Roles of the 29–138 Disulfide Bond of Subtype A of Human α Interferon in Its Antiviral Activity and Conformational Stability†

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ABSTRACT: Human α (leukocyte) interferons contain two disulfide bonds between Cys-1 and Cys-98 and between Cys-29 and Cys-138. Reduction of interferon under native conditions leads to irreversible loss of antiviral activity; reduction in denaturant, followed by oxidation in native conditions, leads to restoration of activity. This behavior, unusual for disulfide-containing proteins, was studied by using a thiosulfonate derivative of subtype A of human α interferon (IFN-αA). The disulfide-free thiosulfonate formed at 25 °C has essentially no antiviral activity, while maintaining a conformation related to that of native IFN-αA. This species can regain activity after regeneration of its 29–138 disulfide, by thiol–disulfide interchange in native buffer. Incubation of the disulfide-free thiosulfonate under nonreducing conditions at 37 °C generates a monomeric species that has lost its native conformation as well as its ability to regain antiviral activity after thiol–disulfide interchange. These results explain the difficulty in obtaining, under native conditions, a reduced species that regains activity upon oxidation; complete reduction of IFN-αA in 100 mM 2-mercaptoethanol requires 37 °C, a temperature that promotes conformational decay of the disulfide-free form.

The three classes of interferon (α, β, and γ) so far described are polypeptides of similar length (144–166 amino acids) and calculated α-helix potential (Zoon & Wetzel, 1983). α (leukocyte) interferon (IFN-α)[1] is actually a set of proteins arising from the expression of at least 13 homologous genes (Weissman et al., 1982). About 30% of the IFN-α amino acid sequence is conserved in β (fibroblast). γ (immune) interferon is a glycoprotein showing little sequence homology with IFN-α or -β (Gray et al., 1982; De Grado et al., 1982).

The presence of at least one disulfide bond in IFN-α was postulated nearly 20 years ago. Working with crude preparations of animal interferons, Fantes & O'Neill (1964) and Merigan et al. (1965) showed that interferon antiviral activity is irreversibly destroyed by disulfide-directed reducing agents. When the genes of several human IFN-αs were cloned into *Escherichia coli* and sequenced, they were found to possess four highly conserved cysteines (Goeddel et al., 1981; Weissman et al., 1982), suggesting the presence of two disulfides in these molecules. This was confirmed when *E. coli* produced IFN-αA[2] was characterized by HPLC mapping of trypsin digests: this subtype of interferon contains two disulfides, between cysteines-1 and -98 and between cysteines-29 and -138 (Wetzel, 1981; Wetzel et al., 1981).

Disulfide bonds have been shown to play several different roles in protein structure and function (Thorton, 1981). A few disulfides (in thioredoxin and glutathione reductase) are catalytically important, and some disulfides have been implicated in regulatory functions (Freedman, 1979; Namboodiri et al., 1979; Thornton, 1981; Gilbert & Stewart, 1981; Gilbert, 1982). Most protein disulfides for which a function has been clearly defined play a part in structure stabilization (Thornton, 1981), predominantly by lowering the entropy of the denatured form (Anfinsen & Sheraga, 1975).

With the availability of relatively large amounts of single subtype IFN-αA (Wetzel et al., 1981) produced by recombinant DNA methods (Goeddel et al., 1980; Wetzel & Goeddel, 1983), we set out to investigate the influence of the disulfide bonds of this interferon on its activity. We wanted to know the mechanism by which antiviral activity is *irreversibly*[3] lost upon disulfide reduction; at first appearances, this behavior is at odds with the observation of facile reversible formation of native structure upon oxidation of many reduced proteins (Anfinsen, 1972; Freedman & Hillson, 1980). The homologous human IFN-β possesses only three cysteines, at residues 17, 31, and 141 (Taniguchi et al., 1980), and thus can only form a single disulfide. Knowing this, we were also interested in determining whether the unusual response of IFN-αA to reducing agents was associated with a single disulfide bond and, if so, which one.

Some of the results described here have been summarized in two symposium volumes (Wetzel et al., 1982, 1983).

Materials and Methods

IFN-αA was prepared from extracts of *E. coli* (Wetzel et

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[1] Abbreviations: IFN-α, leukocyte interferon; IFN-β, fibroblast interferon; IFN-γ, immune interferon; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; GSH, reduced glutathione; GSSG, oxidized glutathione; HOAc, glacial acetic acid; PVC, poly(vinyl chloride); BSA, bovine serum albumin; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; 2ME, 2-mercaptoethanol; IFN-αA, subtype A of human α (leukocyte) interferon; IFN-αA_{SS1}, IFN-αA containing only a single disulfide, that between Cys-29 and Cys-138; IFN-$\alpha A(SSO_3)_4$, the thiosulfonate derivative of IFN-αA containing no intrachain disulfide bridges; IFN-$\alpha A(SSO_3)_4$*, the heat-inactivated monomeric form of IFN-$\alpha A(SSO_3)_4$; IFN-αA_{SS0}, IFN-αA containing no disulfide bonds.

[2] Leukocyte interferons have been named independently by two groups according to the amino acid sequences predicted from the sequences of cloned genes. Here we use the nomenclature of Goeddel et al. (1981), who use capital letter designations A, B, C, etc. Subtype A differs by one amino acid from subtype α2 in the nomenclature of Weissman et al. (1982).

[3] Tanford (1968) made the distinction between protein denaturation that is reversible, via equilibrium states, by return to starting conditions ("thermodynamically reversible") and denaturation that can be reversed only via further laboratory manipulations. We find it useful to refer to the latter case as "thermodynamic irreversiblity" to distinquish it both from denaturations that are thermodynamically reversible and from those that are generally irreversible (due to oxidation, hydrolysis, etc.).

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al., 1981) by a modification of the procedure of Staehelin et al. (1981b) using an immobilized antibody column (either LI-8, provided by Hoffmann-La Roche, or NK-2, purchased from Celltech). The protein was greater than 95% pure as determined by Coomassie blue staining of $NaDodSO_4$–polyacrylamide gels loaded both in the presence and in the absence of reducing agent.

