Susceptibilities of lactoferrin and transferrin to myeloperoxidase-dependent loss of iron-binding capacity

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Apolactoferrin and apotransferrin lost their ability to subsequently bind iron when exposed to an excess of either HOCl or myeloperoxidase plus H_2O_2 and Cl^- . Apolactoferrin, however, was more resistant than apotransferrin. By oxidizing a mixture of the two proteins, then separating them by immunoprecipitation, the difference in susceptibility was shown to be due to the greater reactivity of transferrin iron-binding groups, rather than protective groups on the lactoferrin molecule. The iron-saturated proteins were much more resistant to oxidative modification than the apoproteins. The greater resistance of apolactoferrin should be advantageous for maintaining its iron binding capacity when co-released with myeloperoxidase and reactive oxygen species from stimulated neutrophils.

INTRODUCTION

Lactoferrin is present in the specific granules of neutrophils, and is released into both the phagosomes and the surrounding medium when the cells are stimulated [1–3]. It is present in the cells mostly in an iron-depleted form [4]. By decreasing the availability of iron, it is an effective inhibitor of bacterial growth [5]. However, its role in the microbicidal action and other inflammatory reactions of neutrophils is not clear [6,7]. Although earlier studies suggested that Fe–lactoferrin catalyses hydroxyl radical production [8], further studies have found that it (and transferrin) have no detectable catalytic activity [9,10]. One possibility is that lactoferrin takes up iron released from killed micro-organisms or ingested tissue debris, and prevents release of free iron and undesirable catalysis of oxidative reactions. However, to do this, it must maintain its iron-binding capacity in the presence of oxidants that are co-released from stimulated neutrophils.

Oxidants released by stimulated neutrophils could also affect the plasma iron-binding protein, transferrin. Oxidation of its binding sites might not only prevent uptake of further iron, but also release iron that was already bound.

When neutrophils are stimulated, they undergo a burst of O_2 consumption with the production of superoxide and H_2O_2 [11]. They also release myeloperoxidase, which converts H_2O_2 and Cl^- to the strongly oxidizing HOCl. We have exposed apotransferrin and apolactoferrin to either myeloperoxidase plus H_2O_2 and Cl^- , or to HOCl, and measured their ability to subsequently bind iron. We have also investigated whether exposure to HOCl results in iron release from the iron-saturated proteins.

EXPERIMENTAL

Human lactoferrin and transferrin (substantially iron free) were obtained from Sigma (St. Louis, MO, U.S.A.). Iron-loaded proteins were prepared by adding a 2-fold excess of FeCl_3 (labelled with ^{59}Fe) to a 10-fold excess of

nitrilotriacetic acid (NTA). Solutions were dialysed at 4 °C overnight against excess 10 M-phosphate buffer, pH 7.4, containing 100 μM -NTA, and then overnight against phosphate buffer. Myeloperoxidase was purified from human neutrophils [12] and had a purity index (A_{430}/A_{280}) > 0.7. ^{59}Fe – FeCl_3 was purchased from New England Nuclear (North Ryde, N.S.W., Australia). Rabbit anti-(human transferrin), -lactoferrin and -IgA antibodies were obtained from Dakopatts A/S (Copenhagen, Denmark). There was no cross-reactivity between the transferrin and lactoferrin antibodies. Sodium hypochlorite was from May and Baker (Dagenham, U.K.). Other biochemicals were from Sigma.

For exposure to HOCl or the myeloperoxidase system, the apoproteins or iron-loaded proteins, at a known concentration between 0.3 and 0.6 mg/ml (7.5–15 μM), were dissolved in 10 mM-phosphate (pH 7.4)/0.14 M-NaCl (phosphate-buffered saline). Concentrations were determined using $A_{280}^{1\%}$ 12.9 and 10.9 for apotransferrin and apolactoferrin respectively [13]. Molar concentrations are expressed as per iron equivalent, taking an M_r of 81000 and two binding sites per molecule. To 0.3–0.5 ml of protein solution, either 20–60 μl of 1.5 mM-HOCl was added with continuous vigorous mixing, or otherwise the solutions contained 0.3 μM -myeloperoxidase with portions of up to 100 μM - H_2O_2 added at 20 min intervals. Iron-preloaded proteins were then analysed for bound iron. Apoproteins were mixed for 1 h with a 5-fold excess of ^{59}Fe – FeCl_3 in a 25-fold excess of NTA (per iron-binding site). Three methods for measuring bound iron were used.

(i) Using ^{59}Fe and dialysis, solutions were transferred to dialysis sacs, counted, and then dialysed for at least 20 h at 4 °C against two changes of phosphate-buffered saline containing 50 μM -NTA. The non-dialysable counts in each sample were determined, and a comparison was made with control proteins that had not been exposed to oxidants, but otherwise treated in the same way. (ii) The same procedure as in (i) was followed except unlabelled Fe–NTA and higher protein concentrations (2–3 mg/ml) were used. After dialysis, bound

Abbreviations used: NTA, nitrilotriacetic acid; MCD, monochlorodimedon.

