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Changes in Antioxidative Properties of Lactoferrin from Women's Milk during Deamidation

S. Belizy, I. N. Nasarova, V. N. Procof'ev, I. A. Sorokina*, N. V. Puschkina, and A. I. Lukach

Department of Biochemistry and Microbiology, School of Biology, Rostov-on-Don State University, Bolshaya Sadovaya ul. 105, Rostov-on-Don, 344006 Russia; fax: (8632) 64-5255; E-mail: bio@rnd.runnet.ru

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Abstract—Spontaneous deamidation of lactoferrin preparations from women's milk was found during incubation for 28 days under simulated physiological conditions (0.85% NaCl, pH 7.0, 37°C). After 28 days of incubation, this deamidation was associated with a 12% decrease in the total amide content in the protein. Addition of deamidated preparation to a suspension of lipoproteins from egg yolk in the presence of Rhodamine 6G decreased the total intensity of rapid and slow emission and also the intensity of the slow emission of Fe^{2+}-induced chemiluminescence by 37, 48, and 53%, respectively, suggesting an increase in the antioxidative activity of lactoferrin during deamidation. Deamidation obviously stimulated the nonspecific interaction of lactoferrin with iron ions and, consequently, increased the antioxidant effect of the protein in this way. This was supported by the finding of decreased antioxidative effectiveness of lactoferrin during its complete saturation with iron under the incubation conditions.

Key words: lactoferrin, deamidation, chemiluminescence, free radical reactions

Various changes occur in proteins during their functioning in the body due to chemical modification of their amino acid residues. Spontaneous, non-enzymatic post-translational modifications are especially interesting. The number of spontaneous modifications reported in the literature is constantly increasing. Spontaneous modifications are suggested to be an important evolutionary adaptation that regulates the "lifetime" of protein molecules [1-3].

Hydrolysis of the amides (deamidation) of asparagine and glutamine in polypeptides is one of the most important non-enzymatic modifications. Post-synthesis deamidation and the associated asparagine-dependent auto-hydrolysis of proteins cause changes in their structure and physicochemical properties and in susceptibility to proteolytic enzymes in tissues [4]. These changes decrease the specific biological activities of the proteins [5, 6]. Lactoferrin, which has antiradical properties, is one of the most important constituents of human milk [7-9]. Nonspecific antioxidant activity of lactoferrin was shown earlier by chemiluminescence in a model system of the Fenton reaction [10].

The present work was designed to study in model experiments the effect of post-translational deamidation on the antioxidant functions of human lactoferrin.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

SDS was from Serva (Germany); chromatography carriers Servacel CM-52 and Toyopearl HW-52 were from Reanal (Hungary) and Toyo-Soda (Japan), respectively; a commercial lactoferrin preparation from women's milk was from Sigma (USA); the fluorescent dye Rhodamine 6G was from Reanal (Hungary). All other reagents were of special purity. Lactoferrin was prepared from fresh women's foremilk (milk of the first four days of lactation) that was stored at −20°C.

Lactoferrin was isolated and purified as described in [11] with certain modifications, including ion-exchange chromatography on a column with Servacel CM-52 and gel filtration on a column with Toyopearl HW-52. The homogeneity of the resulting protein was monitored by N-terminal amino acid residues as described in [12] and also by SDS-PAGE by the method of Laemmli [13] using gels with a 9-25% linear gradient of acrylamide concentration. The purity of the lactoferrin was 95-96%. During the isolation, purification, and determination of homogeneity of lactoferrin, commercial lactoferrin was used as a standard.

The dynamics of lactoferrin deamidation were studied during incubation for 28 days at 37°C in neutral saline containing 1% protein, 0.85% NaCl, pH 7.0, under ster-

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ile conditions provided with 0.22 μ bacterial filters (Schleicher Schull, Germany). On the 14th and 28th days of the incubation, lyophilized lactoferrin preparations were used for quantitative determination of total amide groups (TAG) and of easily and poorly hydrolyzable amide fractions (EHA and PHA), which released ammonia, respectively, within 15 min and 3 h of hydrolysis in 0.5 M H_2SO_4 at 100°C [4]. The free ammonia concentration after hydrolysis of amide groups was determined fluorometrically [14] and expressed in μmole ammoniacal nitrogen per g protein, which was quantitatively determined as described in [15]. The fluorimetry was carried out using a Hitachi 650-50 spectrofluorimeter (Japan) (λ_{ex} = 413 nm, λ_{em} = 476 nm). The content of PHA was calculated by the difference between TAG and EHA. From the contents of these amide fractions, the contents of asparagine and glutamine in proteins can be approximated [14]. All data presented in this work are mean values of 8-10 determinations.

