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THE STATE TRANSITIONS OF NORMAL AND MUTANT ANDROGEN–RECEPTOR COMPLEXES IN HUMAN GENITAL SKIN FIBROBLASTS

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Summary—We have incubated cells from controls and subjects with receptor-defective androgen resistance with ^{3}H-labelled testosterone (T), methyltrienolone (MT), dihydrotestosterone (DHT) or mibolerone (MB) and studied the temperature dependance of the dissociation rate constants of these various androgen–receptor (A–R) complexes both within cells and after they were extracted from them. In control cells, Arrhenius plots for T–, MT–, DHT– and MB–R complexes were linear and formed a hierarchy of dissociation states with energies of state IV > III > II > I, respectively. Relative to this hierarchy, the dissociation states of the MB–, DHT– and MT–R complexes in mutant cells were displaced to higher, androgen-inappropriate energies in a mutant-distinctive pattern. When extracted from cells control or mutant T– or MT–R complexes, and mutant (but not control) DHT– or MB–R complexes lowered their respective dissociation rates by undergoing state transitions in conformity with the hierarchy. Hence we propose that different A–R complexes reach different dissociative states by undergoing sequential transitions along a common pathway, and that these transitions are co-regulated both by the chemical characteristics of the bound androgen and by other cellular non-receptor factors.

INTRODUCTION

In order to regulate gene expression, steroid hormones must first bind to specific intracellular receptor proteins to form steroid–receptor (S–R) complexes. These complexes then interact with regulatory sequences of nucleotides (steroid-response elements) in order to enhance or suppress transcription from nearby promoters. For this interaction to be effective, an initial S–R complex must transform to a state that has increased affinity of steroid for receptor and of S–R complex for DNA. Little is known about the steroid-specific contribution of various steroids to the combinatorial properties of a transformed S–R complex. Usually, there is a positive correlation between the affinity with which a steroid binds to its receptor and the potency with which it regulates gene expression. However, it is not understood why complexes formed by estrogen, progesterone or glucocorticoid antagonists with their respective receptors do not actively regulate hormone-responsive genes, when they are just as competent as normal agonist–receptor complexes in binding to their respective hormone-response elements [1–3].

This paper describes a novel approach to studying the manner in which androgen interacts with the androgen receptor: it exploits the effect of temperature on the dissociation rate constants of the complexes formed between the androgen receptor and different androgens; and it compares the intra- and extracellular dissociative behaviour of the various A–R complexes formed in the cells of controls and subjects with AR-defective androgen resistance. Our data indicate that particular A–R complexes can occupy sequential dissociative states in a hierarchy that is common to various A–R complexes, and that while the choice of states is androgen-restricted, it must also involve a non-receptor factor(s) that interacts with the complex in an unknown way. There is considerable precedent for the action of micro- and macromolecular non-steroid-binding factors on various properties of SR and S–R complexes [4–6]. The present results may bear on the contribution of different androgens to the target-specific properties of different A–R complexes. For instance, it is not understood why T–R complexes suffice in some androgen targets, while DHT–R complexes are required in others [7–12]; nor is it known why T–R complexes are labile, apparently because of

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Abbreviations: T, testosterone: DHT, 5α-dihydrotestosterone; MT, methyltrienolone, 17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one; MB, mibolerone, 7α,17α-dimethyl-19-nortestosterone; AR, androgen receptor; HEPES, N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid; GSF, genital skin fibroblasts.

proteolytic susceptibility of the receptor, under conditions that permit DHT–R complexes to transform normally [13].

MATERIALS AND METHODS

Cell culture

The genital skin fibroblast strains (GSF) were developed in our laboratory from small pieces of labium majus, scrotal or preputial skin obtained from healthy volunteers (controls) with informed consent according to protocols approved by the Hospital Ethics Committee. Monolayers were grown to confluence in 5 cm^2 dishes or 750 cm^2 roller bottles with Eagle's minimal essential medium supplemented with 10% fetal calf serum, 1 mM pyruvate, 10 mg/l garamycin and 60 mg/l each of penicillin G and streptomycin sulphate. The strains from subjects with receptor-defective androgen resistance are classified as: (i) "complete", if their external genitalia are female [KIL (14) and 30285 (15)], (ii) "minimal", if they are male [41560 (16), D3295 & B1016 (17), 5555 (18) and 44218 (unpublished)], and (iii) "partial", if they are morphologically ambivalent [4308 (19) and TCF (14, 20)] to any degree. The strains from the patients with 5α-reductase deficiency have been described previously [8, 9].

