Nucleoside triphosphates promote the transformation of Ah receptor to its DNA-binding form

Andrew J. CARY and John J. DOUGHERTY*

Department of Biochemistry and Biophysics, The University of Rhode Island, Kingston, RI 02881, U.S.A.

When it is bound to a specific ligand such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, mild heating can convert the Ah (aryl hydrocarbon) receptor into a form capable of binding DNA. We found that physiological (1–3 mM) levels of ATP substantially increased the transformation of the receptor to its DNA-binding form. GTP, UTP and CTP had similar effects. ADP also promoted this transformation, but was less effective than ATP at low concentrations. Pyrophosphate too promoted transformation, but AMP had little effect. The process did not require nucleotide hydrolysis, since non-hydrolysable analogues of ATP such as adenosine 5'-[$\beta\gamma$ -imido]triphosphate were nearly as effective as ATP itself. Inhibitors of ATP-stimulated proteases did not significantly affect the ability of ATP to promote receptor transformation, which suggests that the effect of ATP was not mediated by these proteases.

INTRODUCTION

Halogenated polycyclic hydrocarbons have received much attention as environmental contaminants and as carcinogens. The biological responses to these compounds are mediated through their high-affinity binding to a protein known as the Ah (aryl hydrocarbon) receptor (Poland & Knutson, 1982; Whitlock, 1987). After binding of such ligands, the Ah receptor acts to regulate the expression of genes, including specific forms of cytochrome *P*-450 designated *P*₁-450 in the mouse and *P*-450c in the rat. Multiple receptor-binding sites have been identified in the 5' leader regions of both genes (Whitlock, 1987).

After the binding of a polycyclic hydrocarbon such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), mild heating can transform a portion of the receptor to a form capable of binding to DNA (Hannah *et al.*, 1986; Gasiewicz & Bauman, 1987). After steroid binding, mild heating similarly transforms steroid receptors to their DNA-binding forms. Before transformation to their DNA-binding forms, steroid receptors are complexed to dimers of the 90 kDa heat-shock protein (hsp90) (Joab *et al.*, 1984; Dougherty *et al.*, 1984; Denis *et al.*, 1987). For the glucocorticoid receptor, release from hsp90 has been shown to coincide with the appearance of the ability to bind DNA (Sanchez *et al.*, 1987). The Ah receptor also forms a complex with hsp90 (Denis *et al.*, 1988; Perdew, 1988). It has been found that the acquisition of DNA-binding ability for the Ah receptor involves the release of the Ah receptor from a 9–10 S complex, which presumably consists of the receptor and hsp90 (Prokipcak & Okey, 1988; Henry *et al.*, 1989; Dougherty, 1989).

For at least two steroid receptors, the progesterone and glucocorticoid receptors, ATP has been shown to increase DNA binding significantly (Toft *et al.*, 1977; Barnett *et al.*, 1983) and thus the release of the receptor from hsp90. Our investigations have established that the presence of ATP during mild heating can greatly increase the transformation of Ah receptor to the form or forms capable of binding to DNA.

EXPERIMENTAL

Materials

[1,6-³H]TCDD (sp. radioactivity 40 Ci/mmol) and 2,3,7,8-tetrachlorodibenzofuran (TCDBF) were from Cambridge Iso-

tope Laboratories (Woburn, MA, U.S.A.). DNA-cellulose (double-stranded) and cellulose (Sigma Type 50) were from Sigma. Disposable columns (2.5 ml, with plastic filler discs) were from Isolabs (Akron, OH, U.S.A.). Hepes was from Fisher. Nucleotides and other chemicals were from Sigma.

Animals

Male CD rats were purchased from Charles River Breeding Laboratories.

Buffer

Buffer H was 25 mM-Hepes/12 mM-thioglycerol, pH 7.6 at room temperature.

Preparation of labelling of cytosol

Rats were anaesthetized in a CO₂ chamber. Their livers were perfused through the portal vein with 0.9% NaCl. The livers were then transferred to ice-cold buffer H, weighed, and minced in 3 ml of buffer/g of tissue. They were homogenized with four strokes of a Potter–Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 12000 *g* in a Sorvall SS-34 rotor. Lipid was removed by aspiration. The supernatant was then centrifuged for 1 h at 40000 rev./min in a Beckman Ti 50 rotor. Lipid was again removed by aspiration. The cytosol was then removed, taking care to avoid any remaining lipid. Samples were labelled with 3 nM-[³H]TCDD in the absence or presence of 225 nM-TCDBF. These were added in a maximum of 7.5 μ l of dioxan/ml of cytosol.

