REVERSIBLE INACTIVATION OF LACTOSE SYNTHASE BY THE MODIFICATION OF HIS 32 IN HUMAN α-LACTALBUMIN

Melvin Schindler, Nathan Sharon

Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel

and

Jean-Paul Prieels
Laboratoire de Chimie Generale I, Université Libre de Bruxelles, 50 Avenue F. D. Roosevelt, Bruxelles, Belgique

Received January 7,1976

SUMMARY

Treatment of human α-lactalbumin with diethylpyrocarbonate at pH 6.1 resulted in reduction of its activity in the lactose synthase assay. Almost complete loss of activity occurred when the molar ratio of reagent to protein was 15:1. Incubation of the modified protein in 20 mM hydroxylamine for 1 h restored its full activity. The loss of activity was correlated with the ethoxyformylation of one of the two histidine residues of human α-lactalbumin, as measured by the change in absorption at 242 nm. Evidence is provided that histidine is the only amino acid residue modified. Examination of the NMR spectrum of the inactivated protein demonstrated the disappearance of one histidine (C-2 proton) peak, which could be assigned to that of His 32.

INTRODUCTION

Lactose synthase activity has been demonstrated to result from a highly specific interaction between A Protein {β(1→4)-galactosyltransferase} and α-lactalbumin (1,2) but the amino acid residues involved in the association between the two proteins have not been characterized. Three dimensional models (3,4) of bovine α-lactalbumin display an extensive hydrophobic region which was postulated to be involved in the association of A Protein and α-lactalbumin. Since His 32, one of the three histidine residues of bovine α-lactalbumin, is an integral part of this region, we attempted to determine chemically whether this residue was important for association. Although a previous attempt using the carboxymethylation reaction to establish the role of histidine residues in bovine α-lactalbumin was reported, the results were not conclusive (5).

We chose to work with human α-lactalbumin which is homologous with the bovine protein (28% difference in primary structure) but contains only two histidine residues (His 32 and His 107). Modification was carried out using diethylpyrocarbonate (DEP)* (6-8), a reagent which under suitable conditions is specific for histidine.

MATERIALS AND METHODS

α-Lactalbumin (mol.wt. 14,500) (9) and A Protein (mol.wt. 43,000) (10) were isolated from human milk. Diethylpyrocarbonate, 8 M in absolute ethanol, was obtained from Aldrich and was diluted to 0.8 M and 0.08 M using absolute ethanol. [^{14}C]UDP-D-galactose was from Radiochemical Center, Amersham and UDP-D-galactose from Sigma Co.

α-Lactalbumin (0.8 mg in 1 ml) was treated with varying concentrations of DEP (1-80 moles DEP/mole protein; maximal volume of reagent was 25 µl) at room temperature for 1 h in 50 mM sodium phosphate buffer pH 6.1. The final concentration of ethanol in each reaction mixture was under 5%. In some experiments, the reaction mixture (after 1 h) was fractionated on a Sephadex G-25 column (1 x 10 cm) using as eluent 50 mM morpholinopropane sulfonate pH 7.4 (for activity measurements), or 50 mM sodium phosphate buffer pH 6.1 (for spectrophotometric analysis). The same buffers were used for preequilibration of the columns. No difference was observed in the activity or optical properties of the α-lactalbumin taken directly from the reaction mixtures and that obtained after passing through the column.

The extent of modification was followed spectrophotometrically by measuring the increase in optical density at 242 nm using a value of ε = 3,200 M^{-1} cm^{-1} for *N*-carbethoxyhistidyl residues (7). Circular dichroism spectra were recorded in sodium phosphate buffer, 50 mM,

---

*Abbreviation used: DEP, diethylpyrocarbonate.

pH 6.1 with a Cary 60 spectropolarimeter at room temperature using a cell with 0.1 cm optical length.

The assay of lactose synthase activity was performed essentially as described by Khatra *et al.* (11). Aliquots (50 µl) from the reaction mixtures were added to 40 µl of a solution containing 1 µmole $MnCl_2$, 5 µmoles morpholinopropane sulfonate pH 7.4, 0.063 µmole UDP-[^{14}C]galactose (476 cpm/nmole), 1.0 µmole D-glucose, and 10 µl A Protein (0.1 mg/ml). Incubation was for 30 min at 37°C after which the amount of [^{14}C]lactose formed was determined.

Reversibility of the chemical modification was examined spectrophotometrically (decrease in $O.D._{242}$) and by following the regeneration of lactose synthase activity after treatment of the modified protein with 20 mM hydroxylamine, pH 6.1 for 1 h at room temperature.

NMR spectra were run on a Brucker 90 spectrometer equipped with Fourier transform capability.

RESULTS AND DISCUSSION

Addition of increasing amounts of DEP to human α-lactalbumin resulted in the modification of histidine as evidenced by the change in $O.D._{242}$, and in a concomitant loss of enzymatic activity (Fig.1). Regeneration of lactose synthase activity observed after reaction with hydroxylamine (Fig.1) led to the complete abolition of the change in absorption at 242 nm.

