Effect of the Insulin Iodination on the Reactivity of the Inter-chain Disulphide Bonds Towards Sodium Sulphite

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Insulin iodination interferes with the ability of the interchain S-S bonds to react with sulphite at pH 7. In insulin samples containing more than 5 iodine atoms/monomer unit, only one S-S bond/molecule reacts. The effect must be related to the substitution of the iodine into the tyrosyl groups, which probably causes a conformational rearrangement resulting in a steric hindrance of one of the interchain S-S bonds. The effect is removed by increasing the pH or by adding urea (8M) to the reaction mixture. The unreactivity of the S-S bond and the biological inactivation occur at the same 'critical' iodination level, suggesting that a same primary alteration of the molecule is responsible for both the effects.

The reaction between insulin and sodium sulphite has been extensively studied by Cecil & Loening (1960). It was found that, whereas the intrachain S-S bond, linking residues 6 and 11 on the A chain, does not react with sulphite at pH 7, the interchain S-S bonds are broken by the nucleophilic attack of the SO_3^{2-} ions, causing the insulin to split into its component A and B chains.

We have found that iodinated insulin (containing on the average 6 iodine atoms/molecule of mol.wt. 6000) is not split by sulphite at pH 7 into its component chains. This finding suggests that one or both interchain S-S bonds are in some way protected against the nucleophilic attack of the SO_3^{2-} ions. A more detailed investigation of the dependence of the S-S bonds' reactivity on the extent of the insulin iodination has been therefore carried out. The results are reported in the present paper.

EXPERIMENTAL

Reagents. Crystalline ox insulin was supplied from Hoechst A.-G., Frankfurt/M, Germany (batch 43161/f). [^{125}I]Iodide, carrier- and sulphite-free, was a special preparation from Commissariat à l'Énergie Atomique (C.E.A., Saclay, France). Solutions of phenylmercuric hydroxide were prepared and potentiometrically titrated as described by Allison & Cecil (1958). Other products were of normal reagent grade.

Iodination technique. A special iodination method developed by us and widely applied to proteins was used (Rosa *et al.* 1964; Rosa, 1964).

Elemental iodine is formed at pH 7.5 by constant-current electrolysis of a solution containing KI, insulin and NaCl as

supporting electrolyte. The rate of formation of the elemental iodine depends on the value of the electrolysis current. With a proper choice of the electrolysis current, the anodic discharge of the I^- ions, occurring with a 100% current yield, is the rate-controlling step of the entire iodination process. In this way, accumulation of elemental iodine in the solution, which is the possible cause of unwanted chemical effects, is prevented. This method allows the iodination to be carried out under easily controlled and reproducible conditions, avoiding any chemical oxidizing agent other than elemental iodine.

The preparation of the iodoinsulins was performed under the following experimental conditions: insulin (100mg.) was dissolved in 25ml. of a solution 0.1M in NaCl, 0.2–1.25mM in KI (labelled with 50 μCi of ^{125}I), at pH 7.5–7.8; 10ml. of this solution was electrolysed at a constant current of 100 μA , for a time depending on the required ID* value.

A standard electrolytic cell, described by Rosa *et al.* (1964), was used. At the end of the iodination, the reaction mixture was dialysed against several changes of twice-distilled water whose pH was brought to 7.8 with a few drops of 0.1N-NaOH. By using samples of the reaction mixture before dialysis, the amount of the initial iodine bound to the insulin was evaluated by paper radioelectrophoresis. The insulin concentration in the final solution was measured by the micro-Kjeldahl method and the ID was calculated and expressed as the number of iodine atoms bound/monomer unit, assuming mol. wt. 6000.

Reaction between insulin and sulphite. The procedure given by Cecil & Loening (1960) was used. The extent of the reaction between insulin and Na_2SO_3 occurring at pH 7 was measured by determining the amount of the SH produced. When the reaction equilibrium was reached, the pH was adjusted to 2 and the titration was carried out with phenylmercuric hydroxide by using the dropping-mercury

* Abbreviation: ID, iodination degree.

electrode. The amperometric titration must be carried out at pH 2 as the equilibrium reaction is 'frozen' at this pH value.

