The folding and assembly of platelet-derived growth factor (PDGF), a potent mitogen involved in wound-healing processes and member of the cystine knot growth factor family, was studied. The kinetics of the formation of disulfide-bonded dimers were investigated under redox reshuffling conditions starting either from unfolded and reduced PDGF-A or B-chains or an equimolar mixture of both chains. It is shown that in all cases the formation of disulfide-bonded dimers is a very slow process occurring in the time scale of hours with a first-order rate-determining step. The formation of disulfide-bonded PDGF-AA or PDGF-BB homodimers displayed identical kinetics, indicating that both monomeric forms as well as the dimerized homodimer have similar folding and assembly pathways. In contrast, the formation of the heterodimer occurred three times more rapidly compared with the formation of the homodimers. As both monomeric forms revealed similar renaturation kinetics, it can be concluded that the first-order rate-determining folding step does not occur during monomer folding but must be attributed to conformational rearrangements of the dimerized, not yet disulfide-bonded protein. These structural rearrangements allow a more rapid formation of intermolecular disulfide bonds between the two different monomers of a heterodimer compared with the formation of the disulfide bonds between two identical monomers. The preferential formation of disulfide-bonded heterodimers from an equimolar mixture of unfolded A- and B-chains is thus a kinetically controlled process. Moreover, similar activation enthalpies for the formation of all different isoforms suggest that faster heterodimerization is controlled by entropic factors.

Platelet-derived growth factor (PDGF)\(^{1}\) is a potent mitogen for cells of mesenchymal origin, i.e. smooth muscle cells, connective tissue cells, or blood cells (1–3). It is released by platelets upon wounding and plays an important role in stimulating adjacent cells to grow and thereby heal the wound (4). PDGF is a non-glycosylated protein that belongs to the family of dimeric cystine knot growth factors (5). The PDGF family consists of different gene products. The most prominent and long known members of this family are PDGF-A and -B. More recently, two new less abundant members, PDGF-C and -D, have been discovered (6, 7).

The two different homologous monomers of PDGF, denoted as A- and B-chains, are known to exist in the three natural occurring dimeric isoforms PDGF-AA, -AB, and -BB (8, 9). These different isoforms have apparently distinct biological functions indicated, e.g. by their different binding affinities to the two different types of PDGF receptors (10). However, the majority of PDGF purified from human platelets is the disulfide-bonded heterodimeric growth factor (11), suggesting that heterodimerization is favored when both genes are coexpressed. Also, PDGF-AB disulfide-bonded heterodimers are almost exclusively formed from an equimolar mixture of unfolded and reduced A- and B-chains when renaturation is carried out under conditions that allow disulfide bond reshuffling (12–14).

From all potential isoforms of the PDGF dimer, only the structure of the BB homodimer has been determined so far (Fig. 1; Ref. 15). PDGF-BB is an all-β sheet protein of about 30 kDa and composed of two very flat subunits arranged head-to-tail and linked together by two intramolecular disulfide bonds (5, 15). In addition to intramolecular disulfide bonds, each monomer contains an unusual knot-like arrangement of three intramolecular disulfide bridges where one disulfide bond threads through a loop formed by the two other disulfide bonds (5, 15). As all members of the cystine knot growth factor family share strong structural homology, it is most likely that the other PDGF isoforms are of almost identical structure as the BB isomer.

The folding pathways of oligomeric proteins frequently exhibit very complex profiles, since unimolecular folding reactions and bimolecular association steps are involved (16, 17). In case of PDGF, the folding and association process becomes even more complex through the additional requirement for the formation of the unusual knot-like arrangement of the three intramolecular disulfide bridges and the formation of the two intermolecular disulfide bonds. Although many members of the cystine knot growth factor family are of enormous medical importance (e.g. PDGFs, transforming growth factors, bone morphogenetic proteins), almost no knowledge exists about the mechanisms governing their folding and assembly. Some more detailed studies have been carried out on the folding and association kinetics of brain-derived neurotrophic factor (18) and nerve growth factor (19). Both factors belong to a subgroup of the cystine knot growth factor family where the subunits are not connected by intermolecular disulfide bonds in the dimeric protein. Kinetic studies on the folding and assembly of those growth factors of the cystine knot family where the subunits are connected by disulfide bonds are scarce (20).
bonds in the native protein are missing so far.

