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Reversible Addition of Thiols to Steroid α,β-Unsaturated Carbonyl Groups: Possible Role in Steroid–Protein Interactions

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The equilibrium constant (M^{-1}) for addition of 2-mercaptoethanol at 25°C was found to be 7×10^3 with 2-cyclohexenone, to be near 2 with 3-methyl-2-cyclohexenone, 19-norprogesterone, and 19-nortestosterone, and to be in the range of 0.01–0.03 with progesterone and testosterone. The results demonstrate that thiol addition to steroid α,β-unsaturated carbonyl groups is sufficiently favorable to be of potential importance in steroid–protein interactions.

INTRODUCTION

A substantial body of evidence shows that binding of steroids to intracellular protein receptors can be dependent upon sulfhydryl groups present in the receptor (Refs. (*1–3*) and literature cited therein). Such sulfhydryl groups also occur in corticosteroid-binding globulin (*4*), CBG, which is unusual since extracellular proteins do not ordinarily contain sulfhydryl groups, these being unstable in the oxidizing extracellular environment (*5, 6*). For CBG (*7*), and also for 20β-hydroxysteroid oxidoreductase (*8*), it has been shown by labeling with 6β-bromoprogesterone that a cysteine sulfhydryl group is present in the binding site at a location adjacent to the position of the steroid A–B ring juncture. The role played by such sulfhydryl groups has not been established. It has been proposed that reversible thiol addition to steroid α,β-unsaturated carbonyl groups might play a role in steroid–protein interactions (*9*), but the actual reactivity of such groups toward thiols has not been established. The purpose of the present study was to determine whether or not steroid A-ring α,β-unsaturated carbonyl groups react with thiols. Use of 2-mercaptoethanol, which has reasonable solvent properties, as the thiol permitted studies at high thiol concentration, thereby allowing the detection of reactions involving unfavorable equilibria.

MATERIALS AND METHODS

2-Mercaptoethanol (2-*ME*) from Calbiochem was vacuum distilled, sealed under nitrogen in ampoules, and stored at 4°C until used. 2-Cyclohexenone (*C*), 3-methyl-2-cyclohexenone (*M*), and *N*,*N*-dimethylethanolamine (*DMEA*) were

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from Aldrich. Progesterone (*P*) and 19-norprogesterone (*NP*) came from Syntex, and testosterone (*T*) and 19-nortestosterone (*NT*) were obtained from Sigma Chemical Company. Reduced glutathione was from Calbiochem. All solutions were prepared with degassed solvents or buffers and stored under nitrogen in sealed containers.

Nuclear magnetic resonance (nmr) spectra were recorded on Varian T-60, EM-390 or HR 220 spectrometers. Ultraviolet (uv) measurements were made in 2- or 10-mm cells using a Beckman DU monochrometer fitted with a Gilford Model 2220 adapter and 222A photometer. Extinction coefficients for enone and *2-ME* solutions were measured directly while those for the adduct were obtained from analysis of the equilibrium absorbance values, corrected for *2-ME* absorbance, as a function of *2-ME* concentration.

Addition to *C* was followed by mixing stock solutions of the reactants in 0.1 *M* phosphate buffer (pH 7) in a quartz cuvette under nitrogen and monitoring the absorbance at 229 nm until it stabilized (5 hr). The results in Table 1 represent the average of 12 runs with varying *C* and *2-ME* concentrations. Addition to *M* was carried out similarly in 95% methanol containing 20 m*M* NaOH, the decrease in absorbance being monitored at 307 nm. Elimination reactions were conducted by diluting 2-hr equilibrated solutions containing *2-ME* (7 *M*) and *M* (0.24–0.59 *M*) to the desired final concentrations and following the increase in absorbance at 307 nm. Addition reactions with *NT* were conducted in an analogous fashion.