A 3 ml. sample of the iodinated insulin solution (corresponding to approx. $0.8 \mu\text{mole}$ of insulin) was used for each experiment. The solution at pH 7 was kept at 37° and made air-free by bubbling nitrogen, whereupon an air-free Na_2SO_3 solution at pH 7 was added, giving a Na_2SO_3 /insulin molar ratio about 200; 0.2 M-KNO_3 was used as supporting electrolyte and care was taken to exclude Cl^- ions from the reaction mixture. After 2 hr. at 37° , the reaction mixture was acidified to pH 2 by adding N-HNO_3 . The titration was carried out with a mM -phenylmercuric hydroxide solution, at -0.15 V versus the saturated calomel electrode. In separate experiments it was proved that no deiodination occurs during the reaction with SO_3^{2-} ions; this was controlled with special care by paper radioelectrophoresis, as I^- ions strongly interfere with the titration. Moreover, the absence of I^- ions was checked in each experiment. Control experiments on the native insulin were performed before and after each titration of iodinated insulin, with the same cell and electrode set. Ten samples, five of the iodoinsulin solution and five of an insulin blank, were alternately titrated in each run. The titrations of the insulin blank were used to standardize internally the results of each set of experiments.

Some titrations of the reaction mixture were carried out at pH 7 or at pH 9 in the presence of an excess of phenylmercuric hydroxide. The same apparatus and procedure described above were used, except that the supporting electrolyte was NaCl . The dropping-mercury electrode was polarized at -0.5 V versus the saturated calomel electrode; the intersection of the residual-current line and the excess-of-reagent line was taken as the final titration point. The residual-current line was found in separate experiments.

In some experiments the reaction at pH 7 was performed in the presence of 8 M-urea ; after a reaction time of 120 min., the titrations were carried out at pH 2 according to the usual procedure.

Under the above experimental conditions no deiodination occurs; this was controlled by paper electrophoresis.

Bioassay of the iodoinsulins. The biological activity of the various iodoinsulins was evaluated by the rat epididymal fat-pad assay. With each run five to ten assays were carried out on each iodoinsulin sample, and the activity was determined by comparison with a blank insulin standard solution. The insulin concentration in both solutions was evaluated by the micro-Kjeldahl method.

RESULTS

Reaction of iodinated insulin containing 6 iodine atoms/molecule with sulphite. A ^{125}I -labelled iodoinsulin at an average substitution degree corresponding to 6 iodine atoms/molecule (mol.wt. 6000) was prepared according to the procedure given above. A sample of the product was submitted to total enzymic hydrolysis followed by paper chromatography. About 90% of the activity was recovered under the form of monoiodotyrosine and di-iodotyrosine (23.2% as monoiodotyrosine and 67.8% as di-iodotyrosine); free iodide and incom-

Fig. 1. Time-course (●) of the reaction between insulin iodinated at ID 6.4 and Na_2SO_3 at pH 7 and 37° . The time-course (▲) of non-iodinated standard insulin is also reported. The concentrations of insulin and Na_2SO_3 were 0.176 mM and 31 mM respectively. The titrations were carried out at pH 2, with mM -phenylmercuric hydroxide.

pletely hydrolysed material on the starting point accounted for the remaining activity. These results prove that the tyrosine iodination was the only occurring substitution process and agree well with the fact that the elemental iodine was consumed during the iodination process according to the stoichiometry required by the tyrosine substitution reaction.

The time-course of the reaction between iodinated insulin and sodium sulphite was followed at pH 7 and 37° .

Samples from the reaction mixture were removed at intervals, acidified to pH 2 and titrated under the conditions given above. The results are shown in Fig. 1. The reaction is rapid and a true equilibrium is reached in about 50–60 min., corresponding to one-third of the S·S bonds split/molecule; under the same conditions two-thirds of the S·S bonds/molecule of native insulin are split, in agreement with the results of Cecil & Loening (1960). The reaction was followed for up to 250 min. without detectable changes in the equilibrium value.

On the same preparation of iodinated insulin the reaction with sulphite was performed at pH 7 and at pH 9 in the presence of an excess of phenylmercuric hydroxide (3.5 moles/mole of insulin). The titrations were carried out at the reaction pH. At pH 7, in agreement with the results of Cecil & Loening (1960), 2.56 ± 0.15 (7) S·S bonds/molecule react in native insulin; in iodoinsulin, only 1.58 ± 0.09 (7) S·S bonds/molecule were titrated, even after a reaction time of 150 min. This proves that one S·S bond remains unreactive at pH 7 even in the presence of phenylmercuric hydroxide. At pH 9, both iodinated and native insulin behave similarly,

giving the following results: 3 ± 0.01 (10) and 2.98 ± 0.01 (10) S·S bonds/molecule respectively.

When the reaction with sulphite was performed at pH 7 in the presence of 8 M-urea and the titration was carried out at pH 2, it was found that iodinated and native insulin react similarly, 2.44 ± 0.01 (5) and 2.35 ± 0.03 (5) S·S bonds/molecule being respectively split.

