3. Grimm, S. & Baeuerle, P. A. *Biochem. J.* **290,** 297–308 (1993).
4. Sen, R. & Baltimore, D. *Cell* **47,** 921–928 (1986).
5. Osborn, L., Kunkel, S. & Nabel, G. J. *Proc. natn. Acad. Sci. U.S.A.* **86,** 2336–2340 (1989).
6. Baeuerle, P. A. & Baltimore, D. *Cell* **53,** 211–217 (1988).
7. Baeuerle, P. A. & Baltimore, D. *Science* **242,** 540 546 (1988).
8. Shirakawa, F. & Mizel, S. *Molec. cell. Biol.* **9,** 2424–2430 (1989).
9. Ghosh, S. & Baltimore, D. *Nature* **344,** 678 682 (1990).
10. Kerr, L. D. *et al. Genes Dev.* **5,** 1464–1476 (1991).
11. Haskill, S. *et al. Cell* **65,** 1281–1289 (1991).
12. Brown, K., Park, S., Kanno, T., Franzoso, G. & Siebenlist, U. *Proc. natn. Acad. Sci. U.S.A.* **90,** 2532–2536 (1993).
13. Sun, S. C., Ganchi, P. A., Ballard, W. & Greene, W. C. *Science* **259,** 1912–1915 (1993).
14. de Martin, R. *et al. EMBO J.* (in the press).
15. Bomsztyk, K. *et al. Cell. Regul.* **2,** 329 337 (1991).
16. Meichle, A., Schütze, S., Hensel, G., Brunsing, D. & Krönke, M. *J. biol. Chem.* **265,** 8339 8347 (1990).
17. Schütze, S. *et al.* Cell **71,** 765 776 (1992).
18. Schreck, R., Rieber, P. & Baeuerle, P. A. *EMBO J.* **10,** 2247–2258 (1991).
19. Schreck, R., Albermann, K. & Baeuerle, P. A. *Free Rad. Res. Commun.* **17,** 221–237 (1992).
20. Schreck, R., Meier, B., Männel, D., Dröge, W. & Baeuerle, P. A. *J. exp. Med.* **175,** 1181–1194 (1992).
21. Meyer, M., Schreck, R. & Baeuerle, P. A. *EMBO J.* (in the press).
22. Baeuerle, P. A., Lenardo, M., Pierce, J. W. & Baltimore, D. *Cold Spring Harb. Symp. quant. Biol.* **53,** 786 798 (1988).
23. Zabel, U., Henkel, T., dos Santos Silva, M. A. & Baeuerle, P. A. *EMBO J.* **12,** 201–211 (1993).
24. Zabel, U. & Baeuerle, P. A. *Cell* **61,** 255 265 (1990).
25. Hochstrasser, M. & Varshavsky, A. *Cell* **61,** 697–708 (1990).
26. Baeuerle, P. A. & Baltimore, D. in *Molecular Aspects of Cellular Regulation* Vol. 6 (eds Cohen, P. & Foulkes, J. G.) 409–432 (Elsevier/North Holland Biomedical, 1991).
27. Zabel,U., Henkel, T., dos Santos Silva, M. A. & Baeuerle, P. A. *EMBO J.* **12,** 201–211 (1993).
28. Henkel, T. *et al. Cell* **68,** 1121 1133 (1992).
29. Wall, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **83,** 295–298 (1986).
30. Schreck, R. & Baeuerle, P. A. *Meth. Enzym.* (in the press).

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Efficient catalysis of disulphide bond rearrangements by protein disulphide isomerase

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PROTEIN disulphide isomerase (PDI)[1,2] is a highly abundant and ubiquitous eukaryotic protein that is essential for viability in yeast[3,4]. Although PDI is thought to catalyse disulphide bond formation and isomerization during protein biosynthesis, PDI has been found previously to have only moderate effects (~25-fold) on the rate of oxidative folding of proteins *in vitro*. In addition, PDI has been implicated in several apparently unrelated cellular functions[3]. For example, PDI is the *β*-subunit of prolyl 4-hydroxylase[5] and is part of the trigylceride transfer complex[6]. The oxidative folding of bovine pancreatic trypsin inhibitor (BPTI) is slow and inefficient *in vitro*[7–11]. Here we report that PDI increases by a factor of 3,000–6,000 the rates of folding of kinetically trapped BPTI folding intermediates, in which native structure impedes disulphide bond formation. By contrast, PDI has only small effects on the rate of disulphide bond formation in intermediates that are oxidized readily in the absence of PDI. These results suggest that an important function of PDI is to catalyse disulphide bond formation and rearrangements within kinetically trapped, structured folding intermediates.

