Determination and comparative analysis of the catalytic subunit of adenosine 3',5'-cyclic phosphate-dependent protein kinase by an enzyme-linked immunosorbent assay*

Gerhild SCHWOCH† and Alf HAMANN‡

†Institut für Physiologische Chemie and ‡I. Medizinische Klinik der Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany

(Received 20 May 1982/Accepted 2 July 1982)

A specific antiserum against bovine heart catalytic subunit was used for the determination of the catalytic subunit in an enzyme-linked immunosorbent assay. Under the conditions elaborated the assay has a lower detection limit for catalytic subunit of 0.25 pmol/ml. In crude bovine heart extracts the concentration of catalytic subunit was determined by this method to be $0.18 \pm 0.02 \mu\text{mol/kg}$ wet wt. The immunochemical comparison of various animal species and cells, including organisms like amoebae and yeast, shows the broad applicability of the assay and provides evidence that the catalytic subunit is a highly conserved molecule.

Since it became known that most, if not all, effects of cyclic AMP are mediated by the activation of cyclic AMP-dependent protein kinases (Kuo & Greengard, 1969; Krebs, 1972), the study of these kinases has found increasing interest. The enzyme is ubiquitously distributed in the form of two isoenzymes which are different in their cyclic AMP-binding regulatory subunits but seem to be identical in their catalytic subunits (Corbin *et al.*, 1975; Hofmann *et al.*, 1975).

Hitherto used determinations of the cyclic AMP-dependent protein kinase which rely on the measurement of its enzymic activity can be biased by several factors causing an alteration of the activity (Sugden *et al.*, 1976; Kupfer *et al.*, 1980; Alhanaty *et al.*, 1981) as well as by the presence of other, cyclic AMP-independent, protein kinases. The latter problem becomes important especially in cellular fractions or cell types in which the percentage of cyclic AMP-dependent protein kinase is relatively low compared with other protein kinases.

These problems could be largely avoided by the use of specific immunochemical techniques. The production of specific antisera and their application in sensitive radioimmunoassays had been reported for the regulatory subunit of the type I (Kapoor *et al.*, 1979) and of the type II (Fleischer *et al.*, 1976) cyclic AMP-dependent protein kinase isoenzymes as well as for cyclic GMP-dependent protein kinase

(Walter, 1981). A corresponding method for the determination of the catalytic subunit of cyclic AMP-dependent protein kinase has not been described until now. However, a separate detection of this part of the holoenzyme seems to be valuable, not only in view of a possible different localization of the kinase subunits (cf. Jungmann & Russell, 1977; Corbin & Keeley, 1977), but also in face of recent findings (Prashad *et al.*, 1979; Walter *et al.*, 1979) which indicate a different regulation of the regulatory and catalytic subunits of cyclic AMP-dependent protein kinases.

In a foregoing publication (Schwoch *et al.*, 1980) we described the production of a rabbit antiserum against the catalytic subunit from bovine heart. The antiserum was characterized by immunotitration of the enzymic activity of cyclic AMP-dependent protein kinase and was found to react specifically with the catalytic subunit regardless whether it was bound to the regulatory subunit of the type I or type II enzyme or not. Here we describe the use of the antiserum in an enzyme-linked immunosorbent assay (ELISA), which can serve to detect and quantify the catalytic subunit independently of its activity and without the use of radioactive material in relatively crude extracts of distantly related animal tissues and cells.

A preliminary account of this work has been presented (Schwoch & Hamann, 1981).

Materials and methods

Materials

Mixed histones were obtained from Sigma (St. Louis, MO, U.S.A.; type II-A). [γ - ^{32}P]ATP was

* Dedicated to Prof. Dr. J. Kühnau on the occasion of his 80th birthday.

Abbreviation used: ELISA, enzyme-linked immunosorbent assay.

prepared from [^{33}P]phosphoric acid (New England Nuclear, Boston, MA, U.S.A.) by the method of Glynn & Chappell (1964) as modified by Walsh *et al.* (1971). The heat-stable inhibitor of cyclic AMP-dependent protein kinase was prepared from bovine skeletal muscle as described by Gilman (1970). Polystyrene microtitre plates (flat bottom) were obtained from Greiner (Nürtingen, Germany). Alkaline-phosphatase-conjugated goat anti-(rabbit immunoglobulin) was purchased from Orion (Helsinki, Finland) and *p*-nitrophenyl phosphate was from Merck (Darmstadt, Germany).

