Biochem. J. (1983) **210**, 15–19
Printed in Great Britain

Lactoferrin-catalysed hydroxyl radical production

Additional requirement for a chelating agent

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(*Received 27 April 1982/Accepted 27 September 1982*)

The ability of lactoferrin to catalyse hydroxyl radical production was determined by measuring ethylene production from methional (2-amino-4-methylthiobutyraldehyde) or 4-methylthio-2-oxobutyrate. Lactoferrin, isolated from human milk and saturated by adding the exact equivalents of Fe^{3+}–nitrilotriacetic acid and dialysing, give little if any catalysis of the reaction between H_2O_2 and either $O_2^{-\cdot}$ or ascorbic acid at either pH 7.4 or pH 5.0. However, in the presence of chelating agents such as EDTA or nitrilotriacetic acid that can complex with lactoferrin, hydroxyl radical production by both mechanisms was observed.

Lactoferrin is found in the specific granules of neutrophils (Baggiolini *et al.*, 1970; Baggiolini, 1980). Because of its high affinity for iron, iron-deplete lactoferrin is bacteriostatic, but whether this is its function in the neutrophil or whether it is more actively involved in microbial killing is not certain (Arnold *et al.*, 1977; Bullen *et al.*, 1978; Aisen & Listowsky, 1980). Production of $O_2^{-\cdot}$ and H_2O_2 is essential for efficient microbicidal function (Roos, 1980), and this may be because they can react to form the highly reactive hydroxyl radical ($OH^{\cdot}$). There is some evidence that $OH^{\cdot}$ is formed by activated neutrophils (Tauber & Babior, 1977; Klebanoff & Rosen, 1978; Weiss *et al.*, 1978; Green *et al.*, 1979), but the mechanism of formation is uncertain (Roos, 1980). $OH^{\cdot}$ production from $O_2^{-\cdot}$ and H_2O_2 requires a metal catalyst (Halliwell, 1981), and it is an attractive proposition that lactoferrin released from neutrophil granules on activation could act in this way, thus contributing to the microbicidal armament of the cell.

I have recently investigated this possibility and found no evidence for lactoferrin being able to catalyse $OH^{\cdot}$ production (Winterbourn, 1981). In contrast, Ambruso & Johnston (1981) have concluded that lactoferrin is an extremely efficient catalyst of the reaction. A possible explanation for this difference stems from my subsequent finding that addition of complexing agents such EDTA or NTA confers on lactoferrin solutions the ability to catalyse $OH^{\cdot}$ production from H_2O_2 and either $O_2^{-\cdot}$ or ascorbate. Studies of these reactions form the basis of the present paper.

Abbreviations used: NTA, nitrilotriacetic acid; MOB, 4-methylthio-2-oxobutyrate ('α-keto-γ-methiol-butyric acid').

Methods

All reactions were carried out in 0.05 M-sodium phosphate buffer, pH 7.4, or 0.05 M-sodium acetate buffer, pH 5.0. To obtain acceptably low background rates of $OH^{\cdot}$ production, it was necessary to remove traces of iron or other contaminant metal ions picked up from glassware or present in the buffer salts. All glassware was acid-washed and the phosphate buffer was passed through a column of Chelex resin (Na^+ form) before use. All solutions were prepared in deionized distilled water. Unless these precautions were taken, solutions contained the catalytic equivalent of up to 1.5 μM-iron, and addition of EDTA (0.1 or 1 mM) stimulated the rate of ethylene production by up to 10 times. With purified buffers, EDTA had little effect.

Lactoferrin, a generous gift from Dr. S. V. Rumball and Mrs. H. Baker (Massey University), was purified from human milk by the method of Querinjean *et al.* (1971). As isolated, it was 10–25% saturated with iron. It was completely saturated by adding the exact equivalents of Fe^{3+}–NTA in a 2-fold excess of NTA and then dialysed for at least 48 h against three changes of 0.01 M-sodium phosphate buffer.

