FEBS 15911 FEBS Letters 370 (1995) 273–277

Characterization of in vitro oxidized barstar

C. Frisch, G. Schreiber, A.R. Fersht*

Cambridge Centre for Protein Engineering, Medical Research Council Centre, Hills Road, Cambridge, CB2 2QH, UK

Received 13 July 1995

Abstract **The polypeptide inhibitor of the ribonuclease barnase, barstar, has two cysteine residues in positions 40 and 82. These have been proposed to form a disulfide bridge leading to an increase in stability without changing the inhibitory activity of the protein. Barstar and a mutant (E80A) were oxidized in vitro and the biochemical and physico-chemical properties of the oxidized monomers were analysed. The oxidized proteins show no inhibition of barnase using a plate assay and are significantly destabilized. CD spectra indicate a loss of secondary structure. The amino acid substitution E80 → A stabilizes the oxidized barstar to about the same extent as it does the reduced protein, indicating, however, that the helical region which it is in is intact.**

Key words: Disulfide bond formation; Protein stability; Thermal denaturation

1. Introduction

Barstar is the natural intracellular polypeptide inhibitor of the extracellular ribonuclease barnase from *Bacillus amyloliquefaciens*. Barstar has 90 residues (M_r = 10342) including the N-terminal methionine which is not cleaved in our preparations. The inhibitor is necessary for survival of barnase-producing cells, since intracellular barnase activity is lethal to the organism. The genes for both barnase and barstar have been cloned and expressed in *Escherichia coli* [1,2]. Barstar inhibits barnase by binding to its active site, forming a 1:1 complex [3,4]. The inhibitor has two cysteine residues at positions 40 and 82 (Fig. 1). It was suggested that formation of an internal disulfide bond between these two cysteines leads to a more stable protein [5] which binds to barnase as tightly as reduced barstar [6]. In another report, it was stated that most of the barstar molecules do not have free sulfhydryl groups but they all bind to and inhibit barnase [2] suggesting that disulfide bond formation occurs to a considerable extent in the cytoplasm of *Escherichia coli*. There has been, however, no detailed study about the binding or physico-chemical properties of oxidized barstar.

The three-dimensional structure of reduced barstar is known from NMR studies in solution [7] and the crystal structure of the complex of barnase with the barstar(C40A/C82A) double mutant which has been solved independently by Guillet et al. [8] to 2.6 Å resolution and by Buckle et al. [4] to 2.0 Å resolution. The distance between the α-carbon atoms of the alanines in position 40 and 82 in the two crystal structures of the barstar (C40A/C82A) double mutant is 11.4 Å [8] and 11.7 Å [4], respectively. In the solution structure of barstar that was determined by NMR [7] (Fig. 1), the distance between the α-carbon atoms of the cysteines is 11.5 Å. These α-carbon distances are outside the normal range for a disulfide bond, which is usually from 4.4 to 6.8 Å [9]. Therefore, local conformational changes would have to take place upon disulfide bond formation [4,7,8]. This should involve a rotation of helix 2 where most of the residues that bind barnase are located [4] (Fig. 1). Hence, the mode of interaction of barnase with barstar$_{ox}$ should be different from that of barnase with barstar [4]. We have reinvestigated, therefore, the properties of oxidized barstar. We were unable, despite considerable effort, to isolate homogeneous barstar$_{ox}$ from expression in different strains of *E. coli*. We have found conditions for oxidizing barstar in vitro.

2. Materials and methods

2.1. Protein expression and purification

The expression and purification of barstar and barstar(E80A) was as described using TG2[pML2bs] and TG2[pML2bsE80A] cells, respectively [7,10]. The mutant barstar(E80A) has been described [6]. Barstar was also expressed in the *E. coli* strains DHB4 and AD494. The strain AD494, which is a derivative of DHB4, lacks thioredoxin reductase and was shown to allow disulfide bond formation in the cytoplasm of *E. coli* [11]. These strains were generous gifts from Jonathan Beckwith (Harvard Medical School, Department of Microbiology and Molecular Genetics). Expression and purification of barstar in these strains were the same as for TG2[pML2bs] cells. The concentrations of barstar and barstar(E80A) and their oxidized forms were determined by measuring the absorption at 280 nm [12] using extinction coefficients estimated by the method of Gill and von Hippel [13]: barstar, 22171; barstar(E80A), 22062; barstar$_{ox}$, 20774; barstar(E80A)$_{ox}$, 21201. Electrospray mass spectroscopy and N-terminal sequencing revealed that all barstar molecules still have the N-terminal methionine. Only the formyl group had been cleaved. The molecular masses obtained by electrospray mass spectroscopy agreed to within ± 1 Dalton with the expected molecular masses. The molecular masses of the oxidized proteins are 2 ± 1 Daltons (barstar$_{ox}$) and 1 ± 1 Dalton (barstar(E80A)$_{ox}$) lower than the masses of the reduced proteins. Polyacrylamide gel electrophoresis was performed on Phast System (Pharmacia) using 20% homogeneous Phast Gels.

