THE ACCESSIBLE CYSTEINE RESIDUE OF HUMAN TRANSCORTIN

Evidence for oxidation of the sulphydryl group

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1. Introduction

Human transcortin (corticosteroid binding globulin), that specifically binds glucocorticoid hormones in blood plasma, consists of a single polypeptide chain and several osidic chains accounting for 27% (w/w) of the molecule [1]. The polypeptide chain contains two cysteine residues and no disulphide bridge [1–3] but heat or acid inactivation of transcortin results in an aggregation and a disulphide bridge dimer formation which occur concomitantly with an important modification of the secondary structure [1,4].

In the native conformation, only one of both sulphydryl groups is shown to react with sodium *p*-chloromercuribenzoate (PCMB) [2] or with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [5]. This is in disagreement with our own observation since we were not able to detect any thiol group on native transcortin (free or bound to cortisol) [6].

The purpose of the present work is to provide an explanation of this apparent discrepancy, which is essential to the study of the cortisol binding site. Indeed, Khan and Rosner have observed the modification of the accessible cysteine residue by a hormone analogue, 6β-bromoprogesterone; since the modification is accompanied by a loss of cortisol binding, they have claimed that the accessible cysteine residue is present in the binding site [5]. In this paper, it will be shown that the accessible cysteine sulphydryl group seems to be partially oxidized to sulphenic acid. This cysteine residue had the same accessibility in free transcortin as in transcortin–cortisol complex; it could be modified by ethylene imine without loss of binding activity. Thus it cannot be considered as an essential amino acid for cortisol binding even if it is present in the binding site as suggested in a recent work [5].

2. Materials and methods

Cortisol was obtained from Steraloids and [1,2-^{3}H]-cortisol (30 Ci/mmol) from Radiochemical Centre, Amersham. DTNB, dithiothreitol (DTT) and ethylene imine were purchased from Pierce Chemicals. All other used materials were of Analytical Reagent grade of purity and all solutions were made with deionized and glass distilled water.

2.1. *Cortisol-free transcortin*

Transcortin was obtained by affinity chromatography as an equimolar transcortin–cortisol complex [1,7]. The freeze-dried powder was kept at −70°C until use. After solubilization, its molar concentration was determined from $A_{280\ nm}$ measurements taking a mol. wt 49 500 ($E^{1\ mg/ml}_{1\ cm}$ at 280 nm = 0.70) [1]. Cortisol was assayed by fluorimetry [8].

Cortisol-free transcortin was prepared, just before use, in the appropriate buffer for chemical modification. For that purpose, a dextran-coated charcoal suspension was made of 10 mg/ml charcoal Norit (Prolabo) and 1 mg/ml dextran T-70 (Pharmacia) in the same buffer as in the transcortin solution. To one volume of transcortin–cortisol complex solution (2–4 mg/ml) was added 1 vol. suspension. After a 30 min incubation at 37°C, the mixture was cen-

trifuged at 2000 × g, 10 min and the supernatant filtered through Whatman no. 1, if necessary to get a clear solution. This method was shown to efficiently remove most of the cortisol (about 95%) from transcortin without denaturation.

2.2. *Sulphydryl group determination*

Titration of free or cortisol bound transcortin with DTNB was performed in 0.1 M Tris–HCl buffer, pH 8.0, containing 0.001 M disodium EDTA. Transcortin solution (0.02 mM) with or without denaturant, was incubated with 35 μl DTNB (4 mg/ml) in final vol. 1 ml for 15 min and the $A_{412\ nm}$ was determined along with appropriate reagent and sample blanks. When 6 M guanidine hydrochloride was included in the incubation mixture, a 60 min preincubation at 50°C took place before addition of DTNB; when 2% sodium dodecylsulfate was used as denaturant, the transcortin solution was boiled during 3 min before addition of DTNB. A molar extinction coefficient of 13 600 M^{-1} cm^{-1} was assumed to calculate the number of sulphydryl groups titrated in absence of sodium dodecyl sulphate (SDS) [9]. This coefficient was corrected when the reaction was carried out in the presence of 2% SDS: the titration was found to be linear with cysteine hydrochloride in the usual concentrations (0–10^{-4} M) but the molar absorption coefficient exhibited a lower value, 12 100 M^{-1} cm^{-1} which agreed with the reported data in the presence of detergents (12 500 M^{-1} cm^{-1}) [10].