Therefore at pH 9, or at pH 7 in the presence of urea, both the interchain bonds react with sulphite in iodinated insulin.

The most significant result of the experiments reported in this section is that one-third of the S·S bonds do not react with sulphite at pH 7 under conditions in which two-thirds react in the original insulin.

Reaction of insulins iodinated at different extent with sulphite. A series of experiments similar to those described above were performed on preparations of iodinated insulin at different ID. In one case an iodinated insulin at ID 6 was prepared by a chemical procedure with iodine monochloride, according to the method given by Izzo, Bale, Izzo & Roncone (1964a).

The results are reported in Fig. 2. The s.d. value was calculated on the basis of 24 titrations of native insulin: these gave 1.93 ± 0.04 (24). The 2 s.d. range was taken to evaluate the significance of the results of the iodoinsulin titrations. It was found that the substitution process in the tyrosyl groups does not affect in a significant way the S·S bonds reactivity up to ID about 2.

Fig. 2. Extent of the reaction between iodoinsulins and Na_2SO_3 . The time-course of the reaction at pH 7 and 37° was determined as shown in Fig. 1 and the maximum extent of the reaction plotted against ID.

As the ID increases from 2 to 4–5, the amount of S·S bonds reacted decreases from two-thirds to one-third/insulin molecule. The chemically iodinated insulin behaved identically with that electrolytically iodinated at the same ID, proving that the observed effect is independent of the iodination procedure.

Analysis of the reaction products of insulin and sulphite. The reaction products were analysed in an attempt to establish whether one S·S interchain bond is actually unreactive in each monomer unit of highly iodinated insulin or whether this value results from a mixture of products averaging one unreactive S·S bond.

Cecil & Loening (1960) proved that A chain, derived from the reaction between insulin and sulphite at pH 7, is insoluble at pH 5, whereas B chain remains in solution. By using an iodoinsulin at ID 0.2, labelled with ^{125}I ($5 \mu\text{C}/\text{mg.}$), we found B chain in the soluble fraction when the reaction mixture of insulin and sulphite was acidified to pH 5, and the precipitate was separated by centrifuging. In agreement with the results reported by Cecil & Loening (1960), some B chain was found in the precipitate, which contained all the A chain.

The same experiment was performed with an iodoinsulin at ID 5, labelled with ^{125}I ($5 \mu\text{C}/\text{mg.}$), which was allowed to react with sulphite at 37° into the polarographic cell. The composition of the reaction mixture was as follows: iodoinsulin (0.18 mM), hydrochloric acid (0.04 M), sodium sulphite (0.1 M), pH 6.9. At the end of the reaction the pH was lowered to 5 by addition of acetic acid. A precipitate formed, which was separated by centrifuging. It was found that 90% of the activity had been carried by the precipitate. This was carefully washed, then dissolved in a solution containing 8 M-urea, 18 mM-sodium sulphite, 10 mM-sodium tetrathionate and acetic acid up to pH 7.6, according to the procedure suggested by Bailey (1957) to split completely any S·S bond. The reaction mixture was kept at 5° for 5 hr., then submitted to paper electrophoresis in 20% acetic acid and 8 M-urea, pH 3.2 (Whatman no. 1 paper; 16 hr.; 4 v/cm.).

The spots of the S-sulphonated A and B chains were identified by radioautography and counted for ^{125}I activity in a well-type scintillation counter. The A chain/B activity ratio was 3.12; the same ratio was found when the original preparation of iodoinsulin was directly submitted to Bailey's (1957) splitting procedure. The precipitate is therefore composed of equal amounts of the two chains and contains the largest part of the protein material; no significant amount of soluble B chain is formed in the reaction. To account for the titration data, showing that one-half of the interchain S·S bonds do not react with sulphite in iodoinsulin at ID 5, it seems

reasonable to postulate that the precipitate is composed of partially split insulin molecules still containing one intact interchain sulphur bridge.

The only alternative possibility still in agreement with the titration data might be that B chain, derived from highly iodinated insulin, is insoluble at pH 5, so that the precipitate would be composed of a mixture of intact insulin and separated chains, averaging one intact S·S bond/molecule. The major objection against such a hypothesis is that, if an insulin species completely unreactive towards the sulphite were formed in the iodination of the native insulin, its relative amount would increase as soon as the ID increases. However, we have found that one interchain S·S bond still reacts with sulphite in iodinsulin at ID of about 8. Anyway, the absence of intact insulin in the precipitate was checked by paper electrophoresis.

