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Characterization of the phosphorylated form of the insulin-stimulated cyclic AMP phosphodiesterase from rat liver plasma membranes

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Incubation of intact purified rat liver plasma membranes with insulin, cyclic AMP and ATP led to the activation of the peripheral 'low-K_m' cyclic AMP phosphodiesterase. When [γ-^{32}P]ATP was included in the incubation mixture, after purification of this enzyme to homogeneity it was found to contain 1 mol of alkali-labile ^{32}P/mol of enzyme. Treatment of the homogeneous phosphorylated enzyme with alkaline phosphatase released all of the ^{32}P from the protein while restoring its activity to the native state. The reversibility of the activation that is achieved by the phosphorylation of this enzyme could also be demonstrated with a high-speed supernatant from rat liver. This restored the activity of the activated membrane-bound enzyme to its native state. The K_a for the cyclic AMP-dependence of this process (1.6μM) was unaffected by a range of ATP concentrations (1–10mM) and by a range of membrane protein concentrations (0.2–2mg/ml). Adenylyl imidodiphosphate could not substitute for ATP, and concanavalin A could not substitute for insulin, as essential ligands in the activation process. The purified activated enzyme exhibited K_m 0.6μM, V_{max} 10.9 units/mg of protein and Hill coefficient (h) 0.47. The $V_{max.}$ for this activated enzyme was much higher than that of the native enzyme, yet h was much lower.

It is generally accepted that treatment of intact adipocytes with insulin causes the activation of a 'low-K_m' membrane-bound cyclic AMP phosphodiesterase (Zinman & Hollenberg, 1974; Wells & Hardman, 1977; Thompson & Strada, 1978; Makino *et al.*, 1980). This process appears to require either metabolic energy or ATP (Zinman & Hollenberg, 1974; Kono *et al.*, 1977), as indeed does the insulin-mediated activation of glucose transport into adipocytes (Siegel & Olefsky, 1980). In liver or hepatocytes the activation of a cyclic AMP phosphodiesterase has proved to be more elusive (Westwood *et al.*, 1979). However, Loten *et al.* (1978) have described a particulate enzyme whose activity is increased after treatment of hepatocytes with either insulin or glucagon. Furthermore Thompson *et al.* (1973) have shown that after insulin injection the amount of a particulate liver cyclic AMP phosphodiesterase is increased in diabetic rats.

Although it has been claimed that insulin, when added alone to intact rat liver plasma membranes, could activate a cyclic AMP phosphodiesterase (House *et al.*, 1972; Tria *et al.*, 1976), this effect has unfortunately not been able to be reproduced in other laboratories (Pilkis *et al.*, 1974; Thompson & Strada, 1978; Loten *et al.*, 1978; Westwood *et al.*, 1979; Marchmont & Houslay, 1980*b*). We have shown that two enzymes provide the cyclic AMP phosphodiesterase activity of rat liver plasma membranes (Marchmont & Houslay, 1980*a*). These are a low-K_m peripheral enzyme, which exhibits apparent negative co-operativitiy, and a high-K_m integral enzyme, which exhibits normal Michaelis kinetics. When intact plasma membranes are incubated *in vitro* with insulin together with cyclic AMP and ATP, the activity of the peripheral enzyme is doubled (Marchmont & Houslay, 1980*b*,*d*). However, no such activation is seen if either any one ligand alone or combinations of any two of these ligands is added to the incubation mixture. The presence of all three ligands is apparently essential for the activation process to ensue (Marchmont & Houslay, 1980*b*). It was suggested that the activation of this peripheral enzyme was due to the insulin-mediated cyclic AMP-dependent phosphorylation of this enzyme. This may be related to the ability of insulin to affect a number of phosphorylation and dephosphorylation reactions within the cell (see Houslay, 1981).

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The present paper describes the identification of a phosphorylated peripheral cyclic AMP phosphodiesterase from rat liver plasma membranes.

Materials and methods

The materials used in the present study are detailed in the accompanying paper (Marchmont *et al.*, 1981).