High-performance liquid chromatography (HPLC) was performed on a system composed of two M-6000 pumps, an M-720 system controller, a Wisp 710B autoinjector (all from Waters Associates), a Perkin-Elmer LC-75 detector, and a Beckman 160 detector. Output from the detectors was sent to a Kipp and Zonen BD-41 dual-pen recorder. Intact interferon derivatives were chromatographed on a 250 × 4.1 mm or a 250 × 7.6 mm RP-P (300 Å) column from Synchrom (Linden, IN). Tryptic maps were developed on a 250 × 4.6 mm Ultrasphere Octyl column from Beckman. Buffers were prepared from distilled water sieved through a Merck Lobar LiChrosorb RP-8 column and HPLC-grade acetonitrile (Burdick and Jackson), each made 0.1% in trifluoroacetic acid (Pierce). Aqueous buffers were adjusted to pH 2.5 with ammonium hydroxide. Gel permeation HPLC was performed on a TSK 2000 SW column purchased from Beckman-Altex using 0.2 M potassium phosphate, pH 6.8.

Circular dichroism spectra were obtained on a Jasco J-500A spectropolarimeter interfaced with a Bascom-Turner 4110 electronic recorder.

$NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Reduced and nonreduced samples loaded on the same gel were separated by at least one empty lane, since artifacts can arise from diffusion of reducing agent into adjacent lanes during electrophoresis.

Antiviral activity was assessed by a microtiter plate cytopathic effect (CPE) assay using vesicular stomatitis virus (VSV) on cultured bovine kidney cells, as described by Wetzel et al. (1981). The enzyme-linked immunosorbent assay (ELISA) utilized two monoclonal anti-interferon antibodies: free LI-9 (Staehelin et al., 1981a) and peroxidase-linked LI-1. Both were kindly provided by Hoffmann-La Roche, Inc. The use of these monoclonal antibodies in an interferon radioimmunoassay has been described (Staehelin et al., 1981c).

Preparation of Disulfide Variants of IFN-αA. Oxidative sulfitolysis of IFN-αA was accomplished by the procedure of Katsoyannis et al. (1967) using sodium tetrathionate (Eastern Chemical, Hauppange, NY) as the oxidizing agent. A solution of sodium sulfite (200 mg/mL, 1.6 M) and sodium tetrathionate (100 mg/mL, 0.37 M) was centrifuged several minutes in an Eppendorf microfuge to remove insoluble tetrathionate decomposition products. A 150-μL aliquot of the supernatant was added to 1.5 mL of a solution of IFN-αA (1.5 mg/mL, 77 μM) in 0.1 M Tris-HCl (pH 8)–1 mM EDTA, and the mixture was allowed to stand at room temperature for 7 h. HPLC analysis (see Figure 3) showed the reaction to be complete. The reaction mixture was dialyzed in Spectropor 2 tubing for 24 h at 4 °C against 3 L of 5 mM ammonium carbonate, with one change of dialysis buffer, then aliquoted into several Eppendorf tubes, and frozen. Alternately, the product was collected from a preparative-scale HPLC column, lyophilized, and resuspended in 5 mM ammonium carbonate. Frozen stocks of IFN-$\alpha A(SSO_3)_4$ were thawed at no higher than room temperature.

Refolding reactions were performed in siliconized Pierce Reactivials. The more voluminous components of reaction buffers were saturated with nitrogen gas immediately before use, and the reaction vials were sealed under a nitrogen atmosphere, but no greater efforts were made to exclude oxygen.

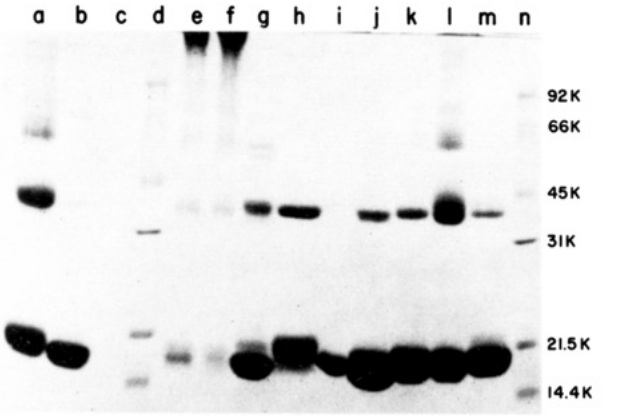

FIGURE 1: Polyacrylamide gel electrophoresis of IFN-αA reduced and carboxymethylated under a variety of conditions. Reduction reactions were conducted under the following conditions with 100 μg of IFN-αA in 500 μL of 50 mM Tris-HCl, pH 9, in Eppendorf tubes: lanes a and e, 1 mM DTT, 37 °C; lane f, 1 mM DTT, 6 M urea, 37 °C; lane g, no additions, 37 °C; lane j, 10 mM 2ME, 25 °C; lane k, 100 mM 2ME, 25 °C; lane l, 100 mM 2ME, 37 °C; lane m, 100 mM 2ME, 5 M guanidine hydrochloride, 37 °C. After 17-h incubation reactions were cooled to 4 °C and terminated by addition of 80 μL of 1 M sodium iodoacetate. After 30 min at 4 °C, reaction mixtures were dialyzed against 5% aqueous HOAc overnight at 4 °C. 150-μL aliquots (20–25 μg) of each were dried down in a Savant Spin Vac. Samples were suspended in gel loading buffer and heated at 90 °C 2 min before loading. Samples run in lanes a and b contained 20 mM 2-mercaptoethanol in loading buffer prior to heating. Lanes d–n were nonreducing. A blank lane (c) was required to prevent artifacts owing to diffusion of 2-mercaptoethanol. Standards and controls: lane b, IFN-αA; lanes d and n, M_r 10000–100000 standards (Bio-Rad; lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B); lane h, IFN-αA_{SS1} reaction mixture (see Materials and Methods); lane i, IFN-$\alpha A(SSO_3)_4$.

Carboxamidomethyl-IFN-αA_{SS1}, which contains only the 29–138 disulfide, was prepared as follows: A 0.3 mg/mL solution of IFN-$\alpha A(SSO_3)_4$ in 0.1 M Tris, pH 8, was incubated at 0 °C for $3^1/_2$ h with 1 mM GSH and 0.1 mM GSSG. At this time 5 μL of 1 M iodoacetamide (in 50% aqueous ethanol) was added and the reaction was continued for 1 h at 0 °C and then for 1 h at room temperature. The product was purified by reverse-phase HPLC, lyophilized, and resuspended in water.