$OH^{\cdot}$ radical production was determined by measuring ethylene production from 1 mM-methional (2-amino-4-methylthiobutyraldehyde) or 1 mM-MOB (Beauchamp & Fridovich, 1970). Reactions were carried out in a total volume of 1.5 ml in 12 ml

0306-3283/83/010015-05$2.00

rubber-stoppered tubes. Gas samples (0.8ml) were removed at intervals and ethylene concentrations were determined by g.l.c. (Winterbourn, 1979). These were quantified by comparing g.l.c. peak areas with the areas of peaks obtained by oxidizing known amounts of methional or MOB with excess OH• produced from H_2O_2 and $FeSO_4$. Production of OH• from H_2O_2 (0.35mM) and either ascorbic acid (0.12mM) or $O_2^{-\bullet}$ generated from xanthine (0.24mM) and xanthine oxidase (5.6m-units/ml at pH 7.4 or 22m-units/ml at pH 5) was measured.

All biochemicals were obtained from the Sigma Chemical Co., St Louis, MO, U.S.A., except superoxide dismutase (Diagnostic Reagents, Oxford, Oxon, U.K.).

Results

The reaction of H_2O_2 with either $O_2^{-\bullet}$ generated by xanthine oxidase or ascorbate, in Chelex-treated sodium phosphate buffer, pH 7.4, gave only a very low rate of ethylene production from methional (Fig. 1*a*). On addition of 5 μM-Fe^{3+}–EDTA the reaction rates were at least 30-fold increased, demonstrating the known requirement for a metal ion catalyst. On addition of 5 μM-lactoferrin (10 μM with respect to iron), with both $O_2^{-\bullet}$ and ascorbate, the rate was either unchanged or very slightly increased (Fig. 1*a*). In seven experiments, provided the lactoferrin had been dialysed for at least 48h against several buffer changes, the increase was in no case greater than 1% of that given by 5 μM-Fe^{3+}–EDTA. From the near-linear dependence of reaction rate on Fe^{3+}–EDTA concentration in this range (Fig. 2), the catalytic ability of lactoferrin was therefore no greater than 1% of the equivalent concentration of Fe^{3+}–EDTA. Less extensively dialysed lactoferrin did, however, give up to 5% of the ethylene production seen with an equivalent concentration of Fe^{3+}–EDTA. Spectral examination showed no loss of specifically bound iron on extensive dialysis. Prior dialysis against 1mM-$NaHCO_3$ in 0.01M-sodium phosphate buffer, or addition of 0.1mM-$NaHCO_3$ directly to the reaction mixture, was without effect.

Measurement of ethylene production from MOB produced similar results to the experiments with methional (Fig. 1*b*). The very slight increase in rate that was observed with 5 μM-lactoferrin was no more than what would be caused by 0.05 μM-Fe^{3+}–EDTA.

The poor catalytic ability of lactoferrin was also apparent in experiments in which iron-deplete lactoferrin was added to phosphate buffer that had not been subject to Chelex treatment. The basal rates of ethylene production were two to ten times those shown in Fig. 1, and with both ascorbate and