The deviation from linearity of the plot of activity versus moles of histidine modified (Fig.1, insert) is a reflection of the non-linearity of the dependence of lactose synthase activity on α-lactalbumin concentration in the concentration range of our experiments (11). Extrapolation of the data (Fig.1, insert) shows that complete loss of activity is the result of modification of a single histidine residue. Much higher concentrations of DEP were required to modify the second histidine. Thus, modification of 1.5 histidine residues was observed at a molar ratio of

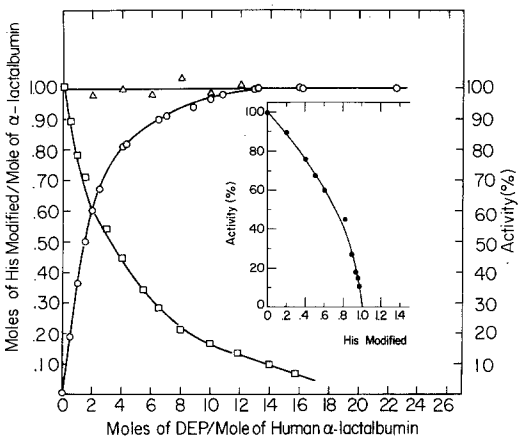

Fig. 1. Reaction of diethylpyrocarbonate with human α-lactalbumin in sodium phosphate 0.05 M, pH 6.1, at room temperature: (□) lactose synthase activity (for assay conditions see text); (o) disappearance of histidine (from change in $O.D._{242}$); (Δ) recovery of lactose synthase activity after incubation at room temperature for 1 h in presence of 20 mM hydroxylamine, pH 6.1. Specific activity of A Protein (100%) was 1.12 mU. Insert: Loss of activity in relation to fraction of moles histidine modified per mole of α-lactalbumin.

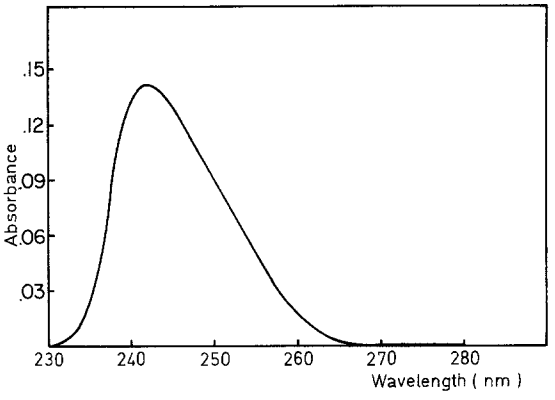

Fig. 2. Ultraviolet difference spectrum of a solution of human α-lactalbumin after reaction with a 15:1 molar excess of diethylpyrocarbonate in sodium phosphate buffer pH 6.1 for 1 h, versus native α-lactalbumin in the same buffer. Protein concentration was 0.62 mg/ml.

DEP to α-lactalbumin of 40:1 while complete modification of two histidine residues required an 80:1 molar excess.

A difference spectrum of α-lactalbumin in which a single histidine was modified (at 15:1 molar excess of DEP) versus native α-lactalbumin

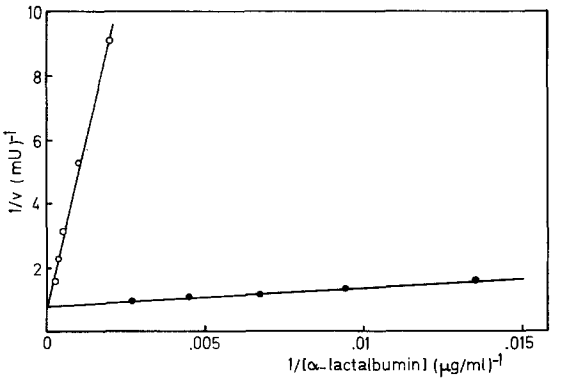

Fig.3. Effect of human α-lactalbumin concentration on lactose synthase activity. Assay conditions as described in text, except that concentration of α-lactalbumin, native or modified, was varied. (o) Native α-lactalbumin; (•) human α-lactalbumin after reaction of diethylpyrocarbonate (same conditions as in Fig.2 except that concentrations of α-lactalbumin up to 8 mg/ml were used). K_m values were computed with a least square program on a Hewlett Packard 9820 desk computer equipped with a peripheral data plotter.

