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ROLE OF THIOL GROUP(S) IN THE ENZYMATIC ACTIVITY OF THE MEMBRANE-BOUND Δ^5-3β-HYDROXYSTEROID DEHYDROGENASE FROM BOVINE ADRENAL CORTEX MICROSOMES

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Summary

The effect of different sizes of thiol groups reagents on the kinetic parameters of the membrane-bound Δ^5-3β-hydroxy-steroid dehydrogenase (3(or 17)-β-hydroxysteroid:NAD(P)$^+$ oxidoreductase, EC 1.1.1.51) from bovine adrenal cortex microsomes was examined. Iodoacetamide and parachloromercuribenzoate increased the maximum velocity 1.6 times and also the K_m for androst-5-en-3β-ol-17-one by factors of 1.5 and 5.5, respectively. Hydrophobic reagents of larger size led to a large decrease of the maximum velocity and further increase in the K_m for androst-5-en-3β-ol-17-one. Androst-5-en-3β-ol-17-one hindered the activating effect of iodoacetamide.

Similar results were found with the NAD$^+$ analogue, 3-chloro-acetyl pyridinium adenine dinucleotide, an electrophilic reagent. A decrease of the maximum velocity was observed (38% inhibition at the highest concentration used). As with other SH reagents, the K_m for androst-5-en-3β-ol-17-one was increased 5 times. Preincubation of membranes with iodoacetamide prevented the enzyme from inhibition by 3-chloroacetyl pyridinium adenine dinucleotide. Protection experiments with androst-5-en-3β-ol-17-one, oestr-1,3,5(10)6,8-pentaen-3-ol-17-one and NAD$^+$ demonstrated that the steroids were more effective than NAD$^+$ in preventing the enzyme from inhibition. In all cases, the K_m for NAD$^+$ was unaltered.

From these results, it is suggested that thiol reagents, including 3-chloroacetyl pyridium adenine dinucleotide, substitute an SH group in the vicinity of the steroid-binding site of the enzyme.

Abbreviations: Dehydroepiandrosterone: androst-5-en-3β-ol-17-one: DHA; equilenin: estr-1,3,5,(10)6,8-pentane-3-ol 17-one; dehydrogenase: Δ^5-3β-hydroxysteroid dehydrogenase; Progesterone: pregn-4-en-(3,20)-dione; 17α-hydroxyprogesterone: pregn-4-en-17α-ol-(3,20)-dione.

Introduction

Among the numerous enzyme systems embedded in the membrane structure, the Δ^5-3β-hydroxysteroid dehydrogenase · 3-oxosteroid-Δ^4-Δ^5-isomerase complex from bovine adrenocortical microsomes has only been studied to a limited extent, mainly due to its instability towards solubilization [1]. The biological role of this system is the conversion of Δ^5-3β-hydroxysteroids to the corresponding Δ^4-3-oxo derivatives, which are either active hormones (androstenedione) or intermediary products for the biosynthesis of gluco- and/or mineralocorticosteroids (progesterone and 17α-hydroxyprogesterone) [2]. In addition to this role, the membrane location of this system raises several questions about the influence of the diffusion of substrates and products on the membrane structure [3,4]. A study of these phenomena and of the interaction of the proteins with their lipidic and proteic environment requires probing of the lipidic phase and covalent labelling of the enzymatic sites. We have first investigated the effect of thiol groups reagents on the activity of the Δ^5-3β-hydroxysteroid dehydrogenase since the involvement of such groups has been described in the activity of several enzymes of this type [5—7].

Iodacetamide, parachloromercuribenzoate, *N*-(anilino-naphthyl-4)maleimide and *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylene diamine were used and their effects on the kinetic parameters of the enzyme were determined. Moreover, the inhibition parameters are presented for the cosubstrate analogue, 3-chloroacetylpyridinium-adenine dinucleotide, an affinity label for some dehydrogenases [8,9].

Materials and Methods

Membrane preparation. The preparation of a microsomal fraction from bovine adrenal cortex has been described previously [10]. Membranes were stored in liquid N_2 and thawed only once for use.