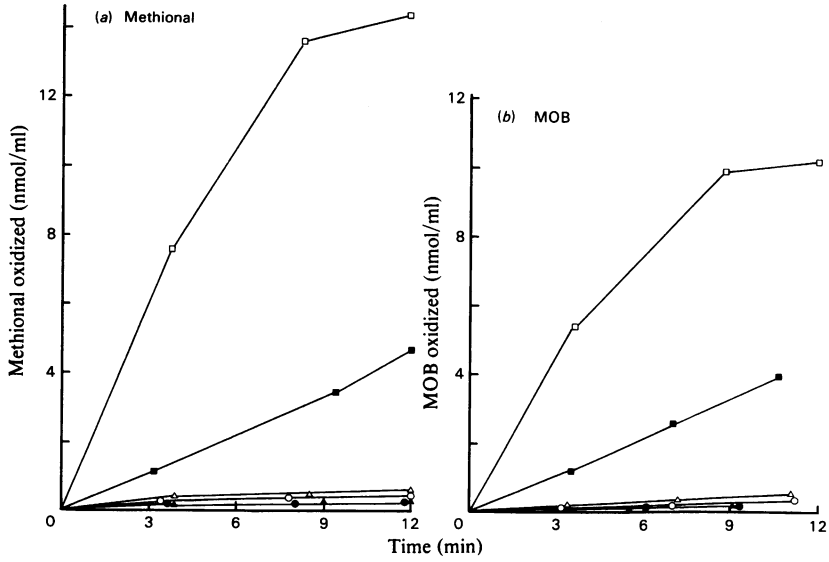

Fig. 1. *Effects of Fe^{3+}-EDTA and lactoferrin on the rates of ethylene production from methional and MOB*
●, Xanthine, xanthine oxidase and H_2O_2; ▲, xanthine, xanthine oxidase, H_2O_2 and 5 μM-lactoferrin; ■, xanthine, xanthine oxidase, H_2O_2 and 5 μM-Fe^{3+}–EDTA; ○, ascorbate and H_2O_2; △, ascorbate, H_2O_2 and 5 μM-lactoferrin; □, ascorbate, H_2O_2 and 5 μM-Fe^{3+}–EDTA. Xanthine/xanthine oxidase was generated from xanthine and xanthine oxidase. Reactions were carried out in phosphate buffer, pH 7.4. Other experimental details are given in the Methods section. Results are means of duplicates obtained from a single experiment. Other experiments gave similar results.

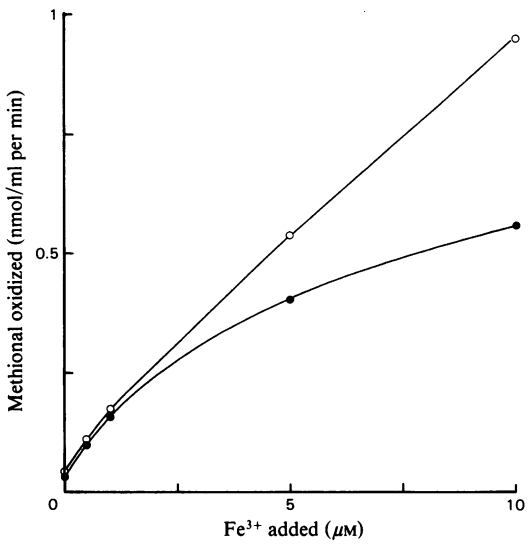

Fig. 2. *Dependence of ethylene production from methional on Fe^{3+}–EDTA concentration*

Reactions were carried out in phosphate buffer, pH 7.4, containing 0.1 mM-EDTA. Other conditions were as described in the Methods section. ○, Ascorbate and H_2O_2; ●, xanthine, xanthine oxidase and H_2O_2.

Table 1. *Effects of lactoferrin and chelating agents on the rate of ethylene production from methional in phosphate buffer, pH 7.4*

Results were obtained from duplicate experiments and are expressed as initial rates determined from curves similar to those in Fig. 1. Experimental details are given in the Methods section.

Additions	Methional oxidized (pmol/ml per min) Ascorbate/ H_2O_2	Xanthine/xanthine oxidase/H_2O_2
None	22	14
Lactoferrin (5 μM)	35	21
EDTA (100 μM)	18	18
Lactoferrin (3.5 μM)/ EDTA (0.05 mM)		83
Lactoferrin (3.5 μM)/ EDTA (0.1 mM)	390	135
Lactoferrin (3.5 μM)/ EDTA (1 mM)		175
Lactoferrin (2 μM)/ EDTA (0.1 mM)	230	80
Fe^{3+} (3.5 μM)/EDTA (0.1 mM)	1220	270
NTA (100 μM)	11	5
Lactoferrin (3.5 μM)/ NTA (0.1 mM)	84	45
Fe^{3+} (5 μM)/NTA (0.1 mM)	300	150

$O_2^{-\cdot}$, 10%-saturated lactoferrin (5 μM) decreased these by about 50%.

