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Reduction of Ferrylmyoglobin by β-Lactoglobulin

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Reduction of iron (IV) in ferrylmyoglobin in the presence of β-lactoglobulin in aqueous solution is the result of two parallel reactions: (i) a so-called autoreduction, and (ii) reduction by β-lactoglobulin in a second-order-reaction resulting in bityrosine formation in β- lactoglobulin. In the pH-region investigated (5.4–7.4), the rate of reduction increased for both reactions with decreasing pH. The second order-reaction had for non-denatured β-lactoglobulin the activation parameters: $\Delta H^{\neq} = 45$ kJ·mol^{-1} and $\Delta S^{\neq} = -93$ J·mol^{-1}·K^{-1} at pH = 7.0 and ionic strength 0.16 (NaCl). Reduction of ferrylmyoglobin by β-lactoglobulin denatured by heat (86°C for 3 min) or by hydrostatic pressure (300 MPa for 15 min) resulted in formation of higher molecular weight species as detected by size-exclusion chromatography and by SDS-PAGE. No molecular weight changes were observed for reduction of ferrylmyoglobin by native β-lactoglobulin. Detection of bityrosine in the native β-lactoglobulin fraction after oxidation with ferrylmyoglobin indicated intra-molecular bityrosine formation. In heat-denatured β-lactoglobulin bityrosine formation could be of intra-molecular and/or of inter-molecular origin, the latter being confirmed by size- exclusion chromatography.

Key words: Ferrylmyoglobin, β-lactoglobulin, cross-linking, bityrosine.

INTRODUCTION

Myoglobin and haemoglobin are oxygen storage and transport proteins. These haem proteins will, in the presence of peroxides or peroxide-generating systems, also catalyze oxidation through intermediate formation of hypervalent iron compounds.[1] Metmyoglobin, MbFe(III), reacts with peroxides in a two-electron transfer process forming the so-called perferrylmyoglobin, •MbFe(IV)=O, a short-lived protein radical with iron in the +4 state, and in an one-electron transfer process forming the more long-lived ferrylmyoglobin, MbFe(IV)=O.[2,3,4] The protein radical in perferrylmyoglobin may be formed directly or generated by an electron transfer from the protein moiety to an initially formed porphyrin radical cation, as has been suggested for cytochrome c peroxidase.[5] Myoglobin and haemoglobin are considered to be 'pseudo' peroxidases because they are both modified by reaction with H_2O_2[6] making them less effective in a cyclic generation of the perferryl-form.[2] As a consequence, the catalytic power of H_2O_2-activated myoglobin and haemoglobin in reaction with traditional peroxidase substrates is much less than for 'normal' peroxidases.[7]

The high abundance of myoglobin and haemoglobin in mammalian tissue has, however,

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prompted a series of investigations on the catalytic action of H_2O_2-activated myoglobin and haemoglobin using a wide range of low molecular weight reductants.[8–14] In addition, H_2O_2-activated myoglobin and haemoglobin have been shown to oxidize proteins resulting in cross-linkage of the oxidized protein.[15–18] Hanan & Shaklai[16] suggest that oxidation of proteins by H_2O_2 is catalyzed more effectively by myoglobin than by horseradish peroxidase. Most studies on protein oxidation by H_2O_2, catalyzed by myoglobin or haemoglobin have used large excess of H_2O_2 making it possible to transfer both oxidation equivalents to the reducing substrate and to regenerate the perferryl species several times. The protein radical is required for H_2O_2-activated myoglobin to cross-link myosin according to the findings of Hanan & Shaklai.[16] A similar requirement for the second oxidation equivalent is likewise observed for epoxidation of styrene mediated by H_2O_2-activated myoglobin,[19,20] in marked contrast to what has been found for the oxidation of linoleic acid.[21]

The present kinetic study explores the reactivity of H_2O_2-activated myoglobin towards proteins in homogenous solution under conditions for which the ferrylmyoglobin rather than the perferrylmyoglobin is the dominant reactant. Both hypervalent forms of myoglobin are of importance for oxidative modifications of other biomolecules, and while •MbFe(IV)=O may be more reactive, MbFe(IV)=O is kinetically more stable, in effect allowing it to react on a longer time scale. β-Lactoglobin was chosen as the reducing protein as it is highly soluble in water and it is well-characterized also with respect to the thermodynamics of pressure denaturation,[22] allowing the effect of denaturation to be studied without precipitation of the protein.