Binding of [^{3}H]MB, [^{3}H]DHT, [^{3}H]MT and [^{3}H]T to androgen receptors within intact GSF

Replicate monolayers in 5 cm^2 dishes were incubated with 3 nM [17α-methyl-^{3}H]methyltrienolone (87 Ci/mmol), [17α-methyl-^{3}H]mibolerone (75 Ci/mmol), [1,2,4,5,6,7-^{3}H]5α-dihydrotestosterone (147 Ci/mmol) or [1,2,6,7-^{3}H]testosterone (105 Ci/mmol) alone (to measure total binding) or together with a 200-fold excess of the respective radioinert androgen (to measure non-specific binding) in serum-free medium buffered to pH 7.4 with 15 mM HEPES. GSF from patients with 5α-reductase deficiency were used in assays with T. After incubations for 2 h in a humidified 37°C incubator supplied with 5% CO_2:95% air, the dishes were placed on a bed of ice, washed twice with 5 ml of Tris–HCl (20 mM, pH 7.4) containing 0.15 M NaCl and 0.2% bovine serum albumin (BSA) and twice with the same buffer lacking BSA. The monolayers were then treated for 5 min at room temperature with 0.1% trypsin. The loosely adherent cells were scraped with a rubber policeman and centrifuged (4°C) at 200 *g* for 5 min. The cells were resuspended in Tris buffer lacking BSA and recentrifuged. The final cell pellets were solubilized with 0.5 N NaOH (1.5 ml) and sampled for protein [21] and radioactivity, the latter in 10 ml of a toluene solution containing Omniflor (4 gm/l; New England Nuclear, Markham, Canada) and counted in an LKB 1217 RACKBETA scintillation counter at an efficiency of 60%.

Dissociation of A–R complexes within intact cells

To determine the dissociation rate of A–R complexes within cells that had been incubated at 37°C for 2 h with [^{3}H]MB, [^{3}H]DHT, [^{3}H]MT or [^{3}H]T, the assay medium in some dishes was replaced by a "chase" medium containing 0.6 μM of the respective radioinert androgen. After various periods of time at temperatures between 20 and 42°C, the activity remaining was determined and the results plotted semi-logarithmically. Rate constants were calculated from slopes of regression lines fitted to these data. In all assays, the concentration of specific A–R complexes formed or remaining at each time was computed by subtracting the non-specific binding measured in duplicate from total binding in triplicate. Arrhenius plots were constructed by plotting the natural logarithm (−ln) of the dissociation rate constant versus the reciprocal of the absolute temperature [$1/T = 1/(C + 273)$] where C = degrees centrigrade. The activation energies for the dissociation reactions were calculated from the slopes of these lines.

Dissociation of A–R complexes in soluble extracts of labelled cells

After incubation for 2 h with [^{3}H]MB, [^{3}H]DHT, [^{3}H]MT or [^{3}H]T (3 nM), the monolayers in five 750 cm^2 roller bottles were scraped into 10 ml Tris–HCl buffer, and centrifuged (200 *g*, 10 min, 4°C). The pellets were resuspended in 1 ml of 5 mM phosphate buffer (pH 7.4) containing 0.4 M KCl, 1.5 mM mercaptoethanol and 50 mM dithiothreitol and homogenized by 50 strokes of a Dounce apparatus (4°C). The homogenate was centrifuged (100,000 *g*, 1 h, 4°C) in a Beckmann Ti-50 rotor. The supernatant was applied at 4°C to a Sephadex G-75 (0.9 × 30 cm) column packed with the phosphate buffer lacking KCl and the void volume was collected. This was sampled for protein and an aliquot was resuspended in 1 ml of a 0.5%, charcoal, 0.05% dextran T-70 suspension in phosphate buffer. The mixture was vortexed, centrifuged (2000 *g*, 10 min, 4°C) and the supernatant was counted by radioscintillation as above. The remainder was placed in a circulating water bath set to various temperatures, and sampled periodically as above. Non-specific binding, determined on material extracted from cells labelled with [^{3}H]androgen in the presence of 0.6 μM radioinert androgen, was found to be less than 10% of [^{3}H]androgen bound in its absence.