Titration of cortisol-free transcortin with PCMB was performed in 0.05 M sodium phosphate buffer, pH 7.0 [11], with or without 2% SDS. Titration of transcortin–cortisol complex was not carried out since cortisol has a high molar absorbance (ϵ at 258 nm = 15 900 M^{-1} cm^{-1} [12]) which interferes with the thiol reaction product (ϵ at 250 nm = 7600 M^{-1} cm^{-1}) [11].

2.3. *Activity assay of transcortin*

The transcortin–cortisol complex solution (0.02–0.04 mM) in the buffer used for chemical modification was added together with 0.5 μCi/ml [^{3}H]cortisol (30 Ci/mmol) and incubated 90 min at 37°C to reach equilibrium. When cortisol-free transcortin was used, [^{3}H]cortisol (40 Ci/mol) was added to a final molar concentration slightly higher than that of transcortin (0.025–0.050 mM) before the activity assay.

Binding activities were tested on 0.1 ml aliquots at 0°C in duplicate, by the dextran-coated charcoal method described in [6]. A systematic comparison was made between chemically modified transcortin samples, a reference sample was treated in a similar manner but without reagent and native transcortin.

2.4. *General methods*

Polyacrylamide gel electrophoreses were performed with 7.5% acrylamide and Tris–glycine buffer, pH 8.3 by the method in [14].

Metal ions were determined on a 0.02 mM transcortin solution in 0.05 M sodium phosphate buffer, pH 7.4, with an Atomic absorption spectrophotometer IL 151.

Amino acid analyses were performed with a Jeol 5 AH amino acid analyser. Samples for acid hydrolysis were treated at 110°C for 24 h with 5.6 N HCl in evacuated, sealed tubes.

3. Results

3.1. *Sulphydryl group determination*

The number of sulphydryl groups present in transcortin was determined under various conditions; the results are shown in table 1. No thiol group was

Table 1
Titration of the sulphydryl groups of transcortin

Conditions	–SH groups/mol transcortin	
	not reduced	reduced with 5 mM DTT
Native	0[a,b]	0.88[a]
2% SDS	0.91[a]–1.15[b]	1.79[a]

[a] Determination with DTNB
[b] Determination with PCMB

Reduction with DTT was performed in 0.1 M Tris–HCl buffer, pH 8, for 1 h in the dark. The solution (1 ml) was then desalted through a Bio-gel P-6 column (0.8 × 18 cm) equilibrated with the same buffer. The eluted protein peak fractions were pooled and concentrated to 1 ml under nitrogen at 4°C in an Amicon micro-ultrafiltration apparatus equipped with Diaflo membrane (PM 10 type). Titration of sulphydryl groups was carried out as described in section 2. Each number represents the average value of 4 determinations with different preparations of transcortin

detected on native transcortin (free or bound to cortisol) but 1 –SH group/mol was titrable in the presence of 2% SDS. Reduction of transcortin with 5 mM dithiothreitol under native conditions led to an –SH content increase without change of the cortisol binding activity: 1 –SH group/mol was titrable in native conditions, 2 groups in the presence of 2% SDS. Determination in 6 M guanidine hydrochloride gave similar results as in 2% SDS.

Since transcortin contains 2 cysteine residues/mol, we may assume that one is sterically hindered and denaturation is necessary for titration of its –SH group. The second residue is available under native conditions but its –SH group is blocked and can be determined only after reduction.

3.2. *Nature of the blocking agent on the available sulphydryl group*

A number of different divalent metals were assayed. Mercury, iron and zinc were not detectable; traces of lead and copper, 0.09 and 0.08 atom/mol transcortin were detected, respectively, but these quantities are insufficient to explain the blockage of 1 –SH group/mol.

Another possibility explored was a disulphide formation in hemolysed blood plasma with a thiol peptide (such as glutathione) or with free cysteine. For this investigation, 2×10^{-7} mol were reduced in 1 ml volatile buffer (0.2 M ammonium bicarbonate buffer, pH 8.0) with 1% 2-mercaptoethanol under nitrogen, in the dark during 16 h; the solution was then desalted through a Bio-gel P 6 column equilibrated with 0.02 M ammonium bicarbonate buffer, pH 8.0 and the salt fraction examined with an amino acid analyser. No amino acids were observed either before or after acid hydrolysis although the available –SH group was effectively regenerated.

The presence of both cysteic acid and half-cystine residues was detected in unreduced transcortin after acid hydrolysis: 0.86 and 0.77 residues/mol respectively. The occurrence of cysteic acid in native transcortin cannot be assumed since that degree of oxidation cannot be reversed by DTT; the question then arises as to the presence of sulphenic (–SOH) or sulphinic ($-SO_2H$) acid which can be reduced to thiol [15]. It has been suggested that sulphenic acids, unlike disulphides, may be reduced to thiols by sodium arsenite, a very mild reductant [16,17]. The effects of arsenite and of another mild reductant, sodium ascorbate, were therefore studied. It was found that arsenite or ascorbate regenerated the available –SH group, like DTT (table 2).