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iron was determined by measuring A_{465} of each solution. (iii) By immunoprecipitation: to approx. 25 μg of control or oxidant-exposed ^{59}Fe -labelled protein were added 30 μl of specific anti-lactoferrin or anti-transferrin serum, 4 mg of protein A-Sepharose and 0.4 mg of casein in 200 μl of phosphate-buffered saline containing 50 μM -NTA. Samples were gently rotated at 4 $^{\circ}\text{C}$ overnight in sealed Eppendorf tubes. Sepharose-bound immunoprecipitates were spun down, washed twice and counted. A similar procedure was carried out with anti-IgA as the antiserum to act as a negative control. The usual immunoprecipitation buffers containing detergent and guanidine hydrochloride were avoided because of the risk of dissociating bound iron, and casein was included to minimize non-specific iron binding to the immunoglobulins. With the IgA controls, immunoprecipitated iron was always < 1 % of the total counts. Using this procedure, > 85 % of the iron bound to native Fe-transferrin or Fe-lactoferrin was immunoprecipitated with the appropriate antiserum, and with apotransferrin exposed to HOCl, all the non-dialysable ^{59}Fe was also immunoprecipitated. This shows that the dialysis and immunoprecipitation methods give similar results, and discounts the possibility that differences measured by immunoprecipitation, reflect oxidant effects on antibody binding.

RESULTS

Exposure of apolactoferrin and apotransferrin to HOCl caused concentration-dependent impairment of the ability of the proteins to subsequently bind iron. Apolactoferrin was less susceptible than apotransferrin (Fig. 1). Most of the results in Fig. 1 are for iron binding determined by adding ^{59}Fe -NTA followed by dialysis, but A_{465} measurements carried out with apotransferrin (shown by triangles in Fig. 1) gave the same results. Different susceptibilities were also observed when the two proteins were exposed to myeloperoxidase, H_2O_2 and Cl^- . A series of additions of 100 μM - H_2O_2 were made, because at this concentration or less, there is efficient conversion to HOCl by myeloperoxidase [14]. With a 12-fold excess of H_2O_2 , apotransferrin showed a 42 % loss in subsequent iron binding (measured by immunoprecipitation), compared with a 30 % loss with apolactoferrin.

The myeloperoxidase system caused much greater impairment of iron binding than H_2O_2 alone. Exposure of 7 μM -apotransferrin to two additions of 70 μM - H_2O_2 (added at 0 and 20 min of a 40 min incubation) caused a 12 % decrease in iron-binding capacity, compared with 60 % for the same system in the presence of 0.1 μM -myeloperoxidase. Peroxidative inactivation independent of Cl^- was much less efficient than HOCl-dependent inactivation. Under the same conditions but with no Cl^- added, the decrease in apotransferrin iron-binding capacity was only 17 %.

The iron-saturated proteins were much less susceptible than the apoproteins to destruction of their iron-binding sites by HOCl. Exposure of Fe-lactoferrin to up to a 30-fold excess of HOCl caused no loss of bound iron on subsequent dialysis, and Fe-transferrin was approx. three times more resistant than apotransferrin (Fig. 2).

The decreased susceptibility of lactoferrin compared with transferrin could mean either that the Fe-binding

Fig. 1. Effect of HOCl on the ability of apotransferrin and apolactoferrin to subsequently bind iron

Oxidant-exposed proteins were mixed with ^{59}Fe -NTA and bound ^{59}Fe was determined after dialysis. Molar protein concentrations are expressed per iron-binding site. □, Apolactoferrin; ●, apotransferrin; ▲, apotransferrin with bound iron determined from A_{465} measurements.

Fig. 2. Iron loss from transferrin and lactoferrin following exposure to HOCl

The iron-saturated proteins were prepared by adding ^{59}Fe -NTA and were extensively dialysed before adding HOCl. Iron loss was measured following dialysis. (□), Lactoferrin; (●), transferrin; (▲), transferrin with bound iron determined from A_{465} measurements.

residues of transferrin are more reactive with HOCl, or alternatively, that other groups on the lactoferrin molecule (or other constituents of the lactoferrin preparation) are highly reactive and protect the lactoferrin Fe-

Fig. 3. Relative susceptibilities of apotransferrin and apolactoferrin to myeloperoxidase, H₂O₂ and Cl⁻

A mixture of 4.5 μM -apotransferrin and 2.8 μM -apolactoferrin was prepared in phosphate-buffered saline containing 65 nM-myeloperoxidase. It was divided into four portions, and either 0, 1, 2 or 3 stepwise additions of 65 nmol of H₂O₂/ml were made to each at 20 min intervals. After addition of 40 μM -⁵⁹Fe-NTA and standing for 1 h, five 20 μl portions were removed, two for immunoprecipitation with anti-transferrin, two with anti-lactoferrin, and one with anti-IgA as control, as described in the Experimental section. (●), Transferrin; (□), lactoferrin. The range of duplicates fell within the symbols, except where indicated with a bar.