Animals and cells

Bovine heart was purchased from a local slaughterhouse and yeast (*Saccharomyces cerevisiae*) from a local bakery. Rat liver was taken from female Wistar rats and frog liver from female *Xenopus laevis*. Crayfish muscle was prepared from the abdominal muscle of *Astacus leptodactylus*. All tissues were quick-frozen in liquid N_2 and stored at -80°C . Human fibroblasts (WI-38) were kindly supplied by Dr. J. Remacle, University of Namur, Belgium. *Acanthamoeba castellanii* cells were kindly given by Dr. H. Jantzen, University of Heidelberg, Germany. Both cell types were obtained in the lymphilized form.

Preparation of cell and tissue extracts

Frozen tissues were minced under liquid N_2 and homogenized with 6 (crayfish), 8 (frog) or 25 (bovine, rat) vol. of 0.25 M-sucrose/3 mM-imidazole, pH 7.4, in a glass/Teflon homogenizer at 1100 rev./min. The homogenate was centrifuged at 20000 g for 30–60 min at 4°C and aliquots of the supernatant were used for the assay either directly or after treatment with 6 M-urea for 30 min at 30°C . In the comparative analyses all tissue extracts were additionally sonicated five times for 5 s at 0°C with a Branson sonifier B-12 (Branson, Danburg, CO, U.S.A.), set at position 10. Fibroblasts and *Acanthamoeba* were suspended in homogenization buffer containing 6 M-urea, sonicated five times for 5 s at 0°C and centrifuged at 8000 g for 2 min at 4°C . Yeast extract was prepared by homogenization with 5 vol. of 10 mM-potassium phosphate/1 mM-EDTA, pH 7.0, in an MSK tissue homogenizer (Braun, Melsungen, Germany) and centrifuged at 20000 g for 30 min at 4°C . The supernatant was treated with 6 M-urea for 30 min at 30°C . Urea-treated samples were diluted to 2 M-urea or less before reaction with the antiserum.

Determination of protein kinase activity

Protein kinase activity was determined by measuring ^{33}P incorporation from γ -labelled ATP into histone. The reaction mixture (0.11 ml final volume) contained 50 mM-Hepes [4-(2-hydroxyethyl)-1-pi-

perazine-ethanesulphonic acid], pH 6.8, 7.5 mM-magnesium acetate, 73 μg of mixed histones, 0.05 mM- $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (sp. radioactivity approx. 200 c.p.m./pmol), 1.8 μM -cyclic AMP (when used) and dilutions of the extracts which had been found to be linear in the enzyme activity with respect to time and protein concentration.

Preparation of catalytic subunit of cyclic AMP-dependent protein kinase

Bovine heart catalytic subunit was purified as described by Peters *et al.* (1977). The product yielded a single band in dodecyl sulphate/polyacrylamide-gel electrophoresis. Rat liver catalytic subunit was prepared by homogenization of 250–300 g of liver in 2.5 vol. of 4 mM-EDTA/1 mM-dithioerythritol, pH 7.0, followed by centrifugation at 20000 g for 20 min at 4°C and isolation of the enzyme directly from the supernatant according to Beavo *et al.* (1974a).

Antiserum

The antiserum against the catalytic subunit from bovine heart was produced in rabbits after coupling the enzyme to keyhole limpet haemocyanin. The antibodies reacted equally well with the free subunit as with the catalytic subunit bound in the type I or type II protein kinase holoenzyme and did not cross-react with cyclic AMP-independent protein kinases, as was shown by immunotitration of the protein kinase activity (Schwoch *et al.*, 1980).

Enzyme-linked immunosorbent assay

Catalytic subunit was diluted with 10 mM-potassium phosphate (pH 7.4)/140 mM-NaCl/0.02% NaN_3 (phosphate-buffered saline) and allowed to adsorb to microtitre plates for 2 h at room temperature. Residual absorption sites on the plastic surface were saturated by treatment with 1% bovine serum albumin in phosphate-buffered saline for 2–3 h at room temperature. Then, the plate was washed three times with phosphate-buffered saline containing 0.05% Tween 20 and incubated for 2 h at 4°C with the diluted antiserum (0.1 ml/well) which had been preincubated for 2 h with or without the antigen-containing samples. At least duplicate determinations were performed. After washing, the alkaline phosphatase-conjugated antibody, diluted 1:500 with phosphate-buffered saline containing 1% bovine serum albumin, was added and left overnight at 4°C . The substrate, *p*-nitrophenyl phosphate, was dissolved (1 mg/ml) in 0.1 M-diethanolamine/HCl (pH 9.8)/1 mM- MgCl_2 and applied (0.2 ml/well). Absorption of the coloured hydrolysis product was measured, after incubation for 1–6 h at room temperature, at 405 nm in a Titertek Multiscan photometer (Flow Laboratories, Meckenheim, Germany). All values were corrected for the blank values obtained in wells containing no antigen.