Alkylated, disulfide-free interferons with ELISA positive conformations were made as follows. A solution of IFN-αA (0.33 mg/mL) in 5.2 guanidine hydrochloride, 30 mM Tris-HCl, pH 9, and 0.1 M 2-mercaptoethanol was incubated at 37 °C in a polypropylene Eppendorf tube. After 6 h the solution was divided into two equal portions of 725 μL each and cooled to 0 °C. Iodoacetamide (100 μL of a 1 M solution in 50% aqueous ethanol) was added to one aliquot and sodium iodoacetate (100 μL of a 1 M aqueous solution) to the other. Both were incubated at 0–4 °C in the dark for 2 h and then dialyzed overnight at 4 °C against 2 L of 50 mM Tris-HCl, pH 8. The clear solutions were stored frozen and thawed at no higher than room temperature.

Results

Figure 1 and Table I summarize the results of a series of reduction reactions on IFN-αA. Figure 1 is a polyacrylamide gel indicating that conditions used to break IFN-αA disulfides can produce monomeric IFN-αAs with hydrodynamic radii larger than that of native IFN-αA (P. Johnston and R. Wetzel, unpublished experiments), as well as IFN-αA oligomers that are stable in the reducing milieu employed. Table I lists

Table I: Antiviral Activities of IFN-αA Reduction Products[a]

	solution from gel lane					
	e	f	g	k	l	m
before carboxymethylation	1.2	0.6	75	75	0.3	100
after carboxymethylation	0.8	<0.1	37.5	18.3	<0.1	0.3

[a] Expressed as relative antiviral units in the reaction mixture compared to those in the starting material. Aliquots of the reactions described in Figure 1 were diluted into BSA–PBS before and after carboxymethylation and assayed for their ability to induce an antiviral state.

Table II: Properties of Disulfide Variants of IFN-αA

	rel ELISA act.	rel antiviral act.	
		MDBK cells	HeLa cells
IFN-αA	100	100	100
carboxamidomethyl-IFN-αA_{SS1}	90 ± 9	85 ± 45	ND[a]
IFN-$\alpha A(SSO_3)_4$	111 ± 11	<1	ND
carboxymethyl-IFN-αA_{SSO}	104 ± 10	<0.0002	0.007
carboxamidomethyl-IFN-αA_{SSO}	28 ± 3	0.2	0.01

[a] ND = not determined.

activities measured on the same samples. The interferon used in these experiments contained a small amount of disulfide-linked dimer, M_n 38 000 (P. Johnston and R. Wetzel, unpublished experiments), as seen by comparing untreated IFN-αA run in nonreducing (lane g) or reducing (lane b) conditions. Treatment of IFN-αA with 2ME can produce monomeric forms (10 mM, 25 °C, lane j; 100 mM, 25 °C, lane k) or monomer plus oligomers (100 mM, 37 °C, lane l). Reduction with 100 mM 2ME yields material that either does (25 °C, lane k; 5 M guanidine hydrochloride, 37 °C, lane m) or does not (37 °C, lane l) display activity after dilution into oxygenated assay buffer.

In Table I, carboxymethylation is seen to block recovery of activity after reduction in some cases (100 mM 2ME, 5 M guanidine hydrochloride, 37 °C, lane m) while producing little effect in others (100 mM 2ME, 25 °C, lane k; compare to lane g material). This suggests that a critical disulfide is (lane m) or is not (lane k) being efficiently reduced prior to carboxymethylation. It is observed that treatment with dithiothreitol (1 mM; 50:1 DTT:protein disulfides) with or without 6 M urea (lanes f and e) leads to the formation of inactive high molecular weight oligomeric IFN-αA. These can decay to a series of monomer and lower molecular weight oligomers upon reduction in $NaDodSO_4$ gel loading buffer (lane a).

Table II shows that the oxidative sulfitolysis product of IFN-αA retains immunochemical activity but loses antiviral activity. Figure 2 shows that this loss is reversible in the presence of 40 μM 2ME at 4 °C and that recovery of antiviral activity is accompanied by generation of a chromatographically distinct (Figure 3) species of IFN-αA.

The structural characterization of various disulfide derivatives of IFN-αA is shown in Figure 4. The HPLC profile of a trypsin digest of native IFN-αA is shown in Figure 4a. The peptides containing disulfide bonds, as identified by sensitivity to reducing agent, amino acid composition (Wetzel, 1981), and sequence analysis (W. Kohr, personal communication), are indicated by arrows. The tryptic map of the final product of oxidative sulfitolysis is shown in Figure 4b; both disulfide peptides have disappeared and are replaced by peptides eluting elsewhere in the chromatogram. This suggests that this derivative is the tetrakis(thiosulfonate), IFN-αA-$(SSO_3)_4$. Figure 4c shows the tryptic map of IFN-αA_{SS1},

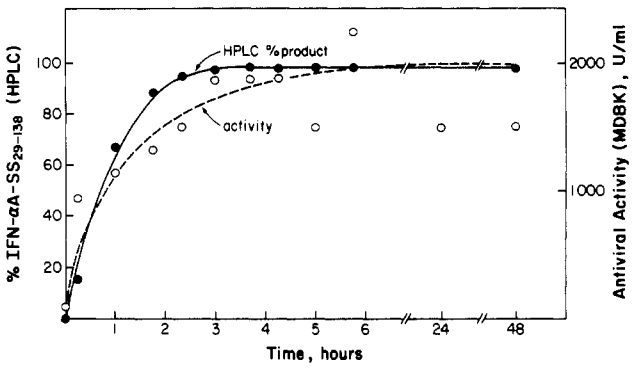

FIGURE 2: Formation of single disulfide interferon IFN-αA_{SS1}. IFN-$\alpha A(SSO_3)_4$ was diluted from a 1 mg/mL stock solution into nitrogen-purged 0.1 M Tris-HCl, pH 9, in a plastic tube to a concentration of 0.2 mg/mL (10 μM), final volume 2.5 mL, and equilibrated at 0 °C. The reaction was initiated with 20 μL of freshly prepared 5 mM 2-mercaptoethanol (40 μM), blanketed with nitrogen, and capped. Aliquots were withdrawn and quickly adjusted to 10 mM iodoacetamide, pH 9 (phosphate). After a 2-min incubation samples were (a) frozen for later HPLC analysis and (b) diluted into BSA–PBS for biological assays. ELISA activity is unchanged across the reaction profile. Adjusted for the dilutions, 2000 units/mL corresponds to 100 megaunits/mg. HPLC analysis was performed in the system described in Figure 3.