MATERIALS AND METHODS

Chemicals

MbFe(III) (horse heart, type III) was obtained from Sigma (St. Louis, MO, USA). Hydrogen peroxide 30% pro analysis was purchased from Merck (Damstadt, FGR) and β-lactoglobulin was kindly supplied by MD Foods Ingredients, Danmark Protein (Videbæk, Denmark). Other chemicals were of analytical grade and double deionized water were used throughout. All buffers in experiments concerning molecular changes were passed through a Chelex-100 column (Sigma, St. Louis, MO, USA) in order to remove any free metal ions.

pH Measurements

For the kinetic studies, pH was measured relative to titrated hydrochloric acid solutions in the actual salt medium as concentration standards with a Radiometer PHM82 pH-meter (Radiometer, Copenhagen, Denmark) and a combination glass electrode with 3 $mol \cdot L^{-1}$ KCl in the reference part.

Formation and Reduction of Ferrylmyoglobin

MbFe(III) was dissolved in 5 mmol/l phosphate buffer (pH = 7.0) and purified on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. MbFe(IV)=O was prepared by mixing 2.2 ml 0.13 mmol/l MMb (ε_{525} = 7700 $l \cdot mol^{-1} \cdot cm^{-1}$)[23] with 0.30 ml 7.6 mmol/l H_2O_2 (ε_{240} = 39.4 $l \cdot mol^{-1} \cdot cm^{-1}$).[24] The resulting solution was allowed to react for 1 min and then cooled in ice for 1 min. Excess H_2O_2 was removed by applying the solution onto a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated and eluted with cold (approximately 5°C) 5 mmol/l phosphate buffer (pH = 7.0) resulting in a concentration of MbFe(IV)=O in the effluent solution in the range of 0.09–0.11 mmol/l. The MbFe(IV)=O solution was thermally equilibrated for 2 min in a waterbath to the desired temperature. 500 µl MbFe(IV)=O solution was mixed with 500 µl 1.0–4.0 mmol/l β-lactoglobulin (Mw = 18 kD)[25] dissolved in 100 mmol/l phosphate buffer ($5.4 < pH < 7.4$, I = 0.32 adjusted with NaCl). The final concentrations in the reaction mixtures were 0.045–0.055 mmol/l ferrylmyoglobin and 0.5–2.0 mmol/l β-lactoglobulin in 50 mmol/l

phosphate buffer (I = 0.16 adjusted with NaCl). Blanks were made by substitution of the β-lactoglobulin solution with phosphate buffer solution. Spectral changes ($450 < \lambda < 700$) during the reduction of MbFe(IV)=O in the presence of 1 mM β-lactoglobulin (25°C; pH = 5.5 or pH = 7.0) was obtained using a HP8452 UV-VIS diode array spectrophotometer (Hewlett Packard Co., Palo Alto, CA, USA). For all other reactions the reduction of MbFe(IV)=O was followed at 588 nm using a Shimadzu UV-2101PC spectrophotometer (Shimadzu Corp., Tokyo, Japan) equipped with a temperature- controlled cuvette compartment preset at the desired temperature. pH in the samples was measured after completion of the reaction. All rate constants reported are the mean of two independent determinations.

Calculations

Rate constants were calculated by a least squares method fitting the three parameters b, a and k_{obs} to the integrated rate expression: $A_{588\ nm,\ t} = b + a \cdot \exp(-k_{obs} \cdot t)$, initiating the algorithm with random parameter values. The temperature dependence of the second order reduction of MbFe(IV) = O by β-lactoglobulin was described by the Arrhenius equation, $\ln k = a + b/T$ (k = second-order rate constant, a = ln A (frequency factor), b = Ea/R) and converted to enthalpy of activation ($\Delta H^{\neq}$) and entropy of activation ($\Delta S^{\neq}$) using transition state theory.[26] Confidence intervals (95%) for $\Delta H^{\neq}$ and $\Delta S^{\neq}$ were estimated according to Zar.[27] All equations were implemented in MATLAB version 4.2a (Mathworks Inc., Natick, MA, USA)

Effect of Denaturation of β-Lactoglobulin on Reaction between β-Lactoglobulin and MbFe(IV)=O

MbFe(IV) = O was formed and reduction was followed as described above. β-lactoglobulin (4 mmol/l) was dissolved in 20 mmol/l Tris/HCl buffer (pH = 8.0). The β-lactoglobulin solution was denatured at 300 MPa for 15 min at 25°C in a closed 750 µl quartz bottle with teflon lid for pressure transmission using a temperature-controlled HPSC-3K pressure cell (SLM Instruments Inc., Urbana, IL, USA). The protein solution was used 30 min after pressure release as protein denaturation by pressure may be reversible.[28] The reaction was started by mixing 500 µl MbFe(IV)=O, 250 µl 200 mmol/l phosphate buffer (pH = 6.5, I = 0.64 adjusted with NaCl) and 250 µl native or pressure-denatured β-lactoglobulin in a cuvette placed in the temperature-controlled cuvette compartment. The final concentrations in the reaction mixture were 0.045–0.055 mmol/l MbFe(IV)=O and 1.0 mmol/l β-lactoglobulin in 50 mmol/l phosphate buffer (pH = 6.8, I = 0.16 adjusted with NaCl).

