Antiserum against the catalytic subunit of adenosine 3':5'-cyclic monophosphate-dependent protein kinase

Reactivity towards various protein kinases

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An antiserum against the catalytic subunit C of cyclic AMP-dependent protein kinase, isolated from bovine heart type II protein kinase, was produced in rabbits. Reaction of the catalytic subunit with antiserum and separation of the immunoglobulin G fraction by Protein A–Sephadex quantitatively removed the enzyme from solutions. Comparative immunotitration of protein kinases showed that the amount of antiserum required to eliminate 50% of the enzymic activity was identical for pure catalytic subunit, and for the holoenzymes type I and type II. The reactivity of the holoenzymes with the antiserum was identical in the absence or the presence of dissociating concentrations of cyclic AMP. Most of the holoenzyme (type II) remains intact when bound to the antibodies as shown by quantification of the regulatory subunit in the supernatant of the immunoprecipitate. Titration with the antibodies also revealed the presence of a cyclic AMP-independent histone kinase in bovine heart protein kinase I preparations obtained by DEAE-cellulose chromatography. Cyclic AMP-dependent protein kinase purified from the particulate fraction of bovine heart reacted with the antiserum to the same degree as the soluble enzyme, whereas two cyclic AMP-independent kinases separated from the particle fraction neither reacted with the antiserum nor influenced the reaction of the antibodies with the cyclic AMP-dependent protein kinase. Immunotitration of the protein kinase catalytic subunit C from rat liver revealed that the antibodies had rather similar reactivities towards the rat liver and the bovine heart enzyme. This points to a relatively high degree of homology of the catalytic subunit in mammalian tissues and species. Broad applicability of the antiserum to problems related to cyclic AMP-dependent protein kinases is thus indicated.

Cyclic AMP-dependent protein kinases catalyse the phosphorylation of a wide variety of protein substrates and play a central role in the regulation of physiological processes by cyclic AMP. The enzymes are classified as type I and type II protein kinases. They differ in their regulatory subunits (R) but seem to possess an identical, or nearly identical, catalytic subunit (C). The catalytic subunit is inhibited when bound in the quaternary complex R_2C_2 of the holoenzyme, and released for full catalytic activity after binding of cyclic AMP to the regulatory subunit R (for a review see Nimmo & Cohen, 1977).

The high significance of protein kinases in metabolic regulation, in growth, differentiation and malignant processes has become apparent in recent

years. Still, little information is available concerning the mechanisms by which cyclic AMP-dependent protein kinases mediate tissue-specific responses to the various stimuli, especially regulation of macromolecular synthesis. In view of the existence of multiple cyclic AMP dependent and independent protein kinases with similar specificities (for review see Rubin & Rosen, 1975) or problems of translocation (cf. Jungmann *et al.*, 1975; Corbin *et al.*, 1977), the availability of specific antibodies appears to offer a powerful tool for the elucidation of these and related problems.

Although production of antibodies against the regulatory R subunits of the cyclic AMP-dependent protein kinase has been reported (Fleischer *et al.*, 1976; Hofmann *et al.*, 1977; Kapoor *et al.*, 1979),

production of antibodies against the catalytic subunit proved to be difficult. Low antigenicity of the catalytic subunit C is also indicated by the observation that injection of protein kinase holoenzyme resulted in antibodies directed exclusively against the regulatory subunit (Fleischer *et al.*, 1976). To our knowledge, only Steiner *et al.* (1978) reported the use of antisera against the catalytic subunit in immunofluorescent studies. However, no detailed description of the immunization procedure or a characterization of the antibodies has been given so far.

In the present study, we report the production of specific antibodies against the catalytic subunit C from bovine heart by using the conjugation of the subunit C with haemocyanin to overcome the low immunogenicity of the antigen. The antiserum obtained was characterized with respect to free subunit C, and subunit C in both holoenzyme forms, and analysed for cross-reactivity with protein kinases from other sources.