The extent of hydrolysis of lactoferrin was assessed by the increase in the content of soluble Folin-positive products [15].

Lactoferrin was saturated with iron in buffer containing 0.1 M sodium citrate and 0.1 M $NaHCO_3$ (buffer A, pH 8.5). Lactoferrin (1.1%, 1.2 ml) was titrated with 20 μl of $FeSO_4 \cdot 7H_2O$ solution in buffer A containing 20 mM Fe^{3+}. The unbound Fe^{3+} was removed by dialysis against buffer A. Changes in the color intensity were followed spectrophotometrically at 470 nm. Iron contents in the lactoferrin preparations were determined with phenanthroline reagent using the test for iron concentration in blood serum [6].

The antioxidative activity of lactoferrin was determined by its effect on the Fe^{2+}-induced chemiluminescence of yolk lipoproteins (YLP) from hen's egg in the presence of Rhodamine 6G [16]. Chemiluminescence was studied using a device for recording induced chemiluminescence that was constructed in the Research Institute of Biology, Rostov-on-Don State University, based on a 22028 scintillation spectrometer (VEB RFT Messelektronik Otto Schön, Germany) with a FEU-37 photomultiplier as the detector. The yolk lipoprotein suspension was prepared by dilution 1 : 10 of hen's egg yolk with distilled water. To enhance the Fe^{2+}-induced chemiluminescence, the fluorescent dye Rhodamine 6G was used as an activator. The yolk suspension (200 μl), 50 μl of 1 mM Rhodamine 6G, 3 ml of 25 mM potassium phosphate buffer (pH 7.7, 37°C), and 100 μl of lactoferrin solution (5 mg protein per ml) were placed into a thermostatted measuring cuvette. The mixture was kept at 37°C with stirring for 100 sec, and the background luminescence was recorded; then the system was supplemented with 500 μl of 25 mM $FeSO_4$ to initiate chemiluminescence, and the development of chemiluminescence was recorded.

The antioxidant effect of lactoferrin was determined by the total intensity of the rapid emission (E_r), which characterizes the content of lipid peroxides, and of the slow emission (E_s) over the first 1000 sec of the latter, which characterizes the branching of free radical processes, by the slow emission amplitude (*I*) and the duration of the latent period of chemiluminescence (*Y*) depending on the prooxidant/antioxidant ratio. *I* was measured from the recording chart and expressed in mm; *Y* is in sec.

RESULTS AND DISCUSSION

Spontaneous deamidation of the lactoferrin preparation was found during its incubation under simulated physiological conditions, with a gradual decrease in the amide content by 12 (TAG) and 18% (EHA), respectively (Table 1). Note that the decrease in the amide level was principally of the EHA fraction, which consists mainly of asparagine [4], and this is consistent with literature indicating the predominant deamidation of asparagine and indirectly confirms that the deamidation is non-enzymatic [17]. The spontaneity of the deamidation was also confirmed by the high purity of the lactoferrin preparation as determined by SDS-PAGE [13], by the contents of N-terminal amino acids [12], and by the absence of prote-

Table 1. Amide level and content of TCA-soluble Folin-positive products after incubation of lactoferrin preparations for 14 and 28 days under conditions simulating physiological "aging" (0.85% NaCl, pH 7.0, 37°C)

Time of incubation, days	Amides, μmole N/NH_3 per g protein			TCA-soluble products of protein destruction, mg tyrosine per g protein
	TAG	EHA	PHA	
0 (control)	813.4 ± 19.5	424.9 ± 6.3	388.4 ± 15.6	0.43 ± 0.05
14	797.2 ± 12.0	384.5 ± 8.8*	412.7 ± 4.7	0.66 ± 0.1**
28	720.0 ± 31.4*	349.1 ± 12.6*	370.9 ± 21.5	1.3 ± 0.1**

* Significant difference ($p < 0.05$) relative to 0 h of lactoferrin incubation.
** Significant difference ($p < 0.05$) relative to control.

olytic enzyme admixtures and of microbial contamination.