After labelling for 2 h on ice, samples were transferred to tubes containing pellets made from an equal volume of 3% charcoal/0.3% dextran. They were vortex-mixed and incubated for 20 min on ice. Next, the charcoal/dextran was pelleted for 20 min at 1500 *g* and the supernatant was removed.

Chromatography on DNA-cellulose

BSA (1 mg/ml) was dissolved in buffer H containing 50 mM-NaCl to make low-salt buffer. Equal weights of DNA-cellulose and cellulose were equilibrated in the above buffer, and 0.5 ml columns were poured. These were washed with 1 ml of low-salt buffer before sample loading. The columns were loaded with

Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; Ah receptor, aryl hydrocarbon receptor; hsp90, 90 kDa heat-shock protein; hsp70, 70 kDa heat-shock protein.

* To whom correspondence should be addressed.

0.15 ml portions of sample. Loosely bound material was removed by a wash with 2.5 ml of low-salt buffer. Receptor bound to DNA was eluted with a 2.5 ml wash with buffer containing 1 mg of BSA/ml and 500 mM-NaCl. All columns were run in pairs consisting of samples labelled in the absence or presence of excess TCDBF, and then subjected to the same experimental treatments. The fraction of the receptor transformed to the DNA-binding form was estimated by comparing the specific binding eluted by the high-salt wash to the specific binding loaded on the columns.

Precautions

Gloves, laboratory coats and bench-top paper were extensively used to avoid contact with TCDD and TCDBF. All resins and disposable glassware exposed to [3 H]TCDD were discarded as radioactive waste.

RESULTS AND DISCUSSION

Several groups have reported that ATP promotes the transformation of the progesterone and glucocorticoid receptors to their DNA-binding forms (Toft *et al.*, 1977; Barnett *et al.*, 1983; Moudgil *et al.*, 1986). We decided to investigate whether ATP had a similar effect on the Ah receptor. Liver cytosol was prepared, labelled with [3 H]TCDD and treated with charcoal/dextran. The abilities of different concentrations of ATP and ADP to promote receptor transformation on incubation at 22 °C were compared (Fig. 1). At 3 mM and 10 mM concentrations, both nucleotides substantially increased transformation. ATP appeared to promote DNA binding more effectively at the lowest concentration tested (1 mM). This was confirmed in another experiment using conditions similar to those of Fig. 1. Incubation in the absence of added nucleotides caused $21 \pm 2\%$ ($n = 4$) transformation. In the presence of 1 mM-ADP there was $25 \pm 2\%$ transformation, and in the presence of 1 mM-ATP $34 \pm 3\%$. We also examined the ability of 1 mM-ADP to promote Ah-receptor transformation in the presence or absence of an ATP-generating system (Table 1). In this experiment, the ADP alone and the ATP-generating system alone had little effect on receptor transformation. However, the combination of ADP with the phosphocreatine and creatine kinase of the ATP-generating system substantially increased receptor transformation.

To investigate whether ATP could completely transform Ah receptor, the effects of multiple ATP additions and extended incubation times were checked. Cytosol was incubated at 22 °C and ATP was added at 0, 30, 60 and 90 min. Each portion added a final concentration of 3 mM-ATP. After 2 h the samples treated with ATP showed $91 \pm 6\%$ ($n = 3$) transformation, whereas those treated with buffer showed $21 \pm 6\%$ transformation. Thus ATP can convert nearly all of the Ah receptor into a form which binds DNA-cellulose and is eluted by 500 mM-NaCl. In the same experiment, cytosol samples which had not been treated with ATP were incubated overnight at 22 °C. After 6 h, transformation was $31 \pm 5\%$, and after 20 h, it was $31 \pm 3\%$. Thus, in the absence of ATP, most of the receptor failed to be transformed, even on extended incubation.

The abilities of ADP, AMP and pyrophosphate to promote receptor transformation were compared. At 3 mM, AMP had no detectable effect on receptor transformation. At this concentration, ADP and pyrophosphate caused substantial and similar increases in receptor transformation (Table 2). Pyrophosphate has been shown to promote glucocorticoid-receptor transformation (Moudgil *et al.*, 1986). Its resemblance to the two phosphate groups of ADP is obvious. However, in at least one case, the mitochondrial F_1 -ATPase, the effects of pyrophosphate resemble those of ATP rather than ADP (Harris, 1978; Kalashnikova *et al.*, 1988).

