Human Leukocyte Interferon: a Role for Disulphide Bonds

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SUMMARY

Some activity (1 to 10%) was recovered from partially purified leukocyte interferon which had been reduced by mercaptoethanol and allowed to oxidize in air. The recovery was complete if the reduced interferon was unfolded by guanidine hydrochloride or urea. Oxidation in the continued presence of these denaturants lead to incomplete recovery (3%). Carboxymethylation of reduced interferon permanently destroyed all activity. Sodium dodecyl sulphate did not cause any loss of interferon activity but did hinder successful re-oxidation in the presence of urea. The prime importance of at least one disulphide bond in interferon structure is indicated. Further confirmation was obtained by oxidative cleavage of disulphide bonds. The lack of effect of p-chloro-mercuribenzoate suggests that free thiol groups are not important for antiviral action.

INTRODUCTION

Human leukocyte interferon is now available in sufficient quantity for clinical studies (Merigan et al. 1973; Strander et al. 1973) and may be concentrated to high potency (Cantell & Pyhälä, 1973) and partially purified to high specific activity (Fantes, 1970; K. Cantell, S. Hirvonen & S. Graff, unpublished observations). Although such material is still impure, we believe that an investigation of those physical and chemical factors that clearly affect antiviral activity can provide useful insights into the structure of interferon. Fantes & O'Neill (1964) attempted a comprehensive study of the effects of group specific reagents on partially purified preparations of chick interferons. Treatments known to cause scission of protein disulphide bonds led to an irreversible loss of interferon. Merigan, Winget & Dixon, (1965) confirmed these results for chick interferon but Ke & Ho (1968) found limited adverse effect of such reagents upon rabbit interferon. Marshall, Pitha & Carter (1972) reported loss in the activity of human interferons with concentrations of reagent that are known to catalyse sulphhydryl–disulphide exchange (Jensen, 1959; Smithies, 1965). The present study offers detailed evidence that the disulphide bond plays an important part in the structure of human leukocyte interferon.

METHODS

Production and assay of interferon. Leukocytes were purified from ‘buffy coats’ (Strander & Cantell, 1966) and, after priming, induced with Sendai virus (100 H.A.U./ml, 10⁷ cells/ml) in the presence of human serum from which most of the globulins had been removed by ammonium sulphate as described by Cantell & Pyhälä (1973). Threefold serial dilutions of
interferon were titrated by the reduction of vesicular stomatitis virus plaques on U (human amnion) cells (Strander & Cantell, 1966). All assay results are given in terms of the unit assigned to the research preparation H 69/19 (International Symposium on the Standardization of Interferon and Interferon Inducers, London, 1969). Estimates of precision indicate that threelfold differences in activity lie close to the 95% confidence limits and tenfold differences exceed the 99% limits.

**Purification.** Batches of crude interferon were concentrated by potassium thiocyanate precipitation at pH 3.5 (Cantell & Pyhälä, 1973). Dissolved in 94% ethanol at pH 4.2, inert protein was selectively precipitated and removed as the pH was raised (K. Cantell et al. unpublished observations). Interferon was recovered from protein precipitated between pH 6 and 7. The method routinely yields interferon of specific activity 1 to 6 × 10^6 units/mg of protein (Lowry et al. 1951). Such preparations were dialysed against phosphate-buffered saline, pH 7.3 (PBS), and centrifuged at 25,000 g for 60 min before further use. Unless otherwise stated, all experiments were performed with such interferon; and repeated with different batches of it.

**Reagents and reactions.** Interferon was treated in rubber-stoppered glass tubes, with reagents under specified conditions (given in parentheses for each reagent). After reaction the samples were exhaustively dialysed against PBS and then assayed. No attempts were made to exclude air during reaction or dialysis.

Guanidine hydrochloride (guanidine HCl; Fluka, puriss.) was dissolved to give a clear colourless solution. To obtain an 8 M solution, the solid was dissolved directly in the reaction mixture (with attendant twofold increase in vol.). For lower concentrations, dilutions were made from an 8 M stock.

Urea (Baker, recrystallized) was dissolved directly in reaction mixtures to give final concentrations in the range 8 to 10 M. At these concentrations it was as effective as 5 M-guanidine HCl.