Protein determination

Protein was precipitated with trichloroacetic acid and quantified by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

ELISA assay of the catalytic subunit

Of the different variations of enzyme linked immunosorbent assays (Engvall, 1980), we chose one in which the antigen is determined by its inhibition of the binding of a limited amount of antibody to antigen adsorbed on the plastic surface of microtitre plates. The antibody bound to the antigen in the solid phase is detected by an alkaline-phosphatase-coupled second antibody.

To reach a high sensitivity in this system the amount of antigen adsorbed on the plate should be low and the concentration of antibody should not exceed its binding capacity. Thus, we initially examined the reaction of various concentrations of the rabbit antiserum with bovine heart catalytic subunit coated at different concentrations on the microtitre plate (Fig. 1). The amount of antigen leading to a suitable response is remarkably low (<10 ng of catalytic subunit/well) and linear binding of the antibody takes place with a concentration of less than 1 μ l/ml.

According to this result, in the standard assay the plates were coated with a concentration of 30 ng/ml of catalytic subunit (0.1 ml/well) and incubated with an antiserum dilution of 1:2000 (0.1 ml/well). Routinely performed controls with equally diluted preimmune serum did not exceed the blank values in wells containing no antigen, except in experiments with higher concentrations of *Acanthamoeba* extract. In this case the values obtained with pre-immune serum were subtracted.

Preincubation of the antiserum with various dilutions of the purified bovine heart catalytic subunit and reaction of the mixture with the adsorbed antigen leads to a typical S-shaped inhibition curve (Fig. 2) of antibody binding to the coated antigen.

However, in view of the amount of material adsorbed (≤ 3 ng/well), the amount of soluble antigen leading to half-maximal inhibition seems to be relatively high (approx. 20 ng/well). Similarly, in first experiments with bovine heart extract as competitor, only unexpectedly high tissue concentrations elicited a significant decrease in the amount of antibody detected on the plate. Thus one could suggest that differences in the structure or molecular accessibility between the solid-phase-bound and the soluble antigen were responsible for this observation.

Fig. 1. Antibody binding to catalytic subunit adsorbed onto a plastic surface

Different concentrations of bovine heart catalytic subunit were coated onto polystyrene microtitre plates (0.1 ml/well) and incubated with 0.1 ml/well of diluted antiserum (○) or preimmune serum (●) for 2 h at 4°C. After overnight incubation at 4°C with alkaline-phosphatase-conjugated anti-(rabbit immunoglobulin G) immunoglobulin, bound antibody was determined by the photometric assessment of nitrophenyl phosphate hydrolysis, incubating for 15–120 min at room temperature. For further details see the Materials and methods section.

Effect of urea treatment on the determination of catalytic subunit by ELISA

In an attempt to overcome the observed insensitivity of the reaction with the tissue extract a pretreatment of the samples with urea was found to enhance strongly their reactivity. Under these conditions inhibition of antibody binding could be observed already at low concentrations of bovine heart (1 mg of tissue/ml) and inhibition increased at higher tissue concentrations far more steeply than was observed with untreated samples (Fig. 3). The effect of the urea was maximal at concentrations of ≥ 6 M (Fig. 4) and a preincubation time of 30 min at 30°C was found to be optimal in the treatment of differently concentrated extracts (results not shown). Before the antibody was added, the urea concen-

Fig. 2. Inhibition of antibody binding to catalytic subunit in the solid phase by soluble catalytic subunit

Bovine heart catalytic subunit was coated on the wells of a microtitre plate (0.1 ml/well at a concentration of 30 ng/ml). Antiserum at a dilution of 1:2000, preincubated with or without different amounts of catalytic subunit, was added (0.1 ml/well) and allowed to react with the solid phase antigen for 2 h at 4°C. Antibody bound to the coated antigen was detected after incubation with the second antibody as described in the Materials and methods section.