The best characterized disulphide folding pathway of a protein is for the *in vitro* folding of BPTI[7–11] (Fig. 1*a*, *b*). During folding at neutral pH, BPTI accumulates rapidly as one of two intermediates. These intermediates, termed N* and N′, both contain two native disulphide bonds, [5–55; 14–38] and [30–51; 14–38], respectively. About half of the molecules become trapped as N*, which is a dead-end intermediate that is stable for weeks. N′ rearranges slowly (hours) to N*, and also to a third native two-disulphide intermediate ([30–51; 5–55]), termed N^{SH}_{SH}, which is oxidized readily to native BPTI (N).

Formation of the final native disulphide bond in N* and N′ is hindered by native structure in these intermediates which constrains and buries the remaining free thiols[8,9]. Recently, a naturally occurring amino-terminal pro- region (ref. 12; J. Li, S. Olson and D. A. Walz, personal communication) was shown to increase both the rate and yield of folding of BPTI[11]. Nonetheless, even in this model of pro-BPTI, folding is slow and ~25% of the molecules accumulate as the dead-end intermediate N*.

TABLE 1 Kinetic parameters for catalysis by PDI

Transition	k_{cat} (min^{-1})	K_m (μM)	k_{uncat}† (min^{-1})	Fold ‡ acceleration
N′→N	5	7	1.4×10^{-3}	3,500
N*→N	0.3	30	5×10^{-5}	6,000
proN*→proN	0.9	35	2×10^{-4}	4,500

BPTI folding experiments were done at 25 °C, 2.0 mM GSH, 0.5 mM GSSG, pH 7.3.

† k_{uncat} is the rate in the absence of PDI. The values for N* and proN* are reliable only to within a factor of 2, owing to difficulties associated with measuring a rate over a period of several days under anaerobic conditions[10]. Michaelis–Menten parameters (K_m and k_{cat}) for the catalysis of N′, N* and proN* were determined by measuring the initial rate of formation of native BPTI or pro-BPTI by HPLC. Initial velocity measurements were made at five or six substrate concentrations that ranged from 1.5 μM to 100 μM for N′ and from 5 μM to 150 μM for N* and proN*. The concentration of PDI used ranged from 0.17–0.7 μM. Kinetic constants were calculated from Lineweaver–Burk plots. The initial velocity measurements were made at times in which less than one third of the starting material had rearranged. The values of k_{cat}/K_m were confirmed by measuring the rate of formation of native BPTI or Pro-BPTI for the entire time course of the reactions at initial substrate concentrations at least fivefold below K_m. Catalysis of proN′ does not appear to follow Michaelis–Menten kinetics; the rate of formation of native BPTI is not constant during initial rate measurements. In addition, several well populated intermediates are present and the level of these intermediates, relative to proN′, increases throughout most of the reaction. Qualitatively, however, the rate of folding of proN′ in the presence of PDI is comparable to that of N′ from mature BPTI.

‡ Fold acceleration is k_{cat}/k_{uncat}. Under optimal redox conditions (1.0 mM GSH, 0.2 mM GSSG, pH 8.0, 25 °C) k_{uncat} for the regeneration of reduced RNase A is 0.02 min^{-1} and k_{cat} is 0.46 min^{-1} (ref. 16).

Earlier studies[13,14] found that PDI increased the rate of oxidation and reduction of BPTI in the presence of dithiothreitol, but had little effect on folding when the physiological[15] redox reagent glutathione was used. In those studies, however, N* was not distinguished from native BPTI (N). In addition, folding was done in the absence of a reducing agent. Subsequently it has been shown, at least for the folding of RNase A in the presence of glutathione, that catalysis of disulphide bond formation by PDI requires a redox buffer that contains reduced and oxidized components[16].

