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Historical Perspectives on Protein Phosphorylation and a Classification System for Protein Kinases

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Historical perspectives on protein phosphorylation and a classification system for protein kinases

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Work on the phosphorylation of proteins as a dynamic process involved in the regulation of biological processes started in the 1950s with the finding that phosphorylase *a* and phosphorylase *b* are phospho and dephospho forms of the same enzyme. The field expanded sharply in the late 1960s with the discovery of the cyclic-AMP-dependent protein kinase, and it is now clear that phosphorylation–dephosphorylation constitutes a major type of regulation almost as common as allosteric control. The protein kinases, which catalyse the phosphorylation step in various phosphorylation–dephosphorylation systems, can be divided into two main classes, the serine–threonine protein kinases and the tyrosine protein kinases. Each class can be subdivided into groups or entities depending on the nature of the agent(s) that regulate activity.

Introduction

It is now well recognized that the phosphorylation and dephosphorylation of proteins is an important regulatory process applicable to many different cellular functions. The holding of this meeting and the organization of numerous other international symposia on protein phosphorylation during the past several years attest to this fact. How did a general appreciation of protein phosphorylation as a control mechanism develop? What is our present understanding of the diversity and properties of the enzymes that catalyse protein phosphorylation and dephosphorylation? Are there sets of principles that can serve as guidelines to investigators probing the possible role of protein phosphorylation in different systems? What is the future of the field? These and other general questions will constitute the theme of this paper, which is meant to serve as an introduction to this meeting.

Historical aspects

Early work on glycogen metabolism

Before the 1950s the only known phosphoproteins were casein, phosvitin and related proteins involved in the feeding of the young or in early embryonic development. One exception was the proteolytic enzyme pepsin, which was known to contain a stoichiometric amount of phosphate (Northrop 1930). (In a sense it is ironical that the first phosphoenzyme should be one in which there is no known function of the bound phosphate, i.e. pepsin is an example of 'silent phosphorylation'.) The first developments that foreshadowed current concepts of protein phosphorylation as a dynamic process involved in regulation grew out of work on glycogen metabolism.

In the early 1940s Carl and Gerti Cori together with Arda Green had shown that the enzyme phosphorylase, which catalyses the first step in glycogen breakdown, exists in active and inactive forms (Cori & Green 1943; Cori & Cori 1945). They named these forms phosphorylase *a* and phosphorylase *b* and succeeded in demonstrating that phosphorylase *a* could be converted

to phosphorylase *b* *in vitro*. They were unable to achieve conversion of phosphorylase *b* to *a* *in vitro* but did obtain evidence that this reaction occurred *in vivo*. Thus the first 'interconvertible enzyme' system was established. The chemical nature of the interconversion reactions involving the forms of phosphorylase was not then appreciated.

In the early 1950s Earl Sutherland and his collaborators (Wosilait & Sutherland 1956; Rall *et al.* 1956), working with liver phosphorylase, and E. H. Fischer and myself (Fischer & Krebs 1955; Krebs & Fisher 1956), working with the muscle enzyme, discovered that the active form of phosphorylase is a phosphoenzyme and that the interconversion reactions involve enzyme-catalysed phosphorylation and dephosphorylation steps. The enzyme catalysing the phosphorylation of phosphorylase by ATP was named phosphorylase kinase, and the enzyme catalysing the hydrolytic dephosphorylation step was named phosphorylase phosphatase. These reactions, incorporating our present knowledge regarding stoichiometry, are as follows:

$$\text{phosphorylase } b + 2\ \text{ATP} \xrightarrow{\text{phosphorylase kinase}} \text{phosphorylase } a + 2\ \text{ADP}; \quad (1)$$

$$\text{phosphorylase } a + 2\ \text{H}_2\text{O} \xrightarrow{\text{phosphorylase phosphatase}} \text{phosphorylase } b + 2\ \text{P}_\text{i}. \quad (2)$$

Shortly after the elucidation of the biochemical mechanism of the phosphorylase interconversion reactions it was found that phosphorylase kinase is itself activated under phosphorylating conditions in a process that is stimulated by cyclic AMP (Krebs *et al.* 1959, 1964). Then in 1963 it was reported that glycogen synthase exists in two forms that are interconverted by phosphorylation and dephosphorylation (Friedman & Larner 1963). The phosphorylation of glycogen synthase, like that of phosphorylase kinase, was shown to be stimulated by cyclic AMP (Belocopitow 1961; Huijing & Larner 1966). It was of particular interest that, whereas phosphorylation had been shown to increase the activity of phosphorylase and phosphorylase kinase, both of which are involved in glycogen breakdown, phosphorylation decreased the activity of the biosynthetic enzyme, glycogen synthase. This reciprocal relation has been found to hold for other enzymes involved in biosynthesis and degradation and it would appear to be one of the 'principles' that has been established for the regulation of enzymes by phosphorylation–dephosphorylation. Philip Cohen, one of the organizers of this meeting, has effectively emphasized this point in recent years.