Fig. 4. Protection of apotransferrin by MCD

Apotransferrin (15 μM) was exposed to a 15-fold excess of HOCl in the presence of varying concentrations of MCD. Iron binding was determined as in Fig. 1(a).

binding site. To distinguish between these possibilities, a mixture of the two apoproteins was exposed to the myeloperoxidase system, ⁵⁹Fe-NTA was added, and separate immunoprecipitations using anti-lactoferrin and anti-transferrin sera were performed. As shown in Fig. 3, the greater resistance of apolactoferrin was still apparent in the mixture. Since reactive groups on the lactoferrin not involved in iron binding would be equally protective of both proteins, this result demonstrates a greater susceptibility of the transferrin iron-binding site to HOCl.

Most biochemical compounds react readily with HOCl. To relate the reactivity of apotransferrin with that of other compounds, the ability of monochlorodimedon (MCD) to prevent loss of iron-binding capacity was measured. Fig. 4 shows that > 200 μM -MCD was sufficient to protect 15 μM -apotransferrin (0.6 mg/ml).

DISCUSSION

This study has shown that apolactoferrin and apotransferrin can be modified by HOCl, so that they can no longer effectively bind iron. Similar findings were obtained with reagent HOCl and the myeloperoxidase system which is consistent with HOCl being responsible for myeloperoxidase-dependent inactivation. Although some inactivation by H₂O₂ alone was observed, it was very much less. Inactivation of both apoproteins required an excess of HOCl (approx. 12-fold for apotransferrin and 30-fold for apolactoferrin for 50% loss of binding capacity) implying that groups that do not influence iron binding are also modified. Considerably more oxidant was required to cause loss of iron already bound to the proteins. An inspection of the lactoferrin structure [15] shows that the iron is buried in the centre of each of the two lobes of the molecule, so it is not surprising that the binding regions are resistant to oxidation. The greater oxidant susceptibility of the apoproteins suggests a more open structure.

A major point of interest is that apolactoferrin is much less sensitive to HOCl than apotransferrin. By exposing a mixture of the two proteins to the myeloperoxidase system, this was shown to be due to enhanced susceptibility of the transferrin binding site rather than protective groups on the lactoferrin molecule. This could be a result of either the critical groups in lactoferrin being less reactive with HOCl, or their being protected by other groups in close proximity in the lactoferrin molecule. Comparing the sequences of the two proteins [16] in relation to the crystal structure of lactoferrin [15] does not reveal an obvious explanation for this difference. The iron-binding residues are identical in both proteins. Cysteines and methionines are particularly susceptible to HOCl [17,18] yet there are no free thiols in either protein, and transferrin does not have additional methionines in positions equivalent to the binding regions of lactoferrin.

Physiologically, for lactoferrin to take up iron released when neutrophils attack targets, it must not be inactivated by oxidants released by the cells. Our results indicate that lactoferrin is better suited than transferrin to this role. Indeed, this became apparent in our investigation (A. L. Molloy & C. C. Winterbourn, unpublished work) of whether apotransferrin or apolactoferrin present during exposure of bacteria to myeloperoxidase, H₂O₂ and Cl⁻ (at pH 7.4) could take up

bacterial iron, which is released by this treatment [19]. Whereas 40% of the released iron was recovered associated with lactoferrin (regardless of whether the lactoferrin was added before or after the oxidants), significant binding to transferrin occurred only if it was added after the oxidant reaction was complete.

The resistance of the iron-loaded proteins to HOCl should be desirable in not favouring iron release in the vicinity of stimulated neutrophils. It should be noted, however, that even the apoproteins are not unduly susceptible to HOCl. Almost all biological compounds react readily with HOCl, and the extent to which any is inactivated depends on competing reactions [17,18]. Our findings with MCD enable comparison with other compounds whose reactivities relative to MCD have been determined [18]. Thus, with 200 μ M-MCD protecting 0.6 mg of apotransferrin/ml, the normal plasma concentration (3 mg/ml) would require 1 mM-MCD. Equivalent protection should be given by approx. 6 mg of albumin/ml, 200 μ M-ascorbic acid, or 30 μ M-methionine. Hence, only a small proportion of myeloperoxidase-derived oxidants generated in plasma should react with transferrin, and without a large oxidant excess, inactivation should be minimal. However, where excess oxidants may be formed, e.g. within phagosomes or close to stimulated neutrophils, oxidant inactivation of iron-binding proteins could be more extensive. It is interesting, therefore, that neutrophil lactoferrin does appear able to take up iron released from phagocytosed *Escherichia coli* (A. L. Molloy & C. C. Winterbourn, unpublished work).

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