Cyclic AMP phosphodiesterase activity was assayed by a modification of the two-step method of Thompson & Appleman (1971) as described in detail previously (Marchmont & Houslay, 1980*a*). Curve fitting was as described previously (Marchmont & Houslay, 1980*a*). Corrections were made to account for the incomplete binding of [^{3}H]-adenosine to the Dowex 1 resin (see Marchmont & Houslay, 1980*a*; Marchmont *et al.*, 1981). In all assays less than 10% of the substrate was converted over the time-course. When 5′-AMP was used as an inhibitor, it was checked that the snake-venom nucleotidase converted all of the [^{3}H]AMP into [^{3}H]adenosine. Linearity of cyclic AMP degradation was assessed in all assays which were all carried out at 30°C at pH 7.2. All errors are given as S.D. with $n-1$ degrees of freedom. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the reduction and carboxymethylation of samples and the slicing and elution of gels was as described in detail by Marchmont & Houslay (1980*c*). Protein concentration was determined, with bovine serum albumin as a standard, with Bio-Rad Protein Assay Kit (Bradford, 1976; Spector, 1978) for the soluble enzyme and by a modification (Houslay & Palmer, 1978) of the micro-biuret method of Goa (1953) for the membranes.

Treatment of plasma membranes with [γ-^{32}P]ATP

[γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964). Rat liver plasma membranes isolated by a modification (Marchmont *et al.*, 1981) of the method of Pilkis *et al.* (1974) were treated with a phosphorylation mixture, containing insulin, cyclic AMP and ATP, as described in detail by Marchmont & Houslay (1980*b*). Briefly, intact plasma membranes (final concn. 5.0 mg of protein/ml) were incubated for 5 min at 30°C in a mixture containing final concentrations of 3 mM-[γ-^{32}P]-ATP (100–150 mCi/mmol), 100 μM-cyclic AMP, 1 μM-insulin, 5 mM-$MgCl_2$, 100 μM-$CaCl_2$ and 20 mM-Tris/HCl, final pH 7.4. The reaction mixture (5 ml) was then cooled to 4°C and the membranes were pelleted by centrifugation at 14000 $g_{av.}$ for 10 min. The membranes were then washed twice with 20 ml of ice-cold 1 mM-$KHCO_3$, pH 7.2, before being subjected to high-ionic-strength treatment.

This washing procedure removed radioactivity that was not incorporated into the membrane. It was also crucial to the purification procedure as the membranes used in this process had been stored in liquid nitrogen. Their freeze–thawing can lead to the solubilization of a small fraction of the peripheral enzyme. Such solubilized enzyme is unable to be activated or presumably phosphorylated in the activation procedure (Marchmont & Houslay, 1980*b*). Subsequently the peripheral phosphodiesterase activity of these membranes was purified to apparent homogeneity by the procedure of Marchmont *et al.* (1981).

Time-course of ^{32}P incorporation into the peripheral cyclic AMP phosphodiesterase

Plasma membranes at a final concentration of 0.5 mg of protein/ml were incubated in 25 μl of the phosphorylation mixture detailed above. At specific time intervals the reaction was quenched by adding 1 ml of ice-cold 1 mM-$KHCO_3$, pH 7.2. The membranes were then pelleted as above and washed with 3 × 1 ml of ice-cold 1 mM-$KHCO_3$, pH 7.2. The peripheral proteins were then liberated by high-ionic-strength treatment (Marchmont & Houslay, 1980*a*), and samples (20 μl) taken both for assay of cyclic AMP phosphodiesterase activity and for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Gels were sliced and the radioactivity in the M_r-52000 peak (P1) was assessed as described previously (Marchmont & Houslay, 1980*c*).

Treatment of the purified phosphorylated enzyme with alkaline phosphatase

A series of samples each containing 10 pmol of the purified [^{32}P]phosphorylated enzyme was mixed with 10 mg of bovine serum albumin in 100 μl of 5 mM-$MgCl_2$/20 mM-Tris/HCl, pH 7.4. To each was added 100 μl of 5 mM-$MgCl_2$/20 mM-Tris/HCl, pH 8.2, containing 20 μg of alkaline phosphatase. The reaction mixture was incubated at 30°C, and at various time intervals the reaction of particular samples was terminated by the addition of 1 ml of ice-cold 10% (w/v) trichloroacetic acid. The mixture was vortex-mixed and left on ice for 5 min before centrifugation at 14000 $g_{av.}$ for 2 min. Samples of the supernatant and of the resuspended pellet, in 200 μl of 5 mM-$MgCl_2$/20 mM-Tris/HCl, pH 8.2, were taken for radioactivity counting.

