Effect of Chaotropic Salts and Protein Denaturants on the Thermal Stability of Mouse Fibroblast Interferon

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SUMMARY

Altering the aqueous environment, especially with agents that affect hydrogen bonds, markedly affects the stability of mouse L cell interferon. Low pH stabilizes interferon whereas high pH labilizes it; heavy water further enhances interferon thermostability at pH 2 but not at pH 9. Exposure to the protein denaturants, 4 M-guanidine hydrochloride and 6 M-urea, significantly decreases the activity of interferon at pH 2 and pH 9; however, the residual interferon activity is relatively thermostable. Certain chaotropic salts protect interferon against thermal destruction, and in terms of effectiveness, their sequence is in the order SCN⁻ > I⁻ ≥ Cl⁻ = ClO₄⁻ = Br⁻ > NO₃⁻. Interferon becomes more stable to heat as the NaSCN concentration is increased from 0.25 M to 2.0 M. Molecular sieve chromatography of interferon in the presence of 1.5 M-NaSCN at pH 7 shows a shift in its apparent mol. wt. from 25,000 to 42,000. Unlike most proteins, the unfolded conformation of interferon appears to be more stable to heat than the molecule with a smaller Stokes' radius.

INTRODUCTION

Interferons are considered to be glycoproteins that are stable to low pH or heat. Since heating has frequently been used to probe the structure and behaviour of proteins under various other environmental conditions (Tanford, 1968), we have analysed several physicochemical factors affecting the thermal inactivation of murine interferon (Jariwalla, Grossberg & Sedmak, 1975). The idea that at least a portion of the molecule may interact with the aqueous environment in such a way as to diminish the rate of heat inactivation led us to study low mol. wt. compounds affecting the solvation of proteins, such as protein denaturants and chaotropic salts, i.e., salts whose anions increase the solubility of the non-polar regions of proteins (Hatefi & Hanstein, 1969).

Protein denaturants, like guanidine hydrochloride (GuHCl) and urea, have been reported to have various effects on the biological activity of interferons. Thus, 8 M-urea destroyed the activity of mouse interferon, but neither chicken (Merigan, Winget & Dixon, 1965) nor human leukocyte interferons (Mogensen & Cantell, 1973) were adversely affected. In the latter report, it was shown that 4 to 8 M-GuHCl protected the interferon from thermal inactivation. Urea or GuHCl (Kadri & Kohlhage, 1969) completely destroyed virus-induced high mol. wt. interferon in the serum of rabbits, but only partly inactivated the smaller mol. wt. species also present. The present study has attempted to determine if reagents that affect the hydrogen-bonded structure of proteins or alter their hydrophobic interactions change the resistance of mouse L cell interferon to thermal inactivation.
METHODS

Cell cultures. The cultural conditions for the L_{929} mouse fibroblast line have been described (Jariwalla et al. 1975).

Preparation, purification and assay of L cell interferon. The preparation of interferon in L cells with Newcastle disease virus and its partial purification using zeolite elution have been described (Jariwalla et al. 1975). Interferon activity was measured by haemagglutinin (HA) yield-reduction bioassay (Oie et al. 1972) with GD-VII virus as the challenge, the endpoint being a 0.5 log (70%) reduction in virus HA yield. When samples of the National Institutes of Health mouse reference standard preparation G002–904–511 (Grossberg, Jameson & Sedmak, 1974), with an assigned potency of 12000 units/container, was assayed by our method, the mean titre obtained was 12000, with a standard deviation of ±61%. Zeolite-purified interferon preparations had a sp. act. of 5.9 to 7.7 × 10^5 units/mg protein.

Heat-inactivation tests. Interferon samples containing the different reagents were stored at 4 °C for 24 h and then subjected to heat-inactivation in a linear non-isothermal (LNS) test (Zoglio et al. 1968; Greiff & Greiff, 1972) and also a static temperature test conducted at 68 °C. For such tests 1 ml quantities of interferon distributed in 15 × 48 mm screw-cap glass vials were submerged in water baths. In the LNS test the bath temperature was continuously raised at a rate of 4 °C/h between 40 and 90 °C over a period of 13 h by a motor-driven attachment on the magnet of the thermoregulator (Constant Temperature Circulator, Model ED 'Unithermal'). Samples were taken when the bath reached 40, 50, 60, 70, 80 and 90 °C; at each temperature it was checked with a thermometer that the temperature inside a vial was the same as that of the water bath.