It was commonly accepted that the presence of the cystine knot is a prerequisite for the dimerization of proteins belonging to the cystine knot growth factor family (5). However, recent studies on the structure and stability of vascular endothelial growth factor, a member of PDGF superfamily of cystine knot growth factors, revealed that cystine deletion mutants lacking one of the two disulfide bonds forming the outer ring of the knot motif are still able to form disulfide-bonded dimers (20). Surprisingly, these mutants even revealed an increased thermodynamic stability although their thermal stability was severely reduced (20). However, the formation of the cystine knot appears to be indispensable for the biological activity of PDGF (21), while the intermolecular disulfide bridges have a stabilizing but non-essential effect on the biological activity (21, 22). PDGF is very prone to aggregation when renaturation is initiated by diluting unfolded and reduced monomers into a buffer, which allows refolding and disulfide bond reshuffling (14). Once folded, however, PDGF is a very stable protein withstand- ing temperatures of up to 100 °C (23).

Previously, we have presented a renaturation method based on the utilization of size exclusion chromatography, which circumvents aggregation during refolding and allows renaturation of PDGF at high protein concentrations (14). In this study, we present a kinetic analysis of the formation of disulfide-bonded dimers and propose a model for the folding pathway of the different isoforms of PDGF. The unfolded and reduced monomers of PDGF were subjected to size exclusion chromatography under renaturing conditions and the formation of disulfide-bonded dimers starting either from pure A- or B-chains, or an equimolar mixture of the two chains) were subjected to SEC under conditions that allow refolding and reshuffling of disulfide bridges as described previously (14). Standard renaturation conditions by SEC were: 1 mol l⁻¹ Tris-Cl (pH 7.8), 0.5 mol l⁻¹ guanidinium hydrochloride (GdnHCl), 10 mmol l⁻¹ glutathione reduced (GSH), 0.25 mmol l⁻¹ glutathione oxidized (GSSG). Under these conditions, the eluted PDGF monomers were able to dimerize in the eluate fraction to yield the dimeric, disulfide-bonded, and biologically active growth factor (14). Aggregation of PDGF during the renaturation procedure was not observed unless otherwise indicated. The formation of disulfide-bonded dimers was followed in aliquots taken from the reaction mixture in the eluate fraction through disulfide trapping by irreversible blocking of free thiol groups and subsequent separation of monomeric and dimeric PDGF by gel electrophoresis under non-reducing conditions. Blocking of the free thiol groups by the addition of iodoacetate and gel electrophoresis was carried out as described previously (14). Gels were stained with Coomassie Brilliant Blue, and quantification of the monomeric and dimeric fraction of PDGF was carried out by densitometry (Hirschman elscript 400).

The putative reaction order for the rate-limiting step during dimerization of PDGF was determined from the slopes of the linearized kinetic equations assuming either a first-order (Equation 1),

\[ \ln[A]_0 = \ln[A]_0 - [\Sigma v_i] k_1 t \]  
(Eq. 1)

or a second-order rate-determining reaction (Equation 2),

\[ \frac{1}{[A]_0} - \frac{1}{[A]_0} = [\Sigma v_i] k_2 t \]  
(Eq. 2)

where [A]₀ and [A]₀ are the monomer concentrations at times t and zero, respectively, \( \Sigma v_i \) is the sum of the stoichiometric factors, and \( k_1 \) and \( k_2 \) are the rate constants for a first- or second-order reaction, respectively.
By rearranging the kinetic Equations 1 and 2, the monomer turnover $U$ can be simulated assuming either a first-order (Equation 3) or a second-order rate-determining reaction (Equation 4).

\[
1 - \frac{[A]}{[A]_0} = U = 1 - e^{-\kappa[A]t} \\
1 - \frac{[A]}{[A]_0} = U = 1 - \frac{1}{1 + \frac{[R]}{[A]_0} k_{2}[A]_{2}} 
\]

In case of a unimolecular rate-limiting reaction, the monomer turnover with time should be independent of the initial monomer concentration (Equation 3), while a second-order rate-limiting reaction should be reflected by an increased monomer turnover with increasing initial monomer concentration (Equation 4). Best-fit simulations of the data from kinetic experiments for the determination of the rate constants and modeling of the monomer turnover were carried out using standard software.