Exchange reaction

Cells in roller bottles incubated with radioinert MT as above were washed twice with buffer containing and once with buffer lacking BSA. Replicate monolayers were incubated for an additional 2.5 h (equivalent to 2.6 half-lives) with 3 nM [^{3}H]DHT or [^{3}H]T. Cells from replicate roller bottles were combined and

from the cell pellet a 100,000 *g* high-speed supernatant was prepared and dissociated as above.

RESULTS AND DISCUSSION

The hierarchical dissociative states of various A–R complexes in control cells

Rates of dissociation for control T–R complexes > MT–R complexes > DHT–R complexes > MB–R complexes (Fig. 1). Rate constants calculated from the slopes of these lines and from those determined for T–R [8], MT–R [20], DHT–R [22] and MB–R [16] complexes at other temperatures are plotted by the method of Arrhenius in Fig. 2. These rate data yielded in hierarchy of energy states (IV . . . I). In order a initiate the dissociation of androgen from each of these states, an identical activation energy is required ($\Delta H = 28$ Cal/mol).

Sequential state transitions out of control cells

At all temperatures studied, extracted MB–R complexes in state I [15] or DHT–R complexes in state II [22] dissociated exactly as they had inside cells (data not shown): monophasically and at an unchanged rate. In contrast, we have previously reported [8] that at 37°C, T–R complexes extracted from 5α-reductase-deficient cells begin to dissociate even faster than they do in cells, but then undergo a time- and temperature-dependent transition to a form with a slower rate. Rate constants for the slow component of the biphasic curves at 37 and 35°C (Fig. 3A; 0.014 and 0.011/min, respectively) and that of the single component at 29°C (0.005/min) are now recognized as typical of normal *intracellular* MT–R complexes in state III. We have previously explained [9,22] the temperature-dependent change in the character of these dissociation profiles by proposing that the rate constant for the extracellular transition of T–R complexes to state III is less temperature-dependent than is the rate constant of dissociation for T–R complexes before the transition [21]. According to this explanation, at 37°C a large fraction of the parental population of complexes dissociates before transition

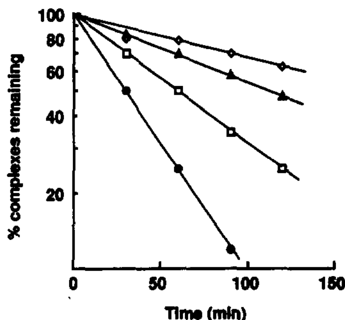

Fig. 1. Dissociation of A–R complexes within intact cells. Control cell monolayers were labelled with 3 nM [^{3}H]T (●), [^{3}H]MT (□), [^{3}H]DHT (▲) and [^{3}H]MB (◇) for 2 h at 37°C, and the A–R complexes chased at 37°C with a 200-fold excess of the respective radioinert androgen.

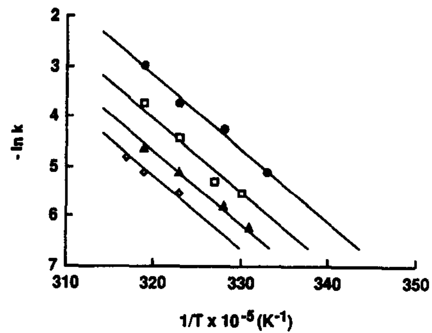

Fig. 2. Energy states of the normal androgen receptor. Rate constants calculated from the slopes of rate data for T–R (●), MT–R (□), DHT–R (▲) and MB–R (◇) complexes at various temperatures plotted by the method of Arrhenius.

to state III can occur. At 35°C, a smaller fraction of this population dissociates before transition occurs to state III. Finally, at 29°C, dissociation does not begin until all of the parental complexes have undergone transition to state III.