3.3. *S-aminoethylation of the available sulphydryl group*

Direct evidence by amino acid analysis of *S*-aminoethylcysteine could not be obtained. Indeed, *S*-aminoethylcysteine was eluted in amino acid chromatograms in a narrow space between lysine and histidine which are present in transcortin acid hydrolysate with, respectively, a 15-fold and a 9-fold molar excess. However, indirect evidence for –SH modification was given by titration of sulphydryl groups before and after aminoethylation.

Although 2 –SH groups/mol were determined in 2% SDS solution of reduced transcortin, a single –SH group was detected after aminoethylation in native conditions. Results were essentially the same for

Table 2
Effect of reductants and aminoethylation in native conditions on sulphydryl content of transcortin

Reductants	–SH groups/mol transcortin (determination in 2% SDS)	
	before aminoethylation	after aminoethylation
None	0.85	–
0.01 M dithiothreitol	1.78[c]	0.87[a] 0.91[b]
0.20 M arsenite	1.67	–
0.20 M ascorbate	1.81	–

S-aminoethylation was performed in native conditions with ethylene imine [13]. Free transcortin solution, 0.04 mM in 1 M Tris–HCl buffer, pH 8.5 containing 0.001 M disodium EDTA, was reduced with 10 mM DTT in the dark over 1 h; it was then divided into three 1 ml portions. [a] The first was mixed with ethylene imine during 2 h (6 additions of 10 µl at 10 min intervals over 1 h. [b] To the second portion, [^{3}H]cortisol (40 Ci/mol) was added to final conc. 0.05 mM before reaction with ethylene imine. [c] The third portion was untreated. Reduction by arsenite and ascorbate was carried out in 0.1 M Tris–HCl buffer, pH 8.1, containing 0.001 M disodium EDTA for 60 min in the dark at 30°C. 0.1 ml aliquots of all reaction mixtures were removed for testing the binding activity and the remaining solutions were desalted and titrated as in table 1

unbound and cortisol bound transcortin (table 2). As no –SH group was detectable in the absence of SDS, the remaining thiol group after aminoethylation corresponds to the buried cysteine residue which was not available to ethylene imine during the reaction. *S*-aminoethylation of transcortin in native conditions thus permitted selective modification of the accessible cysteine residue. This modification induced no loss of binding activity.

4. Discussion

The experimental evidence presented here strongly indicates that the available sulphydryl group of transcortin is converted to a stabilized sulphenic acid. Fries acid (anthraquinone-1-sulphenic acid) [18] and two other closely related compounds [19,20] are the only successfully isolated sulphenic acids. It has been proposed that the stability of these particular sulphenic acids is probably due to hydrogen bonding or to other strong interactions of the oxidized sulfur with the aromatic 9,10 quinone system [20]. Aliphatic sulphenic acids lack this stabilization and therefore cannot be isolated. The stability of the sulphenic derivative in transcortin can be attributed to its steric and chemical environment.

The presence of cysteine sulphenic acid has been described in glyceraldehyde 3-phosphate dehydrogenases and its oxidation is suggested to occur during the isolation procedure [16]. The occurrence of stabilized sulphenic acid is also suggested in papain [17]. Oxidation of the accessible cysteine residue of transcortin that we isolated may occur during collection of biological samples of serum. Indeed, the pool of serum which was often hemolysed was usually kept at room temperature over 2 days and at 4°C over 1 week for collection before freezing at –20°C.

The question arises as to the role of this accessible cysteine residue in cortisol binding affinity of transcortin. Three amino acids have been identified in different regions of the site by affinity labelling: we have suggested the presence of an histidine residue and of a methionine residue [6], a third amino acid is the accessible cysteine residue which is suggested to be present in the vicinity of the carbonatom 6 of cortisol by Khan and Rosner [5]. However, this residue was shown here to be non-essential for cortisol binding. Khan and Rosner also showed that PCMB does not inactivate transcortin. Recently Stroupe et al. [21] have observed that the degree of ionization of cysteine residue is unlikely to affect the dissociation rate constant of transcortin–steroid complexes in contrast to histidyl and arginyl residues. All these observations strongly suggest that the accessible cysteine residue of transcortin may be present at the binding site between the cortisol crevice and the outside of the molecule so as to be available from both faces.

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