The mechanism of action of cyclic AMP in the regulation of phosphorylase kinase activation, and glycogen synthase inactivation, both resulting from enzyme phosphorylation, was not entirely clear at first. In connection with phosphorylase kinase, it was apparent that cyclic AMP enhanced the rate of phosphorylation and activation of the enzyme, but it was not known whether it interacted with phosphorylase kinase itself to stimulate an autophosphorylation reaction or whether a second protein kinase, i.e. a cyclic-AMP-dependent phosphorylase kinase kinase, was involved (Krebs *et al.* 1965). In connection with glycogen synthase, cyclic AMP also enhanced the rate of phosphorylation of the enzyme, and in this case, in that the substrate in the phosphorylation reaction is not a protein kinase, it was reasonably safe to conclude that the target for the regulatory agent was the synthase kinase itself (Huijing & Larner 1966*a*, *b*). A less likely possibility was that the synthase might have contained two interacting kinases involved in a cascade. These uncertainties were resolved when it was determined that a separate phosphorylase kinase kinase existed (Walsh *et al.* 1968) and when later it was shown that this enzyme and glycogen synthase kinase are the same enzyme (Schlender *et al.* 1969); Soderling *et al.* 1970).

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The cyclic-AMP-dependent phosphorylase kinase kinase was not referred to by that name, but was instead called a cyclic-AMP-dependent protein kinase, since it was realized immediately that it could catalyse the phosphorylation of more than one protein (Walsh *et al.* 1968).

Because of widespread interest in cyclic AMP and its multiple functions, the finding of a cyclic-AMP-dependent protein kinase served to focus attention on the process of regulation by protein phosphorylation–dephosphorylation. Before this development many investigators had thought of the process as probably being limited to the control of glycogen metabolism, but it now appeared that it might have widespread implications. Noting an almost ubiquitous distribution of the cyclic-AMP-dependent protein kinase in various cell types, Kuo & Greengard (1969) postulated that *all* actions of cyclic AMP might be mediated by this enzyme. (This hypothesis is still valid for eukaryotic cells but does not hold for prokaryotes.) Another important finding that served to broaden interest in protein phosphorylation was the discovery by Lester Reed and his collaborators that pyruvate dehydrogenase is regulated by phosphorylation–dephosphorylation (Linn *et al.* 1969). Here was a clearcut example of a phospho–dephospho enzyme system well beyond the confines of glycogen metabolism.

Present status of protein phosphorylation–dephosphorylation

When investigators became aware of the 'new' type of metabolic regulation and broadened their horizons to look beyond the confines of glycogen metabolism, work on the phosphorylation and dephosphorylation of enzymes expanded rapidly. This is illustrated in figure 1, in which the total number of enzymes known to undergo phosphorylation–dephosphorylation, or to contain covalently bound phosphate as isolated, is shown as a function of time. By early 1982 more than 40 enzymes belonged in this category. Table 1 lists the individual enzymes and literature citations for each entry.

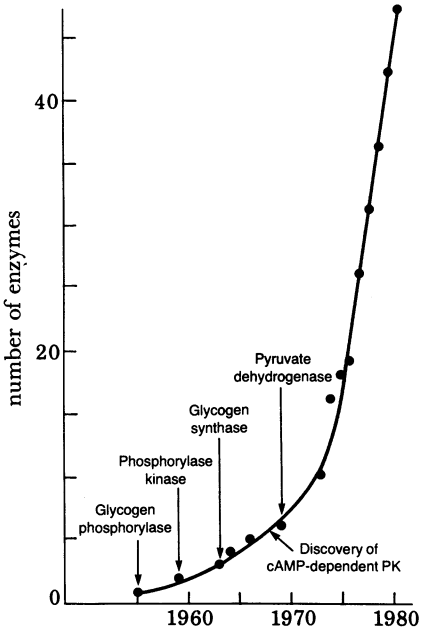

FIGURE 1. Enzymes reported to undergo phosphorylation–dephosphorylation. Abbreviations: cAMP, cyclic AMP; PK, protein kinase.