2.2. In vitro oxidation of barstar

In vitro oxidation of barstar using either oxidized DTT or oxygen as oxidizing agents was successful only under denaturing conditions, whereas oxidation using the disulfide form of glutathione proceeds under native conditions also. Oxidation by glutathione (0.1 mM barstar incubated at room temperature for 15 min in 50 mM Tris-HCl buffer, pH 8, 2.1 M GdnHCl, 40 mM GSSG) led to barstar with two covalently bound glutathione molecules, as shown by electrospray mass spectroscopy and SDS-PAGE. The same result was obtained after incubation of 0.1 mM barstar in 50 mM Tris-HCl buffer, pH 8, 5 mM GSSG, 20 mM GSH at 50°C for 24 h. The same was observed when 0.1 mM

*Corresponding author. Fax: (44) (1223) 402 140.

Abbreviations: barstar$_{ox}$, barstar with intramolecular disulfide bridge; barstar(E80A), barstar mutant with amino acid substitution glutamate80 → alanine80; barstar(C40A/C82A), barstar mutant with both cysteines substituted by alanines; CD, circular dichroism; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; GSH and GSSG, thiol and disulfide form of glutathione; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; Tris-HCl, Tri(hydoxymethyl)aminomethane-hydrochloride; UV, ultraviolet.

SSDI 0014-5793(95)00839-X

barstar was incubated under conditions that favour native protein (50 mM Tris-HCl, pH 8, 0.1 M GSSG at room temperature), the reaction being almost complete after 48 h. Incubation of 0.5 mg/ml barstar at room temperature in 0.1 M Tris-HCl buffer, pH 8, 4 M GdnHCl, using 0.1 M oxidized DTT or oxygen as oxidizing agents led to almost 100% oxidation of barstar, with more than 50% of the protein being monomeric. Oxidation by oxygen was done by saturating the buffer with oxygen (heavy bubbling for 15 min), then adding barstar and further bubbling of oxygen through the sample for about 48 h. Purification of monomers was performed by gel filtration using a Superdex 75 column (Pharmacia).

The thiol content of the protein samples was determined using the Ellman [14] assay as described by Creighton [15]. Measurements were made in 6 M GdnHCl since both cysteines have low solvent accessibilities in native protein [7].

2.3. Barstar activity assay

The inhibition of barnase activity by barstar was measured qualitatively by an RNA plate assay [3]. Barnase mixed with various amounts of barstar or barstar$_{ox}$ was incubated at 37°C for 15 min on agar plates containing 2 mg/ml yeast RNA (obtained from BDH) and 0.1 M Tris-HCl buffer, pH 8. The RNA was then precipitated using 10% trichloroacetic acid.

2.4. Spectroscopic methods

CD spectra were recorded using a Jasco J-720 spectropolarimeter interfaced with a refrigerated water bath (RTE-110, Neslab Instruments Inc., Newington, N.H.). For far and near UV CD measurements, water-jacketed cuvettes of 0.1 cm and 1 cm path length, respectively, were used. Buffers used were 50 mM sodium phosphate, pH 8 for measurements of the far UV CD spectra and 50 mM Tris-HCl, pH 8 for measurements of the near UV CD spectra. Protein concentrations were about 20 μM (far UV) and 40 μM (near UV). 0.5 mM DTT was added to the buffer when measuring the spectra of the reduced proteins. The results are expressed as mean residue ellipticity, $[\Theta]_{MRW}$ [16], which is defined as $[\Theta]_{MRW} = (\Theta \times 100 \times MRW)/(c \times d)$. Θ is the measured ellipticity in degrees, c is the protein concentration in mg/ml, d is the path length in cm and MRW the mean residue weight of all amino acids (119.4 g/mol). For temperature unfolding measurements, the water bath was programmed to increase from 10°C to 90°C (95°C) at the steady rate of 50 degrees per hour, while the CD at 222 nm (or 270 nm) was recorded at 0.2 degree intervals. Some denaturation curves of barstar(E80A) were recorded to 95°C. The T_m value, the temperature at which the protein is half unfolded, was determined as the value at which the first derivative of the heat denaturation curve exhibits a maximum. This method was chosen because of the lack of a steady pre-transition baseline for the oxidized proteins which is probably due to cold unfolding. Usually for thermal denaturation, 50 mM Tris-HCl buffer, pH 8 was used. The pH of Tris buffer changes significantly with temperature, decreasing about 0.0185 pH units per degree between 25 and 85°C. Therefore, some thermal denaturation measurements were performed in 50 mM sodium phosphate buffer, pH 8. For measurements of the reduced proteins, 0.5 mM DTT was added to the buffer.