Molecular Weight Changes during the Reaction between β-Lactoglobulin and MbFe(IV)=O

MbFe(III) was dissolved in 50 mmol/l phosphate buffer (pH = 6.5, I = 0.16 adjusted with NaCl) and purified on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. β-lactoglobulin (0.067 mmol/l) was dissolved in 5.0 mmol/l phosphate buffer (pH = 7.0, I = 0.16 adjusted with NaCl) except for samples intended for pressure denaturation for which β-lactoglobulin was dissolved in 20 mmol/l Tris/HCl (pH = 8.0), as pressure decreases the pH of phosphate buffers significantly. Heat denaturation of the β-lactoglobulin solution was performed in a waterbath (86°C) for 3 min. Pressure denaturation of β-lactoglobulin was performed as described above. A chromatographic procedure was performed for each of the three forms of β-lactoglobulin (i.e. native, heat-denatured and pressure-denatured). Four different reaction media in 50 mmol/l phosphate buffer (pH = 6.5, I = 0.16 adjusted with NaCl) were made for each chromatographic procedure: (i) 0.010 mmol/l MbFe(IV)=O, 0.0083 mmol/l β-lactoglobulin; (ii) 0.010 mmol/l MbFe(III), 0.0083 mmol/l β-lactoglobulin; (iii) 0.010 mmol/l MbFe(IV)=O; (iv)

0.015 mmol/l H_2O_2, 0.0083 mmol/l β-lactoglobulin. MbFe(IV)=O was generated in the solution from H_2O_2 (0.015 mmol/l) and MbFe(III) (0.010 mmol/l) by reaction for 5 min prior to addition of β-lactoglobulin. The reaction mixtures were then incubated for 10 min at room temperature and analyzed using the Pharmacia FPLC system. (Pharmacia, Uppsala, Sweden). A size-exclusion column (Superdex-75 HR10/30; Pharmacia, Uppsala, Sweden) was equilibrated with 50 mmol/l phosphate buffer (pH = 6.5, I = 0.16 adjusted with NaCl). After application of 200 µl sample the column was eluted with a flow rate of 0.40 ml/min. SDS-PAGE was performed for the experiment employing heat-denatured β-lactoglobulin using a Multiphor II and ExcelGel, SDS, gradient 8–18 (Pharmacia, Uppsala, Sweden). The protein bands were visualized using a standard silver staining procedure (Pharmacia, Uppsala, Sweden).

Detection of Bityrosine in β-Lactoglobulin after Reaction with MbFe(IV)=O

Samples were prepared with native or heat-denatured β-lactoglobulin as described in the previous section with the exception that the total sample volume was increased to 10 ml. The concentration of MbFe(III), H_2O_2 and β-lactoglobulin were in addition increased to 0.080 mmol/l, 0.12 mmol/l and 0.033 mmol/l, respectively. After incubation, the phosphate buffer was substituted with a Tris/HCl buffer by applying the sample onto a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 20 mmol/l Tris/HCl buffer (pH = 8.3). Myoglobin and β-lactoglobulin were separated using the Pharmacia FPLC system with an ion-exchange column (Resource Q, Pharmacia, Uppsala, Sweden) equilibrated with buffer A (20 mmol/l Tris/HCl, pH = 8.3). After application of 10 ml sample, elution was performed using the following procedure: 3 min with buffer A, 0–60% buffer B (1 mol/l NaCl in buffer A) for 25 min, 60–100% buffer B for 2 min, and finally 3 min at 100% buffer B. The β-lactoglobulin fraction was collected and freeze dried. The protein was hydrolyzed in 0.70 ml 6 mol/l HCl for 24 hours (110°C). 20 µl of the hydrolyzed sample was injected onto a HPLC column (Nucleosil 120–5 C18, 250 × 4 mm, Macherey-Nagel Duren, Düren, FGR), which was equilibrated with 4% acetonitrile in aqueous 0.10 M citric acid (pH 2.55), with a flow of 1 ml/min. Chromatographic separation was performed on a HP 1090 liquid chromatograph equipped with a HP 1046A fluorescence detector (Hewlett Packard Co., Palo Alto, CA, USA). Tyrosine was eluted within 4.2 min and quantified by excitation at 275 nm and fluorescence emission at 310 nm. Bityrosine was eluted after 4.2 min and quantified by excitation at 283 nm and fluorescence emission at 410 nm.[29] The amount of tyrosine was used to determine the total protein content using a standard curve with β-lactoglobulin as standard after HCl-hydrolysis.

