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CHEMICAL PHOSPHORYLATION OF PROTEINS BY ZINC-ATP

Bruce W. Jarvis, Laurie L. Chinander, Jacque R. Wettlaufer, and David A. Bernlohr

Department of Biochemistry, University of Minnesota,
Gortner Laboratory, St. Paul, Minnesota 55108

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During an examination of *in vitro* phosphorylation of the adipocyte lipid-binding protein (ALBP) by the insulin receptor, we detected insulin receptor-independent, chemical phosphorylation of ALBP. This activity was present in ALBP purified to homogeneity from murine 3T3-L1 cells and in recombinant murine ALBP purified from expressing *E. coli* cultures. Phosphoamino acid analysis revealed that chemical phosphorylation of ALBP occurred primarily on Ser residues. The phosphorylation activity occurred in the alkaline pH range from 8 to 11 and exhibited a broad temperature dependence. The reaction rate was linearly dependent upon the ATP concentration and exhibited a biphasic kinetic profile. Eight of twelve other proteins tested also underwent chemical phosphorylation. Zn^{+2}, Mg^{+2}, or Mn^{+2} promoted optimal phosphorylation of different proteins. We conclude that many proteins are capable of undergoing chemical phosphorylation.

Murine ALBP (also referred to as p422 (3) or aP2 (11)) is a 15 kDa polypeptide expressed in adipose tissue and cultured adipogenic cell lines (2, 3, 4, 19). This protein is thought to be involved in the intracellular solubilization and trafficking of fatty acids. We have studied the phosphorylation of the murine adipocyte lipid-binding protein by the insulin receptor. Recently, Hresko *et al.* (10) and Bernier *et al.* (1) reported that in cultured 3T3-L1 cells ALBP was phosphorylated on tyrosine[19] in response to insulin.

In order to examine the effect that tyrosine[19] phosphorylation of ALBP has upon fatty acid - protein interactions, we have isolated the 3T3-L1 adipocyte insulin receptor and characterized the *in vitro* phosphorylation of ALBP[1]. During the course of this study we detected a low level of insulin receptor-independent phosphorylation of ALBP occurring in the presence of [γ-^{32}P]ATP and divalent cations. In an attempt to understand the functional significance of insulin receptor-independent phosphorylation, we performed "control" phosphorylation reactions with several other test substrates. Surprisingly, of the twelve additional proteins examined for receptor-independent phosphorylation, eight exhibited zinc-ATP dependent phosphorylation. As *in vitro* observations are frequently equated with *in vivo* function, we report here the characterization of an *in vitro* chemical phosphorylation system and discuss the implications of these findings for the correlation of *in vitro* phosphorylation events with *in vivo* function.

[1] Chinander, L. L. *et al.*, Manuscript in preparation.

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Materials and Methods

Reagents

[γ^{32}-P]ATP (3,000 Ci/mmol) and [γ^{32}-P]GTP (>10 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, IL. Spectrapor dialysis tubing with 6,000 to 8,000 molecular mass cut-off was purchased from Spectrum Medical Industries, Los Angeles, CA. Phosphoamino acid standards and Sephadex G-75 were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade. All water used was distilled and deionized.

Protein Sources

Murine ALBP was purified from 3T3-L1 adipocytes by the methods of Matarese and Bernlohr (15). The recombinant ALBP (rALBP) was purified from expressing bacterial cultures by the methods of Chinander and Bernlohr[2].

Bovine serum albumin (BSA), glutamate dehydrogenase, glycerokinase, lysozyme, and myoglobin were obtained from Sigma. Alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Liver fatty acid binding protein (LFABP) was a gift from M. Dempsey, University of Minnesota. Cellular retinol binding protein (CRBP), cellular retinol binding protein type IIa (CRBPIIa), CRBPII with a blocked amino terminus (CRBPIIb), and cellular retinoic acid binding protein (CRABP) were gifts from D. Ong, Vanderbilt University, Nashville, TN. Intestinal fatty acid binding protein (IFABP) was a gift from J. Gordon, Washington University, St. Louis, MO. Myelin P2 was a gift from M. Weise, Medical College of South Carolina, Charleston, SC.