Samples were cooled by placing them at room temperature for 30 min, and then stored at −70 °C until assayed. The time of cooling from 68 °C (the static temperature test usually employed) to room temperature was approx. 15 min (Jariwalla et al. 1975).

Buffer solutions. Interferon samples at pH 2 were prepared in 0.15 M-KCl-HCl buffer solutions, and at pH values 7 and 9 in 0.1 M-Na_2HPO_4-NaH_2PO_4.

Reagents. The following chemicals, of reagent grade or higher purity, were dissolved directly in, or added to, the interferon preparation to give the desired final concentration: NaSCN, NaClO_3, NaNO_3, NaBr, NaI, NaCl, urea – free of any contaminating cyanate (Mallinckrodt Chemical Works, St Louis, Missouri), GuHCl (Eastman Organic Chemicals, Rochester, New York) and heavy water (deuterium oxide D_2O from Matheson, Coleman & Bell, Cincinnati, Ohio). The reagents were removed by dialysis at 4 °C before the assay of interferon activity.

Molecular weight estimation. The mol. wt. of mouse interferon were estimated by molecular sieve chromatography using Sephadex G-100 columns at pH 7 in the presence or absence of 1.5 M-NaSCN. Columns were exhaustively washed and equilibrated with 0.1 M-Na_2HPO_4-NaH_2PO_4 (pH 7) at 4 °C or in 0.1 M-tris-HCl, 1.5 M-NaSCN (pH 7) at room temperature. The void volume was determined with blue dextran 2000. The columns were calibrated with markers of known mol. wt., namely, ovalbumin, soybean trypsin inhibitor and lysozyme or cytochrome c, selected since they resist conformational changes under denaturing conditions such as exposure to pH 2 (Tanford, 1968; R. J. Jariwalla et al. unpublished observations). Zeolite-purified mouse interferon was dialysed against 0.1 M-sodium phosphate (pH 7) or 0.1 M-tris-HCl, 1.5 M-NaSCN (pH 7) at 4 °C before being applied to each column. Fractions (2.0 ml) were diluted and assayed for interferon activity without dialysis; preliminary experiments showed that concentrations up to 0.05 M-NaSCN had no deleterious effect on either cell cultures or virus HA yield.
Thermal stability of mouse interferon

Table 1. The effects of some additives on the stability at 68 °C of partially purified L cell interferon

<table>
<thead>
<tr>
<th>Additive</th>
<th>pH</th>
<th>0°</th>
<th>60</th>
<th>150</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>7600 (100)†</td>
<td>3000 (39.5)</td>
<td>—</td>
<td>750 (9.9)</td>
</tr>
<tr>
<td>Urea, 6 M</td>
<td>2</td>
<td>2160 (28.4)</td>
<td>1350 (17.8)</td>
<td>—</td>
<td>1350 (17.8)</td>
</tr>
<tr>
<td>None</td>
<td>9</td>
<td>7600 (100)</td>
<td>60 (&lt; 0.8)</td>
<td>—</td>
<td>&lt; 50 (&lt; 0.7)</td>
</tr>
<tr>
<td>Urea, 6 M</td>
<td>9</td>
<td>500 (6.6)</td>
<td>&lt; 50 (&lt; 0.7)</td>
<td>—</td>
<td>&lt; 50 (&lt; 0.7)</td>
</tr>
</tbody>
</table>

Expt 2

<table>
<thead>
<tr>
<th>Additive</th>
<th>pH</th>
<th>0°</th>
<th>60</th>
<th>150</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7</td>
<td>7000 (100)†</td>
<td>—</td>
<td>—</td>
<td>480 (6.9)</td>
</tr>
<tr>
<td>NaSCN, 2 M</td>
<td>7</td>
<td>6590 (94.1)</td>
<td>—</td>
<td>5980 (85.4)</td>
<td>3660 (52.3)</td>
</tr>
<tr>
<td>NaI, 2 M</td>
<td>7</td>
<td>7110 (101.6)</td>
<td>—</td>
<td>3720 (53.1)</td>
<td>1490 (21.3)</td>
</tr>
<tr>
<td>NaCl, 2 M</td>
<td>7</td>
<td>6600 (94.3)</td>
<td>—</td>
<td>3360 (48.0)</td>
<td>1000 (14.3)</td>
</tr>
<tr>
<td>NaClO₄, 2 M</td>
<td>7</td>
<td>7200 (102.9)</td>
<td>—</td>
<td>1820 (26.0)</td>
<td>910 (13.6)</td>
</tr>
<tr>
<td>NaBr, 2 M</td>
<td>7</td>
<td>8960 (128.0)</td>
<td>—</td>
<td>4030 (57.6)</td>
<td>1070 (15.3)</td>
</tr>
<tr>
<td>NaNO₃, 2 M</td>
<td>7</td>
<td>11750 (167.9)</td>
<td>—</td>
<td>3390 (48.4)</td>
<td>520 (7.4)</td>
</tr>
</tbody>
</table>