Pure adduct of 2-ME with *NT* was obtained by equilibration of 0.28 g of steroid in 1 ml 2-ME containing 0.1 *M* NaOH for 3.5 days at room temperature. The reaction mixture was then slowly added to 20 ml of cold 0.1 *M* phosphate buffer (pH 6) with rapid stirring. The resulting precipitate was collected, washed, and dried under vacuum (75% yield). The 90-MHz nmr spectrum (5% in CD_3COOD) gave: 3.80 (*m*, 1H), 3.72 (*t*, J = 7 Hz, 2H), 2.61 (*t*, J = 7 Hz, 2H), 1.05–2.30 (broad mults., ~25H), 0.80 (*s*, 3H). After recrystallization from nitromethane the adduct had mp 185.0–185.5°C (uncorr.) and gave an ir spectrum (KBr pellet) having a strong peak at 1720 cm^{-1}. Reaction of an 11.4 m*M* solution of adduct in 95% EtOH containing 40 m*M* DMEA resulted in the slow release ($t_{1/2}$ ~ 1 day) of 1 eq of thiol as determined by the method of Ellman (*10*).

Nuclear magnetic resonance studies were conducted in a similar fashion, reactant solutions being prepared in nmr tubes using deuterated solvents. With *M* and *NP* equilibrations were complete in several hours to 1 day, whereas reactions of *P* and *T* required several days to reach equilibrium. The enone concentration was assessed from the resonance of the vinylic methyl group or the vinyl proton using the $-CH_2O-$ resonance of *2-ME* or the steroid C-13 methyl group to calibrate the integration; adduct concentration was calculated by difference.

With *P* and *T* a precipitation (PPT) technique was also used to estimate adduct formation. Samples of reaction solutions were mixed rapidly with cold 0.1 *M*, pH 6 phosphate buffer. The precipitate was collected and dried under vacuum giving 90% steroid recovery by weight. The ratio of adduct to enone in recovered steroid was determined from nmr measurements in $CDCl_3$. Alternatively, *2-ME* was eliminated from the adduct by incubation with NaOH in ethanol under nitrogen and in the presence of reduced lipoamide linked to glass beads (Pierce) to protect

against thiol oxidation. Released thiol was titrated by the method of Ellman (*10*). Little or no bound thiol was detected in the first minutes after initiating reaction of *2-ME* with enone but the value increased steadily with time, reaching a stable value after several days.

Reaction of *C* and *NT* with glutathione (GSH) was also examined. When 0.135 m*M* *C* was reacted with 2 m*M* GSH in 0.1 *M* phosphate buffer (pH 7.5) the absorbance at 229 nm (2 m*M* GSH as reference) declined in exponential fashion ($t_{1/2} \sim 10$ min) and reached a final value less than 5% that of the starting enone. When 0.2 ml 100 m*M* GSH in water (pH 8.3) and 1.8 ml 12 m*M* *NT* 90% EtOH were mixed no loss in absorbance at 305 nm occurred in 4 days (reference cell contained 10 m*M* GSH without *NT*).

RESULTS

The structures of the compounds studied are shown in Fig. 1, and results are summarized in Table 1. In the case of 19-nortestosterone the adduct was isolated in pure form and shown to be formed by addition of 1 eq of thiol to the steroid. The absence of vinylic proton resonances in the nmr spectrum, the presence of a normal carbonyl peak in the infrared spectrum, and the absence of titratable thiol in the adduct show that the adduct arises from thiol addition across the olefinic double bond. By analogy, the adducts with the other enones studied are taken to