Exposure of plasma membranes to a 'high-speed' supernatant

Either native plasma membranes or membranes that had been washed after treatment with an unlabelled phosphorylation/activation mixture (see below and Marchmont & Houslay, 1980*b*) in order to activate the peripheral cyclic AMP phosphodiesterase were used. These membranes (2 mg of protein/ml) in 250 μl of 1 mM-$KHCO_3$, pH 7.2, were then treated with 250 μl of a 100000 $g_{av.}$ × 60 min rat

liver supernatant (Marchmont & Houslay, 1980*a*) containing 1–2 mg of protein, at 30°C for various time intervals. At such points 1 ml of ice-cold 1 mM-$KHCO_3$, pH 7.2, was added to each 0.5 ml sample. The membranes were then pelleted by centrifugation at 14000 $g_{av.}$ for 10 min and washed with 3 × 1 ml of ice-cold 1 mM-$KHCO_3$, pH 7.2, to remove adsorbed cytosol enzyme (Marchmont & Houslay, 1980*a*). The pellets were finally resuspended in 260 μl of 1 mM-$KHCO_3$, pH 7.2, and 20 μl samples were taken for evaluation of cyclic AMP phosphodiesterase activity. A substrate concentration of 0.4 μM-cyclic AMP was employed.

Standard activation procedure

This was as published previously (Marchmont & Houslay, 1980*b*). Briefly, purified rat liver plasma membranes (final concn. 500 μg of protein/ml) were incubated for 5 min at 30°C in a mixture containing final concentrations of 5 mM-$MgCl_2$, 100 μM-$CaCl_2$, 3 mM-ATP, 100 μM-cyclic AMP, 1 μM-bovine insulin and 20 mM-Tris/HCl, final pH 7.4, and final volume 250 μl. After this time 1 ml of ice-cold 1 mM-$KHCO_3$, pH 7.2, was added and the mixture vortex-mixed before centrifugation at 14000 $g_{av.}$ for 10 min. The pellet was resuspended and washed twice with 1 mM-$KHCO_3$, pH 7.2, before final resuspension in this buffer (250 μl).

Analysis of alkali-labile phosphate content

This was performed with 20 pmol of ^{32}P-labelled enzyme by the method of Nimmo *et al.* (1976). In some instances bovine serum albumin (10 mg) was added as a carrier.

Activation of the re-bound purified peripheral cyclic AMP phosphodiesterase

For this, 20 pmol of the purified enzyme (Marchmont *et al.*, 1981) was incubated at 4°C for 1 h with the washed high-salt-treated membranes (2 mg of protein) (Marchmont & Houslay, 1980*a*) in a solution containing final concentrations of 5 mM-$MgCl_2$, 0.8 mM-$KHCO_3$ and 20 mM-Tris/HCl, final pH 7.2. The membranes were washed twice in 1 mM-$KHCO_3$, pH 7.2, by centrifugation at 14000 $g_{av.}$ for 10 min, and subjected to activation by the standard procedure described above.

Results

Isolation of a phosphorylated enzyme

After incubation of the plasma membranes in the phosphorylation mixture, containing [γ-^{32}P]ATP, the purified peripheral enzyme (Marchmont *et al.*, 1981) on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis exhibited a single protein-staining band that coincided with the single band of radioactivity (Fig. 1). This phosphorylated peripheral enzyme contained 1·02 ± 0·04 mol of alkali-labile ^{32}P/mol of M_r-52000 protein (four preparations).

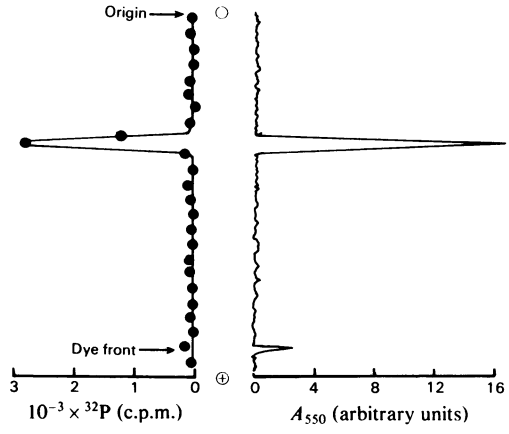

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified phosphorylated peripheral cyclic AMP phosphodiesterase from rat liver plasma membrane*

This Figure compares a scan of a gel stained for protein with a similar gel monitored for ^{32}P. Samples of the purified protein (7 μg) were loaded on the gels, which after electrophoresis were either stained with Coomassie Blue or immediately sliced into 1.5 mm sections and incubated overnight with 100 μl of 0.1% sodium dodecyl sulphate/5 mM-2-mercaptoethanol/40 mM-Tris/HCl, pH 7.4, before samples were taken for counting (see Marchmont & Houslay, 1980*c*, for details).

