cAMP Binds with High Affinity and Specificity to the Androgen Receptor from Murine Skeletal Muscle†

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ABSTRACT: cAMP binding of the androgen receptor (AR) from murine skeletal muscle was studied. Testosterone affinity chromatography yielded androgen receptor with about 4000-fold purification. Determination of the cAMP binding in the affinity eluate, by adsorption of protein–cAMP complexes to cellulose ester filters or removal of unbound cAMP by dextran-coated charcoal, was not possible, as the observed binding was not stable during the assays. Displacement studies suggest that this is due to a very fast dissociation kinetics of the binding. The problem could be solved by assaying the components of affinity eluate immobilized to a testosterone affinity resin that stabilizes the cAMP–protein complexes. The cAMP binding found in the affinity eluate shows an upward concave Scatchard plot and is compatible with a model containing two independent binding sites with dissociation constants of 7 and 58 nM. However, a larger number of binding sites or negative cooperativity cannot be excluded. Sixteen cAMP binding sites were observed per testosterone binding site. The binding affinity of cAMP exceeds that of cGMP 200-fold, that of cCMP 2000-fold, and that of AMP and 2′,3′-cAMP more than 10000-fold. Results indicate that cAMP is bound by the AR, although it only represents about 1% of the total protein in the affinity eluate: (i) Specific testosterone and cAMP binding of affinity eluate was copurified by affinity chromatography, density gradient centrifugation, and gel filtration. The ratio of cAMP to testosterone binding in each peak was about 16:1, identical with that found in the total affinity eluate. (ii) The AR bound specifically to cAMP–agaroses, whereby the binding efficiency depended on the linkage between cAMP and the agarose matrix. tRNA can displace the cAMP binding, but other forms of RNA and DNA have low affinity to the cAMP binding site.

In the cell several connections are known between the action of cyclic nucleotides and steroids: steroids have been shown to alter cAMP[1] regulation [extensively reviewed by Szego and Pietras (1981)] at the level of the cAMP phosphodiesterase activity (Elks et al., 1983), the adenylate cyclase activity (Johnson & Jaworski, 1982; Lai et al., 1982), and other enzymes involved in cAMP action (Liu et al., 1981). On the other hand, cAMP regulation of steroid hormone receptor levels and function has been reported (Fleming et al., 1983; Oikarinen et al., 1984). Some of the latter effects, e.g., the regulation of glucocorticoid receptor function in murine lymphoma cell lines (Gruol et al., 1986), appear to be due to cAMP-dependent protein kinase. Recently, we described the in vitro transformation of oligomeric non-DNA-binding 8–9S androgen receptor into a 4–5S DNA-binding form by physiological concentrations of cAMP (Ofenloch-Hähnle et al., 1986). Our results, although indirect, suggested that the transformation was due to a direct interaction between cAMP and the receptor.

The present study was conducted to determine whether cAMP binds specifically to the androgen receptor and—if this is the case—to characterize this binding. We tried to elucidate in regard to a possible regulative role of this binding whether there is a connection between cAMP binding and the binding of androgen receptor to DNA or RNA, as both DNA and RNA binding are important for androgen receptor function. DNA binding has been considered the sine qua non for the gene regulatory function of steroid hormone receptors. The interest for RNA binding of steroid hormone receptors and its possible posttranscriptional regulative role, however, has recently been revived by the group of Vedeckis, who described a specific interaction of tRNA with the glucocorticoid receptor (Ali & Vedeckis, 1987a,b).

Results presented below show not only a specific binding of cAMP to the androgen receptor from murine skeletal muscle but also a competition between tRNA and cAMP for the same binding site.

Materials and Methods

Solutions and Buffers. KPP: 10 mM potassium phosphate, 2 mM EDTA, 1 mM DTT, and 0.1 mM phenylmethanesulfonyl fluoride, adjusted to pH 7.4 at 22 °C. MoKPP: KPP containing 10 mM sodium molybdate, adjusted to pH 7.4 at 22 °C. T_K: testosterone (Serva) solution saturated at 4 °C in KPP. DCC suspension: 4% charcoal Norit A (Serva), 0.1% dextran 60 (Serva) in KPP. Scintillation fluid: 700 mL of toluene containing 5% PPO (2,5-diphenyloxazole) and 0.2% POPOP [2,2′-*p*-phenylenebis(5-phenyloxazole)] mixed with 300 mL of Triton X-100.

Affinity Resin. Testosterone 17β-hemisuccinyl-[[(ω-aminoundecanoyl)amino]ethyl]cellulose (T-resin; 13.5 μmol of testosterone/g of resin) was used for affinity chromatography and T-resin assay of cAMP binding. Synthesis and

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[1] Abbreviations: AR, androgen receptor; T-resin, testosterone 17β-hemisuccinyl-[[(ω-aminoundecanoyl)amino]ethyl]cellulose; BGG, bovine γ-globulin; BSA, bovine serum albumin; cAMP, adenosine cyclic 3′,5′-phosphate; DCC, dextran-coated charcoal; DTT, dithiothreitol; IEF, isoelectric focusing; KPP, phosphate buffer; T_K, saturated solution of testosterone.

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properties of the resin are described elsewhere (Eisele et al., 1984).