We show here that PDI increases dramatically both the yield and rate of formation of native BPTI in a physiological redox buffer[15] (Fig. 1*c*, *d*). The rate of formation of the two kinetically trapped native intermediates, N′ and N*, is increased moderately (~3-fold) by PDI. The striking feature of the PDI-catalysed folding reaction is that the N′ and N* intermediates appear to be converted readily to native BPTI (N).

Working with purified, reversibly trapped species[9], we examined directly the effect of PDI on the folding of N′ and N*. In

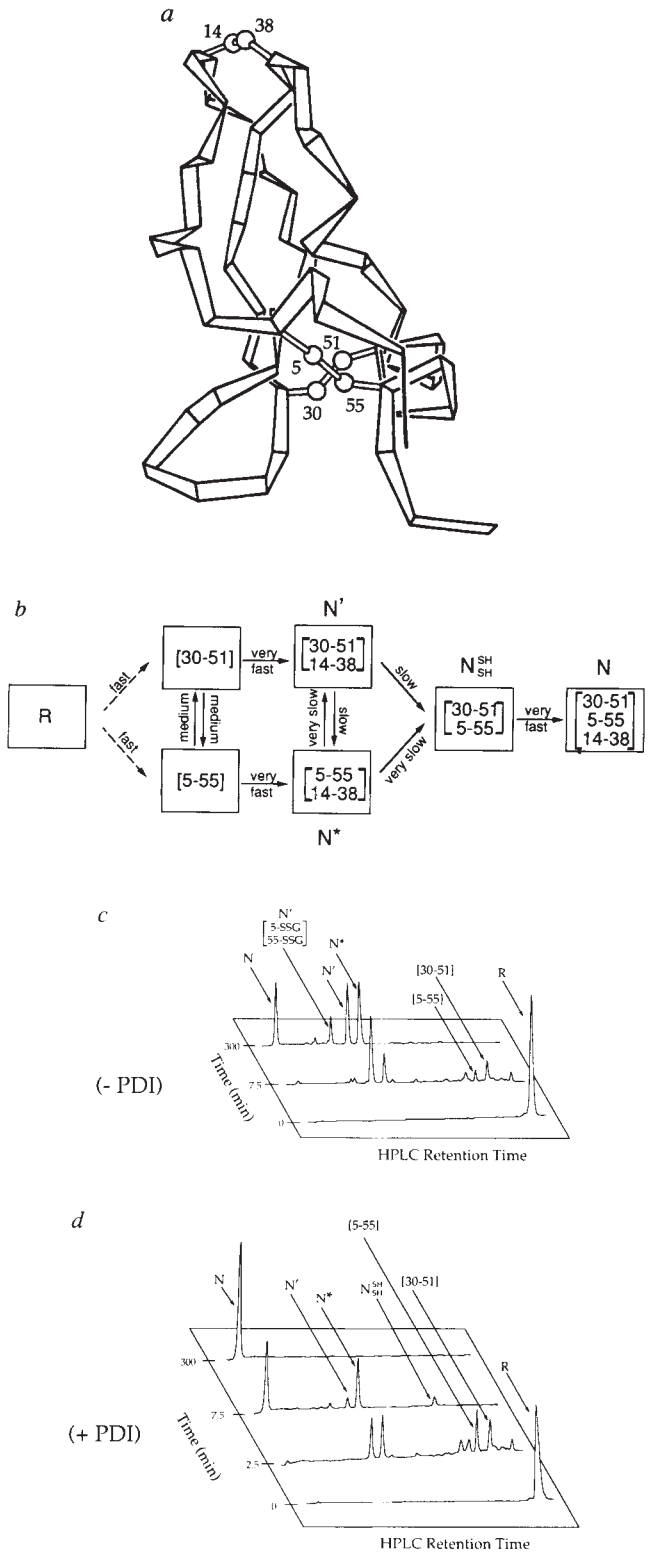

FIG. 1 *a*, Schematic representation of the crystal structure for BPTI[21,22]. The residues involved in disulphide bonds are labelled. The [14–38] disulphide bond is accessible to solvent, exposing ~50% of its total surface area, whereas the [30–51] and [5–55] disulphide bonds are inaccessible, exposing 0% of their total surface area[23]. *b*, Schematic representation of the kinetically preferred pathway for the folding of BPTI at pH 7.3, 25 °C[9,10]. Intermediates are designated by the disulphide bonds they contain. Native BPTI (N) is [30–51; 5–55; 14–38]. N* denotes [5–55; 14–38]. N′ denotes [30–51; 14–38]. N^{SH}_{SH} denotes [30–51; 5–55]. R denotes the reduced protein. X-ray crystallographic and nuclear magnetic resonance (NMR) studies indicate that N^{SH}_{SH} (ref. 24), N* (refs 25, 26), and N′ (refs 9, 27; A. A. Kossiakoff, personal communication; B. A. Schulman and P.S.K., unpublished data) are folded into structures very similar to that of native BPTI. Qualitative descriptions of the relative rates[11] of the intramolecular transitions associated with each step are indicated. N* is a kinetically trapped intermediate that is stable for weeks under these conditions. The dotted arrows indicate that R is oxidized initially to a broad distribution of one-disulphide intermediates, which then rearrange rapidly to [30–51] and [5–55]. *c*, *d*, Time course of folding of reduced BPTI in the absence (*c*) or presence (*d*) of 1.5 μM PDI. A folding reaction was initiated by the addition of redox buffer to reduced BPTI (10 μM). At the indicated times, a portion of the sample was quenched with acid, and analysed by high-performance liquid chromatogrpahy (HPLC). N′[5–SSG; 55–SSG] denotes the double mixed-disulphide derivative of N′, in which Cys 5 and Cys 55 are each disulphide-bonded to glutathione.