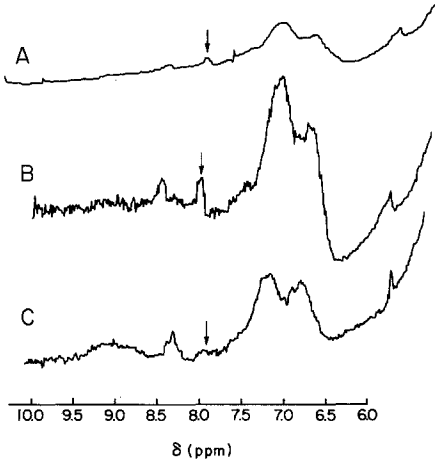

Fig.4. NMR spectra at 90 MHz of the aromatic region of unmodified and modified α-lactalbumin. Two samples of α-lactalbumin (30 mg) were each dissolved in 1 ml 0.05 M sodium phosphate buffer and adjusted to pH 6.1 with 1 M NaOH. The samples were then lyophilized and redissolved in D_2O. Diethylpyrocarbonate was added to one sample (15:1 molar excess of reagent). After 1 h the pH of the modified and unmodified samples was adjusted with 1 M NaOD to 6.7. A and B are the spectra of unmodified α-lactalbumin (A is at lower sensitivity) while C is of the modified protein. An external standard of tetramethylsilane in carbontetrachloride was used.

shows a positive peak at 242 nm (Fig.2). No spectral change is observed at 278 nm, demonstrating that no modification of tyrosine had occurred (6). As the modified α-lactalbumin was reactivated by hydroxylamine, we

conclude that modification of lysine, even if it had occurred (6), was not responsible for the loss of activity observed.

The K_m of A Protein for α-lactalbumin modified at a single histidine residue is 6.30 mg/ml, as compared to that of 8.4 x 10^{-2} mg/ml for the unmodified lactalbumin (Fig.3). This marked decrease (by a factor of 75) in the affinity of α-lactalbumin for A Protein may be taken as evidence that the modified histidine is in the combining site of the lactose synthase. Circular dichroism measurements in the region 200-250 nm indicate that the modification of a single histidine did not introduce gross conformational changes in the protein. Kronman *et al.* (12), using bovine α-lactalbumin demonstrated that there is an increase in the ellipticity of the negative 208 to 209 nm band with the number of tyrosyl residues acylated by *N*-acetylimidazole. Such effects are not seen in our modified protein, providing further support to the proposal that modification of histidine is responsible for the loss of "specifier" activity of α-lactalbumin.

The data given in Fig. 4 clearly demonstrate the presence of two histidine protons in the spectrum of human α-lactalbumin, one of which (δ 7.95) disappears upon treatment of the protein with a 15:1 molar excess of DEP. On the basis of the NMR studies of bovine α-lactalbumin of Bradbury and Norton (13), the histidine protons can be assigned to His 107 (δ 8.24) and His 32 (δ 7.95). As human α-lactalbumin contains only two histidine residues, the proton of His 68 (δ 8.06) found in the NMR spectrum of bovine α-lactalbumin (13), is absent from our spectra. We therefore conclude that the histidine modified by DEP is at position 32.

Previous carboxymethylation studies (5) with bovine α-lactalbumin, demonstrated that the reactivity of His 32 towards iodoacetic acid was much higher than that of His 107. Indeed, at very high molar excesses of DEP (80:1) we find that the second histidine, His 107, is modified with a resulting denaturation of the protein.

ACKNOWLEDGEMENTS

The authors wish to express their sincere appreciation to Dr. S. Blumberg of the Department of Biophysics, Weizmann Institute of Science, for his suggestions and criticism, and to thank EMBO for the short term fellowship to JPP. This study was supported in part by a contribution from a friend of the Weizmann Institute in Buenos Aires, Argentina.

REFERENCES

1. Brodbeck, U., Denton, W.L., Tanahashi, N., and Ebner, K. (1967) J.Biol.Chem. 242, 1391-1397.
2. Brew, K., Vanaman, T.C., and Hill, R.L. (1967) Proc.Nat.Acad.Sci. USA 59, 491-497.
3. Browne, W.J., North, A.C.T., Phillips, D.C., Brew, K., Vanaman, T.C., and Hill, R.L. (1969) J.Mol.Biol. 42, 65-86.
4. Warme, P.K., Momany, F.A., Rumball, S.V., Tuttle, R.W., and Scheraga, H.A. (1974) Biochemistry 13, 768-782.
5. Castellino, F.J., and Hill, R.L. (1970) J.Biol.Chem. 245, 417-424.
6. Mühlrad, A., Hegyi, G., Toth, G. (1967) Acta Biochim.Biophys.Acad. Sci.Hung. 2, 12-29.
7. Ovadi, J., Libor, S., Elödi, P. (1967) Acta Biochim.Biophys.Acad. Sci.Hung. 2, 455-458.
8. Blumberg, S., Holmquist, B., and Vallee, B.L. (1973) Biochem.Biophys. Res.Commun. 51, 987-992.
9. Findlay, J.B., and Brew, K. (1972) Eur.J.Biochem. 27, 65-86.
10. Prieels, J.P., Maes, E., Dolmans, M., and Leonis. J. (1975) Eur.J. Biochem., in press.
11. Khatra, B.S., Herries, D.G., and Brew, K. (1974) Eur.J.Biochem. 44, 537-560.
12. Kronman, M.J., Holmes, L.G., and Robbins, F.M. (1971) J.Biol.Chem. 246, 1909-1921.
13. Bradbury, J.H., and Norton, R.S. (1975) Eur.J.Biochem. 53, 387-396.