The precipitate obtained as described above was dissolved in 8M-urea and 20% acetic acid, pH 3·2, and analysed by electrophoresis with the same buffer and operative conditions previously given for the analysis of the S-sulphonated chains. A single spot, moving slightly towards the anode, was located by radioautography. No intact insulin was found.

On the other hand, the A chain/B chain activity ratio being 3·12, the largest part of the B chains in a iodinsulin preparation at ID 5 must contain no more than 1 iodine atom. The experiment with iodinsulin at ID 0·2, whose labelled B chains were certainly monoiodinated, has shown that such an iodination level does not affect the solubility of the B chain.

The failure to observe the formation of soluble B chain among the reaction products, as well as the absence of intact insulin in the precipitate at pH 5, strongly suggests that each monomer unit actually contains an unreactive S·S bond.

Reaction between fibrous insulin and sulphite. The results reported by Gruen, Laskowski & Scheraga (1959) indicate that neither a change in the state of aggregation nor apparent gross modifications in the molecular size and shape occur in fully iodinated insulin. On this basis, the possibility that the effect we have observed is due to a change in aggregation state of the insulin, occurring during the iodination process, could be ruled out.

Further, if iodinsulin aggregate and some monomer units were 'masked', as far as the reaction with sulphite is concerned, the 'unmasked' ones would be split, and the reaction mixture would contain the separated A and B chains. In the preceding section it was proved that this is not the case. However, it was decided to study the reaction between sodium sulphite and fibrous insulin. This was prepared according to the method given by Waugh (1944), and allowed to react with sulphite at pH 7. It was found that the reaction reaches a true

equilibrium corresponding to two-thirds of the S·S bonds split/molecule in 30–40 min. As far as the reactivity towards sulphite is concerned, fibrous insulin therefore behaves as the native one, proving that the accessibility of the S·S bonds is not appreciably affected by the state of aggregation.

Effect of the progressive iodination on the biological activity of insulin. When the results reported in Fig. 2 were compared with those given by Izzo, Roncone, Izzo & Bale (1964b) about the dependence of the insulin biological activity on the extent of the iodination, it was observed that inactivation and the failure of the S·S bonds reactivity occur in the same ID range. Both effects could be induced by the same unknown alteration of the insulin molecule caused by the iodination of the tyrosyl groups. As it has been suggested (Fraenkel-Conrat & Fraenkel-Conrat, 1950) that the extent of inactivation at a given ID value may depend on the conditions under which the iodination reaction has been performed, we preferred to measure again the biological activity of the iodinsulin series. The results, reported in Fig. 3, are in good agreement with those measured by Izzo *et al.* (1964b) with assays both *in vitro* (fat-pad and diaphragm) and *in vivo* (mouse convulsions).

The evaluation of the limiting ID beyond which the inactivation occurs obviously depends on the accuracy of the bioassay. This is rather poor, and the results must be accepted within a confidence

Fig. 3. Effect of incorporation of increasing number of iodine atoms into insulin on its biological activity, as measured by CO₂ production on rat epididymal fat pad. The 2s.d. range evaluated on 20 assays of native insulin is taken as the criterion to establish the significance of the decrease in biological activity.

limit of 20%. Despite such a limitation in the reliability of the bioassay, a strong similarity exists between the trend of the two processes reported in Figs. 2 and 3.

DISCUSSION

The object of this work was to find the extent to which the reactivity of the insulin S·S bonds towards sulphite is modified by the iodination of the protein. It has been found that at pH 7, where the reaction is effectively confined to the interchain S·S bonds, the extent of the reaction decreases as the iodination degree is increased from 1·5 to 5 iodine atoms/insulin molecule. In iodoinsulins at ID 5 or more, only one-third of the S·S bonds react with sulphite. This is not due to a mixture of products averaging one unreactive S·S bond/molecule, since neither intact insulin nor soluble B chain have been identified among the products of the reaction between insulin at ID 5 and sulphite. Moreover, if an insulin species completely unreactive towards sulphite were formed in the iodination of insulin, its relative amount would increase as soon as the ID increased. However, we have found that one interchain S·S bond still reacts with sulphite at pH 7 also in iodoinsulin at ID of about 8.

On the same basis the possibility is ruled out that the observed effect is due to a sort of masking of one-half of the monomer units caused by aggregation of the iodinated insulin. Further, fibrous insulin is completely split by sulphite at pH 7, and the reaction proceeds at the same rate as for the native insulin. On the other hand, one S·S bond fails to react in insulin substituted at ID 5 or more, even after a tenfold longer reaction time in the presence of an excess of sulphite.

