Biochemical and Biophysical Research Communications 430 (2013) 865–867

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mini Review

Cellular regulation by protein phosphorylation

Edmond H. Fischer *

Department of Biochemistry, University of Washington, Seattle, USA

ARTICLE INFO

Article history:
Available online 9 October 2012

ABSTRACT

A historical account of the discovery of reversible protein phosphorylation is presented. This process was uncovered in the mid 1950s in a study undertaken with Edwin G. Krebs to elucidate the complex hormonal regulation of skeletal muscle glycogen phosphorylase. Contrary to the known activation of this enzyme by AMP which serves as an allosteric effector, its hormonal regulation results from a phosphorylation of the protein by phosphorylase kinase following the activation of the latter by Ca^{2+} and ATP. The study led to the establishment of the first hormonal cascade of successive enzymatic reactions, kinases acting on kinases, initiated by cAMP discovered by Earl Sutherland. It also showed how two different physiological processes, carbohydrate metabolism and muscle contraction, could be regulated in concert.

This minireview provides a brief historical account of how we got started with my close friend and colleague, the late Edwin Krebs, on our studies of glycogen phosphorylase nearly 60 years ago [1], studies that led to the discovery of reversible protein phosphorylation as a regulatory mechanism. Ed had been a post-doc with the Coris in St. Louis where he worked on muscle phosphorylase. I had obtained my Ph.D. degree with Kurt Meyer at the University of Geneva, and, among other things, we had isolated phosphorylase from potato. So having worked in the same area, we had much to speak about and, naturally, we spoke a lot about phosphorylase and it's possible mode of regulation. It must be remembered that in those days, 55 years ago, one knew essentially nothing about the mechanism of enzyme regulation, and terms such as Signaling or Signal Transduction that are so commonly used today would not have been understood.

We were particularly intrigued by a problem that had not been solved regarding phosphorylase, that is, the role adenylic acid (5′AMP) played in its activation. Indeed, when glycogen phosphorylase was discovered in the mid 1930s by Parnas in Poland [2] and Carl and Gerty Cori [3] in the US, the muscle enzyme was shown to have an absolute requirement of AMP for activity, and the Coris assumed that it served the role of a coenzyme. But in 1943, Arda Green in Cori's lab crystallized phosphorylase in a new form that was fully active without added AMP [4]. They called this new form phosphorylase a and, very logically, assumed that it contained AMP covalently bound as a prosthetic group. They further thought that it had to be the native form of the enzyme because, when crude muscle extracts were left standing, it was rapidly converted to the old species that required AMP, which they now called phosphorylase b. It was a logical hypothesis but, if correct, AMP would have to have been released during the reaction and they found none. Furthermore, no AMP could be detected in the "native" enzyme, using the most sensitive microbiological assays available at that time. So the Coris knew that the enzyme existed in two forms but did not know how these two forms differed and, strange as it might seem today, they actually dropped the problem.

As a post-doc of the Coris, Ed assumed, like they did and like most people did, that AMP played the role of some sort of coenzyme or prosthetic group. On the other hand, the potato phosphorylase we had isolated in Geneva showed no requirement whatsoever for AMP. Even though biochemistry was still in its infancy in those days, it had been well established that coenzymes were conserved throughout species, so it seemed unlikely that AMP would serve as a coenzyme for muscle phosphorylase but not for the potato enzyme (though, admittedly, potatoes are pretty dumb when compared to muscles). So, we decided to take a crack at this problem. As it turned out, we never solved the AMP problem either. For that, we had to wait about 7 years for Monod et al. [5] to come up with their brilliant allosteric model of enzyme regulation. But we soon found out that, in reality, the activation of phosphorylase involved a totally different kind of mechanism.

We cleared a bench in the corner of a lab and got started, just the two of us, working side by side, much more as two close friends than as two colleagues in a department. The Coris had assumed that phosphorylase a was the native form of the enzyme in muscle because its purification from fresh muscle extracts yielded the active enzyme. Their purification procedure was archaic. They extracted ground rabbit muscle with water then squeezed the gunk through cheesecloth. To clarify the very turbid crude extract obtained, they passed it through a battery of filter papers. In their defense, it should be said that no good centrifuge existed at that

* Fax: +1 206 616 4799.
E-mail address: efischer@u.washington.edu

0006-291X/$ - see front matter © 2012 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2012.10.024

time. On the other hand, when we started our studies in the early 1950s, the high capacity refrigerated Sorval centrifuge had become available, which enabled us to replaced the very cumbersome filtration step by a centrifugation. But by doing so, we could never obtain the active enzyme, only the so-called ''degraded'' inactive b form. Until, in desperation, we felt that we had to go back to Cori's procedure, following it to the letter, paper filtration and all, checking every step, analyzing every fraction from beginning to end. And, to our total amazement, we found that the very first crude muscle extract did not contain active phosphorylase a as we had expected but the inactive b form. Yet, when the same extract was passed through filter paper, the solution that emerged was active. As if some kind of conversion of phosphorylase b to a had taken place during the filtration process.