MT–R complexes in state III were extracted from cells. They also dissociated biphasically at the highest temperature (40°C) but linearly at the lowest temperature studied (35°C) (Fig. 3B). Again, in a pattern of behaviour paralleling that of T–R complexes, at 35°C the extracellular MT–R complexes dissociated half as fast as MT–R complexes dissociate in cells (0.005/min vs 0.009/min). This rate is equal to the one at which DHT–R complexes dissociate, at 35°C, whether in or out of cells. From these data, in aggregate, we conclude that T–R and MT–R complexes have the intrinsic potential to undergo transitions to lower energy (slower dissociation) states in the hierarchy, but that some quality of the intracellular environment prevents them from realizing this potential. We offer this interpretation for the following set of circumstantial reasons: (a) the complexes are formed *in cells* under conditions of time and temperature that ensure they are "transformed"

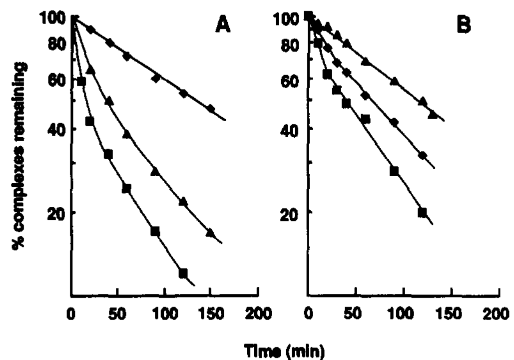

Fig. 3. Extracellular dissociation of A–R complexes. Control monolayers labelled for 2 h at 37°C with [^{3}H]T or [^{3}H]MT were extracted with a phosphate buffer containing 0.4 M KCl. The supernatants from high-speed ultracentrifugations were desalted and the T–R [panel A; 29°C (◆), 35°C (▲) and 37°C (■)] and MT–R complexes [panel B; 35°C (▲), 37°C (◆) and 40°C (■)] dissociated.

as this is classically defined for steroid–receptor complexes; and (b) the step-down in energy state is first observed after they begin to dissociate. Direct support for this interpretation comes from the observation that mutant MT–R (discussed below), DHT–R [22] or MB–R [15] complexes that dissociate linearly, but at abnormally fast rates in cells (at rates equal or faster than that of normal MT–R complexes) also lower their rates of dissociation upon extraction from cells, just as normal T–R or MT–R complexes do. In other words, an A–R complex that starts at a point high enough in the hierarchy has an intrinsic capacity to undergo transition to a lower energy state, whether its starting point has been set by the union of a normal receptor with a restrictive androgen, or an abnormal receptor with a permissive androgen.

Sequential transitions in control cells

Evidence that sequential transitions along a common pathway is the mechanism by which different A–R complexes reach their respective hierarchical positions, in cells, comes from exchange experiments. Radioinert state III MT–R complexes were formed within cells grown to confluence in roller bottles and then exchanged with [^{3}H]DHT (Fig. 4a). The [^{3}H]DHT–R complexes so formed were extracted, and allowed to dissociate. In 6 experiments the DHT–R complexes dissociated from state II; they were thus indistinguishable from complexes extracted from cells labelled directly with [^{3}H]DHT. In an equal number of experiments, however, the [^{3}H]DHT–R complexes dissociated non-linearly and faster than expected. The dissociation profiles of these atypical DHT–R complexes resembled the out-of-cell dissociation profiles of state III MT–R complexes. We conclude from these results that DHT–R complexes can exist intracellularly in states II or III. On the other hand, [^{3}H]DHT–R complexes formed by exchange as above but permitted to dissociate within

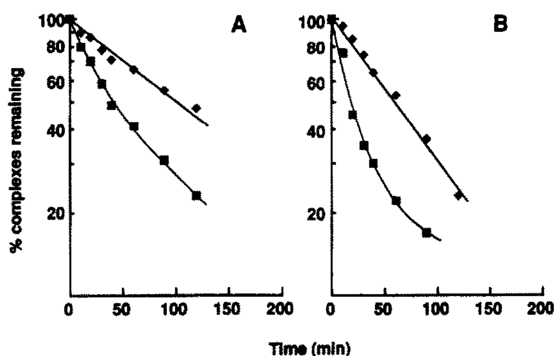

Fig. 4. Variation in the extracellular behaviour of A–R complexes formed within cells by an exchange reaction. Monaloyers labelled at 37°C for 2 h with 3 nM radioinert MT were washed and the intracellular MT–R complexes exchanged for an additional 2 h with: Panel A: 3 nM [^{3}H]DHT. Results of 5 trials (■) were distinguished from the expected profiles (◆, $n = 6$). Panel B; 3 nM [^{3}H]T. Result of one trial (◆) was distinguished from the expected profile (■, $n = 2$).

