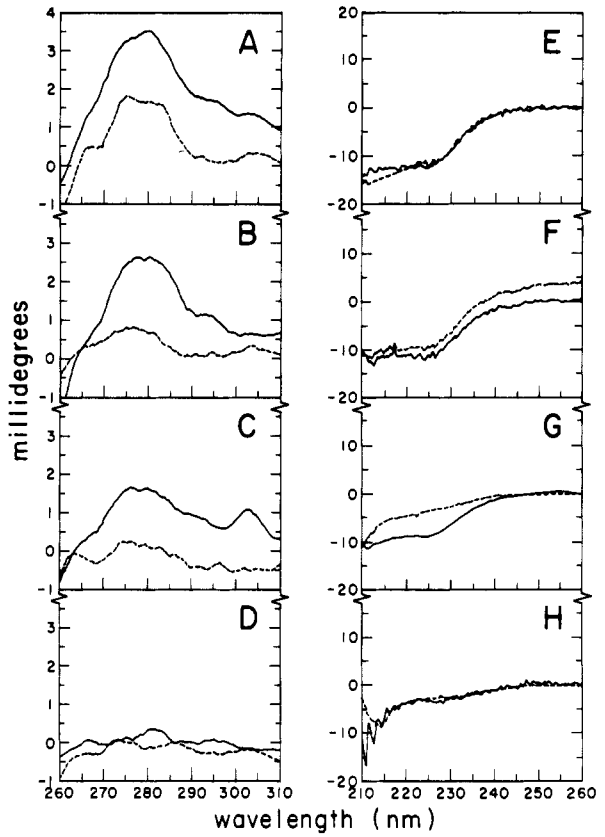


FIGURE 1: Circular dichroism spectra of T4 thioredoxin in the reduced and oxidized forms. The solid lines indicate spectra taken before reduction and the dashed lines spectra taken after reduction. Panels A–D show the near-UV CD measurements and panels E–H the far-UV CD data. The guanidine hydrochloride concentrations for the sample pairs shown in each panel were as follows: A and E, 0 M; B, 1.20 M; F, 0.98 M; C, 1.60 M; G, 1.45; D and H, 3.0 M. The protein concentrations for the near-UV CD samples were approximately 35 μM and 20 μM for those in the far-UV CD.

properties were remeasured. The dilution of protein in these samples was never more than 0.1%. For equilibrium and manual-mixing fluorescence studies, protein concentrations ranged from 1 to 3 μM. However, for stopped-flow experiments 10-μm samples were used due to the lower sensitivity of the photomultiplier tube in that instrument. Todd Schuster at the University of Connecticut kindly offered us the use of his stopped-flow fluorometer. Far-UV CD and near-UV CD samples ranged from 30 to 60 μM in protein. After the physical measurements were completed, DTNB assays (Crestfield, 1963) were carried out to ensure that the protein was still reduced.

With T4Trx one should note that the signals observed in the near-UV CD and fluorescence were monitoring only tyrosine residues and thus were much smaller than with EcTrx which contains tryptophans. The signal to noise ratio in T4Trx is lower, and it was much more difficult to make accurate measurements. When similar studies were carried out on EcTrx with comparable protein concentrations, the difference in fluorescence between the native and denatured forms was approximately 10 times greater than in T4Trx. The near-UV CD suffered similar problems especially in reduced T4Trx where the native protein has already lost half of the ellipticity of the oxidized form even before unfolding.

Results

In Figure 1, panels A and E show the CD properties of the native state in both oxidized and reduced forms; the far-UV CD spectra are nearly identical. However, there is a substantial change in structure in the near-UV CD region upon reduction of the disulfide (Figure 1, panel A). From panel E it is clear that the secondary structure is largely unaffected by the presence of the disulfide bond. There appears to be the same degree of structure in the denatured states of both the oxidized and reduced forms (Figure 1, panels D and H).