To speak of a let down would be to put it mildly. Indeed, of all the marvelously sophisticated mechanisms to activate an enzyme one could have dreamed of (perhaps we could discover a new factor, a new coenzyme or vitamin), filtration through paper was really the pits. In fact, the notion that the native form of the enzyme in muscle was active phosphorylase a was so entrenched, seemed so logical and was so universally accepted, that we were convinced that the inactive enzyme we were dealing with was not real phosphorylase b. We thought we might be dealing with some kind of inhibited form of phosphorylase a; perhaps a large complex with other cellular components that masked its activity and which, somehow, could be disrupted or removed by filtration through paper. To such an extent that in our lab books, we referred to this material as ''false phosphorylase b''.

But this was not the case. We soon found out that it was not the filtration *per se* that caused its activation, but contamination of the filter papers by trace amounts of calcium ions. Enough was picked during these multiple filtrations, to bring about the conversion. If the filter papers were pre-washed with dilute acid, no conversion occurred. What clenched the problem is when we ashed 20 filter papers in a muffle furnace, and simply sprinkled the ahes in the extract rather than filtering it. Then we rapidly found that calcium did not work alone: Mg-ATP always present in fresh muscle extracts was also required.

This then strongly suggested that we were dealing with a phosphorylation reaction but we did not know what had become phosphorylated because, in those days, there was no radioactive ATP^{32}. You had to synthesize the material yourself. But we knew Art Kornberg had prepared some that he needed for his beautiful studies on the biosynthesis of DNA. So we called him and he immediately sent us a sample of γ-labeled ATP^{32} with which we could demonstrate that the radioactive P^{32} had been incorporated into a protein fraction we could isolate and which turned out to be phosphorylase. Only then could we proposed that for the conversion of phosphorylase b to a, inactive phosphorylase b would be phosphorylated and converted into active phosphorylase a in a reaction that required Ca^{2+}, Mg^{2+} and ATP and an enzyme which we called ''phosphorylase kinase''. Obviously, the reverse reaction had to be catalyzed by a ''phosphorylase phosphatase''.

While that reaction would be viewed today as a most ordinary if not trivial reaction came nevertheless as an enormous surprise because, in those days, one knew essentially nothing about phosphoproteins. Only two had been identified: casein from milk and ovovitellin from egg yolk, and their only function was thought to be for the feeding of the young. So that nobody could have imagined that the phosphorylation of an enzyme could be implicated in its regulation. However, the reaction we had found rapidly turned out to be more complicated than just indicated because while calcium was absolutely required for the conversion of phosphorylase b to a in crude muscle extract, it was not involved in that reaction *per se*. It turned out to be required for the activation of phosphorylase kinase which, just like phosphorylase, was found to also exist in an inactive and active form. So that, at this point, we knew that we were dealing with a cascade of two successive enzymatic reactions, two enzyme acting on one another, both activated through a phosphorylation process to bring about the degradation of glycogen.

During that same period of time, Earl Sutherland's laboratory with Ted Rall and Wally Wosilait [6] had obtained good evidence that the regulation of liver phosphorylase they were working with also involved a phosphorylation–dephosphorylation reaction. But an epochal finding that grew out of these studies was the discovery by Earl and Ted Rall of cAMP [7] which, by an unknown mechanism, shifted the balance between the two species of phosphorylase toward the active enzyme. Earl and Ted Rall immediately supplied us with a sample of cAMP with which we could determine that its action was directed towards the activation of phosphorylase kinase, either by accelerating the rate of its autophosphorylation or by allowing yet another enzyme (it had to be another protein kinase) to do so. We called it at first a ''kinase kinase'' for want of a better term, in spite of the objections of one reviewer of our submitted paper who first doubted that yet another enzyme might be involved. He wrote ''when will it stop'' but added ''if indeed another enzyme were involved, surely the authors could chose a better term than a ''kinase kinase''. Anyway, this latter hypothesis was confirmed 4–5 years later by the isolation of the cAMP-dependent protein kinase by Don Walsh and Ed Krebs. And since, by that time, Earl Sutherland and his group had demonstrated the production of cAMP by the adenylate cyclase system at the membrane level, the entire cascade for the phosphorolysis of glycogen was established.