TABLE 1. ENZYMES REPORTED TO UNDERGO PHOSPHORYLATION–DEPHOSPHORYLATION

enzyme	references
glycogen phosphorylase	Fischer & Krebs (1955), Sutherland & Wosilait (1955), Rall *et al.* (1957)
phosphorylase kinase†	Krebs (1959)
glycogen synthase†	Friedman & Larner (1963)
hormone-sensitive lipase†	Rizak (1964), Corbin *et al.* (1970), Huttunen *et al.* (1970)
fructose 1,6-bisphosphatase†	Mendicino *et al.* (1966), Rious *et al.* (1977)
pyruvate dehydrogenase	Linn *et al.* (1969)
hydroxymethylglutaryl-CoA reductase†	Beg *et al.* (1973)
myosin light chain	Perrie *et al.* (1973)
acetyl-CoA carboxylase†	Carlson & Kim (1973)
casein kinase II	Walinder (1973)
RNA polymerase I†	Jungman *et al.* (1974), Martelo & Hirsch (1974)
RNA polymerase II†	Jungman *et al.* (1974), Martelo & Hirsch (1974)
pyruvate kinase (liver)†	Ljungström *et al.* (1974)
cholesterol ester hydrolase†	Trzeciak & Boyd (1974)
type II cyclic-AMP-dependent protein kinase regulatory subunit†	Erlichman *et al.* (1974)
tyrosine aminotransferase	Lee & Nickol (1974)
phosphofructokinase 1†	Brand & Söling (1975)
tyrosine hydroxylase†	Morgenroth *et al.* (1975)
phenylalanine hydroxylase†	Milstein *et al.* (1976)
haem-inhibited eIF-2 kinase	Farrell *et al.* (1977)
cyclic-AMP-dependent protein kinase catalytic subunit†	Bechtel *et al.* (1977)
double-stranded RNA-dependent protein kinase	Farrell *et al.* (1977)
cyclic-GMP-dependent protein kinase	deJonge *et al.* (1977)
tryptophan hydroxylase	Hammon *et al.* (1977, 1978)
type I cyclic-AMP-dependent protein kinase regulatory subunit	Steinberg *et al.* (1977)
SV40 large T antigen (an 'ATPase')	Tegtmeyer *et al.* (1977)
NAD-dependent glutamate dehydrogenase (yeast)	Hemmings (1978)
glycerophosphate acyltransferase†	Nimmo & Houston (1978)
hydroxymethylglutaryl-CoA reductase kinase	Gibson & Ingebritsen (1978)
myosin light chain kinase†	Adelstein *et al.* (1978)
pp60^{src}†	Brugge (1978)
branched-chain ketoacid dehydrogenase	Parker & Randle (1978)
calmodulin-dependent cyclic nucleotide phosphodiesterase†	Eldik & Watterson (1979), Sharma *et al.* (1980)
ATP citrate lyase†	Linn & Srere (1979)
poly(A) polymerase	Rose & Jacob (1979)
isocitric dehydrogenase (*Escherichia coli*)	Garnak & Reeves (1979)
casein kinase I	Hathaway & Traugh (1979)
EGF receptor-associated kinase	King *et al.* (1980)
P120$^{gag\text{-}abl}$	Witte *et al.* (1980)
P90$^{gag\text{-}yes}$	Kawai *et al.* (1980)
P140$^{gag\text{-}fps}$	Feldman *et al.* (1980)
insulin-stimulated low K_m cyclic AMP phosphodiesterase†	Marchmont & Houslay (1980)
glucose-6-phosphatase†	Burchell & Burchell (1980), Begley & Craft (1981)
phosphofructokinase 2†	van Schaftingen *et al.* (1981), Furuya *et al.* (1982), El-Maghrabi *et al.* (1982)
glycogen synthase kinase 3 (phosphatase activator, F_A)	Hemmings *et al.* (1981)
ornithine decarboxylase (slime mould)	Atmar & Kuehn (1981)
Ca^{2+}-transport ATPase	Caroni & Carafoli (1971)
cholesterol 7α-hydroxylase	Sanghri *et al.* (1981)
aminoacyl tRNA synthetase complex	Damuni & Cohen (1982)
poly(A)-endoribonuclease	Tsjapalis *et al.* (1982)

† Cyclic-AMP-dependent protein kinase involved.

