The State of Phosphorylation *in vivo* of Membrane-Bound Phosphoproteins in Rat Brain

By MALCOLM WELLER and RICHARD RODNIGHT

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation,
University of London), De Crespigny Park, London SE5 8AF, U.K.

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The alkali-labile P content of membrane protein prepared from rapidly frozen rat brain was measured, CuSO_4 being used to inhibit protein phosphatase activity during subcellular fractionation. The P content of the membrane fraction was significantly increased (+12%) over the control value by incubation of homogenates with ATP before fractionation. This suggests that the membrane protein in rat brain is normally only partially phosphorylated.

This paper attempts to answer the question whether the membrane-bound phosphoproteins in the brain of normal quiescent rats are fully phosphorylated or are held in a partially dephosphorylated state. The question has implications for the physiological role of these macromolecules in membrane functioning. Most studies on the metabolism of brain phosphoproteins have used isotopic-labelling methods that do not distinguish between net phosphorylation of protein and turnover of protein P (Rodnight, 1971); therefore changes in the rate of incorporation of ^{32}P into protein as a result of altered functional state could result from one or other of these processes. Change in the turnover rate of fully phosphorylated proteins might be taken to imply their involvement in a carrier mechanism, whereas change in phosphorylation state would be more consistent with a role in determining the conformation of membrane structures.

In a study of the turnover *in vitro* of membrane-bound P in ox brain Weller & Rodnight (1971, 1973) showed that membrane fragments prepared from cerebral cortex contained partially dephosphorylated phosphoproteins that could be fully phosphorylated by intrinsic protein kinase activity, stimulated by cyclic AMP, on incubation with 1 mM-ATP for about 15 min. However, no conclusion about the state of phosphorylation *in vivo* can be drawn from this observation because (a) dephosphorylation of membrane protein may have occurred during preparation of the subcellular fractionation and (b) protein kinase activity in the cell membrane in the intact tissue may be controlled by factors not operating in isolated membrane fragments. Investigation of the problem became feasible when it was found that intrinsic protein phosphatase activity in the membrane fragments from brain is effectively inhibited by Cu^{2+} (Weller & Rodnight, 1971). We now present evidence to support the conclusion that the membrane phosphoproteins *in vivo* are in a partially dephosphorylated state.

Experimental

Preparation of membrane fraction. Rats (Wistar strain) were killed either by decapitation or by immersion in liquid N_2 . In the former case the forebrain was removed and homogenized within 30 s of death in 10 ml of the appropriate ice-cold medium. The heads of deep-frozen rats were placed in a cryostat at -20°C for about 2 h, after which the forebrain was removed and homogenized as indicated above. In all cases homogenization was done in a Teflon-glass test-tube homogenizer (type B; A. H. Thomas Inc., Philadelphia, Pa., U.S.A.) by using six passes with the pestle rotating at about 800 rev./min. The usual medium was 0.3 M-sucrose adjusted to pH 7.0 with Tris and containing, where stated, 1 mM- CuSO_4 ; in experiments involving phosphorylation of brain protein, the tissue was homogenized in water (see below).

Homogenates from three or four rats were combined and centrifuged for 10 min at $1.25 \times 10^4 g$ in the GSA rotor of a Sorvall RC-2B centrifuge. The supernatant was then centrifuged for 30 min at $7.8 \times 10^4 g$ in a Spinco centrifuge. The pellets were re-suspended in 40 ml of 1 mM- CuSO_4 and centrifuged again for 30 min at $7.8 \times 10^4 g$. The pellet was re-suspended in about 7 ml of 1 mM- CuSO_4 , and a sample was taken for determination of protein; alkali-labile P was then determined on 2 ml samples in triplicate as described below.

Phosphorylation of brain protein. Forebrains (from four rats) were homogenized in 20 ml of deionized water at 0°C , and immediately 14 ml of an appropriate solution was added to give final concentrations in the mixture of 30 mM-Tris-HCl buffer, pH 7.4, 1 mM- MgSO_4 and 1 mM-ATP (Tris salt). The homogenate was gently stirred at $22-25^\circ\text{C}$ for 30 min, and then 6 ml of 2 M-sucrose and 0.04 ml of 1 M- CuSO_4 were added (giving final concentrations of 0.3 M and 1 mM respectively). Subcellular fractionation was done as described above.

Determination of alkali-labile P. To samples containing at least 7 mg of membrane protein 10 M- HClO_4

was added to a final concentration of 0.5M and the precipitate was spun down at 2500g for 10min. The precipitate was washed in the centrifuge twice by resuspension in 4ml of 0.5M-HClO₄, once with 1 ml of ethanol-diethyl ether (1:1, v/v) and once with 1 ml of chloroform-methanol (2:1, v/v). The residue was then resuspended in 2ml of 5% (w/v) trichloroacetic acid and heated for 15min at 100°C. After centrifugation for 10min at 2500g the protein residues were washed once by suspension in 1 ml of ethanol-diethyl ether (1:1, v/v) and then incubated at 37°C for 18h in 1 ml of 1M-NaOH. Standards and blanks containing 0.05 μ mol of KH₂PO₄ in 1M-NaOH and 1M-NaOH alone respectively were incubated at the same time. After incubation 1.6ml of 1.33M-HClO₄ was added to each tube, the tubes were cooled in ice and 0.6ml of silicotungstate reagent (McIlwain & Rodnight, 1962) was added dropwise to each with constant agitation. The tubes were then centrifuged at 2500g for 10min, 3ml of each supernatant was taken and 0.3ml of 5% (w/v) ammonium molybdate was added. The phosphomolybdate complex was extracted and P_i determined by the procedure of Martin & Doty (1949).