The involvement of calcium in the regulation of carbohydrate metabolism was totally unexpected. The physiologists had known for many years, of course, that Ca^{2+} released in response to a nerve impulse could trigger muscle contraction but nothing was known about its possible involvement in carbohydrate metabolism. To show that it might really be so, that these two processes might be physiologically linked, we had to show that they occurred at the same concentration of calcium. In collaboration with Glenn Kerrick from our Dept. of Physiology [8], who used single skeletal muscle fibers stripped of their membrane and attached to a tension transducer, we could show that tension developed as a function of increasing Ca^{2+} concentration followed almost exactly the activation of phosphorylase kinase.

Just like contraction, activation of phosphorylase by phosphorylase kinase in the presence of Ca^{2+}, Mg^{2+} and ATP was instantaneously blocked by addition of purified sarcoplasmic reticulum, and resumed by re-addition of excess calcium. A few years earlier, Setsuro Ebashi and Fritz Lipman in New York [9], and Hasselbach and Makinose in Heidelberg [10] had shown that the SR could serve as a Ca^{2+} pump. The finding that the same concentrations of Ca^{2+} that triggered muscle contraction could also trigger glycogenolysis ultimately leading to the production of ATP needed to maintain contraction demonstrated for the first time how these two different physiological processes could be coupled and regulated in concert.

At the beginning, of course, we did not know whether this reversible phosphorylation reaction was a unique occurrence restricted to the control of these two enzymes only. Would the enzymes of nitrogen metabolism be regulated by amidation/deamidation, or lipid metabolism by acetylation/deacetylation? As luck would have it, reversible protein phosphorylation turned out to be one of the most prevalent mechanisms by which cellular events are regulated, being implicated in gene transcription/translation, the immune response, cell development and differentiation, oncogenic transformation, transport, programmed cell death, etc. In fact, it would be difficult to find a physiological event that would not be regulated, directly or indirectly, by this kind of reaction. It is

implicated in innumerable hereditary diseases or pathological conditions including diabetes, Alzheimer's and Parkinson's disease, myelogenous leukemia; in viral diseases such as small pox; bacterial diseases such as cholera and plague; and very prominently in cancer.

Coming back to the activation of phosphorylase by AMP mentioned earlier, it became eventually evident from the work of Monod at the Pasteur and that of others that it was a classical allosteric reaction. But one might wonder why organisms found it necessary to develop two separate mechanisms to regulate the activity of their enzymes, i.e. by Allosteric and Covalent modification when, in both instances, these led to the same result, namely, an active conformation? According to the 1965 allosteric model of Monod, Wyman and Changeux [11], the enzyme undergoes a change in conformation in response to effectors that are generated during the normal maintenance of the cell and reflect mostly its overall internal condition: whether it is proliferating or quiescent, actively metabolizing or not, its energy balance (i.e., the ratio of AMP to ADP + ATP), its redox state and so forth. According to the rule that most enzymes are subjected to end-product or feed-back inhibition, phosphorylase would be expected to be inhibited by G6P rapidly produced during its reaction, and by ATP, the ultimate end product of carbohydrate and energy metabolisms. By the same token, it would be expected to be activated by AMP, which is indeed the case. Of course, the same behavior would apply to many enzymes of glycolysis and of the Krebs cycle: just like phosphorylase, they are all activated by AMP and inhibited by ATP. Therefore, if one had to rely solely on an Allosteric activation, and unless one had strict intracellular compartmentation, all these enzymes would be similarly affected. In contrast, covalent modifications are mediated by regulatory enzymes that are extremely specific. Phosphorylase kinase hits phosphorylase and no other enzyme. It provides the possibility of affecting one single step, of opening one single door, without disturbing any other.