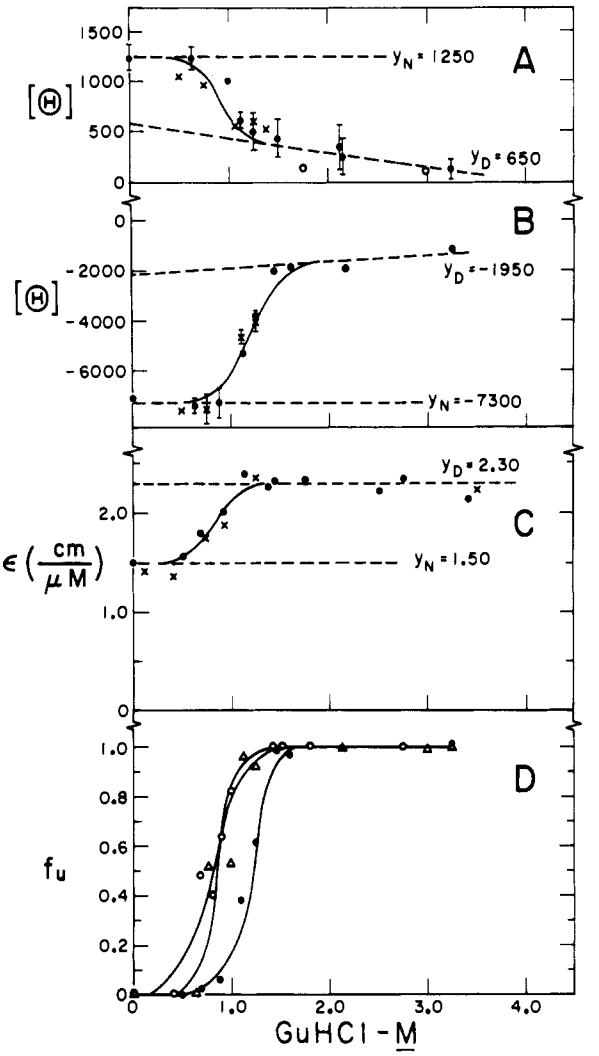


FIGURE 2: Equilibrium measurements obtained by near-UV CD (panel A), far-UV CD (panel B), and fluorescence (panel C). These data plotted in normalized form as the fraction of unfolded protein are shown in panel D. Measurements were made at 25 °C for fluorescence and at ambient temperature (20–25 °C) for CD. In panels A–C, (×) represents data from refolding experiments and the solid circles (●) data from unfolding experiments. In panel D: solid circles (●), far-UV CD data; open triangles (△), near-UV CD data; open circles (○) fluorescence data. The ordinate for panel A is molar ellipticity per tyrosine residue; for panel B, molar ellipticity per residue; and for panel C, intensity from the chart recorder normalized by protein concentration. Standard deviations are shown by error bars. Duplicate runs were made at each GuHCl concentration.

Equilibrium measurements at various concentrations of GuHCl were made on samples allowed to sit overnight in reducing conditions (Figure 2). When the measurements are normalized to the same protein concentration, one can see that the fluorescence of the native reduced protein (y = 1.50, Figure 2C) is only slightly greater than that of the oxidized form (y = 1.18; Borden & Richards, 1990; Figure 1C). In contrast to its effect on the tryptophan fluorescence in EcTrx (Kelley & Stellwagen, 1987), the disulfide bond apparently has only a small quenching effect on the tyrosine fluorescence in the oxidized form of T4Trx. Fluorescence measurements at 25 °C indicated that folding was reversible and that the midpoint

