Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 74-80

CHEMICAL MODIFICATION OF THE RECOMBINANT HUMAN αA- AND β-INTERFERONS

Sergey I.Borukhov and Alexander Ya.Strongin*

Institute of Molecular Genetics, USSR Academy of Science,
Kurchatov square, 123182 Moscow, USSR

Received January 8, 1990

Chemical modification has been used to map the residues essential for the antiviral activity of the recombinant human αA- and β-interferons. Modification of His residues with diethylpyrocarbonate and Nα-tosyl-L-lysyl chloromethylketone does not inhibit both interferons, whereas Nα-tosyl-L-phenylalanyl chloromethylketone significantly suppressing the activity of β-interferon does not affect the activity of αA-interferon. After the modification of 1, 2 and 3 Lys residues from 11 ones with 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester αA-interferon reveals 100%, 50% and 10% of the initial activity, respectively. Modification of Trp residues with H_2O_2, 2-nitrobenzenesulfenyl chloride or 2-hydroxy-5-nitrobenzylbromide inactivates αA- and β-interferons completely. Presumably Trp residue(s) is essential for the antiviral activity of α- and β-interferons.

Several biological activities have been attributed to IFNs. They include antiviral, antiproloferative and immunoregulatory effects. The chemical characterization of IFNs is necessary to understand their action at the molecular level and to identify the functionally important amino acid residues within the IFN polypeptide chain. Site-directed mutagenesis of the corresponding structural genes and chemical modification of the proteins revealed the functional importance of Cys residues for IFN-αA and IFN-β (1-3). The residues, which determine the species specificity of IFN-αA and IFN-αD, have been identified by (4). Earlier it has been shown also that the modification of His residue inactivated β-IFN (5). The attempts of Wetzel et al. (6) and Fantes (7) to identify a role of Trp residues in the

*To whom correspondence should be addressed.

Abbreviation: IFN, interferon; DEPC, diethylpyrocarbonate; TLCK, Nα-tosyl-L-lysyl chloromethylketone; TPCK, Nα-tosyl-L-phenylalanyl chloromethylketone; SPDP, 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester.

0006-291X/90 $1.50

mechanism of IFN action have led to the controversial results, which await to be confirmed. The functional role of other residues within the polypeptide chains of IFN-α and IFN-β remains still undefined. In the present study we have used the chemical modification of His, Lys and Trp residues to determine the functional role of these particular residues in the antiviral action of IFN-αA and IFN-β.

MATERIALS AND METHODS

Electrophoretically pure IFN-αA (antiviral sp.act. 2×10^8 IU/mg) and IFN-β (antiviral sp.act. 5×10^7 IU/mg) have been isolated from recombinant Pseudomonas sp. and E.coli strains, respectively (8, 9). Monomeric form of IFN-αA was further separated from the S-S-oligomeric forms of the protein, as well as forms with non-reduced Cys residues by FPLC on a Mono Q column and gel-exclusion HPLC (10, 11). The concentration of the proteins was determined by the method of Spector (12), using bovine serum albumin as a reference.

IFN titers were determined by a cytopathic inhibition assay on human diploid fibroblast cell cultures using vesicular stomatitis virus, strain Indiana, as a challenge virus (13). The titers were calibrated against the international human leukocyte IFN reference MRC B69/19. Solid-phase ELISA and RIA were also used to measure the IFN concentration and its ability to react with the specific antibodies after the chemical treatment. Murine monoclonal antibodies (Celltech), rabbit polyclonal antibodies (Toray) and goat anti-rabbit polyclonal antibodies, conjugated with horseradish peroxidase, (Sigma) were applied for ELISA of IFN-β, whereas for RIA of IFN-αA murine monoclonal antibodies NK2 (Celltech) and [^{125}I]-labelled rabbit polyclonal antibodies were utilized (14).