* Sample held for 24 h at 4 °C before heating.
† Figures in parentheses show the residual activity as % of unheated control without additive.

RESULTS

Effects of protein denaturants and chaotropic salts on stability

The stability of murine interferon was tested in the presence of urea, GuHCl and chaotropic salts – agents that break the hydrogen-bonded structure of proteins (Table 1). After 24 h exposure to urea at 4 °C, interferon lost 70% to 93% of its original activity. The interferon activity remaining after heating at 68 °C for 240 min at pH 2 was twofold greater in the presence of urea than in its absence. Thus, although urea decreased the original activity of interferon at pH 2, it enhanced the stability of the residual interferon during subsequent heating at this pH. However, urea did not protect interferon during heating at pH 9, possibly due to the decomposition of urea to cyanate upon heating at high pH (Tanford, 1968). Some chaotropic ions had greater protective effects on the heat stability of interferon than others. Treatment of interferon with these salts for 24 h at 4 °C and pH 2 or pH 7 or pH 9 did not significantly alter activity. When subsequently heated at 68 °C for 240 min, the control interferon sample at pH 7 retained < 1% of its original activity as compared to 52% for NaSCN, 21% for NaI, 14% for NaCl, 13% for NaClO₄, 15% for NaBr and 7% for NaNO₃. Thus, in terms of their effectiveness in stabilizing interferon against heat inactivation, these chaotropic ions ranked in the order: SCN⁻ > I⁻ > Cl⁻ = ClO₄⁻ = Br⁻ > NO₃⁻.

The stabilizing effect of some chaotropic salts at pH values 2 and 9 is seen in Fig. 1. Samples of interferon were first treated for 24 h at pH 2 or pH 9 and 4 °C, and this did not alter their activity. At pH 2 the residual interferon activity after 240 min at 68 °C was 10% for the control, 25% for NaSCN and 5% for NaI, but less than 1% for NaNO₃ (Fig. 1a). At pH 9, the residual interferon activity after 240 min at 68 °C was < 1% for the control, 4% for NaSCN, 2.2% for NaI, and < 1% for NaNO₃ (Fig. 1b). NaSCN increased the stability of interferon at pH 9 but not at pH 2. At pH 7, the stability of heated interferon increased as the NaSCN concentration was increased from 0.25 M to 2.0 M (Fig. 2).
Fig. 1. Stability at 68°C of partially purified interferon in 2 M-chaotropic salts at (a) pH 2 and (b) pH 9. ○ — ○, Control; ●—●, NaSCN; ▲—▲, NaI; Δ—Δ, NaNO₃.

Fig. 2. Stability at 68°C of partially purified interferon as a function of NaSCN concentration. Salt molarity: □ — □, 2 M; ■—■, 1·5 M; Δ — Δ, 1 M; ▲—▲, 0·5 M; ●——●, 0·25 M; ○——○, control.

In addition to stabilizing the L cell interferon in liquid preparations, NaSCN also enhanced the stability of freeze-dried interferon. Interferon prepared in 0·5 M-NaSCN at pH 7 was freeze-dried and heated in the LNS test at a rate of 1·5 °C/h, followed by cooling to room temperature. After progressive heating to 85°C, the sample in NaSCN retained 27·2% of its original activity as compared to 2·2% for the control.
Thermal stability of mouse interferon

Table 2. The effect of GuHCl and heavy water (D₂O) on the heat stability of partially purified L cell interferon in the linear non-isothermal stability test