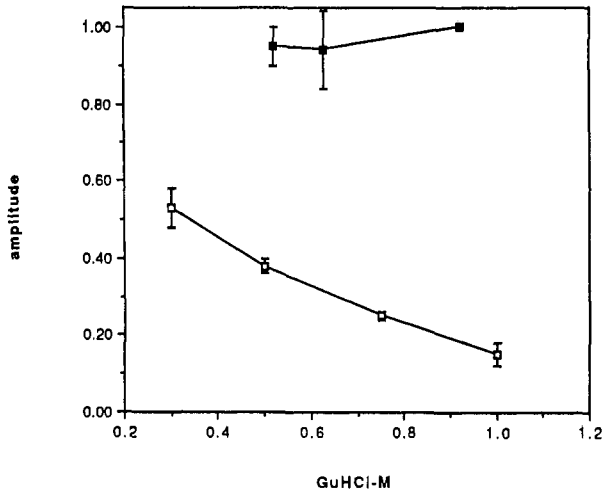


FIGURE 3: Dependence of the amplitude on the final denaturant concentration. The slow-phase amplitude, α_1, is shown by open squares (□), and the fast-folding phase, α_2, is shown by solid squares (■). Each point represents the average of three measurements. The slow phase was determined via manual-mixing experiments while the fast phase was obtained from stopped-flow fluorescence measurements, all at 25 °C; error bars show standard deviations in replicate runs.

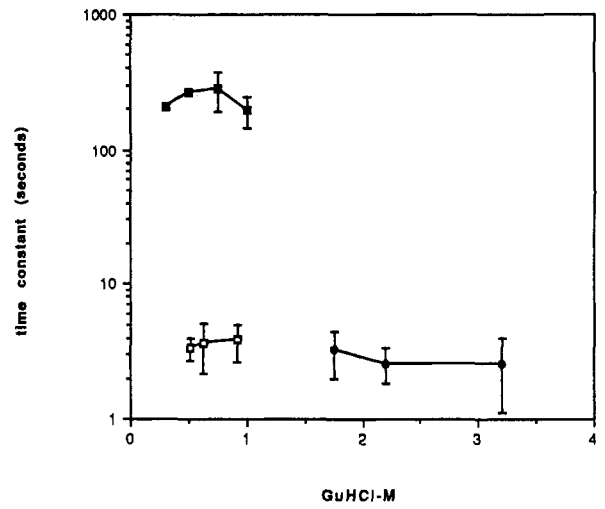


FIGURE 4: Dependence of time constants on final denaturant concentration. Each point is the average of three measurements. Solid boxes (■) represent the slow-folding phase, open squares (□) the fast-folding phase, and solid circles (●) the single unfolding phase.

was 0.9 M GuHCl (Figure 2) with the transition occurring between 0.5 and 1.25 M GuHCl. The cooperativity appears to be lowered by reduction. The slope of the corresponding transition for the oxidized form is substantially larger (Borden & Richards, 1990).

The far-UV CD spectrum of native reduced protein is very similar to that of the oxidized form. The molar ellipticity per residue at 225 nm of the native reduced form, -7300 ± 300 deg·cm^2/dmol, is slightly less than that reported for the oxidized form, which is -8200 ± 400 deg·cm^2/dmol (Borden & Richards, 1990; Figure 1B). This may indicate a slight loss of ordered secondary structure in the reduced state. The transition midpoint for unfolding is 1.1 M GuHCl (Figure 2), and renaturation experiments again indicate that folding is reversible. Reduction causes a small decrease in stability as the midpoint for the oxidized form occurs at 1.4 M GuHCl. However, the unfolding appears more cooperative since the slope in the transition zone is steeper than that for the oxidized protein.

The ellipticities per tyrosine in the near-UV were smaller in magnitude than those found in the oxidized protein (Figure 1). The native value for the oxidized protein is approximately 2000 deg·cm^2/dmol at 277 nm and 1250 deg·cm^2/dmol at 274 nm for the reduced sample (Figure 1A). However, the midpoint of the unfolding transition was shifted from 1.6 to 1.0 M GuHCl on reduction, and the cooperativity was also significantly lowered.

Because of the small signals discussed above, we sought confirmation of the apparent changes in stability caused by reduction, i.e., to confirm the differences between the data in Figure 2 of this paper and Figure 1 in Borden and Richards (1990). For a fixed GuHCl concentration, spectra of the same sample were collected before and after reduction. Examples of the data are shown in Figure 1, panels B and F and panels C and G. These data confirm the observations made in the separate experiments on different oxidized and reduced samples reported in this and the earlier paper.