Fluorescence measurements were performed using a Hitachi F-4500 fluorescence spectrophotometer. Emission spectra from 295 nm to 450 nm were recorded at 25°C upon excitation at 280 nm. The bandwidth of both the excitation and the emission monochromator was 5 nm.

3. Results and discussion

Expression of barstar in the *E. coli* strains TG2, DHB4 and AD494 gave in all cases about 25 mg of pure protein/l cell culture that was at least to 90% reduced as judged by an Ellman assay. The fraction of disulfide-containing material is most likely dimers and multimers since, after incubation with DTT at room temperature, higher molecular mass bands disappeared from SDS-polyacrylamide gels (data not shown). Oxidized monomers, dimers and multimers were obtained from in vitro oxidation of barstar and barstar(E80A) using oxidized DTT or oxygen as oxidizing agents. Oxidation using oxygen was slower but there was less dimer and multimer formation. The purified monomers were shown by an Ellman [14] assay to be almost 100% oxidized. As expected, the oxidized proteins run faster on a SDS-polyacrylamide gel, indicative of a more compact state (Fig. 2). Oxidized monomers obtained from oxidation by oxygen or using oxidized DTT have the same biochemical and physico-chemical properties.

The amino acid substitution Glu80 → Ala has been shown to stabilize barstar by 2.1 kcal/mol [6]. The mutant barstar(E80A) was also studied to see whether oxidation has a similar effect on this mutant as on wild-type barstar.

All oxidized proteins were tested for inhibitory activity by a RNA plate assay. No inhibition of barnase activity could be observed, even when barstar$_{ox}$ was added in 100-fold molar excess over barnase. Purified dimers are also inactive. Incubation of 20 μM barstar$_{ox}$ or barstar(E80A)$_{ox}$ at room temperature for 30 min in 50 mM Tris-HCl buffer, pH 8, 10 mM DTT led to fully active proteins, indicating that the disulfide bridge is solvent exposed.

The far and near UV CD spectra of barstar and barstar(E80A) at pH 8 and at 25°C are virtually identical (Fig. 3). The far and near UV CD spectra of barstar$_{ox}$ and barstar(E80A)$_{ox}$ are very similar and have a similar shape to those of the reduced proteins (Fig. 3). However, the intensity of the spectra of the oxidized proteins is much lower. This indicates a loss of some secondary and tertiary structure in the oxidized proteins.

Thermal denaturation followed by far and near UV CD revealed that the oxidized proteins are less stable than the reduced proteins (Fig. 4). Moreover, the degree of cooperativity of unfolding of the oxidized proteins is considerably decreased. The increase of the CD signal at 270 nm of both oxidized proteins at low temperature (Fig. 4b) suggests a cold unfolding