cells grown to confluence in small (5 cm) petri dishes did so exclusively from state II ($n = 8$, data not shown). The only way we can explain these apparently disparate results is by assuming that periodic exposure of cells to hormone in roller cultures causes their A–R complexes to have slower rates of state transitions than stationary cultures.

When radioinert state III MT–R complexes were exchanged with [^{3}H]T within 5α-reductase-deficient cells (Fig. 4B) and then extracted, they dissociated from state IV in two experiments but from state III in one experiment. This indicates that intracellular androgen receptors complexed with T can exist in one or another of two sequential energy states.

Additional support for the hypothesis that sequential transitions along a common pathway is the mechanism by which A–R complexes reach their respective hierarchical positions comes from a re-evaluation of the two-state model that we developed to predict the time- and concentration-dependence of DHT, MT and MB binding to the AR within human GSF [23]. This model explained the increased "apparent" affinity and linearization of experimental Scatchard plots simply by allowing for a time-dependent redistribution of intracellular A–R complexes between a single pair of high-energy (low-affinity) or low-energy (high-affinity) states. In light of our present data which indicate that MB–R complexes, for example, mature to state I by first passing through states IV (T-like), III (MT-like) and II (DHT-like), this "two-state model" is not completely valid. However, it is still useful as an approximation and can be expected to be completely valid in the case where one of the rate constants for the three postulated state transitions is rate limiting.

Effects of androgen receptor mutations on states of A–R complexes

The effect of temperature on the dissociation rate constants of A-R complexes within cells from subjects with androgen resistance are shown in Figs 5–8. In each figure results are plotted as symbols that are superimposed on the hierarchy of energy states for A–R complexes in control cells. Other effects of these mutations on androgen binding, thermolability and up-regulation have been described [14–20].

The data in Fig. 5 represent rate constants obtained from the cells of three unrelated families with minimal androgen resistance, two previously published [16–18]. At each temperature the dissociation constants for the various A–R complexes of the different patients were identical; hence they were combined in order to simplify the Arrhenius plot. Mutant MB–R and DHT–R complexes dissociated from states II and III, respectively; thus each type of complex was in an atypical energy state, one step higher than normal. In contrast, mutant MT–R and T–R complexes dissociated normally from states III and IV, respectively. Therefore, within cells androgens are unable to induce normal state transitions

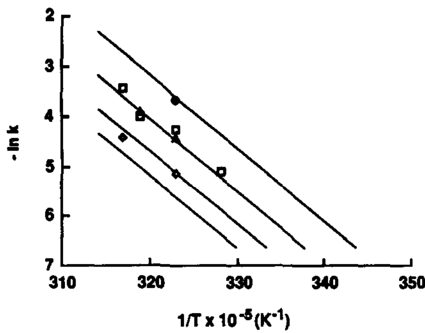

Fig. 5. Arrhenius plot of dissociation constants from patients with minimal androgen insensitivity. Monolayers labelled with [³H]T (●), [³H]MT (□), [³H]DHT (▲) and [³H]MB (◇) were chased at various temperatures. Rate constants calculated from the slopes of linear binding data are plotted against the reciprocal of temperature (T). The lines represent the hierarchy of states for A–R complexes in control cells. MB–R complexes (state I) are closest to the T–R complexes (state IV) furthest from the origin.

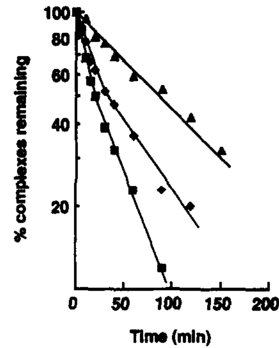

Fig. 7. Extracellular dissociation of TCF's MT–R complexes. Cell monolayers labelled for 2 h at 37°C with [³H]MT were extracted with a phosphate buffer containing 0.4 M KCl. The supernatant from a high-speed ultracentrifugation was desalted and the MT–R complexes dissociated at 30°C (▲), 35°C (◆) and 37°C (■).

of these mutant receptors beyond state III. When extracted from cells, however, state III DHT–R complexes can transform to a lower androgen-appropriate energy state: indeed, at 37°C, they dissociate linearly, and normally, from state II (data not shown). This behaviour will be described more fully below.