Fig. 3. Inhibition of antibody binding to solid phase bovine heart catalytic subunit by bovine heart extracts pretreated with or without urea

Heart extract (20000 g supernatant) was treated with or without 6 M-urea for 30 min at 30°C, diluted to 1 M-urea, incubated with antiserum (1:2000) and then applied to a microtitre plate coated with bovine heart catalytic subunit (30 ng/ml). Inhibition of antibody binding to the immobilized antigen by various tissue concentrations was determined as outlined in the Materials and methods section.

Fig. 4. Effect of different concentrations of urea used for pretreatment of bovine heart extract on the inhibition of antibody binding to catalytic subunit in the solid phase

Heart extract (20000 g supernatant) was incubated in the presence or absence of urea for 30 min at 30°C. The samples were diluted to 1 M-urea and a content of 5 mg of tissue/ml and reacted with antiserum (dilution: 1:2000) for 2 h at room temperature. Subsequently 0.1 ml of the mixture was applied to the antigen-coated (30 ng/ml) wells of the microtitre plate and the bound antibody was determined as described in the Materials and methods section.

tration was diluted to 2 M or less. Under these conditions no significant impairment of the antigen-antibody reaction was observed (results not shown).

Urea treatment obviously does not produce any alteration in the components of the tissue extract which could have non-specific influences on the determination of the catalytic subunit. This was confirmed by an experiment in which the amount of the catalytic subunit in differently concentrated bovine heart extracts was determined either directly or after the addition of a constant amount of pure catalytic subunit as an internal standard (Table 1). The increase obtained in the amount of antigen in the tissue samples was equivalent to the quantity added. This finding indicates not only the applicability of the urea treatment but also the specificity of the detection of the catalytic subunit in the presence of the various other constituents (e.g. proteinases, inhibitors, other protein kinases) in the crude heart extract.

Determination of the amount of catalytic subunit in bovine heart supernatant by ELISA

Competition curves with purified catalytic subunit and with 20000g supernatant from bovine heart tissue both pretreated with urea are shown in Fig. 5.

Incubation with increasing concentrations of catalytic subunit leads to a sharp decrease in the amount of antibody bound to the plate. Half-maximal binding was found with 30ng/ml (=3 ng/well) of catalytic subunit. This concentration is identical to the coating concentration of the antigen and therefore indicates a similar reactivity of surface-bound and urea-treated free antigen. As compared with the untreated samples (Fig. 2), the application of urea has increased the sensitivity of the method about 7-fold. The least detectable dose as determined according to Hunter & Greenwood (1964) is ≤ 1 ng of catalytic subunit (95% probability). This value corresponds to about 25 fmol of the molecule.

The competition curve with bovine heart extract, which was obtained under the same conditions, parallels the curve with the pure antigen, underlining the specificity of the antigen-antibody reaction. This allows the quantitative determination of the catalytic subunit in bovine heart supernatant. The value found is $0.18 \pm 0.02 \mu\text{mol/kg}$ wet wt. Similar concentrations of the catalytic subunit were calculated in different rabbit tissues on the basis of enzyme activity measurements (Beavo *et al.*, 1974b; Hofmann *et al.*, 1977).

Comparative analysis of various animal species and cells by ELISA

As previously described (Schwoch *et al.*, 1980), the antiserum against the catalytic subunit from bovine heart could be also successfully applied in the immunotitration of the enzyme activity of cyclic AMP-dependent protein kinase from rat liver and rabbit skeletal muscle. This led us to assume a high

immunochemical relatedness of the catalytic subunit among different mammalian tissues and species. With the ELISA system described here it is possible to examine these suggestions more quantitatively and to extend a comparative analysis to further species, including sources in which the protein kinase can hardly be determined by measuring phosphate incorporation into protein.

Fig. 5. Inhibition of antibody binding to solid phase bovine heart catalytic subunit by urea-pretreated pure catalytic subunit or bovine heart extract. Catalytic subunit (●) or the 20000g supernatant from bovine heart homogenates (○) was incubated with 6 M-urea for 30 min at 30°C, diluted to 2 M-urea and allowed to react with the antiserum (1:2000). The mixture was then applied to the antigen-coated (30 ng of bovine heart catalytic subunit/ml) plate and the bound antibody was detected as described in the Materials and methods section.