TABLE 1

EQUILIBRIUM CONSTANTS FOR ADDITION OF 2-ME TO ENONES AT 25°C

ene	solvent (base)	[ene]	[2-ME]	Method[a]	K^b (M^{-1})
C	0.1 *M* phosphate (ph 7)	30–120 μM	0.1–1 m*M*	uv-229-A	7×10^3
M	95% MeOH (20 m*M* OH^-)	0.12 *M*	1.4–7 *M*	uv-307-A	1.6 ± 0.3
				uv-307-E	1.6 ± 0.3
M	95% CD_3OD (40 m*M* OD^-)	0.25–2.5 *M*	0.25–2.5 *M*	nmr-A	1.5 ± 0.3
		0.25–1 *M*	0.25 *M*	nmr-E	1.8 ± 0.3
NP	2-ME, benzene-d_6 (20–60 m*M* OH^-)	0.31–1 *M*	3–10 *M*	nmr-E	1.7 ± 0.3
NT	95% EtOH (40 m*M* DMEA)	12 m*M*	0.5–2 *M*	uv-305-A	2.1 ± 0.2
P	2-ME (80 m*M* OH^-)	1 *M*	10 *M*	PPT-A	0.015 ± 0.005
				nmr-A	0.013 ± 0.005
T	2-ME (0.1 *M* OH^-)	1 *M*	10 *M*	PPT-A	0.008 ± 0.002
				nmr-A	0.014 ± 0.002

[a] Numbers are wave lengths in nm. Equilibria were established by addition (A) of thiol to ene or elimination (E) of thiol from adduct.

[b] Uncertainties reflect the range of values obtained in experiments spanning the range of concentrations shown or in at least three replicate experiments.

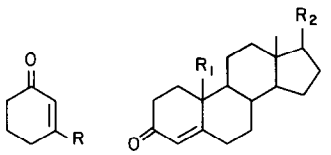

FIG. 1. Structure of enones. *C*, R = H; *M*, R = CH_3; *P*, R_1 = CH_3, R_2 = $COCH_3$; *NP*, R_1 = H, R_2 = $COCH_3$; *T*, R_1 = CH_3, R_2 = OH; *NT*, R_1 = H, R_2 = OH.

be those resulting from Michael addition. The adducts cannot be thiohemiketals, since these would certainly undergo rapid decomposition in the presence of base (*11*) whereas the adducts formed in the present study decompose only slowly with base. The present results do not allow an assignment to be made for the stereochemistry of attachment of the sulfur at C-5 in the steroid series.

Addition to 2-cyclohexenone occurred at 1 m*M* 2-*ME* and neutral pH with a half-life of about 55 min. Addition to 3-methyl-2-cyclohexenone was slower, necessitating higher base concentrations. The half-life was 16 min for 20 m*M* NaOH and was independent of *2-ME* below 3.5 *M*. Values of *K* for *M* determined by uv and nmr methods, and by approach to equilibrium from different directions, were in acceptable agreement. Similar values for *K* were obtained with 19-norprogesterone and 19-nortestosterone.

Addition to progesterone and testosterone was even less favorable; and an indirect experimental approach, involving precipitation of steroid from the equilibrium mixture obtained in neat *2-ME*, was used with *P* and *T* to check the nmr method. Detection of adduct in the recovered steroid by nmr or by titration of thiol released by base gave the data needed to calculate *K*. However, the values thus obtained must be considered lower limits, since only 90% recovery of steroid was obtained and it is possible that the adduct is more soluble than the enone and is preferentially lost. An upper limit for *K* can be estimated assuming that all of the lost steroid was adduct. This treatment gives values for *P* and *T* about twice those reported in Table 1, so that the range of *K* as estimated by the precipitation method can be given as 0.01–0.03 for these two steroids. The values obtained by direct nmr determination at 220 MHz were within this range.

A limited examination of the reaction of glutathione with *C* revealed that *C* reacts rapidly ($t_{1/2}$ ~ 10 min) and completely (>95%) with 2 m*M* GSH near neutral pH. In contrast, *NT* exhibited no detectable reaction (<3%) after 4 days with 10 m*M* GSH at slightly basic pH. This finding is consistent with the results of Table 1 if *2-ME* and GSH behave similarly.