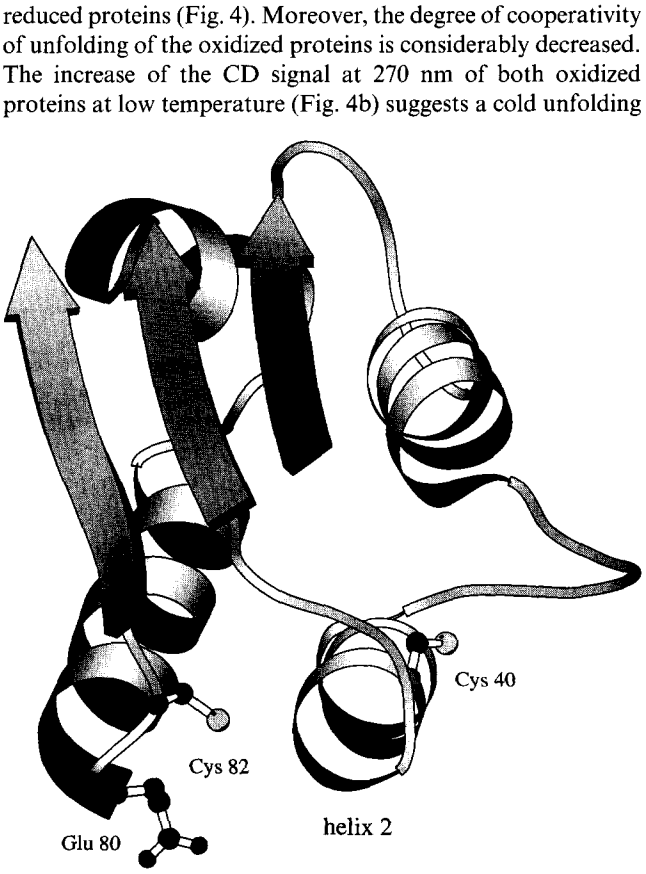

Fig. 1. Structure of barstar drawn with MOLSCRIPT [23]. The C_α atoms and the side chains of amino acid residues 40, 80 and 82 are indicated.

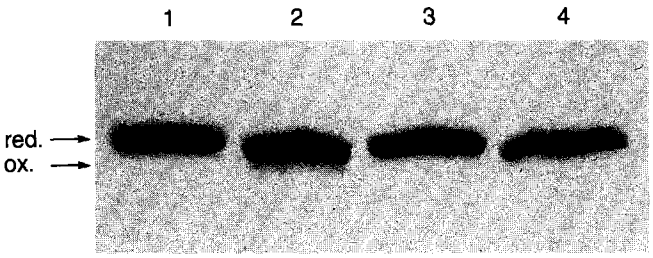

Fig. 2. Reduced and oxidized forms of barstar and barstar(E80A) were run on a 20% homogeneous Phast Gel with SDS buffer strips using Phast System (Pharmacia). The protein concentration was about 0.5 mg/ml for all samples. Lane 1, barstar; lane 2, $barstar_{ox}$; lane 3, barstar(E80A); lane 4, $barstar(E80A)_{ox}$. red., reduced proteins; ox., oxidized proteins.

of the proteins [17]. The susceptibility of wild-type barstar to cold unfolding has been reported [18]. Judged by these denaturation curves, the oxidized proteins would be most stable between about 25°C and 35°C. Table 1 shows the T_m values obtained from the thermal denaturation curves. The stabilizing effect of the amino acid substitution E80 → A [6] was confirmed by thermal denaturation, the T_m of barstar(E80A) being 8.5°C higher than the T_m of wild-type barstar. A stabilizing effect of this amino acid substitution was also observed when both proteins were oxidized. The T_m of $barstar(E80A)_{ox}$ is about 7°C (measured in the far UV CD) and 8°C (measured in the near UV CD) higher than that of $barstar_{ox}$. A comparison of the T_m values of the oxidized and reduced proteins is not feasible, because their cooperativity of unfolding is different. The data indicate, however, that oxidation of barstar destabilizes its structure significantly and only a partly ordered tertiary structure could be detected (Fig. 3B). On the other hand, $barstar_{ox}$ is stabilized by an amino acid substitution that stabilizes the reduced form. This suggests that the structure of barstar near the C-terminus of helix 4 is not altered to a large extent by disulfide bond formation. While the thermal denaturation curves of reduced barstar and barstar(E80A) obtained by far and near UV CD are virtually identical, the tertiary structure in both oxidized proteins melts before the secondary structure. The T_m values of the thermal denaturation curves followed by CD at 270 nm are about 5° ($barstar_{ox}$) and 4° ($barstar(E80A)_{ox}$) lower than the T_m values of the thermal denaturation curves followed by CD at 222 nm.