Table 1. Retrieval of catalytic subunit after addition of bovine heart extract in urea-pretreated samples. Bovine heart supernatant was treated with 6 M-urea (30 min at 30°C) and was then diluted to 2 M-urea and different tissue concentrations. A constant amount of equally treated pure catalytic subunit was added or not and the samples were assayed by ELISA as described in the Materials and methods section. The amount of enzyme was calculated from a standard curve with pure bovine heart catalytic subunit performed under identical conditions.

Amount of catalytic subunit (ng)			
(a) In heart extract	(b) Pure subunit added	(c) Heart extract + pure subunit	Difference (c) – (a)
1.1	4.6	5.3	4.2
2.1	4.6	7.9	5.8
5.4	4.6	9.8	4.4
7.8	4.6	12.7	4.9
13.5	4.6	17.5	4.0
4.7 ± 0.7 (s.d.)			

Fig. 6 shows the competition curve for bovine heart supernatant in comparison with the competition exerted by similarly prepared extracts from rat liver, frog liver and crayfish muscle. All curves run in parallel to each other, as is compatible with the assumption of an immunological identity of the antigen determinants in the different species. However, the relative quantities of tissue leading to the same degree of inhibition of antibody binding are identical only for bovine heart and rat liver, but significantly higher for case of the frog and crayfish tissue. This could be an argument against the assumption mentioned above or it could simply reflect a different concentration of the antigen in the samples examined.

To prove the latter, the enzyme activity of cyclic AMP-dependent protein kinase was determined in the various tissue extracts and compared with the relative amounts of the catalytic subunit as calculated by the ELISA (Table 2).

The specific activity of the protein kinase as measured in the presence of cyclic AMP and corrected for a small amount of kinase activity which could not be inhibited by increasing concentrations of the heat-stable inhibitor of cyclic AMP-dependent protein kinase was similarly high in the bovine and rat tissue. Frog liver contained only about 25% and crayfish muscle less than 5% of the mammalian enzyme activity. These values are in accordance with the relative quantities of the catalytic subunit as determined by the immunochemical method. Provided that the enzymes have comparable catalytic-centre activities, one can therefore assume that the catalytic subunit in the different species is immunologically identical.

In further experiments the application of the ELISA was extended to organisms which are more distantly related, like amoebae and yeast. The results

Fig. 6. Inhibition of antibody binding to rat liver catalytic subunit by extracts from rat liver, bovine heart, frog liver and crayfish muscle

Urea-pretreated extracts (20 000 g supernatant) from rat liver (○), bovine heart (●), frog liver (■) and crayfish muscle (▲) were incubated with antiserum (1:2000) and 0.1 ml/well was applied to a microtitre plate which had been coated with rat liver catalytic subunit (0.1 ml/well; 14 ng/ml). Bound antibody was determined as described in the Materials and methods section.

obtained are documented in Fig. 7, together with the competition curve obtained with human fibroblasts as an additional example of mammalian cells. Enzyme from human cells was found to show a complete cross-reactivity with that from beef or rat tissue in preliminary Ouchterlony experiments (A. Hamann & G. Schwoch, unpublished work).

Table 2. Cyclic AMP-dependent protein kinase in different animal species as determined by enzyme activity and by ELISA

Tissue homogenates were centrifuged for 60 min at 20 000 g and the protein kinase was determined in the supernatant. The enzyme activity measured in the presence and absence of 1.8 μ M-cyclic AMP is given as the mean value $\pm$ S.D. ($n=4$). Determinations were also made in the presence of cyclic AMP and at four increasing concentrations (0.3–1.2 mg of protein/ml) of the heat-stable inhibitor and the residual activity was documented. The part of the total protein kinase activity which was suppressed by the inhibitor is expressed relative to the value found with bovine heart tissue and compared with the corresponding values obtained by the immunochemical determination of the catalytic subunit. These latter values were calculated from duplicate competition curves (compare Fig. 6) whose values had coefficients of variation better than 10%.

Tissue	Protein kinase activity (pmol/min per mg of tissue)			Cyclic-AMP dependent protein kinase (%) as determined by:	
	–Cyclic AMP	+Cyclic AMP	+Inhibitor	Enzyme activity	ELISA
Beef heart	29 $\pm$ 2	195 $\pm$ 13	5	100	100
Rat liver	39 $\pm$ 2	192 $\pm$ 18	21	90	100
Frog liver	17 $\pm$ 1	63 $\pm$ 2	5	25	24
Crayfish muscle	10 $\pm$ 1	18 $\pm$ 2	7	4	5

Fig. 7. Inhibition of antibody binding to bovine heart catalytic subunit by extracts from human fibroblasts, *Acanthamoeba* and yeast cells

Urea-pretreated extracts from Wi-38 cells (●), *Acanthamoeba castellanii* (○) and baker's yeast (■) were incubated with antiserum (1:2000) and then applied to a microtitre plate coated with 0.1 ml/well of bovine heart catalytic subunit (30 ng/ml). The antibody bound to the solid phase was determined as outlined in the Materials and methods section.

