NUCLEAR MAGNETIC RESONANCE STUDIES ON RABBIT UTEROGLOBIN

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Received 29 July 1977

1. Introduction

Uteroglobin is a uterine protein of the rabbit, which is normally present in the uterine lumen during the preimplantation phase of pregnancy [1,2]. Uteroglobin synthesis is induced by progesterone [3,4], and the protein binds progesterone and other progestins with high affinity and specificity [5,6]. We have recently shown that uteroglobin is composed of two very similar, if not identical, subunits of some 75 amino acids each held together by two disulfide bonds and other non-covalent iteractions [7]. In the course of our studies on the interaction of progesterone with uteroglobin we observed that the steroid binding is dependent on the reduction of the disulfide bonds, and that this reduction results in conformational changes of the protein [8,9].

In this paper we present nuclear magnetic resonance (NMR) spectra of uteroglobin in the native and reduced states. It is shown that reduction of the disulfide bonds produces conformational changes in the protein that keeps in part its tertiary and secondary structure. The structure is more modified if reduction is done by carboxymethylation than by reducing agents such as DTT or DTE. The reduced protein is more sensitive to denaturation by urea than the native protein, the latter retains some structure even at 6 M urea.

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2. Materials and methods

Uteroglobin was purified from the uterine flushes of rabbits treated sequentially with estradiol and progesterone, according to a previously described procedure [7].

Reduction was achieved by incubation of 5 mg protein in 1 ml 10 mM DTT, 10 mM phosphate, pH 7.0, at 37°C for 15 min. The sample was then dialyzed to achieve a final concentration of 1 mM DTT, 10 mM phosphate, pH 7.0. Carboxymethylation was carried out as described [4,7].

The lyophilized powder of the protein was dissolved in ^2H_2O (MSD isotopes 99.96% ^2H), left at 37°C for 1 h and lyophilized again. 5 mg of the different samples were dissolved in 0.35 ml of ^2H_2O (14 mg/ml) and introduced in 5 mm diameter NMR tubes. pH was checked before every measurement. NMR spectra were recorded in a Bruker WH 270 spectrometer in the Fourier Transform mode with water elimination. 5000 scans were accumulated and in most cases convolution difference methods were used for resolution enhancement [10]. Temperature of the sample was 18°C. Urea-d_4 was from MSD isotopes.

Integration of NMR peaks has been made by weighing. Two internal references have been taken, the protons at higher field from 1.25 ppm taken as 96 protons, and the protons in the aromatic region at higher field from 7.6 ppm taken as 16 protons.

The observed error using this method is lower than 20%.

Circular dichroism spectra were recorded in a JASCO J-20 spectropolarimeter. Helicity was calculated using the parameters of Chen et al. [11].

3. Results and discussion

On fig.1 are presented the high field NMR spectra of uteroglobin in 10 mM phosphate buffer, pH 6.5 (E), in 10 mM phosphate buffer, 1 mM DTT, pH 7.0 (D), in 10 mM phosphate, pH 7.0, after carboxymethylation (C), in 10 mM phosphate, 6 M urea, pH 7.1 (B) and in 10 mM phosphate, 1 mM DTT, 6 M urea, pH 7.7 (A).

Clear evidence for a globular structure in the protein is present in this figure. Spectrum A is that would be expected for a random-coil protein with the composition of uteroglobin [7]. In the other spectra we find evidence of tertiary structure. This is specially true for native uteroglobin in 10 mM phosphate, pH 6.5 (spectrum E). The larger effects are observed in the region corresponding to the signals from methyl groups of valine, leucine and isoleucine residues. The effects observed in this region indicate the existence of a well-defined tertiary structure. First the appearance of signals at high field from 0.9 ppm, which can be interpreted as ring-current effects of aromatic residues on protons from apolar residues. The area of some of these peaks can be measured and is a multiple of 3 protons except for the best resolved resonance at 0 ppm which has an area corresponding to 1.3 protons. Peaks with an area lower than 3 protons may be due either to the shift of a single proton (from a CH_2 group of an apolar residue) or to a methyl from only one of the two chains of uteroglobin [7]. In the latter case one would observe an area of 1.5 protons. Another possibility would be an equilibrium between a structured and a non-structured state. This is probably not the case because in these conditions the protein is considered 'native' [6,7] and because of the features of the rest of the spectrum that will be discussed later.

A second effect visible in the region of the apolar residues is that very few protons remain at 0.9 ppm, in the position of random-coil methyl groups. This is an effect already observed in other globular proteins [12,13] and may be a consequence of the change in polarity of the environment of the apolar residues with formation of the tertiary structure.