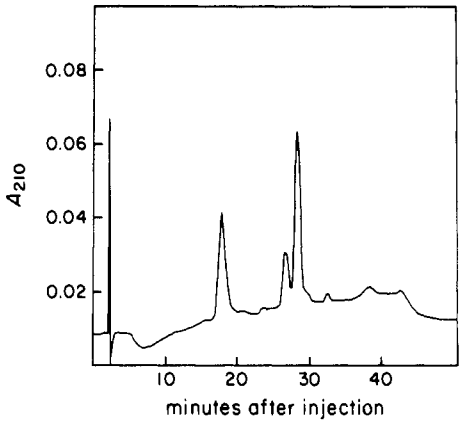

FIGURE 3: Separation of disulfide derivatives of IFN-αA on reverse-phase HPLC. A mixture of purified derivatives, each characterized by HPLC tryptic mapping (Figure 4), was injected on a Synchropak RP-P column and eluted at 1.0 mL/min with a linear gradient of 45–53% CH_3CN in 30 min. See Materials and Methods. IFN-αA elutes at 18 min, IFN-αA_{SS1} at 26.5 min, and IFN-αA-$(SSO_3)_4$ at 28.5 min. The single disulfide form was prepared as described in Figure 2.

obtained by refolding (Figure 2; Materials and Methods) IFN-$\alpha A(SSO_3)_4$; only one of the native disulfide peptides is observed, that derived from a bond between Cys-29 and Cys-138.

Conditions were found that promote re-formation of both native disulfides. With the weak reducing conditions used in the experiment in Figure 2, higher temperatures (25–37 °C) promote formation of the second disulfide. Alternatively, higher concentrations of thiol in a "redox buffer" (Saxena & Wetlaufer, 1970) produce native IFN-αA even at 4 °C (data not shown).

Figure 5a shows the far-UV circular dichroism spectra of IFN-αA (Bewley et al., 1982) and freshly prepared IFN-$\alpha A(SSO_3)_4$. The ellipticity of the strong α-helix bands is essentially identical. Figure 5b displays the near-UV CD spectra of IFN-αA, its thiosulfonate, and a difference spectrum. The conversion of native IFN-αA to thiosulfonate causes a general reduction in ellipticity throughout the region sensitive to aromatic side chains, as indicated by the broad

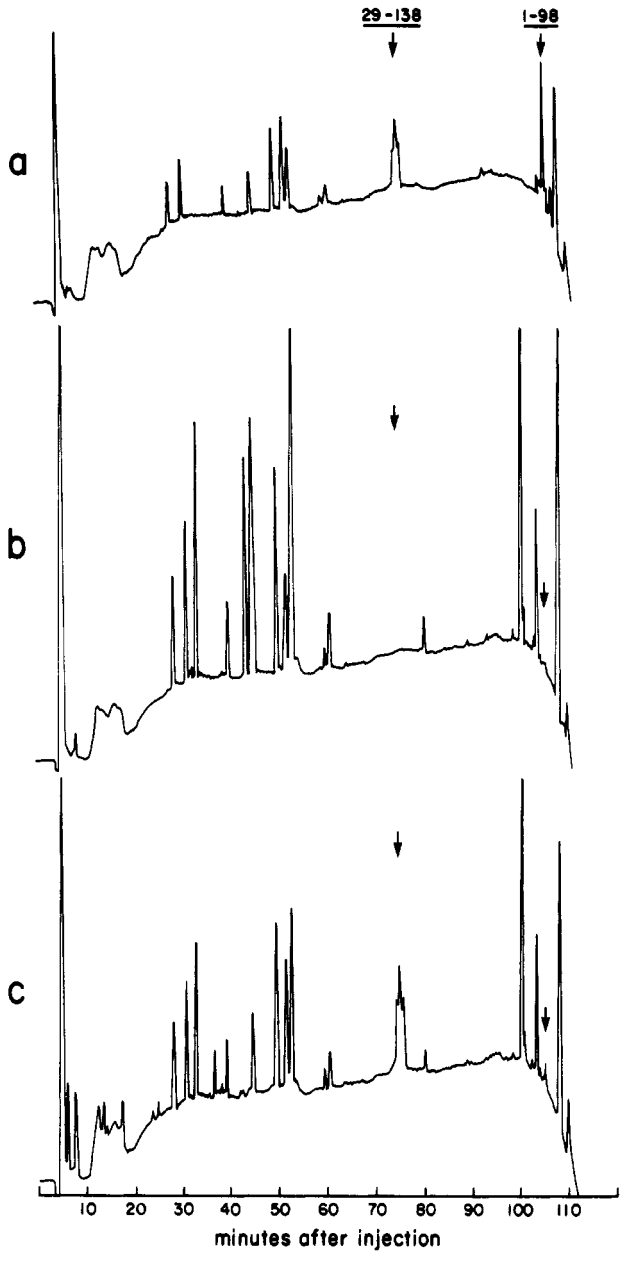

FIGURE 4: HPLC tryptic maps of various disulfide forms of IFN-αA. Interferon derivatives (0.2 mg/mL) in 0.2 M Tris-HCl, pH 9, were incubated with trypsin (TPCK treated, Worthington) at a 1:100 enzyme:substrate ratio for 16 h at room temperature. Reactions were terminated by acidifying with HCl and freezing. Analysis was on a Beckman Ultrasphere Octyl column (250 × 4.6 mm) in a 120-min multilinear gradient of 0–90% CH_3CN in pH 2.5 (TFA) water at a flow rate of 1.0 mL/min. (a) IFN-αA; (b) reaction product from oxidative sulfitolysis of IFN-αA [IFN-αA$(SSO_3)_4$]; (c) reaction product of the conditions described in the legend to Figure 1 [IFN-αA_{SS1}]; the reaction mixture at 24 h was dialyzed against aerated 0.1 M Tris-HCl, pH 9, at room temperature for 24 h before reaction with trypsin. An identical profile was obtained from HPLC-purified carboxamidomethyl-IFN-αA_{SS1} (see Materials and Methods). Arrows indicate observed elution positions for the Cys-29 to Cys-138 and Cys-1 to Cys-98 disulfide peptides.

difference spectrum. No frequency shifts are observed.