Analysis of the rate of incorporation of ^{32}P into this enzyme demonstrated that it followed the activation of the enzyme (Fig. 2).

Kinetic properties of the phosphorylated enzyme

The purified phosphorylated enzyme exhibited non-linear kinetics (Fig. 3), yielding kinetic constants of $K_m = 0.6 \pm 0.15$ μM, $V_{max.} = 10.9 \pm 0.2$ units/mg of protein and Hill coefficient (h) = 0.47 ± 0.05 (three preparations with duplicate assays). The limit values of K_m and $V_{max.}$ are given in Table 1. The catalytic-centre activity, assuming one active site per molecule (M_r-52000) of enzyme, is 34170.

This activity was inhibited by 5′-AMP, which caused a small decrease in h, being 0.42 at 1 mM-AMP and 0.4 at 3 mM-AMP (Fig. 3). The limit values for K_m and $V_{max.}$ are given in Table 1. 5′-AMP appeared to act as a competitive inhibitor of the low-affinity state of the enzyme. However, it acted as a mixed inhibitor against the high-affinity state of the enzyme, with large changes in V_1 and small changes in K_1 (see Table 1). The con-

Table 1. *Limiting values for the kinetic constants of the phosphorylated purified peripheral cyclic AMP phosphodiesterase from rat liver plasma membranes*

Results are means ± S.D. for three experiments with assays carried out in duplicate ($n = 3$). The kinetic analysis was carried out over a concentration range of 0.4 μM–1 mM-cyclic AMP. K_1 and V_1 are respectively K_m and $V_{max.}$ values for the high-affinity state, and K_2 and V_2 those for the low-affinity state.

Inhibitor	K_1 (μM)	V_1 (μmol/min per mg of protein)	K_2 (μM)	V_2 (μmol/min per mg of protein)
None	0.58 ± 0.08	2.03 ± 0.20	30 ± 6	10.83 ± 0.9
1 mM-AMP	0.44 ± 0.06	1.62 ± 0.09	36 ± 8	10.42 ± 1.0
3 mM-AMP	0.40 ± 0.03	1.08 ± 0.20	48 ± 9	9.80 ± 0.9

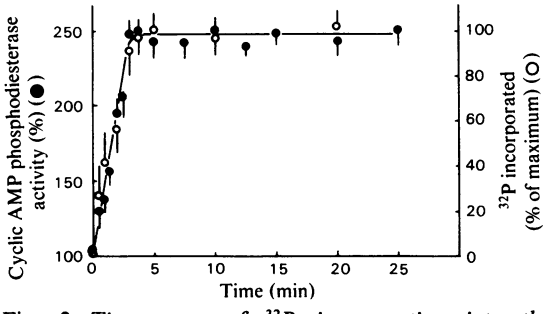

Fig. 2. *Time-course of ^{32}P incorporation into the peripheral cyclic AMP phosphodiesterase*

This Figure compares the time-course of activation of the peripheral enzyme (●) with the incorporation of ^{32}P into the M_r-52000 peripheral enzyme (O) identified by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a high-salt extract of phosphorylated plasma membranes. This experiment was carried out as described previously (Marchmont & Houslay, 1980*b*). The peripheral enzyme was assayed at 0.4 μM-cyclic AMP. Results are means ± S.D. for three different membrane preparations carried out in duplicate.

centration (I_{50}) of 5′-AMP which caused 50% inhibition of the activity of the enzyme was 55 ± 10 mM when assayed at 5 μM-cyclic AMP and 3.5 ± 0.5 mM at 0.4 μM-cyclic AMP (three determinations).

Reversibility of the activation

When the purified phosphorylated enzyme was treated with alkaline phosphatase, the activity of the enzyme, assayed at 0.4 μM-cyclic AMP, fell to approach that exhibited by the native enzyme. This fall in the activity of the phosphorylated enzyme paralleled the release of ^{32}P from the enzyme (Fig. 4). The amount of ^{32}P released from the enzyme was calculated as 0.93 ± 0.05 mol/mol of enzyme. The addition of alkaline phosphate had, however, no effect on the activity of the purified native enzyme. Similar effects could be demonstrated with the purified phosphorylated enzyme that had been re-bound to the plasma membrane.