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Several points in relation to figure 1 and table 1 warrant discussion. Based on what is currently known, only about half of the enzymes in table 1 meet any substantial number of the criteria (Krebs & Beavo 1979) that should be satisfied to establish that a given enzyme undergoes physiologically significant phosphorylation–dephosphorylation from a regulatory standpoint. For instance, many of the enzymes listed undergo 'silent' phosphorylation, a term that has been used by Cohen (1982) to describe the phosphorylation of enzymes at sites that do not influence enzyme activity; examples of silent phosphorylation include the phosphorylation of tyrosine amino-transferase, 6-phosphofructo 1-kinase and ATP-citrate lyase as well as that of a number of the protein kinases. It is known that the last group, protein kinases, characteristically catalyse their own phosphorylation, and in most instances the 'autophosphorylation' reaction has no demonstrable effects on enzyme activity. Also, for several of the enzymes listed in table 1 it has not actually been demonstrated that phosphorylation and dephosphorylation occur *in vivo*. Finally, even for enzyme that meet many of the criteria of physiological relevance, careful work has not always been carried out to correlate the state of phosphorylation of the enzyme *in vivo* with a physiological event that is presumably being regulated by phosphorylation–dephosphorylation. It should also be noted that several of the entries in table 1 are based on preliminary reports and may not withstand the test of time.

The regulation of cellular function as a result of protein phosphorylation–dephosphorylation reactions involves many proteins in addition to those that have been identified as having enzymic activity. For example, even as the role of this process in the control of glycogen metabolism was unfolding, it was recognized that non-enzymic nuclear proteins undergo dynamic phosphorylation–dephosphorylation (Kleinsmith *et al.* 1966), and a 'specific' histone kinase was discovered as early as 1967 (Langan & Smith 1967). After the finding of a cyclic-AMP-dependent protein kinase (Walsh *et al.* 1968), it was shown that this enzyme and the histone kinase are apparently identical (Langan 1968). In addition to early studies on the phosphorylation of non-enzymic nuclear proteins, pioneering work had also been carried out on the rapid turnover of protein-bound phosphate in brain (Heald 1958) and in cell membranes in relation to ion transport (Judah & McLean 1962). It is possible that some of the protein phosphorylation observed in these later studies may have involved the transient phosphoenzyme intermediate of the Na^+–K^+ATPase and would not be germane to protein kinase-catalysed phosphorylation.

During the 1970s the number of reports on the phosphorylation of non-enzymic proteins underwent a dramatic upward surge that in some ways was even more impressive than that which occurred for enzymes. If space had permitted, it would have been of interest to consider in detail some of the countless examples of such proteins. They include cytosolic proteins, cell membrane proteins, nuclear proteins, ribosomal proteins, contractile proteins, cytoskeletal proteins and proteins of highly specialized structures such as nerve endings. Some of these topics will be discussed in other papers in this symposium.

Classification of protein kinases

Until recently it was thought that only serine or threonine residues could serve as the phosphate acceptors in protein kinase substrates, but in 1979 it was shown that an activity phosphorylating tyrosine was present in polyoma T antigen immunoprecipitates (Eckhard *et al.* 1979). Shortly thereafter it was found that $pp60^{src}$, the transforming protein of the Rous

sarcoma virus (Brugge & Erikson 1977), which was known to be associated with protein kinase activity (Collet & Erikson 1978), was a tyrosine protein kinase (Hunter & Sefton 1980; Collet *et al.* 1980). Tyrosine protein kinase activity was then found to be associated with the epidermal growth factor (EGF) receptor (Ushiro & Cohen 1980). In addition, it has been shown that PDGF, the platelet-derived growth factor (Ek *et al.* 1982; Ek & Heldin 1982; Nishimura *et al.* 1982) and insulin (Kasuga *et al.* 1982*a*, *b*) stimulate the phosphorylation of tyrosine in certain proteins.

It appears that tyrosine protein kinases can be treated as a separate class of specific enzymes distinct from serine–threonine protein kinases. In this connection, however, it is of interest that $pp60^{src}$ and the catalytic subunit of the cyclic-AMP-dependent protein kinase have been shown to be structurally related (Barker & Dayhoff 1982). In addition to serine–threonine and tyrosine protein kinases it is possible that a third class of protein kinases, enzymes manifesting activity toward histidine and lysine residues, may exist; however, work on these enzymes has been limited (Smith *et al.* 1974).

Many serine–threonine protein kinases (table 2) other than the cyclic-AMP-dependent protein kinase(s) are regulated by specific effectors, a property that has been useful in their classification (Krebs & Beavo 1979). So far, enzymes of this class have been described that are regulated by (1) cyclic AMP, (2) cyclic GMP, (3) Ca^{2+}, (4) diacylglycerol, (5) double-stranded RNA, (6) haemin, (7) acetyl CoA, NADH, pyruvate and ADP, and (8) polyamines.