2-Mercaptoethanol (Fluka, puriss.) was used to reduce disulphide bonds. It was estimated, qualitatively, by spotting on to a ground mixture of 1 part sodium nitro-prusside and 2 parts anhydrous sodium carbonate. The absence of purple colour was taken to indicate the absence of mercaptoethanol during carboxymethylation. Re-oxidation of mercaptoethanol-reduced disulphide bonds was encouraged during dialysis. The liquid vol. was less than 1/10 of the total vol. of the dialysis bag (27/32; Arthur H. Thomas Co., Philadelphia, USA) and dialysis was continued for at least 48 h (Anfinsen et al. 1961).

Iodoacetamide (Fluka, purum) was added to reaction mixtures as a solid. The pH of the reaction was kept above 8.5 by the addition of 2 N-NaOH. Carboxymethylation and the subsequent dialysis were always carried out in the dark and the solutions remained colourless.

SDS was recrystallized from 95% ethanol and dissolved in appropriate buffers free of potassium salts.

p-Chloromercuribenzoate (sodium salt; Fluka) was dissolved in 0.05 M-glycine/NaOH, pH 8.5, to give a clear 20 mM solution.

Performic acid (1/20 mixture of 30% hydrogen peroxide in 98% formic acid, that had stood for 2 h at 1 °C) was added to an equal vol. of an interferon solution (0.05 mg/ml of protein in 0.1 M-phosphate, pH 7.0). After 2 h at 1 °C, reaction was terminated by dilution into, and dialysis against, 0.1 M-phosphate buffer, pH 7.0.
Disulphide bonds in interferon

RESULTS

Purity and heterogeneity

The results of cellulose acetate strip electrophoresis (Fig. 1) show that even our purest preparation (specific activity 10 million units/mg protein) is still not free of inert protein and that interferon is associated with proteins of different electrophoretic mobilities. Such studies suggest that a significant contaminant is human albumin. The lipid and carbohydrate content of these preparations has not been investigated.

Cleavage of disulphide bonds in the presence of guanidine HCl

Guanidine HCl is an unfolding agent for proteins (Tanford, 1968). It has no irreversible effect on interferon under any of the conditions reported here. We observed an absence of effect which is independent of pH (between 1 to 13), time of incubation and temperature, below 56°C (e.g. 7·2 M-guanidine HCl, pH 5·1, 56°C, 3 days). We have found (Mogensen & Cantell, 1973) that partially purified human leukocyte interferon is exceptionally stable towards heat and can, for example, survive 3 days at 56°C (pH 7·2) without loss of activity.

Fig. 2 shows the effect of guanidine HCl on the inactivation of interferon by mercaptoethanol. As the concentration of guanidine HCl increases from 2 to 5 M, the inactivating effect of mercaptoethanol disappears. Above 5 M-guanidine HCl, it is not possible to tell whether mercaptoethanol was without effect or if such an effect was entirely reversible. The
curve obtained is, however, typical for the effect of guanidine HCl on proteins (Tanford, 1968) and therefore implies that the result is a consequence of protein unfolding. Table I shows that the response described in Fig. 2 is reproducible for the range of pH over which thiol groups are ionized. At pH 1.8 thiols are practically unionized and the reduction of disulphide bonds should be minimal (Haber & Anfinsen, 1962). Thus the insensitivity of interferon to mercaptoethanol at pH 1.8 supports the contention that disulphide bonds in interferon can be reduced and re-oxidized again. Incubation with mercaptoethanol in the presence of 5 to 8 M-guanidine HCl has been carried out some 20 times between pH 4 and 7 and has never led to inactivation; 8 to 10 M-urea proved to be just as effective as 5 M-guanidine HCl. These results suggest that unfolding is necessary either for the complete cleavage of disulphide bonds and/or for their correct recombination.
Table 2. Carboxymethylation by iodoacetamide (IA) of a potent interferon reduced by 2-mercaptoethanol (ME), in the presence of guanidine HCl