Δ^5-3β-hydroxysteroid dehydrogenase assay. In the membrane fraction, the low level of activity of the enzyme required a sensitive technique for assay. NADH formation was followed by fluorescence at 470 nm when excited at 340 nm [11]. With this method, the initial velocity was directly recorded after mixing of the reagents and was zero order for about 5 min under the assay conditions. Routinely, the composition of the assay medium was as follows: 8.3 μM dehydroepiandrosterone, 220 μM NAD^+ and 2—10 μl membrane suspension in 20 mM Tris · HCl (pH 8.5) in a final volume of 2 ml at 36°C. Unless otherwise stated, the reaction was started by the addition of the membrane suspension. A 2 mg · l^{-1} NADH solution in 20 mM Tris · HCl (pH 8.5) was used as a standard for calibration of the fluorescence signal. The measurements were carried out in 1 × 1 cm quartz window cuvettes with the spectrofluorimeter Fica 55 equipped with an Ifelec 3802 recorder. Two blanks were performed, one with microsomal suspension alone the other one with all the reagents except the membrane suspension.

All kinetic data were calculated using a least squares linear regression analysis.

Chemicals. Steroids were gifts from Roussel-Uclaf and were dissolved in

ethanol (Uvasol Spectroscopic grade, Merck, Darmstadt GFR) at a concentration of 1 mg · ml^{-1}. Iodoacetamide, NAD^+ and NADH were from Sigma Chemicals (St. Louis, U.S.A.). Parachloromercuribenzoate (Nutritional Biochemical Corporation), *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylene diamide (Aldricht), *N*-(anilino-naphthyl-4)-maleimide (Teika Seiyaku, Toyama, Japan) were used as supplied. 3-chloroacetyl pyridinium adenine dinucleotide was synthesized as described [12] and stored in liquid N_2. The inactivation efficiency was assayed with yeast alcohol dehydrogenase.

Results

Iodoacetamide activation of Δ^5-3β-hydroxysteroid dehydrogenase: effect of dehydroepiandrosterone. Incubation of the microsomal membranes with iodoacetamide increased the activity of Δ^5-3β-hydroxysteroid dehydrogenase. When incubations were performed in the presence of dehydroepiandrosterone, no increase of activity was observed. No such "protection" effect was observed with NAD^+ (Table I).

Effect of other SH reagents and determination of kinetic parameters. Δ^5-3β-hydroxysteroid dehydrogenase activity was also increased 1.6 times by parachloromercuribenzoate. On the other hand larger-sized reagents of hydrophobic character (*N*-(anilino-naphthyl-4)-maleimide and *N*-iodoacetyl-*N'*(5-sulfo-1-naphthyl) inhibited almost completely the activity (80% inhibition). It can be seen (Table II) that the K_m for NAD^+ did not vary significantly whatever reagent was used. K_m for dehydroepiandrosterone was modified, such that with a larger sized reagent, a higher K_m value was determined.

Inactivation of the enzyme by 3-chloroacetyl pyridinium adenine dinucleotide. Fig. 1a and b summarizes the data of the inactivation of Δ^5-3β-hydroxysteroid dehydrogenase by 3-chloroacetyl adenine dinucleotide.

Since inactivation of the enzyme was limited to a maximum value of 38%, even at the highest available reagent concentration (3.5 mM), the representation of Kitz and Wilson [13] was modified as follows:

$$\log \frac{A_0(1-\alpha)}{(A-A_0\alpha)} = \frac{kt}{1+K/I} \qquad (1)$$

where A, A_0, k, K and I have the classical meaning; α is the ratio of final to initial activity (α = 0.62).

TABLE I

The effect of iodoacetamide on the enzymatic activity of Δ^5-3β-hydroxysteroid dehydrogenase and protective effect of NAD^+ and dehydroepiandrosterone. Incubations were performed with 1.8 mg · ml^{-1} membrane proteins (determined by the Lowry method [14] in 50 mM Tris · HCl (pH 8.5) at room temperature in the dark for 2 h. Reaction was stopped by diluting the assay by 200 times.