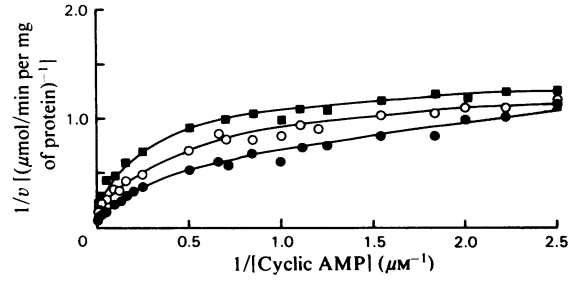

Fig. 3. *Kinetics of the phosphorylated peripheral phosphodiesterase*

Lineweaver–Burk plot of the activity of the purified phosphorylated enzyme in the absence (●) and presence of 1 mM-(O) and 3 mM-(■) 5′-AMP.

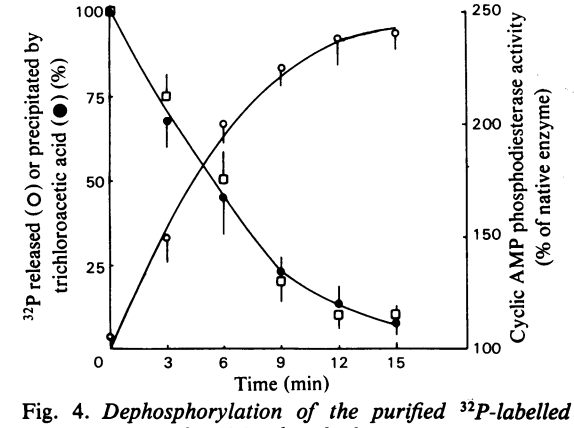

Fig. 4. *Dephosphorylation of the purified ^{32}P-labelled cyclic AMP phosphodiesterase*

The enzyme was treated with alkaline phosphatase as described in the Materials and methods section. The phosphodiesterase activity of the enzyme assayed at 0.4 μM-cyclic AMP is followed at various times (□) together with the ^{32}P label in the enzyme (●) and the ^{32}P released (O). Results are means ± S.D. for three experiments.

Exposure of native membranes to a 'high-speed' supernatant had no effect on the endogenous cyclic AMP phosphodiesterase activity of the membranes (Fig. 5). However, when membranes that had been

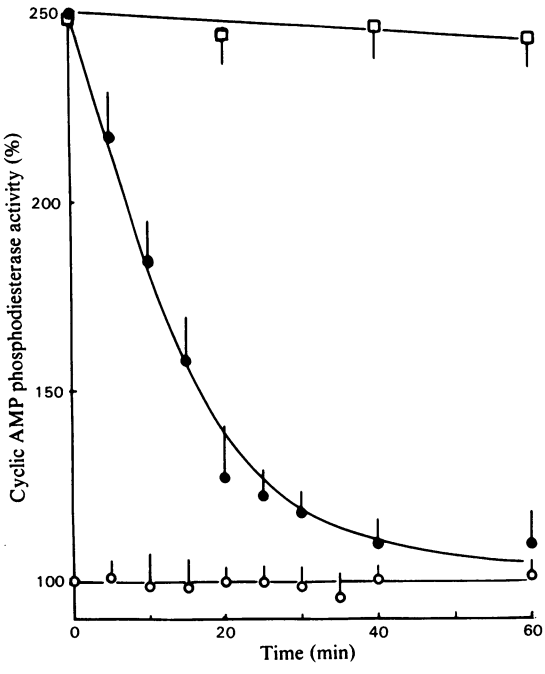

Fig. 5. *Reversal of the activation of the phosphodiesterase with high-speed supernatant*

Rat liver plasma membranes were activated by the standard procedure (see the Materials and methods section). Plasma membranes (500μg/ml) in 20mM-Tris/HCl, pH 7.4, after activation were incubated alone (□) or in the presence of 200μg of protein (●) of a high-speed supernatant (100000$g_{av.}$ × 60min) from rat liver. Plasma membranes that had not been preactivated (○) were similarly treated with the high-speed supernatant.