The data in Fig. 6 represent previously published and unpublished rate constants from the cells of two unrelated patients with partial androgen resistance: 4308 (19) and TCF (14, 20, 21). The most stable complexes in the cells of patient 4308 were formed by MB: they were in state II (Fig. 6B). Complexes formed with DHT were in state III, and those with MT in state IV. Thus, mutant complexes formed with MB, DHT and MT were in atypical energy states one step higher than normal. The A–R complexes of patient TCF (Fig. 6A) were more severely affected than those of 4308. An Arrhenius plot of the rate data for this patient's MT–R complexes was linear but appeared to describe a distinctive energy state. In accord with their unusually high starting point in the intracellular hierarchy, TCF's MT–R complexes dissociated outside cells about as fast as control state IV T–R complexes (Fig. 3A) and, yet, they were able to undergo transition to a lower energy state (Fig. 7). Not surprisingly this patient's DHT–R complexes yielded an Arrhenius plot typical of control T–R complexes in state IV; thus they were two energy-state levels above normal. However, we were surprised by the behaviour of this patient's MB–R complexes. At one temperature (33°C) they dissociated at the rate expected of normal MT–R complexes; at a higher temperature (37°C) their rate constants departed from expected linearity and they dissociated more like T–R complexes than MT–R complexes. This aberrant behaviour of mutant complexes—the ability to "jump" between adjacent energy states at various temperatures—will be described in greater detail below.

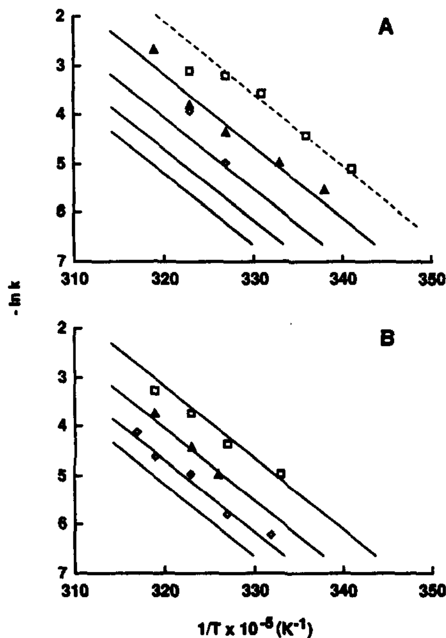

Fig. 6. Arrhenius plots of dissociation constants from a patients TCF (panel A) and 4308 (panel B). Monolayers labelled with [³H]MT (□), [³H]DHT (▲) and [³H]MB (◇) were chased at various temperatures. Rate constants calculated from the slopes of linear binding data are plotted against the reciprocal of temperature (T). The lines are defined in the legend to Fig. 5.

Previously published and unpublished rate constants have been used to construct the Arrhenius plot for the cells of two individuals with complete androgen resistance: KIL [14] and 30285 [15]. In a manner reminiscent of TCF's MB–R complexes, DHT–R complexes formed in KIL's GSF (Fig. 8A) at 37°C dissociated from state III at 40°C, from a higher energy state (state IV) at 27°C, and apparently from a mixture of both states at 37°C. At 37°C KIL's MB–R complexes apparently dissociated from a mixture of states II and III. 30285's A–R complexes