The effects of the four nucleoside triphosphates on Ah-receptor transformation were compared. At 2 mM levels all caused substantial and similar rises in transformation (Table 3). It seems likely that the charcoal treatment to which the samples had previously been exposed would have greatly depleted their supply of endogenous nucleotides. Thus the observed effects probably represented the direct action of the added nucleoside triphosphates rather than the phosphorylation of endogenous nucleoside diphosphates by the added triphosphates. If this is the case, the site at which they act has little nucleoside specificity.

Non-hydrolysable ATP analogues were used to test the possible role of nucleotide hydrolysis in promoting transformation (Table 4). Both of the analogues with non-hydrolysable substitutions

Fig. 1. Effects of various concentrations of ATP and ADP on receptor binding to DNA-cellulose

Liver cytosol was labelled with [3 H]TCDD in the absence or presence of a 75-fold excess of TCDBF. After charcoal treatment, samples were incubated briefly at 22 °C. ATP (Δ) or ADP (●) was then added at the indicated concentrations, and the incubations were continued for $\frac{1}{2}$ h. Samples were then placed briefly on ice, and then were chromatographed on DNA-cellulose columns. The Figure shows means $\pm$ S.D. of data from three experiments.

Table 1. Effect of an ATP-generating system on Ah-receptor transformation

Liver cytosol was labelled with [3 H]TCDD at 0 °C, and charcoal-treated. Samples were incubated for $\frac{1}{2}$ h at 22 °C with the indicated additions. One unit of creatine kinase (CK) will transfer 1.0 μ mol of phosphate from phosphocreatine (PCr) to ADP/min at pH 7.4 and 30 °C. Values represent means $\pm$ S.D. of 3 determinations.

Additions	Binding to DNA-cellulose (%)
None	18 ± 1
1 mM-ADP	20 ± 1
20 mM-PCr and 20 units of CK	20 ± 3
1 mM-ADP, 20 mM-PCr and 20 units of CK	31 ± 2

Table 2. Effects of nucleotides and pyrophosphate on Ah-receptor transformation

Labelled charcoal-treated cytosol was incubated for $\frac{1}{2}$ h at 22 °C with the additions indicated below. Values represent means $\pm$ S.D. of 4 determinations.

Substance added	Binding to DNA-cellulose (%)
None	30 $\pm$ 5
AMP (3 mM)	32 $\pm$ 5
ADP (3 mM)	65 $\pm$ 25
Pyrophosphate (3 mM)	62 $\pm$ 16
Pyrophosphate (10 mM)	88 $\pm$ 15

Table 3. Effects of nucleoside triphosphates on Ah-receptor transformation

Labelled charcoal-treated cytosol was incubated for $\frac{1}{2}$ h at 22 °C in the presence of the indicated nucleotides. Values represent the averages of duplicate determinations.

Nucleotide (2 mM)	Binding to DNA-cellulose (%)	
	Expt. 1	Expt. 2
None	23	19
ATP	61	45
GTP	55	47
CTP	51	49
UTP	57	43

between their β - and γ -phosphate groups effectively increased transformation. Thus mechanisms such as protein phosphorylation, which would involve cleaving ATP at this position, may be ruled out.

In another experiment under the conditions described in Table 4, we tested the ability of a non-hydrolysable analogue of ADP to promote transformation. Incubation for $\frac{1}{2}$ h at 22 °C in the absence of nucleotide gave 17 $\pm$ 1 % transformation to the DNA-binding form. Incubation with 2 mM-ADP gave 24 $\pm$ 3 % transformation. Incubation with 2 mM-adenosine 5'-[α,β -methylene]diphosphate gave 29 $\pm$ 1 %. It had seemed possible that ADP promoted transformation only because endogenous adenylate kinase converted it into ATP. However, this enzyme cleaves two ADP molecules to yield one of ATP and one of AMP (Noda, 1973). Since adenosine 5'-[α,β -methylene]diphosphate, which could not be used in this fashion to generate ATP, promoted transformation at least as well as ADP did, it seems likely that ADP itself can promote transformation. However, since the intracellular level of ADP is approx. 0.5 mM (Gupta & Yushok, 1980), it seems questionable whether Ah receptors are exposed to ADP concentrations high enough to promote transformation significantly.