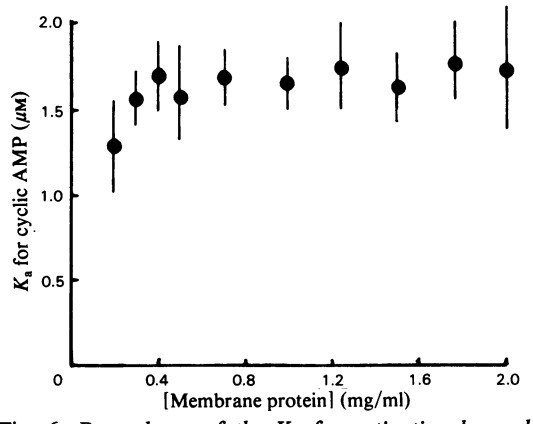

Fig. 6. *Dependence of the K_a for activation by cyclic AMP on membrane concentration*

Under the standard assay conditions a K_a value for cyclic AMP was obtained by using ten different cyclic AMP concentrations. This was carried out at a series of different membrane concentrations.

preincubated in the phosphorylation mixture, in order to activate the peripheral enzyme, were treated with the 'high-speed' supernatant (Fig. 5), the phosphodiesterase activity fell rapidly. Indeed, the activity decreased to a value approaching that demonstrated by the native membrane.

K_a for the cyclic AMP-dependence of the activation process

In the standard activation mixture, the K_a for activation by cyclic AMP, in the presence of saturating (1μM) insulin concentrations, is about 1.6μM (Marchmont & Houslay, 1980*b*). A series of experiments was carried out to determine the K_a for the cyclic AMP-dependence of the activation process, by using the procedure described previously (Marchmont & Houslay, 1980*b*), at different concentrations of membrane protein (Fig. 6).

Alterations in the ATP concentration over the range 1–10mM had little significant effect on the K_a value for cyclic AMP of 1.6μM observed at a concentration of 500μg of membrane protein/ml.

When the ATP analogue adenylyl imidodiphosphate (3mM) was used to replace ATP in the activation mixture, no activation of the peripheral enzyme ensued.

Concanavalin A at either 1 or 20μg/ml neither substituted for insulin in causing the activation of the peripheral enzyme nor inhibited the activation of the peripheral enzyme in the standard activation mixture.

Activation of the re-bound purified enzyme

The purified peripheral cyclic AMP phosphodiesterase (Marchmont *et al.*, 1981) was re-bound to membranes which had been pre-treated with the high-salt solubilization mixture in order to remove peripheral proteins (Marchmont & Houslay, 1980*a*). The activity of the re-bound purified cyclic AMP phosphodiesterase could be increased to 220 ± 9.5% ($n = 3$), when assayed at 0.4μM-cyclic AMP, after pre-treatment with the standard activation mixture. This effect was dependent on the presence of insulin, cyclic AMP and ATP all being present in the activation mixture.

Discussion

Insulin has been demonstrated to cause the activation of a peripheral plasma-membrane cyclic AMP phosphodiesterase from rat liver by a mechanism dependent on cyclic AMP and ATP. It has been suggested that this was achieved through the

phosphorylation of the enzyme (Marchmont & Houslay, 1980*b*). Consistent with such a proposal is our ability to isolate an activated enzyme containing 1 mol of $^{32}P_i$/mol of enzyme. This activated enzyme can be dephosphorylated with alkaline phosphatase, whereupon the loss of $^{32}P_i$ parallels the fall in cyclic AMP phosphodiesterase activity (Fig. 4). The complete removal of $^{32}P_i$ restores the enzyme to its native activity state. Such an effect can also be achieved (Fig. 5) by using a high-speed supernatant from rat liver which contains endogenous phosphatase activity (Brandt *et al.*, 1974; Nimmo & Cohen, 1977). This implies that a reversal mechanism for the activation (phosphorylation) process is present within the liver. Conversely, the activation of the enzyme parallels the incorporation of $^{32}P_i$ into this M_r-52000 protein (Fig. 2).

The activity of the phosphorylated enzyme, although still exhibiting non-linear kinetics (Fig. 3), is very different from that observed with the native enzyme (Marchmont *et al.*, 1981). The parameters that are particularly affected are the $V_{max.}$, which is increased from 9.1 to 10.9 units/mg of protein (20%), and the Hill coefficient (*h*), which is decreased from 0.62 to 0.47, on phosphorylation. There is also a small decrease in the K_m from 0.71 to 0.60μM. Indeed it is this decrease in the Hill coefficient that makes the activation effect of the phosphorylation modification more effective over the physiological range (0.3–5μM) of cyclic AMP concentrations seen within the cell.

This can be seen readily from the limit values for K_m and $V_{max.}$ of the native (Marchmont *et al.*, 1981) and phosphorylated enzymes (Table 1). Upon phosphorylation, the $V_{max.}$ of the high-affinity state of the enzyme is increased by some 64%, whereas that of the low-affinity state only increases by some 20%. The changes in K_m are less dramatic, although both are decreased, that of the high-affinity state by 18% and that of the low-affinity state by 25%.