<table>
<thead>
<tr>
<th>First treatment*</th>
<th>Second treatment*</th>
<th>Interferon (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>2000000</td>
</tr>
<tr>
<td>ME (0.2 M, pH 5.0, 37 °C, 24 h)</td>
<td>None</td>
<td>2000000</td>
</tr>
<tr>
<td>ME (0.2 M, pH 5.0, 37 °C, 24 h)</td>
<td>IA (0.25 M, pH 9.0, 25 °C, 15 min)</td>
<td>600</td>
</tr>
<tr>
<td>ME (0.2 M, pH 5.0, 37 °C, 24 h)</td>
<td>IA (0.25 M, pH 9.0, 25 °C, 90 min)</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>IA (0.25 M, pH 9.0, 25 °C, 15 min)</td>
<td>ME (0.2 M, pH 9.0, 25 °C, 90 min)</td>
<td>200000</td>
</tr>
</tbody>
</table>

* 7 to 8 M-guanidine HCl present.

Carboxymethylation of reduced disulphide bonds

To confirm that essential disulphide bonds were being reduced by mercaptoethanol, we attempted to prevent re-oxidation by treating the reduced interferon with iodoacetamide under conditions that should lead to the complete carboxymethylation of all cysteine residues. To avoid spontaneous oxidation, mercaptoethanol was left in contact with interferon and iodoacetamide was added in an excess, sufficient to carboxymethylate all the mercaptoethanol (nitroprusside test). The results in Table 2 show that this reaction caused complete and irreversible inactivation of interferon, with a measured decline in excess of 5 log units of activity. Furthermore, the reaction is complete within 90 min and the kinetics therefore suggest carboxymethylation of cysteine rather than other susceptible amino acids (Gurd, 1967). We were not able to demonstrate any effect with p-chloromercuribenzoate (<0.1 M) on interferon, further suggesting that free thiol groups are not an important feature.

Table 3 shows that the addition of guanidine HCl, long after mercaptoethanol was first added, can effect the complete recovery of interferon. If, however, the addition of guanidine HCl was deferred until after iodoacetamide treatment, then no further activity could be recovered. Fig. 3 demonstrated clearly that the unfolding effect of guanidine HCl is not necessary for the successful reduction of interferon by mercaptoethanol.

These studies with iodoacetamide indicate that at least one essential disulphide bond is easily exposed to mercaptoethanol in these preparations, and that complete unfolding of the protein is necessary only for the successful reoxidation and not for reduction.

Performic acid oxidation

Reduction is not the only means of cleaving disulphide bonds. Performic acid (Hirs, 1967) will achieve the same with resultant oxidation of exposed cysteine pairs and methionine side-chains. While the reaction is not completely specific for these residues, the conditions employed should minimize oxidation of other amino acids (Hirs, 1967). The results in Table 4 show that performic acid causes a permanent loss of interferon exceeding at least 3 log units of activity. Interferon does not appear to be affected by hydrogen peroxide, which will oxidize methionine by itself. Thus the loss in activity caused by performic acid may be attributed mainly to the oxidation of disulphide bonds, and serves to confirm their importance to interferon.

Re-oxidation of interferon

Table 5 shows that, while dialysis against PBS results in successful oxidation and full recovery, oxidation in the presence of 8 M-urea results in lowered recovery (3 %, 72 h); as expected, the loss is reduced at acid pH (Haber & Anfinsen, 1962). Table 6 shows that, while SDS (0.1 M) is normally without effect on interferon, it does hinder recovery during
oxidation of the reduced form in the presence of urea (the guanidinium salt of dodecyl sulphate is insoluble). The binding of SDS to proteins is very strong (Foster, 1960; Tanford, 1968) and is not reversed by dialysis. That SDS interacts with interferon may be inferred from its protective effect at 70 °C (Table 6). These results suggest that once the disulphide bonds have formed correctly, the secondary and tertiary structure of interferon molecules in solution may be of little consequence.
Table 4. The effects of performic acid, formic acid and hydrogen peroxide on interferon at 1 °C for 2 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Interferon (units/ml) recovered after dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20000</td>
</tr>
<tr>
<td>Performic acid</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>Performic acid and Gu. HCl (4 M)</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>Formic acid (98 %)</td>
<td>6000</td>
</tr>
<tr>
<td>Hydrogen peroxide (1·5 %)</td>
<td>11000</td>
</tr>
</tbody>
</table>