Materials and methods

Materials

Mixed histones were obtained from Sigma (St. Louis, MO, U.S.A.; type II-A), casein from Merck (Darmstadt, Germany), bovine serum albumin from Behring-Werke (Marburg/Lahn, Germany), and keyhole limpet haemocyanin from Schwarz/Mann (Orangeburg, NY, U.S.A.). Freund's adjuvant was a product of Difco Laboratories (Detroit, MI, U.S.A.). CNBr-activated Sepharose 4B and Protein A-Sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden), goat antibodies against rabbit immunoglobulins were from Nordic (Byck-Mallinckrodt, Dietzenbach, Germany), pig antibodies against rabbit immunoglobulin G were from Orion (Helsinki, Finland), DEAE-cellulose (type DE-32) and phosphocellulose (type P-11) were from Whatman (Maidstone, Kent, U.K.). Cyclic [^3H]AMP (sp. radioactivity 32 Ci/mmol) was obtained from Buchler-Amersham (Braunschweig, Germany). [γ - ^{33}P]ATP was prepared from [^{33}P]-phosphoric acid (NEN, 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).

Preparation of antigen and immunization procedure

The catalytic subunit C of protein kinase (type II) was purified from bovine heart muscle by the method of Peters *et al.* (1977). Homogeneity of the product was shown by gel electrophoresis by the method of Laemmli (1970), with the Tris/glycine system (kindly performed by W. Weber). Part of the

purified subunit C was dialysed against phosphate-buffered saline (20 mM-potassium phosphate/140 mM-NaCl, pH 7.4) and then coupled to a 10-fold excess of keyhole limpet haemocyanin by the procedure of Avrameas & Ternynck, (1969). The conjugate was again dialysed against phosphate-buffered saline, pH 7.4. Free catalytic subunit or subunit C coupled to haemocyanin were dissolved in phosphate-buffered saline and emulsified to a stable water-in-oil emulsion with an equal volume of complete Freund's adjuvant by using the double-syringe method. A portion (2 ml) of the emulsion containing 0.5 mg of catalytic subunit C was injected into 6-month-old male rabbits (Loh; Dwingen, Göttingen, Germany) contralaterally, intradermally and intramuscularly at multiple sites. Booster injections with 0.1–0.2 mg of the antigen in incomplete Freund's adjuvant were performed through the same routes 1, 3 and 4 months after the first immunization. Sera were collected from the lateral ear vein, prepared by standard methods and stored at -30°C .

Ouchterlony test

Double immunodiffusion was performed for 48–72 h at 4°C and the precipitation lines were detected by enzyme-linked immunostaining. After diffusion the gels were washed for 1 day in phosphate-buffered saline, pH 7.4, and further incubated for 4–12 h at 4°C with alkaline phosphatase conjugated to pig anti-(rabbit immunoglobulin G) antibodies. After washing for 24 h at 4°C , staining was achieved by incubation with a solution containing 50 mg of α -naphthyl phosphate and 100 mg of Variamin Blue salt in 100 ml of 2% (w/v) barbital/NaOH buffer, pH 9.4.

Immunotitration of protein kinases

Appropriate dilutions of antiserum were incubated with protein kinase antigens in phosphate-buffered saline, pH 7.4, containing 0.25% (w/v) bovine serum albumin and 5 mM-dithioerythritol (for purified subunit C) or 2.5% (w/v) bovine serum albumin $\pm$ 5 μM -cyclic AMP (for holoenzymes) in a volume of 40 μl at 4°C overnight.

Protein A-Sepharose CL-4B (20 μg of protein) in a volume of 50 μl of phosphate-buffered saline was then added, the suspension shaken for 1 h at 5 – 7°C , centrifuged for 2 min at 8000 g, and portions of the supernatant were tested for protein kinase activity. Controls were performed with preimmune serum or with sera from non-immunized rabbits, which served equally well as controls.

Protein kinase assay

Protein kinase activity was determined by measuring ^{33}P incorporation from γ -labelled ATP into histone or casein. The reaction mixture (0.11 ml

final volume) contained 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 6.8, 7.5 mM-magnesium acetate, 73 µg of mixed histones or 140 µg of casein, and 0.05 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity approx. 200 c.p.m./pmol). The mixture was incubated at 30°C with or without 1.8 µM-cyclic AMP. Portions (50 µl) were analysed for protein-bound radioactivity as described by Corbin & Reimann (1974). One unit of protein kinase activity is the amount that catalyses incorporation of 1 µmol of ^{32}P into histone (casein) in 1 min.