5′-AMP inhibits the reaction and also decreases the Hill coefficient (*h*), although this effect is less marked than for the native enzyme (Marchmont *et al.*, 1981). The form of the inhibition, by 5′-AMP, of the high- and low-affinity states of the enzyme is also similar to that of the native enzyme. This implies, for reasons discussed in the accompanying paper (Marchmont *et al.*, 1981), that the non-linear kinetics exhibited by this apparently homogeneous enzyme may be due to it obeying a mnemonical type of mechanism. However, the relative insensitivity of the enzyme to inhibition by 5′-AMP makes it unlikely that this will be of importance in regulating the enzyme *in vivo*.

Little is known about membrane-bound kinases (see Houslay, 1981). However, soluble cyclic AMP-dependent protein kinases, which consist of regulatory (R) and catalytic (C) subunits (Nimmo & Cohen, 1977; Krebs & Beavo, 1979), appear to be tetrameric (R_2C_2). Swillens *et al.* (1974) have pointed out that, as the activation of cyclic AMP-dependent protein kinase depends upon the dissociation of the complex (R_2C_2) into its constituent subunits, the K_a for activation by cyclic AMP will be related to the equilibrium constant for the dissociation process. This means that the K_a for the cyclic AMP-dependence of the activation will be dependent on the concentration of the kinase in the assay. However, as we are investigating a membrane-bound protein kinase, the relevant parameter that will affect the value of K_a for activation by cyclic AMP is the concentration of the kinase in the membrane itself. This means that the K_a for cyclic AMP will be unaffected by the concentration of membranes in our activation mixture (Fig. 6), for the kinase concentration in them will be constant. Furthermore, we can expect this assessment of the K_a for activation by cyclic AMP to reflect that observed *in vivo*. Indeed, the value of K_a that we observe is about 1.6μM. This is rather important, for, as we have suggested previously (Marchmont & Houslay, 1980*b*), basal hepatocyte cyclic AMP concentrations (0.3–0.5μM; Exton *et al.*, 1973; Smith *et al.*, 1978) will be insufficient to allow the activation of the enzyme by insulin. However, after exposure to glucagon, intracellular cyclic AMP concentrations rise to 2–4μM (Jefferson *et al.*, 1968; Blackmore *et al.*, 1979), which is sufficiently high to allow insulin to activate this enzyme. Such a mechanism, which has the K_a value for cyclic AMP at such a crucial point, may explain, at least in part, why insulin can antagonize the action of glucagon in increasing cyclic AMP concentrations, yet does not alter basal cyclic AMP concentrations (Jefferson *et al.*, 1968). However, we expect that the K_a for cyclic AMP would be sensitive to the concentration of the kinase within the membrane. This implies that manipulation at this point could be expected to affect the action of insulin on the target cell. Indeed, that the particulate cyclic AMP phosphodiesterase activity in intact adipocytes can be increased by insulin (see Wells & Hardman, 1977; Thompson & Strada, 1978) suggests that if a similar mechanism exists, the K_a for cyclic AMP is below the intracellular cyclic AMP concentration.

That the re-bound peripheral enzyme could still be activated suggests that not only is the catalytic unit of the kinase an integral protein, but so also is its regulatory unit, for high-ionic-strength treatment causes the dissociation of soluble kinases (Krebs & Beavo, 1979), yielding a catalytic unit that is active in the absence of cyclic AMP. This clearly is not the case here, as the activation process remained dependent on cyclic AMP. Membrane-bound kinases where both the regulatory and catalytic units

are integral proteins have been reported before (Scott & Dousa, 1978), although in the erythrocyte (Rubin, 1979) it would seem that the kinase is anchored to the membrane through its regulatory unit only. Further evidence for the involvement of a cyclic AMP-dependent protein kinase in the process is the observation that adenylyl imidodiphosphate could not substitute for ATP in the activation mixture.

Interestingly, the plant lectin concanavalin A failed either to mimic or to inhibit the action of insulin in this process. This contrasts with the ability of concanavalin A to mimic certain of insulin's actions (Czech, 1977) in intact cells. However, distinct populations of insulin receptors, only certain of which bind concanavalin A in a manner that blocks insulin's ability to bind, have been reported in erythrocytes (Herzberg *et al.*, 1980).