Table 5. Re-oxidation of reduced interferon* in the presence of urea at pH 7·3 and 2·5

<table>
<thead>
<tr>
<th>Time of dialysis† of reduced interferon in 8 M-urea (h)</th>
<th>Interferon (units/ml) recovered after dialysis in 8 M-urea:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7·3</td>
</tr>
<tr>
<td>0</td>
<td>20000</td>
</tr>
<tr>
<td>24</td>
<td>3500</td>
</tr>
<tr>
<td>48</td>
<td>1100</td>
</tr>
<tr>
<td>72</td>
<td>600</td>
</tr>
<tr>
<td>pH 2·5</td>
<td></td>
</tr>
<tr>
<td>20000</td>
<td></td>
</tr>
<tr>
<td>3500</td>
<td></td>
</tr>
<tr>
<td>1100</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td></td>
</tr>
</tbody>
</table>

* Interferon (20000 units/ml)+urea (10 M)+mercaptoethanol (0·2 M, pH 7·3, 37 °C, 24 h).
† 50 vol. of fresh 8 M-urea every 24 h.

Table 6. The effect of sodium dodecyl sulphate (SDS) on interferon (60000 units/ml) and its reduction by 0·2 M-mercaptoethanol (ME)

<table>
<thead>
<tr>
<th>Conditions of treatment with SDS</th>
<th>Interferon (units/ml) recovered after treatment with SDS at pH 4·5</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 °C, 2 h</td>
<td>35000</td>
</tr>
<tr>
<td>37 °C, 24 h</td>
<td>60000</td>
</tr>
<tr>
<td>37 °C, 24 h + ME</td>
<td>20000</td>
</tr>
<tr>
<td>37 °C, 24 h + ME + 9 M-urea</td>
<td>60000</td>
</tr>
</tbody>
</table>

DISCUSSION

To attempt structural studies on a substance which is probably less than 1 % pure (Ng & Vilček, 1972) is a hazardous undertaking. We have, necessarily, assumed that our interferon is a protein and that the reagents we use behave accordingly. While final proof must await the complete purification of human interferon, we feel that none of the results invalidate our assumptions.

It is important to emphasize that no single experiment in this study permits a distinction between the total absence of discernible effect and the existence of an effect that is totally reversible by a return to the initial conditions. Thus mercaptoethanol is only shown to be reversible when oxidation is prevented by carboxymethylation. We have exposed potent preparations of leukocyte interferon to various chemical and physical treatments (unpublished results). It is only when treatment may be expected to result in permanent cleavage of the original disulphide bonds that we are unable to demonstrate residual activity.

Fantes & O’Neill (1964) attempted, without success, to recover activity by bubbling air
through a solution of mercaptoethanol-reduced chick interferon. Merigan et al. (1965) were similarly unsuccessful, though they reported that air oxidation would lead to a gain in activity if reduction was incomplete. Our results suggest that reduction by mercaptoethanol is completely reversible when non-covalent structures have been reduced to a minimum. Anfinsen et al. (1961) were the first to establish that re-oxidation under such conditions could lead to the recovery of the native form of a protein. Their work on ribonuclease suggested that all the information for the higher orders of protein structure reside in the primary amino acid sequence.

As the purification of interferon involves treatment with 94% ethanol; and as SDS has no adverse effect on interferon, it seems probable that non-covalent bonds are not important if the disulphide bonds are intact. Albumin is a significant contaminant in our interferon preparations (Fig. 1). Of all proteins, it is particularly likely to participate in sulphydryl–disulphide interchange reactions as it possesses a sulphhydryl group as well as many disulphide bonds (Foster, 1960). Furthermore such activity is often enhanced by denaturing conditions (Jensen, 1959). It is therefore remarkable that interferon disulphide bonds either do not engage in, or are unaffected by, such exchanges; particularly as they are so readily accessible to mercaptoethanol.

The results presented here lead us to propose that the integrity of at least one disulphide bond in the interferon molecule is an essential feature of its antiviral activity. The stability of interferon towards denaturation, when this bond is intact, suggests that it may lie close enough to an ‘active site’ to counteract the destruction or alteration of the non-covalent bonds.

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REFERENCES


Disulphide bonds in interferon


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