DISCUSSION

It is important at the outset to consider several uncertainties regarding the values of *K* reported in Table 1. First, in most of the studies the *2-ME* concentration was varied over a sufficiently wide range that the solvent composition is significantly changed. To the extent the *K* is solvent sensitive the range of uncertainty reported may reflect a variation in *K* with solvent. Second, for the

steroids it has not been established to what extent each of the two possible stereoisomers is formed, so that K reflects a composite for the two processes. The largest error occurs if the two stereoisomers were to be formed in equal amount, in which case the reported K would be twofold larger than the correct value for a specific reaction. Finally, experimental uncertainties discussed under Results lead to a factor of two uncertainty in the values of K for the addition to P and T. While it would be desirable to have more precise values, the results obtained suffice to answer the questions posed in this study.

The present results confirm and extend the previously observed effect of β-alkyl substitution in lowering reactivity of α,β-unsaturated carbonyl compounds toward thiols (*12, 13*). They demonstrate the reversibility of such addition with the A-ring enone group of steroids and the potential sensitivity of this reaction to variations in steroid structure. Thus, removal of the C-19 methyl group increases K by 100-fold, a feature which is interesting in view of the fact that 19-norsteroids are often more active than their 19-methyl analogs and bind more readily to receptors (e.g., progesterone receptors (*14*)).

The relationship of the present results to the problem of steroids binding to proteins having a sulfhydryl group at the binding site (PSH) is best seen by viewing the binding process in two steps as follows:

$$\text{PSH} + \text{steroid} \rightleftharpoons (\text{PSH} \cdots \text{steroid}) \overset{K_1}{\rightleftharpoons} \text{P-adduct.}$$

The equilibrium for the hypothetical conversion of noncovalent protein–steroid complex to a covalent complex is a unimolecular process. Favorable entropy factors could easily cause K_1 to be 10^3 larger than K for the bimolecular reaction of *2-ME* (*15*) and a factor approaching 10^8 is theoretically possible (*16*). On this basis K_1 for P and T could be 10–30 or larger and K_1 for NP and NT could be ≥ 2000. Reversible formation of covalent steroid–receptor complexes as the result of thiol addition reactions is therefore clearly feasible from an equilibrium standpoint.

We may now consider whether such reactions are likely to occur on a time scale which could be important in biological systems. From the approximate half-life (10^5 sec) for reaction of P and T at thiolate anion concentrations near 0.1 M we can estimate a bimolecular rate constant for addition of $10^{-4}\ M^{-1}\ \text{sec}^{-1}$. Conversion of a noncovalent complex to covalent adduct would be a unimolecular process subject to analogous entropy effects to those applied above to the equilibrium constants. Using a minimal value of 10^3 we estimate that the half-life for conversion of noncovalent complex in which the thiol group is ionized would be about 10 sec. The thiol group of cysteine and related compounds is ionized to the extent of 1–10% near neutral pH; and if this were to hold for the thiol of the noncovalent complex, the effective half-life would become 2–20 min. This period is slower than the time scale of most biological binding reactions. However, the factor of 10^3 could be orders of magnitude low and the binding site could contain a group which functions as a general base in catalyzing the thiol addition. Thus, the kinetics need not be a critical factor limiting the importance of reversible thiol addition in the binding of steroids to proteins.

Finally, we comment on the significance of the results for the reaction of enones

with glutathione. Glutathione is the major thiol component of most cells, usually occurring at concentrations of 1–10 m*M* (*5, 9*). Assuming that glutathione behaves like *2-ME*, we would expect that 2-cyclohexenone would be rapidly and almost completely converted to adduct when it enters cells. In accord with this surmise, the observed half-life at 2 m*M* GSH was 10 min and reaction went essentially to completion. Since cells contain enzymatic activities which catalyze such conjugation reactions (*17*), the actual half-life under physiological conditions could be much lower. In contrast with 2-cyclohexenone, the results would predict that none of the other compounds of Table 1 would react with GSH at typical cellular concentrations. In accord with this expectation, no reaction between *NT* and GSH could be detected at neutral pH and 10 m*M* GSH. Thus, while intracellular glutathione can be expected to conjugate the more reactive enones which enter cells, it will not bind *NT* or less reactive steroids. These should be fully available for binding at receptors.

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