The temperature dependence of the rate of dimerization was described by an Arrhenius relationship, i.e. a plot of $\ln k$ versus $1/T$,

\[
k = A \exp\left(\frac{\Delta S^o}{R}\right) \exp\left(-\frac{\Delta H^o}{R} - \frac{1}{T}\right) 
\]

where $k$ is the experimentally determined rate constant, $A$ is a constant in the activated complex theory, $\Delta S^o$ and $\Delta H^o$ the entropy and enthalpy of activation of the reaction, respectively, $T$ the temperature, and $R$ the universal gas constant. If there is linearity for the temperature dependence of the rate constant, the enthalpy and entropy of activation can be determined from the slope and the $y$ intercept of Equation 5, respectively.

RESULTS

Kinetics of Formation of Disulfide-bonded PDGF-AB Dimers from Unfolded and Reduced PDGF-A and -B Monomers—The kinetics of the formation of disulfide-bonded dimers of PDGF were investigated under redox reshuffling conditions starting from an equimolar mixture of completely unfolded and reduced A- and B-chains (Fig. 2A). The kinetic data are well described either by assuming a first- or a second-order rate-limiting reaction and the putative rate constants extracted from the slopes of the linearized kinetic equations (cf. experimental procedures) were determined to be $k_1 = 1.5 \times 10^{-5} \text{ s}^{-1}$ or $k_2 = 2.5 \text{ mol}^{-1} \text{ s}^{-1}$, respectively (Fig. 2B).

To discriminate between a first- and a second-order rate-limiting reaction controlling the formation of disulfide-bonded
off-pathway folding products Mmisfolded, incompetent for the formation of PDGF-AB dimers additionally including the formation of non-native monomers.

PDGF-AB dimers from unfolded and reduced PDGF-A and -B disulfide-linked dimers was not detectable (data not shown).

products (Fig. 4 leading to the irreversible formation of misfolded off-pathway netic model was further refined by including an additional step off-pathway products, which are not able to form native PDGF-AB. To account for the incomplete monomer turnover, the ki-

PDGF dimers, renaturation experiments were carried out with varying initial monomer concentrations (Fig. 3). The kinetic analysis revealed an independence of the monomer turnover at given time points on the initial monomer concentration (Fig. 3A), thus clearly excluding a second-order rate-limiting step in the renaturation of PDGF-AB. In addition, kinetic modeling revealed that the formation of the disulfide-bonded dimer is best described by a first-order rate-determining reaction (Fig. 3B). Deviation of the predicted monomer turnover from the experimental data originates from the formation of soluble off-pathway products, which are not able to form native PDGF-AB. To account for the incomplete monomer turnover, the kinetic model was further refined by including an additional step leading to the irreversible formation of misfolded off-pathway products (Fig. 4A). The experimental data are now well described by the productive first-order reaction with the rate constant of \( k_1 = 1.5 \times 10^{-5} \text{ s}^{-1} \) yielding the native disulfide-bonded PDGF-AB dimer and an unproductive reaction with a rate constant of \( k_1' = 7.5 \times 10^{-6} \text{ s}^{-1} \) leading to non-native off-pathway products (Fig. 4B).

A variation of the GdnHCl concentration between 0.25 and 1.5 mol l\(^{-1}\) in the renaturation buffer revealed a strong decrease in the rate of the formation of the disulfide-linked dimer with increasing concentrations of the chaotropic agent (Fig. 5). The graphic representation of the rate constants in a “chevron plot” revealed a linear dependence on the GdnHCl concentration by anticipating a first-order rate-limiting reaction (Fig. 5B). Final yields of disulfide-linked PDGF-AB dimers increased from 13 to 75% by decreasing the concentration of GdnHCl from 1.5 to 0.25 mol l\(^{-1}\). At 2 mol l\(^{-1}\) GdnHCl, formation of disulfide-linked dimers was not detectable (data not shown).
Experimental conditions were the same as described in the legend to Fig. 7. 

**Table I**

| Temperature (°C) | First-order rate constant, $k_1$ | Final yield |
|-----------------|---------------------------------|-------------|
|                 | PDGF-AA | PDGF-BB | PDGF-AB | PDGF-AA | PDGF-BB | PDGF-AB |
| 4               | 0.03 ± 0.005 | 0.05 ± 0.005 | 0.06 ± 0.015 | 18 | 17 | 17 |
| 15              | 0.13 ± 0.005 | 0.17 ± 0.01 | 0.5 ± 0.1 | 25 | 45 | 55 |
| 25              | 0.5 ± 0.1 | 0.5 ± 0.05 | 1.5 ± 0.1 | 50 | 50 | 61 |
| 35              | 1.0 ± 0.1 | 1.0 ± 0.05 | 3.0 ± 0.2 | 50 | 51 | 59 |
| 45              | 1.0 ± 0.05 | 0.5 ± 0.05 | 2.0 ± 0.15 | 50 | 47 | 57 |