Phosphorylation Conditions

The standard phosphorylation conditions used 50 mM Tris (Tris(hydroxymethyl)amino methane) pH 8.5, 10% glycerol, 10 μM protein, and 100 μM ATP. Cations, pH, and ionic strength were as described in the text. The usual incubation was at 37° C for 2 hours unless otherwise stated. Phosphorylation reactions were initiated by the addition of ATP and were stopped by addition of an equal volume of sodium dodecylsulfate (SDS) sample buffer (13).

Electrophoresis

After the phosphorylation reactions were completed, phosphoproteins were separated by electrophoresis on 5 to 15% gradient gels (13) and visualized by staining with silver (16). Autoradiography was performed at -70° C with Kodak XOMAT AR5 film with a DuPont Cronex Lightning Plus intensifying screen. Intensity of exposure was quantitated with a Hoefer G-300 scanning densitometer.

Phosphoamino Acid Analysis

The procedure of Lampe *et al.* (14) for chromatographic separation of the phosphoamino acids was used. Phosphorylated protein samples were separated by electrophoresis as discussed above and excised from a region of the gel corresponding to ALBP, as determined using prestained protein markers (Bio-Rad, Richmond, CA). The excised gel was minced, placed into an Elutrap (Schleicher and Schuell, Keene, NH), and the protein was electro-eluted at 200 V for 1.5 hours at 4° C into SDS electrode buffer (25 mM Tris, 192 mM glycine pH 8.3, 0.1% SDS) (13). The eluted protein was dialyzed overnight against 1 liter of water at room temperature, lyophilized, and then hydrolyzed *in vacuo* with 100 μl of 6 N HCl (constant boiling; Bio-Rad) for 1 hour at 100° C. The sample was dried in a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY) for 2 hours, redissolved in a solution containing the phosphoamino acid standards, and separated by paper electrophoresis at 750 V for 1 hour in a pH 3.5 buffer consisting of water: acetic acid: pyridine in the ratio 945: 50: 5 (14). The phosphoamino acids were visualized with ninhydrin and subsequent heating. The radiolabeled spots were identified by autoradiography.

Results

During our investigation of the insulin receptor-dependent phosphorylation of the murine 3T3-L1 adipocyte lipid binding protein (ALBP) we performed control phosphorylations lacking the insulin receptor. We routinely noted that a small amount of radioactive phosphate was covalently or tightly associated with the murine ALBP (Figure 1, lanes 3 and 5). An identical result was obtained when recombinant ALBP (rALBP) was used as the substrate (Figure 1, lanes 2 and 4).

[2] Chinander, L. L. and Bernlohr, D. A., Manuscript submitted.

This suggested that a second kinase did not contaminate our preparations, but rather that a chemical phosphorylation was occurring. Therefore, we chose to characterize this chemical phosphorylation using a standard buffer consisting of 25 mM Tris-HCl at the indicated pH value, divalent cation, ATP, protein, and 10% glycerol (9).

The pH profile of the chemical phosphorylation of rALBP is shown in Figure 2. A markedly greater rate of phosphorylation was exhibited as the pH was increased from 7.5 to 9.0. Then the rate decreased as the pH was increased further to 11.0. It was not clear if the decrease in activity at pH 11.0 was due to alkaline denaturation of the protein or to base catalyzed hydrolysis of the ATP. To avoid these difficulties in interpretation, a standard pH of 8.5 in Tris-HCl containing buffers was adopted.

In order to assess the relative effectiveness of cations in promoting phosphorylation, several metal ions were examined. Figure 3 demonstrates that Zn^{+2} was clearly the most effective cation for phosphorylation of rALBP. Zn^{+2} at 10 mM was more effective than at 1 mM. This suggests that with 100 μM ATP, Zn^{+2} was acting not only to coordinate ATP but also to catalyze the reaction. Mg^{+2}, Ca^{+2}, K^{+}, and Na^{+} were moderately effective in promoting phosphorylation, but high levels (10 mM) of these cations were rarely as effective as 100 μM Zn^{+2}. Also, Mn^{+2} at 10 mM promoted 30% of the activity observed with Zn^{+2} at 10 mM.