The rapidity, reversibility and the magnitude of the K_a values for the dependence of the process on both insulin and cyclic AMP suggest that the activation of this enzyme by insulin may be of physiological relevance (see Marchmont & Houslay, 1980*b*). In agreement with other investigators (Pilkis *et al.*, 1974; Thompson & Strada, 1978; Westwood *et al.*, 1979), we have been unable to observe any effect *in vitro* of insulin itself on the cyclic AMP phosphodiesterase activity of rat liver plasma membranes. However, such a stimulation has been observed by certain investigators (House *et al.*, 1972; Tria *et al.*, 1976). It is highly likely that these two groups were working with a different enzyme preparation from that described by us. They used a low-density 'microsomal' fraction, which would have been enriched in membranes from the bile-canalicular and blood-sinusoidal faces of the hepatocyte as compared with the preparation used by us (Pilkis *et al.*, 1974), which was a fraction obtained from a low-speed pellet of a hypo-osmotic homogenate, containing membranes from the contiguous and blood-sinusoidal faces of the liver cell (see Wisher & Evans, 1975). House *et al.* (1972) and Tria *et al.* (1976) also washed their membranes twice in 50mM-Tris/HCl containing 0.1M-NaCl, which is of a sufficient ionic strength to elute from the membrane a considerable fraction of the peripheral enzyme that we have described (Marchmont & Houslay, 1980*a*), and furthermore, prolonged preincubation, up to 30min with insulin, was necessary in order to see maximal stimulation of the enzyme in the experiments of House *et al.* (1972) and Tria *et al.* (1976). These factors may indicate that there is more than one type of cyclic AMP phosphodiesterase in rat liver whose activity can be augmented by insulin. Clearly this activation is achieved by different mechanisms.

Loten *et al.* (1978, 1980) have described a solubilized high-affinity cyclic AMP phosphodiesterase preparation derived from an EDTA extract of total cell membranes. The cyclic AMP phosphodiesterase activity associated with this extract was increased after pre-treatment of intact hepatocytes with insulin, glucagon, agents that increased intracellular cyclic AMP, and dibutyryl cyclic AMP. These effects were extremely rapid and occurred within 5min. This EDTA extract of a total membrane pellet presumably removes peripheral proteins (see Houslay, 1981) from a variety of membranes. However, the major peak of cyclic AMP phosphodiesterase activity investigated in the preparation of Loten *et al.* (1978, 1980) was clearly different from the peripheral enzyme that we described, for although it had a low K_m for cyclic AMP (0.3μM), displayed non-linear kinetics and was not activated by calmodulin, it exhibited a much lower K_m for cyclic GMP (0.4μM) than the peripheral enzyme, had a different molecular weight (122000), sedimentation coefficient and heat sensitivity (see Marchmont & Houslay, 1980*a*, for discussion). However, the preparation of Loten *et al.* (1978) undoubtedly contained more than one cyclic AMP phosphodiesterase, a minor one of which may well have been the peripheral plasma-membrane enzyme that we have described (Marchmont *et al.*, 1981). Indeed this could well explain why Loten *et al.* (1978) observed that the treatment of hepatocytes with insulin and glucagon together, which might be expected to lead to the activation of the enzyme that we have described (Marchmont & Houslay, 1980*b*), has a greater effect on cyclic AMP phosphodiesterase activation than does either hormone added alone.

It is possible that other cyclic AMP phosphodiesterases are regulated by phosphorylation mechanisms. This has been suggested as an explanation for the cyclic AMP-dependent activation of an enzyme from myoblasts (Ball *et al.*, 1980) and could explain the dependence on ATP for the insulin-mediated activation of a particulate low-K_m enzyme in intact adipocytes (Zinman & Hollenberg, 1974; Kono *et al.*, 1977). Indeed, the activation of cyclic AMP phosphodiesterases by phosphorylation may well be a property that is only associated with high-affinity peripheral membrane forms, as the phosphorylation of low-affinity soluble species from brain has little apparent effect on its kinetic properties (Sharma *et al.*, 1980).

It remains to be seen whether insulin achieves these effects by directly activating a specific kinase, by altering the conformation of the protein in order to make it susceptible to phosphorylation or by indirectly activating a kinase through the release of a 'peptide-like' factor (Jarett & Seals, 1979; Larner *et al.*, 1979; Seals & Czech, 1980). Insulin has been shown to affect a number of cyclic AMP-dependent phosphorylation and dephosphorylation reactions

within the cell, suggesting that this is one important factor of its action (see Houslay, 1981).

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