RESULTS

Reduction of ferrylmyoglobin, MbFe(IV)=O, in aqueous solution was found to be accelerated by the presence of β-lactoglobulin as seen from the insert in Figure 1. The product spectrum showed that MbFe(IV)=O was reduced to MbFe(III) by β-lactoglobulin and the constant wavelengths of the isosbestic points throughout the reaction indicate minimal alteration of the myoglobin during the reaction at pH = 7.0 (Figure 1) and at pH = 5.5 (spectra not shown). This does however not exclude initial modification of the globin part in myoglobin by reaction with H_2O_2 due to decay of the protein radical and/or autoreduction of ferrylmyoglobin.[2,6] The disappearance of MbFe(IV)=O could for excess concentrations of β-lactoglobulin be described by first-order kinetics for all reaction conditions investigated. The observed pseudo-first order rate constant, k_{obs}, was further found to depend linearly on the β-lactoglobulin concentration as shown in Figure 2, in agreement with a β-lactoglobulin dependent reaction occuring

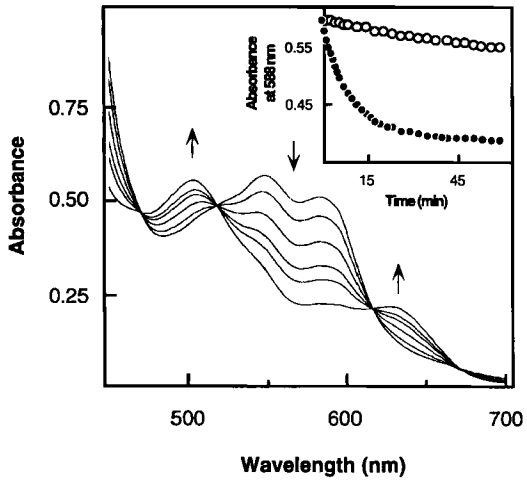

FIGURE 1 Spectral changes during the reduction of 0.05 mmol/l MbFe(IV)=O in the presence of 1.0 mmol/l β-lactoglobulin in phosphate buffer (pH = 7.0, I = 0.16 adjusted with NaCl) at 25.0°C. Spectra obtained 0, 2, 6, 12, 18 and 60 min after initiation of the reaction. Insert: absorbance change at 588 nm for the same reaction in absence (O) or in presence (●) of β-lactoglobulin.

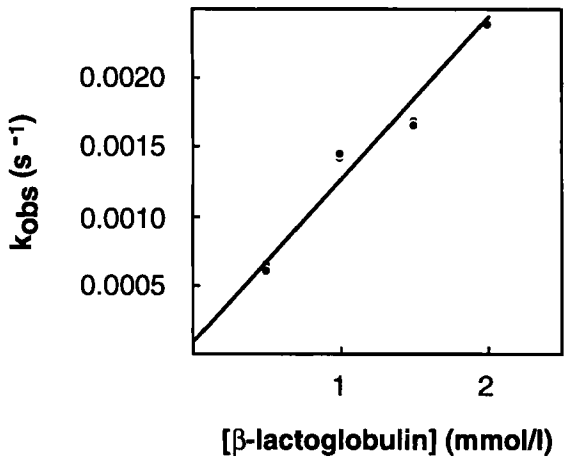

FIGURE 2 Pseudo-first order rate constant; k_{obs}, for the reduction of MbFe(IV)=O in the presence of 0.50–2.0 mmol/l β-lactoglobulin in 50 mmol/l phosphate buffer (pH = 7.0, I = 0.16 adjusted with NaCl) at 25.0°C. Values are the mean of two independent determinations.

simultaneously with an autoreduction reaction. At pH = 7.0, I = 0.16 (NaCl) and 25.0°C, the extrapolated rate constant was $1.5 \cdot 10^{-4}$ s^{-1} (95% confidence interval: ± $2.9 \cdot 10^{-4}$ s^{-1}) for zero β-lactoglobulin concentration (Figure 2), which is in agreement with the value $1.4 \cdot 10^{-4}$ s^{-1} obtained for similar reaction conditions in a separate study of the autoreduction reaction.[12] This confirms the presence of two independent reduction paths. The autoreduction reaction has previously been shown to be specific acid catalyzed,[12] i.e. a decrease in pH by 1 increases the reaction rate by a factor 10. The reduction by β-lactoglobulin was also found to depend on pH as may be seen from Figure 3. However, the direct reduction by β-lactoglobulin cannot be described as a specific acid catalyzed reaction and is rather described by a reaction model including an acid-base equilibrium of MbFe(IV)=O with an acid dissociation constant around 10^{-5} mol/l, as was also found for reduction of MbFe(IV)=O by NADH.[12] The protonated form of MbFe(IV)=O reacts faster than the form present at neutral pH. The temperature dependence of the reaction of the latter form could be described by the Arrhenius equation as shown in Figure 4 (linearity accepted on a 5% significance level). The second-order reaction:

