Reagents which Inhibit Disulphide Bond Formation Stabilize Human Fibroblast Interferon

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SUMMARY

Fibroblast interferon may be stabilized against many inactivating influences by the addition of certain simple sulphhydryl reagents. The use of such easily removable and relatively non-toxic stabilizers should help in the preparation and purification of fibroblast interferon for clinical use.

The purification and characterization of interferon derived from cultured human embryo fibroblasts (FIF) have been seriously hampered by its unusual lability. FIF, unlike that derived from either leucocytes or lymphoblasts is rapidly inactivated when subjected to ultrafiltration, salt precipitation, chromatography, sterilizing filtration, or heating. Treatment with such common stabilizers as glycerol or serum albumin does not always improve the interferon yield, particularly when interferon is inactivated by mechanical stress, to which it is particularly sensitive (De Somer et al. 1973; Edy et al. 1974). We have used a mechanical stress system to study the inactivation of interferon, since this seems more relevant to the inactivation occurring during purification than other methods, such as heating, commonly used to study protein inactivation.

Crude and purified FIF were prepared, assayed and subjected to mechanical inactivation as described in the preceding paper.

Investigation of the effect of sulphhydryl reagents on FIF showed that N-acetyl cysteine had a marked ability to inhibit shear-induced inactivation of interferon and greatly improved the yield of interferon concentrated by ultrafiltration. The effect was strongly concentration-dependent and only became maximal at 100 mM. We postulated that N-acetyl cysteine acts by inhibiting disulphide bond formation by maintaining the equilibrium in favour of keeping the sulphhydryl groups of the protein in a reduced condition. This led us to examine the stabilizing effect of other thiols, particularly those known to be more potent reducing agents than N-acetyl cysteine and which might thus be expected to stabilize interferon at much lower concentrations. This simple prediction was not entirely fulfilled. Some potent reducing agents gave complete protection at lower concentration as predicted (e.g. dithiothreitol at 10 mM) while others (e.g. mercaptoethanol and dimercaptopropanol at 0.1 to 1.0 mM) not only failed to protect interferon from shear, but actually inactivated unheared interferon samples. To summarize our findings: reduced glutathione, thioethanolamine, thiodiglycol, thioacetic acid, monothioglycerol and cysteine all produced some stabilization at 100 mM. N-acetyl cysteine produced complete stabilization at 100 mM as did dithiothreitol at 10 mM. The best thiol stabilizer found was DL-thioctic acid which stabilized interferon well at concentrations as low as 0.1 mM and almost completely at 1.0 mM (Fig. 1a).

Further evidence that thiol stabilizers function by influencing the equilibrium between protein sulphhydryl groups and disulphide bonds is derived from the reversibility of the phenomenon. The thiol reagents may be simply and completely removed by dialysis. When this is done, previously stabilized interferon reverts to its original labile condition. The process may be repeated by re-adding the thiol reagent and again producing enhanced stability.
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Fig. 1. Stabilization of FIF by thiol reagents. (a) Thiocetic acid (1·0 mM): FIF samples were subjected to shear inactivation in its presence (○—○) or absence (△—△). (b) p-Chloromercuribenzoate (0·1 mM): FIF was treated with 0·1 mM-p-chloromercuribenzoate for 1 h to block accessible sulphydryl groups. Samples were then subjected to shear inactivation either with (●—●) or without (○—○) previous exhaustive dialysis against phosphate-buffered saline. An untreated control FIF preparation was included for comparison (△—△).

of the interferon preparation. Thus, a FIF preparation which lost 95 % of its activity during 15 min shearing was completely protected by the addition of 100 mM-N-acetyl cysteine. Removal of the stabilizer by dialysis resulted in 93 % loss of interferon activity during a further shear stress experiment, but addition of the thiol at 100 mM again gave complete stabilization. Similar results were obtained with the other thiol stabilizers investigated.

If interferon is protected by preventing the formation of disulphide bonds, it should be possible to produce an equivalent effect with reagents which covalently block protein sulphydryl groups. In a preliminary study this approach has given mixed results. Interferon preparations were treated with either 1·0 mM-p-chloromercuribenzoate or 1·0 mM-iodoacetate, compounds capable of blocking protein sulphydryl groups irreversibly. They were then dialysed extensively to remove excess blocking reagent, subjected to shear inactivation, and then assayed for antiviral activity. Treatment with p-chloromercuribenzoate had no effect on interferon potency before shearing and significantly stabilized the interferon against shear inactivation. As predicted by the postulated mechanism, this stabilization was not reversible by dialysis (see Fig. 1b). Iodoacetate treatment could not be investigated
Fig. 2. Effects of thiol reagents on the thermal inactivation of FIF at 56 °C. ○—○, Unheated control; △—△, FIF heated with no additives; △—△, FIF heated + N-acetyl cysteine (100 mM); ●—●, FIF heated + DL-thioctic acid (1 mM).

since, even after extensive dialysis, the treated interferon was toxic for the assay cells for reasons which are not clear.