SPDP (Pharmacia) was used for Lys residues modification according to (15). 100 μl of 0,1 M Na-phosphate buffer, pH 7,2, containing 100 μg of IFN-αA monomeric form, was mixed with 0,5-5 μl of 20 mM SPDP solution in ethanol, and after 40 min incubation at room temperature the reaction was terminated by the addition of the excess of lysine. Modified proteins were separated from the reagents by Sephadex G-25 gel-filtration. The number of modified Lys residues was estimated after the addition of 30 μl of 0,1 M dithiothreitol water solution (Sigma) to IFN solution and by measuring the A_{343} value. To plot a calibration curve SPDP samples with concentrations from 40 to 4000 μM were treated with 5 mM dithiothreitol /molar extinction coefficient at 343 nm = 8080 $M^{-1}x\ cm^{-1}$/(15).

Trp residues were modified by the treatment of IFN-αA and IFN-β (0,05-0,2 mg/ml in 0,7 ml 0,5 M Na-bicarbonate buffer, pH 8,4) with 77 μl of 10 mM H_2O_2 in distilled dioxane (16) at 20°C. After 1-180 min the aliquots were withdrawn from the reaction mixture and the modification was stopped by the addition of 50 mM tryptophan in the same buffer. The reaction was monitored by measuring the UV spectra of IFNs with Shimadzu UV-240 spectrophotometer. The number of modified Trp residues was determined by the decrease of $A_{282\ nm}$ value /molar extinction coefficient of oxidized Trp at 282 nm =3490 M^{-1} x cm^{-1}/. Trp residues in IFNs were also modified by the treatment with o-nitrophenylsulfenyl chloride and 2-hydroxy-5-nitrobenzyl bromide according to (17, 18).

For modification of His residues 50 μg of IFN-αA or IFN-β in 0,7 ml of 0,1 M Na-phosphate buffer, pH 6,1, was treated with 100 μl of DEPC (19), TLCK and TPCK (5) solutions in methanol or ethanol for 0,5-3 h at 20°C at a protein/reagent ratio 1:10-1:1000, mole/mole. The aliquots were withdrawn and the reaction was stopped by the addition of 10 μl of 0,1 M histidine solution to the reaction mixture, and then IFN activity was estimated after an appropriate dilution. The amount of His residues modified with DEPC in IFN-αA was determined according to the increase of $A_{242\ nm}$ of the reaction mixture /molar extinction coefficient of His-ethoxycarbonyl complex at 242 nm = 3200 $M^{-1} \times cm^{-1}$/(19).

RESULTS AND DISCUSSION

Lys residues of the monomeric SH-group free form of IFN-αA have been modified with a bifunctional reagent, SPDP. The insertion of one 3-(2-dithiopyridyl)-propionate moiety per IFN-αA molecule does not lead to any loss of antiviral activity, whereas the modification of 2 and 3 Lys residues suppresses the activity 2- and 10-fold, respectively (Fig. 1). This indicates, that 3-4 Lys residues from 11 ones within IFN-αA polypeptide chain are accessible for the reagent, being presumably located on the surface of protein globule. It should be noted that in the course of modification the ability of IFN-αA to react with specific monoclonal and polyclonal antibodies, as determined by RIA, decreases slowly in comparison with the loss of the antiviral activity of the protein. Presumably, some of the modified Lys residues are important for the binding of IFN-αA with cell surface receptor and displaying the antiviral activity of the protein. The results of RIA support this and show that the denaturation of IFN-αA does not occur in the course of the modification.

It has been reported that Cys_{17} is not essential for the antiviral activity of IFN-β (20). This feature of IFN-β as well as the ability of IFN-αA with one modified Lys to display full activity allows construction of the mixed cross-linked dimer IFN-αA(Lys)-SPDP-(Cys_{17})IFN-β, in which the second functional group of SPDP has been utilized for the binding with free SH-group of Cys_{17} of IFN-β. The dimer possesses high specific activity, as well as the ability to bind simultaneously monoclonal antibodies raised against IFN-β and polyclonal antibodies raised against IFN-αA (data not shown). This hybrid is of significant interest for studying and distinguishing the effects, characteristic for IFN-β and IFN-αA, and the corresponding research is in progress.