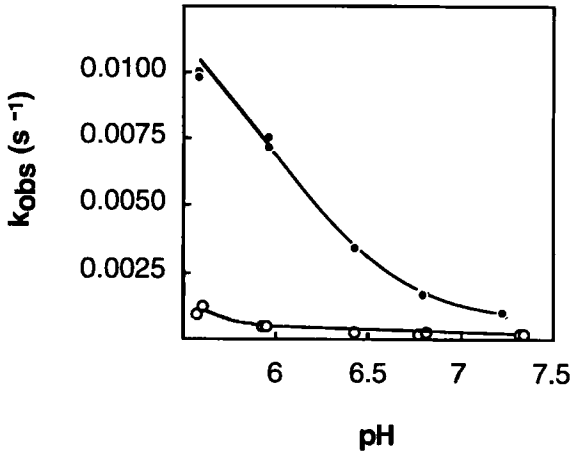

FIGURE 3 Pseudo-first order rate constant, k_{obs}, for the reduction of MbFe(IV)=O in absence (O) or in presence (●) of 1.0 mmol/l β-lactoglobulin in 50 mmol/l phosphate buffer (5.4 < pH < 7.4, I = 0.16 adjusted with NaCl) at 25.0°C. Values are the mean of two independent determinations.

MbFe(IV)=O + β-lactoglobulin → MbFe(III) + oxidized β-lactoglobulin

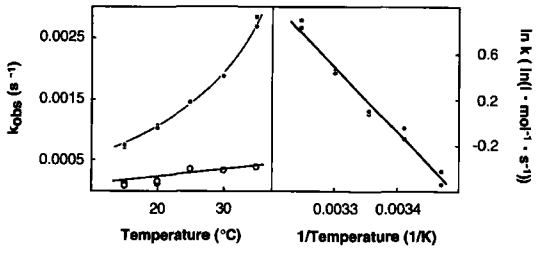

FIGURE 4 Temperature dependence of the reduction of MbFe(IV)=O. A: Pseudo-first order rate constant, k_{obs}, for reduction of MbFe(IV)=O in the absence (○) or the presence (●) of 1.0 mmol/l β-lactoglobulin in 50 mmol/l phosphate buffer (pH = 7.0, I = 0.16 adjusted with NaCl) at 15–35°C. B: Arrhenius plot of second-order rate constants for the reduction of MbFe(IV)=O by β-lactoglobulin corrected for autoreduction. All values are the mean of two independent determinations.

was found to have the following activation parameters with 95% confidence intervals: $\Delta H^{\neq}$ = 45 kJ·mol^{-1} ± 6 kJ·mol^{-1} and $\Delta S^{\neq}$ = – 93 J·mol^{-1}·K^{-1} ± 20 J·mol^{-1}·K^{-1}, at pH = 7.0, I = 0.16 (NaCl) and 25.0°C.

Pressure-denaturation of β-lactoglobulin increased the rate of reduction of MbFe(IV)=O, as shown for a β-lactoglobulin concentration of 1.0 mmol/l at pH = 6.8, I = 0.16 (NaCl) and 25.0°C. k_{obs} (k_{obs} = 2.23·10^{-4} s^{-1}) for pressure-denatured β-lactoglobulin was significantly ($P<0.05$) larger than k_{obs} (k_{obs} = 1.99·10^{-4} s^{-1}) for native β-lactoglobulin.

The oxidative changes in the protein moieties of the reactants were investigated using size-exclusion chromatography. The chromatograms of these reaction mixtures (A: MbFe(IV)=O/β-lactoglobulin; B: MbFe(IV)=O/heat-denatured β-lactoglobulin; C: MbFe(IV)=O/pressure-denatured β-lactoglobulin) are shown in Figure 5. Myoglobin and β-lactoglobulin where both eluted as single peaks. β-Lactoglobulin had the shortest elution time compared to myoglobin in agreement with its higher molecular weight. Heat-denatured β-lactoglobulin was eluted as 3 peaks corresponding to molecular weight species of 22.8, 36.1 and 47.5 kD. The pressure denatured β-lactoglobulin gave similar chromatograms but with indistinct peaks. The results show that it is only possible to