Since Tris buffer was used for thermal denaturation, the pH decreased from pH 8 at 25°C to about pH 7 at 80°C. However, thermal denaturation followed by CD at 222 nm performed in 50 mM sodium phosphate buffer, pH 8 gave almost identical

Table 1
Midpoints of thermal denaturation curves monitored by CD at 222 nm and 270 nm

Protein	T_m far UV (°C)	T_m near UV (°C)
Barstar	71	71.4
$Barstar_{OX}$	61.3	56.4
Barstar(E80A)	79.5	79.7
$Barstar(E80A)_{OX}$	68.4	64.6

The standard errors (2σ) of the T_m values calculated from 4 thermal denaturation curves of barstar and from 4 curves of $barstar_{ox}$ monitored at 222 nm were ± 0.6 degrees and ±1.6 degrees, respectively. All measurements were done in 50 mM Tris-HCl buffer, pH 8 (see section 2).

results: the T_m values of the oxidized proteins are the same within experimental error, while the T_m values of the reduced proteins are about 2 to 3° lower (data not shown). The average T_m value of the thermal denaturation curves followed by far and near UV CD in 50 mM Tris-HCl buffer, pH 8 for barstar wild-type of 71.2 ± 0.3°C (2σ standard error calculated from 7 measurements) agrees well with the value of 71.5 ± 0.5°C reported by Nath and Udgaonkar [19] at pH 7.

Denaturation of $barstar_{ox}$ and $barstar(E80A)_{ox}$ by urea measured by fluorescence confirmed the results obtained by thermal denaturation. The oxidized proteins are significantly destabilized with $barstar(E80A)_{ox}$ being more stable than $barstar_{ox}$. It was, however, not possible to analyse the urea denaturation curves quantitatively, since there are no defined pre-transition baselines and the slopes of the transitions were different from the reduced proteins and from each other (data not shown).

Since it is possible that the in vitro oxidized barstar is kinetically trapped in an inactive conformation [20] and converts only very slowly to an active oxidized form, an experiment was performed where barnase was used as a 'folding template'. The thermodynamic stability of the barnase-barstar complex is higher than that of the individual proteins ([21], Schreiber, G. and Fersht, A. R., unpublished results). The concentration of urea at which half of the barnase-barstar complex is denatured, $[urea]_{1/2}$, is about 6 M urea, while the $[urea]_{1/2}$ values for barnase and barstar are 4.6 M [22] and 4.2 M [6], respectively. Conditions were chosen in which barnase is mostly in its native

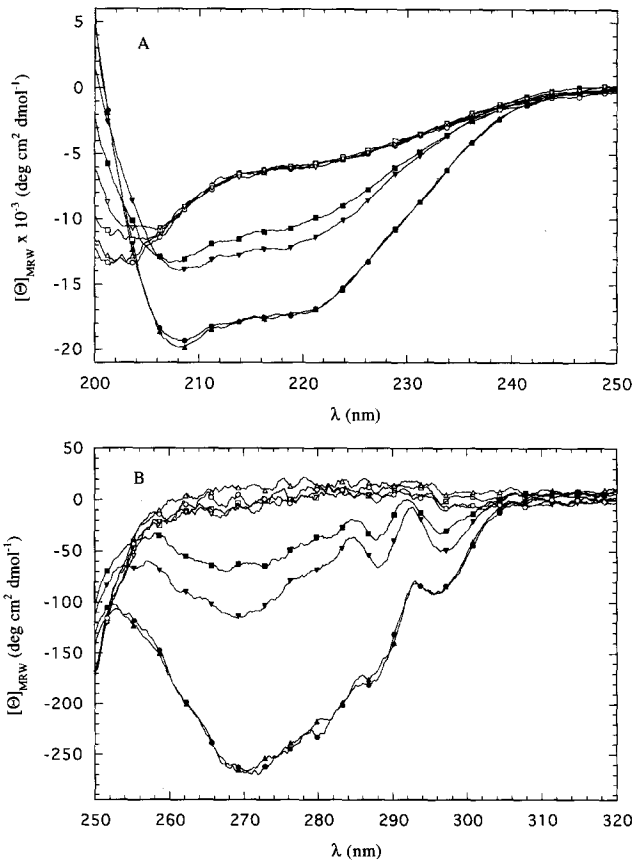

Fig. 3. Far (A) and near UV (B) CD spectra of barstar (●), $barstar_{ox}$ (■), barstar(E80A) (▲) and $barstar(E80A)_{ox}$ (▼) at 25°C (filled symbols) and 90°C (open symbols) at pH 8. The graph was generated using KaleidaGraph on a Macintosh computer.