With Tris-HCl pH 8.5, 10 mM $ZnCl_2$, and 100 μM ATP we determined the time course of the reaction. PhosphoALBP production as a function of time was biphasic (Figure 4). An initial rapid phase (0 to 60 sec) was followed by a slower, linear phase that proceeded for several hours. During this slower phase the velocity of phosphorylation in the presence of 100 μM ATP was 0.2

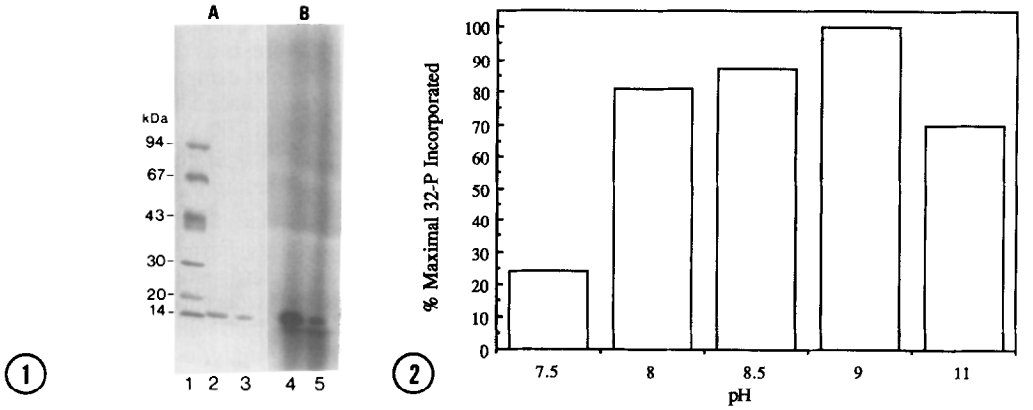
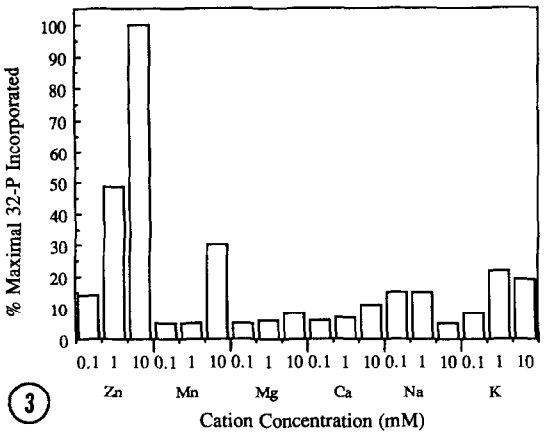


Figure 1. Phosphorylation of 3T3-L1 ALBP and rALBP. Three μg of either homogeneous 3T3-L1 ALBP or of rALBP were incubated in 25 mM Tris-HCl pH 8.5 with 10 mM $ZnCl_2$ and 100 μM [γ^{32}-P]ATP for two hours at 23° C. The samples were subjected to electrophoresis in a linear gradient of 5 to 15% polyacrylamide. Panel A represents the silver-stained gel, while panel B is the corresponding autoradiogram. 3T3-L1 ALBP is shown in lanes 3 and 5. The rALBP is in lanes 2 and 4. On the autoradiogram below each band corresponding to ALBP is an apparent band arising from free [γ-^{32}P]ATP migrating at the dye front.

Figure 2. Phosphorylation of rALBP as a function of pH. The rALBP (10 μM) was dialyzed into 50 mM Tris-HCl at the specified pH and then was phosphorylated for 2 hours at 23° C in the presence of 10 mM $ZnCl_2$. The samples were analyzed by SDS-PAGE and autoradiography. The extent of phosphorylation at each pH was quantitated by densitometric scanning of the autoradiogram using a Hoefer G-300 scanning densitometer.