Even the *Acanthamoeba* and yeast extracts react with the antiserum against bovine heart catalytic subunit. Both inhibition curves are parallel with that from the human fibroblasts. However, the effective concentrations, especially for yeast, are very high. We were not able to determine unequivocally the cyclic AMP-dependent protein kinase activity in the amoebae, nor in yeast, where the bulk of the kinase activity was cyclic AMP-independent. Thus, the question remains open whether the quantitative differences in the immunoreactivity of these cells are indicative of structural differences in the catalytic subunit in the lower organisms or only of differing concentrations of the enzyme. However, it is clear that the molecule is highly related, even in evolutionarily far-distant species.

Discussion

The ELISA described here offers the possibility to detect and quantify cyclic AMP-dependent protein kinase with respect to its catalytic subunit in terms of mass, independently from factors like other kinases, which could impair its determination by measurement of the enzyme activity. The high specificity of the assay is due to the use of pure

catalytic subunit as the antigen in the solid phase and the specificity of the antiserum applied. The specificity of the serum against the catalytic subunit has been shown by immunotitration studies (Schwoch *et al.*, 1980) and by immuno-blotting experiments (S. M. Walter-Lohmann, G. Schwoch, R. Port, G. Reiser & U. Walter, unpublished work). In the latter, crude homogenates of rat tissues were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, transferred to nitrocellulose paper and incubated with the antiserum and ^{125}I -labelled protein A. The antiserum was bound to only one protein running at a position (M_r 40000) identical to that of purified catalytic subunit used as a reference. In the ELISA experiments this high specificity is reflected by the complete competition exerted by crude heart extracts which parallels the competition by the pure catalytic subunit.

The sensitivity of the ELISA is in the range of fmol and therefore comparable with that of sensitive radioimmunoassays. The use of urea additionally decreased the detection level and made it possible to measure the antigen in relatively crude tissue extracts. Only suggestions can be made on the mechanism of action of the urea. In the extracts it

could cause an improvement of the access of the antibody to the antigen. An influence on the dissociation-association state of the catalytic subunit in the protein kinase holoenzyme seems less feasible, since immunotitration experiments had shown an identical reaction of the antiserum with the free enzyme and the enzyme bound to the regulatory subunit of the type I or type II protein kinase (Schwoch *et al.*, 1980). However, it has been also reported (Peters *et al.*, 1977) that pure catalytic subunit molecules tend to associate with one other, an effect which could be reversed by urea treatment. In this context it should be remarked that the antiserum was not produced against the native catalytic subunit but against catalytic subunit coupled to haemocyanin. Thus, the structure of the molecule as altered by the urea treatment could resemble more the structure of the immunogen and therefore react with the antibody with a higher affinity.

Comparative analysis of tissues and cells from various species did show the high degree of homology of the catalytic subunit, or at least its antigenic determinants, in mammals and even in distantly related sources. This confirms also our assumption (Schwoch *et al.*, 1980) of immunotolerance as the basic phenomenon causing the weak immunogenicity of the unmodified catalytic subunit.

The immunochemical identity of the molecules observed in the multicellular organisms offers the possibility to quantify the catalytic subunit by ELISA in a variety of experimental systems without the necessity to purify the catalytic subunit from the corresponding tissue as a standard. Beyond it, the results with amoebae and yeast may suggest an immunological similarity of the catalytic subunit even in much lower organisms. In case of yeast, comparable immunological studies are also known for the cyclic AMP binding subunit of its cyclic AMP-dependent protein kinase. Antibodies raised against the yeast molecule and antibodies against the regulatory subunit from bovine heart protein kinase did not cross-react with the bovine heart antigen or the yeast antigen (Hixson & Krebs, 1980). With respect to the catalytic subunit of the yeast protein kinase previous non-immunological studies reported an apparent identity to the mammalian enzyme in its functional properties (Takai *et al.*, 1974a), but differences in its physical and kinetic properties (Takai *et al.*, 1974b). In the immunochemical studies described here we could not find support for such a diversity. Thus, the antibody against the catalytic subunit seems to recognize a highly conserved site in the molecule. Similar conservative structures have been reported for calmodulin (Chafouleas *et al.*, 1979), a regulatory protein of comparable significance to the cyclic AMP-dependent protein kinase