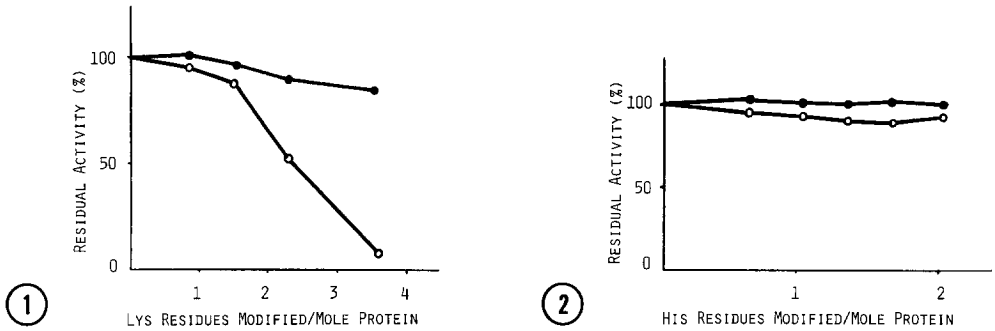

Fig. 1. Modification of Lys residues in IFN-αA with SPDP. -●- ability of IFN-αA to bind mono- and polyclonal antibodies as determined by RIA, -o- antiviral activity.

Fig. 2. Modification of His residues in IFN-αA with DEPC. -●- ability of IFN-αA to bind mono- and polyclonal antibodies as determined by RIA, -o- antiviral activity.

His residues of both IFNs have been treated with DEPC at a protein/reagent ratio from 1:10 to 1:1000. Under the experimental conditions only 2 His residues from 3 ones occurring in IFN-αA polypeptide chain at the positions NN 7, 34, 57 have been modified. This did not affect the antiviral activity of the protein or its ability to bind mono- and polyclonal antibodies in RIA (Fig. 2). The analogous results have been obtained for IFN-β,though the number and positions of His in this protein (NN 91, 95, 129, 139) differ from those of IFN-αA (21). When two other His-specific reagents, TLCK and TPCK, had been used at the same protein/reagent ratio, no inhibition of the antiviral activity of IFN-αA was noticed, but IFN-β lost its activity when

Table 1. Modification of His residues with DEPC, TLCK and TPCK

Concentration of reagents, μM		Residual antiviral activity of IFN-αA	IFN-β
TPCK	100	100%	100%
	400	100%	50%
	800	75%	10%
TLCK	100	100%	100%
	400	100%	100%
	800	80%	80%
DEPC	100	100%	100%
	400	100%	100%
	800	80%	80%

In these experiments the concentration of IFN-αA and IFN-β was 5 μM.

treated with high amount of TPCK (Table 1). This supports the earlier finding and assumption of McCray & Weil (5) that the interaction of tosyl-phenylalanyl moiety of TPCK with the surroundings of the modified His residue might lead to the inhibition of IFN-β.

According to the known sequence of IFN genes, IFN-αA contains 2 Trp residues at the positions NN 77 and 141; for IFN-β the positions of 3 Trp residues are NN 20, 77 and 141 (21). Trp_{141} is invariant for all leukocyte IFNs. To investigate the functional role of Trp we have used the selective oxidation of IFNs with H_2O_2 in 10% dioxane at pH 8,4 (16). Fig. 3 shows the changes in protein spectra in the course of modification of Trp residues of IFNs, as the method allows the spectrophotometric monitoring of the modification process. The oxidation of 2 Trp residues is accompanied with the alteration of the spectra curves, for which two isobestic points at 273 and 293 nm are characteristic, as well as with the decrease in the absorbance value at 282 nm of the modified proteins. This indicates the selective character of Trp modification. IFN-αA with 1 Trp per molecule being