It appears therefore that each monomer unit in insulin iodinated at ID 5 or more contains an interchain S·S bond protected in some way against the nucleophilic attack of SO_3^{2-} ions at pH 7.

The actual iodination level at which the above effect occurs on the single molecule cannot be predicted on the basis of our results in the absence of any possibility of evaluating the intermolecular distribution of the iodine atoms. Since the insulin molecule (mol.wt. 6000) contains 4 tyrosine residues, i.e. eight substitution sites, it is theoretically possible for a given iodinated insulin preparation to be composed of different molecular species containing from 0 to 8 iodine atoms. On the other hand, De Zoeten & Havinga (1961) have found that, up to a substitution degree of about 3 iodine atoms/molecule, the tyrosine residues in A chain are almost selectively substituted. This strongly lowers the probability that molecules containing more than 4 iodine atoms are contained in iodoinsulin within the ID range from 0 to 3. The molecular species containing one S·S bond un-

reactive towards sulphite begin to interfere on the results of the S·S titrations in iodoinsulins containing on the average 1·5–2 iodine atoms/molecule. According to the above discussion, these iodoinsulin preparations are probably composed of molecules containing 1–2 and 3 iodine atoms, with a smaller amount of molecules uniodinated or containing 4 or more iodine atoms. Viewed against this background the effect we have observed may be tentatively attributed to the attainment of an iodination level of the single insulin molecule higher than 1 and probably than 2 iodine atoms; however, the possibility that the intramolecular distribution of the iodine also plays a relevant role makes the validity of the above conclusions very tenuous.

However, the failure of one S·S bond to react with sulphite is to be considered as a side effect induced by the iodination of the tyrosyl groups, which is certainly selective, and very probably by the iodination of those located in the A chain.

The iodine being nearly equal in size to the benzene ring, substituted tyrosines must interfere on their immediate environment. Therefore it cannot be excluded that a conformational rearrangement, involving the whole molecule or a part of it, occurs as a consequence of the iodination; this could give rise to an effect of steric hindrance on the S·S bond or this could favour a more stable configuration of this latter, in the sense indicated by Fava, Iliceto & Camera (1957). The pK of the phenolic hydroxyl groups is substantially lowered in substituted tyrosines, a well-known consequence of such an effect being the increase of the insulin electrophoretic mobility due to the increased negative charge of the molecule. Therefore a negatively charged environment could make more difficult the approach of SO_3^{2-} ions to the S·S bond. The fact that phenylmercuric hydroxide at pH 7 cannot reverse the reaction towards the splitting proves that the protective effect on the S·S bond is very strong. However, the occurrence of the complete splitting at pH 9 could be attributed to configurational changes associated with the increase of the pH, for it has been suggested on theoretical grounds and it has been shown experimentally with synthetic polyelectrolytes that the extent of helical folding in proteins should vary with pH and that the folding would be maximal around the isoelectric region (Laskowsky, Leach & Scheraga, 1960). Such an effect has been invoked to explain the increased reactivity of the tyrosines in the insulin B chain (B16 and B26) towards iodine when the iodination is performed at pH 9; moreover, this effect could account for the removal of any protective effect on the S·S bond when the reaction with sulphite is carried out at the same pH value.

The occurrence of the complete splitting at pH 7 when the reaction with sulphite is performed in

8M-urea proves that unfolding of the protein removes the interfering effect.

The observation that both the loss of the biological activity and the inability of one S·S bond to react with sulphite occur within the same ID range suggests that both effects occur when the insulin molecule has attained the same 'critical' level of iodination.

If one accepts the hypothesis that iodination of the tyrosyl groups interferes with the S·S bond's reactivity 'via' a conformational rearrangement, one cannot exclude that inactivation occurs as a consequence of such a conformational change. It has been suggested by Fraenkel-Conrat & Fraenkel-Conrat (1950) that the biological inactivation resulting from the reaction of insulin with specific group substituents, such as iodine, is probably to be attributed to an induced alteration of the whole molecule rather than to an actually 'essential' role of the groups that have been modified.

As a matter of speculation, a different interpretation could be suggested for the above results. It is conceivable that the unreactivity of the interchain S·S bond is not limited to sulphite alone; the nucleophilic attack of one of the interchain bonds, for instance through a thiol-disulphide exchange reaction, could be equally hindered in insulins iodinated beyond the 'critical level'. This could suggest a cause-and-effect relationship between the unreactivity of the interchain S·S bond and the

biological inactivation, provided that the cleavage of the insulin at the tissue receptor sites or at least the accessibility of the interchain S·S bonds were needed for full expression of biological activity.

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