Preparation of Cytosol. Cytosol from skeletal muscle of adult male C57 BL6J mice was prepared in KPP as previously described (Ofenloch-Hähnle et al., 1986). Protein content was determined according to the method of Lowry et al. (1951).

Testosterone Binding Assays. In samples containing more than 0.1 mg of protein/mL of testosterone binding assay was performed as previously described (Ofenloch-Hähnle et a., 1986). Aliquots of 0.5 mL were incubated either only with 10 nM [1,2,6,7,16,17-^{3}H]testosterone (NEN, specific activity 141 Ci/mmol) (total binding) or in the presence of 1.5 μM radioinert testosterone (nonspecific binding) for 6 h at 4 °C if not otherwise indicated. At the end of incubation, unbound testosterone was separated by DCC treatment (1.2% charcoal in the assay) for 2 min. After centrifugation, and aliquot of the supernatant was examined for radioactivity in scintillation fluid (10 mL/0.5 mL of supernatant).

For samples containing less than 100 μg of protein/mL, the assay was modified as follows: After incubation (described above) with labeled and radioinert testosterone, BGG (final concentration 0.1 mg/mL) was added to all samples. The samples referring to total binding were mixed with the same amount of radioinert testosterone, as were the samples referring to nonspecific binding before incubation. Volume was adjusted by addition of KPP to the latter samples. Thereafter, the DCC treatment was immediately performed as described above but with less DCC (0.2% charcoal in the assay). A time lapse of less than 10 s was observed between the addition of radioinert testosterone and the start of DCC treatment. This guaranteed a minimal dissociation of specifically bound labeled testosterone.

Testosterone Affinity Chromatography. AR was purified by a modification of testosterone affinity chromatography described previously (Ofenloch-Hähnle et al., 1986). All purification steps were carried out at 4 °C. Cytosol in KPP was incubated for 2–3 h with T-resin (5 mg/mL of cytosol). Incubation was terminated by centrifugation at 10000g for 10 min. The pellet was washed five times with 1 mL of KPP/100-mg of resin and resuspended in 0.2 mL of T_K/mg of resin and incubated for 2 h with gentle shaking to elute the receptor from the resin. The suspension was centrifuged at 10000g for 10 min, and the supernatant was filtered with a glass microfiber filter (Whatman, GF/F) and incubated three times with DCC (pellet of 0.125 mL of DCC suspension/mL of supernatant) for 2 min each to remove free testosterone. DTT (0.5 mM) was added to the final supernatant (filtered before use), since DTT was lost by DCC treatment. The resulting receptor solution was called affinity eluate. Protein content was determined by the "silver-stain" method (Krystal et al., 1985).

Combined Affinity Chromatography and Isoelectric Focusing. The agar gel was composed of 1% agarose (IEF agarose from Pharmacia), 12% sorbitol, and 2 mL of ampholines (pH 5–8, Pharmacia) per 30 mL and contained 50 nM testosterone and 0.1 mM DTT. Twenty milligrams of T-resin was incubated with cytosol, centrifuged, and washed with KPP as described under Testosterone Affinity Chromatography. A suspension of the resin was layered onto a filter paper (about 10 mg of resin/cm^2). After removal of the excess fluid, the filter paper carrying the wet T-resin was applied to the agar gel (size: 122 × 115 × 2 mm) 3 cm apart from the cathode. Focusing was achieved at 2.9 kV (constant power of 15 W, 2.5 h, 10 °C); the filter paper was removed from the gel after 2 h. At the end of focusing the pH gradient was measured with a surface electrode. The gel strip was cut into 30 equally sized slices at 0–4 °C. These slices were homogenized and eluted with 1.8 mL of MoKPPF buffer by gentle shaking for 15 h at 0–4 °C. Subsequently, testosterone was removed by adding 50 μL of DCC suspension to the content of each vial under vigorous shaking. After 2 min, the vials were centrifuged at 10000g for 5 min. Specific testosterone and cAMP binding in aliquots of the supernatant was determined as described below.

For AR preparation, gel slices showing specific testosterone binding were pooled, homogenized, and eluted with 0.3 mL of MoKPP/cm^2 gel. Removal of testosterone was performed as described above. The resulting supernatant is called the IEF affinity eluate.

cAMP Binding Assays. (*a*) *Filtration Assay.* Aliquots of the affinity eluate were incubated either only with the indicated [2,8-^{3}H]cAMP concentrations (NEN, 33.5 Ci/mmol) (samples referring to total binding) or in at least a 100-fold excess of radioinert cAMP (Sigma, sodium salt) for 2 h (referring to nonspecific binding). Incubation was terminated by filtration of the samples through a cellulose ester filter (Sartorius, SM 11307) according to the method of Gilman (1970). The filter was washed twice with 4 mL of KPP for 30 s and its radioactivity determined in 10 mL of scintillation fluid after storage for 20 h at 4 °C.