<table>
<thead>
<tr>
<th>Additive</th>
<th>pH</th>
<th>Interferon activity (units/ml)</th>
<th>60 °C</th>
<th>80 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>before heating*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2</td>
<td>4900 (100)†</td>
<td>1950 (39:8)</td>
<td>230 (47)</td>
</tr>
<tr>
<td>GuHCl, 4 M</td>
<td>2</td>
<td>640 (13:1)</td>
<td>225 (4:6)</td>
<td>90 (18)</td>
</tr>
<tr>
<td>Heavy water (D₂O), 50 %</td>
<td>2</td>
<td>3900 (79:6)</td>
<td>3900 (79:6)</td>
<td>740 (15:1)</td>
</tr>
<tr>
<td>None</td>
<td>9</td>
<td>3800 (100)</td>
<td>95 (2:5)</td>
<td>&lt; 50 (&lt; 1:3)</td>
</tr>
<tr>
<td>GuHCl, 4 M</td>
<td>9</td>
<td>900 (23:0)</td>
<td>225 (5:9)</td>
<td>90 (2:4)</td>
</tr>
<tr>
<td>Heavy water (D₂O), 50 %</td>
<td>9</td>
<td>345 (9:1)</td>
<td>&lt; 50 (&lt; 1:3)</td>
<td>&lt; 50 (&lt; 1:3)</td>
</tr>
</tbody>
</table>

* Sample held for 24 h at 4 °C.
† Figures in parentheses show the residual activity as % of unheated control with no additive.

Another protein denaturant, GuHCl, was effective in stabilizing residual interferon activity against heat inactivation in the LNS test, but the large loss of activity, before heating, 77 to 87 % after 24 h at 4 °C (Table 2), limits its usefulness as a stabilizing agent. However, the residual activity appeared more resistant to heating in the presence of GuHCl than in its absence. For example, after being heated progressively to 60 °C, interferon in GuHCl at pH 9 maintained 25 % of the residual activity present before heating as compared to 2:5 % in the absence of GuHCl.

The effect of heavy water

Heavy water (deuterium oxide, D₂O) can strengthen hydrogen bonds of proteins or alter their hydrophobic interactions (Wiberg, 1955; Aleksandrov et al. 1966). Interferon prepared in 50 % D₂O and kept at 4 °C for 24 h, showed no significant change in activity at pH 2 before heating (Table 2), whereas 91 % of the original activity of interferon was lost at pH 9. When interferon in D₂O at pH 2 was progressively heated to 60 °C in the LNS test, it lost virtually none of its activity, but it lost 60 % of its activity in the absence of D₂O. At pH 9, D₂O did not stabilize interferon during heating.

Effect of NaSCN on Stokes' radius and/or mol. wt. of interferon

When L cell interferon was chromatographed on a Sephadex G-100 column at pH 7, its activity was recovered in a single peak corresponding to an apparent mol. wt. of 25000. However, on a pH 7 column containing 1-5 M-NaSCN, 90 % of its activity was recovered on a single peak in the region of mol. wt. 42000 (Fig. 3). Thus, the apparent mol. wt. and/or Stokes’ radius of mouse fibroblast interferon increased in the presence of NaSCN.

DISCUSSION

An analysis of environmental factors affecting the thermal inactivation of mouse fibroblast interferon showed that acid conditions, i.e. pH 2 to pH 4 (which labilize most proteins) stabilized interferon, apparently by unfolding it and increasing its mol. wt. or Stokes’ radius (Jariwalla et al. 1975). The present study further substantiates this finding by demonstrating that interferon is protected against inactivation by heat in the presence of reagents, such as GuHCL or urea, which can unfold globular proteins. Low pH can deform the higher (secondary, tertiary or quaternary) orders of protein structure, primarily by affecting
Fig. 3. Molecular sieve chromatography of partially purified L cell interferon through a column of Sephadex G-100. (a) Interferon (1 x 10⁶ units in 1 ml) was applied to a 72 x 1.5 cm column in 0.1 M-sodium phosphate, pH 7, at 4°C. Recovery of interferon activity was about 60%. (b) Interferon (4.5 x 10³ units in 0.5 ml) was applied to a 53 x 1 cm column in 0.1 M-tris-HCl, 1.5 M-NaSCN, pH 7, at room temperature. Recovery was about 90% of interferon activity.

electrostatic interactions. Thus, the observations that interferon was also stable to heating in solutions of other deforming agents, such as NaSCN, urea or GuHCl, suggested that conformational state(s) of interferon resistant to thermal inactivation may be generated. Alternatively, since denatured proteins are commonly aggregated (Joly, 1965), these agents, which also act to dissociate proteins (Tanford, 1968; von Hippel & Schleich, 1969), may stabilize interferon by preventing aggregation during heating.