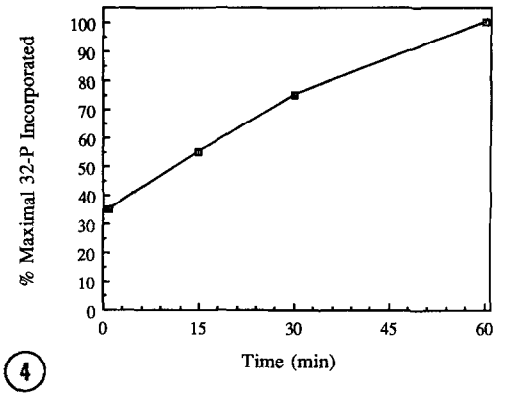


Figure 3. Divalent metal dependence of phosphorylation. The rALBP (10 μM) was incubated at pH 8.5 with 100 μM [γ-^{32}P]ATP at 37° C in the presence of the indicated cation for 2 hours. The extent of phosphorylation was assessed by SDS-PAGE, autoradiography, and densitometry as described above.

Figure 4. Time course of rALBP phosphorylation. The rALBP (10 μM) was incubated at 23° C and pH 8.5 with 100 μM [γ^{32}-P]ATP and 10 mM $ZnCl_2$. At the indicated times a reaction was stopped by adding Laemmli sample buffer (13) containing 1% SDS. The extent of phosphorylation was determined by densitometric analysis of the autoradiogram following SDS-PAGE.

pmol min^{-1} mg^{-1} of ALBP. The reaction exhibited a broad temperature optimum between 37° and 90° C (Figure 5) and was specific for ATP. GTP did not support the activity (result not shown).

We considered the possibility that the phosphorylation we observed utilizing SDS polyacrylamide gel electrophoresis (PAGE) represented a tightly but noncovalently bound phosphate. To address the chemical nature of the phosphate - ALBP interaction, we performed phosphoamino acid analysis. As shown in Figure 6, following electroelution and partial acid hydrolysis we detected phosphoserine as the predominant phosphoamino acid. A small amount of phosphotyrosine was also observed. However, the amount of $^{32}PO_4$ migrating as phosphotyrosine was always less than 10% of the phosphoserine intensity. Incubation of *in vitro*

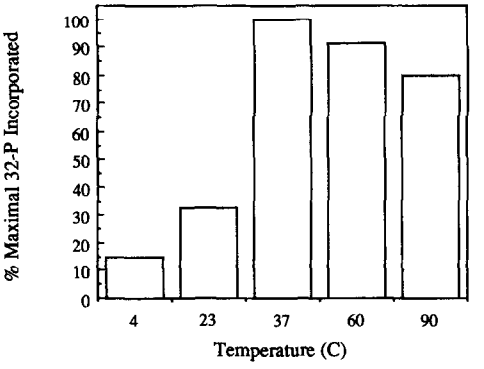


Figure 5. Temperature dependence of rALBP phosphorylation. The rALBP (5 μM) was incubated at pH 8.2 in 63 mM HEPES, 100 μM [γ-^{32}P]ATP, and 10 mM $ZnCl_2$ at the indicated temperature for 2 hours. The extent of phosphorylation was determined by densitometric analysis of autoradiography of SDS polyacrylamide gels.