Incubation with	Relative activity (%)
No addition	100%
4 mM iodoacetamide	155 ± 5
4 mM iodoacetamide + 140 μM dehydroepiandrosterone	105 ± 5
4 mM iodoacetamide + 32 mM NAD^+	155 ± 5

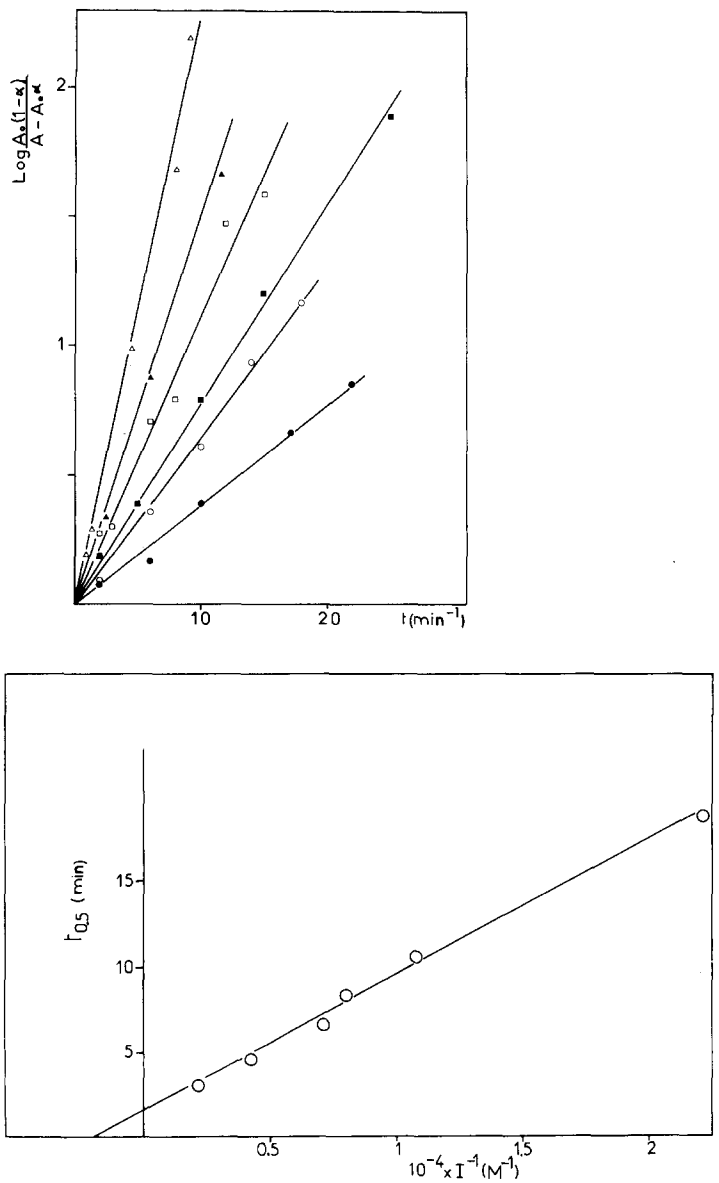

Fig. 1. Inactivation of Δ^5-3β-hydroxysteroid dehydrogenase by 3-chloropyridinium adenine dinucleotide. (a) plot of log $A_0(1-\alpha)/A\text{-}A_0\alpha$ as a function of time. Microsomal protein concentration: 3 mg · ml^{-1}. 3-chloroacetyl pyridinium adenine dinucleotide concentrations: ●, 46 μM; ○, 92 μM; ■, 115 μM; □, 140 μM; ▲, 230 μM; and △, 460 μM. Incubations were performed in 50 mM HEPES/NaOH (pH 7.5) at room temperature. (b) Determination of the kinetic constants of 3-chloroacetyl pyridinium adenine dinucleotide: plot of $t_{0.5}$ (min) vs. $1/I$.

The plots of enzyme activity vs. time according to Eqn. 1 were linear (Fig. 1a) and apparent inactivation rate constant could be determined. The classical double reciprocal plot (Fig. 1b) then allowed graphical determination of the alkylating NAD^+-analogue kinetic constants: K (dissociation constant): 560 μM and k (alkylation rate constant): $7.2 \cdot 10^{-3}$ s^{-1} (for an affinity labelling).