In the rest of the spectrum changes are observed in the region of CH_3 groups of alanine and threonine residues. An interesting fact is the shift of the methionine CH_3. In this case it is difficult to carry out an accurate integration due to the overlapping of other resonances, but from the apparent height it appears that three of the four methionines are implicated in the globular structure. At high field the other conformational effects visible are on the α-CH region which is very sensitive to secondary structure formation, but no detailed study of this region is possible for the moment.

The effect of reducing the disulfide bridges can be studied on spectra D and C. From spectrum E to spectrum D the overall spectrum does not change appreciably, but some significant changes can be observed as compared with the native protein. In the region of the ring-current shifts some of the peaks suffer a loss of area, transferred to the main peak of this region. It is interesting that a peak appears at 0.44 ppm which was not present in the spectrum of the native protein. The same phenomenon occurs at 1.57 ppm where a peak, probably a shifted alanine CH_3, appears. These effects may be indications that the loss of some structured parts in the protein which follows the reduction of the disulfide bonds leads to some rearrangements of the structure giving rise to contacts between residues that did not exist in the native structure. The loss of some structure upon reduction of the disulfide bridges can be observed also in the methionine CH_3 peaks where one of these resonances shifts to the random-coil position.

More dramatic changes can be observed in the spectrum of the carboxymethylated protein (fig.1C). Even if the globular structure is not lost as is evident for instance from conserved ring-current shifts and from the shifts of methionine CH_3 signals, the peak at 0.9 ppm is now intense. It suggests that most of the methyl resonances are in conditions similar to those of the random-coil state; therefore the structure appears to be more open than in the native state. The difference between this spectrum and

spectrum D indicates that carboxymethylation disrupts to a larger degree the structure of uteroglobin. It is probably the effect of either the more energetic method of reduction or the introduction of charged groups in the cysteine residues.

If native uteroglobin is dissolved in 6 M urea-d_4 dramatic changes occur in the spectrum (fig.1B) indicating that an important part of the protein loses its structure. However, the main ring-current shifted peaks are still present. The area of the peak at 0 ppm is of 1.4 protons which is, within the limits of error, equal to the area of the same peak in the native protein. Evidence for remaining structure is also found in the methionine CH_3 peak where one of the four residues remains at 2.23 ppm.

If the protein in the reduced state is dissolved in 6 M urea (spectrum A) the spectrum is that of a random-coil protein with no evidence at all of ring-current shifts and the peaks are located at the positions that can be predicted from literature data [14].

The low field spectra of uteroglobin in the same conditions as in fig.1 are presented in fig.2. Two main features can be observed in these spectra. First, the complex pattern of the aromatic residue signals in spectra C, D and E, corresponding to the fact already observed at high field that a globular

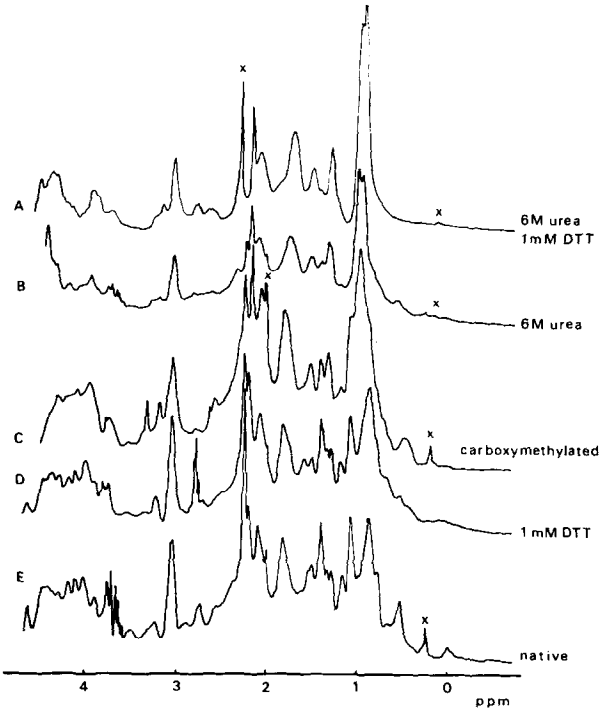

Fig.1. High-field 270 MHz NMR spectra of rabbit uteroglobin in: (A) 1 mM DTT, 6 M urea, 10 mM phosphate, pH 7.7; (B) 6 M urea, 10 mM phosphate, pH 7.1; (C) the carboxymethylated derivative in 10 mM phosphate, pH 7.0; (D) 1 mM phosphate, pH 7.0; and (E) 10 mM phosphate, pH 6.5. Protein concentration: 14 mg/ml, number of scans: 5000. Temperature 18°C. Spectra (D) and (E) are convolution difference spectra. Signals marked with an x are considered coming from impurities.