**DISCUSSION**

Subunit association can be a very fast process with rate constants in the order of $10^5$ mol$^{-1}$ l s$^{-1}$ (e.g., Ref. 25) that are encountered in diffusion-controlled reactions. However, when folding and association is connected with the formation of intramolecular disulfide bonds, e.g., during renaturation of antibody fragments, the regain of the biological activity can occur in the time scale of hours to days (26).

The formation of disulfide-bonded dimers of PDGF is also a very slow process occurring in the time scale of hours. Studies on the folding and association of brain-derived neurotrophic factor (18) and nerve growth factor (19), growth factors where the subunits are not connected by intermolecular disulfide bonds, also revealed slow renaturation kinetics, although, in general, little information exists on the folding and association pathways of dimeric proteins of the cystine knot growth factor family.

The rate-limiting step during the renaturation of a dimeric protein can either be a first-order step resulting from unimolecular conformational changes or a second-order step originating from the encounter and assembly of the subunits. The experimental results clearly show that the formation of the PDGF dimer is a process controlled by a first-order reaction, thus proving that the encounter of the monomeric chains to form the dimeric growth factor is not the rate-limiting step in the renaturation of PDGF. A first-order rate-determining reaction during the renaturation of a multimeric protein is not unusual. For example, the kinetic analysis of the renaturation of the homodimeric mitochondrial malate dehydrogenase revealed a second-order association reaction (27), whereas the renaturation kinetics of the cytoplasmic enzyme, also a homodimer, were governed by a first-order rate-limiting step (28).

A first-order rate-limiting reaction can result from folding events on the monomer level or from structural rearrangements of an already dimerized protein. The comparative analysis of homo- and heterodimerization revealed identical kinetics for the formation of the disulfide-bonded AA or BB homodimers, suggesting that their renaturation pathways do not differ significantly, e.g., that folding of the two different monomeric chains into association competent molecules, association of these monomers into homodimers, and, finally, formation of intermolecular disulfide bridges do not exhibit significantly different pathways when renaturation was started either from the unfolded and reduced pure A- or B-chains. In contrast, the AB heterodimers were formed three times more rapidly compared with the formation of the homodimers. As-

**Fig. 7.** Arrhenius plots of the rate constants of the formation of disulfide-bonded PDGF dimers of the different PDGF isoforms from unfolded and reduced PDGF monomers. The rate constants of dimerization of the three different PDGF isoforms PDGF-AA, PDGF-AB, and PDGF-BB were determined at temperatures ranging from 4 to 45 °C assuming a first-order rate-determining reaction. The initial monomer concentrations were 0.8 μmol l$^{-1}$ PDGF-A (□), 9.25 μmol l$^{-1}$ PDGF-A and -B (●), and 3.5 μmol l$^{-1}$ PDGF-B (○). Experimental conditions were the same as described in the legend to Fig. 2, except for the temperatures.

Either indicate a rate-limiting folding reaction on the level of the monomeric chain or structural rearrangements on the level of the dimeric not yet disulfide-bonded growth factor. To discriminate between these two possibilities, a kinetic study of the formation of disulfide-bonded dimers was carried out using either the purified A- or B-chains or an equimolar mixture of both chains (Fig. 6). The formation of disulfide-bonded PDGF-AA or PDGF-BB homodimers displayed identical kinetics indicating that both monomeric forms as well as the dimerized homodimer have similar folding and assembly pathways. In contrast, disulfide-bonded heterodimers were formed three times more rapidly ($k_1 = 1.5 \times 10^{-5}$ s$^{-1}$), experimental conditions, cf. Fig. 6) compared with the formation of the two different disulfide-bonded homodimers from either pure A- or B-chains ($k_1 = 0.5 \times 10^{-5}$ s$^{-1}$, experimental conditions, cf. Fig. 6) when renaturation was started from an equimolar mixture of both chains.

At all temperatures ranging from 4 °C to 45 °C heterodimerization occurred more rapidly compared with the formation of homodimers (Fig. 7 and Table I), suggesting a general preference for the formation of the heterodimer. Also, the kinetics of homodimerization of the two different PDGF-AA or -BB isoforms did not show any significant difference in the temperature range studied, supporting the conclusion that homodimerization of either PDGF-AA or -BB follows most likely similar pathways.