conformation, barstar is partially denatured but the complex is still fully formed. The large gain of binding energy may, perhaps, overcome any energy barrier of a transition state from an inactive to the active conformation of barstar$_{ox}$. Barnase was incubated with barstar$_{ox}$ (and barstar as control) at 25°C in 4 M urea, 50 mM Tris-HCl buffer, pH 8 and the fluorescence spectra of these samples were compared with the fluorescence spectra of barnase, barstar and barstar$_{ox}$. The results shown in Fig. 5 indicate that no change of the conformation of barstar$_{ox}$, leading to an active form, occurs. Whereas in the control experiment of barstar + barnase a significant blue-shift of the maximum of the fluorescence spectrum occurs, indicative of refolding of the proteins upon formation of complex, no such shift was observed for barnase/barstar$_{ox}$ even after 24 h of incubation at 25°C.

The data presented in this study suggest that there is no active oxidized barstar. No barstar$_{ox}$ could be obtained in vivo by expression of barstar in different *E. coli* strains and the barstar$_{ox}$ obtained by in vitro oxidation using different oxidizing agents was completely inactive. Physico-chemical analysis of in vitro oxidized barstar suggested that it has a conformation that resembles a molten globule. The signal intensity of both the far and near UV CD is significantly decreased (Fig. 3) as well as the cooperativity of unfolding (Fig. 4). Since the tertiary structure of both oxidized proteins is disrupted before the secondary structure (Table 1), unfolding probably precedes via a second molten globular state with only secondary structure.

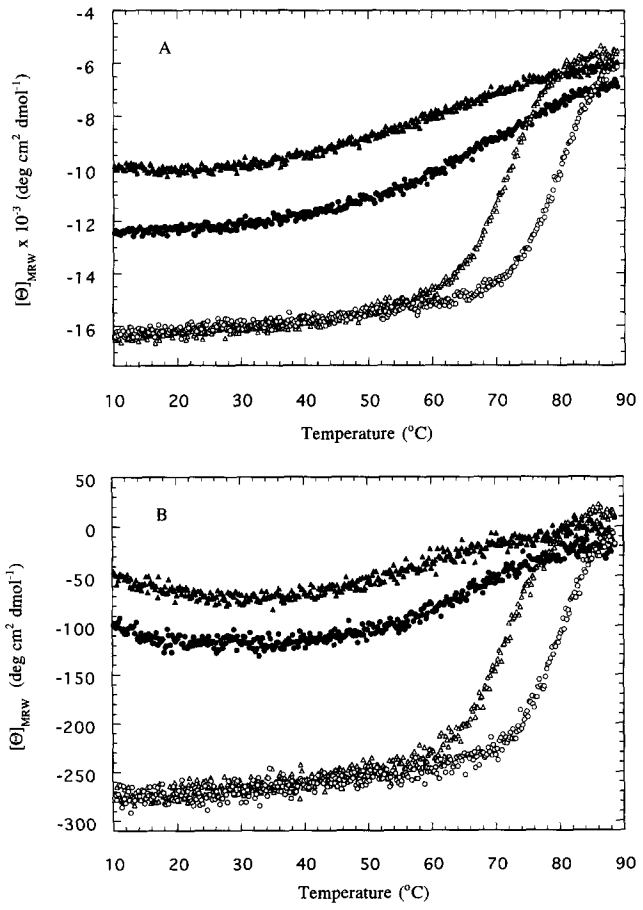

Fig. 4. Thermal denaturation curves of barstar (△), barstar$_{ox}$ (▲), barstar(E80A) (○) and barstar(E80A)$_{ox}$ (●) followed by CD at 222 nm (A) and 270 nm (B). Measurements were done in 50 mM Tris-HCl buffer, pH 8 (see section 2).