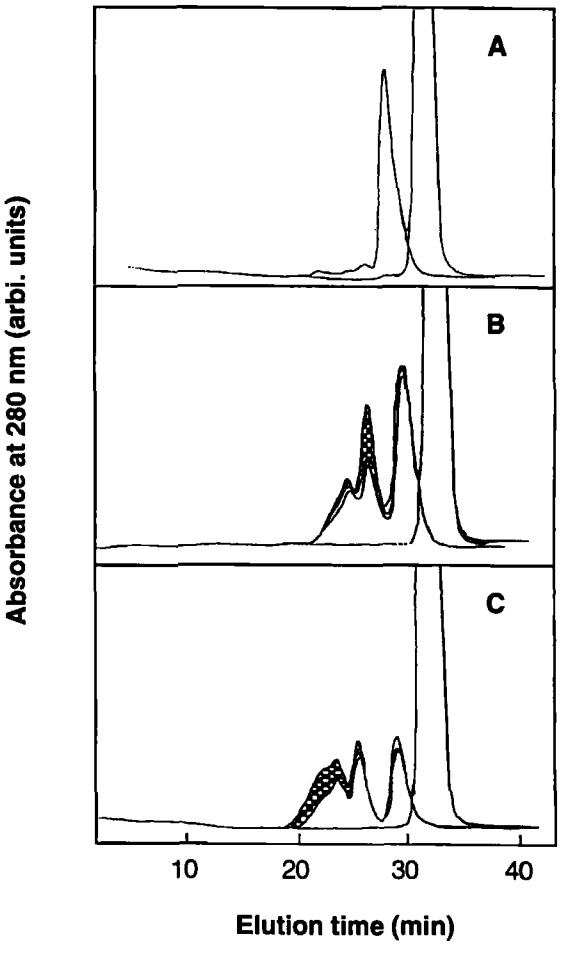

FIGURE 5 Size-exclusion chromatograms for β-lactoglobulin after reaction with MbFe(IV)=O. Each panel shows four chromatograms superimposed and chequered area represents the difference between reaction mixtures containing MbFe(IV)=O/β-lactoglobulin and MbFe(III)/β-lactoglobulin, MbFe(IV)=O or H_2O_2/β-lactoglobulin. A: Native β-lactoglobulin. B: Heat- denatured β-lactoglobulin. C: Pressure-denatured β-lactoglobulin. Reaction conditions: 0.0083 mmol/l β-lactoglobulin, 0.010 mmol/l MbFe(III), 0.010 mmol/l MbFe(IV)=O (prepared from 0.010 mmol/l MbFe(III) and 0.015 mmol/l H_2O_2 5 min prior to incubation) in 50 mmol/l phosphate buffer (pH = 6.5, I = 0.16 adjusted with NaCl) incubated at room temperature for 10 min.

detect changes in the size-exclusion chromatograms if β-lactoglobulin was denatured prior to reaction. The detection of higher molecular weight species by size-exclusion chromatography were further confirmed by SDS-PAGE for heat-denatured β-lactoglobulin (gel scanning results