Contrasting with these findings, lactoferrin in the presence of EDTA caused a marked increase in the rate of ethylene production from methional, with both H_2O_2/ascorbate and $H_2O_2/O_2^{-\cdot}$ (Table 1). The reaction rate increased with increasing lactoferrin concentration, but showed only a slight dependence on EDTA concentration above 0.1 mM. These results suggest that, at saturating EDTA concentrations, lactoferrin–EDTA has about a quarter the catalytic activity (per mol of iron) of an equivalent concentration of Fe^{3+}–EDTA. Lactoferrin also catalysed ethylene production in the presence of NTA. Fe^{3+}–NTA is not as good a catalyst as Fe^{3+}–EDTA, but the efficiency of lactoferrin compared with Fe^{3+} was approximately the same with the two chelating agents. $OH^{\cdot}$ production from MOB was similarly enhanced by lactoferrin in the presence of EDTA or NTA.

The possibility that EDTA or NTA complexed non-specific iron that remained bound to the lactoferrin was considered. Dialysis overnight against 1 mM-EDTA in 0.01 M-sodium phosphate buffer and then for a further 48 h against four changes of 0.01 M-sodium phosphate buffer did not decrease the ability of lactoferrin to catalyse ethylene production in the presence of EDTA.

Ethylene production from methional catalysed by lactoferrin–EDTA was inhibited by catalase (Table 2). Superoxide dismutase inhibited the reaction involving xanthine and xanthine oxidase, but not the ascorbate-dependent reaction. The $OH^{\cdot}$ scavengers formate and benzoate also inhibited. The percentage of inhibition by each was approximately the same as with Fe^{3+}–EDTA as catalyst (results not shown).

If lactoferrin contributes to the bactericidal activity of neutrophils, it is likely to do so within phagosomes, where the pH decreases to 5–6.

Table 2. *Effects of inhibitors and $OH^{\cdot}$ scavengers on ethylene production from methional catalysed by lactoferrin and EDTA (pH 7.4)*

For experimental details, see the legend to Table 1.

Additions	Methional oxidized (pmol/ml per min) Ascorbate/ H_2O_2	Xanthine/xanthine oxidase/H_2O_2
Lactoferrin (4 μM)/EDTA (400 μM)	447	250
Plus catalase (40 μg/ml)	27	5
Plus superoxide dismutase (10 μg/ml)	447	10
Plus benzoate (5 mM)	206	112
Plus formate (10 mM)	250	115

Table 3. *Ethylene production from methional at pH 5.0*
For experimental details, see legend to Table 1.

Additions	Rate of methional oxidation (pmol/ml per min) Ascorbate/H_2O_2	Xanthine/xanthine oxidase/H_2O_2
None	2	103
Lactoferrin (2 μM)	8	103
Lactoferrin (4 μM)	7	115
NTA (100 μM)	18	100
NTA (100 μM)/lactoferrin (1.2 μM)	47	144
NTA (100 μM)/lactoferrin (2 μM)	47	180
NTA (100 μM)/Fe^{3+} (5 μM)	162	470

Catalysis of OH• production at pH 5 was therefore investigated. Lactoferrin was compared with Fe^{3+}–NTA because Fe^{3+}–EDTA at this pH was found not to catalyse OH• production from H_2O_2 and ascorbate, and to have only very low activity with $O_2^{-\bullet}$. As shown in Table 3, with either $O_2^{-\bullet}$ or ascorbate, lactoferrin only very slightly increased the rate of ethylene production from methional above background. In the presence of NTA, however, lactoferrin enhanced the rate of ethylene production, and showed approx. a quarter the catalytic activity (per mol of iron) of an equivalent concentration of Fe^{3+}–NTA.

Discussion

The results of this study indicate that lactoferrin is a very poor catalyst of OH• production from H_2O_2 and either $O_2^{-\bullet}$ or ascorbate. The reaction conditions were such that catalysis by 0.05 μM-Fe^{3+}–EDTA (pH 7.4) or 0.05 μM-Fe^{3+}–NTA (pH 5) could be detected, and the increases due to lactoferrin were less than this. Ethylene production from methional has been used to detect OH•, and the findings with methional have been corroborated by similar results with MOB. Although neither reaction is necessarily specific for OH• (Pryor & Tang, 1978), negative findings provide strong evidence against OH• production. Special efforts were made to minimize contamination by iron or other metal ions capable of catalysing the reaction, to ensure that any effect due to lactoferrin was not obscured by a variable high background reaction. That lactoferrin is a very poor catalyst of OH• production from H_2O_2 and $O_2^{-\bullet}$ or ascorbate is also inferred from the finding that apolactoferrin could inhibit ethylene production, presumably by complexing contaminant iron. This interpretation is consistent with my previous observations (Winterbourn, 1981), and with the finding of Rosen & Klebanoff (1981) that lactoferrin does not support the bactericidal activity of an acetaldehyde/xanthine oxidase system. It is also consistent with the observation of Gutteridge *et al.* (1981) that iron-poor lactoferrin can inhibit lipid peroxidation, but it is not in agreement with the conclusions of Ambruso & Johnston (1981).