(*b*) *T-Resin Assay.* The samples were incubated with T-resin and washed five times with KPP as described under Testosterone Affinity Chromatography. The final resin pellet was resuspended in 1 mL of KPP/100 mg, and aliquots of this suspension were incubated with labeled cAMP in the absence or presence of an excess of unlabeled cAMP as described above (gentle shaking during incubation). After 2 h, the samples were centrifuged for 30 s at 10000g (in a Hettich Mikro-Rapid/K centrifuge at 4 °C). An aliquot of the supernatant was then taken for determination of radioactivity and used for the calculation of the free cAMP concentration in the sample. The resin was washed twice with 1 mL of KPP (suspended for 10 s and centrifuged for 30 s at 10000g and 4 °C). Thereafter, the washed pellet was transferred in 2 × 0.3 mL of KPP into Polyvials (Zinsser); 10 mL of scintillation fluid was added, and the vials were stored for 15 h at 4 °C before determination of radioactivity.

The specifically bound cAMP was related to the AR immobilized on T-resin. The latter had been determined by testosterone binding assay in an aliquot of the sample and the supernatant after incubation with T-resin. From the difference between these two, the amount of receptor bound to T-resin was calculated.

Determination of Ligand Specificity. Aliquots of T-resin preincubated with cytosol and washed as described under Testosterone Affinity Chromatography were incubated for 3 h at 4 °C with 100 nM labeled cAMP and 10-, 100-, 200-, 500-, 1000-, and 2000-fold excess of radioinert cAMP, cGMP, cCMP, 2′,3′-cAMP, and 5′-AMP, respectively (sodium salts from Sigma). At the end of incubation, the unbound nucleotides were separated, and bound [^{3}H]cAMP was determined as described under T-Resin Assay.

Competition Experiments with Polynucleotides. Aliquots of T-resin preincubated with cytosol and washed as described under Testosterone Affinity Chromatography were incubated for 15 h with the indicated concentrations of DNA (calf thymus), polyadenylic acid (pA, potassium salt from Sigma), tRNA (*Escherichia coli*, from Serva), tRNA (rabbit, R-5752 from Sigma), or buffer (control). [^{3}H]cAMP (100 nM) was then added, and the mixtures were incubated for another 3

h. At the end of incubation bound [^{3}H]cAMP was determined as described under T-Resin Assay.

Determination of the Dissociation Kinetics. Aliquots of T-resin preincubated with cytosol and washed five times with KPP were incubated with 100 nM [^{3}H]cAMP for 2 h. Dissociation was started by addition of 10 μM radioinert cAMP and terminated after the indicated time intervals by separating unbound cAMP (see T-Resin Assay). Aliquots were taken for determination of nonspecific binding as described above.

Density Gradient Centrifugation. Aliquots (0.5 mL) of IEF affinity eluate were layered onto the top of 5–35% (w/w) linear glycerol gradients in MoKPP. The tubes were centrifuged at 2 °C for 16 h at 40000 rpm in a Beckman SW 40 rotor. BSA (4.4S) and BGG (6.6S) were used as references. Fractions of 0.45 mL were collected from the bottom of the tube after centrifugation; 2 × 200 μL of each fraction was taken for T-resin assay (1 mg of T-resin/100 μL, 100 nM [^{3}H]cAMP in the sample) for determination of specifically bound cAMP or for determination of specific testosterone binding.

Determination of AR Binding to Immobilized cAMP. Three different 3′,5′-cAMP–agaroses (from Sigma) were used: cAMP attached by C-8 using a 17-atom hydrophilic spacer; cAMP attached by C-8 using a 6-carbon spacer; or cAMP attached by the N^6 amino group using a 8-carbon spacer.

Three milliliters of affinity eluate was applied to a cAMP–agarose minicolumn equilibrated in KPP (0.2 mL of gel; 3 mL/h), washed with 3 mL of KPP and eluted with 3 mL of 15 mM cAMP in KPP (3 mL/h). Specific testosterone binding was determined as described above (protein content less than 10 μg/mL) in testosterone affinity eluate, in the flow through fraction, and in cAMP–agarose eluate.

Gel Filtration. A column of Sepharose CL-6B (Pharmacia) of 200-mL bed volume and 120-cm length was equilibrated with MoKPP. Two milliliters of IEF affinity eluate was applied, and the column was developed at a flow rate of 9 mL/h. Fractions of 2.3 mL were collected; 4 × 400 μL of each fraction was taken for testosterone binding assay (low protein content), and 4 × 100 μL aliquots were taken for cAMP determination by T-resin assay. Thyreoglobulin, katalase, albumin, and ovalbumin were used as markers for calibration.

Results

Modification of Testosterone Affinity Chromatography and Receptor Assay. The purification of the AR by testosterone affinity chromatography has been described previously (Ofenloch-Hähnle et al., 1986). It was modified in some aspects to obtain a better purification of the receptor: the resin was washed five times instead of twice, and the free testosterone was adsorbed with less DCC (see Materials and Methods). These modifications resulted in an increase of receptor purification and only a small decrease in recovery (see Table I). The very high purification is due to the high specificity of testosterone binding, the low nonspecific binding to the T-resin, and the fact that affinity chromatography was performed as the first step of purification. The very low protein concentration (0.4–0.5 μg/mL) in the affinity eluate made modifications of the receptor assay necessary. To minimize protein adsorption to the dextran-coated charcoal, the DCC concentration was reduced from 1.2% to 0.2% and BGG (0.1 mg/mL) was added. Due to the reduced DCC concentration, it was then necessary to have the same steroid concentrations in the total binding and nonspecific binding samples. Therefore, we added—just before DCC treatment—the same amount of radioinert steroid hormone to the total binding samples as had been added to the nonspecific binding samples before incubation with the hormone (see also Materials and Methods). This modified DCC assay yields reliable results, while the nonmodified assay (as used in cytosol) underestimates the receptor amount in samples with low protein concentrations. However, in samples containing more than 5 μg of protein there was no difference between the assays.