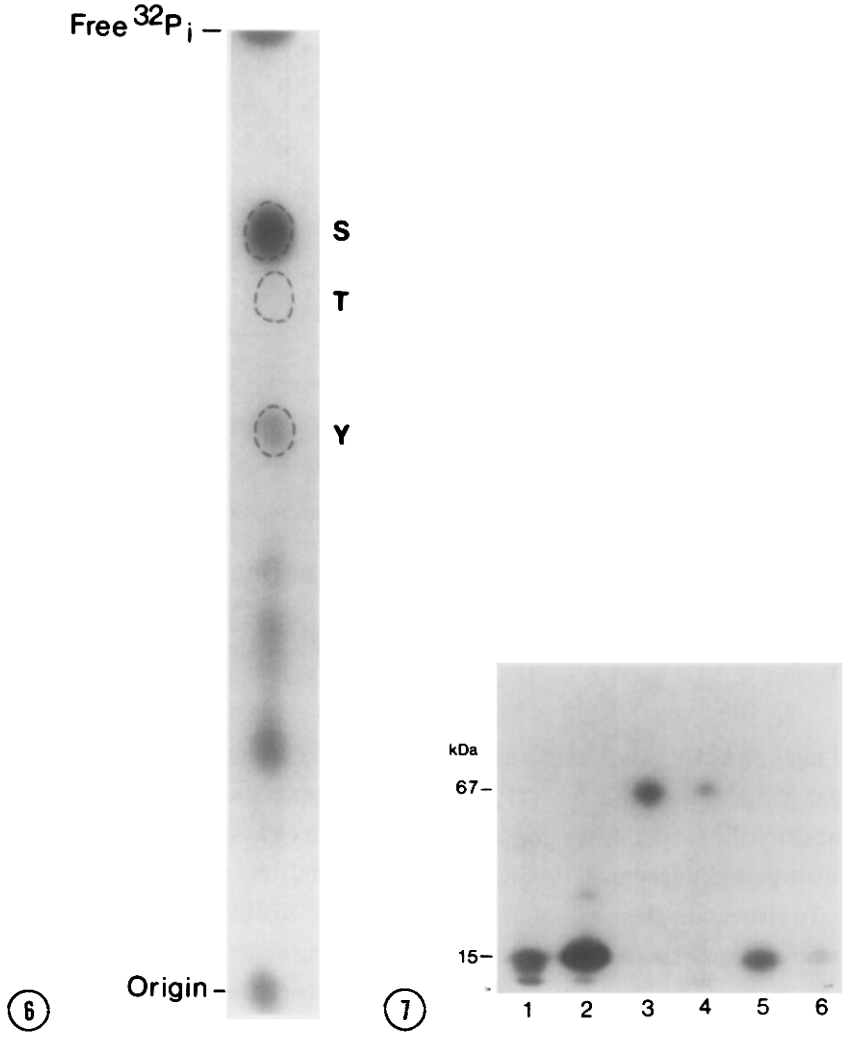


Figure 6. Phosphoamino acid analysis of rALBP. The rALBP was phosphorylated in the presence of 10 mM $ZnCl_2$ and utilizing our standard reaction conditions, eluted from an SDS gel, subjected to partial acid hydrolysis, separated by high voltage paper electrophoresis, and exposed to autoradiography. The autoradiogram is superimposed upon circles indicating the positions of phosphoamino acid standards on the corresponding electrophoretogram. Y is phosphotyrosine; T is phosphothreonine; S is phosphoserine.

Figure 7. Phosphorylation of lysozyme, albumin, and rALBP. Three μg of each protein were incubated at pH 8.5 for 10 hours at 37° C with the indicated cation and then subjected to SDS-PAGE and autoradiographic analysis. Lysozyme (lanes 1, 2), albumin (lanes 3, 4), and rALBP (lanes 5, 6). Lanes 1, 3, and 5 represent samples containing 10 mM $ZnCl_2$, while lanes 2, 4, and 6 represent samples containing 10 mM $MgCl_2$. The commercial preparation of lysozyme used in lanes 1 and 2 contained impurities, some of which were also phosphorylation substrates.

phosphorylated rALBP with calf intestine alkaline phosphatase (pH 8.5 for 2 hours at 37° C) released phosphate from rALBP, as evaluated by SDS-PAGE and autoradiography (results not shown). This suggested that the serine phosphate was a monoester and not a diester (6).

Several other proteins were also phosphorylated in the presence of Zn^{+2}-ATP. Figure 7 demonstrates that lysozyme and albumin were also substrates for *in vitro* chemical phosphorylation. When the metal dependence of lysozyme phosphorylation was tested, enhanced

Table 1. Proteins Tested for Chemical Phosphorylation[1]

Protein	Phosphorylation	Metal Utilized
ALBP	+	Zn>Mn>Mg
BSA	+	Zn>>>Mg
CRBP	+	Zn
CRBPIIa	+	Zn
CRBPIIb	+	Zn
Glutamate dehydrogenase	+	Zn
LFABP	+	Zn≥Mn>>Mg
Lysozyme	+	Mg>Zn
Myoglobin	+	Zn
CRABP	-	ND[2]
Glycerokinase	-	ND
IFABP	-	ND
Myelin P2 (bovine)	-	ND

[1] 3 μg of each protein were incubated for 4 hours at 37° C at pH 8.5 with 10 mM cation.
[2] ND indicates no phosphorylation detected with 10 mM Zn^{+2}.

activity was detected with 10 mM $MgCl_2$ in contrast to 10 mM $ZnCl_2$. However, albumin demonstrated a specificity for $ZnCl_2$. Table 1 shows that of the 13 proteins tested, nine were substrates. However, intestinal fatty acid binding protein, bovine myelin P2 protein, cellular retinoic acid binding protein, and glycerokinase were not substrates under the conditions tested. Interestingly, intestinal fatty acid binding protein, bovine myelin P2 protein, cellular retinoic acid binding protein, and ALBP are members of a multigene family of ligand binding proteins (5, 18). ALBP and myelin P2 protein share nearly 70% sequence identity, especially in the hydrophobic regions (2), and yet exhibited vastly different phosphorylation activities.