During the renaturation of all the three different PDGF isoforms aggregation was not observed up to temperatures of 35 °C. However at 45 °C, partial aggregation of all PDGF isoforms occurred. A summary of the results from the renaturation experiments carried out at the different temperatures is shown in Table I. An estimation of the activation enthalpies from the Arrhenius plots (Fig. 7; only data from 4 to 35 °C) of either homo- or heterodimerization did not reveal a significant difference for the different PDGF isoforms. Assuming a first-order rate-limiting renaturation step, activation enthalpies in the range of 70–80 kJ mol$^{-1}$ were estimated for the formation of PDGF-AA, -AB, or -BB.

The rate-limiting step during the renaturation of a dimeric protein can either be a first-order step resulting from unimolecular conformational changes or a second-order step originating from the encounter and assembly of the subunits. The experimental results clearly show that the formation of the PDGF dimer is a process controlled by a first-order reaction, thus proving that the encounter of the monomeric chains to form the dimeric growth factor is not the rate-limiting step in the renaturation of PDGF. A first-order rate-determining reaction during the renaturation of a multimeric protein is not unusual. For example, the kinetic analysis of the renaturation of the homodimeric mitochondrial malate dehydrogenase revealed a second-order association reaction (27), whereas the renaturation kinetics of the cytoplasmic enzyme, also a homodimer, were governed by a first-order rate-limiting step (28).

A first-order rate-limiting reaction can result from folding events on the monomer level or from structural rearrangements of an already dimerized protein. The comparative analysis of homo- and heterodimerization revealed identical kinetics for the formation of the disulfide-bonded AA or BB homodimers, suggesting that their renaturation pathways do not differ significantly, e.g., that folding of the two different monomeric chains into association competent molecules, association of these monomers into homodimers, and, finally, formation of intermolecular disulfide bridges do not exhibit significantly different pathways when renaturation was started either from the unfolded and reduced pure A- or B-chains. In contrast, the AB heterodimers were formed three times more rapidly compared with the formation of the homodimers. As-
Fig. 8. Basic model for the formation of disulfide-bonded PDGF dimers from unfolded and reduced PDGF monomers. In this scheme M_{misfolded} represents the unfolded and reduced PDGF monomers, which are subjected to SEC under conditions allowing refolding and disulfide bond reshuffling. M* signifies the PDGF monomer recovered directly after SEC in the elution fraction. The reaction includes the transformation of M* into association competent monomers M_{folded}, the subsequent encounter of M_{folded} with another M_{folded} into a non-covalently associated dimer (MM)*, the first-order rate determining structural rearrangements yielding the not yet disulfide-bonded dimer D*, which can be transformed through intermolecular disulfide-bonding into the native growth factor D\textsuperscript{native}. The model additionally includes the formation of non-native off-pathway folding products M_{misfolded} incompetent for the formation of native disulfide-bonded PDGF dimers.