The kinetics of the slow phase of folding were studied by manual-mixing techniques. The effect of varying the final denaturant concentration is shown in Figure 3. The amplitude for the slow phase, α_1, increases with decreasing denaturant concentration as with the oxidized protein (Borden & Richards, 1990; Figure 4). The curve lacks the sigmoidal shape and has a lower amplitude in general than for the oxidized form. In experiments where the final GuHCl concentration is 0.3 M but the starting values are either 6 or 3 M, one obtains different slow-phase amplitudes. The value of α_1 for the 20-fold dilution was 0.37 ± 0.08 and for the 10-fold dilution 0.53 ± 0.05. Because of this observation, all mixing studies reported here started with an initial concentration of 3 M GuHCl. This dependence of α_1 on the initial denaturant concentration was not seen with the oxidized protein. In manual-mixing studies at 4 °C refolding seemed to occur within the deadtime of the experiment, which was approximately 30 s.

The time constants for these reactions are shown in Figure 4. These data suggest that the slow phase is a *cis*-proline–*trans*-proline isomerization. Evidence for this includes the following: (a) time constants are in the correct range, and (b) the time constants are relatively independent of final denaturant concentration (also see below). The time frame for a cis–trans isomerization can vary from 0.2 to 7.7 min according to Brandts et al. (1975) and is specifically 10–100 s for RNase A (Schmid & Baldwin, 1978).

Stopped-flow fluorescence studies were used to examine the unfolding and faster refolding phases. Time constants for both the unfolding and fast refolding were on the time scale of seconds (Figure 4). There seems to be only one fast phase during refolding and only a single unfolding phase. The phase amplitudes for the slow and fast refolding phases account for a fraction unfolded, f_u, greater than 1 (Figure 3), which of course is not possible. One should note that the estimates of α_1 and α_2 in the manual-mixing and stopped-flow fluorescence experiments are entirely independent. While the standard deviations give a measure of the repeatability of the measurement, the absolute accuracy may be less. The small signals cause errors in these amplitude estimates especially in the stopped-flow measurements.

Manual-multimixing experiments were originally reported by Brandts et al. (1975) to determine the rate constant of the *cis*-proline–*trans*-proline isomerization. Extensive studies have been carried out with this technique by Nall et al. (1978) and Schmid and Baldwin (1978) on RNase A. In this study, manual-multimixing experiments were done at the same initial concentration but two different final denaturant concentrations. They yielded different values for α_1 (Figure 5). The slow-

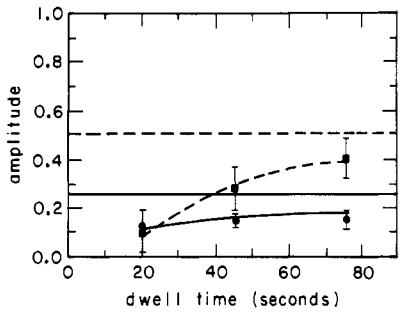
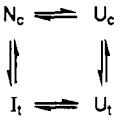


FIGURE 5: Dependence of α_1 on the dwell time. The final guanidine concentration was either 0.3 M (■) or 0.75 M (●). For both cases the initial GuHCl concentration was 3.0 M. The horizontal dashed line indicates the value of α_1 at infinite dwell time for a final GuHCl concentration of 0.3 M, and the solid line represents this value at 0.75 M. Each point is the average of three measurements. The curves drawn through the points are the best single-exponential fits to the data.

Scheme I



phase amplitudes in both cases increase with increasing dwell time. This behavior suggests the accumulation of a slow folding form. For final GuHCl concentrations of 0.3 and 0.75 M the time constants are 40 and 195 s, respectively.