METHODS. The folding reactions of BPTI in the presence or absence of PDI are monitored as described[9–11]. Briefly: (1) folding is initiated by the addition of degassed pH 7.3 folding buffer (150 mM NaCl, 100 mM sodium phosphate, 1 mM EDTA, pH 7.3) containing 2.0 mM GSH, 0.5 mM GSSG, to reduced BPTI; (2) at various times, a portion of the folding reaction is quenched with 0.1 volumes of 3M HCl and; (3) the spectrum of intermediates present at each time is determined by HPLC in acidic conditions (pH 2). The disulphide linkages of the observed intermediates have been determined previously[9]. All folding experiments were done in a circulating water bath at 25 °C. The redox buffer was chosen to mimic the redox conditions of the endoplasmic reticulum[15]. Bovine PDI was purchased from Pierce and was ~90% pure as judged by reverse-phase HPLC and SDS–PAGE. As noted previously[28], two forms of PDI, thought to be monomeric and dimeric species, are resolved by HPLC gel filtration (Toso Haas G2000SW × 1). Using this assay, the preparation of PDI was judged to be ~85% monomeric.

each case, the rate of disulphide bond formation is increased dramatically by PDI (Fig. 2). These enzyme-catalysed reactions appear to follow Michaelis–Menten kinetics (Table 1). Compared to the uncatalysed rates, the k_{cat} values for the PDI-catalysed folding of N′ and N* are 3,500- and 6,000-fold faster, respectively (Table 1). In contrast, PDI increases only moderately the rate of disulphide bond formation in N^{SH}_{SH} (Fig. 2*c*), an intermediate that, unlike N′ and N*, forms native BPTI readily even in the absence of a catalyst. Similarly, PDI affects only moderately the rate of disulphide bond formation in the fully reduced protein, R, and in the single-disulphide intermediates [30–51] and [5–55] (Fig. 1*c*, *d*). Thus, the large effects of PDI are specific to the kinetically trapped intermediates.