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**REFERENCES**

1. Ross, R., Raines, E. W., and Bowen-Pope, D. F. (1986) *Cell* 46, 155–169
2. Heldin, C.-H. (1992) *EMBO J.* 11, 4251–4259
3. Meyer-Ingold, W., and Eichner, W. (1995) *Cell Biol. Int.* 19, 389–398
4. Meyer-Ingold, W. (1995) *Trends Biochem. Sci.* 20, 387–392
5. Isaacs, N. W. (1995) *Curr. Opin. Struct. Biol.* 5, 391–395
6. Gilbertson, D. G., Duff, M. E., West, J. W., Kelly, J. D., Sheppard, P. O., Hofstrand, P. D., Gao, Z., Shoemaker, R., Bukowski, T. R., Moore, M., Feldhaus, A. L., Hames, J. M., Palmer, T. E., and Hart, C. E. (2001) *J. Biol. Chem.* 276, 27406–27414
7. LaRoche, W. J., Jefferis, M., McDonald, W. F., Chillakuru, R. A., Giese, N. A., Lisker, N., Sullivan, C., Beldog, P. L., Yang, M., Vernet, C., Burgess, C. E., Fernandes, E., Deegler, L. L., Rittman, B., Shimkets, J., Shimkets, R. A., Rothberg, J. M., and Lichenstein, H. S. (2001) *Nat. Cell Biol.* 3, 517–521
8. Betscholtz, C., Johnson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M. S., Eddy, R., Shows, T. B., Philpott, K., Mello, A. L., Knott, T. J., and Scott, J. (1996) *Nature* 380, 695–699
9. Hennink, M., and Denuzhko, D. J. (1989) *Biochim. Biophys. Acta* 989, 1–10
10. Heldin, C. H., and Westermark, B. (1989) *Trends Genet.* 5, 108–111
11. Hamburger, A., Hellman, U., Johnson, A., Ostman, A., Gunnarsson, K., Westermark, B., Wasteson, A., and Heldin, C.-H. (1988) *J. Biol. Chem.* 263, 16493–16498
12. Hoppe, J., Weich, H. A., Eichner, W., and Tatje, D. (1990) *Eur. J. Biochem.* 187, 207–214
13. Scheppe, B., Eichner, W., and McCarthy, J. E. G. (1994) *Gene (Amst).* 143, 201–209
14. Muller, C., and Rinas, U. (1999) *J. Chromatogr. A* 855, 203–213
15. Oefner, C., D’Arcey, W., Winkler, F. K., Eggimann, B., and Hosang, M. (1992) *EMBO J.* 11, 3921–3926
16. Jaenicke, R., and Rudolph, R. (1986) *Methods Enzymol.* 131, 218–250
17. Jaenicke, R. (1991) *Biochemistry* 30, 3417–3414
18. Philo, J. S., Rosenfeld, R., Arakawa, T., Wen, J., and Narhi, L. O. (1993) *Biochemistry* 32, 10812–10818
19. Rauenbol, A., Ruppollo, M., Flagiello, A., Monti, M., Vinc, F., Marino, G., Lilie, H., Schwarz, E., and Rudolph, R. (2001) *J. Mol. Biol.* 305, 523–533
20. Muller, Y. A., Hearing, C., Mussenwitz, K., Wellef, K., and Wellef, H. (2002) *J. Biol. Chem.* 277, 43410–43416
21. Kenney, W. C., Haniu, M., Herman, A. C., Arakawa, T., Costigan, V. J., Lary, J., Yphantis, D. A., and Thomason, A. R. (1994) *J. Biol. Chem.* 269, 12351–12359
22. Prestrelski, S. J., Arakawa, T., Duker, R., Kenney, W. C., and Narhi, L. O. (1994) *Int. J. Pept. Protein Res.* 44, 357–363
23. Rames, E. W., and Ross, R. (1985) *Methods Enzymol.* 109, 749–773
24. Seeger, A., Scheppe, B., McCarthy, J. E. G., Decker, W.-D., and Rinas, U. (1995) *Enzyme Microb. Technol.* 17, 947–953
25. Milla, M. E., and Sauer, R. T. (1994) *Biochemistry* 33, 1125–1133
26. Buchner, J., and Rudolph, R. (1991) *Biosci. Reports* 9, 157–162
27. Jaenicke, R., Rudolph, R., and Heider, I. (1979) *Biochemistry* 18, 1217–1223
28. Rudolph, R., Fuchs, I., and Jaenicke, R. (1986) *Biochemistry* 25, 1662–1669
29. Ostman, A., Ball, L., Hammer, A., Worme, M. A., Cott, D., Valenzuela, P., Betscholtz, C., Westermark, B., and Heldin, C.-H. (1988) *J. Biol. Chem.* 263, 16202–16208
30. Scheppe, C., Sehgal, W., and Hultsmeier, M. (1999) *J. Mol. Biol.* 297, 103–115
31. Hinck, A. P., Archer, S. J., Qian, S. W., Roberts, A. B., Sporn, M. B., Weatherbee, J. A., Tsang, M. L.-S., Lucas, R., Zhang, B.-L., Wenker, J., and Torchia, D. A. (1998) *Biochemistry* 35, 8517–8524

The strong dependence of the kinetic constant of the rate-limiting renaturation step on the concentration of the denaturant GdnHCl strongly suggests that general structural rearrangements on the level of the non-covalently associated but not yet disulfide-bonded dimer determine the speed of renaturation. Finally, similar activation enthalpies of hetero- and homodimerization indicate that preferential heterodimerization must be controlled by entropic factors resulting in a more favorable positioning of the cysteines from different chains for the formation of the intermolecular disulfide bonds compared with disulfide bond formation between identical chains.