Some of the properties of various disulfide derivatives of IFN-αA are listed in Table II. All five forms, containing either no, one, or two disulfides, possess good ELISA activity, indicating that at least two native IFN-αA antigenic determinants are retained in all derivatives. Full activity is obtained for all but carboxamidomethyl-IFN-αA_{SS0}. The disulfide-free species IFN-αA$(SSO_3)_4$ and carboxamidomethyl- and carboxymethyl-IFN-αA_{SS0} differ from the single and double di-

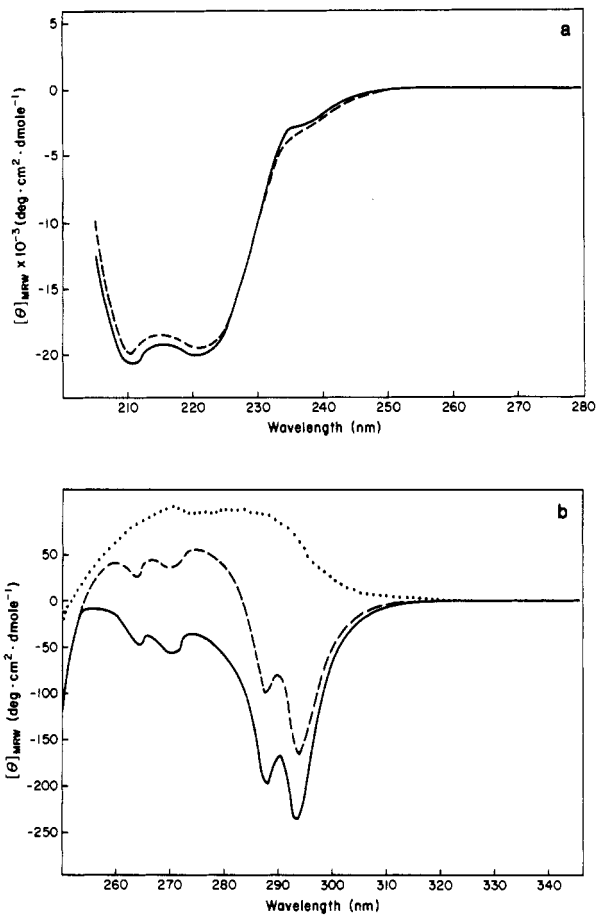

FIGURE 5: Circular dichroism spectroscopy of IFN-αA derivatives. (a) The far-UV region. Freshly prepared IFN-αA$(SSO_3)_4$ was dialyzed overnight at 4 °C against 0.1 M Tris-HCl, pH 7.0. After dialysis, this sample had an A_{280} of 0.85. A sample of IFN-αA in the same buffer, with the same A_{280}, was prepared for comparison. Spectra [IFN-αA (—); IFN-αA$(SSO_3)_4$ (- - -)] were determined in a 1-mm path length cell at room temperature at 10 nm/min. Ellipticities were collected every 0.5 s and stored in a Bascom-Turner electronic recorder, which produced the base-line-subtracted spectra shown here. (b) The near-UV region. Samples described in part a were diluted to fill the sample cell with 0.1 M Tris-HCl, pH 7, to OD_{280} = 0.65 and spectra measured in a 10-mm path length cell at 10 nm/min. Shown are the base-line-subtracted spectra for IFN-αA (—) and IFN-αA$(SSO_3)_4$ (- - -) and the difference spectra (···) produced by subtracting the thiosulfonate spectrum from the spectrum of native IFN-αA.

sulfide forms in their low antiviral activity on bovine kidney and (human) HeLa cells.

Three disulfide variants of IFN [IFN-αA, carboxamidomethyl-IFN-αA_{SS1}, and IFN-αA$(SSO_3)_4$] possessing full activity in the ELISA assay were tested for their stability upon incubation at 37 °C. Figure 6a shows the reaction as followed by ELISA. While IFN-αA is completely stable to this treatment, the single disulfide form decays to about 75% activity after 24 h, and the disulfide-free form decays to less than 10% in the same time. Figure 6b shows that the antiviral activities of IFN-αA and carboxamidomethyl-IFN-αA_{SS1} are retained upon treatment at 37 °C. Figure 6b also shows that the ability of IFN-αA$(SSO_3)_4$ to recover its lost antiviral activity in a redox buffer promoting thiol–disulfide interchange decays in parallel with its loss in ELISA activity (Figure 6a).

Table III shows the effects of incubating heat-inactivated IFN-αA$(SSO_3)_4$ in various buffers. The ELISA results indicate that this reaction product can be renatured by incubation with denaturing levels of urea, guanidine hydrochloride, or $NaDodSO_4$, followed by dilution into PBS. Essentially identical renaturation is shown for urea and guanidine

Table III: Renaturation of Heat-Denatured IFN-αA$(SSO_3)_4$[a]

buffer component	ELISA[b]	antiviral[c]
no addition	<5	<1
7 M urea	35	39
6.4 M guanidine hydrochloride	72	72
0.2% $NaDodSO_4$	90	<1

[a] Aliquots of a 0.2 mg/mL solution of freshly prepared IFN-αA$(SSO_3)_4$ in 50 mM Tris-HCl, pH 7, and 1 mM EDTA were incubated at 4 or 37 °C for 72 h. The heated and unheated thiosulfonate solutions were diluted into 10 volumes of 50 mM Tris-HCl, pH 7, containing the buffer components shown in the left-hand column. These solutions were incubated 72 h at 25 °C, and then a portion was diluted into BSA–PBS and assayed for ELISA. The 72-h solutions were dialyzed 15 h against 0.1 M Tris-HCl, pH 8, 1 mM GSH, 0.1 mM GSSG, and 1 mM EDTA, for refolding, and then against 5% HOAc. The 5% HOAc solutions were diluted into BSA–PBS for antiviral assay. [b] Expressed for the activity recovered from variously treated heat-denatured IFN-αA$(SSO_3)_4$s relative to unheated IFN-αA$(SSO_3)_4$ incubated in $NaDodSO_4$ (=100% = 115 megaunits/mg). [c] Expressed for the activity on MDBK cells, generated by thiol–disulfide interchange on variously treated heat-denatured IFN-αA$(SSO_3)_4$s relative to unheated IFN-αA$(SSO_3)_4$ incubated with guanidine hydrochloride (=100% = 103 megaunits/mg).

treatment when the state of the protein is monitored for the ability of thiol–disulfide interchange to regenerate antiviral activity. While $NaDodSO_4$ treatment under these conditions was not effective, antiviral activity could be regenerated by thiol–disulfide interchange in the *continued presence* of 0.05% $NaDodSO_4$.