Discussion

A four-state model is adequate to describe the kinetic data for a fixed set of unfolding/folding conditions (Scheme I). The slow phase is almost certainly a cis–trans isomerization. In the oxidized form, the fluorescence data suggest, and size-exclusion chromatography confirms, that the slow phase present in manual-mixing experiments is the $I_t \rightarrow N$ transition, i.e., the cis–trans isomerization in the compact state (Borden & Richards, 1990). The dependence of the amplitude on final denaturant concentration (Figure 3) is similar for both oxidized and reduced T4Trx, suggesting that the $I_t \rightarrow N$ accounts for the slow phase in the reduced form as well. Since the disulfide bond is only involved in the formation of a small loop (Cys 14–Cys 17), one might not expect the folding pattern of the reduced state to be altered dramatically.

The disulfide bond is important for the stability of I_t. The magnitude of the slow phase is smaller in the reduced form than in the oxidized form. At 0.3 M GuHCl, α_1 is 0.95 for oxidized and 0.55 for reduced. The slow phase appears to vanish at low temperatures, suggesting that I_t is subject to cold denaturation. In the oxidized form there is also evidence for cold denaturation of I_t; i.e., the α_1 vs denaturant curve is shifted down when the temperature is lowered from 25 to 4 °C (Borden & Richards, 1990). Since there is no slow phase detectable at 4 °C, only $U_c \rightarrow N$ is substantially populated in the reduced form. It is known that the redox potential of the cell is such that disulfides are normally in their reduced form in the cytoplasm. The reduced state seems to hinder the formation of the incorrect isomer, I_t, and thus allows the protein to fold to a completely native state more efficiently than it does in the oxidized form. It may be significant that the *cis*-proline is directly above the disulfide ring and in the active site pocket.

Folding is undoubtedly more complex than the four-state model suggests. This is indicated by both the equilibrium and kinetic data. Equilibrium curves obtained from near-UV CD fluorescence suggest that there is one population of tyrosines in the reduced protein rather than the two seen in the oxidized form (Borden & Richards, 1990). Figures 1 and 2 indicate that the tertiary structure is lost well before the secondary structure, suggesting an expanded but still reasonably compact intermediate often referred to now as a molten globule. While there appears to be a slight difference in stability between the native reduced and oxidized forms and perhaps a differential change in stability between different parts of the structure, there is no evidence for any difference in residual structures between the two forms in the denatured state.

In both T4Trx and EcTrx there appears to be more than one species in the reduced state [see also Langsetmo et al. (1988)]. Since the active sites of both enzymes are so similar with the *cis*-prolines being in an analogous position with respect to the disulfide bond, one would expect a similar effect upon the stability once the disulfide bond is reduced. Furthermore, both proteins seem to be subject to cold denaturation. The problem in comparing these data is that spectral properties only reveal average structures whereas the urea gradient gel approach taken by Langsetmo et al. (1988) can more readily identify different species. For both the oxidized and reduced forms, the four-state model shown (Scheme I) should be used as a framework to build more accurate and thus inherently more complex models.

Acknowledgments

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References

Borden, K. L. B., & Richards, F. M. (1990) *Biochemistry 29*, 3071–3077.

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.

Brown, S., Turner, R., Roche, R., & Stevenson, K. (1987) *Biochemistry 26*, 863–871.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.

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

Hiraoki, T., Brown, S. B., Stevenson, K. J., & Vogel, H. J. (1988) *Biochemistry 27*, 5000–5008.

Holmgren, A., & Brändén, C. I. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2305–2309.

Kelley, R. F., Wilson, J., Bryant, C., & Stellwagen, E. (1986) *Biochemistry 25*, 728–732.

Kelley, R. F., Shalongo, W., Jagannadham, M. V., & Stellwagen, E. (1987) *Biochemistry 26*, 1406–1411.

Langsetmo, K., Fuchs, J., & Woodward, C. (1989) *Biochemistry 28*, 3211–3220.

Lin, T., & Kim, P. (1989) *Biochemistry 28*, 5282–5287.

Nall, B. T., Garel, J.-R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317–330.

Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764–4768.

Söderberg, B.-O., Sjöberg, B.-M., Sonnerstam, U., & Brändén, C.-I. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5827–5830.

Stryer, L., Holmgren, A., & Reichard, P. (1967) *Biochemistry 6*, 1016–1020.