The effect of thiol stabilizers on the inactivation of interferon samples by heating at 56 °C was also studied. In this system they did not protect interferon at all and, indeed, accelerated its inactivation. Interestingly, those reagents which protected most effectively against shearing had the greatest de-stabilizing effect during heating (see Fig. 2).

Similarly, the addition of thioctic acid to interferon stored at 37 °C accelerated its rate of decay. With material stored at 4 °C, however, this was not the case. In a typical experiment, purified FIF (spec. act. \(5 \times 10^7\) units/mg of protein) declined in activity by 90 % after 14 weeks storage at 4 °C. In contrast, thioctic acid treated FIF showed no significant loss over this period.

In view of these results, we now routinely stabilize our interferon preparations with DL-thioctic acid during processing. Although thioctic acid normally gives good protection at 0·1 mM, for consistent results we use it at 1·0 mM. This treatment has raised recovery of FIF to nearly 100 % during the processes of ultrafiltration, salt precipitation, density gradient centrifugation, column chromatography, sterile filtration and ampoule filling. Additionally it enhances stability on storage at 4 °C.

Fantes & O’Neill (1964) and Schonne (1966) concluded that interferon contained no sulphydryl groups necessary for the development of the antiviral activity, and Merigan, Winget & Dixon (1965) showed that interferon contained an essential disulphide bond or bonds whose reduction resulted in destruction of antiviral activity. Superficially our results may appear to be at variance with these conclusions, but they are, in fact, entirely consistent. Our thiol reagents and sulphydryl blockers had no influence on the antiviral activity of static interferon samples suggesting that the sulphydryl groups in interferon contribute nothing to its activity. However, if the interferon was subjected to mechanical inactivation, these reagents dramatically reduced the resulting activity loss. We conclude that interferon does indeed contain sulphydryl groups which play a part in the sequence of events leading to interferon inactivation. Since both sulphydryl blocking agents and competing thiols generally reduce interferon inactivation, the inactivation process probably proceeds via the formation
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of disulphide bonds. It remains to be established whether these are intrachain bonds resulting in the stabilization of inactive interferon conformations, or interchain bonds, producing either aggregates of interferon or complexes between interferon and other proteins in the mixture, or a mixture of both types. The critical requirement for an effective sulphydryl stabilizing agent is that it should react selectively only with these reactive sulphydryl groups of interferon and that it should not cause reduction of the essential disulphide bonds in the interferon molecule. Such involvement of disulphide bond formation in cases of activity loss following mild denaturing treatments has been described in several systems (e.g. Warner & Levy, 1958; Tanford, 1968).

Arrangement of the thiols in order of their reducing power or of the pK of the sulphydryl group gives no indication of their effectiveness. Thus, diithiothreitol, a relatively powerful reducing agent is more effective than the weakly reducing N-acetyl cysteine. On the other hand, the weakly reducing DL-thioctic acid is more effective as a stabilizer than the potent diithiothreitol. The strong reducing agent mercaptoethanol not only fails to protect FIF but actually inactivates it. The effectiveness of a reducing agent may depend on the accessibility of the relevant sulphydryl groups to the agent: mercaptoethanol may readily penetrate into the interferon molecule and disrupt all disulphide bonds while larger molecules such as diithiothreitol may initially act only on the more accessible sulphydryl groups involved in interferon inactivation.

This interpretation is supported by the finding that the thiols which protected interferon against shear inactivation accelerated its inactivation by heating at 56 °C. The conformational changes induced in interferon at this temperature may permit the thiol reagents to gain access to and then reduce the critical disulphide bonds of the molecule, thus facilitating further thermal denaturation (Davidson & Hird, 1967). Similarly, gradual penetration of the molecule may account for the shortened shelf life at 37 °C found in the presence of these reagents.

As in all work of this kind where extrapolations are made from the behaviour of a single component in a complex mixture, it should be pointed out that pure interferon might not behave in the same way in isolation as it does in the mixtures which we have studied. It is possible that the effects reported here depend on the interactions between interferon and other substances present in the system and that sulphydryl reagents exert their primary effect on some molecule other than interferon. The fact that the same effects were observed with crude interferon of spec. act. $5 \times 10^4$ units/mg and with material purified by affinity chromatography (spec. act. in excess of $5 \times 10^7$ units/mg) tends to argue against this.

The practical value of these results is not trivial; we can now protect fibroblast interferon from denaturation during purification and other handling procedures by adding certain sulphydryl stabilizing agents. Their convenience, low active concentration, and low toxicity makes them attractive stabilizers for routine use in the preparation of human fibroblast interferon for clinical studies.

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