Productive folding of pro-BPTI[11] also proceeds through the [30–51; 14–38] intermediate (termed proN′). A significant portion of pro-BPTI also becomes trapped as the dead-end species [5–55; 14–38] (termed proN*). As with N* and N′, PDI increases dramatically the rate of productive disulphide bond

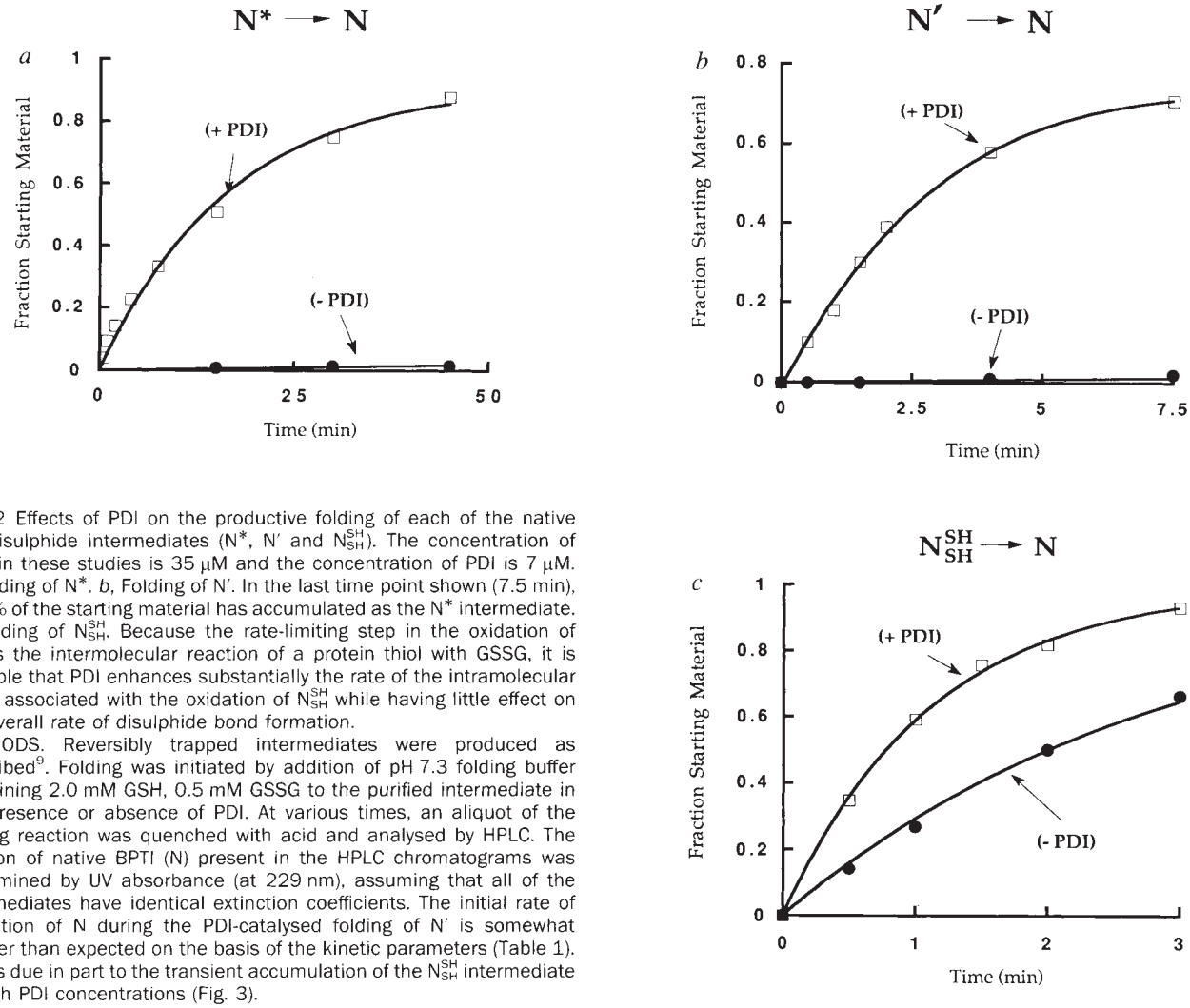

FIG. 2 Effects of PDI on the productive folding of each of the native two-disulphide intermediates (N*, N′ and N_{SH}^{SH}). The concentration of BPTI in these studies is 35 μM and the concentration of PDI is 7 μM. *a*, Folding of N*. *b*, Folding of N′. In the last time point shown (7.5 min), ~20% of the starting material has accumulated as the N* intermediate. *c*, Folding of N_{SH}^{SH}. Because the rate-limiting step in the oxidation of N_{SH}^{SH} is the intermolecular reaction of a protein thiol with GSSG, it is possible that PDI enhances substantially the rate of the intramolecular steps associated with the oxidation of N_{SH}^{SH} while having little effect on the overall rate of disulphide bond formation.