When Ed and I had found that phosphorylase was phosphorylated on a single seryl resdue, that reaction seemed so straightforward and simple that there was no doubt in our minds that it would represent the prototype for these kinds of interconversions. The idea that a single phosphorylation event was all that was needed to alter the state of activity of an enzyme was so ingrained, that when Joe Larner found that glycogen synthase was incorporating perhaps six phosphates per mole [12], we wondered whether his enzyme might contain 6 identical subunits: it might look like an orange! Of course, we know today from his work and that of his collaborator Roach [13], and that of Phil Cohen who discovered GSK3 [14] and determined the involvement of that enzyme in the regulation of the synthase, that whereas phosphorylase is phosphorylated by a single kinase on a single seryl residue, glycogen synthase is phosphorylated by no less than eight different kinases on nine different sites. Furthermore, that those phosphorylation events required the concerted action of several of these kinases that trigger a most complicated program of serial reactions that have to proceed in a strictly defined order. Imagine the incredible mess we would have been in if Ed and I had started to work on that enzyme rather than on phosphorylase: we would never, but absolutely never, have been able to solve that problem.

Ed and I have often been asked whether we realized, at the beginning, that we were dealing with a ubiquitous and, therefore, very fundamental process. Absolutely not. We stayed with it because we felt it was an exciting and obviously important system to investigate but we never could have predicted the incredible developments that followed. Anyway, in those early days, most everybody felt that allostery was it; that allostery was the principal mode of regulation of cellular processes. The question that seemed of concern to most everyone was whether the regulation of a particular system followed the "allosteric" model of Monod [11] or the "induced-fit" model of Koshland [15]. And it was perfectly clear to me, for instance, that Jacques, who was a very close friend, never believed one minute that covalent regulation by protein phosphorylation could play any fundamental role in enzyme regulation. With his passing 36 years ago, that of Dany Koshland 6 years ago and now that of Ed Krebs two Winters ago, we have witnessed the end of an extraordinary era.

References

[1] E.H. Fischer, E.G. Krebs, Conversion of phosphorylase β to phosphorylase α in muscle extracts, J. Biol. Chem. 216 (1955) 121–132.
[2] J.K. Parnas, Bull. Soc. Chim. Biol. 18 (1936) 53.
[3] G.T. Cori, C.F. Cori, The formation of hexosephosphate esters in frog muscle, 116 (1936) 119–128.
[4] G.T. Cori, A.A. Green, Crystalline muscle phosphorylase II. 1. Prosthetic group, J. Biol. Chem. 151 (1943) 31–38.
[5] J. Monod, F. Jacob, J.-P. Changeux, Allosteric proteins and cellular control systems, J. Mol. Biol. 6 (1963) 306–329.
[6] T.W. Rall, E.W. Sutherland, W.D. Wosilait, The relationship of epinephrine and glucagon to liver phosphorylase: III. Reactivation of liver phosphorylase in slices and in extracts, J. Biol. Chem. 218 (1956) 483–495.
[7] E.W. Sutherland, T.W. Rall, The relation of adenosine-3', 5'-phosphate and phosphorylase to the actions of catecholamines and other hormones, Pharmacol. Rev. 12 (1960) 265–299.
[8] E.H. Fischer, H.E. Blum, B. Byers, C. Heizmann, G.W. Kerrick, P. Lehky, D.A. Malencik, S. Pocinwong. Concerted regulation of glycogen metabolism and muscle contraction, in: S. Shaltiel (Ed.), Metabolic Interconversion of Enzymes, Springer-Verlag, Berlin-Heidelberg, 1976, p. 1.
[9] S. Ebashi, F. Lipmann, Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle, J. Cell. Biol. 14 (1962) 389–400.
[10] W. Hasselbach, M. Makinose, ATP and active transport, Biochem. Biophys. Res. Commun. 7 (1962) 132–136.
[11] J. Monod, J. Wyman, J-P. Changeux, On the nature of allosteric transitions: a plausible model, J. Mol. Biol. 12 (1965) 88–118.
[12] C.H. Smith, N.E. Brown, J. Larner, Molecular characteristics of the totally dependent and independent forms of glycogen synthase of rabbit skeletal muscle. II. Some chemical characteristics of the enzyme protein and of its change on interconversion, Biochim. Biophys. Acta 242 (1971) 81–88.
[13] P.J. Roach, Multisite and hierarchal protein phosphorylation, J. Biol. Chem. 266 (1991) 14139–14142.
[14] S.P. Frame, P. Cohen, GSK3 takes centre stage more than 20 years after its discovery, Biochem. J. 359 (2001) 1–16.
[15] D.E. Koshlad, G. Nemethy, D. Filmer, Comparison of experimental binding data and theoretical models in proteins containing subunits, Biochemistry 5 (1966) 365–385.