TABLE 2. SUBCLASSIFICATION OF SERINE–THREONINE PROTEIN KINASES (PKs)

regulatory agent(s)	specific entities
cyclic AMP	type I, type II (heart) and type II (brain) cyclic-AMP-dependent PKs
cyclic GMP	cyclic-GMP-dependent PK
Ca^{2+} (calmodulin)	phosphorylase kinase (glycogen synthase kinase 2)
	myosin light-chain kinase
	liver glycogen synthase kinase (Soderling)
diacylglycerol (Ca^{2+}, phospholipid)	protein kinase C
double-stranded RNA	double-stranded RNA-dependent PK
haemin (inhibition)	eIF_2 kinase
acetyl-CoA, NADH, pyruvate, ADP	pyruvate dehydrogenase kinase
polyamines	polyamine-dependent PK
unknown	casein kinase 1 (NI, Ck-S)
	casein kinase 2 (NII, Ck-TS, glycogen synthase kinase 5)
	glycogen synthase kinase 3 (factor F_A)
	glycogen synthase kinase 4

In addition to the cyclic-nucleotide-dependent enzymes, the protein kinases that are regulated by calcium are especially prominent. Phosphorylase kinase was the first enzyme that was found to be dependent on calcium ions for activity (Meyer *et al.* 1964; Ozawa *et al.* 1967), a property that can now be accounted for by the presence of calmodulin as one of the subunits of the enzyme (Cohen *et al.* 1978). In other members of this group, calmodulin also serves as the component that mediates the calcium effect, but in these enzymes calmodulin is not an integral subunit in the same sense that it is in phosphorylase kinase. It is of interest that the several calcium (calmodulin)-dependent protein kinases (table 2) have distinctly different catalytic subunits and different specificities but share a common regulatory subunit, whereas the different

cyclic-AMP-dependent protein kinases share a common catalytic subunit but have different regulatory subunits. In this meeting we shall be hearing about several of the calcium (calmodulin)-dependent protein kinases and will also be hearing about protein kinase C, which requires calcium but not calmodulin.

A major subset of serine–threonine protein kinases consists of those enzymes for which no regulatory agents or effectors are known to exist. This does not necessarily mean, however, that these enzymes, or the reactions that they catalyse, are not subject to regulation, because regulation could occur by any of several mechanisms other than through the direct interaction of the protein kinase with an effector molecule. For example, regulation could be effected at the level of the protein substrate(s), i.e. it could be brought about through alterations in substrate conformation caused by the binding of effectors. Also, protein kinases for which no known effectors exist might be regulated by covalent modification, e.g. by serving as substrates for other protein kinases. A possible example of this type would be hydroxymethylglutaryl-CoA reductase kinase (Gibson *et al.* 1978). Finally, a protein phosphorylation reaction catalysed by a non-regulated protein kinase might be effectively controlled through regulation of the phosphoprotein phosphatase acting on the phosphorylated substrate.

TABLE 3. SUBCLASSIFICATION OF TYROSINE PROTEIN KINASES

regulatory agent(s)	specific entities
EGF	EGF receptor
PDGF	(PDGF receptor)
insulin	(insulin receptor)
unknown	$pp60^{src}$
	$P140^{gag\text{-}fps}$ and $P105^{gag\text{-}fps}$
	$P90^{gag\text{-}yes}$ and $P80^{gag\text{-}yes}$
	$P120^{gag\text{-}abl}$
	$P85^{gag\text{-}fes}$ and $P110^{gag\text{-}fes}$

Table 3 categorizes the tyrosine protein kinases by the same system that was used for the serine–threonine protein kinases. Thus one can speak of EGF-dependent, PDGF-dependent and insulin-dependent tyrosine protein kinases. For EGF-stimulated tyrosine phosphorylation there is good evidence that the growth factor receptor itself is the protein kinase (Buhrow *et al.* 1982), and this suggests that the PDGF and insulin receptors may also be protein kinases. Supporting this view is the finding that in each case these factors stimulate the phosphorylation of tyrosine in a membrane protein with the molecular mass of the receptor, and it is known (see above) that protein kinases characteristically catalyse an autophosphorylation reaction. The virus-associated tyrosine protein kinases are not known to be regulated by specific effectors and would be analogous to the comparable group of serine–threonine protein kinases. It is of interest in this connection that one of these, $pp60^{src}$, is known to be phosphorylated by the cyclic-AMP-dependent protein kinase (Collett *et al.* 1979), suggesting the possibility of regulation by covalent modification. Table 3 indicates the existence of five types of viral onc-gene related tyrosine protein kinases, which are designated by using a system recently agreed upon by a number of authors (Coffin *et al.* 1981). For each viral-encoded entity listed in table 3 a normal cellular counterpart would also exist, making a total of ten distinct, albeit related, enzymes in this group.

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