Table I: Purification of Androgen Receptor[a]

	cytosol	affinity eluate	IEF affinity eluate
receptor concentration (fmol/mL)	21–25	9–12	50–62
protein content (μg/mL)	3500–4500	0.4–0.5	2.0–2.3[b]
recovery (%)	100	40–50	72–83
purification	1	3900–4500	4000–4700

[a] The androgen receptor from murine skeletal muscle was purified by testosterone affinity chromatography or a combination of affinity chromatography and IEF as described under Materials and Methods. Data refer to six independent preparations. [b] Protein content was determined after separation of ampholines by gel filtration.

Combination of Isoelectric Focusing and Affinity Chromatography. A relatively high purification of AR with one single purification step was yielded by testosterone affinity chromatography. But the AR concentration in the affinity eluate was only 9–12 fmol/mL, which did not allow the use of affinity eluate for density gradient centrifugation or gel filtration, since specific hormone binding cannot be determined with sufficient precision after fractionation.

Hence, we attempted to concentrate the receptor by ultrafiltration and lyophilization of the affinity eluate. However, this resulted in an intolerable decrease of recovery (data not shown). The problem was solved by a combination of testosterone affinity chromatography and agar gel isoelectric focusing (IEF). AR and other cytosolic proteins, which had bound to T-resin, can be removed from the resin in the electric field of IEF. To ameliorate the dissociation of the AR from the T-resin and to prevent inactivation of the receptor, we added testosterone and DTT to the gel. The gel was fractionated after focusing and processed as described under Materials and Methods. We observed that the AR from murine skeletal muscle shows a large p*I* heterogeneity (data not shown), a result also obtained by other groups (Katsumata & Goldman, 1974; Fox & Wieland, 1981). The reasons for this heterogeneity, also observed in chromatofocusing (data not shown), are still unknown. If all gel slices containing receptor were pooled, the purification of AR by IEF affinity chromatography did not exceed that obtained by testosterone affinity chromatography. Since, however, the agar gel could be eluted with a very small volume of buffer, the concentration of AR in the IEF affinity eluate increased about 5-fold compared to the simple testosterone affinity eluate (see Materials and Methods and Table I). This receptor concentration allowed the performance of density gradient centrifugation and gel filtration of the IEF affinity eluate and parallel determination of specific testosterone and cAMP binding with sufficient precision after fractionation. The ampholines did not influence the results, as they are separated from the receptor by these methods.

Determination of cAMP Binding in Affinity Eluate. Determination of cAMP binding in the affinity eluate (see Materials and Methods) by adsorption of cAMP–protein complexes to cellulose ester filters as used for other cAMP binding proteins (Gilman, 1970) was not possible because of the rapid loss of bound cAMP during filtration and wash. This may be due to a very fast off-rate of the observed cAMP binding or to the assay conditions, e.g., insufficient binding of the cAMP–protein complexes to the filter or conformational

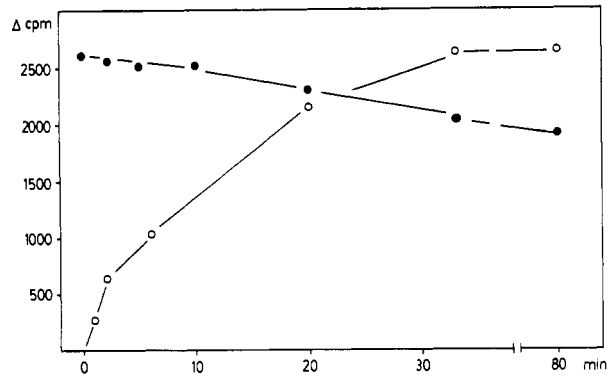

FIGURE 1: On and off kinetics of cAMP binding to immobilized AR. Aliquots of a suspension of T-resin preincubated with cytosol and washed with buffer as described under Materials and Methods were incubated with 100 nM labeled cAMP in the absence or presence of a 100-fold excess of radioinert cAMP at 4 °C for the indicated time. Incubation was stopped by separating free cAMP as described under T-Resin Assay. (O) indicates the specifically bound cAMP; off-kinetics (●) were determined as described under Materials and Methods.