In order to verify that the phosphorylation was independent of the polyacrylamide gel assay, we performed phosphorylation reactions using albumin and separated the [γ-^{32}P]ATP from the albumin by means of Sephadex G-75 chromatography. The albumin protein peak coincided exactly with the early eluting peak of radioactivity indicating that by both column chromatography and SDS-PAGE identical results were obtained (results not shown).

Discussion

The ability to demonstrate protein phosphorylation *in vitro* is widespread. Reports of autophosphorylation include, but are not limited to, casein kinase II from eucaryotes (7) and CheA from *E. coli* (9). Common reaction conditions include the presence of a divalent cation, such as Mg^{+2}, Mn^{+2}, or Zn^{+2}, a neutral to basic pH value, and a nucleotide such as ATP or GTP. The cation, pH, and nucleotide specificity for optimal phosphorylation depend upon the individual protein.

We have observed chemical phosphorylation for nine of thirteen proteins tested in the presence of 10 mM Zn^{+2}. When 10 mM Mn^{+2} was included with LFABP, similar phosphorylation levels were observed. For another protein, lysozyme, 10 mM Mg^{+2} was optimal. These results point out that although Zn^{+2} was generally the most effective divalent cation, other metals will suffice for specific proteins.

It is noteworthy that we have found a correlation between the ability of a protein to undergo zinc-dependent phosphorylation and zinc-induced protein precipitation. The rALBP precipitated in the presence of 10 mM $ZnCl_2$, but not 10 mM $MgCl_2$ or $MnCl_2$, when incubated at pH 8.5 for two hours or more. After centrifugation at 400,000 X g for 15 minutes, the ALBP was found only in the pellet fraction. The insolubility of rALBP in the presence of Zn^{+2} was not dependent upon ATP. The precipitation and concurrent phosphorylation suggest that the process is not enzymatic but chemical.

In the presence of an appropriate divalent cation, many proteins undergo chemical phosphorylation. Hence, by relying on autoradiography, one could mistakenly attribute Mg^{+2}- or Mn^{+2}-dependent phosphorylation to a kinase rather than to a chemical process. Thus, it is important to determine the properties of phosphorylation to rule out a chemical process. It has long been known that BSA undergoes chemical phosphorylation in the presence of high pH, sodium phosphate, $POCl_3$, and CCl_4 (8). Similarly, the occurrence of phosphorylation of rALBP at alkaline pH values and at high temperature (90° C) argues for a nonenzymatic, chemical phosphorylation process. Schieven and Martin (17) reported the "nonenzymatic auto-phosphorylation" of acid-denatured enolase at 80° C in the presence of manganese. They obtained primarily phosphoserine and some phosphotyrosine after acid hydrolysis of the phosphoprotein. Their reaction proceeded well even in the presence of 2% SDS. Our results differ in four respects from those of Schieven and Martin (17). Firstly, our substrates did not need to be denatured prior to phosphorylation. Secondly, all phosphorylations proceeded well at 37° C; elevated temperatures were not necessary. Thirdly, heating to 90° C in 1% SDS blocked the phosphorylation of rALBP, lysozyme, and BSA, unlike their findings with enolase. Fourthly, their rate of nonenzymatic autophosphorylation of enolase at 80° C (17) was ten times the rate of chemical phosphorylation we obtained at 37° C for ALBP.