Lactoferrin in the presence of the complexing agents EDTA and NTA, however, has been shown to be a good catalyst of OH• production from H_2O_2 and either $O_2^{-\bullet}$ or ascorbate. That ethylene production from methional or MOB was due to OH• is strongly implied from the knowledge that other iron complexes catalyse OH• production from H_2O_2 and $O_2^{-\bullet}$ or ascorbate (Diguiseppe & Fridovich, 1980; Winterbourn, 1979), and from the inhibitory effects of benzoate and formate. It is unlikely that the complexing agents acted by removing either specifically or non-specifically bound iron, since prior dialysis against EDTA followed by buffer did not alter the absorption spectrum of the lactoferrin or eliminate its ability to catalyse OH• production when more EDTA was added. Lactoferrin binds iron only in association with an anion. This is normally bicarbonate, and the preparative procedure used in this study would be expected to produce the bicarbonate complex (Masson & Heremans, 1968). Lactoferrin is closely related to, and shows similar binding characteristics to, transferrin, which can complex with either EDTA or NTA in place of bicarbonate (Querinjean *et al.*, 1971; Schlabach & Bates, 1975; Aisen & Listowsky, 1980). The present findings suggest, therefore, that lactoferrin can form complexes with EDTA and NTA that are capable of catalysing OH• production from H_2O_2 and $O_2^{-\bullet}$ or ascorbate. The saturation kinetics seen on increasing the EDTA concentration are consistent with this. In both systems, the lactoferrin complexes were about a quarter as efficient catalysts as the same concentration of the appropriate free iron complex.

Anion replacement in transferrins is a slow process (Aisen & Listowsky, 1980) and the low catalytic activity of some lactoferrin preparations was probably due to incomplete removal of the NTA added to reconstitute the protein. It is possible that this is also the explanation for the positive findings of Ambruso & Johnston (1981). It would not, however, rationalize their conclusion that lactoferrin is a much better catalyst of OH• production than Fe^{3+}–EDTA. This was based on a small enhancement (three times background) of ethylene production from MOB given by 10 mM- or 100 nM-lactoferrin and only a 3-fold enhancement by 50 μM-Fe^{3+}–EDTA. This latter value is surprisingly low for a reaction known to require an iron catalyst, and

compares with 5 μM-Fe^{3+}–EDTA, causing a greater than 30-fold increase over background in the present study.

The results of the present study sound a note of caution that the presence of complexing agents such as EDTA or NTA can affect the properties of lactoferrin, and this must be considered when interpreting experiments *in vitro*. The EDTA or NTA complexes of lactoferrin are unlikely to be important catalysts of OH• production *in vivo*, but the possibility that other complexes of lactoferrin could catalyse the reaction warrants consideration. Other ligands, such as oxalate, malonate, lactate and glycinate, also complex with transferrin iron (Aisen & Listowsky, 1980), and their effects on the catalytic ability of lactoferrin warrant further investigation. Alternatively, lactoferrin in neutrophils may have an opposite function. Although there is some uncertainty about its natural state (Ambruso & Johnston, 1981), there are good indications that it is only partially saturated with iron (Bullen *et al.*, 1978; Bullen & Armstrong, 1979), in which case it could act by complexing iron and preventing reactions such as OH• production and lipid peroxidation (Gutteridge *et al.*, 1981).

I am most grateful to Dr. Sylvia Rumball and Mrs. Heather Baker for their gift of the lactoferrin. This work was supported by a grant from the Medical Research Council of New Zealand.

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