Regulatory subunits R

These were isolated by the method of Weber *et al.* (1979). Quantification was performed as described in the same paper by cyclic $[\text{H}]\text{AMP}$ binding, with reversible denaturation of subunit R by urea to facilitate exchange of any bound cyclic AMP, and to eliminate interference of subunit C with cyclic $[\text{H}]\text{AMP}$ binding. Dissociation of holoenzyme II by reaction with subunit C antibodies was tested by incubation of 10 µl (134 µ-units) of protein kinase II with 50 µl of antiserum, or 50 µl of control serum for 1 h at room temperature and 4 h at 4°C, followed by the addition of 100 µl of Protein A-Sephadex gel. After an additional hour of incubation with shaking, the mixture was centrifuged, and portions of the supernatant were tested for protein kinase activity in the presence of added cyclic AMP, and for subunit R protein, as described above.

Preparation of cyclic AMP-dependent and cyclic AMP-independent protein kinases from bovine heart tissue

Muscle tissue (1.5 g) was homogenized with 4.5 ml of cold 5 mM-Tris/HCl, containing 1 mM-EDTA, 0.5 mM-dithioerythritol, pH 7.4, in an Ultra-Turrax tissue homogenizer (Jahnke and Kunkel, Freiburg, Germany) and centrifuged at 20000g for 20 min at 4°C. A portion of the supernatant containing the equivalent of 1 g of heart was applied to a DE-32 DEAE-cellulose column (0.9 cm × 7 cm) and washed with the homogenization buffer. Cyclic AMP-dependent protein kinases were eluted stepwise with 0.1 M buffered NaCl (type I enzyme), and 0.5 M buffered NaCl (type II enzyme). Fractions (2.75 ml) were collected at 5°C at a flow rate of 16 ml/h.

For separation of particle-bound protein kinase the pellet of the bovine heart homogenate was washed twice with homogenization buffer containing 0.14 M-NaCl. Solubilization of 'insoluble' protein kinases was achieved by sonification (3 times for 5 s; with Branson sonifier set at position 3) in 0.35 M-NaCl. After 30 min at 0°C, the suspension was centrifuged (60 min, 22000g, 4°C) and a portion of the supernatant equivalent to 5 g of heart tissue was applied on a phosphocellulose P-11 column

(0.9 cm × 7 cm) pre-equilibrated with the homogenization buffer containing 0.25 M-NaCl. After washing with the same buffer, elution of the cyclic AMP-independent protein kinases was performed with a linear gradient of 0.25–1.4 M-NaCl at 5°C and a flow rate of 15 ml/h. Fractions (3 ml) were collected, and analysed for histone kinase and casein kinase activity.

Preparation of catalytic subunit C from rat liver

Livers of male Wistar rats (250–300 g) were homogenized in 2.5 vol. of a solution containing 4 mM-EDTA and 1 mM-dithioerythritol, pH 7.0, applying an Ultra-Turrax tissue homogenizer for 1 min. The homogenate was centrifuged at 20000g for 20 min at 4°C, and the catalytic subunit was isolated directly from the supernatant by the method described by Beavo *et al.* (1974).

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 and discussion

1. Preparation of antigen and immunization

The catalytic subunit C of protein kinase type II from bovine heart was purified to apparent homogeneity (Fig. 1) by the procedure described by Peters *et al.* (1977) and Demaille *et al.* (1977).

Raising antibodies against the catalytic subunit proved to be difficult, as described above. The size of the molecule (mol.wt. 39000) is great enough to eliminate this parameter as a likely cause of the low antigenicity. Rather, the failure of subunit C to serve as an effective antigen appeared to be the result of a high degree of homology of the antigen in the donor and the recipient animals, owing to a conserved structure of subunit C during evolution. This is supported by a number of experimental results (cf. Langan, 1973). To overcome the supposed immunological tolerance we used for immunization a subunit C protein coupled to keyhole limpet haemocyanin. This is based on the following considerations. As known from immunological studies with low-molecular-weight haptens, immunological non-reactivity against a given determinant may not necessarily be due to the lack of appropriately reacting B-cell clones, but could be caused by an inability of the antigen to stimulate specific T-cells and thus to initiate T-cell/B-cell co-operation as an essential step in the sequence leading ultimately to antibody production. Since the determinants recognized by T-cells may differ from the B-cell reacting sites, it seemed conceivable to overcome T-cell tolerance against the protein kinase catalytic subunit by conjugating subunit C to

Fig. 1. Gel-electrophoretic analysis of isolated bovine heart catalytic subunit C

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described in the Materials and methods section. Purified regulatory subunit type II (R) ($4\mu\text{g}$) was applied as well as various amounts of subunit C.

haemocyanin as a carrier. With this modified antigen an immunological response was obtained, whereas immunization with the native catalytic subunit failed to elicit a detectable production of antibodies. During a total immunization period of 9 months, antigen binding of the antiserum was maximal 40 days after the first booster injection and then slowly declined in spite of further booster injections (results not shown).