Obviously, the non-enzymatic deamidation was accompanied by conformational disorders in the protein that could not fail to affect the biological properties of the molecule. In our laboratory, the antioxidant protection enzymes of the body, such as catalase, lactoperoxidase, and human blood plasma albumin were shown to significantly lose their antiradical activities depending on their deamidation levels [18]. Thus, 14% deamidation of total amides decreased the activity of catalase by 52%, and 10% decrease in the amide level of the nonspecific antioxidant albumin inactivated it by 22% [18].

The effect of spontaneous deamidation on the antioxidative properties of lactoferrin was studied in model systems during the development of free radical oxidation in lipid substrates in a suspension of lipoproteins from hen's egg yolk (Table 2). Because of the modification of the lactoferrin preparation during 28 days of incubation, the total intensity of the rapid and slow chemiluminescence emissions and the height of the slow emission decreased dramatically compared to the control: by 47.6, 48, and 53.1%, respectively. The latent period concurrently increased by 74.7%. Compared to the native preparation, the total intensity of the rapid and slow chemiluminescence and the height of the slow emission decreased by 29, 29.4, and 25.8%, respectively, and the latent period concurrently increased by 39.3%. We suggest that the increased antioxidant effect of the modified lactoferrin should be explained, on one hand, by its increased ability to bind Fe^{3+} to additional carboxyl groups released during the spontaneous deamidation. This was confirmed by chemiluminescence studies of the effect of saturation of lactoferrin with iron on the antiradical characteristics of the protein (Table 3). Addition of 45% iron-saturated lactoferrin from women's milk to a model system of YLP–Fe^{2+}–Rhodamine 6G decreased by 37.4% the intensity of the rapid chemiluminescence. The antioxidant properties of the completely iron-saturated lactoferrin under the same experimental conditions were decreased by 11.4% compared to the partially saturated lactoferrin, but the intensity of chemiluminescence remained 25.7% lower than in the control reaction medium.

On the other hand, the activation of the antioxidant properties of lactoferrin is probably caused by hydrolysis of the protein, in particular, by auto-hydrolysis resulting in a great number of low-molecular-weight compounds, including so-called medium-molecular-weight molecules that contain peptide constituents with antioxidative properties [19].

Most low-molecular-weight catabolites, including such antioxidants as urea, urate, and medium-molecular-weight molecules display their effects through their interaction with iron ions. The 39.3% prolongation of the chemiluminescence latent period indirectly suggests an increase in the pool of low-molecular-weight antioxidants in the system because the latent period reflects the oxidation rate of Fe^{2+} up to a critical concentration of Fe^{3+} and depends on the prooxidant/antioxidant ratio in the system [20]. Hydrolysis of the peptide chain increases the content of α-carboxyls in the system, and these are likely to interact with iron ions and change their ability to generate free radicals. It is obvious that these fragments of lactoferrin molecules are responsible for the additional quenching of chemiluminescence.

Table 2. Effect of lactoferrin (LF) on development of Fe^{2+}-induced chemiluminescence of a lipoprotein suspension from hen's egg yolk in the presence of Rhodamine 6G

Experiment	Total intensity of the rapid emission (E_r) × 10^3, counts	Latent period (Y), sec	Total intensity of the slow emission (E_s) × 10^3, counts for 1000 sec	Height of the slow emission (I), mm
Control*	434.4 ± 5.7	91.4 ± 6.3	3269.8 ± 31.9	120.2 ± 10.0
LF	320.6 ± 8.9 ($p_1 < 0.001$)	114.6 ± 6.20 ($p_1 < 0.05$)	2405.8 ± 34.71 ($p_1 < 0.001$)	76.1 ± 6.25 ($p_1 < 0.01$)
LF after 14 days of incubation	273.0 ± 11.7 ($p_1 < 0.01$) ($p_2 < 0.01$)	145.2 ± 8.1 ($p_1 < 0.001$) ($p_2 < 0.05$)	2387.3 ± 45.6 ($p_1 < 0.01$)	74.4 ± 8.2 ($p_1 < 0.01$)
LF after 28 days of incubation	227 ± 9.1 ($p_1 < 0.001$) ($p_3 < 0.01$)	159.7 ± 9.5 ($p_1 < 0.001$)	1699.2 ± 32.3 ($p_1 < 0.001$) ($p_3 < 0.001$)	56.5 ± 4.0 ($p_1 < 0.001$) ($p_3 < 0.05$)

Note: p_1) significant difference relative to control; p_2) significant difference between the native lactoferrin and the modified lactoferrin after 14 days of incubation; p_3) significant difference between the native lactoferrin and the modified lactoferrin after 28 days of incubation.