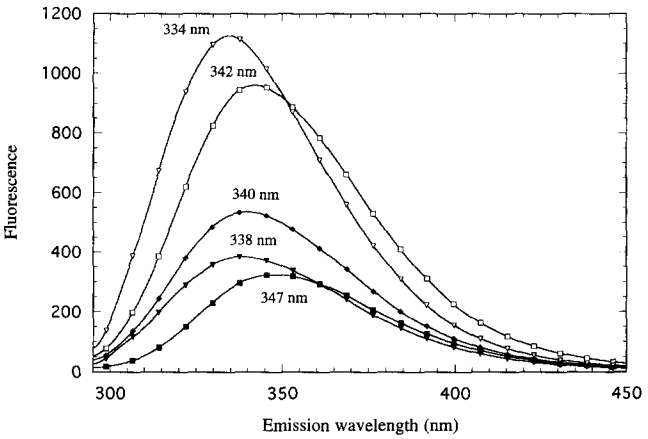

Fig. 5. Fluorescence emission spectra of barnase, barstar, barstar$_{ox}$, barnase+barstar and barnase+barstar$_{ox}$. Excitation was at 280 nm at 25°C. The samples were in 50 mM Tris-HCl buffer, pH 8, 4 M urea and are designated by the following symbols: barnase (◆), barstar (▼), barstar$_{ox}$ (■), barnase+barstar 5 min after mixing (▽), barnase + barstar$_{ox}$ 24 h after mixing (□). The maxima of the emission spectra are given. The maxima of the emission spectra of barnase + barstar$_{ox}$ 5 min and 4 h after mixing were almost the same as the maximum of the spectrum after 24 h. Protein concentrations were 1 μM for each protein. The values for fluorescence are in arbitrary units.

Electrospray mass spectroscopy of the barstar sample which was shown to be oxidized by an Ellman assay and showed a different fluorescence behaviour upon binding to barnase [6] revealed that more than 60% of the molecules have a molecular mass that is about 75 Dalton higher than the one expected. Therefore, it seems that some currently unknown substance is bound covalently to barstar, which might also have been the case for the barstar molecules reported elsewhere to have no free sulfhydryl groups [2,5].

Acknowledgements: We thank Dr. Ralph Golbik for helpful discussions. C.F. is supported by the EC.

References

[1] Hartley, R.W. (1988) J. Mol. Biol. 202, 913–915.
[2] Hartley, R.W. (1989) Trends Biochem. Sci. 14, 450–454.
[3] Hartley, R.W. and Smeaton, J.R. (1973) J. Biol. Chem. 248, 5624–5626.
[4] Buckle, A.M., Schreiber, G. and Fersht, A.R. (1994) Biochemistry 33, 8878–8889.
[5] Hartley, R.W. (1993) Biochemistry 32, 5878–5984.
[6] Schreiber, G., Buckle, A.M. and Fersht, A.R. (1994) Structure 2, 945–951.
[7] Lubienski, M.J., Bycroft, M., Freund, S.M.V. and Fersht, A.R. (1994) Biochemistry 33, 8866–8877.
[8] Guillet, V., Lapthorn, A., Hartley, R.W. and Mauguen, Y. (1993) Structure 1, 165–176.
[9] Richardson, J.S. (1981) Adv. Protein Chem. 34, 167–330.
[10] Schreiber, G. and Fersht, A.R. (1993) Biochemistry 32, 11195–11203.
[11] Derman, A.I., Prinz, W.A., Belin, D. and Beckwith, J. (1993) Science 262, 1744–1747
[12] Drake, A.F. (1994) in: Methods in Molecular Biology, Vol. 22: Microscopy, Optical Spectroscopy, and Macroscopic Techniques. (Jones, C., Mulloy, B. and Thomas, A.H., Eds.) pp. 173–182, Humana Press, Totowa, NJ.

[13] Gill, S.C. and von Hippel, P.H. (1989) Anal. Biochem. 182, 319–326.
[14] Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[15] Creighton, T.E. (1989) in: Protein Function. A Practical Approach. (Creighton, T.E., Ed.) pp. 155–168, IRL Press.
[16] Schmid, F.X. (1989) in: Protein Function. A Practical Approach. (Creighton, T.E., Ed.) pp. 251–284, IRL Press.
[17] Privalov, P.L. (1990) Crit. Rev. Biochem. Mol. Biol. 25, 281–305.
[18] Agashe, V.R. and Udgaonkar, J.B. (1995) Biochemistry 34, 3286–3299.
[19] Nath, U. and Udgaonkar, J.B. (1995) Biochemistry 34, 1702–1713.
[20] Gilbert, H.F. (1994) in: Mechanisms of Protein Folding (Pain, R.H., Ed.) pp. 104–136, IRL Press, Oxford.
[21] Makarov, A.A., Protasevich, I.I., Lobachov, V.M., Kirpichnikov, M.P., Yakovlev, G.I., Gilli, R.M., Briand, C.M. and Hartley, R.W. (1994) FEBS Lett. 354, 251–254.
[22] Clarke, J. and Fersht, A.R. (1993) Biochemistry 32, 4322–4329.
[23] Kraulis, P.J. (1991) J. Appl. Crystallogr. 24, 946–950.