2. Demonstration of antibodies and quantification of antigen-antibody interaction by immunotitration

When the antisera obtained were analysed by the Ouchterlony immunodiffusion procedure, a single precipitation line was obtained with crude and pure preparations of the protein kinase (results not

shown), thus demonstrating the presence of precipitating antibodies.

To quantify the reaction of the antibodies with the antigen, immunotitration was applied. A small amount (2.5 ng) of pure catalytic subunit was incubated with increasing amounts of antiserum at 4°C , centrifuged and the supernatants were assayed for protein kinase activity. As a control, to rule out possible interference by serum factors, preimmune serum was used instead of immune serum. All values were corrected for the (low) protein kinase activity present in the sera. When the residual activity was plotted against antiserum concentration (Fig. 2a), a characteristic sigmoidal curve was obtained that approached zero-activity values at $5\mu\text{l}$ of antiserum. To optimize the separation of bound and unbound antigen, different double-precipitation methods were explored. Use of Sepharose-coupled Protein A, the bacterial adsorbent that specifically binds to the F_C region of immunoglobulin G, was found to be the most effective at relatively low concentrations and therefore was used for all further experiments. Immunotitration of subunit C with the addition of Protein A-Sepharose at the end of the incubation period indicated complete removal of enzyme activity with less than $1\mu\text{l}$ of antiserum and the amount of antiserum required to remove 50% of the antigen was decreased to one-third of the value without Protein A (Fig. 2b).

The neutralization of the antigen by antiserum as seen in the absence of Protein A-Sepharose (Fig. 2a) may be due not only to precipitation reactions, but also to inhibition of the antigen. This is indicated by experiments in which protein kinase activity was determined in re-suspended-versus-centrifuged incubation mixtures of subunit C with limiting amounts of antiserum. Residual activity in the suspension was only slightly higher than in the supernatant obtained by centrifugation (results not shown).

3. Interaction of subunit C antiserum with protein kinase holoenzymes types I and II

In most tissues, the catalytic subunit C can occur in three forms: free catalytic subunit, inactive holoenzyme type I and inactive holoenzyme type II. Although physicochemical and immunological differences in the regulatory subunit of both types of enzyme are well known, the catalytic subunit in the two holoenzymes may also differ to a certain extent, since slight differences in amino-acid composition have been reported (Zoller *et al.*, 1979).

The following experiments were performed to compare the reactivity of the antiserum with the catalytic subunits C from protein kinases I and II and to analyse the interaction with the catalytic subunits when present in the form of the tetrameric holoenzymes.

Fig. 2. Immunotitration of purified catalytic subunit C with antiserum

Purified subunit C (2.5 ng/tube) was incubated with increasing amounts of antiserum followed by a reaction period without (a) or with (b) Protein A-Sepharose. After centrifugation supernatants were analysed for protein kinase activity. For further details see the Materials and methods section. Controls were performed with preimmune serum under otherwise identical conditions. All values were corrected for endogenous protein kinase activity (<10% at 5 μ l of serum); 100% activity corresponds to 2.5 μ -units.

Fig. 3. Immunotitration of holoenzymes types I and II

Type II (a) and type I (b) protein kinases were separated by DEAE-cellulose chromatography from the soluble fraction of bovine heart and incubated with increasing amounts of antiserum in the presence (●) or absence (○) of 5 μ M-cyclic AMP. Total protein kinase activity (inclusion of 1.8 μ M-cyclic AMP in the test) was measured in the supernatant after precipitation with Protein A-Sepharose as immunoadsorbent; 2.5 μ -units (=100%) of protein kinase activity was used in both cases. Heat-stable inhibitor was added to protein kinase II (inset a) and protein kinase I (inset b) holoenzymes respectively, and kinase activity was measured in the presence of 1.8 μ M-cyclic AMP. The data are expressed as the percentage of activity measured without inhibitor (protein kinase I, 30 μ -units/ml; protein kinase II, 690 μ -units/ml).