Data obtained from molecular sieve chromatography show that mouse L cell interferon in neutral 1.5 M-NaSCN has an apparent mol. wt. of 42,000, an increase from the 25,000 measured in neutral, low molarity solutions. A similar shift in apparent mol. wt. occurs at pH 2 (Jariwalla et al., 1975). Since acid pH and molar NaSCN act as strong dissociating agents, it is unlikely that the 42,000 mol. wt. species of interferon resulted from aggregation.

Certain globular proteins containing disulphide bonds, such as lysozyme, ribonuclease, and soybean trypsin inhibitor, are resistant to denaturation at acid pH as measured by optical rotation (Tanford, 1968; Wang & Kassell, 1974). In addition to disrupting the hydrogen-bonded structure of these proteins (Lumry & Eyring, 1954), urea and GuHCl may also weaken their hydrophobic interactions, either directly, resulting in the subsequent hydration of the protein molecule (Haschemeyer & Haschemeyer, 1972), or indirectly, by exerting an effect on the hydrogen-bonded structure of water, similar to the so-called lyotropic or Hofmeister-type effect produced by high concentrations of chaotropic salts (Hammes & Schwann, 1967; von Hippel & Schleich, 1969). Since chaotropic salts also affect hydrophobic interactions, proteins in solutions of these salts acquire a cross-linked, partially unfolded conformation which is not a firmly cross-linked random coil (Tanford, 1968; von Hippel & Schleich, 1969). Thus, the degree of solvation and unfolding may affect the thermal stability of the interferon.
Very few studies have attempted to relate the physical properties of proteins undergoing denaturation and renaturation to their biological activities. Teipel & Koshland (1971a, b) studied the activity of enzymes undergoing denaturation and renaturation during treatment with GuHCl. Regaining of enzymatic activity correlated with the regain of native structure. Some polypeptide chains refolded within 1 min to an inactive structure similar, but not identical, to the conformation of the native enzyme, after which biological activity was slowly regained. Since the resulting amount of enzyme activity related to the proportion of polypeptide chains initially renatured correctly, the energy states in the environment during the cooling phase after thermal denaturation of interferon may determine the residual biological activity. Normal (i.e. relatively slow) cooling may allow time for the protein to refold during renaturation in its most stable, or at least its most biologically active, conformation (Jariwalla et al. 1975).

How D2O enhances the stability of interferon at pH 2 is not clear. It is known that the exchangeable H atoms in proteins are rapidly replaced by deuterium, which forms bonds having greater strength than the hydrogen bond (Wiberg, 1955; Aleksandrov et al. 1966). Protonation of interferon at pH 2 may favour greater substitution of deuterium for hydrogen and/or a lower energy state, requiring the addition of more calories to alter or break the hydrogen bonds. In addition, replacing H2O with D2O alters several properties of the solvent, including the structure of water and the dissociation constants of substances dissolved in it, thereby influencing bonds maintaining the higher order structures of protein molecules.

A model which accounts for various observations during thermal inactivation of mouse fibroblast interferon postulated that the molecule may unfold on prolonged heating and refold to an inactive conformation on cooling (Jariwalla et al. 1975), and that during the cooling at pH 2, a significant fraction of molecules having the unfolded conformation may refold correctly, accounting for their thermal stability. We suggest that this same model may be applied to the observations reported here. Thus, a large fraction of those molecules having extended conformations in a chaotropic salt may refold during cooling and removal or dilution of the salt to the conformation active at pH 7; only a small fraction of those having an unfolded conformation in GuHCl or urea may refold similarly, accounting for the lower residual activities after treatment with these protein denaturants. The direct evidence for such conformational changes could come from measurements by circular dichroism, which will require large amounts of interferon purified to homogeneity.

The studies reported herein demonstrate for the first time that chaotropic salts can stabilize a protein against thermal inactivation. Since these salts can weaken intermolecular or intramolecular hydrophobic interactions of macromolecules (Hatefi & Hamstein, 1969), they may be of use in the elution of interferon bound to immobilized ligands. We have recently applied this principle to the elution of human fibroblast interferon linked non-covalently to concanavalin A-Sepharose (Jariwalla, Sedmak & Grossberg, 1976).

REFERENCES


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