Table IV summarizes results of experiments designed to probe the nature of the thermal denaturation of IFN-αA-$(SSO_3)_4$. Equivalent amounts of native and heat-inactivated IFN-αA$(SSO_3)_4$ were chromatographed by HPLC and the major peaks collected for ELISA assay. In both gel permeation and reverse-phase chromatography, the only molecular form detected in heat-inactivated IFN-αA$(SSO_3)_4$ cochromatographed with IFN-αA$(SSO_3)_4$. While the recovery of the M_r 20 000 form from the 37 °C reaction compared to that of native IFN-αA$(SSO_3)_4$ was 22–33%, this was still considerably higher than 1.6% residual activity in the 37 °C reaction mixture applied to the columns.

Table IV also shows that the monomeric material in the 37 °C reaction, IFN-αA$(SSO_3)_4$*, is less than 2% as active in ELISA as is IFN-αA$(SSO_3)_4$. In contrast, the monomeric material recovered from RP-P chromatography of the 37 °C reaction possesses full ELISA activity, presumably because the solvent conditions are capable of renaturing this species.

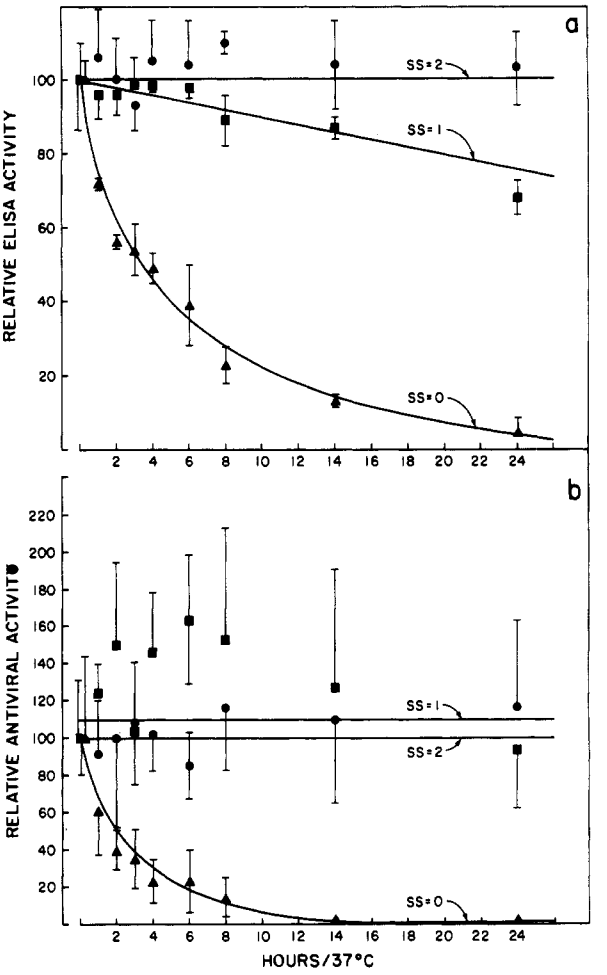

FIGURE 6: Thermal stability of disulfide variants of IFN-αA. IFN-αA (SS = 2), carboxamidomethyl-IFN-αA_{SS1} (SS = 1), and IFN-αA-$(SSO_3)_4$ (SS = 0) were incubated at 37 °C in silanized Pierce Reactivials at 0.1 mg/mL in 0.1 M Tris-HCl, pH 7, and 1 mM EDTA. (a) At each time point, aliquots were diluted into BSA–PBS, stored at 4 °C, and assayed no more than 30 h later by an ELISA assay based on the published radioimmunoassay (Staehelin et al., 1981a). Each value is the mean of all on-scale values from duplicate sets of three different dilutions. (b) Dilutions were also assayed for antiviral activity on cultured bovine kidney (MDBK) cells. Values (curves SS = 2 and SS = 1) are the means of duplicates assayed five consecutive days. Not shown is the curve for direct antiviral activity of IFN-αA$(SSO_3)_4$, which was undetectable at all time points. The curve marked SS = 0 was generated by adding 10-μL aliquots of the 37 °C incubation to 40 μL of 2 mM GSH, 0.2 mM GSSG in 0.1 M Tris-HCl, pH 8, and 1 mM EDTA, incubating overnight, and diluting into BSA–PBS for antiviral activity. These values are means of dilutions of duplicate refolding reactions assayed four consecutive days.

Discussion

The results summarized in Table I and Figure 1 show that IFN-αA's response to reduction is complex, depending both on the nature of the reducing agent and on the presence or absence of denaturant during reduction. These results confirm the experience of others working with crude preparations of IFN-α. As originally observed with animal IFN-α by Fantes & O'Neill (1964), Merigan et al. (1965), and Marshall et al. (1972), complete reduction of IFN-αA under native conditions leads to irreversible loss of antiviral activity. However, when IFN-αA is fully reduced under strong denaturing conditions

Table IV: Characterization of Heat-Denatured IFN-αA$(SSO_3)_4$[a]

		TSK monomer		RP-P monomer	
	before fractionation, ELISA rel sp act.	rel material recovery[c]	rel sp act.[b]	rel material recovery[d]	rel sp act.[b]
IFN-αA$(SSO_3)_4$, 4 °C	100	100	100	100	100
IFN-αA$(SSO_3)_4$, 37 °C	1.6	22	<2	33	105

[a] 500 μL of a 0.2 mg/mL solution of IFN-αA$(SSO_3)_4$ was heated in 50 mM Tris-HCl (pH 7)–1 mM EDTA for 72 h at 37 °C; 500 μL of the same solution was held at 4 °C over this period. Each was titered before fractionation, and then aliquots were chromatographed by HPLC on gel permeation (TSK) and reverse-phase (RP-P) columns. The peak known to be associated with monomeric thiosulfonate was quantitated by UV absorption, collected, and assayed. [b] ELISA; relative specific activity was computed by using measured activity and estimated material recovery. [c] Based on integrated 210-nm peaks. [d] Based on relative heights of 210-nm peaks.

and then returned to native, oxidizing conditions, it recovers its antiviral activity. This confirms for a single subtype of human IFN-α what was previously observed for crude, mixed subtype human IFN-α (Mogensen & Cantell, 1974). The presence of denaturants is often required for *full* reduction of protein disulfide bonds (Means & Feeney, 1971). It is not immediately evident, however, how reduction in the presence of denaturant might lead to maintenance of a protein's native structure, while reduction of the same molecule under native conditions might lead to denaturation.