Protein kinases type I and type II preparations were obtained by DEAE-cellulose chromatography of bovine heart extracts. To study the reactivity of the two catalytic subunits, they were subjected to immunotitration in the presence of dissociating

concentrations of cyclic AMP. As shown in Fig. 3, practically identical amounts of antiserum were required for half-maximal neutralization of subunit C from type II and from type I kinases.

For protein kinase I, a residual activity (approx.

20% of the total activity) did not react with the antibodies. This strongly pointed to the presence in the protein kinase preparation of an immunologically unrelated histone kinase. Indeed, when the two holoenzyme preparations were analysed in the presence of the heat-stable inhibitor of cyclic AMP-dependent protein kinases a similar amount of residual inhibitor-resistant histone kinase activity was found in the type I preparation (Fig. 3*b*, inset), whereas protein kinase type II was completely inhibited (Fig. 3*a*, inset).

Immunoreactivity of the intact holoenzymes with the antibodies was analysed in the absence of cyclic AMP. As shown in Figs. 3(*a*) and 3(*b*) titration curves of the tetrametric holoenzymes were identical with the curves obtained from the C subunits released by cyclic AMP, half-maximal neutralization of the antigen requiring (within the limits of the method) the same amount of antiserum in both cases and for both types of protein kinase.

Since it is known that protein kinase holoenzymes can be dissociated by various factors (cf. Corbin *et al.*, 1975), possible dissociation of R_2C_2 by the binding of antibodies as a cause of the apparently identical immunoreactivity of holoenzyme and of dissociated subunit C was tested. When holoenzyme type II was incubated with enough antiserum and Protein A-Sepharose to remove 95% of the kinase from the supernatant, 90% of the regulatory subunit R was also transferred to the immunoprecipitate. This latter value takes into account a small degree of endogenous dissociation of the holoenzyme preparation, as judged from an activity ratio (–cyclic AMP/+cyclic AMP) of 0.20. Although these data do indicate that some holoenzymes became dissociated during incubation with the antibodies, the majority of the holoenzyme molecules remained intact.

A comparison of the titration curves of type II holoenzyme (Fig. 3*a*) and of isolated catalytic subunit C (Fig. 2*b*) showed that they were identical except for the last part of the curves. The reason for the somewhat decreased binding at low concentrations of unbound antigen in the holoenzyme preparation is not clear. Presence of interfering factors or of a small population of subunit C molecules with decreased affinity for the antiserum could be an explanation.

From the data in this section, we conclude that the C subunits in bovine heart protein kinases I and II are immunologically identical. Further, the antibodies do not discriminate to a significant degree between free subunit C, and subunit C bound to the regulatory subunits RI or RII. However, the antibodies do discriminate perfectly between the cyclic AMP-dependent enzymes and other, so far not described, histone kinase(s) present in the DEAE-cellulose fractions of protein kinase I.

4. Reactivity of the antiserum with various particle-bound protein kinases of bovine heart muscle

Further analysis of the specificity of the C antibodies was performed with three protein kinases extracted from the insoluble fraction of bovine heart. To exclude unspecific binding of the soluble protein kinases (Keeley *et al.*, 1975), the 22000g pellet was washed with buffered saline (0.14M-NaCl), followed by the extraction of the 'insoluble' protein kinases with 0.35M-NaCl. Chromatography of the extract on a phosphocellulose column yielded three peaks of protein kinase activity (Fig. 4). The first peak (designated PK 1) was only slightly active with histones or casein as the substrate. Its histone kinase activity, however, rose 25-fold when tested in the presence of cyclic AMP. Therefore, this enzyme is a cyclic AMP-dependent protein kinase. The two other enzymes (designated as PK 2 and PK 3) eluted by the salt gradient were inactive with histones but did show caseinolytic activity. Their activity was not influenced by the presence of cyclic AMP and remained unchanged when treated with different amounts of the heat-stable protein kinase inhibitor (results not shown). They are thus characterized as cyclic AMP-independent protein kinases.