METHODS. Reversibly trapped intermediates were produced as described[9]. Folding was initiated by addition of pH 7.3 folding buffer containing 2.0 mM GSH, 0.5 mM GSSG to the purified intermediate in the presence or absence of PDI. At various times, an aliquot of the folding reaction was quenched with acid and analysed by HPLC. The fraction of native BPTI (N) present in the HPLC chromatograms was determined by UV absorbance (at 229 nm), assuming that all of the intermediates have identical extinction coefficients. The initial rate of formation of N during the PDI-catalysed folding of N′ is somewhat smaller than expected on the basis of the kinetic parameters (Table 1). This is due in part to the transient accumulation of the N_{SH}^{SH} intermediate at high PDI concentrations (Fig. 3).

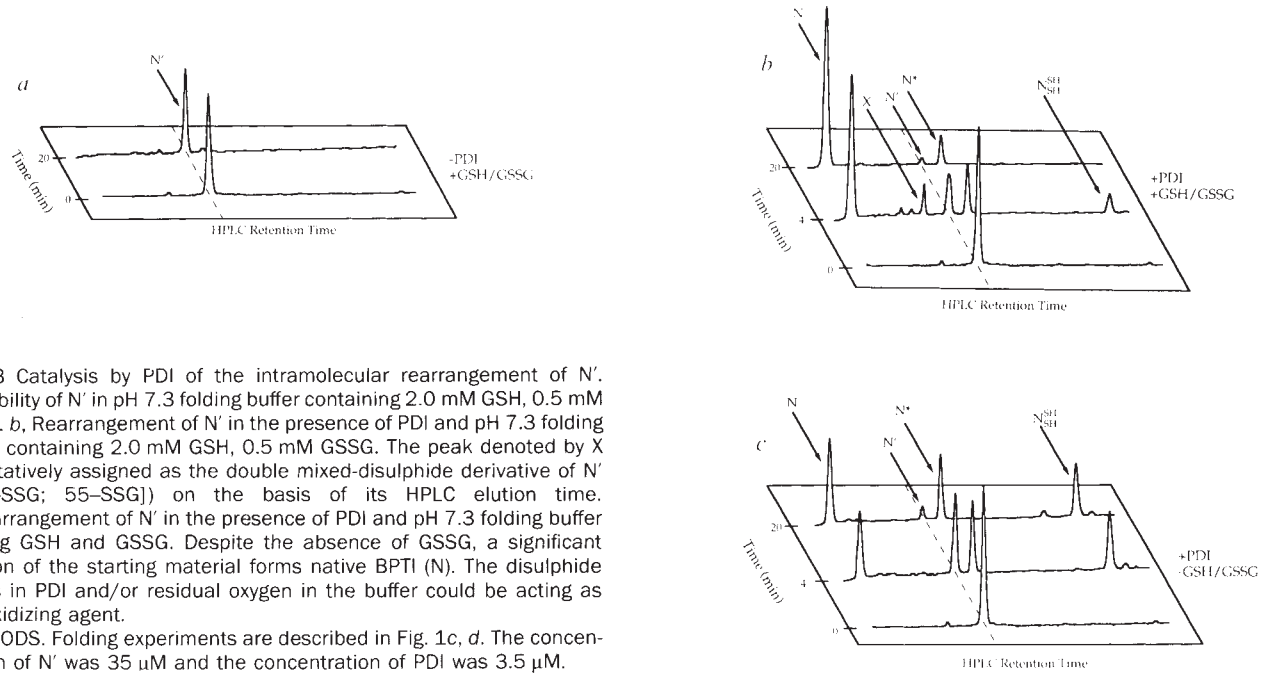

FIG. 3 Catalysis by PDI of the intramolecular rearrangement of N′. *a*, Stability of N′ in pH 7.3 folding buffer containing 2.0 mM GSH, 0.5 mM GSSG. *b*, Rearrangement of N′ in the presence of PDI and pH 7.3 folding buffer containing 2.0 mM GSH, 0.5 mM GSSG. The peak denoted by X is tentatively assigned as the double mixed-disulphide derivative of N′ (N′[5–SSG; 55–SSG]) on the basis of its HPLC elution time. *c*, Rearrangement of N′ in the presence of PDI and pH 7.3 folding buffer lacking GSH and GSSG. Despite the absence of GSSG, a significant fraction of the starting material forms native BPTI (N). The disulphide bonds in PDI and/or residual oxygen in the buffer could be acting as the oxidizing agent.