Since the preparation of a monomeric, immunochemically active, reduced IFN-αA intially proved difficult, we relied chiefly on oxidative sulfitolysis (Means & Feeney, 1971) as a means of reversibly breaking IFN-αA's disulfides. Protein thiosulfonates have been used as starting materials for refolding experiments (Katsoyannis et al., 1967; Chance et al., 1981; Frank et al., 1981) and are relatively stable in the absence of reducing agents. An additional feature of thiosulfonates is their mechanistic compatibility with redox buffer catalyzed thiol–disulfide interchange (Saxena & Wetlaufer, 1970) as a mediator of disulfide formation during protein folding; sulfite is rapidly displaced by reducing agent (Frank et al., 1981).

IFN-αA Disulfides and Antiviral Activity. Some proteins such as ribonuclease and bovine pancreatic trypsin inhibitor (Creighton, 1968) spontaneously decay upon reduction of their disulfide bonds to a state that has been termed random coil; in this state, spectral and chemical properties of amino acid side chains take on properties characteristic of fully solvated, mobile groups, and many immunochemical properties of the parent molecule are destroyed. Clearly, disulfide bonds are not the only forces involved in the formation of the rigid tertiary structure of globular proteins under native conditions, and thus it is not surprising that some proteins such as bovine growth hormone (Li, 1975; Bewley & Yang, 1980) retain considerable structure after cleavage of their disulfide bonds.

IFN-αA seems to lie in this second class of disulfide-containing proteins. An immunochemical assay (Staehelin et al., 1981c) known to be sensitive to conformational changes (Wetzel et al., 1982; Pestka et al., 1983) responds fully to IFN-αA$(SSO_3)_4$. In addition, disulfide-free IFN-αA$(SSO_3)_4$ under native conditions shows only minor changes in its CD spectra (Figure 5). The only significant change observed upon breaking the IFN-αA disulfides is a general reduction in the intensity of the negative bands, associated with side chain absorption, in the near-UV region of the CD. The difference spectrum between IFN-αA and IFN-αA$(SSO_3)_4$ shows that the positive absorbance gained after disulfide bond cleavage is a broad band from 250 to 300 nm. This band has been assigned previously to the protein disulfide bond on the basis of the CD of L-cystine as well as the difference spectrum produced by comparing the oxidized and reduced forms of pituitary growth hormone derivatives (Bewley & Yang, 1980).

Despite the retention of native conformational features suggested by these data, IFN-αA$(SSO_3)_4$ has lost all but a trace of the antiviral activity of the parent molecule. The residual activity (1–5%) occasionally observed in IFN-αA-$(SSO_3)_4$ preparations is probably due to a small degree of refolding during and after purification, since IFN-αA$(SSO_3)_4$ assayed directly from the sulfitolysis reaction mixture is another 10 times less active than the purified thiosulfonate (data not shown). In addition, two disulfide-free, reduced–alkylated IFN-αA derivatives, prepared in a manner that allows retention of a sensitive ELISA positive conformation (see below), display dramatically reduced antiviral activities (Table II). Their loss of antiviral activity is related to a loss of receptor binding activity, since both reduced, alkylated derivatives were unable to compete (at molar concentrations where IFN-αA is competitive) with ^{35}S-labeled IFN-αA in a radioreceptor assay (Wetzel et al., 1983).

IFN-αA regains antiviral activity with the recovery of a single disulfide bond, that linking Cys-29 to Cys-138. This is the more rapidly formed of the two IFN-αA disulfides. The second IFN-αA disulfide (Cys-1 to Cys-98) is clearly not essential for antiviral activity, since carboxamidomethyl-IFN-αA_{SS1}, which is incapable of re-forming additional intra- or intermolecular disulfides, is fully active in the antiviral assay. These results suggest that the Cys-29 to Cys-138 disulfide plays an intimate role in the induction of an antiviral state. The loss of activity in a reduced derivative alkylated with neutral iodoacetamide rules out the possibility that the loss of activity in IFN-αA$(SSO_3)_4$ is due to the introduction of its strongly ionized thiosulfonate groups. We cannot, however, eliminate the possibility that the steric bulk of the sulfhydryl reagents (Smith et al., 1975; Wiedner et al., 1979) plays a significant role in the destruction of activity in disulfide-free IFN-αA.

Since IFN-αA's antiviral activity is highly sensitive to tertiary structure (Wetzel et al., 1982), the required disulfide may serve to preserve some key conformational feature required for receptor binding; the same bond clearly plays a role in conformational stability at 37 °C (see below). In a recent paper, Higashi et al. (1983) showed that mouse IFN-β, while homologous (48% at amino acid level) with human IFN-β, contains only one cysteine, at position 17. This indicates that all type I interferons do not require disulfides for activity and suggests that those that do probably utilize this important disulfide for orientation and support of a receptor-binding region, but not its direct composition.