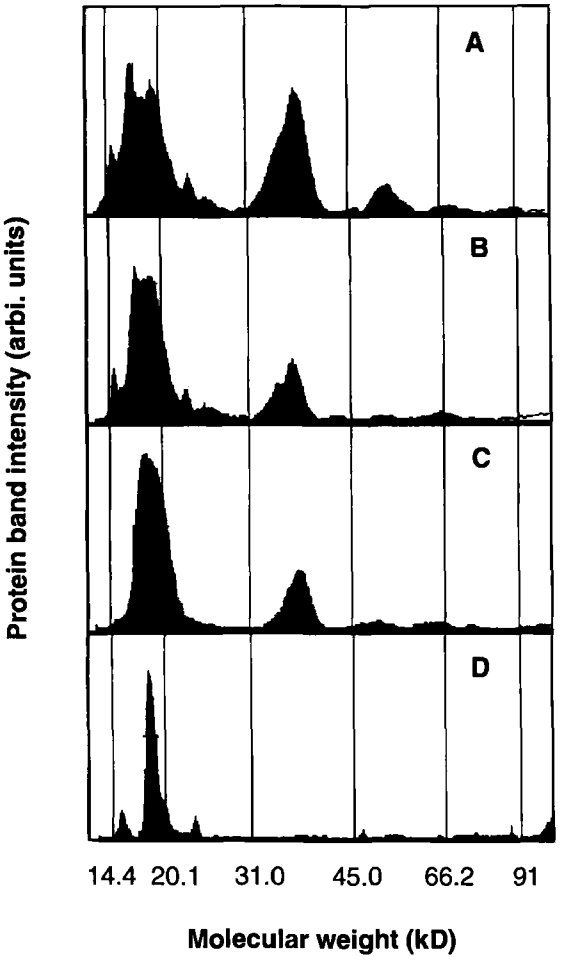

FIGURE 6 Gel scanning of SDS-gel from separation of reaction mixture from reduction of MbFe(IV)=O by heat-denatured β-lactoglobulin. A: MbFe(IV)=O/heat-denatured β-lactoglobulin. B: MbFe(III)/heat-denatured β-lactoglobulin. C: H_2O_2/heat-denatured β-lactoglobulin. D: MbFe(IV)=O. Reaction conditions:0.0083 mmol/l β-lactoglobulin, 0.010 mmol/l MbFe(III), 0.010 mmol/l MbFe(IV)=O (prepared from 0.010 mmol/l MbFe(III) and 0.015 mmol/l H_2O_2 5 min prior to incubation) in 50 mmol/l phosphate buffer (pH = 6.5, I = 0.16 adjusted with NaCl) incubated at room temperature for 10 min.

are given in Figure 6). The reaction mixture containing MbFe(IV)=O and heat-denatured β-lactoglobulin showed an approximate 3 fold increase in the intensity of two protein bands representing molecular weight species of approximately 38 kD and 54 kD compared to the reaction mixture containing MbFe(III)/heat-denatured β-lactoglobulin and the reaction mixture containing H_2O_2/heat-denatured β-lactoglobulin (panel B and C).

From the results presented in Table 1 it may be seen that the reduction of MbFe(IV)=O in the presence of β-lactoglobulin resulted in at least 20 times higher concentration of bityrosine in the β-lactoglobulin fraction compared to the β-lactoglobulin fractions from reaction mixtures containing either MbFe(III)/β-lactoglobulin or H_2O_2/ β-lactoglobulin. These β-lactoglobulin fractions showed traces of bityrosine, but the concentration was below 10 pmol/mg protein, the limit for quantification. Bityrosine detected in β-lactoglobulin exposed to H_2O_2 or MbFe(III) was not formed during the incubation as the β-lactoglobulin used contained bityrosine at a similar level (data not shown). Bityrosine was present in both native and heat-denatured β-lactoglobulin after reaction with MbFe(IV)=O. Approximately 0.15% of the oxidation equivalents present as MbFe(IV)=O (800 nmol in 10 ml reaction mixture) were utilized to form bityrosine (1.2 nmol in 10 ml reaction mixture), provided that all MbFe(IV)=O was converted to MbFe(III) during the reaction and that bityrosine

TABLE 1 Formation of bityrosine in the β-lactoglobulin fraction after oxidation by MbFe(IV)=O.

	Heat-denaturated[a] β-lactoglobulin	Native[a] β-lactoglobulin
	pmol bityrosine/mg protein	
MbFe(IV)=O β-lactoglobulin	180 ± 1.5[b]	208 ± 2.3[b]
MbFe(III) β-lactoglobulin	<10	<10
H_2O_2 β-lactoglobulin	<10	<10

[a]Reaction conditions: 0.033 mmol/l β-lactoglobulin and 0.080 mmol/l MbFe(III) or 0.080 mmol/l MbFe(IV)=O (prepared from 0.080 mmol/l MbFe(III) and 0.12 mmol/l H_2O_2 5 min prior to incubation) in 50 mmol/l phosphate buffer (pH = 6.5, I = 0.16 adjusted with NaCl) incubated at room temperature for 10 min.

[b]Values are the mean of two independent measurements and given with standard deviation.

formation required one oxidation equivalent in a substitution reaction.

DISCUSSION

Our results clearly show that β-lactoglobulin can reduce ferrylmyoglobin in a well defined second-order reaction in homogeneous aqueous solution and that the reaction causes modification of β-lactoglobulin. The observed modifications of β-lactoglobulin are the result of the action of hypervalent iron as no changes in β-lactoglobulin were seen in the absence of either MbFe(III) or H_2O_2. For both experimental designs used to generate MbFe(IV)=O (i.e. 8 fold excess of H_2O_2 followed by removal of non-reacted H_2O_2, or 1.5 fold excess in agreement with the stoichiometry found by Yonetani and Schleyer,[4] Tajima and Shikama[30] and Whitburn[31]), the reaction and/or handling time ensured decay[19,20] of the protein radical initially formed as it has been found to have a half-life of 7–30 s.[3,32] The reactant for the oxidation of β-lactoglobulin may therefore be identified as MbFe(IV)=O rather than the protein radical •MbFe(IV)=O. We have thus obtained evidence for oxidative protein modification mediated by ferrylmyoglobin, MbFe(IV)=O. This conclusion, based on the present study in homogenous aqueous solution, appears to be important, also when compared to the result obtained for cross-linking of myosin which was believed only to occur in the presence of the protein radical •MbFe(IV)=O.[16]