(cf. Klee *et al.*, 1980) and for histones (cf. Isenberg, 1979), one of the substrates *in vivo* of the catalytic subunit (Ord & Stocken, 1966; Langan, 1969). This suggests the catalytic subunit of cyclic AMP-dependent protein kinase to be part of an old regulatory system whose biological efficiency has kept it highly constant throughout evolution.

The support of the work by Professor Dr. H. Hilz and Professor Dr. H.-G. Thiele is gratefully acknowledged. We thank Dr. Eckstein for his help in the preparation of yeast homogenate.

References

- Alhanaty, E., Patinkin, J., Tauber-Finkelstein, M. & Shaltiel, S. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3492–3495
- Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1974a) *Methods Enzymol.* **38**, 299–308
- Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1974b) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3580–3583
- Chafouleas, J. G., Dedman, J. R., Munjaal, R. P. & Means, A. R. (1979) *J. Biol. Chem.* **254**, 10262–10267
- Corbin, J. D. & Keeley, S. L. (1977) *J. Biol. Chem.* **252**, 910–918
- Corbin, J. D., Keeley, S. L. & Park, C. R. (1975) *J. Biol. Chem.* **250**, 218–225
- Engvall, E. (1980) *Methods Enzymol.* **70**, 419–439
- Fleischer, N., Rosen, O. & Reichlin, M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 54–58
- Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305–312
- Glynn, I. M. & Chappell, J. B. (1964) *Biochem. J.* **90**, 147–149
- Hixson, C. S. & Krebs, E. G. (1980) *J. Biol. Chem.* **255**, 2137–2145
- Hofmann, F., Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1975) *J. Biol. Chem.* **250**, 7795–7801
- Hofmann, F., Bechtel, P. J. & Krebs, E. G. (1977) *J. Biol. Chem.* **252**, 1441–1447
- Hunter, W. M. & Greenwood, F. C. (1964) *Biochem. J.* **91**, 43–56
- Isenberg, I. (1979) *Annu. Rev. Biochem.* **48**, 159–191
- Jungmann, R. A. & Russell, D. H. (1977) *Life Sci.* **20**, 1787–1798
- Kapoor, C., Beavo, J. A. & Steiner, A. L. (1979) *J. Biol. Chem.* **254**, 12427–12432
- Klee, C. B., Crouch, T. H. & Richman, P. G. (1980) *Annu. Rev. Biochem.* **49**, 489–515
- Kuo, J. F. & Greengard, P. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1349–1355
- Kupfer, A., Jiménez, J. S. & Shaltiel, S. (1980) *Biochem. Biophys. Res. Commun.* **96**, 77–84
- Krebs, E. G. (1972) *Curr. Top. Cell. Regul.* **5**, 99–133
- Langan, T. A. (1969) *J. Biol. Chem.* **244**, 5763–5765
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Ord, M. G. & Stocken, L. A. (1966) *Biochem. J.* **98**, 888–897
- Peters, K. E., Demaille, J. G. & Fischer, E. G. (1977) *Biochemistry* **16**, 5691–5697
- Prashad, N., Rosenberg, B., Wischmeyer, B., Ulrich, C. & Sparkman, D. (1979) *Biochemistry* **18**, 2717–2725

- Schwoch, G. & Hamann, A. (1981) *Biochem. Soc. Trans.* **9**, 240P
- Schwoch, G., Hamann, A. & Hilz, H. (1980) *Biochem. J.* **192**, 223–230
- Sugden, P. H., Holladay, L. A., Reimann, E. M. & Corbin, J. D. (1976) *Biochem. J.* **159**, 409–422
- Takai, Y., Sakai, K., Morishita, Y., Yamamura, H. & Nishizuka, Y. (1974a) *Biochem. Biophys. Res. Commun.* **59**, 649–652
- Takai, Y., Yamamura, H. & Nishizuka, Y. (1974b) *J. Biol. Chem.* **249**, 530–535
- Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1968–1976
- Walter, U. (1981) *Eur. J. Biochem.* **118**, 339–346
- Walter, U., Costa, M. R. C., Breakefield, X. O. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3251–3255