Protein was determined by the method of Miller (1959).

Results and discussion

It was reasonable to expect that some loss of protein-bound P in the membrane would occur during preparative procedures owing to protein phosphatase activity continuing *post mortem*. Although Weller & Rodnight (1971) found that Cu²⁺ is an effective inhibitor of the intrinsic protein phosphatase activity of membrane fragments, it was necessary to show that Cu²⁺ would also inhibit other phosphatases

that might act on the membrane phosphoprotein before separation of the membrane fraction. To investigate this a membrane preparation was labelled with [³²P]ATP and then incubated with a freshly prepared brain homogenate in the presence and in the absence of 1 mM-CuSO₄. Table 1 shows that the Cu²⁺ completely stabilized the membrane protein-bound P for a period of 1h.

It remained possible that some loss of protein-bound P might occur in the interval between the death of the animal and homogenization of the tissue. In previous work (quoted in Rodnight, 1971) no difference had been found between the total protein P content of the brain of rats killed by rapid freezing and that of rats killed by conventional methods. In the present work, provided that Cu²⁺ was included in the homogenization medium, killing by immersion in liquid N₂ resulted in higher values for the phosphoprotein content of the membrane fraction than did killing by decapitation, although the difference was not statistically significant (Table 2). Confirming the efficacy of Cu²⁺ in stabilizing protein P in the membrane, the values for quick-frozen animals were lower in the absence of Cu²⁺ from the medium (Table 2). It therefore seems reasonable to conclude that the values obtained from animals killed with liquid N₂ where the brain had been homogenized in the sucrose medium containing Cu²⁺ approximate to the true value *in vivo*. To determine whether this represents a state of full or partial phosphorylation of the membrane protein, homogenates were incubated with ATP for 30min before addition of Cu²⁺ and isolation of the membrane fraction for determination of protein P content. In three experiments this procedure resulted in a clear increase in the alkali-labile P in the membrane compared with that in membranes isolated in sucrose medium containing

Table 1. Effect of Cu²⁺ on the loss of ³²P from labelled membrane proteins in brain homogenates

A membrane preparation was labelled at a concentration of 0.5mg of protein/ml by incubation for 1h at 20°C in a medium containing 1mM-[³²P]ATP, 1mM-MgSO₄ and 30mM-Tris-HCl buffer, pH7.4. The membranes were then spun down at 1.25 $\times 10^4$ g for 15min and the pellet was resuspended in water at a concentration of 3-4mg of protein/ml. Samples (2ml) of the suspension were incubated at 20°C with 5ml portions of a freshly prepared homogenate of rat forebrain (12.5%, w/v, in 0.3M-sucrose) in the presence and in the absence of 1mM-CuSO₄. Radioactivity in protein-bound phosphoserine was determined in 0.5ml portions of the mixture by the procedure of Weller & Rodnight (1971), except that it was necessary to carry out electrophoresis for 3h to ensure separation of free phosphoserine because of the high protein content of the sample. Mean values are given $\pm$ S.E.M. with the numbers of observations in parentheses.

Incubation time (min)	Cu ²⁺	³² P present in protein-bound phosphoserine (% of amount present at zero time)
30	Absent	80, 78
60	Absent	60.6 $\pm$ 3.0 (6)
30	Present	100, 100
60	Present	99.2 $\pm$ 0.7 (6)

Table 2. Concentration of alkali-labile P in membrane preparations from rat forebrain prepared under various conditions

In each experiment membrane preparations were made from the pooled homogenates of three or four rat forebrains. Alkali-labile P was determined in each case in triplicate. Homogenates were phosphorylated as described in the text. Mean values are given $\pm$ S.E.M. with the numbers of observations in parentheses. Values that are significantly different from those in the first line in each series are indicated: * $P < 0.001$; † $P < 0.1$; ‡ $P < 0.025$.

Experimental series	Method of killing	Cu ²⁺ in homogenate	Special treatment of homogenate	Alkali-labile P (nmol/mg of membrane protein)
1	Liquid N ₂	Present	None	11.8 $\pm$ 0.6 (12)
1	Liquid N ₂	Absent	None	7.1 $\pm$ 0.7 (6)*
1	Decapitation	Present	None	10.0 $\pm$ 0.9 (9)†
2	Liquid N ₂	Present	None	11.6 $\pm$ 0.6 (12)
2	Liquid N ₂	Absent	Phosphorylated with ATP	13.9 $\pm$ 0.8 (9)‡

Cu²⁺ but that had not been incubated with ATP; the mean value for all three experiments is given in Table 2.

It therefore appears that the membrane protein in rat brain is normally only partially phosphorylated. The extent of the dephosphorylation can be seen from Table 2 to amount to about 12%. The data refer, however, to the total phosphoproteins in the membrane, and, as these are probably a heterogeneous group of proteins, it is possible that in a metabolically active fraction the number of vacant sites able to accept phosphate constitutes a higher percentage of the total available.

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