Titration of PK 1 protein kinase with increasing

Fig. 4. Phosphocellulose chromatography of particulate bovine heart muscle protein kinases

NaCl extract from the particulate fraction equivalent to 5g of heart tissue was applied to a phosphocellulose column, washed and eluted as described in the Materials and methods section. Protein kinase activity was analysed with casein (●) or with mixed histones in the presence of $1.8\mu\text{M}$ -cyclic AMP (○) as the substrates. Histone kinase activity in peak 1 fractions determined in the absence of cyclic AMP did not significantly differ from the values obtained with casein. The various types of protein kinase indicated in the Figure are defined in the text. —, Conductivity.

amounts of antiserum (Fig. 5) resulted in a titration curve that was very similar in shape to the one obtained with the soluble cyclic AMP-dependent holoenzyme type II (Fig. 3a). Also, the amount of antiserum required for half-maximal neutralization of the antigen was nearly identical when the different kinase concentrations (activities) in the two experi-

ments are taken into account. This result indicates that the insoluble histone kinase found in the washed pellet fraction of bovine heart is identical with or closely related to, the soluble protein kinase subunit C. Comparable findings were reported also for other tissues (cf. Spielvogel *et al.*, 1977; Rubin *et al.*, 1979). When peak-2 protein kinase (PK 2) was tested, no decrease in kinase activity was observed even at the highest amounts of antiserum applied (Fig. 5).

Cross-reactivity of the 'insoluble' protein kinases (Fig. 4) was further analysed by adding increasing amounts of the individual enzyme fractions to a constant concentration of antiserum. The results in Fig. 6 show that the antiserum considerably decreased the activity of PK 1, but not those of PK 2 or PK 3. Also, no significant difference in the titration curve of protein kinase PK 1 was seen when protein kinase PK 2 was present during immunoreaction (results not shown). Thus, significant interaction of protein kinases 2 and 3 with the subunit C antibodies is excluded.

5. Applicability of the antiserum to cyclic AMP-dependent protein kinases from other species

A broader use of the subunit-C-specific antiserum for the analysis of cyclic AMP-dependent protein kinases depends on the extent of the cross-reactivity with enzymes from different tissues and different species.

To obtain corresponding information the immunoreactivity of the catalytic subunit from rat liver was studied as an example. When 1.1 μ -units of

Fig. 5. Immunotitration of protein kinases extracted from the particulate fraction of bovine heart. PK 1 and PK 2 protein kinases obtained by phosphocellulose chromatography (see Fig. 4) were titrated with antiserum as described in the Materials and methods section. The 100% values for both enzymes were 4.8 μ -units.

Fig. 6. Activity-concentration curves of particulate bovine heart muscle protein kinases after reaction with a constant amount of antiserum

Particulate protein kinases PK 1 (a), PK 2 (b) and PK 3 (c) (phosphocellulose chromatography, Fig. 4) were incubated with 1 μ l of antiserum (O) or preimmune serum (●) in a total volume of 80 μ l as described in the Materials and methods section. The supernatant obtained after immunoprecipitation with Protein A-Sepharose (20 μ g of protein) was analysed for protein kinase activity by using histones (PK 1) or casein (PK 2 and PK 3) as substrates.

Fig. 7. Reactivity of rat liver protein kinase with the antiserum

Purified liver protein kinase subunit C was incubated with increasing amounts of antiserum and processed as described in the legend to Fig. 2(b). For further details see the Materials and methods section; 100% activity corresponds to 1.1 μ -units.

the rat liver enzyme (equivalent to 44% of the activities in Figs. 2 and 3) was titrated with the antiserum (Fig. 7), the slope of the curve was somewhat less steep than with the bovine antigens, indicating a slightly lower affinity between this antigen and the antibodies. To neutralize 50% (0.55 μ -unit) of the rat liver enzyme, the same amount of antiserum was required as that to eliminate 50% (1.25 μ -units) of the bovine enzyme (cf. Fig. 2b). Thus, it can be concluded that the rat liver enzyme serves almost as well as an antigen in the immune reaction as the authentic antigen (provided the two enzymes have comparable catalytic-centre activities). Furthermore, the antiserum reacted also with partially purified protein kinase subunit C from skeletal muscle of a rabbit strain different from that used for immunization (results not shown).

These data point to a generally high immunological similarity of the catalytic subunit of cyclic AMP-dependent protein kinases in mammalian tissues and species and that could be taken as an indication for a high constancy of this molecule during evolution.

The experiments described in the present paper have shown that the antibodies raised against the catalytic subunit C react equally well with the free antigen and with the antigen bound to subunit R proteins (holoenzymes). The clear discrimination of cyclic AMP-independent histone kinase and cyclic AMP-independent casein kinase by the antibodies,

and their high cross-reactivity with an enzyme from another species, offer the opportunity to study yet unsolved problems of protein kinase function and localization in various systems.

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