METHODS. Folding experiments are described in Fig. 1*c*, *d*. The concentration of N′ was 35 μM and the concentration of PDI was 3.5 μM.

formation in both proN* and proN′ (Table 1). This suggests that PDI is primarily responsible for the rapid rate of BPTI folding *in vivo*.

The conclusion that PDI principally accelerates the folding of kinetically trapped intermediates, such as N′ and N*, provides an explanation for why the effects of PDI on the folding of BPTI are much greater than on RNase A. The folding of reduced RNase A is relatively rapid even in the absence of PDI[16] and therefore does not appear to be retarded substantially by the accumulation of structured intermediates. By contrast, formation of the native state of BPTI is hindered by the accumulation of highly structured intermediates. Thus, although the uncatalysed rate for folding of BPTI is substantially lower than for RNase A, the PDI-catalysed rates for folding are similar in the two proteins (see Table 1 legend).

PDI could help formation of the final disulphide bond in the kinetically trapped intermediates, N′ and N*, either by accelerating the intramolecular rearrangement of these intermediates (to N_{SH}^{SH}) or by catalysing the direct oxidation of a third native disulphide bond. In the case of N′, we find that the other native two-disulphide species (N_{SH}^{SH} and N*) accumulate rapidly in the presence of PDI (Fig. 3*b*). Moreover, PDI catalyses the rearrangement of N′ (to N_{SH}^{SH} and N*) in the absence of redox reagents (Fig. 3*c*). These observations demonstrate that PDI acts largely by increasing the rate of intramolecular rearrangement steps, although it is possible that PDI also accelerates direct oxidation (see also ref. 13).

The mechanism by which PDI catalyses disulphide bond rearrangements in structured intermediates is not known. It is known, however, that addition of high concentrations of denaturant (6 M urea) accelerates the rate of rearrangement of the N′ (ref. 9) and N* (ref. 8) intermediates, suggesting that the rearrangement of these species requires substantial loss of structure[10]. In addition, PDI has been observed to promote the reductive unfolding of structured intermediates of BPTI[13] and retinol-binding protein[17]. Finally, PDI is able to interact with a wide variety of unstructured peptides[18,19], in a manner similar to the Hsp70 family of molecular chaperones[20]. These considerations raise the interesting possibility that PDI functions in part by promoting both local unfolding and disulphide bond rearrangements in structured intermediates. □