The conformation of β-lactoglobulin was shown to be important for the reaction with MbFe(IV)=O. Denatured β-lactoglobulin was thus slightly more effective in reducing MbFe(IV)=O compared to native β-lactoglobulin. In addition, denatured β-lactoglobulin had the ability to cross-link during the reaction with MbFe(IV)=O, in marked contrast to native β-lactoglobulin. The conformational changes of β-lactoglobulin as a result of denaturation apparently expose reducing amino-acid side-chains to the solvent leading to oxidative cross-linking. The cross-linked proteins are most likely dimers and trimers of β-lactoglobulin, but since the molecular weight of myoglobin is very similar to β-lactoglobulin it can not be excluded that the dimers and trimers are heterogeneous. Homogeneous myoglobin dimers and trimers were not considered as equine myoglobin does not cross-link in the presence of H_2O_2.[33] This was also confirmed in our experiment with H_2O_2 and MbFe(III) in the absence of β-lactoglobulin (see panel D in Figure 6). Native β-lactoglobulin at neutral pH will normally exist as a dimer or an octamer,[34] but low concentrations facilitate dissociation into the monomer[25] which may explain the monomer conformation of native β-lactoglobulin in the present study. Denaturation of β-lactoglobulin was performed by either heat or pressure and resulted in formation of higher molecular weight species (dimers and trimers) as also reported by Li *et al.*[35] Denaturation by heat was not used for the high concentration of β-lactoglobulin used in the kinetic experiments due to protein-gel formation. Conformational differences between heat- and pressure-denatured β-lactoglobulin are not well-documented at present,[22,36] and a more qualitative interpretation of any differences in their reactivity will have to await further studies. It should, however, be noted that Rice *et al.*[17] found that H_2O_2-activated haemoglobin induced cross-linkage of native β-lactoglobulin in an experiment using 900 fold excess of H_2O_2 to haemoglobin. Under these extreme conditions haemoglobin may be activated several times by H_2O_2, concomitant with degradation of the haem protein[16,37] and cross-linking of native β-lactoglobulin may therefore happen in two steps. Oxidation may initially change the conformation of the protein and further oxidation may subsequently result in the observed cross-linkage.

Different amino acids such as cysteine and tyrosine have reducing side chains and form dimers on oxidation. Bityrosine was detected in hydrolyzed samples of β-lactoglobulin after oxidation with MbFe(IV)=O under conditions comparable

to those for which oligomerization of β-lactoglobulin was detected. This provides evidence for a significant contribution of bityrosine to the observed cross-linking of β-lactoglobulin. Disulfide formation could be excluded in cross-linking since disulfide bonds will be reduced by the added mercaptoethanol in the SDS-PAGE. The amount of cross-linkage detected on the SDS-gel was comparable to that detected by size-exclusion chromatography where disulfide bridges will not be reduced. It can therefore be concluded that disulfide bridges are of little importance in the present system. Rice *et al.*[17] has likewise detected non-disulfide linkage in various proteins after reactions with H_2O_2-activated haem proteins, while Shaklai *et al.*[18] and Bhoite-Solomon *et al.*[15] found both disulfide and non-disulfide linkage present in cross-linked proteins after reaction with H_2O_2-activated myoglobin or haemoglobin. The low efficiency with which the oxidation equivalent of MbFe(IV)=O is utilized to form bityrosine is not surprising, as a wide variety of amino-acid side-chains will be competing in different reactions. Comparing the bityrosine formation with the results from the size-exclusion chromatography experiments shows that bityrosine can be formed without resulting in cross-linkage of native β-lactoglobulin after reaction with MbFe(IV)=O. This means that bityrosine is present as intramolecular cross-linkage in native β-lactoglobulin following oxidation and that inter-molecular bityrosine may account for the polymerization of heat-denatured β-lactoglobulin during oxidation.

Hypervalent iron is formed in haem proteins during oxidative stress and the present study has demonstrated a direct reduction by β-lactoglobulin in aqueous solution leading to bityrosine formation and for denatured β-lactoglobulin to oligomerization. The mechanism of electron transfer to the Fe(IV)-center of ferrylmyoglobin is speculative, although the moderate enthalpy of activation ($\Delta H^{\neq}$ = 45 kJ·mol^{-1}) and the relatively large negative entropy of activation ($\Delta S^{\neq}$ = – 93 J·mol^{-1}·K^{-1}) for the bimolecular process is in agreement with an outer-sphere electron transfer in an encounter-complex, provided that specific solvent effects are absent.

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