* Control reaction mixture without lactoferrin.

Table 3. Effect of saturation of lactoferrin (LF) with iron on the development of free radical oxidation of yolk lipoproteins induced by the Fe^{2+}–Rhodamine 6G system

Saturation of LF with iron, %	Total intensity of the rapid emission (E_r) × 10^3, counts	Latent period (Y), sec	Total intensity of the slow emission (E_s) × 10^3, counts for 1000 sec	Height of the slow emission (I), mm
Control*	448.8 ± 9.1	98.3 ± 7.1	3386 ± 50.2	115.5 ± 8.6
LF (45)	280.3 ± 6.2 ($p_1 < 0.001$)	128.2 ± 9.9 ($p_1 < 0.05$)	1910 ± 39.5 ($p_1 < 0.001$)	72.6 ± 6.2 ($p_1 < 0.01$)
LF (100)	342.4 ± 7.6 ($p_1 < 0.001$) ($p_2 < 0.001$)	108.1 ± 9.3	2328 ± 44.9 ($p_1 < 0.001$) ($p_2 < 0.001$)	85.5 ± 5.9 ($p_1 < 0.05$)

Note: p_1) significant difference relative to control; p_2) significant difference between the lactoferrin preparations differently saturated with iron.
* Control reaction mixture without lactoferrin.

We studied changes in the contents of Folin-positive TCA-soluble components in the lactoferrin preparations during incubation under simulated physiological conditions (Table 1). The content of TCA-soluble products of lactoferrin destruction increased during deamidation. By the 14th day of incubation in saline, the TCA-soluble product content increased by 53.5%, and after 28 days it was increased more than threefold, in agreement with literature showing that non-enzymatic deamidation loosens the protein conformation and causes occasional breaks in the covalent polypeptide skeleton (asparagine-dependent auto-hydrolysis) [4].

Our findings indicate that the spontaneous deamidation stimulated a nonspecific interaction of the lactoferrin preparation from women's milk with iron ions and thus increased the antioxidative effect of the protein by this mechanism. Thus, lactoferrin has an advantage compared to other protein antioxidants: its antiradical properties increase during deamidation and auto-hydrolysis.

The situation simulated in our experiments is quite probable also for lactoferrin that has circulated for a long time in blood and is shown by various authors to be identical to lactoferrin from women's milk and other human tissues [16, 20]. The blood serum content of lactoferrin is not high, being 0.1 ± 0.02 mg/100 ml [20]. However, like ceruloplasmin, lactoferrin is an acute phase protein, and blood levels of such proteins significantly increase during any inflammation [21, 22]. Therefore, attempts have been made to use the blood lactoferrin level as a diagnostic and predictive test in pneumonia [23]. It is suggested that hyposiderosis observed in acute infections in adults and children should also be associated with an increase in the blood content of lactoferrin [23].

It seems that under extreme conditions lactoferrin can supplement or substitute for transferrin, which has a similar structure but is a poor antioxidant effect compared to other inhibitors [24]. As well as transferrin and along with ceruloplasmin, the more effective lactoferrin seems to be a component of the blood serum antioxidant system [21, 22, 24]. The effects of these proteins seem to be provided by the oxidation of Fe^{2+} and binding of Fe^{3+} and by their interaction with oxygen radicals [6]. About 60% of the amino acid sequence in the lactoferrin molecule of women's milk is homologous to that of transferrin, and, consequently, lactoferrin has similar physicochemical properties [25] and can bind two iron atoms. However, the binding of Fe^{3+} to lactoferrin is very stable, and the affinity of lactoferrin for iron is 300 times higher than the affinity of transferrin [26], and this determines the antioxidant properties of lactoferrin.

Finally, specific features of the structure of milk lactoferrin responsible for the increase in its antioxidant activity during deamidation and fragmentation are suggested to play an important biological role: the antioxidant effect of this protein is suggested to increase during its partial destruction in the gastrointestinal tract of an infant. Peptides with antioxidant effects generated from the milk lactoferrin can be an important constituent of antiradical protection within the first days of life when an infant's own antioxidant systems are not yet completed and its body is subjected to oxidative stress caused by the immediate contact with oxygen [26].

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