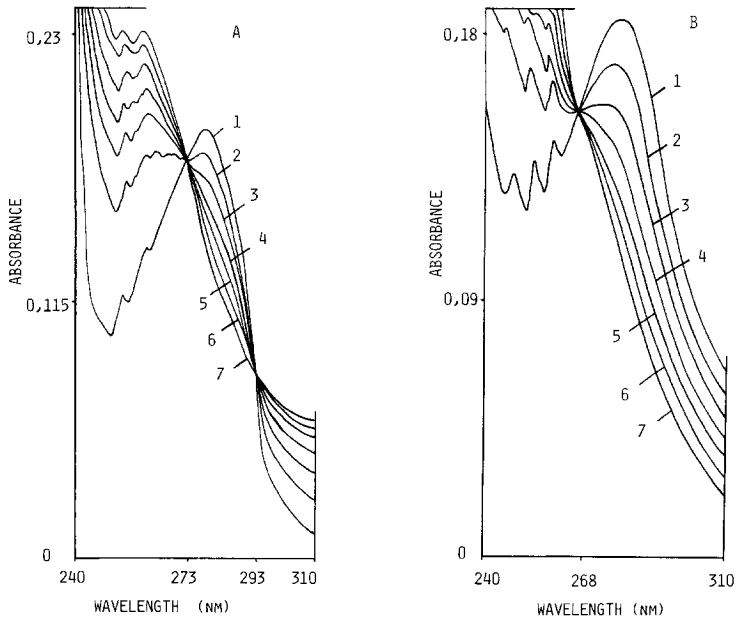

Fig. 3. Absorption spectra of 0,2 mg/ml IFN-αA (A) and 0,1 mg/ml IFN-β (B) in the course of modification of Trp residues with 1 mM H_2O_2 in 10% dioxane. 1, 2, 3, 4, 5, 6, 7 - 0, 25, 50, 75, 100, 125, 150 min after the start of the modification reaction, respectively.

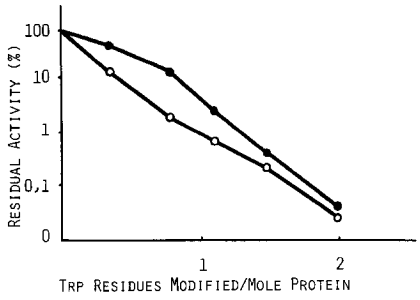

Fig. 4. Modification of Trp residues of IFN-αA with H_2O_2 in 10% dioxane. -●- ability of IFN-αA to bind mono- and polyclonal antibodies as determined by RIA, -o- antiviral activity.

modified, retained less than 1% of its initial antiviral activity, while its ability to bind mono- and polyclonal antibodies, as shown by RIA, was suppressed only 10-fold (Fig. 4). IFN-αA with both modified Trp residues shows less than 0,02-0,05% of its initial activity. These data show that the first modified Trp plays a significant role in the mechanism of antiviral activity of IFN-αA, probably being directly involved in the binding of the protein with cell surface receptor. Nevertheless, it is impossible to exclude that this Trp is also essential for maintaining correct protein folding and conformation. The second oxidized Trp residue might be also important for IFN binding with the receptor or for correct protein folding. The analogous data have been obtained when IFN-αA had been treated with o-nitrophenylsulfenyl chloride and 2-hydroxy-5-nitrobenzyl bromide. The same approach has been used to modify Trp residues of IFN-β. When 1 and 2 Trp residues from 3 ones of IFN-β had been oxidized, the antiviral activity of the protein was reduced 100- and 400-fold, respectively. The alterations of the spectra in the course of modification of IFN-β was similar to those of IFN-αA and is shown in Fig. 4. This indicates the similar functional role and spatial location of one Trp residue, at least, in IFN-αA and IFN-β. The spatial location and surroundings of Trp residues have been studied recently by analyzing the self-fluorescence spectra of IFNs (manuscript in preparation).

Acknowledgments. The authors would like to thank Drs. V.Yurin and Ya.Khesin for measuring the antiviral activity of IFN samples.