IFN-αA Disulfides and Structural Stability. Most disulfide-containing proteins that have been studied can be modified to generate a reduced monomeric species capable of returning, by air oxidation (Sela et al., 1957; Epstein et al., 1963; Ahmed et al., 1975) or chemically (Saxena & Wetlaufer, 1970) or enzymatically (Givol et al., 1964; Steiner et al., 1965) catalyzed thiol–disulfide interchange, to their native, active states (Anfinsen, 1972; Creighton, 1978; Freedman & Hillson, 1980). In most cases in which a disulfide has been implicated in conferring conformational stability, this is toward thermodynamically *reversible*[3] denaturation (Thornton, 1981). One exception is potato protease inhibitor I, which acquires a dramatically increased sensitivity to irreversible thermal denaturation upon reduction/carboxymethylation of its single disulfide bond (Plunkett & Ryan, 1980). As shown above, there is no evidence that the disulfides of IFN-αA play any major role in stabilizing against reversible effects; the disulfide-free molecule at 25 °C displays CD spectra indicating a nativelike tertiary conformation. On the other hand, the results summarized in Figure 1 and Table I suggest that IFN-αA behaves as if conditions necessary to produce complete reduction also produce an irreversibly denatured, inactive conformation.

Fortunately it proved possible, via oxidative sulfitolysis at 25 °C, to obtain a disulfide-free IFN-αA that maintains a proactive conformation; that is, a conformation capable of recovering antiviral activity on thiol–disulfide interchange (Wetzel et al., 1982; see above). When IFN-αA$(SSO_3)_4$ is incubated at 37 °C, it decays to a state that has lost ELISA activity (Figure 6a) as well as the ability to refold in a redox buffer to an antivirally active state (Figure 6b). This behavior is identical with that of the presumed initial product of 2ME

reduction at 37 °C, which decays in situ to an inactive conformation (Figure 1; Table I).

The results shown in Table III indicate that heat-inactivated material can be restored by denaturant or detergent treatment to an ELISA active state. Furthermore, treatment with denaturants restores the molecule's ability to recover antiviral activity after incubation in a redox buffer. *This shows that heat inactivation is not mediated by changes in the chemical status of the cysteines of IFN-αA* but rather is due to conformational effects producing a denaturation that can be characterized as thermodynamically irreversible.[3]

Early in the 37 °C incubation of IFN-αA$(SSO_3)_4$, but not in the incubation of the other interferon derivatives, high molecular weight aggregates were detected by Rayleigh scattering in fluorescence measurements (data not shown); later, protein precipitate was visible. It thus seemed possible that 37 °C inactivation involved a simple rate enhancement of aggregation and/or precipitation of IFN-αA$(SSO_3)_4$. Alternatively, aggregation or precipitation might thermodynamically drive the temperature-induced conformational change of IFN-αA$(SSO_3)_4$. Finally, a conformationally altered monomeric form might itself be more stable than IFN-αA$(SSO_3)_4$ under the conditions employed.

The experiment summarized in Table IV was designed to gain insight into the mechanism of inactivation of IFN-αA-$(SSO_3)_4$. Only 22% monomeric material was recovered from the reaction mixture; the unrecovered material presumably was precipitate and/or aggregates. By measurement of the relative specific activities of the monomeric IFN species collected by sizing HPLC, it was found that the 37 °C derived monomer has $\leq$2% the ELISA *specific activity* of the 4 °C derived monomer. These results indicate that the 37 °C transition involves a conformation change in the monomer and that the inactive monomer, IFN-αA$(SSO_3)_4$*, is thermodynamically favored over IFN-αA$(SSO_3)_4$ under native conditions at 37 °C.

The results obtained with the thiosulfonate derivative of IFN-αA are not related to the addition of the highly ionized sulfonate groups. This is shown by the reduction experiments with native interferon, in which IFN-αA reduced at 37 °C requires the presence of denaturant for regeneration of activity (Figure 1; Table I). This is also shown in work with carboxymethyl and carboxamidomethyl derivatives of reduced IFN-αA, which can be similarly inactivated at 37 °C and their ELISA activities restored by a denaturation/renaturation cycle (data not shown).

While the prevailing view is that proteins fold into a state that is the global free energy minimum for that sequence, there are examples in the literature of the ability of some proteins to decay to lower free energy states associated with ordered, monomeric forms (Ghelis & Yon, 1982). In this paper we show that disulfide-free IFN-αA has this same metastable[4] quality.

This means that the 29–138 disulfide plays a role in the preservation of an important global conformation of the IFN-αA molecule. In this regard it is of interest that disulfide-free mouse IFN-β (Higashi et al., 1983; see above) possesses, in place of human IFN-β's Cys-31, Asn in a recognition sequence for N-linked glycosylation. Glycosylation has been suggested to be another route by which proteins can be stabilized.

[4] It is also possible that the monomeric, solution form of inactivated IFN-αA$(SSO_3)_4$ is kinetically trapped at the conditions used in these experiments. Further work would be required to distinguish between this and a thermodynamic driving force in the conversion.

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Interaction of Aromatic Retinal Analogues with Apopurple Membranes of *Halobacterium halobium*†

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ABSTRACT: Absorption spectral properties of aromatic analogues of retinal with apopurple membrane of *Halobacterium halobium* were studied. The spectra of the all-trans forms were composed of two or more absorption bands. During incubation at 20 °C, an absorption band above 500 nm increased in intensity gradually at the expense of an absorption band in the shorter wavelength region with no isomerization of the chromophore. The longer wavelength species was shown to be the protonated form of the shorter wavelength species by changing the pH of the medium. Upon irradiation with blue light, the bandwidth of the spectrum became smaller with isomerization of the chromophore to its 13-cis form. Irreversible binding of protons on the membrane occurred during this process. The rate of the increase in the longer wavelength absorption band was especially low in the reaction with the all-trans form of retinal analogues having a bulky substituent at the para or meta positions of the phenyl ring. In contrast, the 13-cis isomer of aromatic retinal analogues gave a single absorption peak. The extent of the spectral shift upon binding to apopurple membranes was compared over a series of aromatic retinals, and the results were explained in terms of steric interactions of the chromophore with the protein.

Bacteriorhodopsin (bR)[1] in the purple membrane of *Halobacterium halobium* transports protons unidirectionally across the membrane by using light energy absorbed by its chromophore (Stoeckenius et al., 1979; Stoeckenius & Bogomolni, 1982). Conversion of light energy into chemical energy is most probably carried out in the vicinity of the retinylidene chromophore. In order to investigate the structure of the retinal binding site, experiments with a series of chemically modified chromophores (Nakanishi et al., 1980) have been done along with chemical modification of the protein itself

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[1] Abbreviations: bR, bacteriorhodopsin; λ_{max}, the wavelength of maximum absorption; HPLC, high-performance liquid chromatography.

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