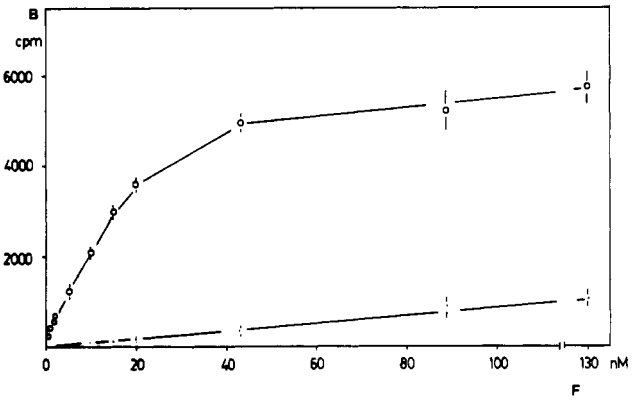

FIGURE 2: cAMP binding curve of AR immobilized on T-resin. Aliquots of a suspension of T-resin preincubated with cytosol and washed with buffer as described under Materials and Methods were incubated with the indicated concentrations of labeled cAMP in the absence or presence of 13 μM radioinert cAMP. Total binding (O) and nonspecific binding (●) of cAMP were determined by T-resin assay. Vertical bars denote SD of four determinations.

changes induced by the cellulose ester binding. However, the same phenomenon was observed when the DCC assay was used for separating bound and free cAMP, as used earlier (Ofenloch-Hähnle et al., 1986). Furthermore, displacement studies of the cAMP binding in the affinity eluate suggest that the loss of cAMP binding during both assays is due to fast off kinetics (80% of the binding is lost after 2 min). Nevertheless, the bound cAMP is underestimated by these binding tests. Our work with affinity chromatography gave us the idea to assay the cAMP binding of the proteins in affinity eluate immobilized on T-resin. Cytosol or fractions from receptor purification steps were incubated with T-resin in the same manner as during affinity chromatography and washed with buffer. Then aliquots of the resin were incubated with labeled cAMP in the absence or presence of radioinert cAMP. Separation of the free ligand was obtained by centrifugation. During this procedure a very slow loss of specifically bound cAMP was observed (Figure 1) in contrast to the filtration and DCC assay described above. This may be due to steric hindrance of cAMP association and dissociation to the immobilized receptor.

The T-resin assay thus allows a more reliable determination of cAMP binding in affinity eluate. The results obtained by the T-resin assay were comparable to those obtained by the filtration assay if the latter were extrapolated back to $t = 0$

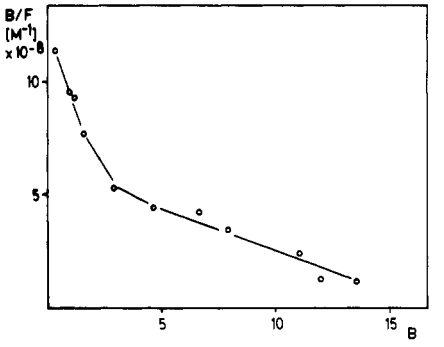

FIGURE 3: Scatchard plot of the specific cAMP binding calculated from Figure 2. B = specifically bound cAMP/testosterone binding site; F = concentration of free cAMP.

Table II: Fitting cAMP Binding Data by a Model with Two Different Independent Binding Sites[a]

	site 1	site 2	total
dissociation constant (nM)	7.3 ± 3.6	58.8 ± 10.7	
bound cAMP/bound testosterone	2.3 ± 0.2	13.2 ± 0.7	15.5 ± 0.7

[a] The cAMP binding data obtained by the T-resin assay of affinity eluate were fitted by an approximation program using the Monte Carlo method [modification of a Pascal program described by Herdt (1985)] based on a model taking two different independent binding sites into account. Data were obtained by five independent determinations. ± indicates SE.

(the extrapolated values showed a low statistical accuracy). T-Resin alone did not specifically bind cAMP. Due to this very low nonspecific retention of cAMP the assay shows a good ratio between the signal and background at cAMP concentrations up to 200 nM and a low SD (see Figure 2). Furthermore, this method allows direct determination of free cAMP concentration in the assay (see Materials and Methods). Summing up, it can be said that the T-resin assay allows a rapid determination of proteins that bind testosterone *and* cAMP, even in crude preparations, since the method includes a very potent purification by affinity chromatography.

cAMP Binding Characteristics of Affinity Eluate. The cAMP binding of the affinity eluate was determined with the T-resin assay. The binding curve and the respective Scatchard plot are shown in Figures 2 and 3. From the Scatchard plot it can be seen that the binding is apparently not due to identical independent binding sites. The binding data can be fitted into a model that assumes two different independent binding sites (see Table II). However, models with more than two binding sites or negative cooperativity are also possible. It is noteworthy that the ratio of testosterone binding to total cAMP binding was constant in different cytosolic preparations (T:cAMP was about 1:16; see Table II) and independent of the receptor content per milligram of protein. The same ratio was observed in experiments with receptor partially inactivated by elevated temperatures. Extensive washing of the resin (with buffer of ionic strength up to 100 mM) with more than five washes described under Materials and Methods did not influence the observed cAMP binding. This is another sign that cAMP binding is not due to proteins nonspecifically bound to the T-resin. As shown in Figure 4, the binding of cAMP is highly specific. Obviously the binding site requires the 3′,5′-diester group as well as the adenine system.