REFERENCES

1. Beilharz, M., Nisbet, I., Tymms, H., Hertzog, P., and Lihnane, A. (1986) J. Interferon Res. 6, 677-685.
2. De Chiara, T., Erlitz, F., and Tarnowski, S. (1986) Methods in Enzymology 119, 403-415.
3. Pestka, S. (1983) Arch. Biochem. Biophys. 221, 1-37.
4. Weber, H., Valenzuela, D., Lujber, G., Gubler, M., and Weissmann, C. (1987) EMBO J. 6, 591-598.
5. McCray, J., and Weil, R. (1982) Proc. Natl. Acad. Sci. USA 79, 4829-4833.
6. Wetzel, R., Levine, H., Estell, D., Shire, S., Finner-Moor, J., Stroud, R., and Beweley, T. (1982) UCLA Symposia on Mol. Cell. Biology, v.25, pp. 365-376, Academic Press, New York.
7. Fantes, K. (1986) Methods in Enzymology 119, 233-241.
8. Kostrov, S.V., Borukhov, S.I., Eremashvili, M.R., Novikov, A.A., Skvortsova, M.A., Sterkin, V.E., Izotova, L.S. and Strongin, A.Ya. (1987) Antibiotics and Med. Biotechnology, 32, 243-247 (In Russian).
9. Mashko, S.V., Lebedeva, M.I., Podkovirov, S.M., Trukhan, M.E., Kozlov, Yu.I., Rebentish, B.A., Ryzhavskaya, A.S., Strongin, A.Ya., Evdonina, L.V., Yurin, V.L., Bibilasvili, R.S., Kuznetsov, V.P, and Debabov, V.G. (1985) Doklady Acad. Nauk USSR 282, 210-214 (In Russian).
10. Borukhov, S.I., Kostrov, S.V., Izotova, L.S., Orlova, M.N., and Strongin, A.Ya. (1988) Biochimiya 53, 332-340 (In Russian).
11. Borukhov, S.I., Izotova, L.S., Korobitsin, L.P., Bumyalis, V.V., and Strongin, A.Ya. (1988) Biochimiya 53, 1718-1727 (In Russian).
12. Spector T. (1978) Analyt. Biochem. 86, 142-146.
13. Familletti, P., Rubinstein, S., and Pestka, S. (1981) Methods in Enzymology 78, 381-394.
14. Kostrov, S.V., Izotova, L.S., Eremashvili, M.R., Galaktionov, K.I., Yurin, V.L., Korobitsin, L.P., Pivovarov, A.P., Pavlov, I.A., Tyagotin, Yu.V., Fridlyanskaya, I.I., Margulis, B.A., and Strongin A.Ya. (1985) Biochimiya 50, 2031-2039 (In Russian).
15. Carlsson, J., Dervin, H., and Axen, R. (1978) Biochem. J. 173, 723-737.
16. Hashimori, Y., Horinishi, H., Kurihara, K., and Shibata, K. (1964) Biochim. Biophys. Acta 93, 345-360.
17. Oza, N., and Martin, C. (1967) Biochem. Biophys. Res. Communs. 26, 7-13.
18. Scoffone, E., Fontana, A., and Rocchi, R. (1966) Biochem. Biophys. Res. Communs. 25, 170-174.
19. Popov, V.O., Rodionov, Yu.V., Egorov, A.M., Berezin, I.V. (1978) Biochimiya 43, 1212-1221 (In Russian)
20. Mark, D., Lu, S., Creasy, R., Yamamoto, R., and Lin, L. (1984) Proc. Natl. Acad. Sci. USA 81, 5662-5666.
21. Henco, K., Brosius, J., Fujisawa, A., Fujisawa, J., Haynes, J., Hochstadt, J., Kovacic, T., Pasek, M., Schambock, A., Shmid, J., Todokoro, T., Walchli, M., Nagata, S., Weissmann, C. (1985) J. Mol. Biol. 185, 227-260.