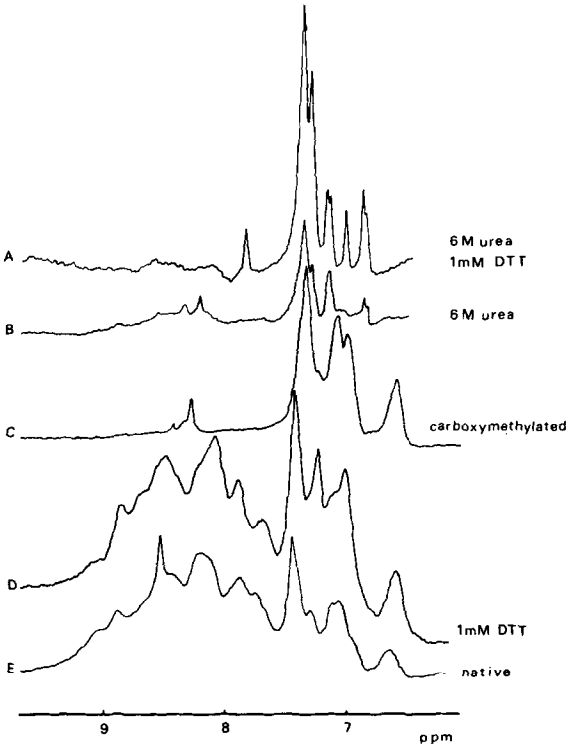

Fig.2. Low-field 270 MHz spectra of rabbit uteroglobin. Same conditions as in fig.1.

structure exists in native and reduced states of uteroglobin. This structure includes the tyrosine residue and at least one phenylalanine residue. Of special interest is the peak most shifted to high field corresponding probably to two protons of the tyrosine residue. The peak has an area close to 2 protons for the three spectra, indicating that the changes in the structural features observed are not due to a slow equilibrium between a structured and a non-structured state, but, at least for part of the chain, because of different structural states.

The second feature in the low field spectra is the presence of a large number of resonances at lower field from 7.5 ppm where resonances of NH groups are found. This is an indication that a number of these protons are forming hydrogen bonds or deeply buried in the structure of the protein. It is interesting to compare these results with those obtained by circular dichroism. The amount of helicity of native uteroglobin is of 41%, when reduced with 10 mM DTE 40%, and when carboxymethylated 34%. The total area of the NH region in the native state is equivalent to 30 protons which would mean a 40% helicity but 10 of these protons are lost by reduction with DTT and all upon carboxymethylation. These results indicate that reduction causes an opening of the structure of uteroglobin, coherent with that observed at high field, and that this effect is larger if reduction is followed by carboxymethylation. It also agrees with the loss of some tertiary structure as seen by ultraviolet difference spectra [9].

4. Conclusions

Uteroglobin has in its native state a well defined tertiary structure in which the majority of the apolar residues (leucine, isoleucine, valine and threonine) are involved. The tyrosine residue and at least one phenylalanine also take part in this structure. The protein has a 40% helicity, corresponding to a similar amount of NH protons exchanging slowly with deuterium. Reduction of disulfide bridges does not produce the disruption of the globular structure, but the structure appears more open as seen by the loss of some non-exchangeable NH protons and changes at high field. This effect is more evident if reduction is followed by carboxymethylation, although in this case part of the structure is lost. These findings are relevant to the observation that the binding of progesterone to uteroglobin is dependent on the reduction of the disulfide bonds [5,8], and could reflect an enhanced accessibility of the steroid binding site otherwise buried in the more compact structure of the native protein. Some rearrangements of the polypeptide chain are observed upon reduction leading to new local structures.

The oxidized form of the protein has a limited amount of structure which is resistant to 6 M urea. This residual structure disappears if the disulfide bonds are reduced.

Acknowledgements

We thank Drs E. M. Bradbury and F. J. Avilés (Portsmouth Polytechnic) and the staff of the Organic Chemistry Department, Technische Universität, Berlin for permission to use their spectrometers and their assistance. Dr Adolfo Ruiz-Carillo, Max-Planck Institut, Berlin, for his help and for the use of his laboratory, The Behringwerke, Marburg for the kind gift of the animals and the Deutsche Forschungsgemeinschaft for financial support (SFB 103-B1).

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