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1. Goldberger, R. F., Epstein, C. J. & Anfinsen, C. B. *J. biol. Chem.* **238,** 628–635 (1963).
2. Venetianer, P. & Straub, F. B. *Biochem. biophys. Acta* **67,** 166–168 (1963).
3. Noiva, R. & Lennarz, W. J. *J. biol. Chem.* **267,** 3553–3556 (1991).
4. Freedman, R. B. in *Protein Folding* (ed. Creighton, T. E.) 481–488 (Freeman, New York, 1992).
5. Pihlajaniemi, T. *et al. EMBO J.* **6,** 643–649 (1987).
6. Wetterau, J. R., Combs, K. A., Spinner, S. N. & Joiner, B. J. *J. biol. Chem.* **265,** 9800–9807 (1990).
7. Creighton, T. E. *J. molec. Biol.* **113,** 275–293 (1977).
8. Creighton, T. E. & Goldenberg, D. P. *J. molec. Biol.* **179,** 497–526 (1984).
9. Weissman, J. S. & Kim, P. S. *Science* **253,** 1386–1393 (1991).
10. Weissman, J. S. & Kim, P. S. *Proc. natn. Acad. Sci. U.S.A.* **89,** 9900–9904 (1992).
11. Weissman, J. S. & Kim, P. S. *Cell* **71,** 841–851 (1992).
12. Creighton, T. E. & Charles, I. G. *J. molec. Biol.* **194,** 11–22 (1987).
13. Creighton, T. E., Hillson, D. A. & Freedman, R. B. *J. molec. Biol.* **142,** 43–62 (1980).
14. Zapun, A., Creighton, T. E., Rowling, P. J. E. & Freedman, R. B. *Proteins* **14,** 10–15 (1992).
15. Hwang, C., Sinskey, A. J. & Lodish, H. F. *Science* **257,** 1496–1502 (1992).
16. Lyles, M. M. & Gilbert, H. F. *Biochemistry* **30,** 613–619 (1991).
17. Kaji, E. H. & Lodish, H. F. *J. biol. Chem.* (in the press).
18. Noiva, R., Kimura, H., Roos, J. & Lennarz, W. J. *J. biol. Chem.* **266,** 19645–19649 (1991).
19. Morjana, N. A. & Gilbert, H. F. *Biochemistry* **30,** 4985–4990 (1991).
20. Flynn, G. C., Chappell, T. G. & Rothman, J. E. *Science* **245,** 385–390 (1989).
21. Deisenhofer, J. & Steigemann, W. *Acta crystallogr.* **B31,** 238–250 (1975).
22. Wlodawer, A., Walter, J., Huber, R. & Sjölin, L. *J. molec. Biol.* **180,** 301–329 (1984).
23. Lee, B. & Richards, F. M. *J. molec. Biol.* **55,** 379–400 (1971).
24. Stassinopoulou, C. I., Wagner, G. & Wüthrich, K. *Eur. J. Biochem.* **145,** 423–430 (1984).
25. States, D. J., Dobson, C. M., Karplus, M. & Creighton, T. E. *J. molec. Biol.* **174,** 411–418 (1984).
26. Eigenbrot, C., Randal, M. & Kossiakoff, A. A. *Protein Engng* **3,** 591–598 (1990).
27. van Mierlo, C. P. M., Darby, N. J., Neuhaus, D. & Creighton, T. E. *J. molec. Biol.* **222,** 353–371 (1991).
28. Morjana, N. A., McKeone, B. J. & Gilbert, H. F. *Proc. natn. Acad. Sci. U.S.A.* **90,** 2107–2111 (1993).

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ADDENDUM

The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation

Marc G. Weisskopf, Robert A. Zalutsky & Roger A. Nicoll

Nature **362,** 423–427 (1993)

SINCE publication of our article, it has become apparent that further reference to some topics covered in this paper is warranted. First, reference should have been given to research in invertebrates establishing functional roles for colocalization of neuropeptides with other neurotransmitters. This area of research is discussed thoroughly in a recent review[1]. Second, although not the major focus of the article, our finding that the opioid receptor antagonist naloxone has no effect on hippocampal mossy fibre LTP conflicts with another report[2]. Apart from the different species, experimental conditions and *in vitro* slice preparation used in our study, rather than an *in vivo* anaesthetized preparation[2], we have no explanation for the difference in results. □

1. Kupfermann, I. *Physiol. Rev.* **71,** 683–732 (1991).
2. Derrick, B. E., Weinberger, S. B. & Martinez, J. L. *Brain Res. Bull.* **27,** 219–223 (1991).

CORRECTION

Pulsed high-energy γ-rays from the radio pulsar PSR1706–44

D. J. Thompson, Z. Arzoumanian, D. L. Bertsch, K. T. S. Brazier, N. D'Amico, C. E. Fichtel, J. M. Fierro, R. C. Hartman, S. D. Hunter, S. Johnston, G. Kanbach, V. M. Kaspi, D. A. Kniffen, Y. C. Lin, A. G. Lyne, R. N. Manchester, J. R. Mattox, H. A. Mayer-Hasselwander, P. F. Michelson, C. v. Montigny, H. I. Nel, D. Nice, P. L. Nolan, K. Pinkau, H. Rothermel, E. J. Schneid, M. Sommer, P. Sreekumar & J. H. Taylor

Nature **359,** 615–616 (1992)

IN Fig. 2 of this letter, the γ-axis should be labelled as Photons $(cm^2\ s\ MeV)^{-1}$ and not Photons $(cm^2\ s\ GeV)^{-1}$ as published. □