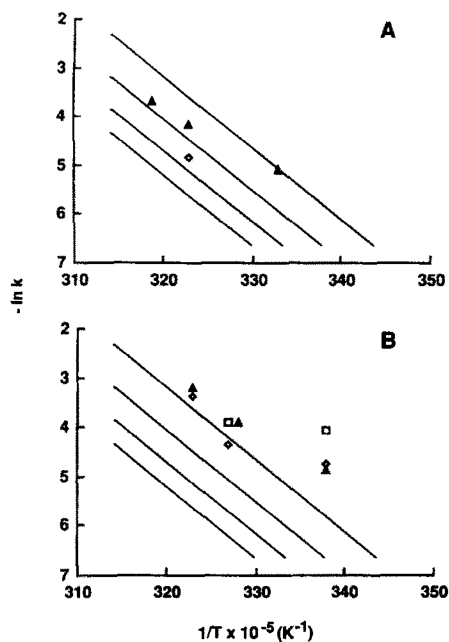

Fig. 8. Arrhenius plots of dissociation constants from KIL (panel A) and 30285 (panel B). Monolayers labelled with [^{3}H]MT (□), [^{3}H]DHT (▲) and [^{3}H]MB (◇) were chased at various temperatures. Rate constants calculated from the slopes of linear binding data are plotted against the reciprocal of temperature (T). The lines are defined in the legend to Fig. 5.

(Fig. 8B) were the most severely affected. Its MB– and DHT–R complexes at 37 and 33°C, and its MT–R complexes at 33°C, dissociated from state IV. At 22°C, however, its MB–R, DHT–R and MT–R complexes dissociated much faster than control state IV T–R complexes. The ability of mutant A–R complexes to "jump" between energy states when dissociated at temperatures other than at which they were formed is remarkable. These temperature-induced changes in the energy state of a given A–R complex must reflect the action of cellular factors that, along with specific androgens, determine the hierarchical state to which particular mutant A–R complexes are processed. In essence, then, these intracellular alterations in the dissociative behaviour of specific A–R complexes are akin to the extracellular transitions seen with control T–R or MT–R complexes and mutant (but not control) DHT–R or MB–R complexes.

A model of A–R interaction

Differences in the dissociation rates of complexes formed between a receptor and various cognate steroids are conventionally assumed to reflect a static steroid-binding domain that has constitutively different avidities for various steroids. Another possibility is that a steroid receptor is conformationally mobile and the different steroids enable its ligand-binding domain to adopt different conformational states. The latter view of A–R interaction is compatible with the data presented in this paper and is summarized

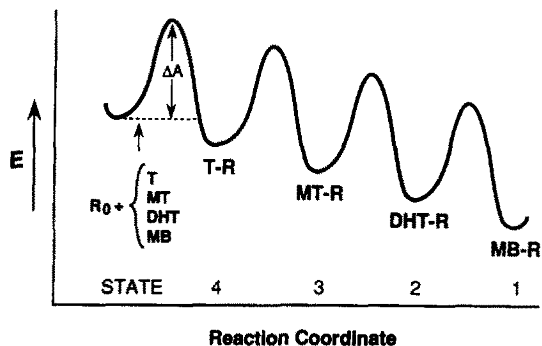

Fig. 9. A model of the state transitions of the androgen receptor. The ordinate is in units of relative energy. The abscissa is the reaction coordinate and is meant to represent the energy changes of a receptor complex as the binding reaction proceeds.

schematically by the energy diagram shown in Fig. 9. According to this scheme A–R complexes are capable of undergoing a series of *temperature*- and *androgen-dependent* transitions along a pathway that is intrinsic to the androgen receptor. These transitions, or changes in conformation, are assumed to be additionally coregulated by non-receptor factors that interact with the A–R complex non-covalently. Within normal cells MB–R complexes, for example, are assumed to be formed initially in energy state IV (or higher) and they ultimately reach state I by undergoing a series of transitions through states III and II. In mutant cells, on the other hand, the MB–R complexes are incapable of undergoing some or all of these state transitions. They thus get "blocked" in androgen-inappropriate states. Predictably, the *androgen-inappropriate* states of these mutant A–R complexes, display kinetic dissociative properties identical to normal A–R complexes brought to *androgen-appropriate* states with different androgens. Thus we conclude that the kinetic properties of an A–R complex in cells are primarily dependent on the state of the receptor rather than on the particular androgen that composes the complex. Furthermore, state transitions can occur, apparently in a *hormone-independent* fashion, when the interaction of non-receptor factors with the A–R complex is disrupted during its extraction from cells. Thus, the biological relevance of studies on *in vitro* temperature-dependent receptor "transformation" can only be evaluated in conjunction with comparative intracellular studies.

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