TABLE II

Modification of the kinetic parameters of Δ^5-3β-hydroxysteroid dehydrogenase by sulfhydryl reagents. Incubations were performed as follows: (1) Iodoacetamide, see Table I for experimental conditions; (2) Parachloromercuribenzoate: membranes were incubated 5 min in the assay buffer at 36°C with 500 μM reagent (protein concentration: 20 $\mu g \cdot ml^{-1}$); (3) *N*-iodo-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylene diamine: microsomal suspension (15 $mg \cdot ml^{-1}$) was incubated 2 h in the dark in 50 mM Tris · HCl (pH 8.5) and 660 μM reagent. Excess fluorescent reagent was eliminated by washing of the membranes with buffer and centrifugation at 100 000 × *g* for 30 min, 3 times; (4) *N*-(anilino-naphthyl-1)-maleimide: incubations were performed in 0.1 M HEPES/NaOH (pH 7.3) and 580 μM reagent (a stock solution of 3.5 mM in ethyleneglycol monoethylether was used). Excess reagent was removed as described above. Kinetic data were shown as Lineweaver-Burk plots.

Incubation with	K_m NAD^+ (μM)	K_m DHA (μM)	V (nmol · $min^{-1} \cdot mg^{-1}$)
No reagent	10 ± 1.5	0.3 ± 0.1	32 ± 3
Iodoacetamide	9 ± 1.5	0.7 ± 0.1	50 ± 5
Parachloromercuribenzoate	8.5 ± 1.5	2.2 ± 0.3	50 ± 5
N-iodo-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine	11.5 ± 3	3.5 ± 1.5	7 ± 1
N-(1-anilino-naphthyl-4)-maleimide	11 ± 3	3.5 ± 1.5	6.5 ± 1

The effect of pH on the alkylation rate was determined between pH 7.1 and 9.4 at constant concentration of 3-chloroacetyl pyridinium adenine dinucleotide. In the pH range 7.1—8, hydrolysis of the chloroketone could be neglected [13]; above pH 8, only data obtained in the first 3 min were used. The half time of alkylation increased with pH, and a p*K* of about 9 pH units was calculated (Fig. 2).

Protection against inactivation by 3-chloroacetyl pyridinium adenine dinucleotide. The data obtained after incubation of membranes (+ inhibitor) in the presence of different effectors of the enzyme are presented in Table III. NAD^+ exhibited a protective effect of about 45% when present in large excess (NAD^+/3-chloroacetyl pyridinium adenine dinucleotide = 28.5). Protection by dehy-

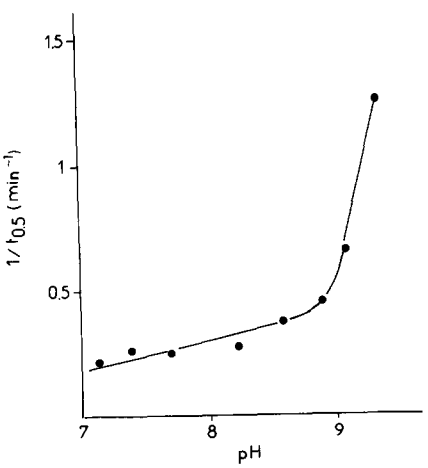

Fig. 2. pH dependence of the inactivation reaction half-time of 3-chloroacetyl pyridinium adenine dinucleotide. Microsome concentration 3 $mg \cdot ml^{-1}$. Inhibitor concentration: 280 μM. In the pH range 7.14—7.71, 50 mM HEPES/NaOH was used. At higher pH, the buffer was 50 mM Tris · HCl. Above pH 8, short-time incubation values were taken into account and graphically extrapolated to a 50% loss of activity.

TABLE III

Protection of Δ^5-3β-hydroxysteroid dehydrogenase against inactivation by 3-chloroacetyl pyridinium adenine dinucleotide. Incubations were performed at room temperature in 50 mM HEPES/NaOH (pH 7.5) with: (I) 460 μM 3-chloroacetyl pyridinium adenine dinucleotide (II) 70 μM 3-chloroacetyl pyridinium adenine dinucleotide or (III) 3.5 mM 3-chloroacetyl pyridinium adenine dinucleotide. Microsome concentration 3 mg · ml^{-1}.

Incubation with	Incubation time (min)	Residual activity (%)	% protection
(I)	20	63 ± 3	—
(I) + 116 μM DHA	20	84 ± 4	58
(I) + 578 μM DHA	20	84 ± 4	58
(I) + 34 μM equilenin	20	98 ± 5	93
(II)	60	78 ± 4	—
(II) + 2 mM NAD^+	60	88 ± 4	45
(III)	20	70 ± 4	—
(III) after preincubation with 1.67 mM iodoacetamide	20	170 ± 8	100

TABLE IV

Modification of the kinetic parameters of Δ^5-3β-hydroxysteroid dehydrogenase by alkylation with 3-chloroacetyl pyridinium adenine dinucleotide. Incubations were performed as in Table III with 460 μM 3-chloroacetyl pyridinium adenine dinucleotide.