The relatively high number of cAMP binding sites per testosterone-binding site and the nonlinear Scatchard plot led to the question of whether the cAMP binding compounds in the affinity eluate are the AR or other proteins copurified with AR. Only about 1% of the total protein in the affinity eluate

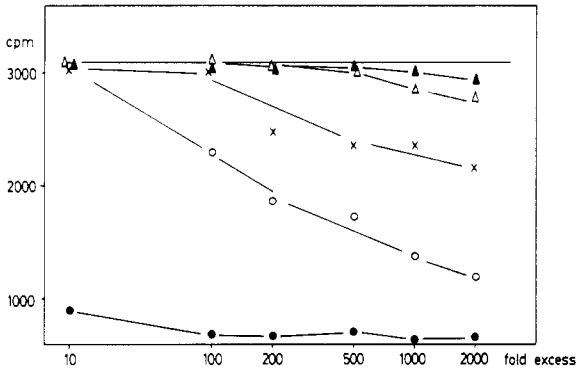

FIGURE 4: Specificity of cAMP binding. The binding of labeled cAMP to T-resin preincubated with cytosol and washed with buffer as described under Materials and Methods was determined by T-resin assay in the presence of varying concentrations of different radioinert nucleotides: (●) 3′,5′-cAMP; (○) 3′,5′-cGMP; (×) 3′,5′-CMP; (△) 2′,3′-cAMP; (▲) 5′-AMP. (—) indicates binding of labeled cAMP in the absence of radioinert nucleotides.

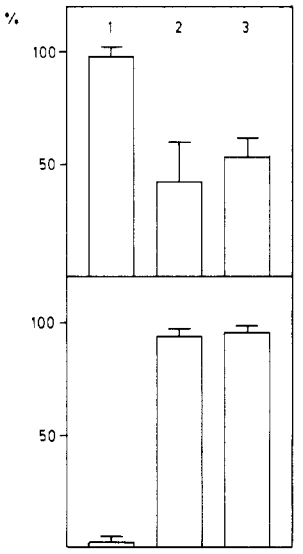

FIGURE 5: Binding of the androgen receptor to cAMP–agaroses. (Top) The binding of AR to N^6-linked cAMP–agarose (1) and C-8 linked cAMP–agarose with a 6-carbon spacer (2) and a 17-carbon spacer (3) was determined as described under Materials and Methods and is given in percentage of the total receptor applied to the column. The bottom panel shows the elution from the respective resins by 15 mM cAMP, given in percentage of the total receptor bound to cAMP–agarose determined as in the top panel. SD for three determinations is indicated.

was active androgen receptor. Therefore, it seemed possible that most of the cAMP binding in the affinity eluate was not due to AR. To determine an answer we examined the binding of AR to immobilized cAMP and the copurification of cAMP and testosterone binding by isoelectric focusing, density gradient centrifugation, and gel filtration.

Binding of AR to Immobilized cAMP. We used three different cAMP–agaroses: a C-8 linked cAMP with a C_6 spacer; a C-8 linked cAMP with a C_{17} spacer; and a N^6-linked cAMP with a C_6 spacer. Figure 5 shows that the androgen receptor in the affinity eluate binds to the cAMP–agaroses under the conditions described under Materials and Methods, while binding to DNA–cellulose is very low (less than 10%). The binding efficiency of the cAMP–agaroses, however, depended on the linkage between cAMP and the agarose matrix: N^6-linked cAMP–agarose bound significantly better than C-8-linked cAMP–agaroses, while the AR, on the other hand, could be more easily eluted from C-8-linked agaroses by 15 mM cAMP than from N^6-linked resins. Obviously, a special

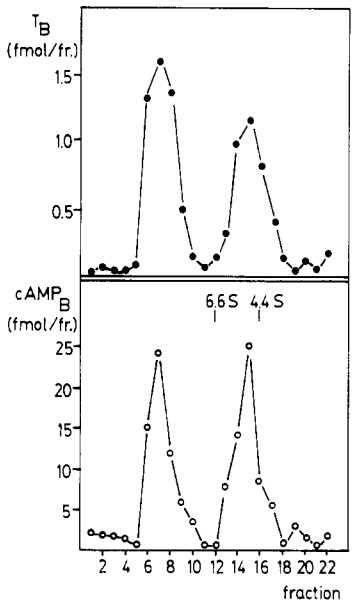

FIGURE 6: Density gradient centrifugation of the affinity eluate. Five-tenths of a milliliter of the affinity eluate was centrifuged as described under Materials and Methods. In each fraction the specific cAMP binding ($cAMP_B$) or the specific testosterone binding (T_B) was determined. The vertical bars indicate the sedimentation references BSA (4.4 S) and BGG (6.6 S).

steric accessibility of cAMP is necessary for AR binding to the immobilized cAMP, which is a hint for a specific binding of AR to cAMP. However, binding of AR to cAMP–agarose is not due to only ionic interaction, as DNA–cellulose, another polyanion, bound AR only to a very low extent under the chosen conditions. These results strengthened our opinion that cAMP binding in the affinity eluate was, at least partially, due to the androgen receptor. If this was the case, testosterone and cAMP should cosediment in density gradient centrifugation.

Density Gradient Centrifugation. IEF affinity eluate was centrifuged in 5–35% glycerol gradients. After fractionation, we determined the specific binding of cAMP and testosterone respectively in each fraction. As shown in Figure 6 cAMP and testosterone binding cosedimented at 4–5 S and 8–9 S. These two receptor forms are known as the untransformed (8–9S) and the transformed (4–5S) cytostolic AR of the skeletal muscle (Ofenloch-Hähnle et al., 1986) and other tissues (Johnson et al., 1987; Rowley et al., 1986). The 1:16 ratio of bound testosterone to cAMP obtained in affinity eluate was confirmed by density gradient centrifugation. Both peaks showed a similar ratio of testosterone to cAMP binding. The cosedimentation of cAMP and testosterone binding is the first hint that the bulk—if not all—of affinity eluate components binding cAMP is AR. Since the resolution achieved by density gradient centrifugation was not very high, we performed gel filtration to determine whether testosterone binding and cAMP binding are also copurified by this method.