Our results indicate that one must exercise caution when extrapolating from *in vitro* phosphorylation events to *in vivo* function. In the presence of neutral to high pH, a divalent cation, and a nucleotide, many proteins may undergo chemical phosphorylation. When identifying proteins as kinase substrates, it is imperative to quantitate by radioactive detection the rate of phosphorylation. The easier method of identification of "phosphospots" on autoradiograms of SDS gels is not sufficient evidence for an enzymatic process. For example, the rate of chemical phosphorylation was 0.2 pmol min^{-1} mg^{-1} of ALBP which is 1% of the rate (20 pmol min^{-1} mg^{-1} of receptor) obtained by Kohanski and Lane (12) for autophosphorylation of the insulin receptor. An experimental distinction can be made between chemical and enzymatic phosphorylation. The chemical phosphorylation we have described here proceeds at high cation concentration (1 to 10 mM), elevated pH (pH 9 to 11), and at high temperature (60° to 90° C), while enzymatic phosphorylations typically do not.

Protein kinases affect the rate and mechanism of substrate phosphorylation. Therefore, protein kinase substrates are capable of undergoing nonenzymatic, chemical phosphorylations, albeit under widely different conditions. Accordingly, the chemical phosphorylation we have noted here for many proteins may in fact have an enzymatically relevant counterpart. We emphasize that the conditions for our reported chemical phosphorylation system are not drastically different from standard protocols for enzymatic phosphorylation. Erroneous conclusions may be drawn both

about the specificity of a protein kinase for a particular substrate and, by implication, about the metabolic significance of the kinase, if one interprets a chemical phosphorylation as an enzymatic one. Hence, we would urge caution regarding the significance of an *in vitro* phosphorylation for a particular protein without careful analysis of the conditions for such a phosphorylation. This caution is critical when correlating observations *in vitro* with presumed function *in vivo* .

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References

1. Bernier, M., Laird, D. M., and Lane, M. D. (1988) *J. Biol. Chem.* **263**, 13626-13634.
2. Bernlohr, D. A., Angus, C. W., Lane, M. D., Bolanowski, M. A., and Kelly, T. J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5468-5472.
3. Bernlohr, D. A., Bolanowski, M. A., Kelly, T. J., and Lane, M. D. (1985) *J. Biol. Chem.* **260**, 5563-5567.
4. Bernlohr, D. A., Doering, T. L., Kelly, T. J., and Lane, M. D. (1985) *Biochem. Biophys. Res. Commun.* **132**, 850-855.
5. Chytil, F. and Ong, D. E. (1987) *Ann. Rev. Nutr.* **7**, 321-335.
6. Cozzone, A. J. (1988) *Ann. Rev. Microbiol.* **42**, 97-125.
7. Hathaway, G. M. and Traugh, J. A. (1983) *in* Methods in Enzymology (Corbin, J. D and Hardman, J. G., Eds.), Vol. 99, pp. 317-331.
8. Heidelberger, M., Davis, B., and Treffers, H. P. (1941) *J. Amer. Chem. Soc.* **63**, 498-503.
9. Hess, J. F., Oosawa, K., Kaplan, N., and Simon, M. I. (1988) *Cell* **53**, 79-87.
10. Hresko, R. C., Bernier, M., Hoffman, R. D., Flores-Riveros, J. R., Liao, K., Laird, D. M., and Lane, M. D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8835-8839.
11. Hunt, C. R., Ro, J. H.-S., Dobson, D. E., Min, H. Y., and Spiegelman, B. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3786-3790.
12. Kohanski, R. A. and Lane, M. D. (1985) *J. Biol. Chem.* **260**, 5014-5025.
13. Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685.
14. Lampe, P. D., Bazzi, M. D., Nelsestuen, G. L., and Johnson, R. G. (1986) *Eur. J. Biochem.* **156**,351-357.
15. Matarese, V. and Bernlohr, D. A. (1988) *J. Biol. Chem.* **263**, 14544-14551.
16. Morrissey, J. H. (1981) *Analyt. Biochem.* **117**, 307-310.
17. Schieven, G. and Martin, G. S. (1988) *J. Biol. Chem.* **263**, 15590-15593.
18. Sweetser, D. A., Heuckeroth, R. O. and Gordon, J. I. (1987) *Ann. Rev. Nutr.* **7**, 337-359.
19. Zezulak, K. H. and Green, H. (1985) *Mol. Cell Biol.* **5**, 419-421.