Cells have been reported to contain two proteases whose activities are regulated by ATP. One is ATP-dependent, and the other has an activity which can be stimulated by ATP (Waxman *et al.*, 1987). Partial proteolysis of the Ah receptor or of hsp90 by such a protease could disrupt the complex between these proteins, and account for the increased DNA binding observed. The protease inhibitor cystatin effectively blocks both of the ATP-regulated proteases (Fagan *et al.*, 1987), so we investigated the effect of cystatin on the ATP-dependent rise in the DNA binding

Table 4. Effects of non-hydrolysable ATP analogues on Ah-receptor transformation

Liver cytosol was labelled with [3 H]TCDD at 0 °C and charcoal-treated. Samples were incubated for $\frac{1}{2}$ h at 22 °C with 2 mM concentrations of the additions indicated. Values are means $\pm$ S.D. of triplicate determinations.

Addition	Binding to DNA-cellulose (%)
None	24 $\pm$ 2
ATP	58 $\pm$ 3
Adenosine 5'-[$\beta\gamma$ -methylene]triphosphate	51 $\pm$ 3
Adenosine 5'-[$\beta\gamma$ -imido]triphosphate	51 $\pm$ 2

Table 5. Effect of the protease inhibitor cystatin on Ah-receptor transformation

Labelled charcoal-treated cytosol was incubated for 20 min at 22 °C in the presence or absence of cystatin. ATP or buffer was then added, and the incubations were continued for $\frac{1}{2}$ h. Values are means $\pm$ S.D. of four determinations.

Inhibitor (100 μ g/ml)	ATP (3 mM)	Binding to DNA-cellulose (%)
None	—	19 $\pm$ 4
Cystatin	—	19 $\pm$ 3
None	+	45 $\pm$ 3
Cystatin	+	45 $\pm$ 5

of Ah receptor (Table 5). Cystatin did not inhibit the ability of ATP to promote DNA-cellulose binding. In other experiments (results not shown), haemin, another inhibitor of ATP-dependent proteolysis (Fagan *et al.*, 1987), was similarly unable to prevent the ATP-dependent rise in DNA-cellulose binding. Thus ATP-stimulated proteolysis does not appear to play an important role in the promotion of receptor transformation by ATP. ATP might act to promote transformation by binding directly to either the Ah receptor or hsp90. There have been reports suggesting the existence of ATP-binding sites on steroid receptors. Cytosolic preparations of progesterone, oestrogen and glucocorticoid receptors bind to ATP-agarose after transformation, but have little binding affinity before transformation (Miller & Toft, 1978; Moudgil & John, 1980).

It is also possible that the observed nucleotide effects may be mediated by the 70 kDa heat-shock protein, hsp70. This has been found associated with progesterone receptor both before and after transformation (Kost *et al.*, 1989). Members of the hsp70 family are known to bind ATP and to use ATP to disrupt protein complexes. hsc70, a member of the hsp70 family, can be found complexed with the oncogene p53. ATP promotes the separation of hsc70 from p53, but non-hydrolysable ATP analogues are ineffective (Clarke *et al.*, 1988). Another member of this family, the clathrin-uncoating ATPase, can use ATP, but not non-hydrolysable analogues, to disrupt clathrin cages (Schmid *et al.*, 1985; Chappell *et al.*, 1986). Since these activities require nucleotide hydrolysis, they do not seem to be closely related to the promotion of Ah-receptor transformation by ATP. Schmid *et al.* (1985) have suggested that the clathrin-uncoating ATPase has a second ATP-binding site which promotes the binding of the protein to clathrin triskelions which have been partially or fully released from cages. Nucleoside triphosphates other than ATP, and non-hydrolysable analogues of ATP, can act effectively at

this second nucleotide-binding site. It seems possible that ATP might promote transformation by binding to such a site on a member of the hsp70 family, and increasing the binding of this protein to one or more sites on the Ah receptor or on hsp90. If the binding of the hsp70 to either of these proteins destabilized the complex between the Ah receptor and hsp90, this would increase transformation.

This work was supported in part by the American Cancer Society, Grant CN-18, and in part by a biomedical support grant from the National Institutes of Health/University of Rhode Island Consortium for Allied Health Research. We thank Michelle Collins for expert secretarial assistance.

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Received 27 July 1990/5 October 1990; accepted 23 October 1990