Gel Filtration. IEF affinity eluate was applied to a Sepharose CL-6B column and developed with MoKPP. After fractionation, the specific tetosterone binding and cAMP binding were determined in each fraction. As shown in Figure 7 the specific testosterone binding peaked at Stokes radii of 4.2 nm (peak 3), 5.6 nm (peak 2), and 8.5–9.0 nm (peak 1). If these Stokes radii are compared with the results of Johnson et al. (1987), peak 1 represents the untransformed receptor, peak 2 represents the transformed receptor, and peak 3 may be a proteolytic fragment, not observed in density gradient centrifugation. Again, the specific cAMP binding was coeluted

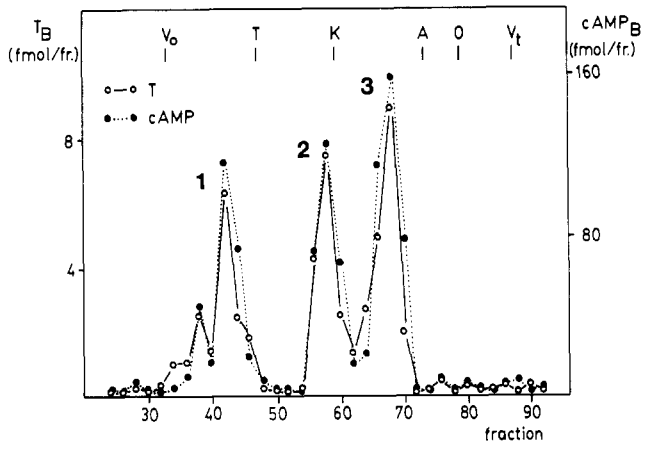

FIGURE 7: Gel filtration of the IEF eluate on a Sepharose CL-6B column. The gel filtration of 2 mL of IEF eluate was performed as described under Materials and Methods. Specific cAMP (●···●) binding and testosterone (O–O) binding were determined in the fractions. The vertical bars indicate external standard proteins: thyreoglobulin (T), katalase (K), bovine serum albumin (A), and ovalbumin (O). V_0 indicates the void volume of the column, V_t the total volume. K_{av} of the references and the peaks binding testosterone were calculated from $(V_t - V_0)/(V_e - V_0)$ (V_e is the elution volume of the protein). Stokes radii of the peaks binding testosterone were estimated from linear regression of the known Stokes radii of external standard proteins versus $(-\log K_{av})^{1/2}$ (Miller et al., 1975; Sherman et al., 1980).

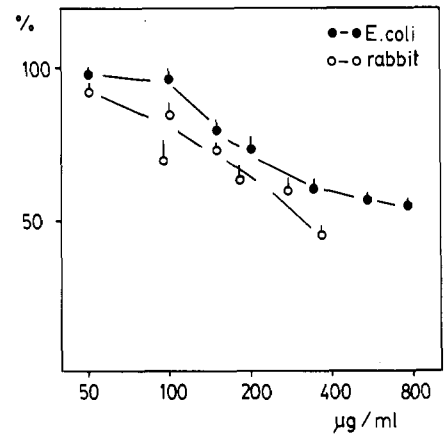

FIGURE 8: Comparison between the displacement of cAMP binding by tRNA from *E. coli* and rabbit. The specific cAMP binding of the affinity eluate was determined by the T-resin assay in the presence of the indicated tRNA concentrations from *E. coli* (●) and rabbit (O). The binding is depicted in percentage of the specific cAMP binding in the absence of tRNA. The SD for three determinations is indicated.

with testosterone binding in a ratio of about 16:1 in each peak. This result strengthens our hypothesis that most of cAMP binding in affinity eluate was due to the androgen receptor.

Displacement of cAMP by Polynucleotides. Because of the ratio of cAMP to testosterone binding sites and the upward concave Scatchard plot of the cAMP binding, we assumed that cAMP binding may bind to the DNA binding site or to the putative RNA binding sites of the AR. This supposition was strengthened by the fact that cAMP competed in DNA–cellulose binding studies with DNA (Ofenloch-Hähnle et al., 1986). The displacement of the cAMP binding by polynucleotides was examined as described under Materials and Methods. If 200 µg/mL of the respective polynucleotide was present in the assay, DNA could displace 12.3 ± 1.2%, polyadenylic acid 7.5 ± 2.0%, a RNA mixture 25.9 ± 1.3%, tRNA from *E. coli* 38.3 ± 0.5%, and tRNA from rabbit 44.4 ± 1.8% of the specific cAMP binding in the absence of any competitor. Obviously, tRNA is the best cAMP competitor. However, there were differences in the displacement efficiency between the two different tRNA forms. Rabbit tRNA had a higher affinity to the cAMP binding site of the AR than *E. coli* tRNA (Figure 8). We do not know yet whether this is due to a different composition of the tRNA amino acid specificity or a species specificity.