Incubation with	K_m NAD^+ (μM)	K_m DHA (μM)	V (nmol · min^{-1} · mg^{-1})
No addition	9 ± 1.5	0.2 ± 0.1	36 ± 4
+ inactivator	10 ± 1.5	1.5 ± 0.3	25 ± 3

droepiandrosterone was more efficient (58%) even at a much lower concentration ratio (dehydroepiandrosterone/3-chloroacetyl pyridinium adenine dinucleotide = 0.25).

The use of equilenin, a competitive inhibitor of the enzyme, ($K_I = 10^{-8}$ μM), fully prevented inactivation of the enzyme at a concentration as low as 34 μM.

In addition, preliminary carboxyamidomethylation prevented the enzyme from inactivation by the NAD^+ analogue.

Modification of kinetic parameters 3-chloroacetyl pyridinium adenine dinucleotide. Table IV presents data on the effect of the NAD^+ analogue on the kinetic parameters of the enzyme. As found for the other SH reagents, the K_m for dehydroepiandrosterone was increased (about 5 times). No change in the K_m for NAD^+ was detected, whereas V was decreased (38% inhibition).

Discussion

It has been demonstrated that several SH group reagents modify to some extent the enzymatic activity of the Δ^5-3β-hydroxysteroid dehydrogenase. Although the effects on V were quite different, these compounds exhibited comparable behavior as far as the K_m of both substrates were concerned: no change of K_m for NAD^+ was detected whereas K_m for dehydroepiandrosterone

was strongly affected. The larger the reagent used, the higher the increase of this parameter. These results are consistent with the protection experiments against the effect of iodoacetamide: whereas the steroid exhibited a strong protective effect, NAD^+ was ineffective. This set of observations leads to the assumption that iodoacetamide as well as the other thiol reagents used in this work were replacing a group near the steroid-binding site of the enzyme. The activation effect observed iodoacetamide and parachloromercuribenzoate remains without clear explanation but a similar observation has been specifically reported in the case of a steroid dehydrogenase [15].

Surprisingly, the 3-chloroacetyl pyridinium adenine dinucleotide, which behaved as an affinity label for the 17β-hydroxysteroid dehydrogenase from human placenta [9], led to similar modifications of K_m for dehydroepiandrosterone with no significant change in K_m for NAD^+. Furthermore the protection experiments performed either with steroids or NAD^+ clearly showed that the steroids, and especially the competitive inhibitor equilenin, were more effective than NAD^+. Finally, a preliminary incubation with iodoacetamide protected the enzyme from inhibition by 3-chloroacetyl pyridinium adenine dinucleotide, which indicated that iodoacetamide and 3-chloroacetyl pyridinium adenine dinucleotide were competitive. This raises some questions about the inactivation mechanism exhibited by 3-chloroacetyl pyridinium adenine dinucleotide. The inactivation parameters as determined by the method of Kitz and Wilson [13] are not essentially different from those with 17β-hydroxysteroid dehydrogenase [9] but this does not prove that in our case the inactivator was acting as a true affinity label. A first binary complex is likely to occur as with protection by NAD^+, however, a precise examination of the inactivation mechanism is difficult as we are dealing with a membrane preparation. The relatively poor inactivation effect of the NAD^+ analogue is also difficult to explain. One possible hypothesis is that in the adrenal microsomes several dehydrogenases are working on different physiological substrates [15, 16]. Although solubilization and purification data are needed to settle this points [17], measurements performed with pregnenolone and 17α-hydroxypregnenolone (preliminary data) indicated no inactivation by 3-chloroacetyl pyridinium adenine dinucleotide when the last steroid was used as substrate, whereas the inactivation levels are comparable when pregnenolone or dehydroepiandrosterone are used as substrates (38%). Whatever the mechanism may be, the existence of an SH-reactive group near the steroid-binding site is interesting for further studies with alkylating steroids which should behave as true affinity labels [7]. This work is now being undertaken in this laboratory with fluorescent steroids.

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