Discussion

The development of a novel cAMP binding assay (T-resin assay) for proteins binding testosterone has allowed us to study the cAMP binding of the cytosolic components that also bind testosterone from murine skeletal muscle purified by affinity chromatography. The rapid loss of cAMP binding during filtration assay or DCC assay did not allow the use of these nonequilibrium standard methods for the separation of bound and unbound ligand. Equilibrium methods, like equilibrium dialysis, could not be used because the concentration of binding sites was far below the dissociation constant of the binding.

The reasons for the difference in the observed off-rate of the cAMP binding are not clear, but it can be assumed that the steric situation of the receptor protein immobilized to the T-resin may retard the dissociation of cAMP from the receptor as well as its association. This must be due to the special steric situation on the resin and not just to the testosterone binding of the receptor, since addition of free testosterone had no effect on the cAMP binding properties. Furthermore, it is important to note that the changed off- and on-rates did not result in a markedly changed dissociation constant, since the binding data obtained by DCC assay or filtration assay correlated to the data obtained with T-resin assay when extrapolated for $t = 0$. Using the T-resin assay, we found that the testosterone binding components of the muscle cytosol showed cAMP binding with a high affinity and a high specificity. Although active AR represents only 1% of the total protein in the affinity eluate, the following results led us to the conclusion that the bulk, if not all, of this cAMP binding is due to the androgen receptor: (i) The specific testosterone binding and cAMP binding of the affinity eluate were copurified by density gradient centrifugation and gel filtration. (ii) Every peak of testosterone binding showed cAMP binding with a ratio of about 1:16 of total testosterone to cAMP binding sites in the affinity eluate and in every peak after density gradient centrifugation and gel filtration. (iii) The testosterone binding peaks observed in density gradient centrifugation and gel filtration are characteristic of the transformed and the untransformed androgen receptor. (iv) Partially purified androgen receptor bound specifically to cAMP–agaroses.

The cAMP and testosterone binding sites are obviously located on the same receptor subunit, since the 4–5S receptor form, recognized as the monomeric form of androgen receptors from several tissues (Johnson et al., 1987), bound cAMP just as all receptor forms detectable by testosterone binding assay.

Both transformed and untransformed AR bound cAMP. This is not surprising, since the untransformed receptor is dissociated to the transformed form by the cAMP concentrations used in the binding experiments (Ofenloch-Hähnle et al., 1986). However, since cAMP binding (dissociation constants between 7.3 and 58.8 nM) parallels the transformation of AR by cAMP, the half-maximum effect could be observed at 20–40 nM cAMP (Ofenloch-Hähnle et al., 1986), it can be concluded that the observed in vitro transformation of AR by cAMP is due to cAMP binding and not to enzyme-mediated processes. Study is presently being conducted to show if this receptor transformation is also physiologically relevance.

Aside from receptor transformation, another regulative role of cAMP binding to AR can be suggested. It is interesting that tRNA was the best of all polynucleotides examined for competition with cAMP for the receptor binding and that there are differences in the binding affinity between different tRNA-mixtures. RNA binding of the androgen receptor has frequently been reported (Liao et al., 1980; Haase et al., 1983; Rowley et al., 1986), but it has not yet been determined which RNA species bind to the AR. For the glucocorticoid receptor, however, the specific binding of tRNA to the untransformed receptor could be demonstrated, and it has been speculated that this binding may be involved in selective receptor degradation, possibly by the ubiquitin pathway (Ali & Vedeckis, 1987a,b). We believe that there may be an interesting parallel between androgen and glucocorticoid receptor. Our further investigations will show which tRNA species have the highest binding affinity to AR, whether there is a connection to receptor degradation at all, and how far the cAMP binding of the AR is involved.

Although DNA mixtures have a very low affinity to the cAMP binding sites of AR, it cannot be excluded that the cAMP binding occurs at the DNA binding site. As is well-known, the binding of steroid hormone receptors to DNA mixtures is generally nonspecific, while specific DNA binding requires special sequences and secondary structure of the DNA. Interestingly, Strähle et al. (1987) described a partially palindromic DNA sequence of *15* base pairs, sufficient for steroid hormone receptor recognition on the chromatin. Further study will show if the size of 15 nucleotides (of the respective single strand) and the number of cAMP binding sites of AR is a mere coincidence or if there is a connection between cAMP binding and DNA binding of the receptor. Beyond these physiological aspects, the cAMP binding of AR offers an additional tool in androgen receptor research. The binding of AR to cAMP-agaroses can be used for further purification of the receptor in a second affinity chromatography step and for assaying the testosterone binding of the receptor by incubation of resin-bound AR with testosterone, analogous to the cibacron blue method of Iqbal et al. (1985). Furthermore, the cAMP binding of the receptor may be used for assaying inactivated purified AR (i.e., receptor that is not able to bind hormone), since cAMP and testosterone binding sites seem to be independent but are located on the same receptor subunit.

Registry No. cAMP, 60-92-4; cGMP, 7665-99-8; cCMP, 3616-08-8; AMP, 61-19-8; 2′,3′-cAMP, 634-01-5; testosterone, 58-22-0.

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